Published in final edited form as:

Expert Opin Med Diagn. 2013 January ; 7(1): 5–16. doi:10.1517/17530059.2012.709232.

Advances in Medical Diagnosis of Intra-Amniotic Infection

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Abstract

Introduction—Intra-uterine infection is a global problem and a significant contributor to morbidity and perinatal death. The host response to infection causes an inflammatory state that acts synergistically with microbial insult to induce preterm birth and fetal damage. Prompt and accurate diagnosis of intra-amniotic infection in the asymptomatic stage of the disease is critical for improved maternal and neonatal outcomes.

Areas Covered—This article provides an overview of the most recent progress, challenges and opportunities for discovery and clinical implementation of various maternal serum, cervico-vaginal and amniotic fluid biomarkers in pregnancies complicated by intra-amniotic infection.

Expert Opinion—Clinically relevant biomarkers are critical to the accurate diagnostic of intra-uterine infection. Front end implementation of such biomarkers will also translate in lower incidence of early-onset neonatal sepsis which is an important determinant of neonatal morbidity and mortality associated with prematurity. However, of the hundreds of differentially expressed proteins, only few may have clinical utility and thus function as biomarkers. The small number of validation studies along with barriers to implementation of technological innovations in the clinical setting are current limitations.

Keywords

Intra-uterine infection; Molecular microbiologic diagnostic tools; infection-induced inflammation; non-invasive diagnostics

1. Introduction

The percentage of preterm birth (PTB) cases in the U.S. has recently declined [1]. While PTB rates in the U.S. have fallen for four consecutive years, the overall rate is 11.9% [1]. The decline in PTB for 2008-2010 was noticeable among infants born at 34-36 weeks (late preterm). The percentage of infants born at less than 34 weeks also dropped, from 3.56% to 3.50% [1]. Declines were seen among most age, race, and ethnic groups. The rate of low birthweight births was unchanged for 2008–2010 at 8.15%. Regretfully, the percentage of newborns delivered at very low birthweight (<1,500 grams) declined only minimally from 1.46% in 2008 to 1.45% in 2010. From the over half a million premature infants born annually, ~60,000 have a birthweight <1,500 grams. The clinical relevance of this observation is that very-low birthweight premature newborns remain a problem due to the highest risk of early death and disability [2].

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Declaration of interest: The authors state no conflict of interest and have received no payment in preparation of this manuscript.

The percentage of infant deaths that are “preterm-related” is ~36% [3]. However, as life support in Newborn Special Care Units has improved, more very-low birthweight infants survive. Thus, it has become clear that improving neonatal outcomes associated with prematurity is vital. About 5% of nearly 55,000 preemies who survive the newborn period exhibit cerebral palsy and up to 25-50% have sensorial, cognitive and behavioral deficits. The immediate impact of PTB on the society is underscored by the multi-billion dollar/year cost of caring for premature infants [4]. Collectively, this data highlights the need for a paradigm shift in prematurity research from the one-sided goal of extending the duration of gestation to concurrently improving neonatal outcomes.

Preterm birth is viewed as a syndrome with multiple pathogenic mechanisms. Specifically, maternal and fetal stress, excessive myometrial stretching, genetic predisposition, decidual hemorrhage and infection/inflammation, are the most recognized [5,6,7]. This article focuses on the process of intra-uterine infection with an emphasis on several recent discoveries in the field of metagenomics. This knowledge carries an unprecedented opportunity for improving the diagnosis of intra-amniotic infection and early-onset neonatal sepsis (EONS) given that these two entities are etiologically intertwined. We will review information supporting the hypothesis that the inter- and intra-species heterogeneity impacts on microbial virulence and thus on the ability of certain bacteria to overcome maternal defense mechanisms and infect and proliferate in the gestational sac. Lastly, we will summarize recent proteomics findings that lead to identification of an array of maternal serum, cervico-vaginal and amniotic fluid biomarkers with diagnostic potential for early and accurate diagnosis of intra-uterine infection in women at risk for PTB.

2. Translational Microbiology Research and Intra-Uterine Infection

Of all pathogenic pathways, intra-uterine infection and subsequent inflammation contributes disproportionately to PTB and to neonatal morbidity and mortality [7]. Infection provides the stimulus for various maternal-fetal signaling events leading to preterm premature rupture of membranes (PPROM), cervical ripening, and premature activation of myometrial contractility [8,9]. Consequently, immature babies first carry the burden of prematurity. Second, following invasion of the gestational sac, intra-amniotic bacteria infect the fetus launching a heightened inflammatory state that acts synergistically with microbial insult to induce damage to vital fetal organs [10]. This is emphasized by the evidence which suggests that the poor outcome of many premature neonates is not entirely dependent on gestational age at birth but rather results from adverse processes triggered antenatally [11].

