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From Cell Biology to Tissue Engineering

Analysis of cell-cell junctions in human amnion and chorionic plate affected by chorioamnionitis

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Summary. Chorioamnionitis is an acute inflammatory reaction associated with the premature rupture of the fetal membranes. It is caused mainly by invasion of bacteria from the vaginal tract that can penetrate the intact membranes and invade the amnion cavity and the decidua. Tight junctions (TJ) and adherent junctions (AJ) are intercellular junctions crucial for epithelia adhesion and permeability regulation in a wide variety of tissues and organs. Our aim is to investigate if TJ and AJ molecules are involved in human chorioamnionitis.

We studied the protein expression (by immunohistochemistry and western blotting) and the mRNA levels (by RT-PCR) of some junction proteins such as Zonula Occludens-1 (ZO-1), occludin, VE-cadherin and β-catenin in fetal membranes from women with chorioamnionitis compared to those membranes derived from idiopathic pregnancies.

Western blotting and immunohistochemical data established that occludin expression was decreased in amnion with chorioamnionitis compared to amnion from idiopathic pregnancies. Samples tested for ZO-1, VE-cadherin and β-catenin (proteins and mRNAs) showed no differences between idiopathic and pathological membranes.

One of the most relevant results is the decrease of occludin in membranes with chorioamnionitis. Since we have previously demonstrated that some cytokines, particularly elevated in the chorioamnionitis, cause the disruption of TJs in placental villi, we suggest that the decrease of occludin in amnion may be the first change that leads to the rupture of the amniotic membrane in this pathology.

Key words: Tight junction, Adherent junction, Amnion, Chorioamnionitis

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Introduction

Fetal membranes represent the structure that contains and protects the fetus during pregnancy. The inner layer of the membranes is the amniotic epithelium and it consists of cuboidal epithelial cells planted on a thick basement membrane (Benirschke et al., 2006; Litwiniuk and Grzela, 2014).

Chorioamnionitis is an acute inflammatory reaction associated with the premature rupture of the fetal membranes and it can cause preterm delivery, high perinatal morbidity and mortality (D'Alquen et al., 2005; Galinsky, 2013; Redline, 2006). Chorioamnionitis can show evident clinical symptoms such as body temperature >37,8°C, uterine tenderness, malodorous vaginal discharge, maternal leucocytosis, maternal tachycardia, fetal tachycardia even if in many cases these clinical symptoms may not appear. It is diagnosed through histological analysis such as microscopic evidence of inflammation, infiltration of polymorphonucleates and other immunocytes (Menon et al., 2010;

Toti et al., 2010). Chorioamnionitis can be caused by microbial invasion and one major pathway of infection is ascending bacteria, protozoa and virus from the vaginal tract (Romero et al., 2002; Spinillo et al., 2014). Bacteria can multiply at this site and penetrate the intact membranes invading the amnion cavity and the decidua (D'Alquen et al., 2005; Menon et al., 2010; Galinsky et al., 2013; Fortner et al., 2014; Spinillo et al., 2014).

The presence of infectious agents, such as Gram negative bacteria, induces an inflammatory response also by amniotic cells that release proinflammatory molecules such as prostaglandins (PGs) into the amniotic fluid (López Bernal et al., 1988; Casey et al., 1989; Romero et al., 1990; van der Elst et al., 1991; Di Giulio et al., 2010; Menon et al., 2010). The huge increase in PGs production could cause preterm labour as occurs in the majority of chorioamnionitis cases (López Bernal et al., 1988; van der Elst et al., 1991).

One of the mechanisms by which pathogens usually invade the sub epithelial tissue is the disruption of junctions present in the tissue, causing inflammation and the weakening of the amnion (Simonovic et al., 2000; Gruenheid et al., 2003; Kobayashi et al., 2010a; Rodríguez-Tirado et al., 2012). Tight (TJs) and adherent junctions (AJs) are intercellular junctions crucial for epithelia adhesion and permeability regulation in a wide variety of tissues and organs. Both TJs and AJs are closely associated with a circumferential belt of actin. TJ is composed of integral membrane proteins, such as occludin, claudin and junctional adhesion molecules (JAMs), which bind cytoplasmic proteins, such as zonula occludens-1 (ZO-1) and zonula occludens-2 (ZO-2). In particular, ZO proteins are scaffolding proteins and they link the actin of the cytoskeleton to the integral membrane proteins. This type of junction is present in placental tissues, such as trophoblast, fetal vessels and amniotic membrane. TJs have an important role in membrane permeability controlling the movement of water, small molecules and immune cells through cellular spaces (Marzioni et al., 2001; Niessen, 2007; Di Tommaso et al., 2014).

