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Změny imunity indukované intraamniálním zánětem

Changes of immune parameters induced by intraamnial inflammation

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Prohlášení autora

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Obsah

Seznam použitých zkratek

ATP	adenosine triphosphate
AUC	area under the curve
CCL	C-C motif ligand
CD	cluster of differentiation
CLU	clusterin
CR3	complement receptor 3
CRP	C-reactive protein
DAMP	damage-associated molecular pattern
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immuno sorbent assay
ER	endoplasmatické retikulum
Fc	fragment constant
FcgammaBP	Fcgamma-binding protein
FIRS	fetal inflammatory response syndrome
G-CSF	granulocyte-colony stimulating factor
GDF-15	growth differentiation factor 15
Gr	granzym
HCA	histologic chorioamnionitis
HMGB1	high-mobility group box 1
HSP	heat shock protein
IAI	intraamnial inflammation
ICAM-1	intercellular adhesion molecule 1
Ig	imunoglobulin
IgGFcBP	IgGFc-binding protein
IGFBP	insulin-like growth factor-binding protein
IL	interleukin
IRF	interferon regulatory factors
ITGAM	integrin alpha M
LPS	lipopolysacharid
Mac-1	macrophage-1 antigen

MALDI	matrix assisted laser desorption/ionization
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MHC	major histocompatibility complex
MIAC	microbial invasion of amniotic cavity
MIP	macrophage inhibitory protein
miRNA	microRNA
NFkB	nuclear factor kappa B
NK	natural killer
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PPROM	preterm premature rupture of membranes
PR3	proteináza 3
PRR	pattern recognition receptors
POCT	point of care testing
PTL	preterm labor
$P2X_7R$	P2X purinoceptor 7
RAGE	receptor for advanced glycation endproducts
RNA	ribonucleic acid
ROC	receiver operating characteristic
ROS	reactive oxygen species
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
TGFβ	transforming growth factor beta
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRPM-2	testosterone-repressed prostate message-2

Souhrn

Intraamniální infekce hraje významnou roli v etiologii předčasného porodu a může vést k vážnému ohrožení zdraví plodu. Diagnostický přístup představuje přímý mikrobiologický průkaz infekčního agens či nepřímý průkaz pomocí stanovení rozličných biomarkerů, jejichž koncentrace se během intraamniální infekce zvyšuje. Vzhledem k povaze infekce probíhá stanovení těchto parametrů z plodové vody, což činí tuto diagnostiku náročnou pro lékaře i rodičku a rutinně nedostupnou. Disertační práce komentuje publikované výsledky vědeckého týmu, jehož cílem bylo vytipování vhodných markerů infekce, stanovení jejich koncentrace v plodové vodě a u pozitivních nálezů otestovat jejich diagnostický potenciál v cervikální tekutině, tedy v biologickém materiálu, jež může být odebrán neinvazivním způsobem. Vzorky plodové vody a cervikální tekutiny pocházely od žen s jednočetným těhotenstvím, které bylo ukončeno předčasným porodem, a které bylo u části kohorty komplikováno intraamniálním zánětem a infekcí. Bylo zjištěno, že z testovaných molekul je u intraamniální infekce statisticky vyšší koncentrace kalretikulinu, katepsinu G, CD11b, FcgammaBP a MIP1α v plodové vodě. V cervikální tekutině byla prokázána významně vyšší hladina FcgammaBP u pacientek s intraamniální infekcí při současném předčasném odtoku plodové vody. Jedná se tak o diagnosticky nejužitečnější nově prokázaný marker intraamniální infekce.

Summary

Intraamniotic infection plays an important role in the etiology of preterm birth and can lead to a serious threat to fetal health. The diagnostic approach is based on direct microbiological detection of an infectious agent or indirect detection by determining various biomarkers, which concentration increases during intraamniotic infection. Due to the nature of the infection, these parameters are determined from amniotic fluid, which makes this diagnosis difficult for both the doctor and the mother and routinely unavailable. The dissertation comments the published results of a scientific team whose aim was to identify suitable markers of infection, determine their concentration in amniotic fluid and test their diagnostic potential in cervical fluid, ie biological material that can be collected non-invasively. Amniotic and cervical fluid samples were taken from women with singleton pregnancies with preterm labor and that were complicated by intraamniotic inflammation and infection in part of the cohort. It was found that among the tested molecules there is a statistically higher concentration of calreticulin, cathepsin G, CD11b, FcgammaBP and MIP1α in amniotic fluid during intraamniotic infection. Significantly higher levels of FcgammaBP were found in the cervical fluid samples from patients with intraamniotic infection with preterm leakage of amniotic fluid. It is thus the most diagnostically useful newly demonstrated marker of intraamniotic infection.

Úvod do problematiky

Charakteristika předčasného porodu

Předčasný porod je v současné době nejzávažnější porodní komplikací v zemích hospodářsky rozvinutého světa. Je spojen s vysokou mírou rizika zdravotních komplikací pro matku i dítě, a jeho včasné rozpoznání je důležité pro nastavení adekvátní péče o rodičku [1]. Předčasný porod je definován jako porod před dokončeným 37. týdnem těhotenství [1][2]. Dle gestačního stáří je rozlišován extrémně předčasný porod (před ukončeným 28. týdnem gestace), velmi předčasný porod (mezi 28. a 31. týdnem gestace) a mírně předčasný porod (mezi 32. a 36. týdnem gestace), přičemž platí, že riziko zdravotních komplikací plodu klesá se stoupajícím gestačním stářím [3]. Hranice viability se pohybuje mezi 22. až 24. týdnem gestace, při poskytování aktivní péče se vychází z pravděpodobnosti přežití a přežití bez závažných zdravotních komplikací [4]. Odhaduje se, že předčasným porodem je ukončeno 5 – 18 % všech těhotenství [5], světová zdravotnická organizace udává hodnotu 10,5 % všech jednočetných těhotenství celosvětově [6]. Ročně dojde ve světě ke zhruba 15 milionům předčasných porodů a přes milion dětí v důsledku předčasného porodu zemře, což z předčasného porodu činí nejčastější důvod úmrtí novorozenců a druhý nejčastější důvod úmrtí dětí do pěti let [7]–[9]. Předčasný porod může rovněž vést ke zvýšenému riziku zdravotních komplikací u dospělých, kteří se narodili předčasně [6], což je pravděpodobně z části způsobeno narušením epigenetického řízení genové exprese plodu při předčasném porodu [10]. V USA byl v posledních letech zaznamenán mírný pokles prevalence předčasného porodu (11,4 %), kterému však předcházel dlouhodobý a konstantní růst po tři desetiletí od roku 1980 s maximem v roce 2006, kdy se předčasně narodilo 12,8 % dětí [11]. Předčasný porod může být aktivně vyvolán z důvodů ohrožení zdraví matky či plodu (iatrogenní předčasný porod) či probíhá spontánně. Spontánní předčasný porod je komplikace častější, představuje zhruba dvě třetiny předčasných porodů [5]. Dle stavu plodových obalů při předčasném porodu je rozeznáván předčasný porod s předčasným odtokem plodové vody (preterm premature rupture of membranes; PPROM) či se zachovalým vakem blan (preterm labor; PTL) [1]. PPROM představuje přibližně jednu čtvrtinu až jednu třetinu předčasných porodů [1][12], v USA bylo v posledních letech zaznamenáno

asi 175 000 porodů komplikovaných PPROM ročně [13]. Na předčasný porod a jeho fenotyp mají vliv i socioekonomické podmínky, stravovací návyky, etnikum či region [14].

Intraamniální zánět

Etiologie předčasného porodu je velmi složitá, a předčasný porod by tak měl být vnímán spíše jako syndrom tvořený různými patologickými událostmi s různými příčinami, než jako jeden přesně definovatelný patofyziologický děj [5]. Do těchto příčin patří jak příčiny neinfekční, tak i intraamniální infekce s jednoznačně prokázanou patologickou souvislostí s předčasným porodem [15]. Společným jmenovatelem těchto jevů je intraamniální zánět (intraamnial inflammation; IAI), který je aktivován jak vnitřními (sterilními), tak vnějšími (nesterilními) noxami. Vzhledem k faktu, že podání antibiotik není dostatečně účinným prostředkem v prevenci předčasného porodu u žen s mikrobiálním nálezem, se lze domnívat, že je to zejména zánětlivá odpověď, která vede k předčasnému porodu a nikoliv přítomnost bakterií sama o sobě [16][17]. Zánět ve svém obecném pojetí by však neměl být vnímán jako jev patologický a nežádoucí. Naopak, koordinované a citlivě regulované zánětlivé změny jsou klíčové pro úspěšné těhotenství. Vědomé zánětlivé procesy jsou zásadní pro všechny fáze těhotenství, od implantace blastocysty do děložní sliznice a placentaci až po samotný porod, kdy snížený imunitní dohled a řízené zánětlivé změny stimulují aktivaci buněk myometria, zkracování děložního hrdla a rozrušení plodových obalů. Zánětlivé procesy se rovněž nezbytně účastní ovulační a menstruační fáze menstruačního cyklu [17][18]. Imunita těhotné ženy je přitom lokálně výrazně regulována a vedena k tolerogennímu nastavení, jež zajišťuje řada mechanismů a buněk se specifickými vlastnostmi v mikroprostředí na rozhraní matky a plodu [19]. Teprve místně a časově nekoordinované zánětlivé procesy, které naruší tuto citlivou rovnováhu, mohou vést k PPROM či PTL s negativními důsledky pro plod. IAI doprovází zvýšená koncentrace mediátorů zánětu v plodové vodě [20][21], v případě intraamniální infekce je prokázána i přítomnost bakterií [22]. Kromě intraamniální infekce mohou zánětlivé změny spustit i signály vnitřního poškození, které infekční příčinu nemají. Jedná se o tzv. zánět sterilní. Při nekróze či zánětlivé stimulaci buněk placenty či plodových obalů se z těchto buněk uvolňují signály vnitřního poškození, tzv. alarminy nebo také DAMP (damage-associated molecular patterns). Jedná se nejčastěji o volnou extracelulární DNA, ATP, IL-1α, high-mobility group box protein 1 (HMGB1), kyselinu močovou či proteiny

teplotního šoku (heat shock proteins; HSP). Tyto signály nebezpečí jsou rozeznávány buňkami trofoblastu, buňkami plodových obalů a leukocyty na feto-maternálním rozhraní, jež stimulují k tvorbě cytokinů a chemokinů skrze aktivaci příslušných PRR receptorů (pathogen recognition receptors), zejména rodiny TLR (Toll-like receptors) [23]. To má za následek migraci dalších leukocytů do místa poškození a amplifikaci zánětu [17]. Schwenkel a kol. představili práci, ve které intraamniálně aplikovali alarmin HSP70 březím myším. Ačkoliv podání HSP70 nemělo vliv na časnost porodu, výrazně zvýšilo riziko podváhy mláďat a riziko jejich úhynu [24]. Příčin, které vedou ke sterilnímu zánětu je celá řada, jedná se např. o oxidační stres, podvýživu, kouření, chronický psychický stres či silné trauma, vaskulární, děložní a cervikální poruchy, špatná implantace trofoblastu, předčasné stárnutí plodových obalů nebo hypoxie [5][17]. Wheler a Oyen dávají i do souvislosti vyšší riziko předčasného porodu a změny atmosferického tlaku při hurikánech [25]. Při zánětu povahy infekční TLR rozpoznávají typické bakteriální, fungální či virové molekulární struktury (pathogen-associated molecular patterns; PAMP). TLR jsou receptory solubilní, ukotvené na buněčné membráně a také se nachází v nitrobuněčných kompartmentech buněk vrozené imunity, ale i buněk amniálního epitelu či mezenchymu, který se tak významně podílí na protiinfekční obraně plodu [23][26]. Stimulace těchto receptorů, ať už cestou sterilního či infekčního prozánětlivého signálu, aktivuje nitrobuněčné dráhy spojené s aktivací transkripčních faktorů, které vedou k přepisu genů pro prozánětlivé cytokiny. Zánětlivý proces tak s sebou nese zvýšení hladiny zánětlivých mediátorů, jako jsou IL-1β, IL-6, IL-8, G-CSF či TNFα, což má za následek infiltraci a aktivaci neutrofilních granulocytů a monocytů spojenou se zvýšenou tvorbu prostaglandinů a matrix-degradujících enzymů. Tyto látky pak stimulují děložní kontrakce a rozrušují mezibuněčnou hmotu plodových obalů, což vede zákonitě k jejich předčasné ruptuře a porodu [27][28]. Významným průvodním jevem doprovázejícím každý zánět je zvýšená produkce reaktivních kyslíkových intermediátů (ROS), jež vede k oxidačnímu stresu. I porod v řádném termínu je charakterizován redoxní dysbalancí a akumulací ROS v plodové vodě a placentě [29]. Patologické i fyziologické signály zvyšující oxidační stres vedou k aktivaci kinázy p38MAPK (mitogen-activated protein kinase) v buňkách plodových obalů, což podpoří jejich stárnutí a prozánětlivé nastavení [30]. Stárnoucí buňky plodových obalů vykazují tzv. se stárnutím asociovaný sekretorní fenotyp, který se projevuje uvolněním cytokinů, chemokinů, růstových faktorů, enzymů a signálů poškození DAMP [31]. Tyto uvolněné látky se vážou na TLR buněk v okolí, vyvolávají aktivaci prozánětlivého

transkripčního faktoru NFκB (nuclear factor kappa B) a sestavení inflamasomu a jsou tak dále stimulovány k jejich prozánětlivému nastavení, čímž se vytváří zpětnovazebná smyčka rozšiřující zánět [23][31]. V případě řádného termínu porodu jsou těmito signály známky zralosti orgánů plodu, které se uvolňují do plodové vody. Jedná se o signály, které informují o zralosti plic, ledvin či mozku plodu [32]. Tyto signály jsou v plodové vodě detekovatelné během celého těhotenství, jejich zvýšená koncentrace, která informuje o vhodné době k porodu, však překoná tolerogenní nastavení imunity a stimuluje prozánětlivé nastavení amniálního epitelu a aktivaci hladké svaloviny myometria [32][33]. Dalším signálem pro fyziologický zánět je zvýšené mechanické napětí plodových obalů, které se zvyšuje s velikostí plodu. Rozpínání plodových obalů, jejichž životnost dosahuje svého limitu, vede k uvolnění prozánětlivých cytokinů spojených s porodem, jako je IL-8 [34] i k aktivaci p38MAPK, byť v menší míře [32]. Změny v enzymech, které se podílí na udržení elasticity obalů a jejich mechanických vlastnostech, mohou vést k předčasné ruptuře obalů k a porodu před řádným termínem [35]. Dalšími fyziologickými signály mohou být snížené hladiny antioxidantů a zvýšené metabolické nároky plodu, k aktivaci p38MAPK vede také zvýšená hladina fragmentů telomerové DNA, která hlásí, že buňky plodových obalů vyčerpaly svou mitotickou kapacitu [36]. Injekce fragmentů telomer vedla u březích zvířecích modelů k aktivaci p38MAPK, urychlení stárnutí a k předčasnému porodu [37]. I tento průvodní jev zrání plodových obalů je tak součástí orchestru místně a časově koordinovaného signalizačního procesu, který udává vhodnou dobu k porodu. Patologické sterilní i nesterilní signály stimulující produkci ROS a oxidační stres byly popsány výše. Molekulární dráhy vedoucí k aktivaci p38MAPK nemusí být pro různé stimuly identické, avšak její předčasná aktivace může mít za následek předčasný porod z důvodů předčasného navození signálu stárnutí. Behnia a kol. prokázali, že prozánětlivý stimul sterilní vyvolává vyšší úroveň oxidačního stresu spojeného s aktivací p38MAPK a stárnutím buněk plodových obalů, než podnět infekční, který aktivuje zánět preferenčně skrze odlišnou signalizační dráhu a transkripční faktor [38][39]. Ti samí autoři však zároveň uvádějí, že i když bakteriální lipopolysacharid (LPS) zvyšoval hladinu ROS méně než sterilní prozánětlivé podněty (cigaretový kouř, polutanty), i tento infekční stimul aktivoval stárnutí buněk plodových obalů [39]–[41]. Zároveň však nepozorovali významný rozdíl v hladinách markerů zánětu v plodové vodě, pupečníkové či mateřské krvi u zánětu vyvolaném infekčními podněty či oxidačním stresem, což naznačuje, že zánět je klíčovým patologickým procesem bez ohledu na dráhu svého spuštění [42].

V reálném komplexním biologickém systému však bude situace velmi pravděpodobně mnohem komplikovanější. Buňky plodových obalů vykazují schopnost proliferace, migrace a schopnost transformace do různých typů buněk, podobně jako buňky kmenové [43]. Díky těmto vlastnostem je možné udržet plodové obaly pevné a pružné po celé těhotenství.
K pevnosti a pružnosti plodových obalů přispívá významný podíl mezibuněčné hmoty, kterou tvoří především proteoglykany, glykoproteiny, biglykany, kolagen, hyaluronan a dekorin [44][45]. Remodelace a neustálá sebeobnova plodových obalů však vede i k zákonitému odlučování a zániku buněk, což vede ke vzniku mikrofraktur v plodových obalech. Tyto mikrofraktury, spojené s jistou mírou zánětu a oxidačního stresu, jsou však pro remodelaci tkáně nezbytné [46]. Se zráním obalů se schnopnost sebeobnovy buněk zpomaluje a v době porodu je počet i velikost mikrofraktur největší [46]. Rovněž plodové obaly při PPROM vykazují větší množství mikrofraktur, než plodové obaly při porodech bez jejich předčasné ruptury [43]. Hojení těchto mikrofraktur doprovází přeměna epitelových buněk na buňky mezenchymální a jejich následná přeměna zpět, přičemž by mezi epitelovými a mezenchymálními buňkami měl být zachován ideální poměr. Proces hojení mikrofraktur tlumí oxidační stres, který udržuje buňky plodových obalů v mezenchymálním nastavení [43]. Tranzici epitelových buněk na buňky mezenchymální podporuje transformující růstový faktor beta (transforming growth factor beta; TGFβ), jehož koncentrace v plodové vodě stoupá s blížícím se porodem a jehož koncentraci zvyšuje i oxidační stres, přičemž při zablokování TGFβ byla zjištěna i redukovaná aktivita prozánětlivé kinázy p38MAPK [47][48]. Jelikož jsou mezenchymální buňky citlivé na zánět a přítomnost ROS, mezenchymální nastavení přetrvává při fyziologickém porodu, kdy usnadňuje rozrušení plodových obalů [48][49]. Mezenchymální přeměna je asociována s aktivací maxtrix-degradujících enzymů a rozrušením kolagenu, což vede k degradaci bazální membrány plodových obalů a k porodu [48]. Regulace počtu mezenchymálních buněk a jejich tranzice do buněk epitelových vede naopak ke zvýšené produkci kolagenu a zvýšení integrity plodových obalů, přimčemž bylo prokázáno, že tuto přeměnu stimuluje hormon progesteron, který vykazuje obecně protizánětlivé vlastnosti [48]. Při fyziologickém těhotenství tak poměr epitelových a mezenchymální buněk reguluje citlivá rovnováha mezi TGFβ a progesteronem. Tento proces zajišťuje neustálou obnovu a remodelaci plodových obalů, mezenchymální buňky plní svou mechanickou, sekretorní endokrinologickou a imunomodulační úlohu. Zvýšená míra oxidačního stresu při blížícím se porodu udržuje buňky v mezenchymálním nastavení a

usnadňuje rupturu plodových obalů a porod. Vysoká hladina oxidačního stresu před řádným termínem tak může přispět k předčasnému porodu tím, že zvyšuje koncentraci TGFβ a zároveň snižuje hladinu progesteronu. Tato regulační dysbalance vede k předčasnému udržování buněk plodových obalů v mezenchymálním nastavení, což způsobí jejich zvýšenou citlivost k zánětu a sníženou schopnost regenerace mikrofraktur [32]. Menon nicméně uvádí, že zvýšené markery mezenchymální přeměny byly pozorovány zejména u předčasných porodů s intaktními plodovými obaly. U PPROM byly naopak zjištěny vyšší známky zánětlivých markerů, které jsou spojeny s procesem buněčného stárnutí a degradace plodových obalů [32]. Zdá se tedy, že i když rizikové faktory pro PTL a PPROM nejsou odlišné, budou se tyto patofyziologické děje odlišovat v biologických mechanismech, jaké aktivují zánět v buňkách plodových obalů. Obdobně přibývá důkazů o odlišném průběhu sterilního a infekčního zánětu. Behnia informuje o rozdílných aktivačních cestách u zánětu sterilního a u zánětu infekční povahy, kdy každá z cest aktivovala zánětlivé pochody cestou jiného transkripčního faktoru a byla spojena s odlišnou mírou oxidačního stresu [50], Motomura pozoruje významné rozdíly ve transkriptomu buněk plodových obalů během sterilního zánětu a infekce, a také pozoruje rozdíl v intenzitě zánětlivé odpovědi v závislosti na jeho příčině [51].