2.1 The role of microbial cultures in diagnosis of intra-amniotic infection

Larsen et al. provided the first evidence that intra-uterine infection is an important trigger of PTB [12]. Today, there is compelling microbiologic evidence to suggest that infection may contribute to ~25% of PTBs, with bacterial involvement responsible for ~80% for deliveries occurring early in gestation. This proportion declines to ~10% closer to term [13,14]. Limitations of culture techniques as the “gold standard” diagnostic test for infection are well-known [15]. This implies that there is a clear underestimation in diagnosis of intra-amniotic infection and in the frequency with which bacterial pathogens are involved in triggering PTB. There are reasons to believe classic bacteriologic methods of diagnosis will be complemented or even replaced by rapid and less biased approaches such as mass spectrometry and molecular sequencing [15,16]. Until then, however, the identity of the bacterial species involved in the process of intra-amniotic infection and neonatal sepsis is dependent upon bacterial cultures and microscopy studies. Based on microbial cultures alone, most intra-amniotic infections are polymicrobial [17]. The most common microorganisms identified in the fetal membranes and amniotic fluid of patients with infection-associated PTB are *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Gardnerella*

vaginalis, Group B *Streptococcus* (GBS), *Bacteroides* spp., and *Escherichia coli* (*E. coli*) [18,19,20,21]. *Listeria monocytogenes* has also been identified in infrequent cases [22].

2.2 Molecular microbiologic diagnostic tools for investigation of intra-amniotic infection

In clinical microbiology the possibility of identifying the presence and diversity of organisms in biological samples is an appealing approach to enhance the diagnostic abilities of microbiological cultures and provide targeted therapies. Metagenomics aims to study the diversity of microbial communities by analyzing complex genomic datasets. Rapid advancements in the field of metagenomics and the development of sequence-based culture-independent methods, using either cloning or most recently cloning-independent pyrosequencing, proved that the vast majority of environmental and human microbiota is uncultivable [23]. Recognized reasons for the inability to cultivate certain species include low prevalence, slow growth patterns and/or requirement of special conditions [24]. The cornerstone of the culture-independent detection method is sequencing of full length or variable regions of the bacterial 16S ribosomal RNA (16S-rRNA) gene. The growing database of 16S rRNA sequences enables identification of bacteria and/or their phylogenetic relationships. Most 16S rRNA genes are approximately 1,500 base pairs in length and contain regions that are both characteristic of the broad phylogenetic domain (Archaea, Eucarya and Bacteria) and regions specific to more narrow groups such as phyla, families and genera. There is general consensus that microbes holding 97% similarity in 16S rRNA gene analysis belong to the same genus while a similarity of 99% implies the same species [25]. In this way, species' richness and diversity within complex microbial communities can be catalogued in a virtually unbiased fashion. Results of metagenomics studies of amniotic fluid are valuable to understanding the true prevalence of infection in general and of each species in particular [26,27,28]. Notable results emerged including the observation that the bacterial diversity of the amniotic fluid microbiome is richer than appreciated by microbial cultures and includes multiple uncultivated and difficult-to-cultivate species such as *Sneathia*, *Fusobacterium nucleatum*, *Bergeyella*, *Clostridiales*, *Peptostreptococcus*, and *Bacteroides* spp [27,28].

3. Pathogenesis of Intra-Uterine Infection, Screening and Diagnostic Opportunities

3.1 Pathways to intra-amniotic infection and fetal infection

Because most intra-amniotic bacteria are genital tract microorganisms, and the amniotic fluid is normally sterile, the current paradigm of intra-uterine infection advocates that most often bacteria originate from the lower genital tract and invade into the pregnant uterus via an ascending mechanism [29,30]. Once the mechanical and innate immune barrier of the cervix is bypassed, microorganisms infect the decidua and penetrate the fetal membranes to invade the amniotic fluid [31,32]. Finally, microorganisms gain access and infect the fetus [14,31]. The conceptual frame-work outlined above is biologically plausible and is based on studies which demonstrated that: **i)** microorganisms frequently implicated in intra-amniotic infection (i.e. GBS, *Mycoplasma*, *E. coli*) are constituents of the vaginal flora [33] and cohabitants of the amnio-chorionic space [34]; **ii)** presence of *Ureaplasma* and *Mycoplasma* in the amniochorion space incite polymorphonuclear tissue infiltration and higher degree of histological chorioamnionitis in pregnancies complicated by PTB [35]; **iii)** GBS and *E. coli* carry the capacity to attach to the chorioamniotic membranes *in vitro* [36]; **iv)** in animal models of infection-induced PTB, trans-cervical and choriodecidual inoculation of GBS is followed by trans-migration of bacteria from the choriodecidual space to the amniotic fluid cavity, a graded response in amniotic fluid leukocyte infiltration, increased levels of pro-inflammatory cytokines [tumor necrosis- (TNF-), IL-6, IL-1] and prostaglandins (PGE2, PGF2) with initiation of uterine activity [37,38].

A secondary route of intra-amniotic infection is hematogenous spread through transplacental seeding of the fetus with infectious organisms, in particular *Haemophilus influenzae* (gram-negative rod-shaped bacteria) or *Fusobacterium nucleatum* (*F. nucleatum*, Gram-negative anaerobic bacteria) originating in other sites of the body [39,40]. These microorganisms first gain access to the placenta or to the fetus and only later translocate in the amniotic fluid cavity [14]. Iatrogenic infections during invasive procedures such as chorionic villous sampling, amniocentesis and cordocentesis are also considered possible routes of inoculation [41]. Retrograde microbial seeding of the amniotic fluid through the fallopian tubes or colonization of the uterine endometrium prior to implantation are other proposed routes [31]. However, stronger evidence in support of these pathways remains to be provided.