AJs are present in epithelia and endothelia. One of the two basic units that form AJs is the cadherin- catenin complex. Cadherins are transmembrane proteins and are linked inside the cell to a large number of intracellular partners, like catenin, that are connected in turn to cytoskeletal actin. α-catenin binds several actin-binding proteins that could link the cadherin- β-catenin complex to the actin cytoskeleton. VE- cadherin is an endothelial-specific member of the cadherin family. AJs are crucial for the initiation and maintenance of intercellular adhesion in a wide variety of tissues and cell populations (Halbleib and Nelson, 2006; Niessen, 2007; Vestweber, 2008; Dejana and Giampietro, 2012). We hypothesized that TJ and AJ molecules could be altered in human amnion and chorion plate affected by chorioamnionitis as a consequence of the action of inflammatory molecules.

Since no data are available on this matter we evaluated protein and mRNA expression levels of ZO-1, occludin, VE-cadherin and β-catenin in membranes affected by chorioamnionitis compared to membranes obtained from idiopathic preterm birth (IPB) pregnancies.

Materials and methods

Tissue collection

We analysed a total of 15 membranes (amnion and chorionic plate) from human placentas: 8 from preterm deliveries complicated by chorioamnionitis (24-34 weeks of gestation) and 7 from IPB (27-35 weeks of gestation). Pregnant women gave their informed consent to collect placentas and membrane specimens (Division of Obstetrics and Gynaecology, University of Siena). The procedures followed for the collection of samples were in accordance with the Helsinki Declaration of 1975, as revised in 1983. Diagnosis of acute chorio-amnionitis (stage 2 of maternal inflammatory response in Redline et al., 2003) by the histological examination of placentas was defined as the presence of at least ten polymorphonuclear leucocytes (PMNs) per field in ten non-adjacent 400-power fields in membranes and/or placental chorionic plate (Toti et al., 2000).

In three cases, the acute inflammatory infiltrate was also present in the umbilical cord (funisitis; stage 2 of fetal inflammatory response in Redline et al., 2003). We collected samples excluding those having specific exclusion criteria such as relevant identifiable placental pathology (different from chorioamnionitis), as well as maternal and fetal-neonatal diseases (pre-eclampsia, IUGR, placental infarcts, abruption and placenta previa, maternal thrombophilia, diabetes and smoking). Finally, we collected samples with preterm deliveries with chorioamnionitis, on which the infection was assumed to be the cause of preterm delivery. For this study, after the histology was done, we selected areas with a very low amount of inflammatory cells to facilitate data interpretation. IPBs used as controls of the same gestational ages were diagnosed after exclusion of the above mentioned criteria.

Four specimens were taken from each placental membrane. Two samples were used for immunohisto-chemistry and two specimens were taken for biochemical and molecular analysis (RNA and protein expressions). Samples for immunohistochemistry were fixed in 4% buffered formalin at 4°C for 12 h then washed in cold phosphate buffer pH 7,4 for 30 minutes. Thereafter the specimens were dehydrated via a graded series of ethyl alcohol (50°C for 30 minutes, 75°C for 30 minutes, 2x96°C for 75 minutes, 3x100°C for 75 minutes) and two steps in xylene for 60 minutes. Then, they were processed for paraffin embedding at 56°C. The samples for RNA and protein expressions were frozen in liquid nitrogen and stored at -80°C.

Immunohistochemistry

Immunohistochemistry was performed as previously

described (Marzioni et al., 2001).

Primary antibodies used are described in Table 1.

For all reactions, we used peroxidase ABC (Vector laboratories, Burlingame, CA) and 3',3'- diaminobenzidine hydrochloride (Sigma- Aldrich, St. Louis, MO, USA). Pre-treatment by heat in 10 mM citrate buffer pH 6.0 for 20 minutes was used for VE- cadherin and β-catenin and pre-treatment by 0.1% trypsin (Sigma-Aldrich) in distilled water for 10 minutes at 37°C was used for ZO-1 and occludin.