Z uvedených informací souhrnně vyplývá, že fyziologické zánětlivé změny jsou aktivovány a udržovány řadou rozdílných mechanismů na mnoha úrovních, které jsou přesně regulovány v čase a prostoru. K předčasnému porodu pak vede jejich dysregulace a předčasná aktivace, ať už způsobena sterilním či infekčním podnětem. Přibývají však data o rozdílných cestách aktivace zánětlivých změn a nestejné míře zapojení konkrétních zánětlivých mechanismů u jednotlivých kategorií předčasného porodu. Při zkoumání předčasného porodu a tvoření vědeckých závěrů tedy bude nutné citlivě vnímat jeho okolnosti a ptát se na jeho podstatu, neboť se jedná o složitou a velmi komplexní nozologickou jednotku. Jednou porušené plodové obaly již není možné přirozeně zacelit, byť Richter a kol informovali o úspěšném použití „záplaty“ z krevních destiček a zmražené plasmy na poškozené plodové obaly [52][53]. Pokud uvažujeme nad IAI jako nad procesem, při kterém vede uvolnění signálů nebezpečí DAMP a PAMP k jejich vazbě na PRR receptory rodiny TLR s následnou aktivací prozánětlivých transkripčních faktorů a produkcí cytokinů v buňkách plodových obalů, můžeme z této představy i odvodit potenciální strategie „léčby“ předčasného porodu. Tyto nástroje, které snižují intenzitu zánětu, zatím nejsou rutinně zavedené do klinické praxe a

jejich testování bylo provedeno *in vitro* či na zvířecích modelech. Přesto se jedná o nadějné postupy, které odráží celkovou orientaci moderní medicíny na využití nástrojů biologické léčby a imunomodulace. Tyto postupy představuje např. použití monoklonálních protilátek proti TLR [54] nebo aplikace solubilní formy receptoru RAGE (receptor for advanced glycation endproducts) [55]. Dále je možné využít rozličných molekul snižujících koncentraci prozánětlivých cytokinů nebo potlačujících aktivitu TLR [56]–[59]. Možné je zasáhnout přímo genovou expresi pomocí molekul interferujících s prozánětlivými transkripčními faktory [60][61], terapeutický zásah může cílit i na receptor $P2X_7$ pro ATP [23] nebo na Mincle, receptor lipidů, což opět vede k potlačení zánětu [62]. Interferon regulační faktory (interferon regulatory factors; IRF) jsou molekuly, jež jsou fosforylovány při aktivaci TLR, a které následně stimulují přepis genů pro interferony I. třídy a prozánětlivé cytokiny [63]. Jejich blokádou bylo docíleno snížení intenzity zánětu u buněk myometria a zdají se tak být dobrým cílem pro potlačení prozánětlivé signalizace skrze TLR [58]. Byla tak již představena celá řada možných terapeutických cílů a možností jejich ovlivnění. Vzhledem k velmi komplikované, vzájemně provázané a citlivé signalizační síti PRR receptorů a jejich ligandů však bude obtížné najít univerzální přístup k tlumení IAI. Zbývá také ještě mnoho potenciálních terčů a signalizačních cest odhalit, především je ovšem nutné zkoumat nežádoucí efekty těchto postupů a případná rizika pro tělo matky i plodu.

Intraamniální infekce

Intraamniální infekce je definována jako přítomnost mikrobů či jejich nukleových kyselin v plodové vodě (MIAC) při současném zánětu, jež definuje hladina IL-6 v plodové vodě [64]. Intraamniální infekce se vykytuje u cca 25 – 40 % předčasných porodů [28], konkrétně doprovází cca 6 – 35 % předčasných porodů s PTL [65][66] a 32 – 50 % předčasných porodů s PPROM [67][68], a je asociována i s dalšími negativními jevy v těhotenství, jako je vaginální krvácení [69], zkrácení děložního čípku [70], cervikální nedostatečnost [71][72] a chorioamnionitida [73]. Intraamniální infekce může být způsobena výstupem bakterií z dolních částí genitálního traktu skrze děložní hrdlo, krevním rozsevem ze vzdálených infekčních ložisek přes placentu, retrográdně z oblasti peritonea či může být zanesena během invazivního lékařského výkonu, přičemž první zmíněný případ je nejběžnější

[1][15][74][75]. Romero popisuje model, kde v tomto případě intraamniální infekce začíná vzestupem bakterií do děložního hrdla, čemuž může předcházet přítomnost patogenních bakterií ve vagíně nebo zmnožení přirozené bakteriální flóry (bakteriální vaginóza) [15]. Bakterie se následně rozšíří do deciduálního prostoru, odkud mohou infikovat cévy na choriové plotně (choriovaskulitida) či plodové obaly (histologická chorioamnionitida; HCA), ze kterých se mohou uvolnit do plodové tekutiny, což vede k intraamniální infekci. Ruptura plodových obalů pro přechod bakterií z plodových obalů do amniální dutiny není podmínkou, jelikož bakterie jsou schopny překonat i intaktní obaly [76]. Aspirace infikované plodové vody plodem následně způsobuje infekci plodu, která může mít za následek zánět středního ucha, konjuktivitidu či zánět pupečního pahýlu, případě sepsi [15]. V závislosti na stáří plodu se bakterie mohou dostat do těla i skrze spojivku, ucho či respirační trakt, bakterie se mohou do těla plodu dostat i skrze materno-fetální cirkulaci [15][75]. Infekce plodu je bezpochyby nejzávažnější formou intraamniální infekce. Infekce plodu může navodit závažný syndrom fetální zánětlivé odpovědi (FIRS), tedy systémovou imunitní odpověď plodu na patologický stimul, který je způsoben aktivací TLR plodu, rozeznávajících signály vnějšího (PAMP) či vnitřního poškození (DAMP). Tento syndrom doprovází zvýšená koncentrace IL-6 v pupečníkové krvi, histopatologicky FIRS provází funisitida a chorionická vaskulitida, tedy zánět pupečníku a cév chorionu, který je způsoben neutrofily, jež jsou do místa zánětu chemotakticky přitahovány zánětlivými mediátory [77]–[79]. Vznik FIRS je spojen se zvýšeným rizikem zdravotních komplikací plodu, jako jsou vývojové poruchy nervového systému, bronchopulmonární dysplázie, nekrotizující enterokolitida či novorozenecká sepse [77][80]–[83]. Byť intraamniální infekce vede k HCA, nejsou tyto termíny synonymem. HCA totiž vzniká i bez přítomnosti mikroorganismů v plodové vodě, tedy při zánětu sterilním [84]. Dle studie Romera a kol.pouze 12,5 % pacientek s PTL a intraamniální infekcí vykazovalo syndrom klinické chorioamnionitidy [85]. V kontrastu k termínu intraamniální infekce stojí intrauterinní infekce extraamniální, tedy infekce plodových obalů a decidui bez průniku do prostoru amniální dutiny. Jedná se však z hlediska diagnostiky o těžce uchopitelnou jednotku, neboť odběr a kultivace mikrobů z deciduální a chorioamniální tkáně je technicky náročný a riskantnější na kontaminaci než kultivace vzorků plodové vody. Navíc se bude pravděpodobně jednat o předstupeň intraamniální infekce vzhledem k obdobnému složení bakteriálních agens u vzorků z plodové vody a plodových obalů vždy u identické pacientky s infekcí [15]. Plodová voda by měla být za normálních okolností sterilní, proto jakýkoliv

mikrobiální nález lze považovat za bakteriální invazi, a to i bez patrných klinických symptomů [75]. Nejčastěji detekovanými bakteriemi, které způsobují intraamniální infekci jsou bakterie rodu *Mycoplasma*, *Ureaplasma*, *Fusobacterium, Gardnerella* [67][86]–[90] a *Chlamydia*, byť v případě poslední jmenované bakterie se vědecké názory na její vliv na předčasný porod a zdravotní komplikace plodu liší [91]–[94]. Nově je diskutována i role bakterií rodu *Sneathia* v patogenezi HCA, PTL a PPROM [95]. Bakteriální intraamniální infekce jsou však často polymikrobiálního původu [96]. Běžné bakterie vaginální sliznice, jako je např. *Streptococcus agalactiae* způsobují intraamniální infekci vzácně [97], naopak, absence vaginálních laktobacilů a bifidobakterií je spojena se zvýšeným rizikem předčasného porodu [98][99]. Riziko novorozenecké sepse výrazně zvyšuje vaginální dysbióza [100], k rozvoji intraamniální infekce rovněž přispívá zhoršená funkce děložního hrdla [101] a svou roli také hraje gestační věk. Bylo prokázáno, že riziko intraamniální infekce je vyšší s klesajícím gestačním stářím [102], a že se zráním plodu se snižuje intenzita zánětlivé odpovědi [103]. U dříve narozených dětí byla rovněž prokázána větší bakteriální nálož a větší diverzita bakteriálních druhů [104]. Intraamniální infekci však mohou kromě bakterií způsobovat i kvasinky rodu *Candida* [105]–[107], infekčním agens mohou být i viry, byť se jedná o méně časté případy. Otázka incidence virových intraamniálních infekcí však může být podhodnocena, jelikož se z podstaty věci jedná o obtížněji identifikovatelné agens. Nejčastěji se v případě intraamniálních infekcí jedná o adenoviry, cytomegaloviry, Epstein-Barrové virus a enteroviry [108][109]. Vyšší riziko předčasného porodu bylo zaznamenáno u žen s aktivní virovou hepatitidou typu B [110]. Principem virové infekce je invaze viru do buněk trofoblastu, což vede k apoptóze napadených buněk, aktivizaci deciduálních lymfocytů a ke vzniku zánětu, což může vést k předčasnému porodu [111]. Podobně jako u bakteriálních infekcí je však patrně klíčový právě až rozvoj zánětu, neboť prostá detekce virových nukleových kyselin z plodové vody nemusí nutně s předčasným porodem souviset. Baschat a kol. ve své studii informují o běžné izolaci virových nukleových kyselin u nerizikových těhotenství s normálním sonografickým nálezem [109]. Význam virové kolonizace a infekce amniální dutiny je téma aktuální a málo probádané, jež by si zasloužilo větší vědeckou pozornost i vzhledem k proběhnuvší pandemii viru SARS-CoV-2. Garcia-Flores v této souvislosti uvádí, že infekce covid-19 u těhotných žen vyvolala systémovou imunitní odpověď matky, imunitní odpověď byla prokázána i na feto-maternálním rozhraní. Přítomnost viru SARS-CoV-2 však nebyla detekována v placentě ani

nebyla zaznamenána buněčná imunitní reakce plodu či přítomnost specifického IgM v pupečníkové krvi, kde však byla prokázána zvýšená hladina cytokinů [112].

Intraamniální infekce probíhá většinou chronicky a asymptomaticky, bez horečky, bolestí či leukocytózy v periferní krvi pacientky [74]. Vzorek plodové vody se tak logicky jeví jako nejvhodnější materiál pro diagnostiku intraamniální infekce. Odběr plodové vody pro mikrobiologickou analýzu je možný transabdominální amniocentézou. K předčasnému porodu může vést rovněž extrauterinní infekce matky, přičemž riziko předčasného porodu u některých typů infekcí nesnižuje ani kvalitní antibiotická léčba těhotných žen [15]. Tento závěr dokládají výsledky studií zaměřených na riziko předčasného porodu těhotných žen se zápalem plic, pyelonefritidou, malárií, tyfem či periodontitidou [15][113]. První studie o souvislosti pozitivního mikrobiologického nálezu v plodové vodě s předčasným porodem u žen s PTL byla podána v druhé polovině sedmdesátých let [114]. Na začátku devadesátých let pak byl představen levný a citlivý průkaz intraamniální infekce – hladina glukózy v plodové vodě, která je u pacientek s intraamniální infekcí nižší než u pacientek bez infekce [115][116]. Následně přibývaly důkazy o zvýšeném počtu leukocytů v plodové vodě při intraamniální infekci [117], zvýšení koncentrací složky komplementu C3 [118] a o diagnostickém významu dodnes zásadní molekuly IL-6 [119]. Mnohé další studie přinesly informace o zvýšení či snížení koncentrací řady molekul souvisejících s buněčným poškozením či imunitní odpovědí na zánětlivý stimul [20][120]–[122]. Od klasických, z dnešního pohledu nedokonalých, kultivačních technik se přechází na postupy molekulárně-biologické [15][22], diagnostika se přesouvá z laboratoří do místa péče o pacientku [123][124]. Zcela novátorským přístupem je sledování probíhající pyroptózy pomocí stanovení koncentrace molekuly gasderminu D a molekul, jež se účastní sestavení inflamasomu v plodové vodě a v buňkách plodových obalů [125]. Slibným přístupem je i stanovení miRNA z plodové vody. Kiyoshima a kol. představili molekuly miR-4535 a miR-1915-5p, jejichž hladina v plodové vodě významně korelovala s nálezem zánětu a těžké HCA [126]. Svět miRNA je v tomto ohledu málo probádaný a vykazuje slibný potenciál mnoha originálních vědeckých výstupů. I přes řadu velmi dobrých výsledků se však nejedná o markery rutinně stanovitelné, neboť není prakticky možné (ani vhodné) všem těhotným ženám provádět amniocentézu. Stanovení rozličných biomarkerů z vaginální či cervikální tekutiny, nebo dokonce z periferní krve nicméně dlouhodobě nepřináší kýženou spolehlivost. Nadějným přístupem může být stanovení hladiny IL-6 z pupečníkové krve či cervikální tekutiny [78][127] a dále fibronektinu z cervikální či vaginální

tekutiny [128][129], kam se pravděpodobně dostává po svém uvolnění z choriodeciduální basální membrány při infekci. O zvýšeném riziku intraamniální infekce a předčasného porodu u asymptomatických pacientek rovněž informují zvýšené hladiny G-CSF [130], ferritinu [131] a CRP [132] v periferní krvi matky. Jedním z dalších možných přístupů je sledování délky děložního hrdla, jehož zkrácení koreluje s markery infekce a rizikem HCA [133][134]. O riziku předčasného porodu rovněž informuje kombinace sledování délky děložního hrdla spolu s počtem T-regulačních lymfocytů [135], což je však rovněž technologicky nedostupné pro rutinní péči. Galaz pozoroval souvislost mezi výrazně zkráceným cervixem (≤15 mm) a zvýšenou náloží prozánětlivých cytokinů, avšak bez zřetelného zvýšení počtu leukocytů v plodové vodě [136]. Z diagnostického pohledu se tak stále jeví nejlepší molekulou IL-6, jehož koncentrace dnes dobře definuje přítomnost intraamniálního zánětu, je stanovitelný z plodové vody, pupečníkové krve i cervikální tekutiny, a to i v režimu point of care testing (POCT). Nevýhodou IL-6 je jeho nespecifičnost, jelikož zvýšené hladiny IL-6 informují o probíhajícím zánětu, a nikoliv o jeho příčinách. Hladiny IL-6 u pacientek bez zánětu nejsou nulové, neboť IL-6 je v jisté míře konstitutivně exprimován během celého těhotenství a jeho koncentrace obecně vzrůstá při porodu v řádném termínu i při porodu předčasném. Je secernován nejenom buňkami imunitního systému, ale i buňkami plodových obalů [137]. Omere a kol. nicméně prokázali, že i když normální i zvýšené hladiny IL-6 aktivují kinázy reagující na signály stresu, na buněčný cyklus, produkci prozánětlivých cytokinů, apoptózu, stárnutí či mezenchymově-epitelový přechod u buněk plodových obalů samotný IL-6 nemá vliv [138]. Byť je tedy IL-6 považován za spolehlivý marker IAI, jeho samotné působení může být více homeostatické než poškozující [138].

