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Solubilní forma scavenger receptoru pro hemoglobin (sCD163) v těhotenstvích komplikovaných předčasným odtokem plodové vody

Soluble form of the scavenger receptor for hemoglobin (sCD163) in pregnancies complicated by preterm prelabor rupture of membranes

Marian Kacerovský, MD

Supervisor: Associate Professor Jindřich Tošner, MD, PhD
Consultant supervisor: Associate Professor Bo Jacobsson, MD, PhD

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DECLARATION

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SOUHRN

Předčasný odtok plodové vody (PPROM) představuje zhruba 30% všech předčasných porodů. Histologická chorioamnionitida, která se vyskytuje u přibližně 50-80% těhotenství s PPROM, je spojena se zhoršenou perinatální mortalitou a morbiditou.

Scavenger receptor pro hemoglobin (CD163) je transmembránový glykoprotein nalézaný téměř výhradně na monocytech a makrofázích. Jeho hlavní funkcí je vychytávání komplexů hemoglobin-haptoglobin. Také však slouží jako povrchový receptor, který dokáže rozpoznat intaktní bakterie. Navíc se podílí na pozdní, tlumivé, fázi akutního i chronického zánětu. Jeho solubilní forma (sCD163) představuje odštěpenou extracelulární část receptoru, která se uvolňuje do tělních tekutin.

Hlavní cíl disertační práce bylo vyšetřit hladiny sCD163 v plodové vodě a pupečníkové krvi u těhotenství PPROM s ohledem na přítomnost HCA a funisitidy.

První specifický cíl byl zjistit hladiny sCD163 v plodové vodě nekomplikovaných těhotenství, resp. v druhém trimestru a v termínu porodu. Vzorky plodové vody byly získané od 31 žen, které podstoupily aminocentézu z genetické indikace, 21 žen v termínu porodu bez děložní aktivity a 11 od žen s běžícím porodem. Hladiny sCD163 v plodové vodě byly stanoveny pomocí enzyme-linked immunosorbent assay (ELISA) metody. Hladiny sCD163 v plodové vodě klesaly v průběhu nekomplikovaných těhotenství.

Druhý specifický cíl bylo vyšetřit hladiny sCD163 v plodové vodě PPROM těhotenství s ohledem na přítomnost histologická chorioamnionitidy a funisitidy. Hladina sCD163 byla stanovena v plodové vodě od 89 žen ELISA metodou. Hladiny sCD163 byly vyšší v přítomnosti HCA a funisitidy. Likelihood ratio (LR) 5.5 pro přítomnost histologické chorioamnionitidy činí z sCD163 v plodové vodě potencionální marker pro predikci této závažné komplikace.

Třetí specifický cíl byl stanovit hladiny sCD163 v pupečníkové krvi PPROM těhotenství s ohledem na přítomnost histologické chorioamnionitidy a funisitidy. Vzorky pupečníkové krve byly odebrané z pupečníku po porodu plodu. Hladina sCD163 byla stanovena ELISA metodou. Vyšší hladiny sCD163 v pupečníkové krvi byly nalezeny v přítomnosti histologické chorioamnionitidy a funisitidy. Nízké LR pro přítomnost histologické chorioamnionitidy (1.8) a funisitidy (2.3) znamenají, že sCD163 v pupečníkové krvi není vhodný ukazatel pro detekci těchto zánětlivých komplikací.

Čtvrtý specifický cíl byl zjistit distribuci CD163 pozitivních buněk v placentě a plodových obalech z těhotenství komplikovaných PPROM s ohledem na histologickou chorioamnionitidu. Imunohistochemické vyšetření bylo provedeno na placentách a plodových obalech od 52 žen. CD163 pozitivní buňky byly nalezeny ve všech částech placenty a plodových obalů. V přítomnosti histologické chorioamnionitidy byl vyšší počet CD163 pozitivních buněk v choriové plotně a subchoriálním fibrinu.

Hlavní závěr disertační práce je, že přítomnost histologické chorioamnionitidy u PPROM těhotenství je spojena s vzestupem hladin sCD163 v plodové vodě a pupečníkové krvi. Stanovení sCD163 v plodové vodě u těhotenství komplikovaných PPROM může být klinicky aplikovatelná a užitečná metoda k prenatální identifikaci histologické chorioamnionitidy.

SUMMARY

Preterm prelabor rupture of membranes (PPROM) is responsible for approximately 30% of all preterm deliveries. Histological chorioamnionitis (HCA) has been found in 50-80% of PPROM cases and is associated with higher rates of adverse maternal and neonatal outcome.

The scavenger receptor for hemoglobin (CD163) is a transmembrane glycoprotein expressed almost exclusively on monocytes and macrophages. Its main function is the binding of hemoglobin–haptoglobin complexes. CD163 also serves as a surface receptor that recognizes intact bacteria and triggers cytokine production function. Moreover, it participates in the late down-regulatory phase of both acute and chronic inflammation. The soluble form of CD163 (sCD163) most likely represents the extracellular domain of CD163, which can be shed from the surface and released into the body fluid.

The main aim of this thesis was to investigate sCD163 in pregnancy complicated by PPROM and relationships with HCA and funisitis.

The first specific aim was to determine amniotic fluid sCD163 levels in uncomplicated pregnancies. Amniotic fluid samples were taken from 31 women who underwent amniocentesis for genetic testing in the second trimester, as well as from 32 women at term, 21 of whom had and 11 of whom did not have uterine contractions. The sCD163 levels in amniotic fluid were determined with sandwich enzyme-linked immunosorbent assay (ELISA) technique. Amniotic fluid sCD163 levels were inversely related to gestational age.

The second specific aim was to evaluate amniotic fluid sCD163 levels in PPROM pregnancies and relationships with HCA and funisitis. Amniotic fluid was retrieved by transabdominal amniocentesis from 89 women and analyzed with ELISA technique. Amniotic fluid levels of sCD163 were higher when in cases with HCA and further increased in cases with funisitis. The observed likelihood ratio (LR) of 5.5 for the prediction of HCA in PPROM suggested that amniotic fluid sCD163 is a valuable clinical marker.

The third specific aim was to evaluate umbilical cord blood levels in PPROM pregnancies, and relationships with HCA and funisitis. A total of 86 women were enrolled in the study. Umbilical cord blood samples were obtained at delivery and sCD163 levels were determined with ELISA technique. Umbilical cord blood sCD163 levels were higher in cases with HCA and funisitis. The LR of 1.8 for the prediction of histological chorioamnionitis and 2.3 for the

prediction of funisitis prevented them from being useful clinical markers for early postpartum detection.

The fourth specific aim was to examine the distribution of CD163-positive ($CD163^+$) cells in the placenta and fetal membranes in PPROM pregnancies with and without HCA. Placenta and fetal membrane samples from 52 women with PPROM were evaluated by immunohistochemistry. $CD163^+$ cells were found in all compartments of the placenta and fetal membranes, regardless of inflammatory status. HCA was associated with a higher amount of $CD163^+$ cells in subchorionic fibrin and the chorionic plate.

The overall conclusion of this thesis is that HCA and funisitis, in PPROM pregnancies is associated with increased sCD163 levels in amniotic fluid and umbilical cord. Measuring sCD163 in amniotic fluid might be a clinically applicable method for prenatal detection of HCA.

ABBREVIATIONS

AUC	Area under curve
CD	Cluster of differentiation
CD163	Scavenger receptor for hemoglobin
CD163$^+$	Positive for scavenger receptor for hemoglobin
CI	Confidence interval
ELISA	Enzyme-linked immunosorbent assay
Hb-Hp	Hemoglobin-haptoglobin complex
HCA	Histological chorioamnionitis
IL	Interleukin
IQR	Interquartile range
LPS	Lipopolysaccharide
LR	Likelihood ratio
MIAC	Microbial invasion in the amniotic cavity
PAMP	Pathogen-associated molecular pattern
PPROM	Preterm prelabor rupture of membranes
PRRs	Pattern recognition receptors
PTD	Preterm delivery
ROC	Receiver operator characteristic
sCD163	Soluble form of hemoglobin scavenger receptor
SD	Standard deviation
SRCR	Scavenger receptor-cysteine rich
TNF	Tumor necrosis factor
sTLR	Soluble form of Toll-like receptor
TLR	Toll-like receptor

1. BACKGROUND

1.1. PRETERM DELIVERY

Preterm delivery (PTD) is defined by the World Health Organization as delivery occurring at less than 37 gestational weeks or before 259 days (1). According to this definition, the lower limit is not specified and varies by location (2). PTD is the leading cause of perinatal mortality and is associated with up to 75% of long-term perinatal morbidity, such as cerebral palsy, developmental delay, retinopathy of prematurity, and other conditions (3, 4). Despite progress in perinatal medicine and knowledge about risk factors and mechanisms related to this pregnancy complication, PTD rates are generally between 5-9% in Europe and other developed countries (4). In the USA, the rate rose to 12.7% in 2005 (5).

1.1.1. Classification of PTD

PTD can be classified in three categories, based on gestational age at delivery, as follows: about 5% of PTD occur at less than 28 weeks (extreme prematurity), about 15% at 28-31 weeks (severe prematurity), about 20% at 32-33 weeks (moderate prematurity) and 60-70% at 34-36 weeks (near-term) (5) .

There are two clinical types of PTD: iatrogenic (medically indicated) PTD and spontaneous PTD, which in turn consists of spontaneous preterm labor and PPROM (2). Iatrogenic PTD is responsible for about 25% of all PTD, with a wide range: 9-36% (2, 6, 7). Spontaneous preterm labor, defined as regular uterine contractions accompanied by cervical ripening, accounts for at least 50% (range 23-64%) of all PTD (6-8), while PPROM occurs in 25% (range 7-51%) (2, 6, 7).

1.1.2. Pathophysiology of PTD

A plethora of studies have proposed etiological heterogeneity of PTD, based on its multifactorial origin (9, 10). There are multiple epidemiological factors (e.g. previous PTD, multiple pregnancy, bleeding during pregnancy, abnormal placentation, race, parity, body mass index), which can activate one or more of the major pathways leading to membrane activation, myometrial contraction and cervical ripening, with subsequent PTD (10, 11). Pathways involved in PTD include activation of the maternal or fetal hypothalamic pituitary axis, inflammation, decidual hemorrhage and pathological distension of the myometrium (10). These pathways are not mutually exclusive; indeed, there are numerous overlaps.

1.2. PPROM

PPROM occurs in 2-4% of all singleton pregnancies and 7-20% of twin pregnancies (12, 13). It is the leading identifiable cause of PTD, responsible for approximately 20-30% of cases (14, 15) and is an important cause of perinatal morbidity and mortality (12, 16-18).

1.2.1. Definition of PPROM

PPROM is usually defined as the rupture of fetal membranes with leakage of amniotic fluid, occurring before onset of labor (14, 15). However, some authors have specified the interval before onset of labor in more detail and the second most common definition of PPROM is the rupture of fetal membranes with release of amniotic fluid more than one hour prior to the onset of labor (19, 20).

1.2.2. Pathophysiology of PPROM

PPROM is multifactorial; one or more pathophysiological pathways are usually involved in this process (14). Choriodecidual infection, collagen degradation, decreased membrane collagen content, localized membrane defects, stretching of membranes and programmed amniotic cell death are considered to be possible mechanisms underlying PPROM (13, 14, 21, 22). A number of maternal, uteroplacental and fetal risk factors for PPROM have been described. PPROM in a prior pregnancy, antepartum vaginal bleeding, chronic corticosteroid therapy, collagen vascular disorders (e.g. systemic lupus erythematosus), PTD in a prior pregnancy, smoking, cocaine use, anemia, low body mass index, nutritional deficiency and low socioeconomic status are the most common maternal factors (21-23). Uterine anomalies, placental abruption, cervical insufficiency, history of cervical conization, uterine overdistension, intraamniotic infection and repeated vaginal examination are some uteroplacental factors (13, 24-27). There is only one, albeit important, fetal risk factor - multiple pregnancy (13, 21).

1.2.3. Diagnosis of PPROM

PPROM is largely a clinical diagnosis. Over 90% of cases are confirmed based on the “gold standard” for the diagnosis of rupture of membranes: watery vaginal discharge, confirmed on sterile speculum examination visualizing fluid passage from the cervix or pooling in the posterior vaginal fornix, and positive Temesvary test (pH indicator changes from yellow to

green on contact with alkaline amniotic fluid) (14, 15, 28). Diminished intrauterine amniotic fluid volume found on ultrasound examination cannot confirm the diagnosis, but may help to suggest it in the appropriate clinical setting (23). Although these clinical approaches are common, they all have limitations in terms of diagnostic accuracy. For example, the Temesvary test is associated with a high false positive rate, related to contamination with vaginal discharge, urine, blood or semen (29, 30). Therefore, more sensitive and specific commercially available tests are preferable, because of the critical importance of the accuracy of a PPROM diagnosis, especially at lower gestational ages. Insulin-like growth factor-binding protein 1 and placental alpha macroglobulin 1 are detected with the Actim PROM™ and Amnisure® ROM tests, respectively (31, 32). The latter has a sensitivity approaching 99% and a specificity of 87-100%. The sensitivity of the Actim PROM™ test varies between 74% and 100% and the specificity varies between 77% and 98.2% (33, 34).

1.2.4. Therapeutic approach to PPROM

The management of PPROM pregnancies was characterized as enigmatic by Thomas Garite in 1985, and little has changed over the last 26 years (35). There are several areas of controversy concerning the management of PPROM pregnancies, but at least two of the most important management strategies are widely accepted by the broader community of obstetricians: the use of antibiotics to prolong latency (the interval between membrane rupture and delivery) and the administration of corticosteroids to diminish the risk of respiratory disease in newborns (36-40).

The ORACLE I study showed that administration of erythromycin in PPROM pregnancies is associated with prolongation of latency, reduction of surfactant treatment, decreased oxygen dependence at 28 days of age and older, as well as fewer cases of bacteremia and a lower rate of abnormal neonatal brain scans (39). Moreover, the administration of antibiotics in PPROM pregnancies has only minor adverse effects on the health of children at age 7 years (41).

In general, it could be said that once the membranes rupture, delivery is recommended when the risk of ascending infection outweighs the risk of prematurity (15). There is agreement among obstetricians when it comes to the initial management of PPROM: confirmation of amniotic fluid leakage, validation of fetal wellbeing and decision concerning the type of delivery (which in turn depends on fetal presentation, gestational age and cervical ripening) (15, 21, 23, 34, 42). A subsequent choice must be made between expectant and active management. There is a little maternal benefit to expectant management, but there can be

significant neonatal benefit, especially in the late second and early third trimesters (19). The benefits of expectant management are mainly related to prolongation of the pregnancy, leading to the reduction of gestational age-dependent morbidity (42). There is general consensus on some issues, regardless of the choice of management: a woman with PPROM should be evaluated for evidence of fetal distress, placental abruption, clinical chorioamnionitis and advanced labor. In cases with signs of fetal distress, clinical chorioamnionitis, advanced labor and/or significant vaginal bleeding, delivery should be immediate, regardless of gestational age (34). Furthermore, women with genital herpes simplex virus infection or human immunodeficiency virus infection should generally not be managed expectantly (15, 34).