Negative controls were performed by omitting the first or secondary antibody for all the immunohistochemical reactions performed in this study. Non-immune goat or horse serum or isotype antibodies (rabbit IgG: cat.ab27478 Abcam and mouse IgG1: cat.ab 27479 Abcam, Cambridge, UK) were used in the same way (dilution, volume, incubation conditions) as the respective primary antibody. Placenta at term was used as positive control for all antibodies used.

RNA extraction and real-time PCR

Total RNA was extracted from 100 mg of each sample using TRIzol reagent (Invitrogen Life Technologies, Milan, Italy) as per manufacturer's datasheet. RNA extracted was quantified at UV absorption at a wave length of 260 nm. Retrotranscription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA) and 1 μg of total RNA from each sample. Reverse transcriptase reactions were done as written in the manufacturer's protocol. We used Taqman gene expression assay probes (Table 2, Applied Biosystem) in real-time PCR in order to quantify every gene expression that we had in analysis. The program run by the thermocycler worked on the following time and temperature: initial denaturation at 95°C for 10 min, 40 following cycles at 95°C for 15 s and 60°C for 1 min. Reactions were performed using 100 ng cDNA in a final volume of 20 μl.

All results were normalized using β-actin and Hypoxanthine phosphoribosyltransferase (HPRT) as housekeeping genes. Negative controls were performed omitting either the reverse transcriptase enzyme or template RNA to test contamination with genomic DNA or not-specific amplification.

Protein extracts and western blotting

The samples for the protein assay were thawed and washed in PBS 0,1 M pH 7.4. We homogenized 300 mg of each sample with lysis buffer containing 20 mM Tris/HCl pH 8,1% Nonidet-P40, 10% glycerol, 137 mM NaCl, 1 mM CaCl2, 1 mM $MgCl_2$ and protease inhibitors (Protease Inhibitor Cocktail, Sigma-Aldrich) via Ultra-Turrax T8 (IKA-WERKE, Lille, France). The specimens were centrifuged at 20000 g for 20 min at 4°C and the supernatants were aliquoted and stored at -80°C. The proteins concentrations were determined by Bradford protein assay (Biorad Laboratories, Milan, Italy).

For the analysis of ZO-1, supernatants were

Table 1. Primary antibodies used in immunohistochemistry and western blotting.

Antibody	Specificity	Ab conc. for IH‡	Ab conc. for WB§	Source
Rabbit pAb† 61- 7300	Human ZO-1	1:40	1:250	Invitrogen, San Diego, CA
Rabbit pAb V1514	HumanVE-Cadherin	1:150	1:500	Sigma- Aldrich, Milan, Italy
Rabbit pAb 71- 1500	Human Occludin	1:30	1:200	Invitrogen
Mouse mAb* 18- 0226	Human β- catenin	7.5 mg/ml	1.12 mg/ml	Invitrogen
Mouse mAb A5316	Human β-actin	/	1:5000	Sigma- Aldrich

*mAb, monoclonal antibody; †pAb, policlonal antibody; ‡IH, Immunohistochemistry; §WB, western blotting.

Table 2. Probes "TaqMan gene expression assay" used in Real-time PCR.

Gene name	Alias	Gene symbol	Reference sequence	Assay ID	Amplicon length
Catenin (cadherin-associated protein), beta 1	CTNNB; FLJ25606; FLJ37923; OK/SW-cl.35	CTNNB1	NM_001098209.1	Hs00170025_m1	88
Occludin	BLCPMG; FLJ08163; FLJ18079; FLJ77961; FLJ94056; MGC34277; hCG_1988850	OCLN	NM_002538.3	Hs00170162_m1	68
Tight junction protein 1 (zona occludens 1)	DKFZp686M05161; MGC133289; ZO-1	TJP1	NM_003257.3	Hs01551876_m1	64
Cadherin 5, type 2 (vascular endothelium)	7B4; CD144; FLJ17376	CDH5	NM_001795.3	Hs00901463_m1	63
Hypoxantine phosphoribosyltranferase 1	HGPRT; HPRT	HPRT1	NM_000194.2	Hs99999909_m1	100
β-actin	PS1TP5BP1	ACTB	NM_001101.3	Hs99999903_m1	171