Cíle práce

Porodník, který poskytuje péči pacientce, u které hrozí PPROM a předčasný porod, je vystaven dilematu, kdy musí na jedné straně zvážit benefit prodloužení těhotenství, což má za následek snížení zdravotních komplikací spojených s předčasným porodem. Na straně druhé je zde při včasném neukončení těhotenství nezanedbatelné riziko rozšíření intraamniální infekce se všemi negativními důsledky pro dítě i matku. Toto riziko dokládá fakt, že intraamniální infekce byla diagnostikována u 40 % pacientek s PPROM, pokud byly odběry provedeny před porodem. Vzorky plodové vody, které byly odebrány ženám s PPROM v čase porodu, byly pozitivní v 75 % případů [139]. Intraamniální infekce zvyšuje riziko předčasného porodu, přičemž nedoprovází pouze předčasné porody s PPROM ale i s PTL [68]. Včasné a rychlé rozpoznání intraamniální infekce je tedy významné pro posouzení míry rizika předčasného porodu a pro zvážení dalšího medicínského postupu. Kultivační techniky, jež byly zlatým standardem mikrobiologické diagnostiky, jsou časově náročné a mohou poskytovat falešně negativní výsledky vzhledem k obtížnému průkazu bakteriálních rodů, které způsobují intraamniální infekce nejčastěji. Pozornost se v průběhu posledních let přesunula k molekulárně-biologickým technikám průkazu bakterií, případně ke kombinaci obou přístupů. I přes vyšší rychlost a citlivost těchto metod existuje snaha o nalezení vhodného molekulárního markeru, který by poskytoval spolehlivou informaci o probíhající infekci ideálně v režimu „bed-side“ testu. Takový parametr by měl být přirozeně stanovitelný z plodové vody. Jelikož je však amniocentéza invazivní zákrok spojený s riziky a jistou mírou nedůvěry u veřejnosti, bylo by ideální nalézt molekulu, jež by vykazovala dobrou informační hodnotu při stanovení z cervikální tekutiny, či periferní krve. Vzhledem k izolovanému průběhu infekce v amniální dutině, je však stanovení vhodné molekuly z periferní krve prakticky vyloučeno. Na význam hladin markerů zánětu a infekce z cervikální tekutiny nepanuje jednotný názor, dostupné výsledky různých autorů nejsou jednoznačné. Cílem výzkumného záměru, jehož vybrané výsledky disertační práce komentuje, bylo testovat hladiny vybraných molekul, u nichž se předpokládal možný diagnostický potenciál pro stanovení intraamniální infekce. Koncentrace vybraných molekul byly stanoveny v plodové vodě pacientek s PPROM či PTL, přičemž u části žen bylo těhotenství komplikováno IAI či intraamniální infekcí. U výsledků molekul s diagnostickým potenciálem bylo provedeno jejich stanovení i z cervikální tekutiny. Výběr testovaných molekul se zakládal na výsledcích

proběhnuvších proteomických studiích [120][121][140], literárních rešerších a zkušenostech vědeckého týmu. Vedlejším efektem práce pak mělo být lepší porozumění sterilním i nesterilním okolnostem biologie IAI. Síla získaných závěrů tkví v přesně definované kohortě žen s jasně popsaným patofyziologickým nálezem, v současném stanovení IL-6 a v přesné identifikaci původců infekce na molekulární úrovni. Pacientky tak bylo možné rozdělit na podskupiny s ohledem na přítomnost či absenci MIAC a přítomnost či absenci IAI. Slabým místem provedených měření zůstává fakt, že neznáme přesné zdroje detekovaných molekul a z výsledků tak není možné prokazatelně vyvodit, zda a v jaké míře se na produkci daných molekul podílí leukocyty či buňky plodových obalů nebo placenty. Dalším limitem publikovaných výsledků je skutečnost, že takto získané výsledky poskytly informace o koncentracích daných molekul pouze ve chvíli porodu, a že tudíž neznáme dynamiku změn jejich koncentrace během gestace. Z povahy vyšetření je však nemožné tento limitující faktor odstranit.

Metodický přístup

Vědecký tým

Disertační práce shrnuje dílčí výsledky dlouhodobého výzkumného záměru, kterým je hledání nových biomarkerů IAI a intraamniální infekce u žen s předčasným porodem. Smyslem výzkumu je i snaha o větší porozumění biologickým okolnostem IAI a zákonitostem imunitní odpovědi na sterilní a nesterilní prozánětlivé stimuly a jejich vztahu k předčasnému porodu. Výzkumný tým vedou prof. MUDr. Marian Kacerovský, Ph.D. a doc. MUDr. Ivana Kacerovská Musilová, Ph.D. z Porodnické a gynekologické kliniky Fakultní nemocnice Hradec Králové, kteří v čase rozvinuli bohatou mezioborovou spolupráci s řadou lékařů a bioanalytiků napříč celým spektrem medicínských a biologických oborů. Z lékařů Porodnické a gynekologické kliniky se na výzkumu podíleli zejména MUDr. Martin Štěpán, Ph.D., MUDr. Jaroslav Stráník, MUDr. Tomáš Bestvina a další. Za Ústav klinické imunologie a alergologie výzkumnou činnost plánoval a koordinoval prof. RNDr. Ctirad Andrýs, Ph.D., dále se, kromě autora práce, na dílčích činnostech podílely i Mgr. Martina Koláčková, Ph.D. a RNDr. Marcela Drahošová. Veškeré molekulárně-biologické diagnostické procedury a stanovení IL-6 zajišťovaly pracovnice Ústavu klinické biochemie a diagnostiky a Ústavu klinické mikrobiologie, zejména Mgr. Radka Bolehovská, Ph.D. a PharmDr. Lenka Plíšková. Svým expertním názorem přispěla i řada zahraničních expertů, především prof. Bo Jacobsson z University of Gothenburg ve Švédsku.

Soubor pacientek zařazených do studie

Do studie byly zařazeny pacientky přijaté na Porodnickou a gynekologickou kliniku Fakultní nemocnice Hradec Králové v gestačním stáří mezi 24.+0. až 36.+6. týdnem těhotenství. Skupinu tvořily ženy starší 18 let s jednočetným těhotenstvím komplikovaným PTL či PPROM, které souhlasily s účastí ve studii. Ženy, jejichž těhotenství bylo komplikováno růstovou retardací plodu, přítomností vrozených vad plodu či s abnormálním genetickým nálezem, těhotenským i s těhotenstvím nesouvisejícím diabetem, gestační hypertenzí, preeklampsií,

hypoxií plodu nebo s významným vaginálním krvácením, byly ze studie vyloučeny. Studie byly schváleny etickou komisí Fakultní nemocnice Hradec Králové, všechny účastnice studie podepsaly informovaný souhlas, kterému v plném rozsahu porozuměly. Kompletní demografické údaje ke každé studii jsou uvedeny v příslušných přílohách.

Gestační stáří bylo stanoveno pomocí primotrimestrální biometrie plodu. Ženám s **PPROM** před 34. týdnem těhotenství byla podána antibiotika a kortikosteroidy pro indukci plicní zralosti. Po 34. týdnu těhotenství nebyla, kromě antibiotik, podávána další léčba k oddálení porodu. Ženy s prokázanou intraamniální infekcí po 28. týdnu těhotenství dostávaly pouze antibiotika a kortikoidy pro indukci plicní zralosti. Za 24 hodin po dokončené indukci plicní zralosti byl porod buď vyvolán či bylo těhotenství ukončeno císařským řezem dle porodnického nálezu. U ostatních žen bylo postupováno konzervativně. Ženám s **PTL** byly podány kortikosteroidy a tokolytika na 48 hodin. Pacientkám s prokázanou intraamniální infekcí byla intravenózně podávána antibiotika po dobu sedmi dnů, pokud nedošlo k porodu dříve. U obou skupin pacientek byl jako antibiotikum první volby zvolen klaritromycin, terapie byla následně upravena dle konkrétního mikrobiologického nálezu.

PTL byl diagnostikován jako přítomnost pravidelných děložních kontrakcí (nejméně dvě kontrakce každých deset minut) při délce děložního čípku kratší než 15 mm (měřeno pomocí transvaginálního ultrazvuku) nebo při délce děložního čípku 15 – 30 mm při pozitivním výsledku PartoSure testu (Parsagen Diagnostics, Boston, MA, USA) [141].

PPROM byl diagnostikován při vyšetření v zrcadlech, přímým průkazem přítomnosti plodové vody v zadní poševní klenbě. V případě potřeby byl výsledek vyšetření potvrzen testem přítomnosti vazebných proteinů inzulinu podobných růstových faktorů (Insulin-like growth factor-binding proteins; IGFBP) ve vaginální tekutině (ACTIM PROM test, MedixBiochemica, Kauniainen, Finsko).

Odběr plodové vody a cervikální tekutiny

Vzorky plodové vody a cervikální tekutiny byly u pacientek odebrány při přijetí na porodním sále před zahájením antibiotické, tokolytické a kortikosteroidní terapie. Vzorky **cervikální tekutiny** byly odebrány dakronovým tamponem, který byl umístěn do cervikálního kanálu na

dvacet sekund pro dostatečné nasycení. Tampon byl následně přenesen do plastové zkumavky s 1,5 ml fosfátového pufru, která byla s tamponem umístěna na 20 minut na třepačku. Poté byl tampon odstraněn, zkumavka se vzorkem byla umístěna do centrifugy a 15 minut stáčena při pokojové teplotě na 300xg. Získaný supernatant byl následně rozdělen na alikvoty a skladován při -80°C. Pro získání vzorků **plodové vody** byla provedena transabdominální amniocentéza pod ultrazvukovou kontrolou, přičemž byly získány přibližně 2-3 ml plodové vody. Ze 100 µl těchto vzorků byla následně stanovena koncentrace IL-6 pomocí POCT testu. Zbytek odebraného vzorku byl následně rozdělen na dva podíly. První podíl byl odeslán do mikrobiologické laboratoře k PCR detekci DNA mikrobiálních druhů *Ureaplasma* species, *Mycoplasma hominis* a *Chlamydia trachomatis*, k sekvenování genu pro 16S rRNA a aerobní a anaerobní kultivaci. Druhý podíl byl centrifugován (15 min./2000g), supernatant byl alikvotován a zamražen na -80°C pro imunologickou analýzu.

Stanovení koncentrace sledovaných parametrů v plodové vodě a cervikální tekutině

Koncentrace sledovaných látek v plodové vodě a v cervikální tekutině byla stanovena sendvičovým ELISA testem (enzyme-linked immuno sorbent assay) pomocí ELISA soupravy na stanovení dané molekuly podle instrukcí výrobce. Výsledná hodnota absorbance byla měřena při vlnové délce 450 nm za použití readeru Multiskan RC ELISA (Thermo Fisher Scientific, MA, USA). Kompletní informace k jednotlivým diagnostickým soupravám jsou uvedeny v příslušných přílohách.

Stanovení koncentrace IL-6 v plodové vodě a cervikální tekutině

Stanovení koncentrace IL-6 v plodové vodě a v cervikální tekutině bylo provedeno pomocí imunochromatografického testu Milenia® QuickLine IL-6 za použití Milenia®POCScan Readeru (Milenia Biotec, GmbH, Giessen, Německo). Detekční rozpětí metody se nacházelo mezi 50 – 10 000 pg/ml, variabilita v sérii (intra-assay variability) byla 12,1 %, variabilita mezi sériemi (inter-assay variability) činila 15,5 % [124]. Pro účely novějších studií byl použit imunologický analyzátor Cobas e602 (Roche Diagnostics, Basilej, Švýcarsko). Detekční rozpětí

metody se nacházelo mezi 1,5 – 50 000 pg/ml. Variabilita v sérii i mezi sériemi se nacházela pod 10 % [142].

Mikrobiologická analýza

Detekce bakterií *Ureaplasma* species, *Mycoplasma hominis* a *Chlamydia trachomatis* byla provedena pomocí polymerázové řetězové reakce (PCR) ve formě Real-Time PCR. Pro potřeby metody byla z plodové vody izolována DNA pomocí QIAamp DNA Mini Kitu (QIAGEN, Hilden, Německo) podle instrukcí výrobce (použit protokol pro izolaci bakteriální DNA z biologických tekutin). K detekci DNA *Ureaplasma species*, *Mycoplasma hominis* a *Chlamydia trachomatis* byla použita komerční souprava AmpliSense® *Chlamydia trachomatis*/*Ureaplasma*/*Mycoplasma hominis*-MULTIPROME-FRT (Federal State Institution of Science, Central Research Institute of Epidemiology, Moskva, Ruská Federace). PCR bylo provedeno na přístroji Rotor-Gene 6000 (QIAGEN) s použitím standardního laboratorního materiálu. Jako kontrola metody byla provedena PCR amplifikace genu pro β-aktin pro zjištění, zda nejsou při provádění metody ve vzorku přítomny inhibitory polymerázové reakce. Množství DNA (kopie/ml) bylo stanoveno absolutní kvantifikací pomocí kalibrační křivky, k jejíž přípravě sloužila plazmidová DNA o známé kvantitě (pCR4, Invitrogen, Carlsbad, CA) [103][120]. Bakteriální DNA ostatních bakteriálních druhů byla identifikována pomocí amplifikace genu pro ribozomální 16S rRNA PCR metodou za použití primerů:

5`-CCAGACTCCTACGGGAGGCAG-3` (oblast V3) a 5`-ACATTTCACAACACGAGCTGACGA-3` (oblast V6) [143][144]. Každá reakce obsahovala 3 µl vyšetřované DNA, 500 nM primerů a 25 µl Q5 High Fidelity DNA polymerázy (NEB, Ipswich, MA, USA). Reakce byla provedena na přístroji 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) a výsledné produkty byly detekovány na agarózovém gelu po provedení gelové elektroforézy. Pozitivní nálezy, tedy amplifikační produkty o velikost 950 bp, byly následně vyjmuty, očištěny a byla provedena sekvenační PCR formou oboustranné sekvenace vždy s jedním primerem, tedy v první reakci forward primerem, v druhé pak reverse primerem. K sekvenační reakci byl použit komerční kit obsahující DNA polymerázu a značené dideoxynukleotidy – BigDye Terminator kit, version 3.1 (Thermo Fisher Scientific, Waltham, MA, USA). Výsledný produkt byl opětovně pročištěn a byla provedena sekvenační analýza na sekvenátoru ABI 3130

(Applied Biosystems, Foster City, CA, USA). U získaných sekvencí ve formátu FASTA byly porovnány shody se sekvencemi v databázi referenčních kmenů pomocí programu BLAST®, event. SepsiTest™ BLAST. U novějších studií byly prováděny i aerobní a anaerobní kultivace vzorků plodové vody. Vzorky byly kultivovány na Columbia krevním agaru, *Gardnerella vaginalis* selektivním mediu, MacConkey agaru, *Neisseria* selektivním mediu, Sabouraudově agaru a Schaedlerově anaerobním agaru. Půdy byly kultivovány po dobu šesti dnů a denně kontrolovány. Mikrobiální nálezy pak byly specifikovány pomocí MALDI technologie (matricí asistovaná laserová desorpce/ionizace) za použití MALDI Biotyper software (Bruker Daltonics, Brémy, Německo). Detailní popis mikrobiálních nálezů je podán v článcích uvedených jako přílohy práce.

Diagnostikování MIAC, určení probíhajícího IAI a intraamniální infekce

MIAC byla u pacientek diagnostikována na základě pozitivního výsledku PCR analýzy DNA *Ureaplasma* species, *Mycoplasma hominis* a / nebo *Chlamydia trachomatis* a / nebo pozitivního výsledku amplifikace genu pro 16S rRNA. Jako IAI byl u pacientek definován stav, kdy byla v plodové vodě naměřena koncentrace IL-6 vyšší než 745 pg/ml v případě měření na přístroji použití Milenia®POCScan Reader [123][145]. Při použití přístroje Cobas e602 byl IAI stanoven při koncentraci IL-6 v plodové vodě vyšší než 3000 pg/ml [142]. Za intraamniální infekci byla považována situace, kdy byla prokázána současně MIAC a IAI. IAI bez prokázané MIAC definoval sterilní IAI.

Statistické zpracování dat

Demografické charakteristiky souboru pacientek byly porovnány pomocí neparametrických Mann-Whitneyho U testu a Kruskal-Wallis testu pro spojité proměnné. Kategoriální proměnné byly analyzovány pomocí chí-kvadrátu či Fisherova exaktního testu. Normalita rozložení dat byla testována za pomocí D´Agostino-Pearsonova testu, Shapiro-Wilkova testu a Anderson-Darlingova testu. Výsledky koncentrací testovaných látek v plodové vodě nenabývají normálního rozložení, proto byly analyzovány pomocí neparametrických Man-

Whitneyho U testu a Kruskal-Wallis testu. Ke statistické úpravě gestačního stáří při odebírání vzorků byl použit Spearmanův korelační koeficient. Cut-off hodnoty, sensitivita, specificita a plocha pod křivkou AUC (area under the ROC curve) vybraných parametrů byly určeny pomocí ROC (Receiver Operating Characteristic) analýzy. Testování probíhalo na 95% hladině významnosti. Všechny *p*-hodnoty byly získány z dvouvýběrových testů, ke statistické analýze byl použit program GaphPad Prism 6 (GraphPad Software, San Diego, CA, USA) nebo program SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Výsledky

Následující část práce obsahuje komentáře k článkům, které představily vybrané výsledky výzkumné činnosti, na níž se spolupodílel autor disertační práce. Výsledky byly publikovány v časopise The Journal of Maternal-Fetal & Neonatal Medicine (Taylor & Francis Group), výsledky stanovení FcgammaBP byly publikovány v časopise Scientific Reports (Springer Nature). Originální vědecká sdělení jsou uvedena jako přílohy práce.

Komentáře k publikacím

Komentář k práci č. I.

Musilova I, Andrys C, Drahosova M, Soucek O, Kutova R, Pliskova L, Spacek R, Laudanski P, Jacobsson B, Kacerovsky M

Amniotic fluid calreticulin in pregnancies complicated by the preterm prelabor rupture of membranes

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Kalretikulin je pleiotropní protein s mnoha funkcemi. Podílí se na udržení správné intracelulární hladiny vápníku, plní chaperonovou funkci v endoplasmatickém retikulu (ER), zásadním způsobem se podílí na vystavování antigenních peptidů na buněčné membráně přes MHC I a zprostředkovává mnoho rozmanitých funkcí v imunitní signalizaci, jak v solubilní formě, tak vázaný na membránu [146]. Mezi jeho funkce extracelulární patří imunomodulační schopnosti a účast na apoptóze. Kalretikulin byl při svém objevu popsán jako protein lokalizovaný na membráně endoplasmatického / sarkoplasmatického retikula [147], což přímo souvisí s výkonem jeho intracelulárních funkcí. Kalretikulin může být však lokalizován i na cytoplasmatické membráně [148] či secernován do krevního oběhu [149]. Pravděpodobně se kalretikulin nachází i volně v cytoplasmě či v jádře. Vzhledem ke své schopnosti regulovat expresi genů citlivých na přítomnost steroidů je totiž nutné, aby byl schopen vázat se na DNA vazebné domény těchto receptorů [150], jež se účastní signalizačních kaskád. Při apoptóze vysoké koncentrace kalretikulinu v buňce zvyšují intraluminální zásobu vápníku v ER, což vede k jeho snadnějšímu uvolnění do cytoplasmy a k aktivaci kalcineurinu, který kontroluje aktivitu významných transkripčních faktorů ovlivňujících průběh apoptózy a rovněž přímo aktivuje některé z kaspáz a proapoptotických proteinů a transkripčních faktorů [151]. Abnormálně zvýšená produkce kalretikulinu tak zvyšuje citlivost buňky k apoptóze [151]. Kalretikulin dále slouží např. jako receptor složky komplementu C1q (C1q-R) [152], kdy po vazbě této složky komplementu přenáší do buňky aktivační signál vedoucí k individuální imunitní reakci v závislosti na typu buňky, na které je exprimován. Jako receptor tohoto faktoru je kalretikulin rovněž důležitý při rozvíjení

specifické imunitní odpovědi. C1q je totiž produkován jako autokrinní faktor během kostimulační vazby povrchových molekul CD40/CD40L, tedy při poskytování zásadního kostimulačního signálu dendritickými buňkami T lymfocytům [153]. Kalretikulin je dále schopný stimulovat určité subpopulace B lymfocytů k produkci protilátek [154] a stimulovat produkci TNFα a IL-6 v makrofázích přes aktivaci transkripčního faktoru NFκB a MAPK [155]. Kalretikulin uvolněný do prostředí slouží jako tzv. alarmin neboli DAMP, tedy molekula, která iniciuje a udržuje neinfekční zánětlivou odpověď [156]. Toto se děje především za podmínek stresu ER, kdy dochází k úniku vápenatých iontů a akumulaci nesprávně složených proteinů [157]. Kromě toho kalretikulin sám stimuluje fagocytózu během procesu tzv. imunogenní smrti u nádorových buněk, kdy je za stresových podmínek ve vyšší koncentraci vystaven na buněčnou membránu [146]. Tam je za normální situace asociován s molekulou CD47, která fagocytóze brání, jako tzv. „don´t eat me signal“. Kalretikulin se od ní během imunogenní buněčné smrti disociuje, čímž naruší její funkčnost a sám působí jako „eat me“ signál, tedy signál fagocytózu podporující. Navíc u buněk v apoptotickém procesu kalretikulin podporuje tvorbu receptoru pro TRAIL (tumor necrosis factor – related inducing ligand), což je cytokin, který po vazbě na své receptory podporuje procesy buněčné smrti [146].