In the Czech Republic, imminent PPROM at less than 34 weeks of gestation is treated with corticosteroids for lung maturation induction, tocolytics (48 hours) and antibiotics, whereas antibiotics is the only administered treatment after 34 weeks. Management of PPROM in the Czech Republic is not expectant (except at < 28 weeks). Induction of labor or termination of pregnancy is initiated depending on gestational age (within 24 hours at gestational age > 34 weeks, within 48 hours at 32-34 weeks and within 72 hours at 28-31 weeks), fetal status, maternal serum C-reactive protein concentrations, white blood cell counts and cervicovaginal streptococcus β colonization, but is undertaken no later than 72 hours after rupture of the membranes (43).

1.2.5. Transabdominal amniocentesis in management of PPROM pregnancies

Intraamniotic infection, intraamniotic inflammation and chorioamnionitis have been implicated as major etiologic factors in the pathogenesis of, and subsequent maternal and neonatal morbidity related to, PPROM pregnancies (37, 44-46). Therefore, evaluation of amniotic fluid, which mirrors the current status within the amniotic cavity, can yield at least two valuable results on which to base clinical decisions. The first is information about the presence of bacteria or intraamniotic inflammation. The second benefit of amniotic fluid evaluation before 34 weeks is the opportunity to determine lung maturity by means of the lecithin-to-sphingomyelin ratio and phosphatidylglycerol levels (47).

Amniocentesis in the management of PPROM pregnancies remains controversial. Previous studies have reported success rates ranging from 49% to 98%, with an average of 72% (46). On the other hand, it is important to emphasize that the majority of these studies were performed before 1990 (48). Nowadays, ultrasound-guided transabdominal amniocentesis is a

minimally invasive, fast and safe procedure for both fetus and mother. The current success rate is very high, with a possibility of failure only in cases of total anhydramnios (19, 48).

1.3. MICROBIAL INVASION OF THE AMNIOTIC CAVITY IN PPROM PREGNANCIES

The amniotic cavity is considered sterile under physiological conditions. The isolation of microorganisms in the amniotic fluid is a pathological finding defined as microbial invasion of the amniotic cavity (MIAC) (49). Microorganisms may gain access to the amniotic cavity using of any of these pathways: ascending from the vagina or cervix, hematogenous dissemination through the placenta, retrograde from the peritoneal cavity via the fallopian tubes or accidentally during invasive procedures (amniocentesis, cordocentesis, chorionic villi sampling) (49).

MIAC occurs most frequently in pregnancies complicated by PPROM, in which it occurs in approximately 30-40%. Some studies indicate that this rate may be even higher, depending on gestational age and the bacterial detection techniques utilized (14, 46, 50-53). However, at onset of delivery, MIAC was found in approximately 75% of all PPROM pregnancies, suggesting that bacteria ascend from the vagina to the amniotic cavity during latency (44).

The most common bacteria found in the amniotic fluid in PPROM pregnancies are genital Mycoplasmas (*Ureaplasma urealyticum, Ureaplasma parvum* and *Mycoplasma hominis*) (51, 54). The genital Mycoplasmas are the smallest bacteria without a cell wall and are historically considered to be of low virulence. Their presence in the amniotic cavity can either elicit a strong and robust intraamniotic inflammatory response, with an intensity comparable to that generated by other aerobic and anaerobic bacteria, or no inflammatory response whatsoever (55-57).

The pathophysiology and clinical impact of the intraamniotic inflammatory response to bacteria or their products are as yet incompletely understood. Nevertheless, a strong link exists between the intraamniotic cytokine response and adverse sequelae for the mother and her preterm newborn (58-59).

1.4. CHORIOAMNIONITIS

Chorioamnionitis is defined as an inflammation of the placenta, fetal membranes and umbilical cord in response to MIAC or due to other pathological processes (60). It is most prevalent in pregnancies complicated by PPROM and is associated with increased maternal and neonatal long-term adverse outcome (61-63). The latter include perinatal death, preterm delivery, sepsis, retinopathy of prematurity, chronic lung disease and fetal brain injury (intraventricular hemorrhage, cerebral white matter damage), as well as long-term sequelae leading to cerebral palsy (64-70). Puerperal endometritis, wound infection, pelvic abscess, bacteremia and postpartum hemorrhage are among the maternal complications associated with chorioamnionitis (71-73). Chorioamnionitis is traditionally divided into two main classifications: clinical, based on clinical manifestation of local and systemic inflammation, and HCA, based on microscopic evidence of inflammation (neutrophil infiltration) (74-79).

1.4.1. Clinical chorioamnionitis

The term clinical chorioamnionitis is used for the description of a clinical symptom complex based solely on clinical signs, i.e. maternal temperature exceeding 37.5°C in addition to two of the following: uterine tenderness, abdominal pain, maternal tachycardia > 100 beats/min, fetal tachycardia > 160 beats/min, foul or purulent vaginal discharge or white blood cell count > 15 000 cells/mm^3) (80-83). The diagnosis of clinical chorioamnionitis is not a problem for clinicians. This form of chorioamnionitis, which is related to high perinatal morbidity, represents a late stage of chorioamnionitis and is an indication for prompt delivery. Therefore, the main aim for clinicians must be to reveal the subclinical form before the manifestation of clinical symptoms.

1.4.2. HCA

Microorganisms in amniotic fluid can initiate a cascade of inflammatory processes recruiting neutrophils, which represent the first line of defense, as well as other immuno-active cells from the uterine wall into the fetal membranes and placenta (59-60). Different chemokines and chemoatractant proteins play a role in this process. For example, interleukin (IL)-8 is a strong attractant for neutrophils, macrophage inflammatory protein 1α potently recruits macrophages and monocyte chemotactic protein 1 and granulocyte colony–stimulating factor is an attractant for monocytes and dendritic cells. HCA is characterized by the accumulation of neutrophils, along with the invasion of the fetal membranes and placenta by macrophages,

T cells, and dendritic cells. Intense neutrophil infiltration is sufficient for the diagnosis of HCA (76, 78, 79).

The prevalence of HCA decreases with gestational age (from 50-70% below gestational age 28 weeks to 10-15% at term) (84). HCA is commonly found in pregnancies complicated by PPROM at rates of 50-80%. The rate of HCA in PTD is a dramatically lower (approximately 33%), possibly because HCA is associated with MIAC, which is more frequent in PPROM pregnancies (54, 85). Definitions of HCA are inconsistent among studies. Salafia et al showed that low-grade neutrophil infiltration in both the chorion-decidua and the chorionic plate is a relatively common finding (79). More than one focus with at least 5 neutrophils or at least one focus with 5-20 neutrophils in the chorion-decidua and the chorionic plate have been found in 95-98% and 84%, respectively, in uncomplicated term deliveries (79). Therefore, only intense neutrophil infiltration can be regarded as pathological, and definitive for HCA.

HCA reflects the maternal and fetal acute inflammatory response to either microorganisms or endogenous molecules gaining access to the amniotic cavity. Although more than 70% of HCA cases are associated with MIAC, a variety of non-infectious stimuli (for example, fetal hypoxia, meconium and other nonspecific responses) can be causes of HCA as well (77). The initial inflammatory response is maternal in origin and generally occurs within 6-12 hours of the activation of the first line of defense. Neutrophil infiltration initially occurs in subchorionic fibrin because of maternal blood flow in the intervillous space (84). The next involved site is the chorionic layer of the fetal membranes, due to the migration of neutrophils from decidual venules. The amniotic connective tissue is reached over a 12-36-hour period (84).

The fetal inflammatory response (funisitis) appears as neutrophil invasion in the umbilical vein and chorionic plate vessels, considered being the initial stage of fetal inflammation (84). Involvement of the umbilical artery represents the second stage of the fetal inflammatory response (84, 86) With increasing duration of infection; neutrophils migrate from the vessel walls into Wharton's jelly that surrounds the vessels. The fetal inflammatory response is a significant risk factor for white matter damage and is related to neurological impairment in preterm newborns (87, 88) Neutrophils in the placenta (except the chorionic plate) and the fetal membranes are of maternal origin, whereas those found in the umbilical cord and the amniotic fluid are of fetal origin (89-91).

1.5. PATTERN RECOGNITION RECEPTORS

In general, humans are constantly threatened by the invasion of microorganisms and have developed an immune defense system in order to eliminate this threat (92). The innate immune system represents the immunological first line of defense against microorganisms and distinguishes between self and a variety of pathogens through an evolutionarily conserved system of pattern recognitions (93, 94). Limited numbers of specific receptors, known as pattern recognition receptors (PRRs), recognize and bind to highly conserved sequences on the bacterial surface. These specific bacterial patterns are known as pathogen-associated molecular patterns (PAMPs) (95). The most common PAMPs are lipopolysaccharide (LPS) for Gram-negative bacteria, peptidoglycan for Gram-positive bacteria and lipopeptide/lipoprotein for genital Mycoplasmas. PAMPs are crucial for the survival of the microorganisms; it is therefore difficult for the microorganism to alter them (92). Some PRRs have the ability to recognize endogenous molecules released from damaged tissue (for example, heat shock proteins, high mobility box group protein and fibrinogen), called damage-associated molecular patterns (96). The activation of PPRs leads to the triggering of an inflammatory response (97). Although PPRs are expressed predominantly in innate immune cells (including macrophages and dendritic cells), epithelial, endothelial cells, and fibroblasts can contain them as well. Currently, there are four different known families of PRRs, involving families of transmembrane receptors such as Toll-like receptors (TLRs), C-type lectin receptors for extracellular recognition of microorganisms (bacteria, viruses parasites, protozoa, fungi), cytoplasmatic proteins such as retinoic acid-inducible gene-I-like receptors and nucleotide-binding oligomerization domain-like receptors for cytoplasmic recognition of intracellular bacteria or viruses (98, 99).

1.5.1. Pattern recognition receptors and the placenta and fetal membranes

Microorganisms can gain access to the placenta, fetal membranes and amniotic fluid by one of three major routes, the most common of which is ascension from the vagina and cervix. Microorganisms may also cross the fetal membranes, spread in the amniotic fluid and subsequently proceed to infect the fetus or colonize the chorio-decidual space, infecting the placenta and fetal membranes or maternal decidua (46).

PPRs are expressed not only by immune system cells, but also by epithelial and stromal cells in the placenta and fetal membranes. Thus, the placenta and fetal membranes recognize the presence of microorganisms through their own PPRs. This ability is targeted to identify and

resolve intraamniotic infection. The expression of PRRs, particularly TLRs, has been found in both the placenta and fetal membranes (100, 101) The activation of TLR can trigger the release of inflammatory mediators and chemokines, which attract neutrophils and other immuno-active cells to the placenta and fetal membranes, with subsequent development of HCA.

1.6. SCAVENGER RECEPTOR FOR HEMOGLOBIN

1.6.1. Macrophages

Macrophages play an important role in homeostasis as well as under specific pathological conditions, such as infection, chronic inflammation, atherosclerosis and cancer (102). They are capable of phagocytosis, degradation of both endogenous and exogenous material and establishment of interaction among cells and they present antigen and produce inflammatory mediators (103). These specialized functions of macrophages are reflected in their specific "tools", such as the expression of different Fc, complement and scavenger receptors; adhesion molecules and receptors for soluble mediators (102).

1.6.2. Cluster of differentiation

The cluster of differentiation (CD) nomenclature was introduced at the First International Workshop and Conference on Human Leukocyte Differentiation Antigens in 1982. This system is used for the identification of cell surface molecules presented on white blood cells. The CD system is commonly used as cell markers in immunophenotyping, in order to define cells based on the molecules presented on their surface (104). In 2009, 350 different CDs had been identified (104). Cell populations are usually defined using "+" or "-" to mark whether a specific fraction of cells expresses or lacks a CD molecule.

1.6.3. Scavenger receptor cysteine-rich

The first interaction between pathogen and macrophage is mediated by PRRs. Scavenger receptors belong to the PRR family and can recognize a broad spectrum of ligands (105, 106). A specific subgroup of scavenger receptors, transmembrane glycoprotein receptors, form the so-called scavenger receptor cysteine-rich (SRCR) an ancient and highly conserved protein module, divided into group A and group B (106). The scavenger receptors for hemoglobin

(CD163) and M160 occupy a specific position within group B due to their almost exclusive expression on monocytes and macrophages (107, 108). The other group B members are expressed on other immune cells, on non-hematopoietic cells or on mucosal epithelial cells (109).

1.6.4. Description of CD163

CD163 is 130–kDa glycoprotein containing a single transmembrane element, a short cytoplasmatic tail and large extracellular region of 9 SRCR domains (105, 110). CD163, which is also known as RM3/1, M130 or p155, is a member of the SCRC family group B (111, 112). Between 5% and 30% of all monocytes have been identified as $CD163^+$ (113). $CD163^+$ macrophages have been found in the spleen, liver, thymus, brain, lymph nodes, lung, placenta, peritoneum and blood (105). Moreover, $CD163^+$ macrophages have been identified during the healing phase of inflammation and in chronic inflammation, suggesting that they play a key role in the resolution of inflammation (105).

The expression of CD163 is up-regulated by glucocorticoids and anti-inflammatory mediators such as IL-10 and IL-6 (110, 114). Contrarily, pro-inflammatory mediators such as LPS, interferon-γ, and tumor necrosis factor-α (TNFα) suppress CD163 expression (110). The regulation of CD163 by both pro- and anti-inflammatory mediators demonstrates the relationship between CD163 in immune suppression and the resolution of inflammation. The differential response to pro- and anti-inflammatory mediators suggests that CD163 is expressed most highly on so-called alternatively activated macrophages, which mediate wound repair, angiogenesis, scavenging and tumor progression (115).

1.6.5. Functions of CD163

The specific functions of CD163 have been extensively studied during recent years, albeit remaining mostly elusive.

So far, the best-characterized abilities of CD163 are related to binding of the hemoglobin-haptoglobin complex (Hb-Hp) (110, 116). Intravascular hemolysis generates the oxidative and toxic substance free hemoglobin, which is complexed to haptoglobin for clearance by tissue macrophage (117). CD163 has been recognized as a receptor involved in clearance of these complexes, thereby protecting tissue from free-hemoglobin-mediated oxidative damage (117-119). In addition, this endocytosis of Hb-Hp represents a pathway for the uptake of iron in tissue macrophages, which can play important role for the recycling of iron. Ligation of

CD163 by Hb-Hp elicits a direct production of cytokines, including IL-10 (120). IL-10 further stimulates CD163 production (120).