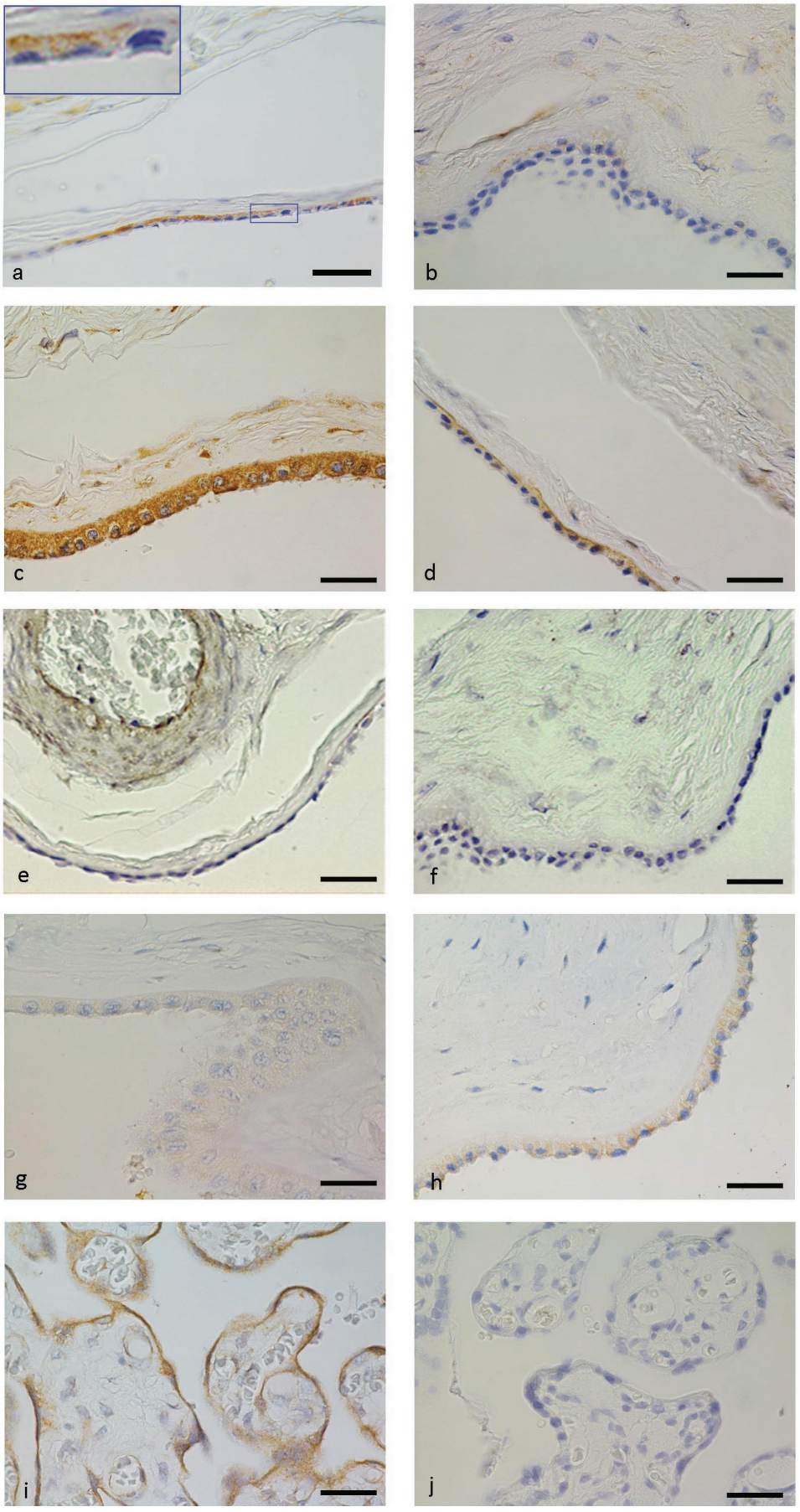

Fig. 1. Immunohistochemistry of amnion and chorionic plate samples from IPB membranes and membranes with chorioamnionitis. Occludin staining in IPB membrane **(a)** and in membrane with chorioamnionitis **(b)**. ZO-1 staining in IPB membrane **(c)** and membrane with chorioamnionitis **(d)**. VE-cadherin staining in the membrane in IPB **(e)** and with chorioamnionitis pregnancy **(f)**. β- catenin espression in IPB membrane **(g)** and in membrane with chorioamnionitis **(h)**. Positive control **(i)** and negative control **(j)** for occludin, third trimester placenta. Scale bars: a-j, 75 μm; Inset, 25 μm.

immune-precipitated (2 mg of total proteins) using a polyclonal anti-ZO-1 antibody (61-7300, Invitrogen, 1 μg) absorbed on protein-G-Sepharose beads (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). The bound proteins were eluted from the bead pellet via incubation with glycine 0.1 M, pH 2.5 for 30 min. Then the eluates were collected and stored at -80°C until use.

