Charles University in Prague
Faculty of Medicine

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

Marian Kacerovský

Abstract of the thesis

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Author:	Marian Kacerovský, MD Department of Obstetrics and Gynecology, Faculty of Medicine in Hradec Králové, Charles University in Prague Faculty Hospital Hradec Králové, Czech Republic
Supervisor:	Ass. Prof. Jindřich Tošner, MD, PhD Department of Obstetrics and Gynecology, Faculty of Medicine in Hradec Králové, Charles University in Prague, Faculty Hospital Hradec Králové, Czech Republic
Consultant-Supervisor:	Ass. Prof. Bo Jacobsson, MD, PhD Department of Obstetrics and Gynecology, Sahlgrenska University, Gothenburg, Sweden
Opponents:	Prof. Pavel Calda, MD, PhD Charles University in Prague, First Faculty of Medicine, Department of Gynaecology and Obstetrics of the First Faculty of Medicine and General Teaching Hospital, Prague, Czech Republic Ass. Prof. Martin Procházka, MD, PhD Department of Obstetrics and Gynecology, Palacky University Hospital, Olomouc, Czech Republic

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SOUHRN

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

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 (LR 1.8) a funisitidy (LR 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.. 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

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

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.

1. BACKGROUNDS

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).

PPROM occurs in 2-4% of all singleton pregnancies and 7-20% of twin pregnancies (6, 7). It is the leading identifiable cause of PTD, responsible for approximately 20-30% of cases (8, 9) and is an important cause of perinatal morbidity and mortality (6, 10-12). PPROM is usually defined as the rupture of fetal membranes with leakage of amniotic fluid, occurring before onset of labor (8, 9). 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 (13, 14).

Histological chorioamnionitis (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 (15, 16). 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 (17). Intraamniotic inflammatory response is elicited by activation of the innate immunity through the pattern recognition receptors. They are able detect the specific motifs on the surface of the microorganism or endogenous molecules and initiate the inflammatory response cascade. Highly conserved sequences on the bacterial surface interact with different host pattern recognition receptors to modulate the severity and character of the inflammatory response (12).

Scavenger receptor for hemoglobin (CD163) is a monocyte/macrophage-restricted transmembrane glycoprotein. The main function of CD163 is the binding of the hemoglobin–haptoglobin complex. It has also been demonstrated CD163 as a macrophage surface receptor can recognize intact Gram-positive and Gram-negative bacteria (18). 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 (19, 20).

The soluble form of CD163 (sCD163), the counterpart to membrane-bound CD163, has been identified in plasma and other body fluids (21, 22) 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 (23, 24).

Like its membrane-bound counterpart, sCD163 can bind to Hb-Hp, but with lower affinity (25, 26). In addition, sCD163 can mediate innate immune defense by sequestering hemoglobin-bound

iron (26, 27). It has been proposed that sCD163 can inhibit the proliferation and activation of lymphocytes, via an as yet unknown receptor (26, 28). The function of sCD163 in bacterial recognition remains to be established (18).

In pregnancy, sCD163 levels become slightly elevated compared with non-pregnant women (29-31). Plasma sCD163 levels have previously been determined in asymptomatic pregnant women during the first trimester and in women with symptoms of preterm labor (30, 32). sCD163 has also been reported in the serum of asymptomatic pregnant women with a history of late spontaneous miscarriage or early spontaneous preterm birth (31).

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.

2. Material and methods

2.1. Patients

2.1.1. 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 10th percentile for the respective gestational age), structural malformations, chromosomal abnormalities or signs of fetal hypoxia on admission were excluded.

2.1.2. 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 10th 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.

2.1.3. 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 10th and 90th 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.

2.1.4. 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 10th 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.

2.2. SAMPLE COLLECTION

2.2.1. 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.

2.2.2. Umbilical cord blood

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.

2.2.3. Placenta and fetal membranes

At delivery, tissue samples were obtained from placenta, umbilical cord, and placental membranes. At delivery, the placenta, the fetal membranes, and the umbilical cord were obtained and fixed in 10% neutral buffered formalin. Tissue samples from placenta (at least 2 samples), umbilical cord (usually 1 sample), and placental membranes (at least 2 samples) were processed and embedded in paraffin. Sections of tissue blocks were stained with hematoxylin and eosin.

2.3. AMNIOTIC FLUID ANALYSES

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).