It has been demonstrated that CD163 as a macrophage surface receptor can recognize intact Gram-positive and Gram-negative bacteria (105). Moreover, $CD163^+$ macrophages are able, after the recognition of bacteria, to trigger cytokine production (105). Therefore, it has been proposed that CD163 serves as a typical innate immune sensor for the detection of bacterial infection. It seems likely that CD163 triggers the initiation of a local inflammatory response, leading to the elimination of infection. These facts suggest that it has a function resembling that of TLRs. Nevertheless, there is at least one difference: TLRs are receptors for soluble, dissociated microbial components (such as LPS, peptidoglycan, lipoprotein/lipopeptide), whereas CD163 recognizes the whole bacterium (105).

CD163 has been also proposed to function in the resolution of inflammation, in the late down-regulatory phase of both acute and chronic inflammation, during which it down-modulates the inflammatory macrophage response process (121, 122).

It has been suggested that CD163 is involved in the adhesion of monocytes to endothelial cells. Nevertheless, the ligand for CD163 on endothelial cells is still unknown (111).

1.7. SOLUBLE FORM OF CD163

The soluble form of CD163 (sCD163), the counterpart to membrane-bound CD163, has been identified in plasma and other body fluids (123, 124) It has been suggested that it is a product of shedding from the cell surface, instead of an alternative splice variant, because the shedding process can be inhibited by proteinases (125, 126).

The shedding of sCD163 from the membrane of either monocytes or macrophages can be induced by TLR activation by LPS, crosslinking of Fcγ receptors, thrombin or oxidative stress (127-130) TNFα-converting enzyme has been identified as an enzyme responsible for cleaving CD163 (130). Recently, neutrophil elastase has also been shown to cleave membrane CD163 and tissue inhibitor of metalloproteinase 3 has been found to be an inhibitor of shedding (126, 131). Generally, an inverse relationship between plasma levels of sCD163 and membrane-bound CD163 levels has been found (132, 133).

The shedding of sCD163 mediated by TLRs occurs within minutes, remaining elevated for 1-2 days (125-127, 134). Nevertheless, several other factors may be responsible for regulation of sCD163 levels. It has been shown that glucocorticoids increase expression of CD163 on the monocyte surface and lead to increased plasma sCD163 levels only after inflammatory stimulus, meaning that glucocorticoids alone do not affect sCD163 levels in plasma (129, 135). Interestingly, small amounts of sCD163 have been found in the systemic circulation in the absence of inflammatory stimulation (136).

1.7.1. Biological functions of sCD163

Although activation of TLR is associated with a rapid release of sCD163 into plasma, the specific biological functions of sCD163 have not yet been determined.

Like its membrane-bound counterpart, sCD163 can bind to Hb-Hp, but with lower affinity (120, 129). In addition, sCD163 can mediate innate immune defense by sequestering hemoglobin-bound iron (129, 137). It has been proposed that sCD163 can inhibit the proliferation and activation of lymphocytes, via an as yet unknown receptor (129, 133). The function of sCD163 in bacterial recognition remains to be established (105).

1.7.2. sCD163 as a clinical biomarker

sCD163 has been proposed as a biomarker, associated with several clinical conditions such as chronic inflammatory conditions, cancer, and other critical illnesses (sepsis, liver failure, disseminated intravascular coagulopathy) (129).

High levels of sCD163 in plasma have also been found in patients with Gaucher's disease (an inherited lysosome storage disease resulting from a deficiency in lysosomal glucocerebrosidase activity) and the hemophagocytic syndrome (hyperactivation of macrophages leads to the secretion of large amounts of proinflammatory cytokines) (138, 139).

Macrophages are an important source of proinflammatory cytokines in rheumatoid arthritis. This is supported by observations that patients with this disease have elevated plasma sCD163 levels (140), a finding described in patients with asthma and scleroderma as well (141, 142).

High levels of sCD163 have also been described in patients with acute myelomonocytic leukemia (122). On the other hand, low sCD163 levels were found in patients with acute myeloid leukemia treated with CD33–targeted chemotherapy (143).

Plasma levels of sCD163 were identified as a better prognostic factor than C-reactive protein in patients with bacteremia in one study (144), while another found that sCD163 levels are associated with a poor prognosis in patients with severe liver disease. A recent study revealed a close association between sCD163 levels and disseminated intravascular coagulopathy (145).

Due to the pivotal role of CD163 in the clearance of hemoglobin during intravascular hemolysis, it is not surprising that increased levels of sCD163 have been found in patients with malaria (146).

1.7.3. sCD163 and pregnancy

In pregnancy, sCD163 levels become slightly elevated compared with non-pregnant women (147-149). Plasma sCD163 levels have previously been determined in asymptomatic pregnant women during the first trimester and in women with symptoms of preterm labor (148, 150). sCD163 has also been reported in the serum of asymptomatic pregnant women with a history of late spontaneous miscarriage or early spontaneous preterm birth (149).

Amniotic fluid levels of sCD163 during uncomplicated pregnancy have not yet been determined. Similarly, there is a lack of knowledge about levels in amniotic fluid and umbilical cord blood in PPROM pregnancies, and relationships with HCA. Moreover, there is no information about its membrane-bound counterpart in the placenta and fetal membranes in these different pregnancy conditions.

2. OBJECTIVE OF THE THESIS

The main aim of this thesis was to investigate sCD163 in women with pregnancies complicated by PPROM and relationships with HCA, with and without funisitis.

Specific aims:

1. To determine sCD163 levels in amniotic fluid during uncomplicated pregnancies. Amniotic fluid samples from the second trimester and late third trimester were evaluated.

2. To evaluate amniotic fluid levels of sCD163 in women and relationships with HCA and funisitis, in pregnancies complicated by PPROM between gestational ages 24+0 and 36+6 weeks. To investigate amniotic fluid sCD163 as a potential marker for prediction of HCA and funisitis.

3. To investigate umbilical cord blood levels of sCD163 and relationships with HCA and funisitis, in pregnancies complicated by PPROM between gestational ages 24+0 and 36+6 weeks. To determine umbilical cord blood sCD163 as a potential marker for early postpartum detection of HCA and funisitis.

4. To examine the distribution of $CD163^{+}$ cells in the placenta and fetal membranes from PPROM pregnancies between gestational ages 24+0 and 36+6 weeks, with and without HCA.

3. SET OF PATIENTS, STATISTICAL ANALYSIS

3.1. PATIENTS

3.1.1. Specific aim 1

A cross-sectional study was conducted. All 63 participants were recruited at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic, between June 2008 and December 2009. The women were divided into the following groups: 1) women in the second trimester of pregnancy (16-19 weeks of gestation) (n=31) who underwent amniocentesis to test for genetic abnormalities with negative results and delivered a healthy neonate at term and 2) healthy pregnant women at term (n=32), with (n=21) and without (n=11) regular uterine activity and cervical ripening. Only women who fulfilled the following criteria were enrolled: singleton pregnancy, maternal age ≥ 18 years and certain gestational age, determined by last menstrual period and confirmed by first-trimester fetal biometry. Cases with multiple pregnancy, treatment for preterm labor or suspected intraamniotic infection but delivered at term, preeclampsia, placenta previa, diabetes mellitus, chronic hypertension, surgical or medical complications, small for gestational age (fetal weight estimated by ultrasound below the 10^{th} percentile for the respective gestational age), structural malformations, chromosomal abnormalities or signs of fetal hypoxia on admission were excluded.

3.1.2. Specific aim 2

A prospective study was conducted and 89 women with PPROM between 24+0 and 36+6 weeks were recruited at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic, between June 2008 and December 2009. Only women who fulfilled the following criteria were enrolled: singleton pregnancy, maternal age ≥ 18 years, and certain gestational age, determined by last menstrual period and confirmed by first-trimester fetal biometry. Cases with multiple pregnancy, preeclampsia, placenta previa, diabetes mellitus, chronic hypertension, surgical or medical complications, small for gestational age (fetal weight estimated by ultrasound below the 10^{th} percentile for the respective gestational age), structural malformations, chromosomal abnormalities or signs of fetal hypoxia on admission were excluded.

PPROM was diagnosed by sterile speculum examination confirming the pooling of amniotic fluid in the vagina and a positive vaginal fluid test for insulin-like growth factor–binding protein (ACTIM PROM test; Medix Biochemica, Kauniainen, Finland), and was managed according to standard department protocols.

3.1.3. Specific aim 3

A prospective cohort study was performed. The study population consisted of 83 women with singleton pregnancies at gestational ages between 24+0 and 36+6 weeks, admitted for PPROM to the Department of Obstetrics and Gynecology in Hradec Kralove between June 2008 and October 2009. Seventy-one of these women were included in the cohort for specific aim 2. Eligibility was defined as follows: maternal age ≥ 18 years, no congenital fetal abnormalities, certain gestational age and an ultrasound-estimated weight between the 10^{th} and 90^{th} percentiles for the respective gestational age. Pregnancies complicated by intrauterine growth restriction, preeclampsia, significant vaginal bleeding, diabetes mellitus, medical or surgical complications or a non-reassuring fetal monitor trace on admission were excluded. All women enrolled in this study were Caucasians. Gestational age was established by last menstrual period and confirmed by first trimester fetal biometry. PPROM was diagnosed by sterile speculum examination confirming the pooling of amniotic fluid in the vagina, together with a positive test for insulin-like growth factor–binding protein (ACTIM PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

3.1.4. Specific aim 4

A retrospective study was performed. A total of 62 women with PPROM between the gestational ages of 24+0 and 36+6 weeks were enrolled in the study. All women were recruited at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic, between September 2008 and June 2009. All 62 and 53 of these women were included in the cohorts for specific aims 2 and 3, respectively. Only women who fulfilled the following criteria were enrolled: singleton pregnancy, maternal age > 18 years, and certain gestational age determined by last menstrual period and confirmed by first trimester fetal biometry. Women with multiple pregnancy, preeclampsia, placenta previa, diabetes mellitus, chronic hypertension, surgical or medical complications, small for gestational age (fetal weight estimated by ultrasound below the 10^{th} percentile for the respective gestational age), structural malformations, chromosomal abnormalities or signs of

fetal hypoxia on admission were excluded.. PPROM was diagnosed by sterile speculum examination confirming the pooling of amniotic fluid in the vagina, together with a positive test for insulin-like growth factor–binding protein (ACTIM PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

3.2. METHODS

3.2.1. Sample collection

3.2.1.1. Amniotic fluid

Amniotic fluid samples from women in the second trimester of pregnancy were collected by transabdominal amniocentesis under ultrasound guidance. Amniotic fluid samples from women at term without uterine activity or cervical ripening were collected just before cesarean section. Amniotic fluid samples from women at term with uterine activity and cervical ripening were collected strictly from the forebag by needle puncture of intact membranes without contamination with cervicovaginal fluid or blood, prior to artificial rupture of membranes. All samples were centrifuged for 15 minutes at 2000 *g* immediately after collection in order to remove debris and cells, divided into aliquots and stored at -70°C until analysis.

Amniotic fluid samples, approximately 5 mL, from women with PPROM were collected by ultrasound-guided transabdominal amniocentesis before administration of corticosteroids, antibiotics or tocolytics. The samples were immediately divided into three polypropylene tubes. The first and second tubes, containing uncentrifuged samples, were immediately transported to the microbiology laboratory for PCR testing for genital Mycoplasmas and for aerobic and anaerobic cultivation, respectively. The third tube was centrifuged for 15 minutes at 2000 *g* to remove cells and debris, divided into aliquots and stored at -70°C until analysis.

3.2.1.2. Umbilical cord blood

Samples of mixed (arterial and venous) umbilical cord blood were obtained from clamped umbilical cords after delivery of the neonates and prior to delivery of the placenta. Samples were collected and centrifuged. The supernatants were stored in polypropylene tubes at -70°C until analysis.

3.2.1.3. Placenta and fetal membranes

At delivery, the placenta, fetal membranes and umbilical cord were collected and fixed in 10% neutral buffered formalin. At least two placenta, usually one umbilical cord and at least two placental membrane tissue samples were processed and embedded in paraffin. Sections of these tissue blocks were stained with hematoxylin and eosin.

3.2.2. Microbial analyses

The first tube, containing an uncentrifuged amniotic fluid sample, was used for the detection of genital Mycoplasmas. DNA was isolated from the amniotic fluid with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instruction (protocol for isolation of bacterial DNA from biological fluids). Real-time PCR was performed on the Rotor-Gene 6000 instrument (QIAGEN, Hilden, Germany) with the commercial kit AmpliSens® C. trachomatis/Ureaplasma/M. hominis-FRT (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect DNA from *Chlamydia trachomatis*, *Ureaplasma parvum/urealyticum,* and *Mycoplasma hominis* in an ordinary PCR tube. Negative and positive controls were included in each amplification experiment. A control included PCR for beta-actin, a housekeeping gene, to test for inhibitors of the PCR reaction.

The second tube was used for aerobic and anaerobic cultivation using Columbia agar with sheep blood, *Gardnerella vaginalis*-selective medium, MacConkey agar, *Neisseria*-selective medium (modified Thayer-Martin medium), Sabouraud agar and Schaedler anaerobe agar.

MIAC was defined as a positive PCR for the genital Mycoplasmas (*Ureaplasma* spp*., Mycoplasma hominis*) and/or *Chlamydia trachomatis* and/or growth of any bacteria in the amniotic fluid except coagulase-negative *Staphylococcus epidermidis*, which was considered to be a skin contaminant (52).

3.2.3. Amniotic fluid analysis

The concentration of sCD163 in amniotic fluid was determined by sandwich ELISA technique with MACRO163™ commercial kits (Trillium Diagnostics, LLC. USA), according to the manufacturer's instructions. The detection limit of this kit is 0.23 ng/mL. The amniotic fluid samples were diluted 1:250. Absorbance values were read at 450 nm by a Multiskan® RC ELISA reader (Thermo Fisher Scientific, USA).

3.2.4. Umbilical cord blood analyses

The concentration of sCD163 in umbilical cord blood was determined by sandwich ELISA technique with MACRO163^{TM} commercial kits (Trillium Diagnostics, LLC, USA). The detection limit of this kit is 0.23 ng/mL. The fetal serum samples were diluted 1:250. The measurement range was 0.275-17.6 ng/mL. Absorbance values were read at 450 nm in an automatic Multiskan® RC ELISA reader (Thermo Fischer Scientific, USA).

3.2.5. Immunohistochemistry and quantification of CD163^{+} cells and neutrophils

Immunohistochemistry was performed on 5-μm, formalin-fixed paraffin sections of the placenta and fetal membranes, using an Ventana BenchMark ULTRA Advanced Staining System (Ventana Medical Systems, Inc., USA) and the ultraView Universal DAB Detection Kit. Analysis was undertaken after heat-induced epitope retrieval at 95°C in the cell-conditioning medium CC1 for 36 minutes, followed by 32 minutes of incubation with primary antibody at 37°C. Mouse monoclonal CD163 antibody, clone 10D6 (Leica Microsystems, UK), dilution 1:100, was used as the primary antibody.