3.2 Host-bacterial interactions and susceptibility to intra-amniotic infection

Answers to questions such as why only certain women develop intra-amniotic infection, or why only specific bacteria have the ability to evade the defense mechanisms set in place to prevent their widespread into the amniotic fluid cavity or infect the fetus are still needed. There is a large body of data to suggest that the cervix, amniotic fluid membranes and placental cytotrophoblast are equipped to fight infection. For example, the endo-cervical epithelium, the amniochorion and amniotic fluid have significant antibacterial and immune functions [42,43]. Antimicrobial polypeptides such as defensins, calgranulins, cathelicidins are few of the primary mechanisms used by the cervical epithelium and fetal membranes in the early stages of immune defense [42,44,45]. Immunity of the cervix and fetal membranes as defense mechanisms against infection was recently reviewed [29,46], while the presence and expression pattern of anti-microbial peptides including defensins and elafin in trophoblasts has been studied and summarized by King et al. [47].

Recognizing how, why and when some microbes manage to evade maternal antimicrobial defense systems remains a significant challenge. Bacterial invasion is a complex phenomenon because many of the factors described above are constituents of the physiologic defense system. Paradoxically, when the defense mechanisms are overwhelmed by microbes, their excessive activation is becoming a mechanism of disease. The ability of pathogenic strains of bacteria including *Ureaplasma* to synthesize and release IgA and IgG proteases, mucinases and neuraminidase facilitates microbial entrance into the lower uterine segment and uterine decidua [48]. Maternal genetic make-up may be important in explaining inter-individual and racial variations in susceptibility to various infectious diseases [49]. For example, genetic variation in key innate immune receptors such as Toll-like receptors (TLRs), has been described as predisposing to sepsis [50]. For example, although not specifically described in fetal membranes, TLR4 genetic variants may result in excessive immune responses to microbial challenges which influence the outcome of bacterial infections [51]. Alterations of the biology of the chorion and/or amnion may also play a role. Winram et al. showed that GBS was capable of transcytosing through the intact chorion without disrupting the intercellular junctions [52]. Interestingly, even though GBS strains COH1 and A909 attached to the confluent amnion cell layers, bacteria could not invade. However, while these data suggest that amnion epithelial cells provide an effective bacterial barrier [52], *GBS* remains a causative microbial agent of intra-amniotic inflammation and early onset neonatal sepsis (EONS), even in women with intact membranes [53].

With respect to *E. coli*, phylogenetic classification according to the *E. coli* Reference Collection (ECOR) identify four main groups (ECOR A, B1, B2, D) [54]. Using this classification, Watt et al. characterized the intra-species genetic diversity of *E. coli*, to identify the strains more frequently isolated in the vagina, amniotic fluid and blood of neonates with sepsis [55]. ECOR B2 strains seemed to be better fit to colonize the vagina, and 84% of these strains were isolates in the amniotic fluid and blood of neonates with EONS. Genes for several virulence factors including *neuC*, *fimH* and *iucC* which are

responsible for transcription of K1-capsule antigen, type-1 pili and aerobactin, respectively, seem to be associated with the increased penetrability and survival ability of the ECOR B2 strains.

Recent studies highlighted the role of E-cadherin, in promoting bacterial attachment and invasion [56,57]. E-cadherin is a cell surface transmembrane protein with a role in maintaining vascular endothelium integrity. It is also expressed on the apical plasma membrane of syncytiotrophoblast and villous cytotrophoblast. Fardini et al. demonstrated that E-cadherin serves as receptor for the *F. nucleatum* surface adhesion molecule FadA [56]. The interaction between E-cadherin and FadA was postulated as being responsible for cellular adhesion and invasiveness of *F. nucleatum*. To their surprise, co-incubation of *F. nucleatum* with *E. coli* enhances the ability of the latter to penetrate the endothelial cell layer. Thus, *F. nucleatum* may facilitate passage of multiple microorganisms across fetal membranes and placenta. If true, this interaction may offer an alternative explanation for the polymicrobial character of intra-amniotic infection, and why *F. nucleatum* is a frequent isolate in such clinical circumstances. Interactions between E-cadherin and the bacterial surface protein, internalin, may also be exploited by *Listeria monocytogenes* to cross the placental barrier and hematogenously infect the fetus [56].

The above data suggest that both host-bacterial and bacterial-bacterial interactions are likely determinants of the susceptibility of particular women for intra-amniotic infection and PTB. One of the best examples is that of bacterial vaginosis. An association [odds ratio (OR): 1.4-2.2] between bacterial vaginosis and spontaneous PTB has been previously reported [58]. A proposed theory is that bacterial vaginosis facilitates an ascending pathway of intra-uterine infection that triggers an inflammatory reaction which in turn increases the risk of PTB. In support of this hypothesis, by using 16S-rRNA molecular tools, Fredricks et al. showed that bacterial vaginosis facilitates overgrowth of facultative bacteria such as *Mycoplasma*, *Gardnerella vaginalis*, *E. Coli*, and *GBS* [59]. Surprisingly, however, treatment of bacterial vaginosis with metronidazole increased even further the incidence of PTB [60]. Thus, in the absence of a better understanding of the pathophysiology of PTB, the translational relevance of microbiological inventories of the maternal genital tract for virulent bacterial subpopulations, remains uncertain.