Koncentrace kalretikulinu v plodové vodě (bez ohledu na přítomný PPROM či jeho absenci) nebyla dosud studována. Provedené měření zjistilo, že (i) ženy s MIAC vykazují vyšší koncentrace kalretikulinu v plodové vodě než ženy bez MIAC ($p = 0,001$), (ii) u žen s IAI byla zjištěna vyšší koncentrace této molekuly než u žen bez zánětu ($p < 0,0001$), (iii) koncentrace kalretikulinu v plodové vodě u žen s intraamniální infekcí byla vyšší než u žen bez MIAC a IAI ($p < 0,0001$). Dále bylo zjištěno, (iv) že koncentrace kalretikulinu v plodové vodě pozitivně koreluje s koncentrací IL-6 (rho = 0,32; $p < 0,0001$) a (v) s mikrobiální náloží *Ureaplasma* species (rho = 0,35; $p < 0,03$). Při analýze hodnot u pacientek rozdělených podle přítomnosti či absence MIAC a IAI bylo zjištěno, (vi) že pacientky s intraamniální infekcí vykazují vyšší koncentraci kalretikulinu v plodové vodě než pacientky se sterilním zánětem, s kolonizací a pacientky bez MIAC a intraamniálním zánětem současně. Hladina kalretikulinu 81,4 ng/ml v plodové vodě se jeví jako ideální pro stanovení intraamniální infekce (AUC = 0,88; $p < 0.0001$).

Vzhledem ke komplexnosti proteinu a jeho funkční diverzitě není jednoduché přesně definovat úlohu kalretikulinu v patogenezi IAI u PPROM. Ve výsledcích byla zvýšená

koncentrace této molekuly zjištěna jak v případě MIAC, tak probíhajícího IAI. Vzhledem k výše uvedeným rozmanitým funkcím této molekuly předpokládáme jeho zvýšenou koncentraci během patologických situací, mezi něž rozhodně IAI či intraamniální infekce patří. Nejvyšší koncentrace zkoumaného proteinu byla zjištěna u pacientek s intraamniální infekcí, jež byla dvakrát vyšší než u pacientek se sterilním IAI či prostou kolonizací. Uvolnění kalretikulinu do extracelulárního prostoru se děje za podmínek buněčného stresu, resp. stresu ER, kdy je v reakci na nepříznivé podmínky během zánětu, spolu s dalšími rezidentními proteiny ER, jako je např. kalnexin a dalšími proteiny teplotního šoku, vystaven na buněčné membráně, či secernován mimo buňku [158]. Zvýšená exprese s následnou extracelulární lokalizací kalretikulinu byla prokázána v průběhu virové infekce [159][160]. Zvýšený přepis genu pro kalretikulin byl rovněž pozorován na zvířecích modelech po inokulaci gram-negativními bakteriemi [161]. Intenzivnější exprese a extracelulární lokalizace kalretikulinu potom pravděpodobně slouží k nastartování opravných mechanismů (protektivní účinek kalretikulinu proti oxidačnímu stresu, regulace koncentrace a lokalizace iontů vápníku a chaperonové funkce), dále k podpoře zánětlivých procesů přes aktivaci transkripčního faktoru NFκB, kdy ztráta funkčnosti kalretikulinu vede ke snížení prozánětlivé signalizace přes tento transkripční faktor [162]. Kalretikulin stimuluje produkci IL-6 a TNFα [155] a stimuluje aktivaci T-lymfocytů [158]. Povrchově lokalizovaný rovněž podporuje fagocytózu [146].
V úvahu připadá i možná zvýšená produkce MHC I na povrchu buněk. Při analýze výsledků byla zjištěna korelace mezi hladinami kalretikulinu a množstvím mikrobiální nálože bakterií rodu *Ureaplasma* (rho = 0,35; p = 0,03). Fakt, že koncentrace kalretikulinu v plodové vodě pozitivně koreluje s množstvím mikrobiální masy a IL-6 pak podporuje představu, že intenzita zánětlivé odpovědi reflektuje množství mikrobiální nálože. Obdobné pozorování prokázal tým prof. Kacerovského ve studii, jež byla zaměřena na sledování souvislosti mezi mírou bakteriální nálože a intenzitou zánětu při infekci genitálními mykoplazmaty [103]. Stavy samotné mikrobiální kolonizace bez přítomného zánětu či zánět definovaný jako sterilní (tedy bez účasti bakterií) statisticky vyšší expresi kalretikulinu nevykazovaly. Až intraamniální infekce se tedy jeví jako stav, kdy dochází ke zvýšení produkce tohoto proteinu (resp. zvýšení jeho extracelulární koncentrace). Přesný zdroj kalretikulinu není znám. Může se jednat o kalretikulin uvolněný z leukocytů podílejících se na imunitní odpovědi, rovněž však jako zdroj této molekuly mohou sloužit odloučené buňky placenty či plodových obalů. Byla prokázána exprese kalretikulinu na povrchu neutrofilních granulocytů [163], zvýšená hladina

kalretikulinu tak může přímo souviset se zvýšenou migrací buněk vrozené imunity do míst zánětu. Výsledky rovněž poukázaly na účast kalretikulinu během intraamniální infekce u pacientek s PPROM. Kalretikulin se tak jeví jako vhodný parametr pro stanovení přítomnosti intraamniální infekce z plodové vody. Výsledky analýzy hladiny kalretikulinu u pacientek s IAI a MIAC v cervikální tekutině však nepřinesly žádné statisticky významné rozdíly [164].

Komentář k práci č. II.

Musilova I, Andrys C, Drahosova M, Soucek O, Pliskova L, Stepan M, Bestvina T, Maly J, Jacobsson B, Kacerovsky M

Amniotic fluid cathepsin-G in pregnancies complicated by the preterm prelabor rupture of membranes

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Katepsin G je endoproteáza patřící mezi serinové katepsinové proteázy [165], strukturně podobná granzymům [166]. Funkce proteinu je enzymatická, podobně jako chymotrypsin a trypsin je enzym schopen pracovat v širokém rozmezí pH. Enzym je, spolu s neutrofilní elastázou a proteázou 3 (PR3), hlavní serinovou proteázou azurofilních granulí neutrofilních granulocytů [167]. Jako součást obranných mechanismů neutrofilů se podílí na odstraňování zbytků dezintegrovaných bakterií, na proteolytických úpravách cytokinů i na odstraňování molekul z povrchu buněčné membrány buňky. Na rozdíl od neutrofilní elastázy, která je schopna proteolytického štěpení buněčného povrchu pouze u gram-pozitivních bakterií [168], je katepsin G, díky svému aktivnímu místu bohatému na arginin, schopný narušovat bakteriální povrch nezávisle na struktuře bakteriální buněčné stěny [169]. Enzym se dále podílí na organizaci neutrofilových extracelulárních pastí [166] a jeho enzymatická aktivita je rovněž důležitá pro chemotaxi a enzymatickou úpravou ligandů chemokinových receptorů [165]. Náš výzkumný tým zjistil statisticky významně vyšší koncentrace katepsinu G v plodové vodě u žen s IAI ($p < 0,0001$) a u žen s MIAC ($p < 0,0001$) oproti ženám bez těchto komplikací. Pacientky s intraamniální infekcí rovněž vykazovaly vyšší hodnoty zkoumaného proteinu ($p < 0,0001$). Statisticky významné nálezy se nezměnily ani po úpravě hodnot s ohledem na gestační stáří. Celková analýza podskupin sledované kohorty pacientek pak ukázala rozdíl mezi skupinou se zánětem (ať už sterilním či nesterilním) a bez zánětu, byť s bakteriální kolonizací ($p < 0,0001$). Mezi hodnotami katepsinu G u žen se sterilním IAI a intraamniální infekcí nebyl zjištěn statisticky významný rozdíl. Byla zjištěna slabá korelace mezi hladinou katepsinu G a IL-6. Jako nejvhodnější hladina katepsinu G informující o probíhajícím zánětu v plodové vodě byla stanovena hodnota 105 ng/ml (AUC = 0,82; $p < 0.0001$) při sensitivitě 50 % a specificitě 92 %. Jedná se však o hodnotu vypovídající o probíhajícím zánětu jako takovém, nikoliv o probíhající intraamniální infekci.

Nejvýznamnějším zdrojem katepsinu G jsou neutrofilní granulocyty, proto vysvětlení tohoto jevu patrně tkví v migraci granulocytů do místa probíhajícího zánětu a jejich aktivní účasti na něm. Koncentrace katepsinu G se statisticky nelišily při porovnání hodnot u žen bez zánětu s bakteriální kolonizací a bez infekce. Dá se tedy předpokládat, že zvýšení hladiny katepsinu G v přítomnosti mikroorganismů v plodové vodě se děje jako následek zánětu, nikoliv jako jeho příčina. Vystavení neutrofilních granulocytů prozánětlivým cytokinům totiž vede k rychlé mobilizaci cytoplasmatických granul a k uvolnění jejich obsahu do prostředí [168][170]. Zvýšená koncentrace katepsinu G se proto jeví jako potenciální marker pro sledování přítomnosti či absence intraamniální infekce v plodové vodě. Představu tohoto modelu umocňuje fakt, že nebyl zjištěn významný rozdíl mezi hodnotami u žen se sterilním IAI a intraamniální infekcí. Zdá se tedy, že vliv IAI na akumulaci neutrofilů a produkci katepsinu G bude obecný a nebude omezen na mikroorganismy coby spouštěče zánětlivé odpovědi.

Komentář k práci č. III.

Andrys C, Musilova I, Drahosova M, Soucek O, Pliskova L, Jacobsson B, Zhong N, Kacerovsky M

Cervical fluid calreticulin and cathepsin-G in pregnancies complicated by preterm prelabor rupture of membranes

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Po provedeném stanovení hladin kalretikulinu a katepsinu G ve vzorcích plodové vody u pacientek s PPROM, které přineslo příznivé výsledky, byla stanovena hladina těchto molekul ve vzorcích cervikální tekutiny u žen s PPROM. Tento přístup měl za cíl zjistit, zda stanovení molekulárních markerů, jejichž dynamika v plodové vodě vykazuje prediktivní potenciál ve vztahu k IAI a intraamniální infekci, bude poskytovat obdobné rozdíly i v cervikální tekutině, jejíž odběr je méně invazivní a náročný. Jak u kalretikulinu, tak u katepsinu G však nebyl zjištěn statisticky významný rozdíl mezi vzorky od pacientek s MIAC či s IAI. Rovněž nebyla prokázána souvislost mezi koncentrací sledovaných molekul a hladinou IL-6 v plodové vodě. Pro stanovení intraamniální infekce z cervikální tekutiny se tak sledované molekuly nejeví jako vhodné. Plodová voda a cervikální tekutina jsou anatomicky i fyziologicky oddělené kompartmenty, jejichž složení je odlišné. Situace v plodové vodě, která reflektuje intraamniální zánětlivou odpověď nemusí být identická v cervikální tekutině, jejíž složení odráží především zánět lokální. K obdobnému zjištění došel tým prof. Kacerovského u molekul TLR2 [171][172] a MIP1α. Obdobně nebyla při úpravě ke gestačnímu stáří prokázána zvýšená koncentrace IL-8 ve vaginální tekutině u pacientek s MIAC a HCA na rozdíl od IL-6, který tak zůstává nejspolehlivějším markerem IAI v plodové vodě, cervikální tekutině i v pupečníkové krvi [78][173][174], což prokázala i práce Stráníka [127] a Musilové [175]. Situace s IL-8 však není jednoznačná. Holst a kol. ve své studii uvádějí, že prediktivní hodnotu ve vztahu k IAI a intraamniální infekci, dle jejich měření, má v cervikální tekutině IL-8 srovnatelnou s IL-6 [176]. Z literatury vyplývá, že výsledky stanovení vybraných parametrů z cervikální a amniální tekutiny ve vztahu k IAI a infekci však mohou být ve vzájemném rozporu. Při PPROM totiž může plodová voda kontaminovat cervikální tekutinu, jejíž složení pak může do jisté míry zrcadlit události v amniálním prostoru. Lee a kol. uvádějí, že zjistili významné statistické rozdíly v koncentracích IL-6, IL-8, MCP-1, MIP1α a MIP1β u pacientek

s a bez mikrobiální invaze jak v amniální, tak v cervikovaginální tekutině [177]. Hladiny studovaných cytokinů v plodové a cervikovaginální tekutině dále vykazovaly vysokou míru vzájemné korelace. Koncentrace studovaných látek v pupečníkové krvi nevykazovaly statistikou významnost. Stejný vědecký tým v novější studii udává význam shodné skupiny cytokinů (s výjimkou MIP1α) pro stanovení IAI u žen s PTL, byť samotné stanovení těchto molekul z cervikovaginální tekutiny nepovažují za dostatečné pro detekci IAI [178].
Z celkového pohledu se tak jeví jako zásadní rozlišovat mezi výsledky stanovení těchto parametrů ve vzorcích cervikální tekutiny od pacientek s PPROM a PTL.

Komentář k práci č. IV.

Musilova I, Andrys C, Drahosova M, Soucek O, Pliskova L, Stepan M, Bestvina T, Maly J, Jacobsson B, Kacerovsky M

Amniotic fluid clusterin in pregnancies complicated by the preterm prelabor rupture of membranes

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Klastrin, apolipoprotein J, sulfatovaný glykoprotein 2, TRPM-2 (Testosterone-Repressed Prostate Message 2) nebo rovněž SP-40, je heterodimerní glykoprotein [179], pojmenovaný podle své schopnosti shlukovat buňky. Je tvořen prakticky ve všech tkáních lidského těla a patří do rodiny chaperonů, proto, stejně jako u kalretikulinu, je jeho hlavním úkolem správná konformační úprava nově syntetizovaných proteinů [180]. Kromě funkce chaperonu však hraje roli i v mnoha patofyziologických procesech jako je apoptóza, oxidační stres, odstraňování buněčného odpadu, transportu lipidů, při zánětlivé odpovědi, buněčném růstu a diferenciaci, obnově buněčné membrány nebo při regulaci aktivace komplementu [179][181]. Vysoká exprese této molekuly je považována za nespecifickou buněčnou odpověď na stres či poškození [179]. Působení klastrinu výrazně ovlivňuje jeho forma. Bylo prokázáno, že alternativním sestřihem mRNA vzniká buď do okolí secernovaný klastrin (sCLU) nebo intracelulárně lokalizovaný klastrin, nacházející se v cytoplasmě (cCLU) nebo v jádře buňky (nCLU) [182]. Intracelulárně lokalizovaný klastrin vystupuje jako aktivátor proapoptotických procesů přes uvolnění cytochromu *c* [181] a reakci s proapoptotickým proteinem Ku70, zatímco secernovaný klastrin se podílí na opravných procesech buněčné membrány, zabraňuje agregaci a precipitaci proteinů, plní svou funkci chaperonu a buňku naopak chrání [183]. O výsledném efektu na buňku pak rozhoduje poměr cCLU:sCLU [184]. Za jakých podmínek však dochází k akumulaci proteinu v cytoplasmě, není zcela jasné, naopak je prokázáno, že secernovaná forma proteinu je exprimována během podmínek buněčného stresu [179], což kromě benefitu pro zdravé buňky přináší i problémy při léčbě nádorů, jelikož molekula klastrinu může interferovat s chemoterapeutiky. Zároveň je tak možným terapeutickým cílem při případném ovládnutí regulace poměru sekretované a cytoplasmatické formy molekuly [184].

Analýza hodnot klastrinu přinesla zjištění, že koncentrace molekuly v plodové vodě je statisticky nižší u pacientek s MIAC oproti ženám bez MIAC ($p < 0,0001$). Stejně tak vzorky od žen s IAI vykazovaly nižší hladinu klastrinu než vzorky od žen bez této komplikace ($p = 0,001$), snížené hodnoty byly rovněž zaznamenány u žen s intraamniální infekcí ($p = 0,008$). Ze vzájemné analýzy jednotlivých podskupin pacientek podle přítomnosti MIAC a probíhajícího IAI vyplynul statisticky významný rozdíl mezi pacientkami s intraamniální infekcí oproti rodičkám bez těchto komplikací, kdy pacientky s prokázanou infekcí vykazovaly nižší koncentraci proteinu v plodové vodě ($p < 0,0001$). Dále bylo prokázáno, že koncentrace klastrinu se snižuje se zvyšujícím se gestačním věkem při odběru vzorků (rho = -0,38; $p < 0,0001$). Pravděpodobné vysvětlení získaných údajů by mohlo spočívat ve vlastnosti klastrinu regulovat aktivaci komplementu vazbou složek C5b a C6, což znemožňuje formaci lytického komplexu [185]. Snížení hladiny koncentrace klastrinu tedy může být následkem jeho vazby na komplement ve snaze regulovat jeho aktivaci, ke které při zánětu přirozeně dochází. Výsledky, které by mohly takovou hypotézu potvrzovat, byly získány při studii zaměřené na koncentraci složky C3 a komplementového faktoru I v plodové vodě u pacientek s PPROM. Zatímco koncentrace složky C3 byla zvýšena za probíhajícího IAI ($p = 0,0004$) ovšem nikoliv během pouhé kolonizace, koncentrace regulačního faktoru I byla při zánětu snížena ($p = 0,03$). Při kolonizaci bez známek zánětu nebyly zjištěny statistické rozdíly [data publikována autorem práce na 5th European Congress of Immunology, Amsterdam, 2018]. Obdobné výsledky byly týmem prof. Kacerovského získány také z analýzy koncentrace molekuly vitronektinu [120], kdy byly jeho nižší koncentrace nalezeny v plodové vodě u pacientek s MIAC a HCA. Vitronektin přitom rovněž vykazuje komplement-inhibiční aktivitu blokádou vazby složek C5b-C7 na buněčnou membránu a zabráněním polymerizace složky C9 [187]. Zdá se tedy, že aktivace komplementu může vést k vyčerpání jeho inhibitorů, či ke snížení schopnosti jejich detekce *in vitro*. Za snížením koncentrace klastrinu může být rovněž schopnost některých bakterií vázat jej na svůj povrch ve snaze vyhnout se imunitní odpovědi [188][189], není však popsáno, že by se tento jev uplatňoval v přítomnosti bakterií rodu *Ureaplasma* či *Mycoplasma*. I přes přínosná zjištění se tak, dle našich výsledků, klastrin nejeví jako vhodný marker pro sledování přítomnosti intraamniální infekce.