The numbers of CD163^{+} cells and neutrophils were both counted by a single pathologist (HH) in the same fields in slides immunohistochemically stained for CD163. Three different high power (400x) fields were chosen in the following locations of the fetal membranes and the placenta: the amnion, chorion, and decidua (including trophoblastic structures below the mesenchymal part of the chorion) for the membranes; and the amnion, chorionic plate, subchorionic fibrin, stem villi and terminal villi (or the most mature villi in placentas from the lower gestational ages), and decidua for the placenta.

3.3. DIAGNOSIS OF HCA

The placenta, fetal membranes and umbilical cord were examined histologically in all PPROM cases. The degree of polymorphonuclear leukocyte infiltration was assessed separately in the free membranes (amnion and chorion-decidua), chorionic plate and umbilical cord, according to the criteria presented by Salafia et al (Table 1) (79). The diagnosis of HCA was determined based on histological grades 3-4 in chorion-decidua and/or 3-4 in the chorionic plate and/or 1-4 in umbilical cord and/or 1-4 in amnion (See Figure 1). Funisitis was diagnosed based on histological grades 1-4 in umbilical cord (see Figure 2) (79).

Histopathological examination was performed by a single perinatal pathologist who was blinded to clinical status.

3.4. ETHICAL CONSIDERATIONS

Amniocentesis was routinely offered for microbial assessment to all women admitted with PPROM. The results of the PCR for genital Mycoplasmas and the aerobic and anaerobic cultivation were used in the clinical management of PPROM. This study was approved by the Institutional Review Board Committee (March 19, 2008; No. 200804 SO1P), and informed consent was obtained from all participants.

3.5. STATISTICAL ANALYSES

3.5.1. Specific aim 1

The demographic characteristics were compared by either ANOVA or the Kruskal-Wallis test for continuous variables and are presented as mean ± standard deviation (SD) and median (range), respectively. Categorical variables were compared using Fisher´s exact test or the chi-square test and are presented as n (%). sCD163 concentrations were compared with nonparametric tests (Mann-Whitney test), given the non-normal distribution of the analyzed substance, and presented as a median [interquartile range (IQR)]. The normality of the data was tested using the D'Agostino-Pearson omnibus normality test and the Shapiro-Wilk test. Differences were considered statistically significant at $p < 0.05$. All p-values were obtained from two-sided tests, and all statistical analyses were performed using GraphPad Prism 5.03 for Mac OS X (GraphPad Software, USA).

3.5.2. Specific aim 2

The demographic and clinical characteristics were compared using either unpaired *t*-tests for continuous variables, and presented as mean ± SD, or the nonparametric Mann-Whitney *U* test, and presented as median (range). Categorical variables were compared using Fisher's exact test or the chi-square test and are presented as n (%). The normality of the data was tested using the D'Agostino-Pearson omnibus normality test and the Shapiro-Wilk test. Because amniotic fluid sCD163 concentrations were not normally distributed, a

nonparametric test (Mann-Whitney *U* test) was used for analysis and variables are presented as median (IQR). Mixed linear models were applied to identify major determinants of sCD163 levels in HCA cases with funisitis, both without and with adjustment for gestational age at sampling. Estimated marginal means of the fitted models were calculated. Differences were considered statistically significant at $p < 0.05$. A receiver-operator characteristic curve (ROC) was constructed to describe the relationship between the sensitivity and the false-positive rate for different amniotic fluid sCD163 levels in the identification of HCA. All *p*-values were obtained from two-sided tests and all statistical analyses were performed using GraphPad Prism 5.03 for Mac OS X (GraphPad Software, USA) and with the SPSS 19.0 for Mac OS X (SPSS Inc., USA).

3.5.3. Specific aim 3

Demographic and clinical characteristics were compared using either unpaired *t*-tests for continuous variables, and presented as mean ± SD, or the nonparametric Mann-Whitney *U* test, and presented as median (range). Categorical variables were compared using Fisher's exact test or the chi-square test and are presented as n (%). The normality of the data was tested using the D'Agostino-Pearson omnibus normality test and the Shapiro-Wilk test. Because umbilical cord blood sCD163 concentrations were not normally distributed, a nonparametric test (Mann-Whitney *U* test) was used for analysis and variables are presented as median (IQR). Mixed linear models to identify major determinants of sCD163 levels in HCA cases with funisitis were performed both without and with adjustment for gestational age at sampling. Estimated marginal means of the fitted models were calculated. Differences were considered statistically significant at $p < 0.05$. ROC curves were constructed to describe the relationship between the sensitivity and the false-positive rate for different umbilical cord blood sCD163 levels in the identification of HCA and funisitis. All *p*-values were obtained from two-sided tests, and all statistical analyses were performed using GraphPad Prism 5.03 for Mac OS X (GraphPad Software, USA) and with the SPSS 19.0 for Mac OS X (SPSS Inc., USA).

3.5.4. Specific aim 4

The demographic and clinical characteristics were compared using either unpaired *t*-tests for continuous variables, and presented as mean ± SD, or the nonparametric Mann-Whitney *U* test, and presented as median (range). Categorical variables were compared using Fisher's

exact test and presented as n (%). The normality of the data was tested using the D'Agostino-Pearson omnibus normality test and the Shapiro-Wilk test. A nonparametric test (Mann-Whitney U test) was used for analysis of the number of $CD163^+$ cells and presented as median (range). Differences were considered statistically significant at $p < 0.05$. All *p*-values were obtained from two-sided tests, and all statistical analyses were performed using either GraphPad Prism 5.03 for Mac OS X (GraphPad Software, USA) or the SPSS 19.0 statistical package for Mac OS X (SPSS Inc., USA).

4. RESULTS

4.1. SPECIFIC AIM 1

4.1.1. Demographic and clinical characteristics of the study population

Table 2 displays the demographic and clinical characteristics of the participants in the second trimester and at term, the latter with and without uterine contractions. The only difference among the subgroups was gestational age at sampling, for obvious reasons. All women were self-reported as Caucasian.

4.1.2. Amniotic fluid sCD163 levels during healthy pregnancy

Measurable levels of sCD163 were detected in all amniotic fluid samples. Women in the second trimester had significantly higher median amniotic fluid sCD163 levels than women at term without uterine activity (307.8 ng/mL, IQR 200.9-460.8 vs. 216.7 ng/mL, IQR 202.9-227.4; $p = 0.04$; Figure 3). No significant difference was found between the median amniotic fluid sCD163 levels in the second trimester and those at term in cases with uterine contractions (307.8 ng/mL, IQR 200.9-460.8 vs. 255.7 ng/mL, IQR 170.2-373.2; $p = 0.29$; Figure 3) or between those at term with and without contractions (255.7 ng/mL, IQR 170.2-373.2 vs. 216.7 ng/ml, IQR 202.9-227.4; $p = 0.19$; Figure 3).

4.2. SPECIFIC AIM 2

4.2.1. Demographic and clinical characteristics of the study population

Demographic and clinical characteristics of the women and neonates, related to HCA and funisitis, are presented in Tables 3 and 4, respectively. HCA was associated with higher rates of MIAC and genital Mycoplasmas in the amniotic fluid and more Apgar scores < 7 at 5 minutes, compared with cases without HCA. Furthermore, HCA was associated with lower gestational age at delivery and birth weight. Funisitis was associated with a lower primiparity rate, body mass index, gestational age at both admission and delivery and birth weight. Other aerobic and anaerobic bacteria were detected more often in women with funisitis. All women were self-reported as Caucasian.

4.2.2. Amniotic fluid sCD163 levels in women with HCA

Women with HCA had a higher median amniotic fluid sCD163 concentration than those without HCA (885.1 ng/mL, IQR 295.3-1779.0 vs. 287.8 ng/mL, IQR 170.0-499.4; $p < 0.0001$; Figure 4).

4.2.3. Amniotic fluid sCD163 levels in women with funisitis

Funisitis was associated with a higher median amniotic fluid sCD163 level, compared to cases without funisitis (1160.0 ng/mL, IQR 833.7-2524.0 vs. 368.7 ng/mL, IQR 213.6-850.6; p=0.001; Figure 4) in the crude analysis, but not after adjustment for gestational age at sampling in the model ($p = 0.09$; Table 5). There was no difference between the median amniotic fluid CD163 level in HCA alone and HCA with funisitis (735.0 ng/mL, IQR 275-1693 vs. 1160.0 ng/mL, IQR 833.7-2524.0; $p = 0.06$; Figure 5)

4.2.4. The predictive value of amniotic fluid sCD163 levels for HCA

The cut-off value for amniotic fluid sCD163 levels, determined by a ROC curve, was 684.3 ng/mL for identification of HCA with 61.4% sensitivity (95% confidence interval (CI): 45.5-75.6), 88.9% specificity (95% CI: 75.9-96.3), LR 5.5 and area under curve (AUC) 0.75 ($p < 0.0001$; Figure 6). The predictive value for identification of funisitis was not calculated because of the non-significant difference found after adjustment for gestational age.

4.3. SPECIFIC AIM 3

4.3.1. Demographic and clinical characteristics of the study population

Demographic and clinical characteristics of the women and neonates, related to HCA with and without funisitis, are presented in Tables 6 and 7, respectively. No differences were found between women with and without HCA. Women with funisitis had higher rates of MIAC and other bacteria in cord blood as well as lower gestational age at admission and at sampling. Furthermore, their babies had lower birth weights than women without funisitis. All women were self-reported as Caucasian.

4.3.2. Umbilical cord blood sCD163 levels in women with HCA

HCA was associated with a higher median umbilical cord blood sCD163 level, compared to cases without HCA (median 1466.0 ng/mL, IQR 1187-1828 vs. median 1168 ng/mL, IQR 887.0 -1595.0; $p = 0.01$; Figure 7).

4.3.3. Umbilical cord blood sCD163 levels in women with funisitis

A higher median umbilical cord blood sCD163 level was found in women with than without funisitis (median 1741.0 ng/mL, IQR 1301.0-3251.0 vs. median 1248.0, IQR 984.5-1626.0; $p = 0.02$; Figure 7) in the crude analysis, as well as after the adjustment for gestational age at sampling ($p = 0.009$; Table 8). No difference was found between HCA alone and HCA with funisitis (median 1379.0, IQR 1173.0-1672.0 vs. median 1741.0 ng/mL, IQR 1301.0-3251.0; $p = 0.13$; Figure 8).

4.3.4. The predictive value of umbilical cord blood sCD163 levels for HCA

The cut-off value for umbilical cord blood sCD163 to identify HCA, determined by ROC curve, was 1343 ng/mL, with 63.2% sensitivity (95% CI: 46.0-78.2), 64.4% specificity (95% CI: 48.8-78.5), LR 1.8 and AUC 0.65 ($p = 0.01$; Figure 9).

4.3.5. The predictive value of umbilical cord blood sCD163 levels for funisitis

The cut-off value for amniotic fluid sCD163 to identify funisitis, determined by ROC curve, was 1467.0 ng/mL, with 77.8% sensitivity (95% CI: 40.0-97.0), 66.2% specificity (95% CI: 54.3-76.8), LR 2.3 and AUC 0.73 ($p = 0.03$; Figure 10).

4.4. SPECIFIC AIM 4

4.4.1. Demographic and clinical characteristics of the study population

A total of 62 women with PPROM were included in the study. Placental and fetal membrane samples were obtained from 52 women (83%). The overall HCA rate was 52% (27/52). Demographic and clinical characteristics of the women and newborns, related to HCA, are presented in Table 9. No differences between women with and without HCA were found. All women were self-reported as Caucasian.

4.4.2. The distribution of sCD163$^+$ cells in the fetal membranes and the placenta

CD163$^+$ cells were identified in all compartments of the placenta and fetal membranes. The lowest number of CD163$^+$ cells was found in subchorionic fibrin and both placental and fetal membranes amnion (see Figures 11C, 12D, and 12F). Contrarily, the highest number of CD163$^+$ cells was found in chorionic fibrin as well as placental and fetal decidua (see Figures 11A, 12A, and 12E).

A significant correlation between the intensity of CD163$^+$ and neutrophil accumulation in the chorionic plate (rho 0.47, $p = 0.001$) and in subchorionic fibrin (rho 0.78, $p < 0.0001$) was observed. No correlations were found in the other compartments.

4.4.3. The distribution of sCD163$^+$ cells in the fetal membranes and the placenta, related to HCA

Higher numbers of CD163$^+$ cells were found in women with HCA, compared to those without HCA, both in the chorionic plate (median 93.3, range 33.0-253.3 vs. median 80.0, range 12.3-133.3; $p = 0.049$) and in subchorionic fibrin (median 10.3, range 1.0-136.7 vs. median 3.0, range 0-71.1; $p = 0.007$) (Figure 13).

5. DISCUSSION

5.1. SPECIFIC AIM 1

sCD163 is known as a marker of alternatively activated macrophages and is shed from the macrophage surface, in response to induction by inflammatory stimuli. CD163 is predominantly expressed on alternatively activated macrophages, a phenotype associated with inhibition of the inflammatory response, and promotes a Th2-type immune response (151). A balanced immune response shift from Th1 to Th2 is important for an uncomplicated pregnancy. Furthermore, healthy first-trimester decidua contains macrophages with an alternatively activated phenotype, and these alternatively activated $CD163^+$ macrophages may be involved in the immunosuppressive biological barrier between mother and fetus (148, 152, 153).

We are aware that we only evaluated amniotic fluid from early and late in uncomplicated pregnancies, thus missing evaluation of amniotic fluid from gestational ages 19+5 to 36+6 weeks. Unfortunately, there was no ethically feasible way to obtain samples from healthy pregnancies in this gestational age interval.

Our results show that sCD163 is a physiological constituent of amniotic fluid during healthy pregnancy. This could be explained by the presence of alternatively activated macrophages in the placenta, the fetal membranes and decidua. Moreover, spontaneous production of IL-10, which induces the increase of sCD163 expression, has been proven in term decidua macrophages (113, 114, 121, 154). The cause of decreasing amniotic fluid sCD163 levels as gestation advances remains unclear. On the other hand, macrophages have displayed the capability to switch from one activation state to another with reversibility of CD163 production, depending on various pro- or anti-inflammatory stimuli (155). This explains the universality of macrophage activation-enhancing tissue and the ability of macrophages to resolve inflammation rapidly without recruitment of new macrophages (155).