4. Intra-Amniotic Inflammation

Inflammation encompasses the array of host defenses in response to a trigger. Although some features are common, the spectrum of the inflammatory reactions is highly dependent on the nature of the inducer (i.e. bleeding, trauma, auto-immunity, infection). The clinical circumstances of intra-amniotic infection and fetal sepsis poses specific challenges for both mother and her unborn fetus. That is, a robust intrauterine inflammatory reaction to infection (chorioamnionitis) remains a major risk for poor fetal outcome [10,32,1]. Consequently, rapid and accurate diagnosis of *in utero* inflammation is vital to develop targeted interventions to improve neonatal outcome.

The mechanisms of infection-induced inflammation have been extensively reviewed [62,63]. To understand why cytokines and several other inflammatory molecules have been proposed as diagnostic biomarkers of intra-amniotic inflammation and/or infection, several remarks are needed.

4.1 General aspects of innate immunity and Pathogen Associated Molecular Patterns (PAMPs) in human pregnancy

Two complementary inflammatory systems to detect and clear pathogens have been described: the innate and the adaptive immune systems. The genetically programmed innate

immune system is the first to be activated by pathogens, and in the majority of cases it is sufficient to clear the infection. Its limitation is inherent in its ability to detect only invariant features of the pathogens (Pathogen Associated Molecular Patterns - PAMPs), which are not unique to a specific microbe [62]. Bacterial PAMPs consist of molecular cell wall entities such as lipopolysaccharide (Gram negative bacteria), peptidoglycan and lipoteichoic acid (Gram positive bacteria), or various lipopeptides [macrophage-activating lipopeptide 2 (MALP-2), P48 and M161Ag] for *Mycoplasma* and *Ureaplasma* spp [64]. Detection of PAMPs by the innate immune system occurs through pattern recognition receptors (PRRs), out of which TLRs are best characterized [62].

4.2 Toll-like receptors (TLRs)

Owing to their strategic positioning at the maternal-fetal interface (decidua), and fetal membranes, TLR2 and TLR4 are considered key players in the fight against infection [65]. TLR4 is recognized as the membrane-bound receptor that triggers LPS signaling of Gram negative microbes [62]. TLR2 is mainly involved in the recognition of lipoproteins, peptidoglycan, glycolipids of Gram positive bacteria, and *Mycoplasmataceae* [66].

While the full spectrum of TLR responses remains to be elucidated, it is known that once engaged by PAMPs, these bacterial sensors trigger a downstream molecular chain of events that leads to the synthesis and release of pro-inflammatory cytokines such as TNF- α , interferon- γ , cytokine IL-12, IL-6, IL-1, and many others via an NF- κ B-mediated mechanism [67]. Key chemokines secreted following TLR activation are IL-8, monocyte chemoattractant protein-1, -2, -3, -4, macrophage inflammatory protein-1 α , -1 and RANTES. Traditionally, it is believed that activation of TLRs induces a T_H1-cytokine type response (i.e. IL-2, interferon- γ , lymphotoxins). However, using genetically engineered animal models and a variety of *in vitro* cell culture systems, Pulendran et al. showed that TLR4 engagement preferentially triggered a T_H2-cytokine reaction consistent with the release of IL-4, IL-5, IL-6, and that of the anti-inflammatory cytokine IL-10, based on bacterial type [68]. The significance of this observation during human pregnancy remains to be clarified, but these results underlines that the balance between pro- and anti-inflammatory cytokine response can dictate the intensity and possible resolution of an infectious process.

Significant advancements have been made to understand the molecular mechanisms responsible for TLRs activation. It is currently recognized that engagement of TLRs by various PAMPs is not the only mechanism controlling an inflammatory response [62]. Soluble factors (i.e. soluble TLR2, soluble TNF- α) released as part of enzymatic membrane shedding of the receptors or alternative splice mechanisms, may play critical roles [69,70]. Endogenous damage-associated molecular pattern molecules (DAMPs) [62,71], also known as “alarmins”, are intracellular proteins that include among others the high-mobility group box-1 (HMGB1 or amphoterin) and S100 proteins. When released into the extracellular compartment secondary to cell activation, injury or necrosis, DAMPs become “danger signals” that specifically activate the receptor of advanced glycation end-products (RAGE) [71]. RAGE is a transmembrane receptor, member of the immunoglobulin superfamily and functions as a receptor for advanced glycation end-products (AGEs), HMGB1 and S100 proteins [71]. Involvement of RAGE, HMGB1 and S100 proteins in the molecular events related to intra-amniotic inflammation has been demonstrated by our group [72,73].