Komentář k práci č. V.

Soucek O, Kacerovsky M, Musilova I, Pliskova L, Bolehovska R, Andrys C

Amniotic fluid CD11b levels in pregnancies complicated by preterm prelabor rupture of membranes

J Matern Fetal Neonatal Med. 2020 May 19:1-9

Protein CD11b, také známý jako integrin alfa M (ITGAM), spolu s molekulou CD18 vytváří heterodimerní komplex alfa-M beta-2 ($\alpha_M\beta_2$), také nazývaný jako komplementový receptor 3 (CR3) nebo makrofág-1 antigen (Mac-1). Molekula CD11b je exprimována na monocytech, makrofázích, granulocytech a NK buňkách a hraje roli při zánětu, zejména při adhezi a migraci leukocytů. Samotný CD11b pak zprostředkovává vazbu integrinů na četné substráty jako je fibrinogen, faktor X, ICAM-1 [190]. Tento protein také zprostředkovává fagocytózu díky své schopnosti vázat inaktivovanou složku komplementu C3b [191]. Stimulace lymfocytů pomocí antiCD3 a IL-2 však také indukuje expresi CD11b na T lymfocytech [192]. Deficit CD11b je spojen se zhoršenou adhezí k vaskulárnímu endotelu, ale je také spojen s akumulací neutrofilů v extravaskulárním prostoru a se zhoršenou apoptózou granulocytů. Expresi CD11b lze proto považovat za homeostatický mechanismus zánětu [193]. Snížená exprese CD11b je také spojena s horším průběhem nebo zvýšenou mortalitou u infekčních onemocnění [194]–[197]. Většina molekul CD11b neutrofilů není exprimována na membráně, ale je uložena ve specifických granulích [198] a jejich membránová exprese a uvolňování je zvýšena během zánětlivých stavů [199]–[202]. Cílem práce bylo zjistit, zda se mění koncentrace solubilního CD11b u žen s PPROM v závislosti na přítomnosti IAI, MIAC či intraamniální infekce, jelikož o vztahu mezi koncentrací CD11b v plodové vodě a PPROM dosud nebyly žádné poznatky. Provedené měření přineslo následující zjištění: (i) ženy s MIAC mají vyšší koncentrace CD11b v plodové vodě než ženy bez MIAC (p = 0,001); (ii) ženy s IAI nemají vyšší koncentrace CD11b v plodové vodě ve srovnání se ženami bez IAI po zohlednění gestačního věku při odběru vzorků (p = 0,37); (iii) existuje slabá korelace mezi koncentrací CD11b v plodové vodě a hladinou IL-6 (rho = 0,26; p = 0,02), (iv) ženy s intraamniální infekcí mají vyšší hodnoty CD11b v plodové vodě než ženy bez těchto komplikací (p = 0,001) i po zohlednění gestačního stáří (p = 0,04).

Nejvyšší koncentrace CD11b byla zjištěna u žen s intraamniální infekcí, zvýšená koncentrace molekuly byla rovněž zjištěna u prostého MIAC, tedy stavu, kdy je prokázána pouze kolonizace bez známek zánětu. Naopak statisticky významný rozdíl v hladinách CD11b nebyl nalezen u rodiček se zánětem bez ohledu na jeho příčinu po zohlednění gestačního věku při odběru vzorků. To by mohlo znamenat, že zvýšení exprese CD11b na rozhraní matky a plodu je indukováno spíše přítomností bakterií než samotným zánětlivým stavem, což je v souladu s pozorováním, že zvýšená exprese CD11b je indukována bakteriálními PAMPy, jako je bakteriální LPS [203] a dále přítomností prozánětlivých cytokinů, jako např. TNFα [204] nebo volnou myeloperoxidázou [205]. Toto zjištění je rovněž ve shodě se skutečností, že CD11b je považován za účinný marker pro diagnostiku novorozenecké sepse s časným nástupem [206]–[208], Genel a kol. také informovali o zvýšené expresi CD11b na neutrofilech a monocytech při infekcích novorozenců [209]. Pokud považujeme za hlavní zdroj molekuly CD11b v plodové vodě neutrofily a monocyty, můžeme uvažovat o jejich vlivu na zvýšení koncentrace CD11b v plodové vodě při zánětu vyvolaném přítomností bakterií, který bude doprovázen akumulací těchto buněk v místě zánětu. Zapojení a význam neutrofilů a makrofágů je však nesporný jak u infekce, tak u zánětu sterilního [210]–[212]. Byť se v případě sterilního zánětu neuplatňuje stimulace imunocytů a buněk plodových obalů skrze PAMPy, i zánět sterilní doprovází zvýšená koncentrace prozánětlivých cytokinů v plodové vodě, která expresi CD11b zvyšuje. Bylo také pozorováno, že hladiny katepsinu G byly zvýšeny při intraamniální infekci i při intraamniálním zánětu jako takovém [213], přičemž za hlavní zdroj katepsinu G považujeme rovněž neutrofilní granulocyty. Na otázku proč není koncentrace CD11b zvýšena i u pacientek se zánětem bez jednoznačně prokázané infekční příčiny by mohla odpovídat práce Behnii a kol., která představuje molekulární rozdíly mezi IAI vyvolaným sterilní a nesterilní noxou [50]. výsledky odhalily aktivaci různých molekulárních drah vedoucích k iniciaci zánětu v závislosti na typu iniciačního podnětu. Ačkoliv tím není zpochybněna samotná povaha zánětu, je nutné vzít v úvahu různé fenotypy IAI, které určují typ poškození buněk. Behnia považuje za rozhodující faktor, který určuje fenotyp IAI, úroveň oxidačního stresu, která povede k poškození DNA a která bude u vnitřního (sterilního) poškození vyšší, přičemž spouštěcím faktorem sterilního IAI může být např. obezita nebo naopak podvýživa, užívání drog či alkoholu nebo rizikové chování, jak bylo uvedeno v úvodu práce. Takový typ zánětu bude doprovázet uvolnění vzorů DAMP sterilního zánětu do extracelulárního prostředí, jako jsou HMGB1, mitochondriální DNA, ATP, HSP či

kyselina močová [214]. Naopak přítomnost bakteriálních PAMP v souvislosti s menší mírou oxidačního stresu a signálů vnitřního poškození buněk vede ke stimulaci klasické zánětlivé dráhy aktivací transkripčního faktoru NFκB. Z hlediska snahy o diagnostickou predikci intraamniální infekce a předčasného porodu je proto nutné zkoumat různé fenotypy zánětu a věnovat pozornost jejich iniciátorům. Zvýšená exprese CD11b u pacientek s MIAC a intraamniální infekcí v kontextu výše uvedeného může naznačovat, že v případě této konkrétní molekuly je to zejména nástup zánětu v přítomnosti mikroorganismů, který ovlivňuje jeho expresi. V kohortě pacientek vybrané pro dané měření však nebylo dostatečně početné množství vzorků plodové vody od pacientek se sterilním IAI, aby mohla být tato hypotéza ověřena. Dále se nabízí otázka, jak se molekula CD11b, standardně vázaná na buněčnou membránu, stane rozpustnou. Každý neutrofil se přesouvá do místa zánětu řadou kroků, které zahrnují konstantní vazbu a odloučení od různých substrátů v extracelulárním prostoru [215]. Jak prokázal Zen a kol., oddělení a uvolňování CD11b z jeho ligandů je přitom zprostředkováno serinovými proteázami, včetně katepsinu G [216], přičemž zvýšená koncentrace katepsinu G byla také zjištěna u žen s IAI a MIAC [213]. Je tak pravděpodobné, že tyto procesy jsou spojeny, aby byl zajištěn hladký pohyb neutrofilů do místa zánětu. Jak je však uvedeno výše, na rozdíl od katepsinu G nebylo u CD11b pozorováno zvýšení koncentrace při zánětu při zohlednění gestačního stáří. U katepsinu G nebyla dále pozorována jeho statisticky významně vyšší koncentrace u pacientek s IAI či infekcí v cervikální tekutině, podobně jako u kalretikulinu [164]. U CD11b tyto údaje zatím chybí. Limitem provedené studie je fakt, že neznáme míru exprese CD11b v buňkách plodových obalů, resp. zda vůbec ke zvýšení koncentrace CD11b v plodové vodě tyto buňky přispívají.

Komentář k práci č. VI.

Spacek R, Musilova I, Andrys C, Soucek O, Burckova H, Pavlicek J, Pliskova L, Bolehovska R, Kacerovsky M

Extracellular granzyme A in amniotic fluid is elevated in the presence of sterile intra-amniotic inflammation in preterm prelabor rupture of membranes

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Granzymy (Gr) jsou serinové proteázy produkované cytotoxickými T lymfocyty a NK buňkami a slouží jako důležitý nástroj při obraně proti nitrobuněčným parazitům a nádorově transformovaným buňkám. Exprese granzymů však byla prokázána i u dalších typů leukocytů jako jsou neutrofilní granulocyty [217], makrofágy [218], dendritické buňky [219] či mastocyty [220]. V současnosti známe pět odlišných typů granzymů: GrA, GrB, GrH, GrK a GrM [221], přičemž v cytotoxických buňkách jsou nejvíce zastoupeny granzymy typu A a B. Granzymy jsou proteolytické enzymy, které štěpí a aktivují tak efektorové molekuly v cílových buňkách, což vede různými cestami k jejich apoptóze [222]. Cytotoxická reakce začíná fúzí cytoplasmatických vezikul obsahujících granzymy a perforiny s buněčnou membránou cytotoxické buňky, což vede k uvolnění těchto látek do extracelulárního prostoru [223]. Perforin následně vytvoří v buněčné membráně cílové buňky zatím ne zcela objasněným mechanismem póry, jimiž prochází granzymy do cytoplasmy [186].

Studie měla za cíl zhodnotit koncentraci granzymu A u žen s PPROM ve vztahu k IAI, MIAC a intraamniální infekci. Vzhledem k imunologické povaze této látky zde nebyl předpoklad vysokých hodnot u zánětu, který je způsobován především bakteriemi, nicméně zvýšené hodnoty granzymu A byly zaznamenány i u bakteriálních infekcí [224]–[227] a septických stavů [228][229] a tato molekula by tedy mohla být ukazatelem zánětu. Rovněž neexistovala studie, která by hodnotila koncentraci granzymu A v plodové vodě ve vztahu k výše uvedeným komplikacím. Důvodem k proměření koncentrace této molekuly u pacientek s PPROM je také možné podcenění významu virové etiologie u intraamniálních infekcí. Tyto infekce jsou vzácnější než bakteriální, mohou ovšem rovněž vést k předčasnému porodu [111]. Nejčastěji se jedná o infekce vyvolané adenoviry, enteroviry a cytomegaloviry [230].

Diagnosticky jsou však tyto případy obtížně zachytitelné, jelikož mikrobiologická analýza je zaměřena na detekci bakteriálních a fungálních agens.

Zhodnocení množství granzymu A v plodové vodě ukázalo velmi nízké hodnoty, hodnotitelné množství molekuly bylo zaznamenáno u 87 % vzorků. Byla zjištěna nižší koncentrace granzymu A u pacientek s MIAC (p = 0,03) i po úpravě vzhledem ke gestačnímu stáří (p = 0,02). Rozdíl mezi pacientkami s IAI a bez IAI bez ohledu na původ zánětu nebyl pozorován (p = 0,39). Analýza podskupin pacientek s ohledem na IAI a / nebo MIAC přinesla zjištění, že statisticky vyšší hodnoty byly zjištěny u pacientek se sterilním IAI (p = 0,02). Mezi hladinou IL-6 a granzymu A nebyla zjištěna souvislost (p = 0,58).

Za hlavní zdroj granzymu A považujeme imunokompetentní buňky, které produkují tuto molekulu při své aktivaci. Do extracelulárního prostoru se granzym A může dostat uvolněním během imunologické synapse, ale rovněž jeho aktivní secernací imunocyty bez ohledu na cytotoxickou reakci. Tento jev, tedy uvolnění granzymů A a B nezávisle na cytotoxické reakci, je např. dobře popsán při intravenózním podání bakteriálního LPS zdravým lidem i při inkubaci plné krve s LPS či bakteriemi [224]. Přítomnost extracelulárního granzymu A byla popsána v krvi a dalších tělních tekutinách [231]–[234]. V případě našich výsledků byla prokázána přítomnost této molekuly v plodové vodě. Zda jsou zde hlavním zdrojem granzymu A leukocyty je ale diskutabilní, jelikož nebyla zjištěna vyšší koncentrace zkoumané molekuly u intraamniální infekce, která je doprovázena zvýšenou přítomností leukocytů [235]. V případě prosté kolonizace amniální dutiny, kterou rovněž doprovází zvýšené zastoupení leukocytů [236], byla zjištěna naopak nižší hladina granzymu A u pacientek s pozitivním nálezem. Zdrojem extracelulárního granzymu A v plodové vodě by tak mohly být i buňky plodových obalů a placenty. Tuto hypotézu potvrzují i studie, které prokázaly přítomnost granzym-exprimujících buněk v placentě a plodových obalech [237]–[239]. Výsledky studie dále poukázaly na fakt, že nejvyšší koncentrace zkoumané molekuly byly detekovány v plodové vodě pacientek se sterilním IAI. Vysvětlení tohoto jevu by mohlo být obdobné jako u molekuly CD11b, tedy že příčinná noxa ovlivňuje fenotyp zánětu, a tedy i obranné mechanismy, které se při jeho rozvoji uplatňují. Je však třeba uvést, že měření bylo provedeno na malém počtu vzorků (n = 8), což může mít vliv na reprezentativnost výsledků. Celkově vzato se granzym A nejeví jako molekula významně zúčastněná v patofyziologii intraamniální infekce a současně ani jako vhodný marker k její predikci.

Komentář k práci č. VII.

Stranik J, Kacerovsky M, Soucek O, Kolackova M, Musilova I, Pliskova L, Bolehovska R, Bostik P, Matulova J, Jacobsson B, Andrys C

IgGFc-binding protein in pregnancies complicated by spontaneous preterm delivery: a retrospective cohort study

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FcgammaBP (IgGFcBP) byl objeven před více než 30 lety jako specifické místo pro vazbu Fc části imunoglobulinu G v epitelu tenkého a tlustého střeva [240]. Toto specifické místo bylo odlišné od dříve rozpoznávaných receptorů pro Fc oblast IgG [240] a bylo později označeno jako FcgammaBP a identifikováno jako protein primárně lokalizovaný ve sliznicích epitelu tenkého a tlustého střeva a vylučovaný do střevního lumen [241]. Na základě současných znalostí je FcgammaBP považován za protein poskytující imunologickou ochranu střevní tkáni a usnadňující interakci mezi střevním hlenem a potenciálně škodlivým podnětem (mikroorganismy, vzory nebezpečí a poškození PAMP a DAMP) [240]–[242]. Jeho přesná biologická funkce však musí být ještě plně objasněna. Produkce FcgammaBP byla popsána v buňkách střevního epitelu, placentě a tkáni štítné žlázy [242][243]. Jeho exprese však nebyla prokázána ve svalech, mozku, sluchovém ústrojí, ledvinách, játrech, plicích a kostech [242]. FcgammaBP byl nalezen v nízkých koncentracích v lidském séru zdravých jedinců [244], jeho sérové koncentrace však byly zvýšeny v přítomnosti autoimunitních onemocnění, jako je Crohnova choroba, ulcerózní kolitida, revmatoidní artritida, systémový lupus erythematodes a progresivní systémová skleróza [244]. FcgammaBP byl dále prokázán v plodové vodě, moči, slinách a mozkomíšním moku [245][246]. Liu a kol. detekovali FcgammaBP jako složku plodové vody ve druhém trimestru nekomplikovaných těhotenství [246]. Kromě toho bylo prokázáno, že FcgammaBP patří mezi nejhojnější proteiny plodové vody [246]. Tým prof. Kacerovského dříve popsal přítomnost FcgammaBP v plodové vodě u těhotenství komplikovaných PPROM a PTL [120][122], a dále zjistil, že koncentrace FcgammaBP v plodové vodě u žen s MIAC a HCA je vyšší než u žen bez těchto komplikací [120]. Koncentrace FcgammaBP v plodové vodě u žen s HCA samotnou nebo pouze s MIAC však nebyla stanovena.

Naše studie byla zaměřena na stanovení tohoto proteinu jak z plodové vody, tak z cervikální tekutiny u žen s PTL i u žen s PPROM. U žen s PPROM bylo zjištěno, že jak infekční, tak neinfekční zánětlivý stimul byl schopen spustit produkci FcgammaBP do plodové vody, protože přítomnost IAI, bez ohledu na přítomnost mikroorganismů v plodové vodě, byla spojena se zvýšením koncentrace FcgammaBP ($p < 0,0001$). Toto zjištění je v souladu s výše uvedeným pozorováním, protože u podskupin žen s intraamniální infekcí a sterilním IAI je vysoké riziko rozvoje HCA [247][248]. Obdobný trend byl zaznamenán u žen s PTL, kde byly nejvyšší hodnoty proteinu detekovány v plodové vodě u pacientek s intraamniální infekcí, hladiny molekuly u pacientek se sterilním zánětem byly nižší, ale stále statisticky významně vyšší než u pacientek bez zánětu. Při stanovení FcgammaBP v cervikální tekutině byla koncentrace FcgammaBP u žen s PPROM zvýšena právě v podskupině žen s intraamniální infekcí ($p < 0,0001$), ale nikoliv u žen se sterilním IAI. Asociace mezi koncentracemi FcgammaBP ve spárovaných vzorcích plodové vody a cervikální tekutiny byla prokázána pozitivní korelací (rho = 0,34; $p < 0,0001$). Pozitivní korelace byla rovněž nalezena mezi koncentrací IL-6 a FcgammaBP v plodové vodě (rho = 0,55; $p < 0,0001$), naopak negativní korelace byla zjištěna mezi obsahem FcgammaBP v plodové vodě a gestačním věkem při odběru vzorků (rho = -0,4; $p < 0,0001$). U kohorty pacientek s PTL byla zjištěna vyšší hladina FcgammaBP v cervikální tekutině u žen s intraamniální infekcí i se sterilním zánětem oproti ženám bez zánětu, avšak statisticky se nejednalo o významný rozdíl. V plodové vodě byly stanoveny cut-off hodnoty pro detekci intraamniální infekce: 60 ng/ml u žen s PPROM (AUC = 0,94; $p < 0.0001$) a 120 ng/ml u žen s PTL (AUC = 0,86; $p< 0.0001$). V cervikální tekutině se jako vhodná hraniční hodnota u žen s PPROM jeví hladina 300 ng/ml (AUC = 0,93; $p < 0.0001$).

Překvapivým pozorováním byla skutečnost, že koncentrace FcgammaBP byly vyšší ve vzorcích cervikální tekutiny než ve vzorcích plodové vody. Vzhledem k technice odběru vzorků cervikální tekutiny (ředění v 1,5 ml pufru) a pětkrát vyššímu ředění vzorků cervikální tekutiny než ředění vzorků plodové vody před stanovením FcgammaBP, je vysoce pravděpodobné, že epitelové buňky endocervikálního kanálu produkují FcgammaBP. Toto zjištění podporuje roli děložního čípku v imunologické ochraně proti vzestupu mikroorganismů z vagíny / děložního čípku směrem k horní části genitálního traktu [249]–[252].