For western blotting assay, all protein samples (50 μg of protein for each sample) were fractionated by 8% SDS- polyacrylamide gels and electrophoretically transferred to PVDF (Polyvinylidene Fluoride) membranes. Membranes were incubated with 5% (w/v) BSA (USB corporation, Cleveland, OH) in TBS/0,05% Tween 20 (TBS-T) in order to avoid non-specific protein binding. Blots were incubated with primary antibodies (see Table 1). After washing, blots were incubated with secondary antibody conjugated with horseradish peroxidase (Amersham Italia s.r.l., Milan, Italy) diluted 1:5000. Detection of antibody binding was performed with the ECL-Western Blotting detection kit (Amersham) and the densitometry analysis of the obtained bands using Chemidoc and Quantity-One program (Bio-Rad Laboratories). The relative quantities were expressed as the ratio of densitometry reading for analysed proteins to β-actin.

Statistical analysis

Expressed proteins (variables) resulted non-normally distributed at the Shapiro test. Indeed, the sample size was calculated to evaluate the protein expression and mRNA levels of ZO-1, occludin, VE-cadherin and β-catenin in placentas with chorioamnionitis compared to that from IPB pregnancies. We estimated that a sample size of 8 observations in each group achieved 80% power to detect a difference of -60.0 intensity/mm of occludin/β-actin in placentas, between chorioamnionitis compared to IPB pregnancies, with group means equal 100.0 under the null hypothesis, standard deviations of 50.0, a significance level of 0.05, using a two-sided two-sample t-test. The same sample size ensures a power for this analysis of about 77%.

A non-parametric approach was used since data resulted non-normal distributed at the Shapiro test; variables were summarized using median, as measure of centrality, and 1st-3rd quartiles, as measure of variability, and graphically represented in boxplots. Comparisons between groups were performed by means of Wilcoxon rank sum test. The R statistical program was used for the analysis and a probability of 0.05 was used to assess the statistical significance.

Results

Occludin immunostaining was visible in chorionic membranes (Fig. 1a,b). Occludin was expressed in amniotic epithelium with chorioamnionitis (Fig. 1b) but its expression was less intense than in IPB membranes (Fig. 1a).

Western blotting confirmed the immunohistochemical data and it was established that occludin expression was decreased in amnion with chorioamnionitis compared to amnion from IPB pregnancies (Figs. 2, 3a).

ZO-1 was highly expressed in both IPB and chorioamnionitis membranes (Fig. 1c and 1d), showing no obvious differences both in immunohistochemistry and quantitative western blotting analysis (Fig.3b).

VE-cadherin was not detected in amnion samples tested by immunohistochemical analysis (Fig. 1e,f). VE-cadherin was instead present in vessels, as endothelial marker, as shown in Fig.1e. The VE-cadherin detected by western blotting was due to the presence of foetal vessels in the chorion plates, although showing no significant differences between chorioamnionitis and IPB (Fig. 3c).

Samples tested for β-catenin showed a very weak immunostaining (Fig. 1g,h) both in IPB and in chorioamnionitis amnion epithelia and no differences were found in western blotting analysis between IPB and pathological membranes (Fig. 3d).

We performed real time PCR analysis of occludin, ZO-1, VE- cadherin, and β-catenin mRNAs in IPB and chorioamnionitis samples in order to verify if the quantitative variation was due to changes in transcription or in post-transcriptional state.

As shown in Fig. 4, there were no significant differences in mRNA expression between chorioamnionitis and IPB membranes. Levels of occludin (Fig. 4a), ZO-1 (Fig. 4b), VE- cadherin (Fig. 4c) and β-catenin (Fig. 4d) mRNAs in chorioamnionitis samples were in fact comparable to values of IPB membranes.