4.2 Decidual inflammation

Accumulating data points toward choriodecidua as a major site of inflammatory processes linked to PTB [5,6]. The inflammatory events occurring within the choriodecidua are key because they may lead to premature activation of myometrial contractility and PTB through

synthesis and release of various prostaglandins and metalloproteases. However, as shown by immunohistochemistry and demonstrated *in vitro* using cultured decidual cells, in the setting of “silent” chorioamnionitis inflammatory cytokines and chemokines released secondary to TLR engagement lead to recruitment of inflammatory cells such as macrophages, dendritic cells and neutrophils, with the final purpose of killing the invading pathogens and halting their spread into the amniotic fluid [5,74]. Studies conducted in fetal membranes of pregnancies complicated by histological chorioamnionitis demonstrated that the leukocytes invading the chorion and amnion are maternal in their origin [75]. Based on these observations it is reasonable to assume that the development of a maternal inflammatory response (deciduitis), has some diagnostic potential, even if the process at the time of evaluation is at subclinical stage.

The process of cell migration into the inflamed decidual tissue is tightly controlled and involves chemotactic factors (chemokines) and cell adhesion molecules (i.e. selectins, integrins). The resultant microenvironment is rich in factors that induce tissue damage which consequently results in translocation of cytokines and chemokines into the amniotic fluid. Nitric oxide, vascular endothelium growth factor (VEGF), and angiopoietins seem to be involved [5,76].

4.3 Amniotic fluid cytokines

In the setting of intra-amniotic infection, a large array of inflammatory cytokines (i.e. IL-6, IL-8, IL-1, granulocyte-macrophage-colony-stimulating factor) are elevated in amniotic fluid. Their source may be the decidua, fetal membranes, the fetus or all of the above. However, independent of the source, these cytokines induce neutrophil chemotaxis, activation and degranulation with release of collagenolytic enzymes. In contrast to the neutrophils in the choriodecidua, those found in the amniotic fluid appear to be of fetal origin [77]. Taken together, these observations underline the fetal involvement in the process of intra-amniotic inflammation and its role in amplification of the inflammatory status of the amniotic fluid and tissue damage in a feed-forward loop fashion [10,14].

4.2 Bacteria and intra-amniotic inflammation

Once present in the amniotic fluid microorganisms can stimulate the production of pro-inflammatory cytokines through activation of fetal membrane TLR receptors [78]. Several microorganisms (i.e. *Ureaplasma*, *Mycoplasma*) are traditionally considered of low virulence [20,79]. Studies describing the presence of *Ureaplasma parvum* and *Mycoplasma hominis* in the amniotic fluid of second trimester asymptomatic women are in support of this concept [79]. Menon et al., demonstrated *in vitro* that *Ureaplasma* has lower pro-inflammatory properties on fetal membranes in comparison with Gram-positive and Gram-negative bacteria [78]. However, isolation of *Ureaplasma* and *Mycoplasma* from the amniotic fluid has been consistently associated with a wide range of adverse outcomes, such as early abortion, stillbirth, prematurity, and neonatal morbidity and mortality [80]. Although an intense intra-amniotic inflammatory response is often encountered at the time of the clinical manifestations of PTB, these studies identified association and not causation [81]. The evidence that the so called “silent” microorganisms carry the capability of triggering an inflammatory response *in vivo* and induce PTB was recently provided by Novy et al. [82]. Inoculation of *Ureaplasma urealyticum* and *Mycoplasma hominis* in the amniotic fluid of Rhesus monkeys was followed by an increase in the myometrial contractile activity that was preceded by an intense pro-inflammatory cytokine response and prostaglandins synthesis [82].

Following invasion of the amniotic fluid sac, Gram positive anaerobes, *E. coli* and *GBS* are well recognized triggers of intra-amniotic inflammation and fetal sepsis [21,53]. Yet, intra-

amniotic inflammation may be present in the absence of a positive amniotic fluid culture results [18,83]. As previously discussed, “uncultivated” or “difficult-to-cultivate” bacteria may play an important role [27]. The extent of the intra-amniotic inflammatory response triggered by each of these “uncultivated” or “difficult-to-cultivate” bacteria, separate or as a group, remains to be determined.

5. Non-Invasive Diagnosis of Intra-Amniotic Infection and Inflammation

5.1 Clinical chorioamnionitis

Clinical chorioamnionitis is traditionally diagnosed by maternal fever, tachycardia, uterine tenderness, leukocytosis, and foul odor of the amniotic fluid. Yet, clinical chorioamnionitis occurs in approximately 18% of pregnancies complicated by intra-amniotic inflammation [18], suggesting that unrecognized intra-uterine infection/inflammation contributes to prematurity and neonatal sepsis [84]. From a diagnostic perspective, recognition of clinical chorioamnionitis is a challenge only when other systemic maternal inflammatory conditions (i.e. pyelonephritis, appendicitis, inflammatory bowel conditions, pneumonia) may complicate clinical presentation. The situation is different when women present with symptoms of preterm labor with intact membranes or PPROM. In the absence of intra-amniotic infection/inflammation, these women can be managed expectantly, especially at an early gestational ages. Thus, over many decades, the attention of many investigators has focused on the development of various clinical diagnostic tests and algorithms aimed to detect intra-amniotic infection/inflammation, in “asymptomatic” women by using non-invasive tests.