5.2. SPECIFIC AIM 2

Our results show that amniotic fluid sCD163 levels are higher in cases with HCA and further enhanced in cases with funisitis. This suggests that stimuli eliciting both a maternal (chorioamnionitis) response and a fetal (funisitis) inflammatory response result in a higher

production of amniotic fluid sCD163. The presence of sCD163 is not typical in the early phase of inflammation because the proinflammatory cytokines present during this phase of inflammation significantly suppress CD163 production. Furthermore, down-regulation of CD163 by bacterial antigens such as LPS may partly affect the number of $CD163^+$ macrophages during this phase of the inflammation process (110). On the other hand, expression of CD163 occurs in late inflammation and the surface occurrence of CD163 is associated with the release of anti-inflammatory products from macrophages (110, 156, 157). Moreover, the expression of IL-6 and IL-10 (induced also by LPS and TNFα) participates in the up-regulation of CD163 in late inflammation (110, 158, 159) Amniotic fluid sCD163 levels in pregnancies with funisitis, the most severe form of HCA, tended to be higher than in those with less severe forms (HCA alone) but the difference did not reach statistical significance (p=0.06). Nevertheless, this concurs with the hypothesis that sCD163 participates primarily in the late inflammation phase. As we expected, women with funisitis had higher amniotic fluid sCD163 levels and lower gestational ages at sampling than those without funisitis. Nevertheless, there was a difference between gestational ages in the groups at sampling. In the model adjusted for gestational age, we did not find this difference between women with and without funisitis. Therefore, we did not calculate the predictive value of sCD163 for funisitis. Gestational age is an important confounding factor for the evaluation of amniotic fluid markers because there is no knowledge about normal amniotic fluid levels throughout advancing uncomplicated pregnancy. Therefore, gestational age should be taken into account when interpreting these analyses (160).

An amniotic fluid sCD163 level of 684.3 ng/mL was found to be best for the prediction of HCA in PPROM pregnancies; the LR of 5.5 suggests that it may be a valuable clinical marker. Amniotic fluid sCD163 is a stronger clinical predictor of HCA than markers tested in our previous studies, i.e. pentraxin 3 and IL-8, with cut-off level LRs of 2.3 and 2.5, respectively, for prediction of HCA (161, 162).

5.3. SPECIFIC AIM 3

Our results demonstrated that sCD163 levels were significantly increased in umbilical cord blood from PPROM pregnancies, in cases with HCA and funisitis. This suggests that stimuli

inducing both maternal and fetal inflammatory response also cause elevated production of umbilical cord blood sCD163.

We hypothesized that the most severe form of intrauterine inflammation (funisitis) would be associated with a higher umbilical cord blood sCD163 level than the less severe form (HCA alone), because sCD163 participates primarily in the late phase of inflammation. However, the expected difference between these groups was not found. A possible explanation for this finding is the power problem, because the group with funisitis was very small (n = 9).

We assumed that the sCD163 in umbilical cord blood was of fetal origin but could not completely rule out a partial contribution of maternal plasma sCD163 crossing the placenta. Nevertheless, previously described maternal plasma sCD163 levels in conditions involving either spontaneous preterm labor or PPROM were approximately 2-3-fold higher than our umbilical cord blood sCD163 levels (148, 149, 163). Based on these results, we could exclude passive diffusion across the placenta (substances crossing the placenta by passive diffusion reach equilibrium between the maternal and fetal compartments) (164). On the other hand, we are aware that there is very little information about active transport of sCD163 across the placenta by the transporter systems, and this possibility could thus not be ruled out.

The next issue is the potential influence of antenatal administration of corticosteroids. It is known that administration of glucocorticoids increases CD163 expression on the monocyte surface, which might lead to the elevation of plasma sCD163 levels. However, sampling at the time of delivery did not allow researchers to obtain umbilical cord blood samples from women that had not been treated prenatally with corticosteroids when the gestational age was less than 34 weeks. Nevertheless, the potential influence of this factor was minimized by the fact that corticosteroid administration rates are notably similar among the subgroups presented here (with HCA 14/39 vs. without HCA 21/45; $p = 0.38$; and with funisitis 7/9 vs. without funisitis 32/74; $p = 0.08$).

The collection of umbilical cord blood at delivery is an effortless, non-invasive procedure that is not associated with an adverse effect on the neonates. It is well known that placental histopathology results are not available immediately after delivery; evaluation of umbilical cord blood sCD163 in pregnancies complicated by PPROM may thus be useful for the early detection of HCA and funisitis in clinical practice. The umbilical cord blood sCD163 cut-off level of 1343 ng/mL was found to yield the best prediction of HCA, but the LR (1.8) prevented its usefulness in clinical practice. Our results concur with our previous study,

presenting LRs for early postpartum HCA detection with umbilical cord blood IL-6 (LR 1.8) and IL-8 (LR 1.3) levels (165). The umbilical cord blood sCD163 cut-off level of 1467 ng/mL was identified as optimal for prediction of funisitis, with a LR of 2.3. While this LR was better than the corresponding LR for HCA, we have previously shown that umbilical cord blood IL-6 and IL-8 had LRs 7.3 and 3.5, respectively, for the early postpartum detection of funisitis (165). This means that umbilical cord blood IL-6 and IL-8 levels, especially the former, are better predictors of funisitis than umbilical cord blood sCD163 levels in clinical practice.

5.4. SPECIFIC AIM 4

Tissue macrophages are derived from blood monocytes. Their general function includes phagocytosis and antigen presentation in response to inflammation and infectious agents (166). According to their function, macrophages are often divided into two groups and called either classically or alternatively activated (167). The latter represent a heterogeneous population with a broad spectrum of functions entailing anti-inflammatory and tissue remodeling properties (168). CD163 is expressed almost exclusively on macrophages and monocytes and has been proposed as a marker of alternatively activated macrophages. However, it is notable that CD163 has also been found on cells of intermediate phenotype between myofibroblast and macrophage, because of co-expression of desmin and procollagen (169). Moreover, CD163 may belong to a subpopulation of hematopoietic stem/progenitor cells (170).

The placental and fetal membranes contain numerous macrophages in all their compartments, but they are most highly accumulated in decidua and villi. The macrophages in the placental villi were described more than 125 years ago and are called Hofbauer cells (171). It is believed that Hofbauer cells represent, together with the trophoblast, the second line of defense to prevent pathogens and toxins from reaching the fetus. Decidual macrophages mediate an immunosuppressive environment in the decidua (172). Moreover, both decidual macrophages and Hofbauer cells have been shown to have an alternatively activated phenotype (171).

Our study showed that decidua, chorionic plate, and villi were the compartments with the highest accumulation of $CD163^+$ cells. However, we do not know the exact cellular origin of

$CD163^+$ cells, but we assume that majority of these cells are macrophages with alternatively activated phenotype. This assumption was supported by the distribution of $CD163^+$ cells, with the maximum in the decidua and the placental villi.

Low occurrence of $CD163^+$ cells in both amnions was expected. The migration from decidua into the amnion, an avascular structure, is known to be difficult because of transmigration across the epithelial layer, chorionic basal membrane and the chorioamniotic interface (91).

A unique finding of this study is the observation that higher numbers of $CD163^+$ cells were found only in subchorionic fibrin and the chorionic plate, but not in the fetal membrane and placental villi, in cases with HCA. We also observed a trend toward a higher number of $CD163^+$ cells in placental amnion, but these differences failed to reach statistical significance (p=0.06). Since subchorionic fibrin and the chorionic plate were the sites for which the correlations between $CD163^+$ cells and neutrophils were found, we hypothesize that both these cells have the same source.

Our study revealed that HCA is not associated with an increase in $CD163^+$ cells in the fetal membranes. This finding is novel and completely unexpected in the light of knowledge regarding neutrophil distribution after inflammatory stimulus. It is traditionally accepted that monocytes and macrophages are recruited from decidua to fetal membranes due to potent chemokine activity, if there is HCA (60). On the other hand, $CD163^+$ cells most likely represent alternatively activated macrophages, which play a role in the late, but not the acute, phase of inflammation. Moreover, the heterogeneity of macrophages in fetal membranes might be another possible explanation. The decidual macrophage population is of maternal origin, but recently Kim et al reported that $CD163^+$ macrophages in the amnio-chorion of fetal membranes are of fetal origin, emanating from the chorioamniotic mesodermal layer, suggested as the reservoir for their production (169).

6. CONCLUSION

sCD163 in amniotic fluid has been found to be a physiological constituent during uncomplicated pregnancy. Amniotic fluid sCD163 levels decrease with advancing gestational age.

Amniotic fluid levels of sCD163 in PPROM are elevated in cases with HCA and further elevated in HCA cases with funisitis. The LR 5.5 for the prediction of HCA in PPROM suggests that amniotic fluid sCD163 is a valuable clinical marker.

Umbilical cord blood sCD163 levels in PPROM are higher in cases with HCA and in cases with funisitis. The LR 1.8 for the prediction of HCA and the LR 2.3 for the prediction of funisitis prevent them from being useful clinical markers for early postpartum detection of these complications.

$CD163^+$ cells were found in all compartments of the placenta and fetal membranes, regardless of inflammatory status. HCA is associated with a higher number of $CD163^+$ cells in subchorionic fibrin and the chorionic plate. An association between the number of $CD163^+$ cells and neutrophils in subchorionic fibrin and the chorionic plate was found.

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APPENDICES

Table 1 Grading system for acute chorioamnionitis, based on Salafia et al. (79)

	Grade 1	Grade 2	Grade 3	Grade 4
Chorion-decidua	One focus of at least 5 neutrophils	More than one grade-1 focus or at least one focus of 5-20 neutrophils	Multiple and/or confluent grade-2 foci	Diffuse and dense acute inflammation
Amnion	One focus of at least 5 neutrophils	More than one grade-1 focus or at least one focus of 5-20 neutrophils	Multiple and/or confluent grade-2 foci	Diffuse and dense acute inflammation
Chorionic plate	One focus of at least 5 neutrophils in subchorionic fibrin	Multiple grade-1 foci in subchorionic fibrin	Few neutrophils in connective tissue or subchorionic fibrin	Numerous neutrophils in chorionic plate, and chorionic vasculitis
Umbilical cord	Neutrophils in inner third of umbilical vein wall	Neutrophils in inner third of at least two umbilical vessel walls	Neutrophils in the perivascular Wharton's jelly	Panvasculitis and funisitis extending deep into Wharton's jelly

Table 2 Maternal and neonatal characteristics related to gestational age and obstetric condition (specific aim 1)

	Second trimester (n = 31)	**Term, without contractions (n = 11)**	**Term, with contractions (n = 21)**	***p*-value**
Maternal age (years)	31.9±4.5	29.1±4.9	31.7±5.7	0.17
Primiparous	8 (26%)	6 (55%)	12 (57%)	0.62
Smoking during pregnancy	8 (26%)	2 (18%)	4 (19%)	0.09
Pre-pregnancy body mass index	21.5 (16.3–38.6)	22.0 (18.9– 30.0)	23.3 (19.3–32.6)	0.78
Gestational age at sampling (weeks+days)	17+5 (16+1-19+3)	39+2 (38+4-40+0)	39+4 (37+0-41+0)	**<0.0001**
Gestational age at delivery (weeks+days)	40+1 (38+6-41+5)	39+2 (38+4-40+0)	39+4 (37+0-41+0)	**0.09**
Birth weight (g)	3617±356	3445±592	3504±451	0.51
Apgar score < 7 at 5 minutes	0 (0%)	0 (0%)	0 (0%)	*
Apgar score < 7 at 10 minutes	0 (0%)	0 (0%)	0 (0%)	*
Puerperal endometritis	0 (0%)	0 (0%)	0 (0%)	*

Continuous variables were compared using the parametric ANOVA (presented as mean ± SD) or the nonparametric Kruskal-Wallis test (presented as median [range]). Categorical variables were compared using the chi square test and presented as n (%).

* *p*-value cannot be calculated because two variables are zero.

The statistically significant results are marked in bold type.

Table 3 Maternal and neonatal characteristics, related to histological chorioamnionitis (HCA) (specific aim 2)

	HCA (n = 44)	**No HCA (n = 45)**	***p*-value**
Maternal age (years)	31.0±6.5	30.6±5.2	0.41
Primiparous	21 (48%)	28 (62%)	0.20
Smoking during pregnancy	11 (25%)	9 (20%)	0.62
Pre-pregnancy body mass index	23.2 (17.0-40.6)	21.0 (16.3-38.6)	0.35
Gestational age at sampling (weeks+days)	31+4 (24+0-36+4)	32+3 (24+2-36+4)	0.14
Gestational age at delivery (weeks+days)	31+6 (24+3-36+6)	32+6 (25+1-36+4)	**0.04**
Microbial invasion of the amniotic cavity	20 (45%)	9 (20%)	**0.01**
Genital Mycoplasmas in amniotic fluid	14 (32%)	6 (13%)	**0.04**
Other bacteria in amniotic fluid	8 (18%)	3 (6%)	0.12
Birth weight (grams)	1710±605	1979±580	**0.03**
Apgar score < 7 at 5 minutes	5 (11%)	0 (0%)	**0.03**
Apgar score < 7 at 10 minutes	2 (5%)	0 (0%)	0.24
Puerperal endometritis	2 (5%)	0 (0%)	0.24

Continuous variables were compared using the parametric t-test (presented as mean ± SD) or the nonparametric Mann-Whitney *U* test (presented as median [range]). Categorical variables were compared using Fisher's exact test and presented as n (%).

The statistically significant results are marked in bold type.

Table 4 Maternal and neonatal characteristics, related to funisitis (specific aim 2)

	Funisitis (n = 12)	**No funisitis (n = 77)**	***p*-value**
Maternal age (years)	29.2±6.1	30.5±5.9	0.76
Primiparous	2 (17%)	47(61%)	**0.005**
Smoking during pregnancy	6 (50%)	14 (18%)	0.03
Pre-pregnancy body mass index	19.6 (17.0-28.0)	22.0 (16.3-40.6)	**0.04**
Gestational age at sampling (weeks+days)	27+5 (24+5-32+3)	32+5 (24+0-36+4)	**0.0003**
Gestational age at delivery (weeks+days)	28+0 (25+0-32+5)	32+6 (24+5-36+2)	**0.0001**
Microbial invasion of the amniotic cavity	9 (75%)	20 (26%)	**0.002**
Genital Mycoplasmas in amniotic fluid	5 (42%)	15 (19%)	0.13
Other bacteria in amniotic fluid	4 (33%)	7 (9%)	**0.04**
Birth weight (grams)	1335±424	1927±588	**0.03**
Apgar score < 7 at 5 minutes	2 (17%)	3 (4%)	0.13
Apgar score < 7 at 10 minutes	0 (0%)	2 (3%)	1.00
Puerperal endometritis	0 (0%)	2 (3%)	1.00

Continuous variables were compared using the parametric t-test (presented as mean ± SD) or the nonparametric Mann-Whitney *U* test (presented as median [range]). Categorical variables were compared using Fisher's exact test and presented as n (%).

The statistically significant results are marked in bold type.

Table 5 Amniotic fluid sCD163 levels, related to funisitis in a model with and without adjustment for gestational age (specific aim 2)

Funisitis	**Number**	**Mean (crude)**	***p*-value (crude)**	**Mean (adjusted)**	**95% CI (adjusted)**	***p*-value (adjusted)**
Yes	12	1649.2	**0.02**	1161.2	694.0-1628.4	0.09
No	77	645.6		721.6	555.3-887.9	

This linear mixed model was adjusted for gestational age at sampling.

The statistically significant results are marked in bold type.

Table 6 Maternal and neonatal characteristics, related to histological chorioamnionitis (HCA)(specific aim 3).