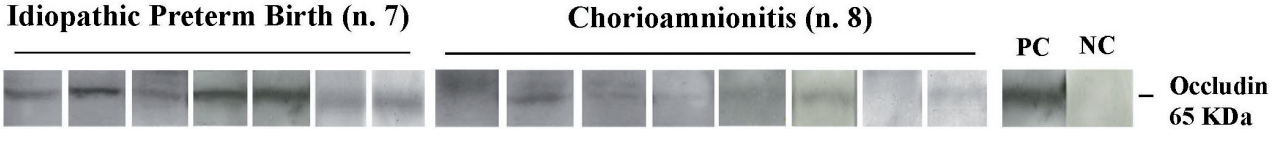

Fig. 2. Representative western blotting bands. Membranes with chorioamnionitis (n.8) at gestational age of 24- 34 weeks, IPB membranes (n.7). Placenta at 39th week of gestation was used as positive control (PC) and fibroblasts were used as negative control (NC). 65 kDa: molecular weight of occludin.

Discussion

In chorioamnionitis, the presence of microorganisms in chorioamnion and, later in the amniotic fluid, brings a chorioamnion infiltration of inflammatory cells (e.g., neutrophil granulocytes) not only in the placenta, but sometimes also in the umbilical cord, causing funisitis. Although the infection of the amniotic fluid can negatively affect the progress of pregnancy, it is unknown how this infection spreads across fetal membranes, in particular across chorioamnion. We have recently shown the important role of cytokines in the destruction of TJs in human placental villi affected by chorioamnionitis (Tossetta et al., 2014). Based on this previous study we hypothesized a reduction of cell-cell junctions in fetal membranes that could be a cause of a potential predisposition to bacterial invasion or gross rupture of amniotic membrane. Normally, micro-organisms spreading through epithelial tissues are blocked by TJs and AJs present on apical and basal lateral surface, respectively. This is the first study that describes the status of both tight and adherent junctions