Po provedených měřeních byl FcgammaBP z plodové vody a cervikální tekutiny identifikován jako potenciální marker intraamniální infekce v těhotenstvích s PPROM, což je výhoda oproti výše zmíněným molekulám katepsinu G a kalretikulinu, které se jeví jako vhodné markery pouze při stanovení v plodové vodě [69][70][115]. Zejména stanovení FcgammaBP z cervikální tekutiny tak může být klinicky relevantním markerem kvůli neinvazivní povaze odběru vzorků. Jedná se o však pouze o kohortu pacientek s PPROM a nikoliv s PTL. Toto zjištění velice pravděpodobně odráží fakt, že složení cervikální tekutiny bude zákonitě jiné u pacientek s PPROM než u pacientek s PTL, jelikož bude obsahovat i příměs plodové vody uniknuvší z předčasně rozrušených plodových obalů. Cervikální tekutina pak do jisté míry může odrážet molekulární nález v plodové vodě.

Přestože byla popsána exprese FcgammaBP v placentě [242], zůstává otázka, která část placenty je zdrojem FcgammaBP a zda plodové obaly produkují FcgammaBP. Existuje řada důkazů, že střevní epitel produkuje FcgammaBP, ale nejsou k dispozici žádná data, zda má tuto schopnost i amniální epitel. Vzhledem k tomu, že amniální epitel je důležitou bariérou proti vzestupu mikroorganismů do amniální dutiny [255]–[257], lze identifikovat některé podobnosti mezi střevním a amniálním epitelem, a sice, že: (i) slouží jako mechanická bariéra [255]–[259]; (ii) má specificky prostorově exprimované TLR [260][261]; (iii) exprese TLR se mění v přítomnosti zánětu [54][162][163]. Můžeme proto předpokládat, že amniální epitel by se mohl podílet na tvorbě FcgammaBP.

Komentář k práci č. VIII.

Soucek O, Kacerovsky M, Stranik J, Musilova I, Pliskova L, Bolehovska R, Matulova J, Andrys C

Macrophage inflammatory protein-1α in amniotic and cervical fluids inspontaneous preterm labor with intact membranes with respect to intra-amnioticinflammation

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Protein MIP1α (také známý jako CCL3) je členem CC rodiny chemokinů a je produkován lymfocyty, monocyty a makrofágy, žírnými buňkami, bazofily, epiteliálními a mezoteliálními buňkami a fibroblasty poté, co jsou tyto buňky vystaveny prozánětlivým molekulám, jako je IL-1β, TNFα nebo bakteriální LPS [263][264]. Chemokin CCL3 se váže na chemokinové receptory na mononukleárních buňkách i na neutrofilních a eosinofilních granulocytech a působí jako jejich chemoatraktant a aktivátor prostřednictvím kaskádové signalizace na jejímž konci je aktivace protein kinázy C. Hraje proto důležitou roli při zprostředkování a udržení zánětu [264]–[266]. Provedená studie měla za cíl stanovit hladiny MIP1α u pacientek s PTL v závislosti na sterilním IAI či intraamniální infekci v plodové vodě a v cervikální tekutině. O MIP1α jako o proteinu, jehož koncentrace se zvyšuje v souvislosti se sterilním IAI a intraamniální infekcí informovali ve své již zmíněné práci Lee [177], dále Romero a kol. [267] a Bhatti a kol. [20]. Dudley udává zvýšené koncentrace této molekuly u pacientek s intraamniální infekcí [268]. Tyto práce hodnotí prediktivní potenciál solubilního MIP1α v plodové vodě, v extracelulárních vesikulech plodové vody a v cervikovaginální tekutině. Jako markery s dobrým potenciálem pro predikci IAI a intraamniální infekce vychází i další testované molekuly rodiny CC chemokinů: MIP1β, MIP3α a MCP-1. Náš tým měl za cíl ověřit prediktivní potenciál této molekuly a stanovit jeho hladiny u vybrané kohorty pacientek i ve vzorcích cervikální tekutiny. V plodové vodě byl v případě stanovení MIP1α při vzájemném porovnání zjištěn významný rozdíl mezi vzorky pacientek s intraamniální infekcí a bez infekce ($p < 0,0001$). Pacientky se sterilním zánětem vykazovaly vyšší hodnoty MIP1α v plodové vodě než pacientky bez zánětu ($p < 0,0001$), po úpravě dat vzhledem ke gestačnímu stáří však rozdíl nebyl statisticky významný ($p = 0,26$). Porovnání hodnot mezi vzorky s intraamniální infekcí a sterilním zánětem ukázalo statisticky vyšší hladiny MIP1α u pacientek s infekcí ($p < 0,0001$). Jako optimální hladina MIP1α pro predikci intraamniální infekce z plodové vody jeví hodnota 1300 pg/ml (AUC = 0,88; $p < 0.0001$) při sensitivitě 75 % a specificitě 95 %. Zde se

nabízí srovnání s hladinou MIP1α v plodové vodě, kterou doporučuje Romero, a sice 500 pg/ml [267], a Lee, který stanovuje jako cut-off hodnotu pouze 12,7 pg/ml (AUC = 0,88) [177]. Obě práce nicméně hodnotí hladinu MIP1α v kontextu s MIAC, a přestože bakteriální kolonizace je z velké části doprovázena zánětem, není to její podmínkou. Přednost komentované práce tedy může být v přesné identifikaci pacientek s prokázanou intraamniální infekcí, což by mohlo vysvětlit i nejvyšší cut-off hodnotu ze srovnávaných prací, které mezi nálezy u pacientek s prostým MIAC a infekcí nerozlišují. V cervikální tekutině byly detekovány zvýšené hladiny MIP1α u pacientek s intraamniální infekcí a sterilním zánětem, po úpravě dat vzhledem ke gestačnímu stáří však rozdíly nebyly statisticky významné. Naše závěry tak nejsou zcela konzistentní se závěry Leeho, který detekoval statisticky významně vyšší hladiny MIP1α v plodové, ale i v cervikovaginální tekutině u pacientek s MIAC a udává vysokou míru korelace hladiny MIP1α s hladinou IL-6 [177]. V případě jeho práce však byly hodnoty stanoveny ze vzorků od pacientek s PPROM a nikoliv s PTL, což mohlo mít významný vliv na rozdílnost výsledků, zejména s ohledem na složení cervikální tekutiny. Podobný rozdíl pozoroval tým prof. Kacerovského u hladin molekuly FcgammaBP, kdy byla při probíhající intraamniální infekci prokázána zvýšená hladina v cervikální tekutině u žen s PPROM a nikoliv s PTL.

Závěr

Potvrzení intraamniální infekce představuje pro kliniky diagnostickou výzvu. Nutnost vyloučit přítomnost mikroorganismů v plodové vodě činí diagnostiku intraamniální infekce časově náročnou a nákladnou, obzvláště pokud se používají techniky identifikace nekultivovatelných nebo obtížně kultivovatelných mikroorganismů. Z pohledu lékaře existuje naléhavá potřeba spolehlivého markeru intraamniální infekce s dobrou citlivostí a specificitou. Cílem disertační práce bylo shrnout a okomentovat vybrané publikované výstupy vědeckého kolektivu, jehož práce byla zaměřena na stanovení diagnostického významu vybraných molekul u intraamniální infekce. Vědecký kolektiv byl složen z pracovníků Porodnické a gynekologické kliniky, Ústavu klinické imunologie a alergologie, Ústavu klinické mikrobiologie a Ústavu klinické biochemie a diagnostiky Fakultní nemocnice Hradec Králové. Smyslem výzkumného záměru bylo vytipovat molekuly, jejichž dynamika by se mohla měnit s ohledem na probíhající intraamniální infekci. Testované molekuly byly vybrány na základě výsledků dříve provedených proteomických studií a literárních rešerší. Koncentrace vybraných molekulárních parametrů byly stanoveny ve vzorcích plodových vod žen s PPROM či PTL přijatých do péče na Porodnické a gynekologické klinice Fakultní nemocnice Hradec Králové, přičemž u části žen bylo těhotenství komplikováno IAI či intraamniální infekcí. U molekul, které vykazovaly slibný diagnostický potenciál, bylo provedeno i jejich stanovení ze vzorků cervikální tekutiny. U všech pacientek přijatých do studie bylo provedeno stanovení IL-6 a mikrobiologické vyšetření, aby bylo možné pacientky přesně stratifikovat do podskupin s ohledem na přítomnost či absenci mikrobiální kolonizace a intraamniálního zánětu. Výsledky, které práce komentuje se týkají měření koncentrace molekul kalretikulinu, katepsinu G, klastrinu, CD11b, granzymu A, FcgammaBP a MIP1α. Stanovení některých dalších vybraných molekul nepřineslo publikovatelné výsledky, nebo výsledky jejich měření nebyly v době přípravy disertační práce připraveny k publikování. Jedná se o molekuly GDF-15, CD64, vybrané složky komplementu nebo molekuly účastnící se purinergní signalizace: adenosin, CD39, CD73 a receptor $P2X_7R$. Hladiny kalretikulinu, katepsinu G, klastrinu, CD11b, granzymu A byly stanoveny u pacientek s PPROM, koncentrace MIP1α byla stanovena u žen s PTL. Hladiny FcgammaBP byly stanoveny jak u žen s PPROM, tak u žen s PTL. Statisticky významné zvýšení koncentrace při probíhající intraamniální infekci bylo pozorováno u kalretikulinu, katepsinu G, CD11b, FcgammaBP a MIP1α. U klastrinu bylo zjištěno statisticky významné

snížení koncentrace této molekuly při intraamniální infekci. Hladiny granzymu A se s ohledem na přítomnost či absenci intraamniální infekce statisticky významně nelišily od výsledků u pacientek bez infekce. U molekul s významným nálezem v plodové vodě bylo provedeno jejich stanovení i v cervikální tekutině. Jednalo se o kalretikulin, katepsin G, FcgammaBP a MIP1α. U kalretikulinu, katepsinu G a MIP1α však v cervikální tekutině nebyl zjištěn významný rozdíl v koncentraci s ohledem na probíhající infekci. U FcgammaBP byla zjištěna statisticky významně vyšší koncentrace i v cervikální tekutině, avšak pouze u žen s PPROM a nikoliv u žen s PTL. Na základě výsledků byly u vybraných molekul stanoveny cut-off hodnoty pro detekci intraamniální infekce. V plodové vodě se jedná o hodnoty 81,4 ng/ml pro kalretikulin a 1300 pg/ml pro MIP1α. V případě FcgammaBP se jedná o hodnotu 60 ng/ml v plodové vodě u pacientek s PPROM a 120 ng/ml plodové vodě u žen s PTL. V cervikální tekutině žen s intraamniální infekcí a PPROM byla stanovena cut-off hodnota 300 ng/ml. Ze získaných výsledků vyplývá, že zejména FcgammaBP by mohl být kvalitním novým biomarkerem intraamniální infekce s dobrou specificitou a věrohodnostním poměrem. Vzhledem k jeho predikčnímu potenciálu z cervikální tekutiny u žen s PPROM by mohl být využit pro sledování dynamiky infekce u žen v období mezi rupturou plodových obalů a porodem.

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ORIGINAL ARTICLE

Amniotic fluid calreticulin in pregnancies complicated by the preterm prelabor rupture of membranes

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Abstract

Objective: This study aimed to determine the amniotic fluid calreticulin concentrations in women with the preterm prelabor rupture of membranes (PPROM) based on the microbial invasion of the amniotic cavity (MIAC), intraamniotic inflammation (IAI) and microbial-associated IAI.

Methods: One hundred sixty-eight women with singleton pregnancies were included in this study. Amniotic fluid samples were obtained by transabdominal amniocentesis and were assayed for calreticulin concentrations by ELISA. IAI was defined as an amniotic fluid interleukin-6 concentration > 745 pg/ml. Microbial-associated IAI was defined as the presence of both MIAC and IAI.

Result: Women with MIAC (with MIAC: median 54.4 ng/ml, versus without MIAC: median 32.6 ng/ml; $p < 0.0001$), IAI (with IAI: median 66.8 ng/ml, versus without IAI: median 33.0 ng/ml; $p < 0.0001$) and microbial-associated IAI (with microbial-associated IAI: median 82.5 ng/ml, versus without microbial-associated IAI: median 33.7 ng/ml; $p < 0.0001$) had higher concentrations of calreticulin than women without these complications. An amniotic fluid calreticulin concentration of 81.4 ng/ml was found to be the best cutoff point for identifying women with microbial-associated IAI.

Conclusions: The presence of microbial-associated IAI is associated with increased amniotic fluid calreticulin concentrations. Calreticulin seems to be a promising marker for the early identification of PPROM complicated by microbial-associated IAI.

Keywords

Bacteria, chaperon, inflammation, preterm delivery

History

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Introduction

The preterm prelabor rupture of membranes (PPROM), defined as the rupture of fetal membranes with amniotic fluid leakage before the onset of regular uterine activity prior at a gestational age of 37 weeks, continues to remain a challenge in current perinatology [1]. PPROM is responsible for approximately one-third of all preterm deliveries, and the prognosis of newborns from PPROM pregnancies is worse than that of newborns from pregnancies complicated by preterm labor with intact membranes [1,2].

Recent studies have suggested that the etiology of PPROM is non-infectious based on the accelerated aging of the fetal membrane [3–5]. However, PPROM is often complicated by infectious and inflammatory conditions, such as microbial invasion in the amniotic cavity (MIAC) and intraamniotic infection (IAI), either microbial-associated (IAI with MIAC) or sterile IAI (IAI without MIAC) [6,7]. However, our recent study did not identify any association between the presence of these inflammatory conditions and worsened neonatal outcome. Thus, infection/inflammation likely affects specific aspects of long-term outcome [7].

The early and prompt identification of infectious complications of PPROM remains a clinical problem because the evaluation of MIAC (either by cultivation or by different non-cultivate techniques) is time consuming and expensive [7,8]. Although many studies have suggested single or multiple potential markers, a robust and clinical applicable marker has not yet been identified [7,9–12].

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Unbiased proteomic studies provide a completely new, complex view of the amniotic fluid composition in PPROM [13–19]. Amniotic fluid has been shown to also contain proteins traditionally considered to only be intracellular [14,17,19]. One of these specific proteins found in the amniotic fluid is calreticulin [14,19], which is a highly conserved protein that is traditionally considered to be an obligate a calcium-binding, endoplasmic reticulum-resident, 60-kDa chaperone [20–28]. Calreticulin has been found on the surface of most mammalian cells, including neutrophils as well [23–28]. Calreticulin is a multifunctional and multicompartmental protein playing a vital role in many cellular functions (e.g. adhesion, blood function, development, endoplasmatic reticulum functions, gene expression and others) [29]. In addition, calreticulin at the cell surface and calreticulin released from the cell to extracellular environment has been shown to be associated with a number of function in physiological and pathological processes [29]. Proposed functions for calreticulin range from the regulation of Ca^{2+} signaling to the modulation cell adhesion via interaction with fibronectin and/or integrins affecting extracellular matrix [29–31].

Our previous proteomic study found higher amniotic fluid calreticulin concentrations in PPROM pregnancies complicated by infectious histological chorioamnionitis [14]. Nevertheless, this finding has not yet been validated in an independent cohort by a complementary antibody-based approach. Moreover, little information is available about amniotic fluid calreticulin in the context of IAI or microbial-associated IAI.

Therefore, the aim of this study was to determine the amniotic fluid concentrations of calreticulin in pregnancies complicated by PPROM based on the presence of MIAC, IAI and microbial-associated IAI. The final goal was to evaluate the potential of amniotic fluid calreticulin to predict the presence of microbial-associated IAI.

Materials and methods

A prospective cohort study of pregnant women between gestational age 24 + 0 and 36 + 6 weeks who were admitted to the Department of Obstetrics and Gynecology of the University Hospital Hradec Kralove in the Czech Republic was conducted between January 2014 and September 2015. Women with a maternal age ≥18 years having singleton pregnancies complicated by PPROM were invited to participate in the study. Women with signs of fetal growth restrictions, the presence of either congenital or chromosomal fetal abnormalities, gestational or pre-gestational diabetes, gestational hypertension, preeclampsia, signs of fetal hypoxia or significant vaginal bleeding were excluded from the study.

Gestational ages were established by first-trimester fetal biometry. Women with PPROM at <34 weeks of gestation were treated with tocolytics for 48 h, antibiotics and corticosteroids to accelerate lung maturation, whereas no treatment except antibiotics was initiated to delay delivery after 34 weeks. Women with proven microbial-associated IAI beyond 28 gestational weeks were actively managed. Actively managed women did not receive tocolytics and were treated only with corticosteroids and antibiotics. Furthermore, labor was induced or an elective cesarean section was performed after finalizing corticosteroid treatment within 72 h of the rupture of membranes. The remaining women were conservatively managed.

PPROM was diagnosed by examination with a sterile speculum to verify the pooling of amniotic fluid in the vagina, and when necessary, it was confirmed by the presence of insulin-like growth factor binding proteins (ACTIM PROM test; MedixBiochemica, Kauniainen, Finland) in the vaginal fluid.

Ultrasound-guided transabdominal amniocentesis was performed at admission but before the administration of antibiotics, corticosteroids and tocolytics, and ~2–3 ml of amniotic fluid was aspirated. A total of 100 µl of non-centrifuged amniotic fluid was used for the bedside assessment of interleukin-6 concentrations. The remaining amniotic fluid was immediately divided into two polypropylene tubes. The first tube, containing uncentrifuged samples, was immediately transported to the microbiology laboratory for polymerase chain reaction (PCR) testing for *Ureaplasma* species, *Mycoplasma hominis*, and *Chlamydia trachomatis* as well as for evaluating the 16S rRNA content. The second tube was centrifuged for 15 min at 2000 *g* to remove cells and debris, divided into aliquots and stored at −70 °C until analysis.

This study was approved by the Institutional Review Board committee (March 19 2008; No 200804 SO1P), and informed consent was obtained from all participants.

Amniotic fluid calreticulin concentrations

The concentrations of calreticulin in the amniotic fluid were determined in duplicate by a sandwich enzyme-linked immunosorbent assay technique (ELISA) using an ELISA kit for Calreticulin (Wuhan USCN Business Co., Ltd, Wuhan, China) according to the manufacturer's instructions. The limit of detection of the calreticulin kit was 0.264 ng/ml. Samples of amniotic fluid were diluted 1:2, and the absorbance values were read at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA).

Amniotic fluid interleukin-6 concentrations

The interleukin-6 concentrations were assessed with a lateral flow immunoassay Milenia® QuickLine IL-6 using the Milenia®POCScan Reader (Millenia Biotec, GmbH, Giessen, Germany). The measurement range was 50–10 000 pg/ml. The intra-assay and inter-assay variations were 12.1% and 15.5%, respectively.