5.2 Clinical usefulness of maternal serum and cervico-vaginal fluid acute-phase reactants and cytokines in “asymptomatic” women with chorioamnionitis

Diagnosis of intra-amniotic infection/inflammation can be potentially augmented by routine laboratory studies [i.e. white blood cell (WBC) count], and assays of various acute phase reactants [i.e. C-reactive protein (CRP), lipopolysaccharide-binding protein] performed on maternal blood and cervico-vaginal secretions. The clinical utility of maternal serum CRP, IL-6, IL-8, TNF- α , G-CSF and defensins was recently reviewed [85,86]. Following evaluation of a large body of literature the main conclusions were: **i)** elevated maternal serum CRP levels are associated with an increased risk of intra-amniotic infection/inflammation, but the overall ORs are modest (~ 2.0); **ii)** in women with intact membranes the cervico-vaginal, but not maternal plasma cytokines (i.e IL-6, IL-8), may carry some diagnostic potential with higher ORs (~ 3.0); **iii)** addition of various screening tests such as cervical length and fetal fibronectin may have additive diagnostic value for intra-amniotic infection; **iv)** the clinical utility of maternal blood CRP and various cytokine levels is limited for diagnosis of intra-amniotic infection/inflammation due to the wide range of cut-offs reported as optimal; **v)** none of the current maternal serum or cervico-vaginal markers alone or in combination seem to offer adequate sensitivity and specificity for the medical diagnosis of intra-amniotic infection/inflammation in “asymptomatic” women; **vi)** diagnostic accuracy studies including women with inflammatory conditions other than intra-amniotic inflammation have yet to be performed.

5.3 Maternal serum biomarkers and intra-amniotic infection

Proteomics-based biomarker identification in reproductive tissues and fluids has registered significant advances for the last decade. The general principles of proteomics in human pregnancy have been extensively reviewed [87]. Several studies have taken an unbiased approach aimed to discover human maternal serum proteomic peptides characteristic of PTB [88,89]. Pereira and colleagues, described the presence of 23 peptides that had the ability to discriminate PTB [88]. Differentially expressed serum peptides associated with PTB

belonged to various functional pathways such as complement/coagulation cascade (i.e. serotransferin, coagulation factors -VII, -XII, -XIII, -1 anti-trypsin, complement components), fetal-placental development (i.e. insulin-growth factor II, apolipoprotein-C, -A, -B), extracellular matrix proteins (i.e. serum amyloid-P, transthyretin, gelsolin, afamin, plasma retinol-binding protein) and inflammation/immune response (i.e. protein S100, apolipoprotein A, haptoglobin, ADAM-12). In a second study, Esplin et al. reported that three peptides belonging to inter- α -trypsin inhibitor heavy chain-4 protein were reduced in women with spontaneous PTB at 24-28 weeks [89]. Candidate serum inflammatory biomarkers included defensins, ferritin and TNF- α receptor type-I. The diagnostic performance of 9 biomarkers reached a sensitivity of up to 86.5% and specificity up to 80.6% at the beginning of the 3rd trimester. The study design for both studies provided evidence that PTB is associated with inflammatory events, but did not allow discovery of maternal serum biomarkers specifically diagnostic for intra-amniotic infection or histological chorioamnionitis.

In a primate model of *GBS*, *Ureaplasma* and *Mycoplasma*-induced intra-amniotic infection/inflammation, Gravett et al. identified S100A9 (calgranulin-B), a fragment of insulin like-growth-factor-binding-protein-1 (IGFBP-1), and several other immuno-regulator proteins as biomarkers characteristic of intra-amniotic infection/inflammation [90]. For clinical translational relevance the authors searched for the presence of the two fragment peptides in the serum of women with “silent” chorioamnionitis. Antibodies developed against IGFBP-1 and calgranulin-B, revealed their presence in human serum, but the small number of analyzed samples did not permit the evaluation of the diagnostic performance of the two biomarkers. In sum, at the current stage of clinical practice, no single or panel of maternal serum biomarkers is sufficiently reliable to allow a non-invasive diagnosis of intra-amniotic infection/inflammation.

5.4 Cervico-vaginal peptide biomarkers and intra-amniotic infection

The endo-cervical mucosa and the cervical mucus plug are positioned strategically in the vicinity of the fetal membranes. The clinical implication is that the presence of specific biomarkers in the cervico-vaginal fluid may reflect activation of signaling pathways secondary to intra-amniotic infection. Given the proximity of the cervix to the gestational sac, cervico-vaginal fluid may prove a better biological sample than maternal blood for a non-invasive diagnosis of intra-amniotic infection in the “asymptomatic phase” of the disease and in the absence of PPROM.

Significant innovations in mass spectrometry have provided a wealth of information about the cervico-vaginal proteome. This subject was recently reviewed by Zegels et. al. [91]. What can be concluded from the large body of literature is that: **i)** the cervico-vaginal proteome consists of thousands of peptide fragments belonging to various immunoregulatory proteins (i.e. lacto-transferin, haptoglobin, lactoferrin, and azurocidin, S100A9, S100A8, defensins, IL-1 receptor antagonist); **ii)** biomarker expression level and identity of the parent protein are highly dependent on the proteomic technique and the various experimental modalities used for their identification and quantitation; **iii)** there is overlap among cervico-vaginal, amniotic fluid and maternal serum proteins, which may offer some diagnostic potential for PTB.