	HCA (n = 38)	**No HCA (n = 45)**	***p*-value**
Maternal age (years)	30.7±6.5	30.6±5.2	0.94
Primiparous	20 (53%)	27 (60%)	0.51
Smoking during pregnancy	8 (21%)	10 (22%)	1.00
Pre-pregnancy body mass index	22.7 (17.5-40.6)	21.4 (16.3-38.6)	0.30
Gestational age at admission (weeks+days)	32+2 (24+0-36+3)	33+3 (25+2-36+4)	0.08
Gestational age at delivery and sampling (weeks+days)	32+4 (24+0-36+4)	33+5(25+4-36+6)	0.09
Microbial invasion of the amniotic cavity	17 (45%)	13 (29%)	0.17
Genital Mycoplasmas in amniotic fluid	11 (29%)	9 (20%)	0.44
Other bacteria in amniotic fluid	8 (21%)	4 (9%)	0.13
Birth weight (grams)	1814±583	2058±590	0.12
Apgar score < 7 at 5 minutes	3 (8%)	1 (2%)	0.33
Apgar score < 7 at 10 minutes	0 (0%)	0 (0%)	*
Puerperal endometritis	3 (8%)	0 (0%)	0.09

Continuous variables were compared using the parametric t-test (presented as mean ± SD) or the nonparametric Mann-Whitney *U* test (presented as median [range]). Categorical variables were compared using Fisher's exact test and presented as n (%).

* *p*-value cannot be calculated because two variables are zero.

Table 7 Maternal and neonatal characteristic, related to funisitis (specific aim 3).

	Funisitis (n = 9)	**No funisitis (n = 74)**	***p*-value**
Maternal age (years)	28.1±6.4	30.9±5.6	0.17
Primiparous	3 (33%)	44 (59%)	0.16
Smoking during pregnancy	2 (22%)	16 (22%)	1.00
Pre-pregnancy body mass index	19.4 (17.5-20.8)	22.1 (16.3-40.6)	0.10
Gestational age at admission (weeks+days)	27+5 (24+5-32+0)	33+3 (24+0-36+4)	**0.002**
Gestational age at delivery and sampling (weeks+days)	28+2 (25+0-32+1)	33+4 (24+0-36+6)	**0.001**
Microbial invasion of the amniotic cavity	8 (89%)	22 (30%)	**0.001**
Genital Mycoplasmas in amniotic fluid	4 (44%)	16 (22%)	0.21
Other bacteria in amniotic fluid	4 (44%)	8 (11%)	**0.02**
Birth weight (grams)	1407±408	2021±641	**0.006**
Apgar score < 7 at 5 minutes	1 (11%)	3 (4%)	0.37
Apgar score < 7 at 10 minutes	0 (0%)	0 (0%)	*
Puerperal endometritis	0 (0%)	3 (4%)	1.00

Continuous variables were compared using the parametric t-test (presented as mean ± SD) or the nonparametric Mann-Whitney *U* test (presented as median [range]). Categorical variables were compared using Fisher's exact test and presented as n (%).

* *p*-value cannot be calculated because two variables are zero.

The statistically significant results are marked in bold type.

Table 8 Umbilical cord blood sCD163 levels, related to funisitis, in a model with and without adjustment for gestational age at sampling (specific aim 3).

Funisitis	**n**	**Mean (crude)**	***p*-value (crude)**	**Mean (adjusted)**	**95% CI (adjusted)**	***p*-value (adjusted)**
Yes	9	1402.4	**0.02**	1408.7	1234.5-1528.9	**0.009**
No	74	2213.3		2160.7	1633.0-2688.4	

This linear mixed model was adjusted for gestational age at sampling.

The statistically significant results are marked in bold type.

Table 9 Maternal and neonatal characteristics, related to histological chorioamnionitis (HCA) (specific aim 4).

	HCA (n = 27)	**No HCA (n = 25)**	***p*-value**
Maternal age (years)	31.1±6.8	28.8±4.6	0.16
Primiparous	12 (44%)	19 (76%)	0.03
Smoking during pregnancy	6 (22%)	4 (16%)	0.73
Pre-pregnancy body mass index	22.4 (17.0-33.2)	21.3 (16.6-38.6)	0.23
Gestational age at sampling (days)	31+4 (24+5-36+4)	32+2 (25+1-35+4)	0.43
Gestational age at delivery (days)	32+1 (25+2-36+6)	32+2 (26+0-35+5)	0.46
Microbial invasion of the amniotic cavity	15 (56%)	7 (28%)	0.06
Genital Mycoplasmas in amniotic fluid	9 (33%)	5 (20%)	0.36
Other bacteria in amniotic fluid	8 (30%)	3 (12%)	0.18
Birth weight (grams)	1650±503	1737±558	0.56
Apgar score < 7 in 5 minutes	2 (7%)	0 (0%)	0.49
Apgar score < 7 in 10 minutes	0 (0%)	0 (0%)	*
Puerperal endometritis	3 (11%)	0 (0%)	0.24

Continuous variables were compared using the parametric t-test (presented as mean ± SD) or the nonparametric Mann-Whitney *U* test (presented as median [range]). Categorical variables were compared using Fisher's exact test and presented as n (%).

* *p*-value cannot be calculated because two variables are zero.

Table 10 $CD163^+$ cells in different compartments, related to histological chorioamnionitis (HCA) (specific aim 4).

	HCA		**No HCA**		***p*-value**
	Cases	Number of $CD163^+$ cells per HFP	Cases	Number of $CD163^+$ cells per HFP	
Fetal membrane - decidua	27	75.7 (4.7 – 200.0)	25	65.0 (1.0 – 178.0)	0.89
Fetal membrane - chorion	27	28.3 (5.0 – 70.0)	25	22.3 (0 – 60.0)	0.13
Fetal membrane - amnion	27	7.8 (0 – 33.3)	25	5.7 (0.7-18.0)	0.74
Placenta - decidua	27	61.7 (20.0 – 133.3)	25	51.7 (0 – 173.3)	0.55
Placenta – term villi	27	91.7 (53.3 – 166.7)	25	100. 0 (51.7 – 163.3)	0.80
Placenta – stem villi	27	60.0 (29.3 – 111.7)	25	60.0 (27.7 – 85.0)	0.61
Placenta – subchorionic fibrin	25	10.3 (1.0 – 136.7)	21	3.0 (0 – 71.7)	**0.007**
Placenta – chorionic plate	25	93.3 (33.0 – 253.3)	21	80.0 (12.3 – 133.3)	**0.049**
Placenta - amnion	24	8.17 (0.7 – 19.3)	20	3.3 (0 – 25.7)	0.06

Continuous variables were compared using the nonparametric Mann-Whitney *U* test (presented as median [range]).

Abbreviations:

$CD163^+$ cell cell positive for scavenger receptor for hemoglobin

HPF high-power field

The statistically significant results are marked in bold type.

Figure 1 Fetal membranes without (A) and with (B) histological chorioamnionitis.

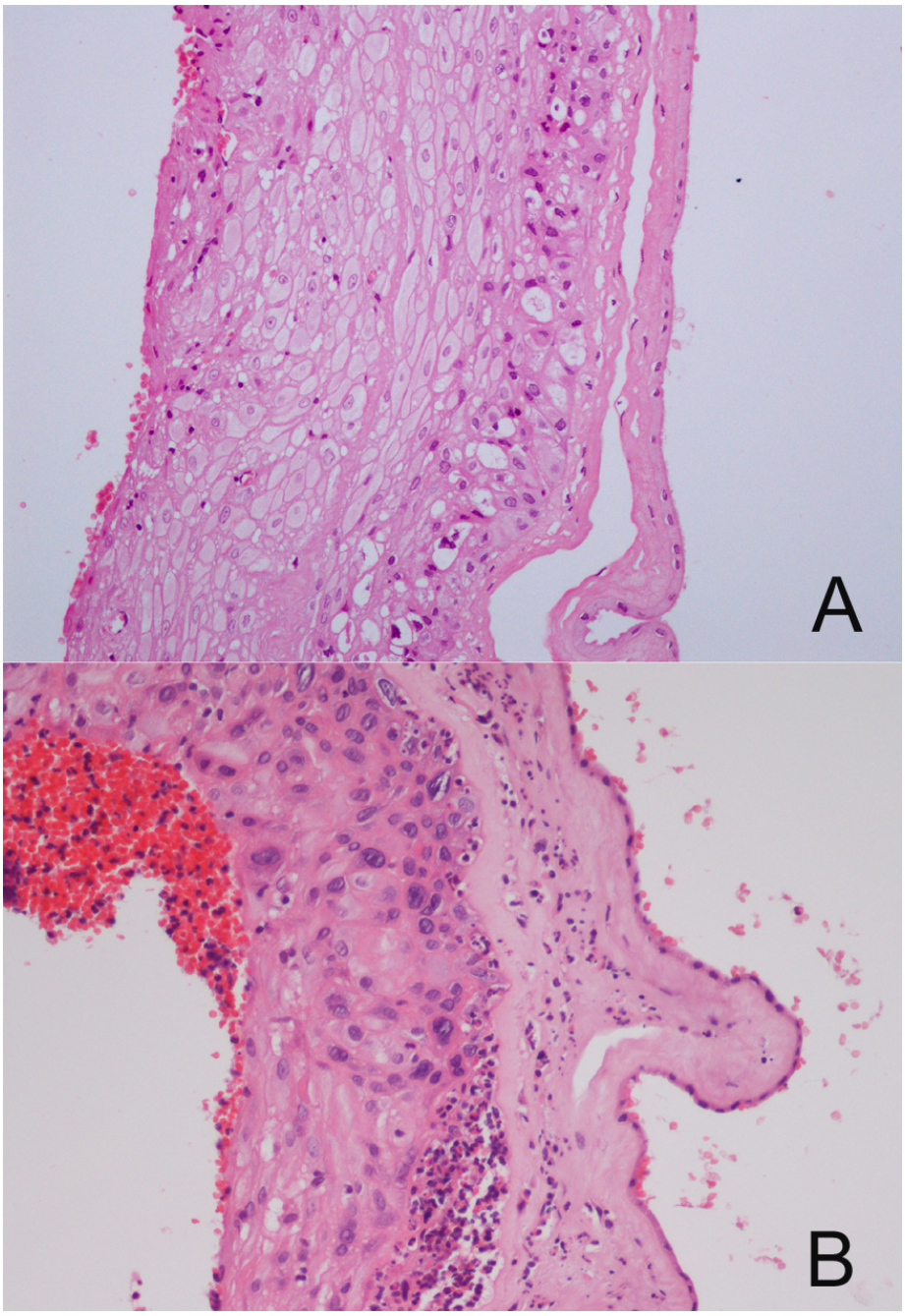

Figure 2 Umbilical cord without (A) and with (B) funisitis.

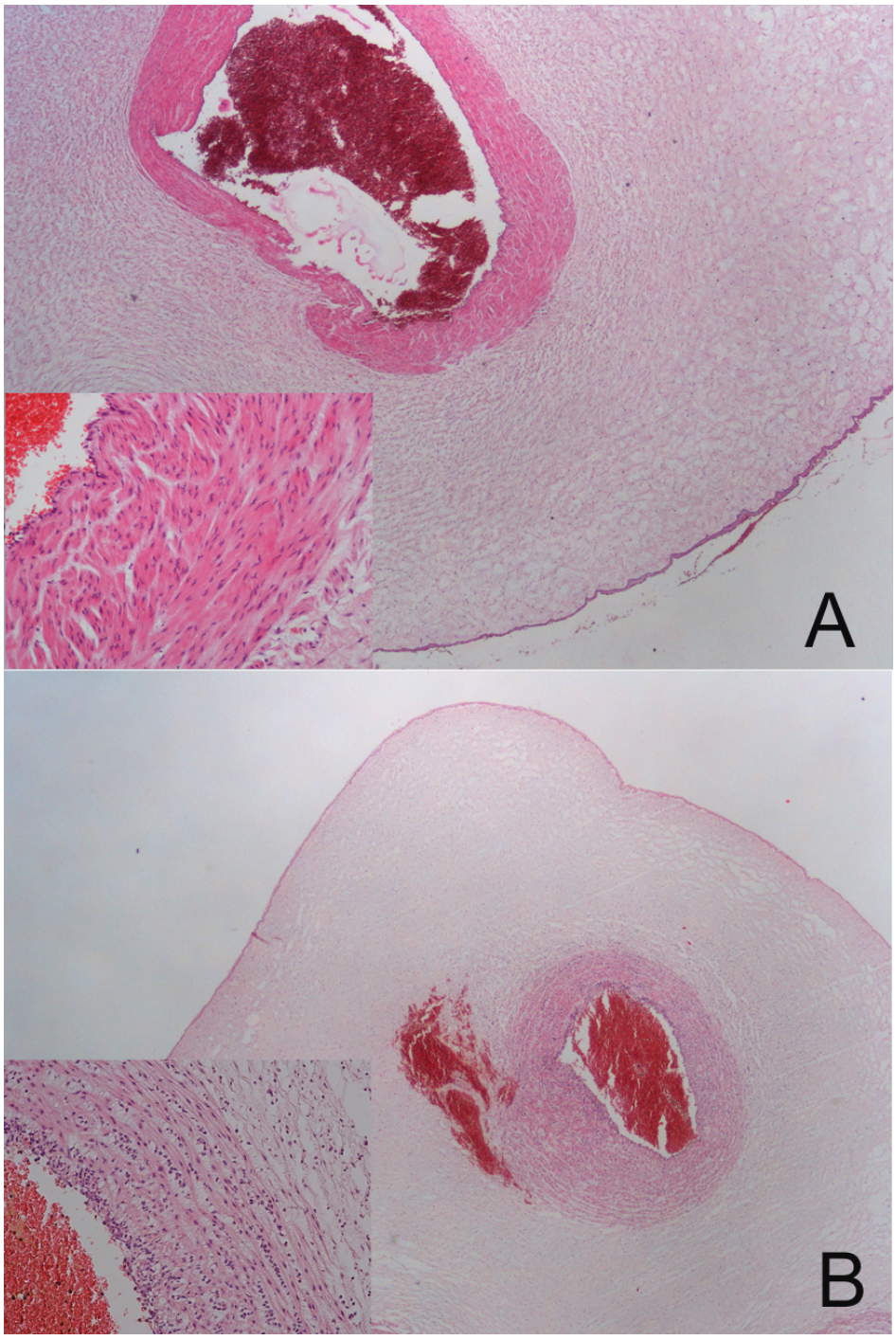

Figure 3 Amniotic fluid sCD163 levels in women in the second trimester and at term, with and without regular uterine contractions. Women in the second trimester had a higher median sCD163 level than those at term without regular uterine contractions. Horizontal bars indicate medians.