Detection of *ureaplasma* species, *mycoplasma hominis* and *chlamydia trachomatis*

DNA was isolated from the amniotic fluid with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (using the protocol for the isolation of bacterial DNA from biological fluids). Real-time PCR was performed on a Rotor-Gene 6000 instrument (QIAGEN) 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 the DNA from *Ureaplasma*

species, *Mycoplasma hominis* and *Chlamydia trachomatis* in a common PCR tube. As a control, we included a PCR run for beta-actin, a housekeeping gene, to examine the presence of inhibitors of the polymerase chain reaction. The amount of *Ureaplasma* species DNA in copies/ml was determined by an absolute quantification technique employing an external calibration curve. Plasmid DNA (pCR4, Invitrogen, Carlsbad, CA) was used to prepare the calibration curve [14,32].

Detection of other bacteria in the amniotic fluid

Bacterial DNA was identified by PCR targeting the 16S rRNA gene with the following primers: 5′-CCAGACTCCTACGGG AGGCAG-3′ (V3 region), 5′-ACATTTCACAACACGAGCT GACGA-3′ (V6 region) [33,34]. Each individual reaction contained 3 µl of target DNA, 500 nM of forward and reverse primers and Q5 High Fidelity DNA polymerase (NEB, Hitchin, UK) in a total volume of 25 µl. The amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The products were visualized on an agarose gel. Positive reactions yielded products of 950 bp, which were subsequently analyzed by sequencing the 16S PCR products were cleaned and used in sequencing PCR reactions utilizing the above primers and the BigDye Terminator kit, version 3.1 (Applied Biosystems, Foster City, CA). The bacteria were then typed using the sequences obtained in BLAST® and SepsiTest™ BLAST.

Definition of IAI

Intraamniotic inflammation (IAI) in PPROM pregnancies was defined as amniotic fluid bedside interleukin-6 concentrations >745 pg/ml [9,35].

Diagnosis of MIAC

The MIAC was determined based on a positive PCR analysis for *Ureaplasma* species, *Mycoplasma hominis* and/or for *Chlamydia trachomatis* and/or by positivity for the 16S rRNA gene.

Statistical analyses

The demographic characteristics were compared using the non-parametric Mann–Whitney *U* test and Kruskal–Wallis test for continuous variables and are presented as median values (range). Categorical variables were compared using the chi-squared test and are presented as numbers (%). The normality of the data was tested using the D'Agostino–Pearson omnibus normality test and the Shapiro–Wilk test. Because the amniotic fluid concentrations of calreticulin were not normally distributed, non-parametric tests (Mann–Whitney *U*-test and Kruskal–Wallis) were used for analyzes. A Spearman partial correlation was performed to adjust for gestational age at sampling. Receiver-operator characteristic curves were constructed to determine the predictive value of calreticulin for the presence of microbial-associated IAI. Differences were considered significant at $p<0.05$. All p values were obtained from two-sided tests, and all statistical analyzes were performed using GraphPad Prism 6 for Mac OS X (GraphPad Software, San Diego, CA) or the SPSS 19.0 statistical package for Mac OS X (SPSS Inc., Chicago, IL).

Results

A total of 168 women with PPROM were included in the study during the study period. MIAC and IAI were identified in 31% (52/168) and 20% (34/168) of women, respectively. The participant demographics and clinical data are shown in Table 1. In women with MIAC, *Ureaplasma* species were the most common microorganism and detected in 69% of women (36/52). Polymicrobial findings were identified in 29% (12/52) of women with MIAC. All microbial findings are listed in Table 2. All women self-reported as Caucasians.

Amniotic fluid calreticulin concentrations according to the presence of MIAC

The concentration of calreticulin in the amniotic fluid was higher in women with MIAC than in women without MIAC upon crude analysis (with MIAC: median 54.4 ng/ml, range 10.9–955.8, versus without MIAC: median 32.6 ng/ml, range 2.3–180.1; $p<0.0001$; Figure 1A), and as well as after adjustment for gestational age at sampling ($p=0.001$). Next, we tested the relationship between the microbial load of *Ureaplasma* species, and the amniotic fluid concentrations of calreticulin. A correlation between the microbial load of *Ureaplasma* species in the amniotic fluid and calreticulin concentration was found (rho = 0.35; $p=0.03$).

Amniotic fluid calreticulin concentrations according to the presence of IAI

The calreticulin concentrations in the amniotic fluid were higher in women with IAI than in women without IAI in crude analysis (with IAI: median 66.8 ng/ml, range 2.3–955.8 versus without IAI: median 33.0 ng/ml, range 7.4–180.1; $p<0.0001$; Figure 1B), and after adjustment for gestational age ($p<0.0001$). A positive correlation between amniotic fluid calreticulin and interleukin-6 concentrations was found (rho = 0.32; $p<0.0001$).

Amniotic fluid calreticulin concentrations according to the presence of microbial-associated IAI

The presence of microbial-associated IAI was associated with higher amniotic fluid calreticulin concentrations in the crude analysis (with microbial-associated IAI: median 82.5 ng/ml, range 22.9–955.8 versus without microbial-associated IAI: median 33.7 ng/ml, range 2.3–180.1; $p<0.0001$; Figure 1C) and in the analysis adjusted for gestational age at sampling ($p<0.0001$). When the women were split into four subgroups based on the presence or absence of MIAC and/or IAI, we observed differences in the amniotic fluid calreticulin concentrations in the crude analysis ($p<0.0001$; Figure 2) as well as after adjustment for gestational age at sampling ($p<0.0001$). The women with microbial-associated IAI (median 82.5 ng/ml, range 22.9–955.8) exhibited a higher median amniotic fluid calreticulin concentration than women with sterile IAI (median 40.8 ng/ml, range 2.3–68.1; $p=0.0007$; Figure 2), colonization (MIAC alone; median 43.5 ng/ml, range 10.9–199.8; $p<0.0001$; Figure 2) and

Table 1. Maternal and neonatal characteristics of PPROM pregnancies according to the presence or absence of MIAC and/or IAI.

Characteristic	The presence of MIAC and IAI (n=26)	The presence of IAI alone (n=8)	The presence of MIAC alone (n=26)	The absence of MIAC and IAI (n=108)	p values
Maternal age [years, median (range)]	30.5 (17–42)	28.0 (21–35)	31.5 (18–42)	31.0 (21–40)	0.36
Prepregnancy body mass index [kg/m^2, median (range)]	24.6 (16.5–38.0)	24.1 (19.3–37.8)	22.4 (16.0–33.5)	22.7 (15.8–39.0)	0.68
Smoking [number (%)]	12 (46%)	1 (13%)	7 (27%)	6 (6%)	**<0.0001**
Gestational age at admission [weeks, median (range)]	31 + 6 (24 + 2–36 + 5)	33 + 6 (25 + 1–36 + 6)	33 + 3 (25 + 3–36 + 6)	33 + 6 (25 + 0–36 + 5)	**0.03**
Gestational age at delivery [weeks, median (range)]	32 + 1 (24 + 5–36 + 5)	34 + 1 (25 + 1–36 + 6)	34 + 0 (26 + 5–36 + 6)	34 + 4 (25 + 2–36 + 6)	**0.006**
Latency from PPROM to amniocentesis [h, median (range)]	9 (1–97)	8 (3–42)	6 (1–35)	6 (1–68)	0.15
Latency from amniocentesis to delivery [h, median (range)]	37 (3–106)	45 (17–768)	104 (4–390)	134 (4–482)	**0.005**
CRP levels at admission [mg/l, median (range)]	15.5 (2.1–106.3)	8.4 (2.5–59.6)	4.5 (0–23.1)	5.5 (0–47.1)	**<0.0001**
WBC count at admission [$\times 10^9$ l, median (range)]	13.8 (9.2–24.4)	15.3 (9.1–20.0)	11.9 (7.6–22.8)	11.4 (6.5–24.4)	**0.001**
Amniotic fluid IL-6 at admission [pg/ml, median (range)]	8478 (831–10 000)	996 (801–1446)	190 (50–673)	197 (50–685)	**<0.0001**
Administration of corticosteroids [number (%)]	23 (88%)	7 (88%)	19 (73%)	87 (81%)	0.32
Spontaneous vaginal delivery [number (%)]	20 (77%)	7 (88%)	21 (81%)	77 (71%)	0.59
Cesarean section [number (%)]	6 (23%)	1 (12%)	5 (19%)	30 (28%)	0.65
Forceps delivery [number (%)]	0 (0%)	0 (0%)	0 (0%)	1 (1%)	0.91
Birth weight [g, median (range)]	1790 (550–2840)	2215 (990–3320)	2225 (780–3250)	2255 (990–3320)	**0.005**
Histological chorioamnionitis [number (%)]	24 (92%)	7 (88%)	18 (69%)	51 (47%)	**<0.0001**
Funisitis [number (%)]	16 (62%)	2 (25%)	10 (38%)	23 (21%)	**0.001**
Apgar score <7; 5 min [number (%)]	2 (8%)	1 (12%)	0 (0%)	2 (2%)	0.12
Apgar score <7; 10 min [number (%)]	1 (4%)	1 (12%)	0 (0%)	1 (1%)	0.08

PPROM, preterm prelabor rupture of membranes; MIAC, microbial invasion of amniotic cavity; IAI, intraamniotic inflammation; CRP, C-reactive protein; WBC, white blood cells; IL, interleukin. Continuous variables were compared using a non-parametric Kruskal–Wallis test. Categorical variables were compared using chi-squared test. Statistically significant results are marked in bold. Continuous variables are presented as median (range) and categorical as number (%).

Table 2. Microorganism identified in the amniotic fluid of women with preterm prelabor rupture of membranes.

The presence of microbial-associated IAI (n=26)	The presence of colonization (n=26)
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Chlamydia trachomatis	*Ureaplasma* species
Haemophilus influenza	*Ureaplasma* species
Fusobacterium nucleatum	*Ureaplasma* species
Streptococcus agalactiae	*Ureaplasma* species
Streptococcus species	*Ureaplasma* species
Leptotrichia amnionii	*Ureaplasma* species
Ureaplasma species+*Veillonella species*	*Ureaplasma* species
Ureaplasma species+*Sneathia sanguinegens*	*Ureaplasma* species
Ureaplasma species+*Chlamydia trachomatis*	*Mycoplasma hominis*
Ureaplasma species+*Chlamydia trachomatis*	*Mycoplasma hominis*
Ureaplasma species+*Leptotrichia amnionii*	*Mycoplasma hominis*
Ureaplasma species+*Mycoplasma hominis*	*Mycoplasma hominis*
Ureaplasma species+*Mycoplasma hominis*	*Mycoplasma hominis*
Ureaplasma species+*Mycoplasma hominis*	*Streptococcus pneumoniae*
Ureaplasma species+*Lactobacillus species*	*Enterococcus faecalis*
Streptococcus agalactiae+*Streptococcus anginosus*	*Bifidobacterium* species
Peptococcus species + Propionibacterium species	*Ureaplasma* species+*Chlamydia trachomatis*

IAI, intraamniotic inflammation.

women without both MIAC and IAI (median 31.1 ng/ml, range 7.4–180.1; Figure 2). The amniotic fluid calreticulin concentrations did not differ among women with sterile IAI, colonization and without both MIAC and IAI (Figure 2).

The predictive value of calreticulin for the presence of microbial-associated IAI

An amniotic fluid calreticulin concentration of 81.4 ng/ml was found to be the best cutoff point in the identification of PPROM women with the presence of microbial-associated IAI. The sensitivity of this cutoff point was 68% [95% confidence interval (CI) 37–77%], its specificity was 95% (95% CI 88–97%), its positive predictive value was 63% (95% CI 41–81%), its negative predictive value was 92% (95% CI 87–96%), its likelihood ratio was 9.1, and the area under receiver-operating characteristic curves was 88% ($p < 0.0001$; Figure 3).

Discussion

Calreticulin, a calcium-binding chaperone, plays a multifunctional role in the immune response. However, its exact role in the pathophysiology of PPROM, a specific phenotype of spontaneous preterm delivery, has not yet been explained. The principal findings of this study are as follows: (i) women with MIAC have higher amniotic fluid calreticulin concentrations than women without MIAC; (ii) the amniotic fluid calreticulin concentrations positively correlated with the microbial load of *Ureaplasma* species; (iii) women with IAI have higher amniotic fluid calreticulin concentrations than women without this complications; (iv) the amniotic fluid calreticulin concentration positively correlates with the interleukin-6 concentration; (v) women with microbial-associated IAI have higher amniotic fluid calreticulin concentrations than women with sterile IAI, colonization and women without both MIAC and IAI and (vi) the amniotic fluid calreticulin concentration of 81.4 ng/ml was found to be the optimal cutoff point for the identifying PPROM pregnancies complicated by the presence of microbial-associated IAI.

Calreticulin is well known for its role as an endoplasmic reticulum chaperone [20–28]. However, recent studies have clearly demonstrated that calreticulin can be expressed on the cell surface, where it is relevant for phagocytic uptake and the immunogenicity of cells [25,29,36]. In other words, calreticulin serves as an ''eat me'' signal on the surface the cells undergoing apoptosis. In addition, calreticulin has been proposed to localize on the neutrophil surface [23]. Therefore, the calreticulin concentrations in the body fluids and tissues may increase in response to pathological conditions that are typically associated with the presence or diffuse infiltration of neutrophils.

In this study, we found that women with MIAC had higher median amniotic fluid calreticulin concentrations than women without MIAC. However, MIAC represents a very heterogeneous group of patients due to the diversity of microorganisms, their microbial loads and the intensity of intraamniotic inflammatory response to different bacteria [7,37]. The differences in the amniotic fluid calreticulin concentrations between two main subtypes of MIAC [microbial-associated IAI (MIAC with IAI) and colonization (MIAC without IAI)] demonstrated that women with microbial-associated IAI had a 2-fold higher median calreticulin concentrations than women with colonization. Thus, the presence of bacteria in the amniotic fluid that elicit an inflammatory response, but not the presence of bacteria *per se*, increases the calreticulin concentrations in

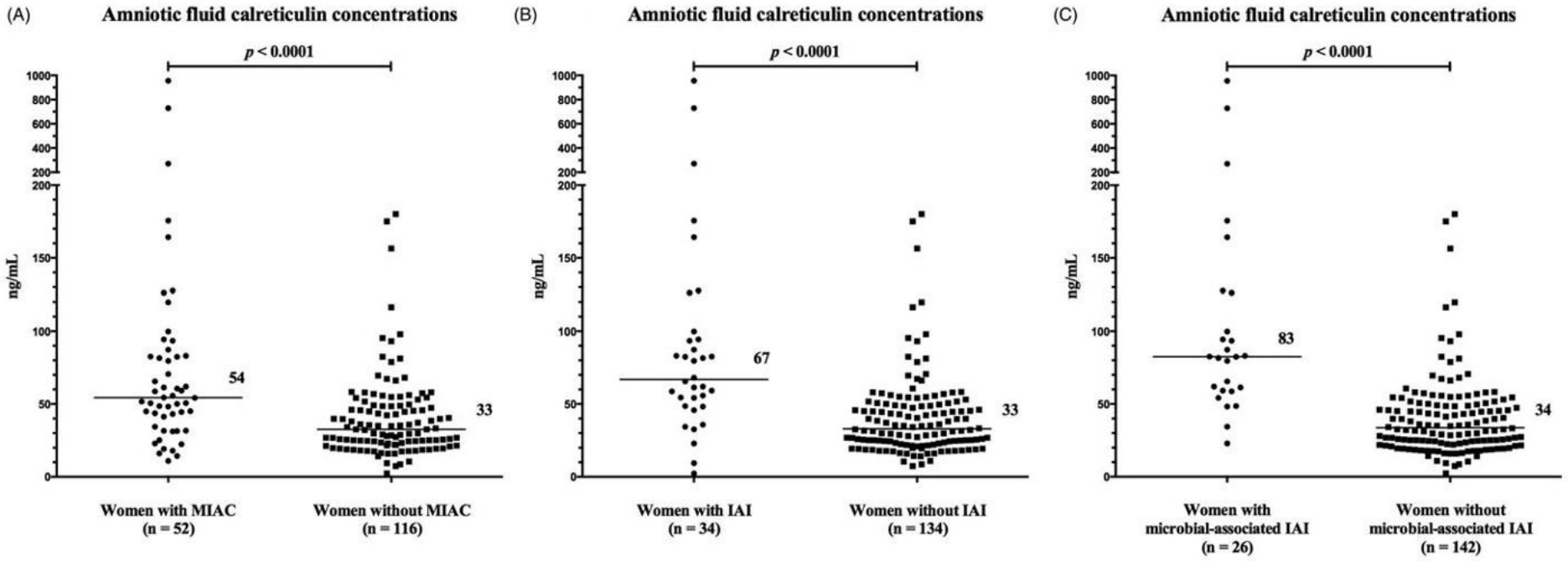

Figure 1. Amniotic fluid calreticulin concentrations with respect to the presence or absence of MIAC (A), IAI (B) and microbial-associated IAI (C). Horizontal bars represent medians. Abbreviations: MIAC, microbial invasion of the amniotic cavity; IAI, intraamniotic inflammation.