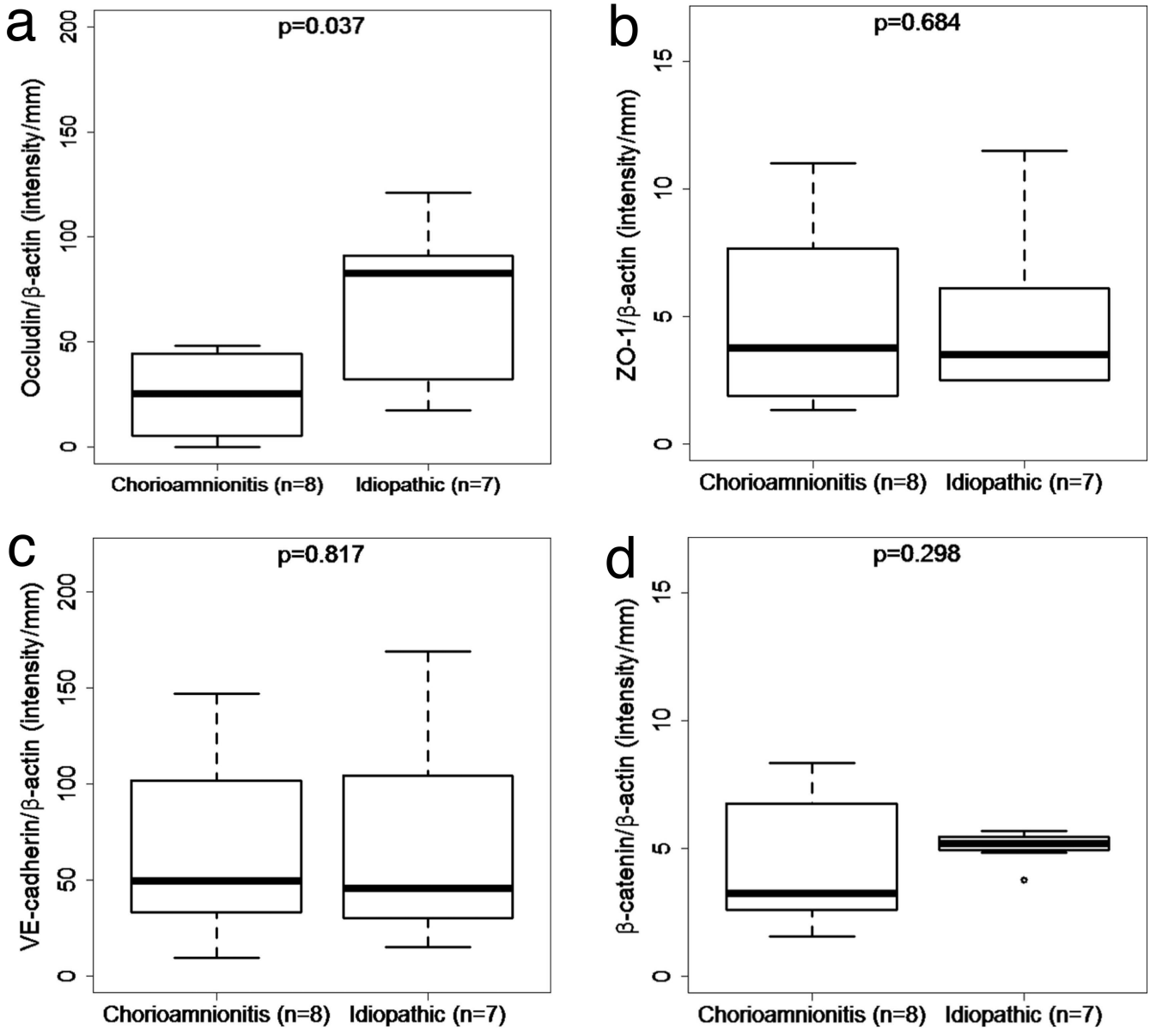

Fig. 3. Representative box plot from quantitative western blot results. Comparison of data obtained from western blot analysis of proteins between IPB and membranes with chorioamnionitis. **a.** Box plot of occludin. **b.** Box plot of ZO-1. **c.** Box plot of VE-cadherin. **d.** Box plot of β-catenin.

in human amnion and chorion plate with chorioamnionitis. We analysed two molecules of AJs, i.e., VE-cadherin and β-catenin, and two molecules of TJs, i.e., ZO-1 and occludin, in this pathology. Concerning the analysis of TJ molecules, the most relevant results are the decrease of protein but not of mRNA expression of occludin and the unaltered, both protein and mRNA ZO-1, expression in samples with chorioamnionitis. These data suggest that occludin, but not ZO-1, expression may be modified by post-transcriptional events.

Since occludin is considered the key molecule of TJ (Simonovic et al., 2000; Raleigh et al., 2011) we hypothesize that it could be destabilized by inflammatory molecules causing a remodelling of the junctional molecules involved in chorioamnionitis. In particular, the alteration of the intermembrane occludin structure could promote barrier deregulation or loss, leaving unaltered the other intracellular molecules such as ZO-1. One possible explanation of the modification of occludin structure can be suggested by previous data demonstrating that the phosphorylation (Raleigh et al., 2011) or de-phosphorylation (Simonovic et al., 2000) of