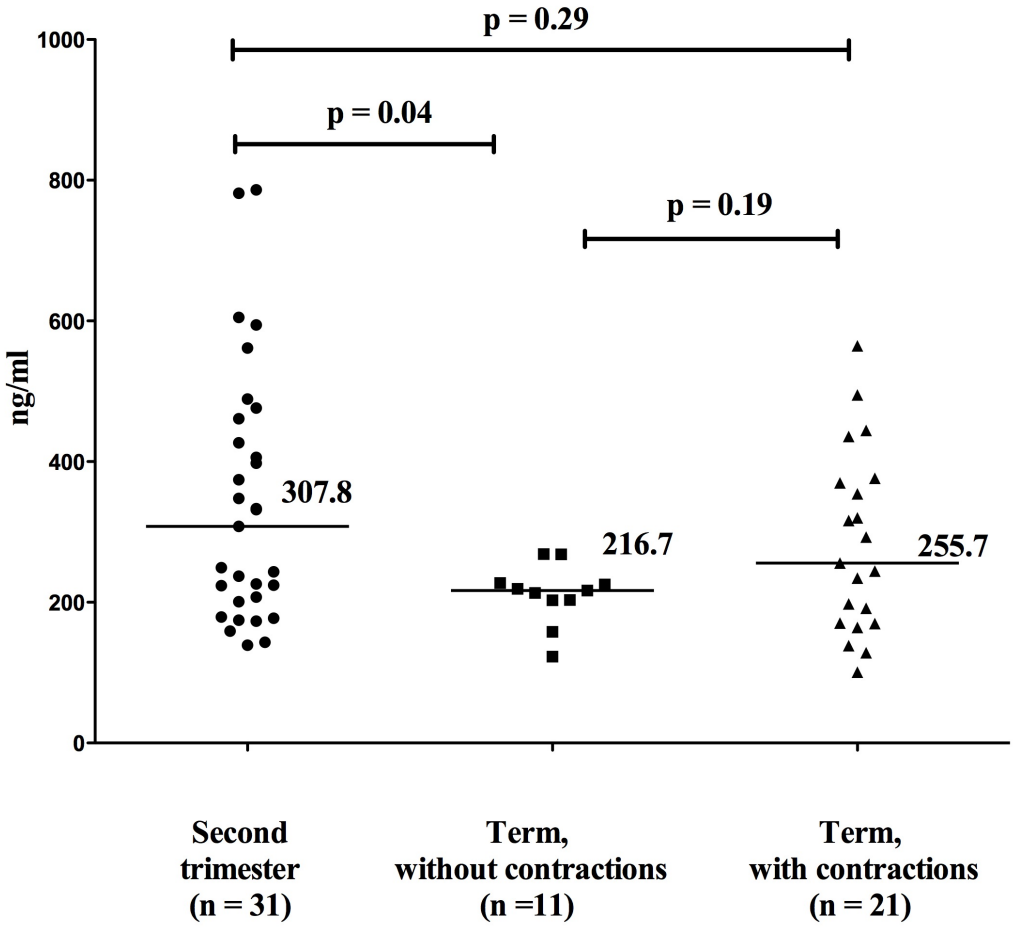

Figure 4 Amniotic fluid sCD163 levels in women with PPROM. Women with HCA had a higher median level than those without HCA. Women with funisitis had a higher median level than those without funisitis. Horizontal bars indicate medians.

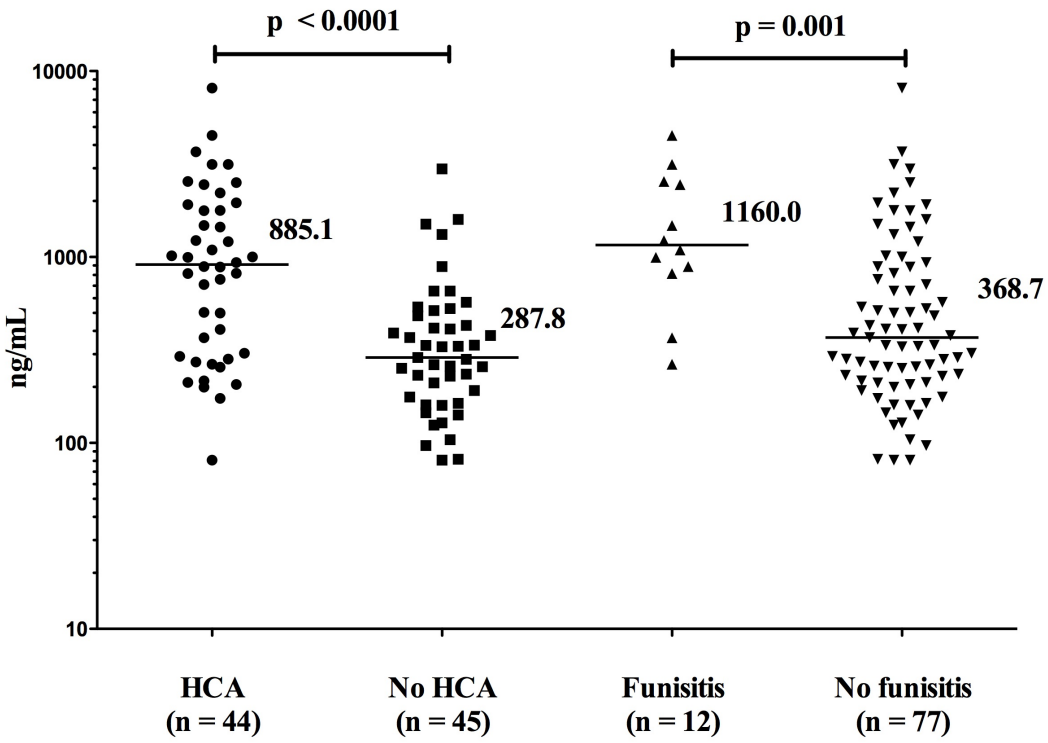

Figure 5 Women with PPROM with HCA alone (without funisitis) did not have a higher median amniotic fluid sCD163 level than women with HCA with funisitis. Horizontal bars indicate medians.

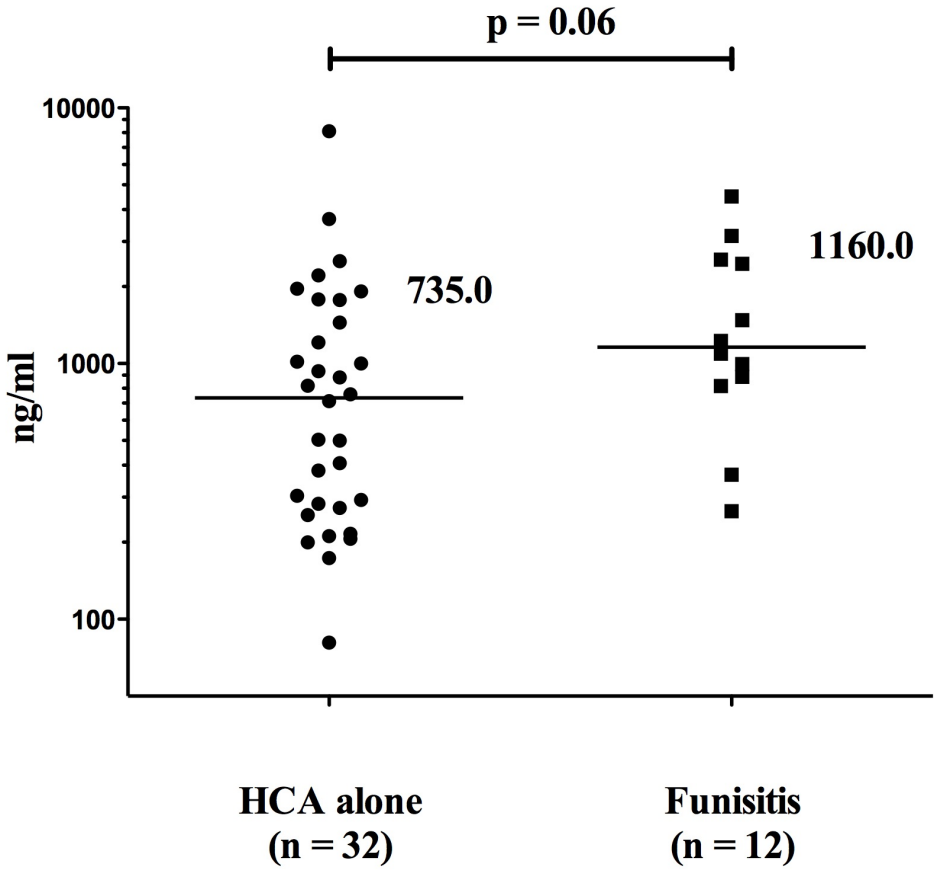

Figure 6 Receiver operating characteristic curve of amniotic fluid sCD163 levels for the identification of HCA (AUC for umbilical cord sCD163 level: 75%, $p < 0.0001$).

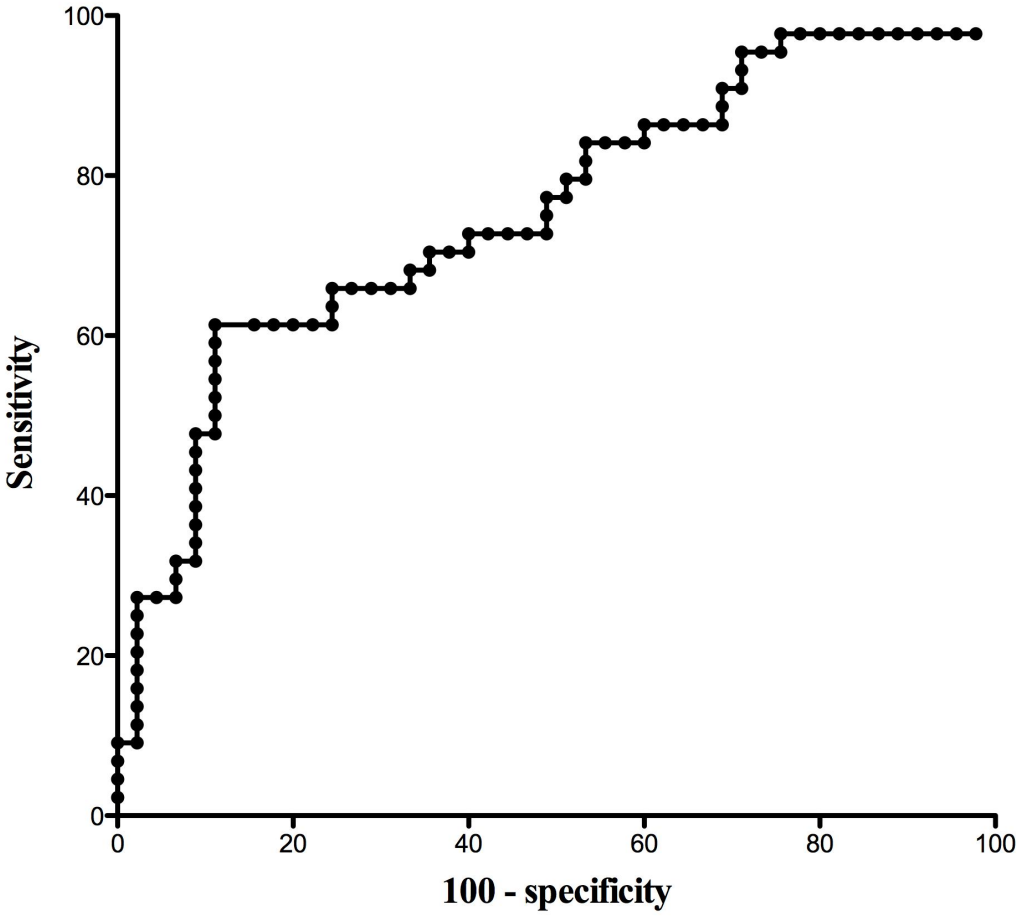

Figure 7 Umbilical cord blood sCD163 levels in women with PPROM. Women with HCA had a higher median level than those without HCA. Women with funisitis had a higher median level than women without funisitis. Horizontal bars indicate medians.

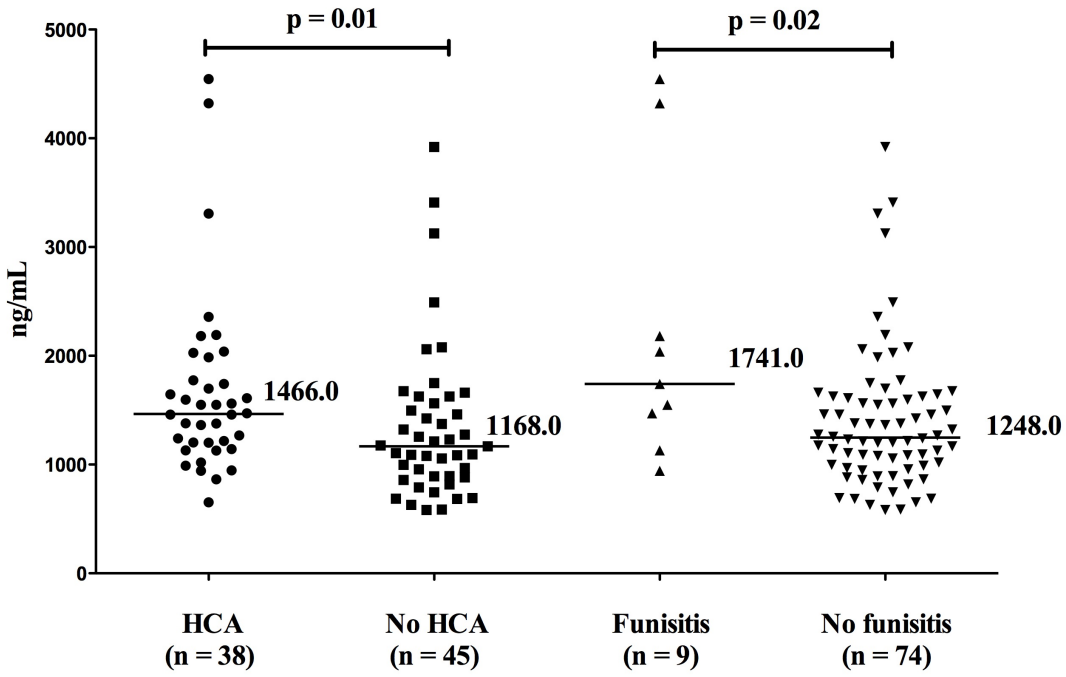

Figure 8 Women with PPROM with HCA alone (HCA without funisitis) did not have a higher median umbilical cord blood sCD163 level than women with funisitis. Horizontal bars indicate medians.