2.4. UMBILICAL CORD BLOOD ANALYSES

The concentration of sCD163 in umbilical cord blood was determined by sandwich ELISA technique with MACRO163™ 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).

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 a 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.

2.6. 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) (33). 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) (33). Histopathological examination was performed by a single perinatal pathologist who was blinded to clinical status.

2.7. 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.

2.8. Statistical analyses

The demographic characteristics were compared by using either parametric (t-test, ANOVA) or non-parametric (Mann-Whitney *U* test, Kruskal-Wallis test) tests for continuous variables and were presented as mean ± standard deviation (SD) and median (range), respectively. Categorical variables were compared using Fischer´s exact test and are presented as n (%). sCD163 concentrations were compared nonparametric tests (Mann-Whitney test), given the non-normal distribution of analyte, and presented as a median [interquartile range (IQR)]. The normality of the data was tested using the D'Agostino and Pearson omnibus normality test and the Shapiro-Wilk test. Mixed linear models to identify major determinants of the sCD163 levels in the presence of funisitis were performed both without and with adjustment gestational age at sampling. Estimated marginal means of the fitted models were calculated. Receiver operator characteristic (ROC) curves were constructed to describe the relationship between the sensitivity and the false-positive rate for different values of sCD163 levels in the identification of HCA and funisitis. Differences were considered statistically significant at $p < 0.05$. All *p*-values were 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. RESULTS

3.1. SPECIFIC AIM 1

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). 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) 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).

3.2. SPECIFIC AIM 2

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; $p < 0.0001$). 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) in the crude analysis, but not after adjustment for gestational age at sampling in the model (p = 0.09). 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). 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 ($p < 0.0001$). The predictive value for identification of funisitis was not calculated because of the non-significant difference found after adjustment for gestational age.

3.3. SPECIFIC AIM 3

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). 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) in the crude analysis, as well as after the adjustment for gestational age at sampling (p = 0.009). 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). 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). 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).

3.4. SPECIFIC AIM 4

$CD163^+$ 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. Contrarily, the highest number of $CD163^+$ cells was found in chorionic fibrin as well as placental and fetal decidua. 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. 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; $p = 0.007$).

4. DISCUSSION

4.1. 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 (34). 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 (30, 35, 36).

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 (19, 37-40). 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 (41). This explains the universality of macrophage activation-enhancing tissue and the ability of macrophages to resolve inflammation rapidly without recruitment of new macrophages (41).

4.2. 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 (42). 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 (42, 43, 44). 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 (42, 45, 46) 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 (47).

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 (48, 49).

4.3. 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 (30, 31, 50). 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) (51). 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 (52). 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 (52). 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.

4.4. 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 (53). According to their function, macrophages are often divided into two groups and called either classically or alternatively activated (54). The latter represent a heterogeneous population with a broad spectrum of functions entailing anti-inflammatory and tissue remodeling properties (55). 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 (56). Moreover, CD163 may belong to a subpopulation of hematopoietic stem/progenitor cells (57).

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 (58). 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 (59). Moreover, both decidual macrophages and Hofbauer cells have been shown to have an alternatively activated phenotype (58).

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 (60).

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 (61). 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 (56).

5. 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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3. Proteomic discovery of potential infection and inflammation biomarkers in Caucasian spontaneous preterm birth patients. Preterm Birth Collaboration – WHO, Young scientist forum. May 2011; Geneva, Switzerland.

4. Amniotic fluid sCD163 in PPROM pregnancy complicated by histological chorioamnionitis. 6th Annual Meeting, The Bridges in Life Sciences. Young scientist forum. April 2011; Bratislava, Slovakia.

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6. Fetal Thymus on Ultrasound. Research Seminar. Sahlgrenska University Hospital. January 2011; Gothenburg, Sweden.

7. The Relationship between Sonographic Transverse Diameter of Fetal Thymus and Intraamniotic Inflammation in Women with PPROM. Research Seminar. Cedars Sinai Medical Center. November 2010; Los Angeles, USA.

8. Pentraxin 3 a New Marker of Subclinical Chorioamnionitis in Preterm Premature Rupture of Membranes. Research Seminar. Cedars Sinai Medical Center. November 2011; Los Angeles, USA.

9. Fetal thymus as a marker of histological chorioamnionitis. 20th World Congress ISUOG. October 2011; Prague, Czech Republic.

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30. Dopplerovská flowmetrie v graviditě. Ultrazvukový workshop. Celostátní konference ČGPS. Květen 2008; Hradec Králové.

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