The proof of concept that non-invasive diagnosis of intra-amniotic infection/inflammation is possible through the analysis of the cervical-vaginal fluid proteome was provided by Gravett et al. [92]. Using a primate model of *Ureaplasma* induced intra-amniotic infection/inflammation, and following a comprehensive survey of the cervical-vaginal fluid proteome the authors identified that calgranulin A and B, azurocidin, IGFBP-1 and possible defensins were upregulated in both amniotic and cervical-vaginal fluids [92]. Our group demonstrated

that calgranulins and defensins are immunoregulatory proteins present in the cervical-vaginal fluid of women with normal pregnancy outcomes [45]. From this perspective, the differential level in expression of these peptides rather than their presence may offer some diagnostic potential that awaits validation.

6. Invasive Diagnosis of Intra-Amniotic Infection and Inflammation

6.1 Rapid diagnostic tests of amniotic fluid infection

Analysis of the amniotic fluid allows for a direct evaluation of the microbial and inflammatory status of the gestational sac. As described, assessment of inflammatory mediators may help clarify the perinatal infectious status of the amniotic fluid because routine microbial cultures and Gram stain may return negative even in the presence of true bacterial infection.

Amniotic fluid WBC count is regarded as the “gold standard” for assessment of intra-amniotic inflammation in the setting of infection. Simplicity of a manual cell count and availability of various automatic techniques make the WBC count an easily available test. Still, the interpretation of the WBC count based on various cut-offs may sometimes prove difficult. WBC lysis by the bacterial inoculum may artificially lower the absolute number of neutrophils. Moreover, in the setting of blood contamination corrective count methods are necessary [93]. The above observations suggested that the amniotic fluid WBC count is best interpreted together with results of other rapid diagnostic tests. Proposed additional markers are glucose concentration, lactate dehydrogenase activity, components of the complement system, matrix-metalloproteases (i.e. MMP-8, MMP-9) or pro-inflammatory cytokines [94,95,96]. The results of one of the largest comparative studies of the diagnostic performance of amniotic fluid pro-inflammatory markers for prediction of intra-amniotic infection were recently communicated by Jacobsson et al [97]. Predictive models based on 27 amniotic fluid proteins (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, soluble IL-6R, interferon- γ , monocyte chemoattractant protein-1, transforming growth factor- β , macrophage inflammatory protein-1 α and -1 β , MMP-9, TREM-1, brain-derived neurotrophic factor, granulocyte macrophage colony stimulating factor, neurotrophin-3, neurotrophin-4, TNF- α , migration inhibitory factor, and RANTES) were created using a stepwise binary logistic regression. The highest predictive values for detection of intra-amniotic infection were attained by IL-18, IL-1, IL-6 and RANTES [97]. In the multivariate model TNF- α and IL-6 contributed significantly to detection of amniotic fluid infection with ORs of 15.9 [95% CI: 2.1–122.2 ng/ml], and 8.3 [95% CI 1.7–40.6 ng/ml], respectively. The combination of the two cytokines had a +LR of 6.0.

6.2 Peptide biomarkers and intra-amniotic infection

Because few interventions utilizing inflammatory cytokines have shown clinical benefit, for the past decade several investigators focused their efforts on the quest for identification of amniotic fluid biomarkers that would allow for an accurate diagnosis of amniotic fluid infection. Most of the studies seeking to identify amniotic fluid diagnostic protein biomarkers have consistently relied on various mass spectrometry technologies [98,99]. Although a large number of peptides were identified, only a few studies implemented a validation phase of the most promising biomarkers [98].

Using on-chip immunoassays and peptide mass fingerprinting our group discovered that 4 amniotic fluid protein biomarkers, neutrophil defensin-2, neutrophil defensin-1, calgranulin-C and calgranulin-A, carry a robust diagnostic accuracy for intra-amniotic infection-induced inflammation [98]. From the perspective of clinical application, we applied a bioinformatics filtering strategy to reduce the proteomics information in each mass spectrometry tracing into a numeric variable that characterized each sample. We named our strategy “Mass

Restricted” scoring. In the original study we developed the MR score which ranged from 0 to 4 depending on the presence or absence of each of these 4 biomarkers [98]. An MR score of 3 or 4 indicated the presence of inflammation, while scores of 0, 1 or 2 were considered to exclude it. The next step was to compare in real time the MR score to previously established or proposed markers of intra-amniotic fluid inflammation/infection: glucose, WBC, lactate dehydrogenase activity, Gram stain, IL-6, and MMP-8 [18]. The key findings of our comparisons revealed that the combination of the Gram stain and MR score performed best in predicting infection (Gram stain: OR=43.9 [95% CI: 9.6-200.5], and MR score: OR=19.6 [95% CI: 6.5-59.0]. Interestingly, five laboratory tests (glucose, Gram stain, LDH, IL-6, MMP-8) were concordant in establishing inflammation in only 21% of the analyzed samples, underlying the difficulties clinicians may face in interpreting discordant results. The MR score alone correctly identified inflammation in 93% of the cases.