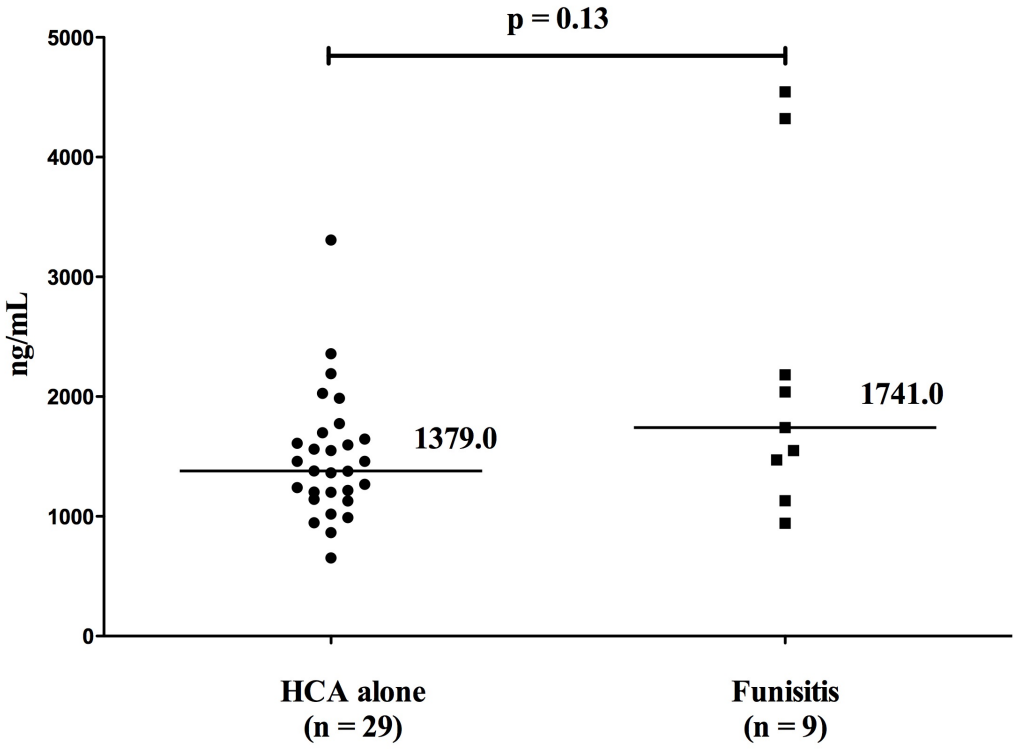

Figure 9 Receiver operating characteristic curve of umbilical cord blood sCD163 levels for the identification of HCA (AUC for umbilical cord sCD163 level: 65%; $p = 0.01$).

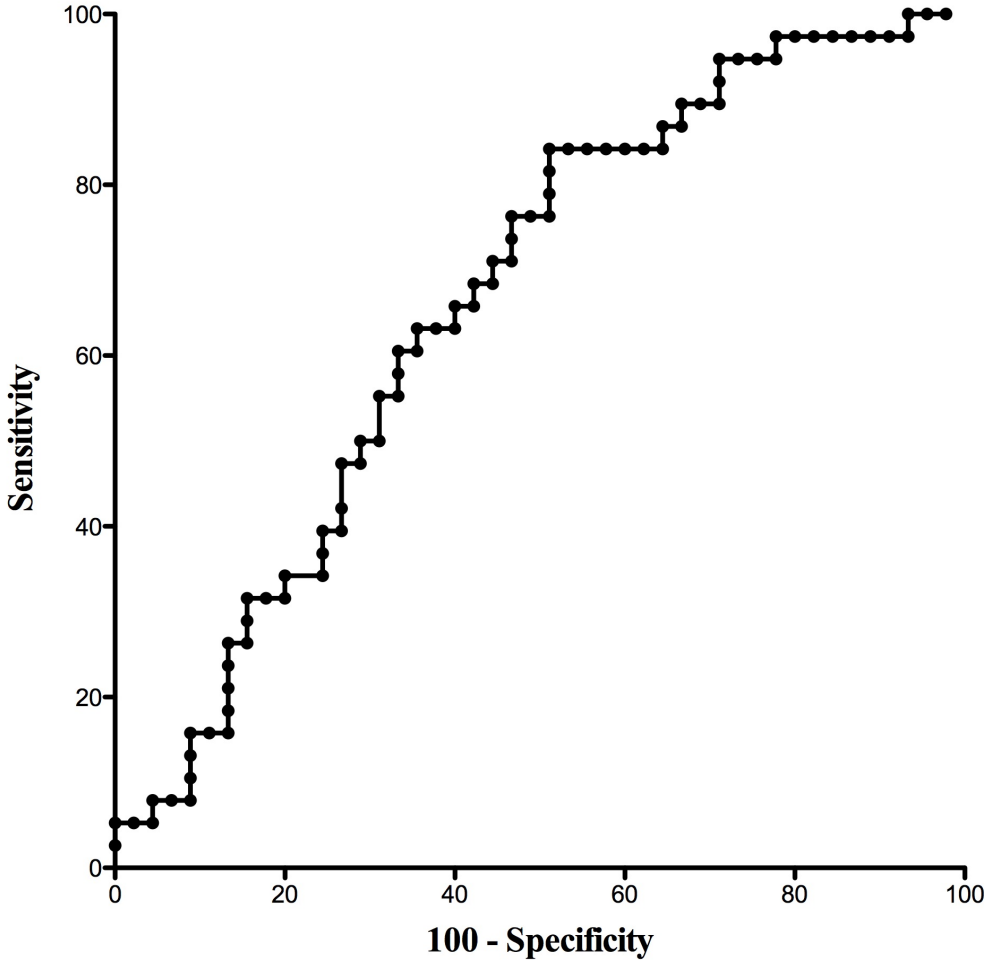

Figure 10 Receiver operating characteristic curve of umbilical cord blood sCD163 levels for the identification of funisitis (AUC for umbilical cord sCD163 level: 73%; $p = 0.03$).

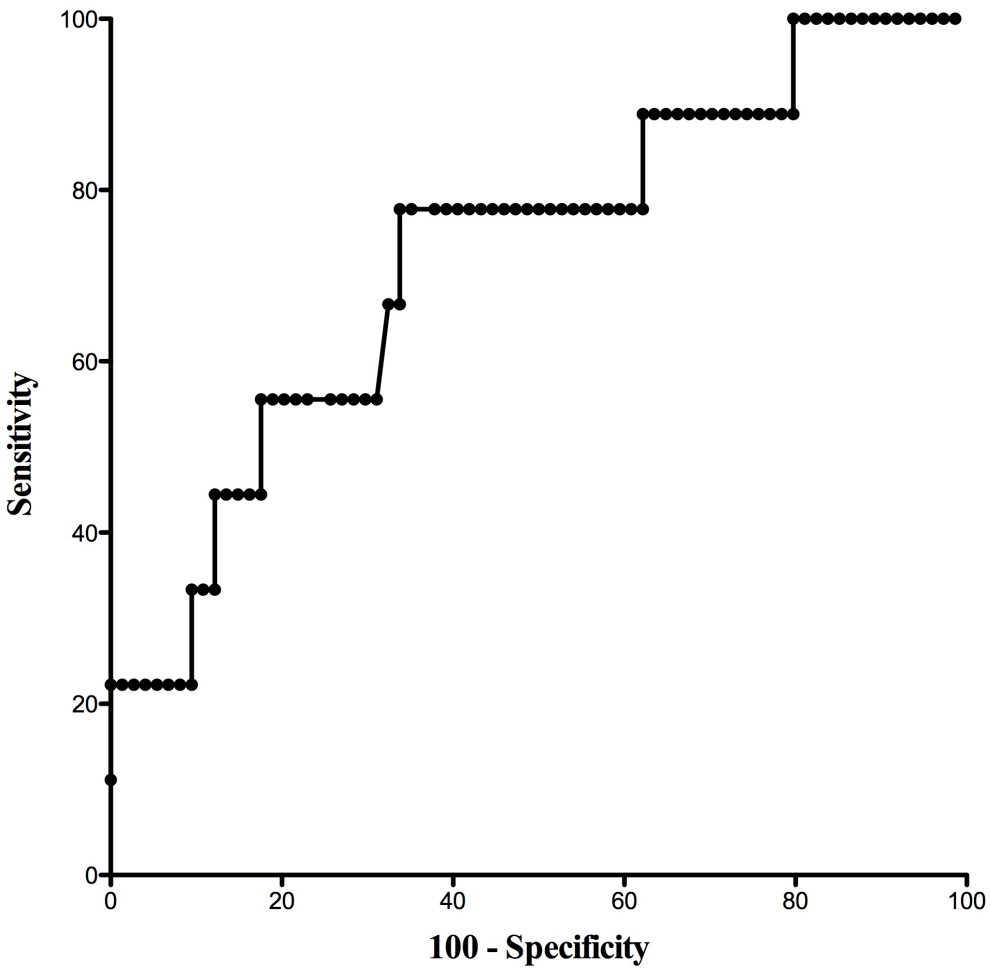

Figure 11 Comparison of the number of $CD163^+$ cells per high-power field in groups with and without HCA in the fetal membranes: decidua (A), chorion (B), and amnion (C)

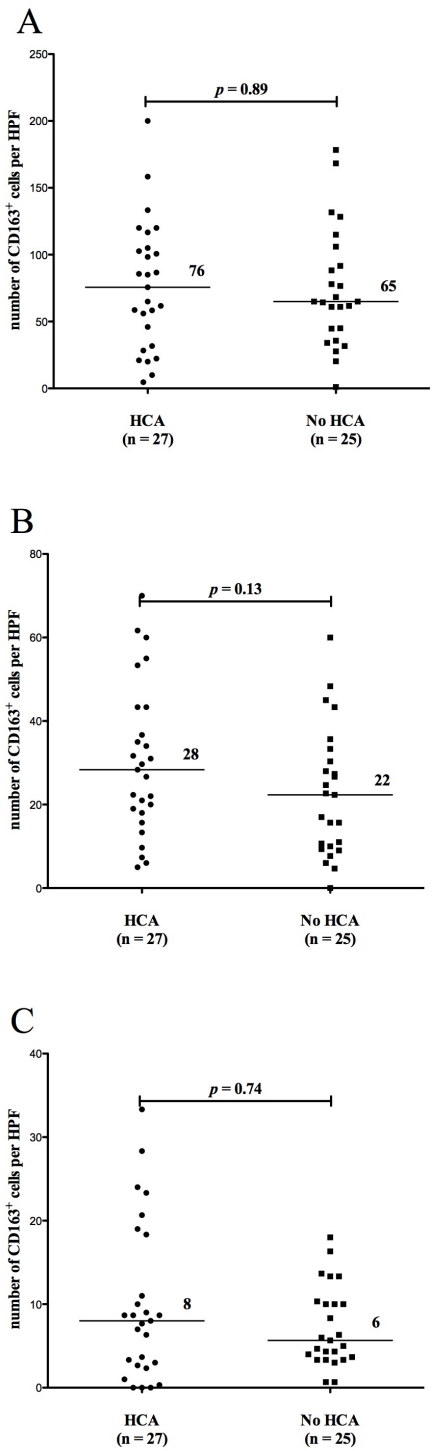

Figure 12 Comparison of the number of $CD163^+$ cells per high-power field in groups with and without HCA in the placenta: decidua (A), term villi (B), stem villi (C), subchorionic fibrin (D), chorionic plate (E), and amnion (F)

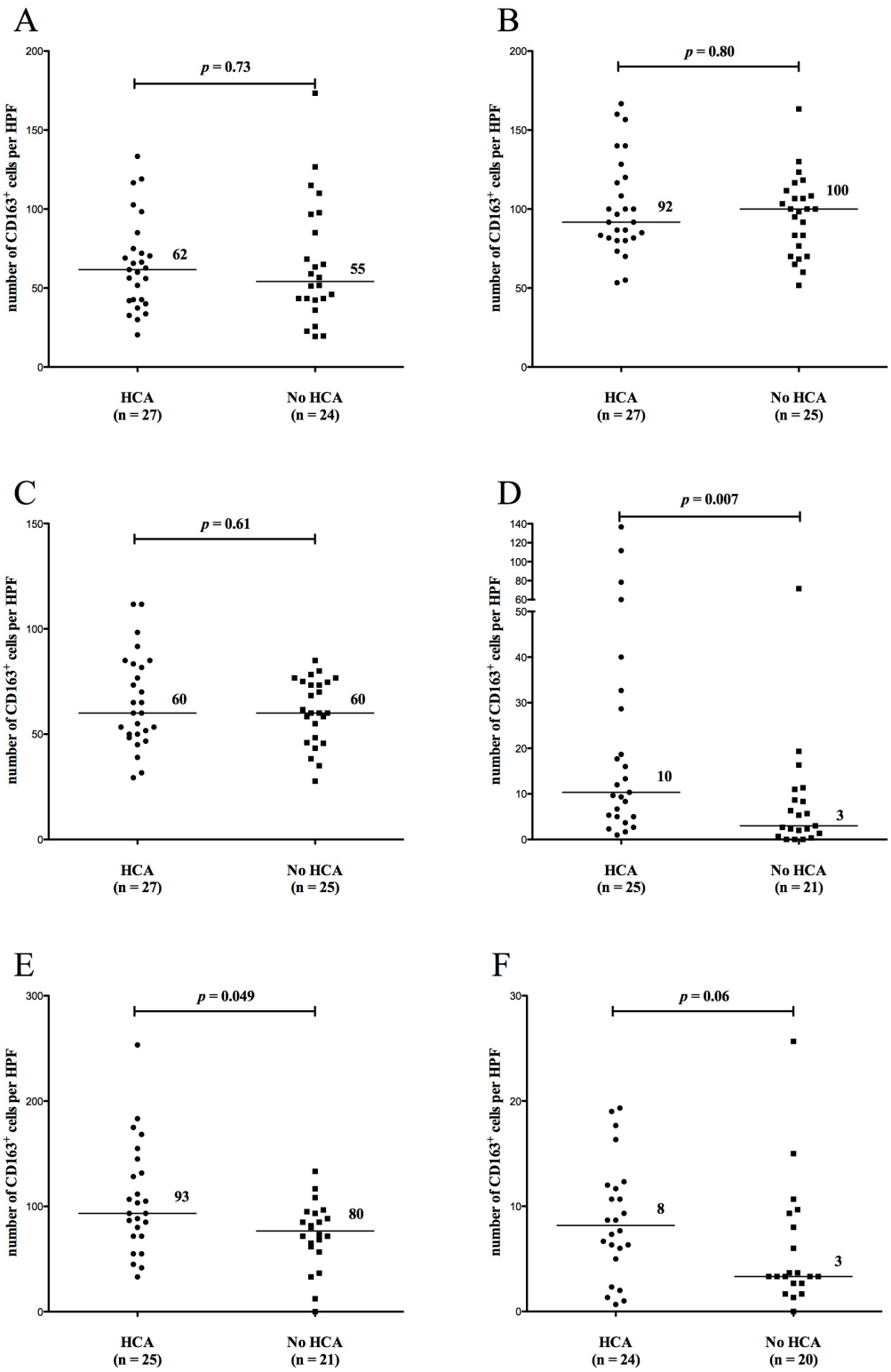

Figure 13 Placenta without inflammation, gestational age 32 weeks, hematoxylin-eosin (A) and CD163 (B); 200 x

Placenta with inflammation, gestational age 32 weeks, hematoxylin-eosin (C) and CD163 (D); 200 x

Arrow indicates amnion. * and ° indicate subchorionic fibrin and the chorionic plate, respectively.