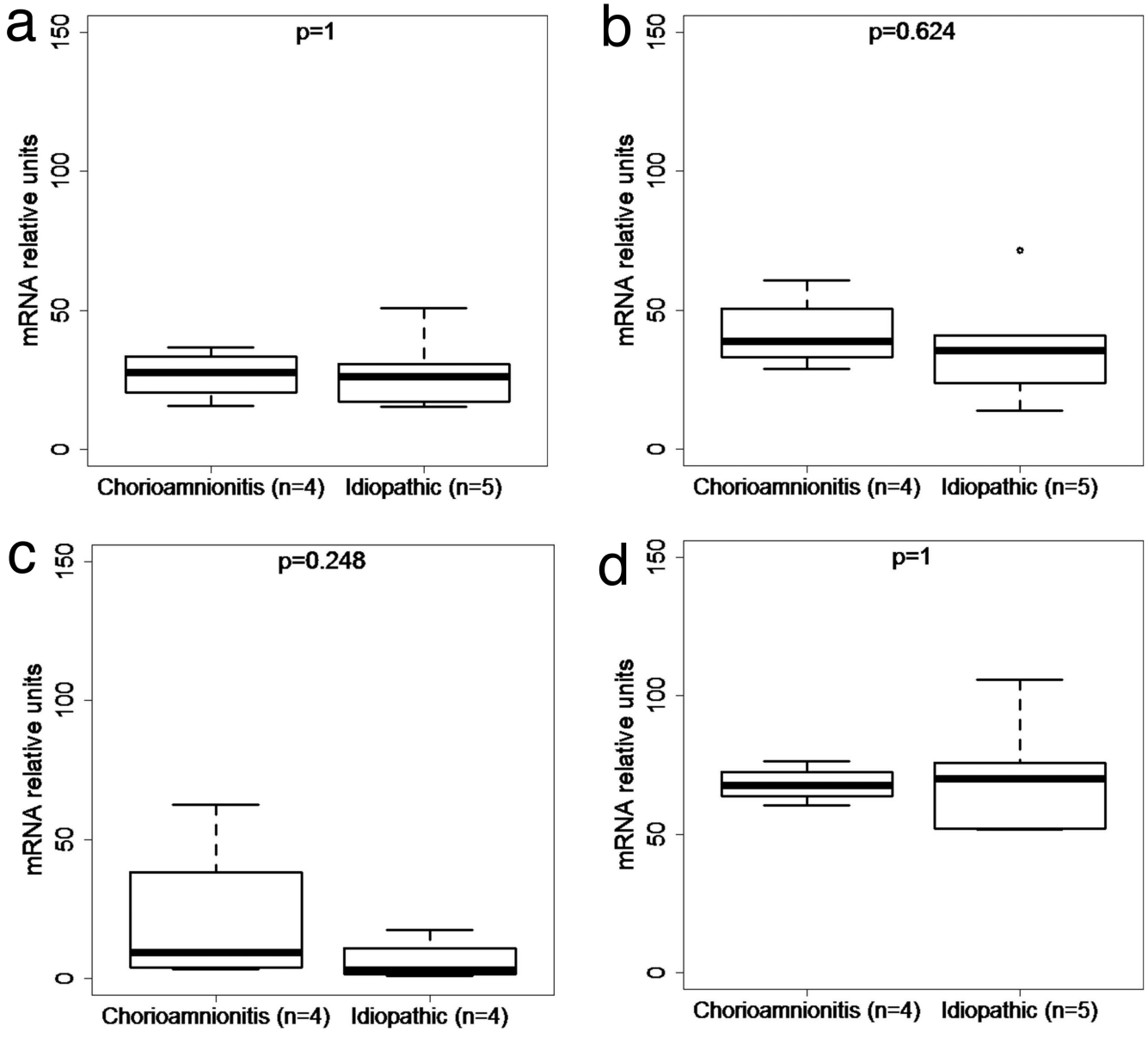

Fig. 4. Box plot from RT PCR results. **a.** Box plot of occludin mRNA. **b.** Box plot of ZO-1 mRNA. **c.** Box plot of VE-cadherin mRNA. **d.** Box plot of β-catenin mRNA.

occludin protein could regulate barrier function in a tissue specific manner.

Kobayashi et al. (2009, 2010b) have shown in mouse fetal membrane that the increase in permeability of the amniotic membranes is mainly attributable to increase in the paracellular permeability when TJs are disrupted. In addition, using a mouse model, it has been demonstrated that the amniotic membrane barrier weakens under inflammatory conditions (Kobayashi et al., 2009). These data support our hypothesis that the disruption of TJs may be due to inflammatory molecules causing a first change that leads to the rupture of the amniotic membrane (Kobayashi et al., 2010a) and consequently to preterm labour in women affected by chorioamnionits. In addition, our previous data (Tossetta et al., 2014) demonstrated that IL-1β and TGFβ1 have broken TJs in HUVEC cultures showing a decreased expression of occludin, suggesting an effective role of these inflammatory molecules on the destruction of TJs. Thus, although regulation of occludin under inflammatory conditions is not fully understood, it is tempting to speculate that the downregulation of specific inflammatory molecules may be of therapeutic use in chorioamnionitis disease. In addition, future investigations should be addressed to understanding the mechanisms that lead to occludin destruction because it could offer additional prophylactic or therapeutic modalities to assure an intact amniotic membrane until parturition. Moreover, we also demonstrate that there are no differences in VE-cadherin and ß-catenin expressions in samples with and without chorioamnionitis. The lack of statistical significance of proteins and mRNAs expression of the majority of the molecules analysed might be due to the small sample size analysed, but our data were recently justified by Du and co-workers (2015). They have demonstrated that the expression of VE-cadherin, following IL-1β treatment, could be modified, or not, using different kinds of cell cultures (Du et al., 2015). Our results show that the adherent junctions do not undergo modifications in chorioamnionitis suggesting that this kind of cell junctions may not be responsive to inflammatory molecules. We can conclude that the molecules of TJs and AJs might have a different behaviour depending on the kind of tissue in which they are located and in response to different inflammatory molecules.

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