7. Conclusion

The field of metagenomics and the development of DNA sequence-based culture independent methods have provided a broader view about the infectious etiology of intra-amniotic inflammation. However, the pathogenic relationships among the presence of various bacteria in amniotic fluid, the nature of the host response, and the mechanisms leading to the initiation of PTB still remain to be characterized. Maternal serum and cervico-vaginal secretions have been exhaustively mined for diagnostic markers of intra-amniotic infection-induced PTB. The presented evidence articulates two vital clinical needs: discovery of biomarkers with true clinical utility and implementation in the clinical laboratories of technological innovations to allow a better transfer of laboratory discoveries into new diagnostic methods. Additionally, enhancing our understanding of how intra-uterine infection/inflammation leads to PTB and antenatal fetal damage remains critical to future development of therapeutic options.

8. Expert Opinion

The ultimate goals of research conducted in the field of intra-amniotic infection are quite ambitious. Specific goals are to identify women at risk, non-invasively diagnose an ongoing asymptomatic intra-amniotic infectious/inflammatory process, recognize fetuses at danger of intra-uterine damage, optimally time delivery, and lastly, develop preventive and targeted therapies. So far, diagnostic efforts have focused on microbiological laboratory techniques identifying amniotic fluid bacteria in culture, cataloguing uncultivable species, understanding the clinical utility of various inflammatory biomarkers in predicting fetal/neonatal outcome, and discovering of biomarkers predictive of intra-amniotic infection amniotic fluid, maternal blood and cervico-vaginal secretions of women with symptoms of PTB. Based on the available literature, what can be concluded is that none of the proposed non-invasive maternal serum or cervico-vaginal biomarkers reached sufficient diagnostic robustness to be implemented alone or in combination into current clinical practice. Thus, it remains clear that a better understanding of the pathophysiology of infection-induced PTB is necessary. Key concepts and ongoing research in order to achieve the aforementioned goals include: rapid and accurate identification of the microbial agents responsible for intra-amniotic infection, development of rapid molecular techniques to screen for colonization with specific microbes prior to infection, incorporation in the routine clinical practice of a battery of tests to allow profiling of the maternal genetic make-up able to forecast susceptibility to infection/inflammation. These achievements should increase our chances to prevent intra-amniotic infection-induced PTB, predict treatment response and prevent fetal damage during intra-uterine development.

Serologic and cervico-vaginal assays that can identify activation of the host response subsequent to microbial invasion of the amniotic fluid cavity are promising, but not yet available clinically. A possible explanation for our difficulties in identifying relevant serum or cervical biomarkers may be related to the time of sampling *vis-a-vis* the natural progression of the disease. The compartmentalization process characteristic to human pregnancy is complex and critical for survival of the mother and fetus. Because the inflammatory process is successfully restrained within the gestational sac in its initial stages, sampling of the maternal and cervico-vaginal compartment may yield a false negative result. Only in advanced stages of the infectious process, when defense mechanisms are overwhelmed, may the various biomarkers become useful for diagnostic purposes. This paradigm may explain the large heterogeneity in the number and nature of biomarkers proposed as having diagnostic value for intra-amniotic infection, as well as the large variance in the cut-off chosen to indicate test positivity for the biomarkers currently used in the clinical practice (i.e., glucose, LDH, WBC count).

Recent data demonstrating that poor neuro-developmental outcome and cerebral palsy may have an inflammatory perinatal origin strengthens the argument that rapid and accurate diagnosis of occult intra-uterine infection is needed [100]. Some advocate for a limited value of amniocentesis in the clinical management of women with preterm labor given that the invasive nature of the procedure and that the evidence-based benefit has yet to come. The first measure of clinical utility for the amniocentesis procedure should be the preventable neonatal morbidity and mortality that will be eliminated through shortening the exposure of the fetus to intra-amniotic infection and/or inflammation. The only way to assess the impact of amniocentesis on preventable neonatal morbidity and mortality is through randomized clinical trials. It is possible that ethical concerns preclude performance of such studies. However, from the perspective of an increased incidence of chorioamnionitis in PPROM women managed expectantly, high frequency of histological chorioamnionitis in women who deliver preterm in the absence of clinical symptoms, and the observation that chorioamnionitis is a known significant risk factor for the development of periventricular leukomalacia and cerebral palsy, rescuing the fetus from an “unfriendly” environment remains an acceptable clinical concept.

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9. Article Highlights

- Biomarkers for diagnosis of intra-amniotic infection/inflammation in a non-invasive fashion are urgently needed.
- Conventional culturing methods alone provide an incomplete picture of the microbial diversity of intra-amniotic infection.
- Metagenomic technologies demonstrated that in intra-amniotic infection the microbiome spectrum is rich and includes uncultivated and difficult-to-cultivate species.
- Susceptibility to intra-amniotic infection may be dependent on maternal genetic make-up and colonization of the genital tract with various bacterial clones.
- Maternal serum and cervico-vaginal inflammatory markers alone or in combination offer limited medical diagnosis usefulness for diagnosis of intra-amniotic infection/inflammation in “asymptomatic” women.
- Mass spectrometry is a revolutionary and powerful technology for diagnosis of intra-amniotic infection/inflammation, but its use as a diagnostic tool carries important caveats likely related to the compartmentalization process of human gestation.
- Implementation of novel technologies is needed for the rapid and efficient translation of discoveries to clinical practice.