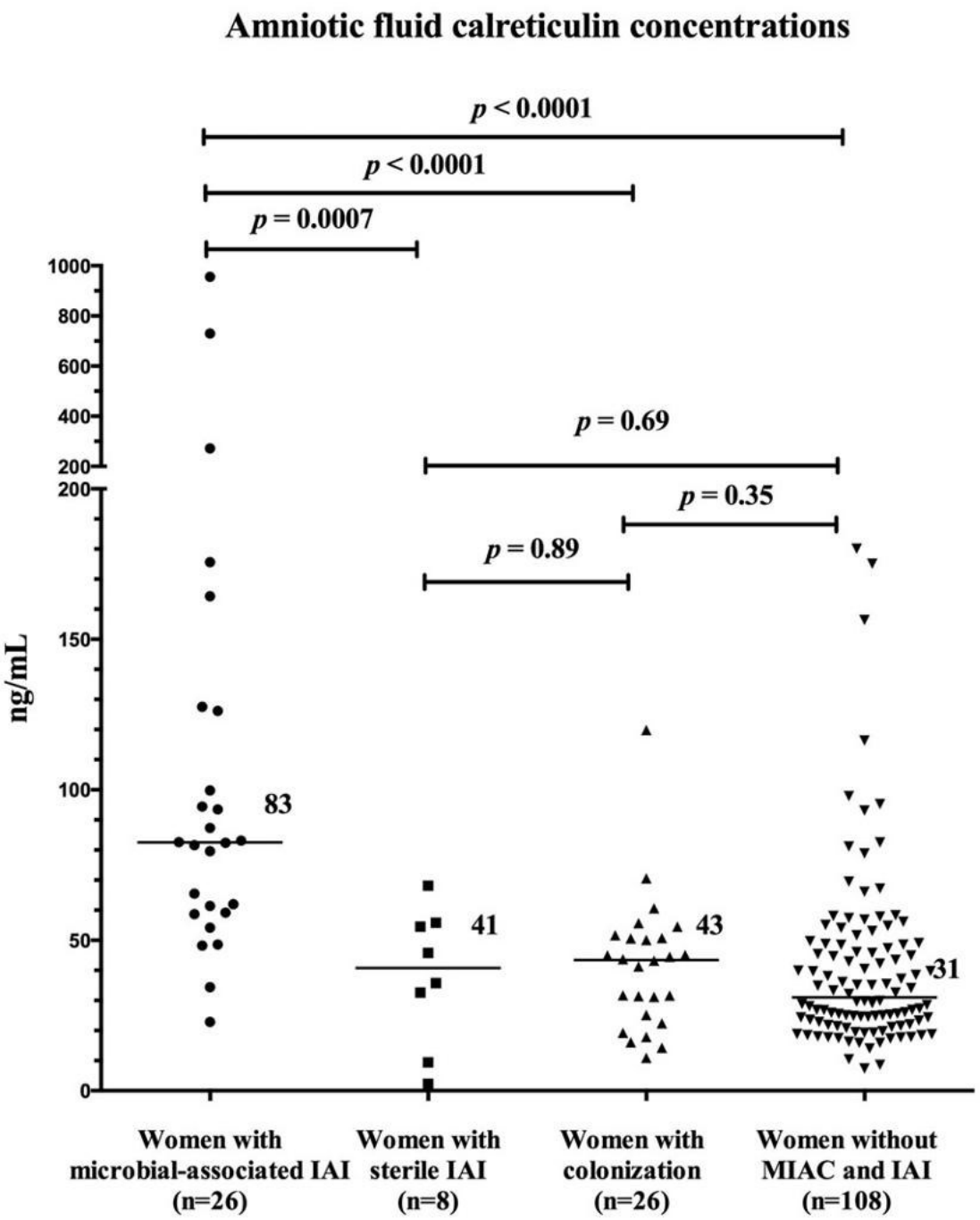

Figure 2. Amniotic fluid calreticulin concentrations in the subgroups of PPROM based on the presence of microbial-associated IAI, sterile IAI, colonization and the absence of both MIAC and IAI. Horizontal bars represent medians. Abbreviations: PPROM, preterm prelabor rupture of membranes; MIAC, microbial invasion of the amniotic cavity; IAI, intraamniotic inflammation.

the amniotic fluid. In addition, we found a correlation between the microbial burden of *Ureaplasma* species in the amniotic fluid and the calreticulin concentrations. This finding corroborates our previous results showing that the intensity of the intraamniotic inflammatory response to *Ureaplasma* species is dose-dependent [7,32,38,39].

The exact definition of IAI remains debated, and a slight controversy persists between ''bed-side'' and ''bench-side'' views on this issue. Although clinicians prefer a single marker with an easy to follow cutoff point, scientists and researchers prefer a more complex and precise definition based on multiple markers. In this study, we used the amniotic fluid IL-6 level as a marker of IAI. We found that women with IAI exhibited higher median amniotic fluid calreticulin concentrations. However, based on the recent publications, IAI consists of two subtypes – microbial-associated IAI and sterile IAI [6,7,40–42]. An analysis of the difference between these two subtypes of IAI indicated that the elevation of calreticulin in the amniotic fluid is primarily associated with microbial-associated IAI (the median calreticulin level in women with microbial-associated IAI was 2-fold higher than in women with sterile IAI). This finding corroborates our previous finding regarding MIAC. PPROM complicated by the presence of bacteria in the amniotic fluid with IAI is a

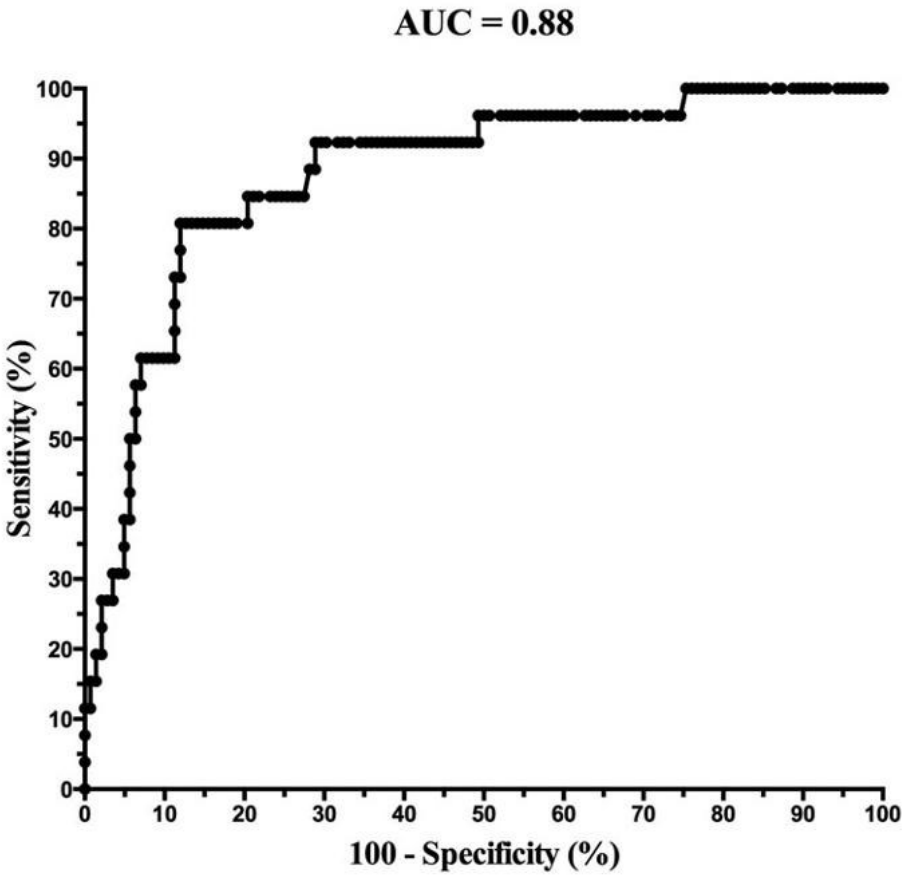

Figure 3. Receiver operating characteristic curve for the presence of microbial-associated IAI (area under curve for calreticulin cutoff point > 81.4 pg/ml: 88%; $p < 0.0001$). Abbreviations: IAI, intraamniotic inflammation; AUC, area under curve.

specific subtype of PPROM with the highest amniotic fluid calreticulin concentrations.

Calreticulin has been shown to induce the production of IL-6 and tumor necrosis factor-α from macrophages due to an induction active mRNA transcription via mitogen-activated protein kinases and nuclear factor kappa-light-chain-enhancer of activated B cells in macrophages [27]. However, we are not able to conclusively identify macrophages as the source of amniotic fluid calreticulin, we only found a correlation between the calreticulin and the IL-6 concentrations.

Our study provides new information regarding the amniotic fluid concentrations of calreticulin in PPROM pregnancies. The amniotic fluid calreticulin concentration cutoff identified in this study can serve as an accurate predictor of microbial-associated IAI in PPROM women, which may have important clinical implications. However, this promising finding needs to be validated on an independent cohort of women with PPROM.

An important strength of this study is that MIAC was identified based on non-cultivation techniques, consists of non-specific PCR (16S rRNA) and specific PCR (*Ureaplasma* species, *Mycoplasma hominis* and *Chlamydia trachomatis*). Furthermore, only women with one well-defined specific phenotype of preterm delivery (PPROM) were included in this study. This study was also subject to some limitations. The exact source of the amniotic fluid calreticulin remains unclear because identifying this source was beyond the scope of this study. We can only speculate on the origin of amniotic fluid calreticulin, i.e. amniotic fluid neutrophils or macrophages. In addition, diffuse infiltration of the placenta and fetal membranes by these cells can be a possible source as well. The conservative management of women without microbial-associated IAI prevents this study from evaluating an association between the amniotic fluid calreticulin concentration and the presence of histological chorioamnionitis in PPROM because of a long latency between an amniotic fluid sampling and delivery. Finally, the analyses of *Ureaplasma* species did not include any information on species or serovars, which may significantly affect the intraamniotic inflammatory response.

In conclusion, the presence of microbial-associated IAI was related to the highest amniotic fluid calreticulin concentrations. Calreticulin seems to be a promising marker for the early identification of PPROM complicated by microbial-associated IAI.

Declaration of interest

This work was supported by Charles University in Prague, Faculty of Medicine in Hradec Kralove, Czech Republic, project ''PRVOUK'' P37/10 and Faculty Hospital in Hradec Kralove (long-term organization development plan). The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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Příloha č. 2: Amniotic fluid cathepsin-G in pregnancies complicated by the preterm prelabor rupture of membranes

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ORIGINAL ARTICLE

Amniotic fluid cathepsin-G in pregnancies complicated by the preterm prelabor rupture of membranes

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Abstract

Objective: The aim of this study was to evaluate the amniotic fluid cathepsin-G concentrations in women with preterm prelabor rupture of membranes (PPROM) based on the presence of the microbial invasion of the amniotic cavity (MIAC) and/or intra-amniotic inflammation (IAI).
Methods: A total of 154 women with singleton pregnancies complicated by PPROM were included in this study. Amniotic fluid samples were obtained by transabdominal amniocentesis. Amniotic fluid cathepsin-G concentrations were assessed by ELISA. MIAC was determined using a non-cultivation approach. IAI was defined as an amniotic fluid bedside interleukin-6 concentration $\geq$ 745 pg/mL.
Results: Women with MIAC had higher amniotic fluid cathepsin-G concentrations than women without MIAC (with MIAC: median 82.7 ng/mL, versus without MIAC: median 64.7 ng/mL; $p = 0.0003$). Women with IAI had higher amniotic fluid cathepsin-G concentrations than women without this complication (with IAI: median 103.0 ng/mL, versus without IAI: median 66.2 ng/mL; $p < 0.0001$). Women with microbial-associated (with both MIAC and IAI) IAI and sterile (IAI without MIAC) IAI had higher amniotic fluid cathepsin-G concentrations than women with colonization (MIAC without IAI) and women without both MIAC and IAI ($p < 0.0001$).
Conclusions: The presence of either microbial-associated or sterile IAI was associated with increased amniotic fluid cathepsin-G concentrations in pregnancies complicated by PPROM. Amniotic fluid cathepsin-G appears to be a potential marker of IAI.

Keywords

Cathepsin-G, preterm delivery, PPROM, inflammation, serine protease, neutrophil

History

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Introduction

Preterm prelabor rupture of membranes (PPROM), defined as a leakage of amniotic fluid before the onset of regular labor activity prior to gestational age 37 weeks, complicates about 3% of all pregnancies [1]. PPROM is responsible for approximately one third of all preterm deliveries and about one-half of spontaneous preterm deliveries [1]. PPROM is associated with serious neonatal morbidity and mortality, which is even worse in lower gestational ages than in spontaneous preterm birth with intact membranes [2–4].

Despite the fact that the pathophysiology of a vast majority of PPROM seems to be noninfectious, PPROM is often associated with infection-related (microbial invasion of the amniotic cavity [MIAC] and microbial-associated intra-amniotic inflammation [IAI] in 34–41% and 21–29%, respectively) and inflammatory complications (IAI and sterile IAI in 25–58% and 5–29%, respectively). [3,5–9]. The presence of MIAC sets off a host-innate immune defense leading to a cascade of inflammatory processes resulting in IAI [8–10]. Nevertheless, endogenous molecules called alarmins, released from necrotic cells, can trigger an intra-amniotic inflammatory response leading to the development of sterile IAI. These conditions are followed by the recruitment of neutrophils and other immune cells from the uterine wall and maternal circulation to the placenta and fetal membranes, as well as the release of fetal neutrophils in amniotic fluid [11,12].

Neutrophils play an essential role in innate immune defense against invading pathogens and serve as the primary mediators of inflammatory response [13]. Neutrophils have the ability to phagocytose and kill microorganisms [13,14]. Moreover, they have the unique capacity to directly and specifically shape the immune response [13,15].

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Neutrophils contain granule-storing proteins that can kill microorganisms and digest tissues [13,16,17]. The neutral serine protease family, which includes neutrophil elastase, cathepsin-G, and proteinase 3, belongs among the proteins stored in the neutrophil granules [17–21]. However, these serine proteases mainly serve as intracellular antimicrobial agents; they are also released from the cells and extracellularly play the role [17,19–21].

One of these serine proteases, neutrophil elastase, has previously been evaluated in the amniotic fluid of women with PPROM with respect to the presence of MIAC and histological chorioamnionitis [22–24]. Amniotic fluid neutrophil elastase concentrations have been shown to be elevated when these complications were present [22–24]. There is a lack of information about cathepsin-G, another member of the serine protease family, in amniotic fluid from women with PPROM.

Therefore, the aim of this study was to determine the amniotic fluid cathepsin-G concentrations in pregnancies complicated by PPROM based on the presence of MIAC and/or IAI. The final goal was to evaluate the potential of amniotic fluid cathepsin-G to predict the presence of IAI.

Materials and methods

A retrospective cohort study of pregnant women between gestational age 24 + 0 and 36 + 6 weeks who were admitted to the University Hospital Hradec Kralove's Department of Obstetrics and Gynecology in the Czech Republic was conducted between December 2013 and May 2015. Women with singleton pregnancies complicated by the presence of PPROM who had a maternal age $\geq$ 18 years were invited to participate in the study. Women with pregnancy and other medical complications such as fetal growth restrictions, the presence of either congenital or chromosomal fetal abnormalities, gestational or pre-gestational diabetes, gestational hypertension, preeclampsia, signs of fetal hypoxia, or significant vaginal bleeding were excluded from the study.

Gestational ages were established by first-trimester fetal biometry. Women with PPROM at less than 34 weeks of gestation were treated with tocolytics for 48 h, antibiotics, and corticosteroids to accelerate lung maturation. Women with a proven microbial-associated IAI (amniotic fluid interleukin [IL] 6 $\geq$ 745 pg/mL and the presence of MIAC) beyond 28 gestational weeks were actively managed. Actively managed women had finished a tocolytic therapy when microbial-associated IAI was proven and were treated only with corticosteroids and antibiotics. Furthermore, labor was induced or an elective cesarean section was performed after finalizing corticosteroid treatment within 72 h of the membranes rupturing. The remaining women with PPROM at less than 34 weeks were conservatively managed, and labor was induced or an elective cesarean section was performed after gestational age 34 + 0 weeks. Women with PPROM after 34 weeks were treated only with antibiotics, and labor was induced or the elective cesarean section was performed after 24 h of the membrane rupturing.

PPROM was diagnosed by examining the women with a sterile speculum to verify the pooling of amniotic fluid in the vagina. If clinical doubts of PPROM, leakage of amniotic fluid was confirmed by the presence of insulin-like growth factor binding proteins (Actim PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

Ultrasound-guided transabdominal amniocentesis was performed at admission but before the administration of antibiotics, corticosteroids, and tocolytics, and approximately 2–3 mL of amniotic fluid was aspirated. A total of 100 µL of non-centrifuged amniotic fluid was used for the bedside assessment of IL-6 concentrations. The remaining amniotic fluid was immediately divided into two polypropylene tubes. The first tube, containing uncentrifuged samples, was immediately transported to the microbiology laboratory for polymerase chain reaction (PCR) testing for *Ureaplasma* species, *Mycoplasma hominis* and *Chlamydia trachomatis* as well as evaluation for the 16S rRNA content. The second tube was centrifuged for 15 min at 2000*g* to remove cells and debris, divided into aliquots and stored at −70 °C until analysis.

Women were split in the subgroups based on the presence and absence of MIAC, as well as based on the presence and absence of IAI. Moreover, women were split into these four subgroups: microbial-associated IAI (the presence of both MIAC and IAI); sterile IAI (IAI without MIAC); colonization (MIAC without IAI); and without both MIAC and IAI.

This study was approved by the Institutional Review Board committee (July, 2014; No. 201407 S14P), and informed consent was obtained from all participants. Amniotic fluid samples from 102 women were used in our previous study of different protein [25].

Amniotic fluid cathepsin-G concentrations

The amniotic fluid cathepsin-G concentrations were determined in duplicate by a sandwich enzyme-linked immunosorbent assay (ELISA) technique using a cathepsin-G (CtSG) ELISA kit (Cloud-Clone Corp., Houston, TX) according to the manufacturer's instructions. The limit of detection of the cathepsin-G kit was 0.065 ng/mL. Amniotic fluid samples were diluted 1:20, and the absorbance values were read at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA).

Amniotic fluid IL-6 concentrations

The IL-6 concentrations were assessed with a lateral flow immunoassay Milenia QuickLine IL-6 using the Milenia POCScan Reader (Milenia Biotec, GmbH, Giessen, Germany). The measurement range was 50–10 000 pg/mL. The intra-assay and inter-assay variations were 12.1% and 15.5%, respectively [26].

Detection of *Ureaplasma* species, *Mycoplasma hominis*, and *Chlamydia trachomatis*

DNA was isolated from the amniotic fluid with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (using the protocol for isolating bacterial DNA from biological fluids). Real-time PCR was performed on a Rotor-Gene 6000 instrument (QIAGEN) 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 the DNA from *Ureaplasma* species, *Mycoplasma hominis* and *Chlamydia trachomatis* in a common PCR tube. As a control, we included a PCR run for beta-actin, a housekeeping gene, to examine the presence of inhibitors of PCR.

Detection of other bacteria in the amniotic fluid

Bacterial DNA was identified by PCR targeting the 16S rRNA gene with the following primers: 5′-CCAGACTCCTACGGG AGGCAG-3′ (V3 region), 5′-ACATTTCACAACACGA GCTGACGA-3′ (V6 region) [27,28]. Each individual reaction contained 3 µL of target DNA, 500 nM of forward and reverse primers, and Q5 High-Fidelity DNA polymerase (NEB, Ipswich, MA) in a total volume of 25 µL. The amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The products were visualized on an agarose gel. Positive reactions yielded products of 950 bp, which were subsequently analyzed by sequencing. The 16S PCR products were cleaned and used in sequencing PCR reactions utilizing the above primers and the BigDye Terminator kit, v3.1 (Thermo Fisher Scientific, Waltham, MA). The bacteria were then typed using the sequences obtained in BLAST® and SepsiTest™ BLAST.

Definition of IAI

IAI in PPROM pregnancies was defined as amniotic fluid bedside IL-6 concentrations $\geq$ 745 pg/mL [29,30].

Diagnosis of MIAC

The MIAC was determined based on a positive PCR analysis for *Ureaplasma* species, *Mycoplasma hominis* and/or *Chlamydia trachomatis* and/or by positivity for the 16S rRNA gene.

Statistical analyses

The demographic and clinical characteristics were compared using a nonparametric Jonckheere–Terpstra test for continuous variables and are presented as median values (range). Categorical variables were compared using a Cochran–Armitage test for trend and are presented as numbers (%). The normality of the data was tested using the D'Agostino–Pearson omnibus normality test and the Shapiro–Wilk test. Because the amniotic fluid cathepsin-G concentrations were not normally distributed, nonparametric tests (Mann–Whitney *U*-test and Jonckheere–Terpstra test) were used for analyses and presented as median values (interquartile range [IQR]). A Spearman partial correlation was performed to adjust for gestational age at sampling. A receiver–operator characteristic curve was constructed to determine the predictive value of cathepsin-G for the presence of IAI. Differences were considered significant at $p < 0.05$. All p were obtained from two-sided tests, and all statistical analyses were performed using GraphPad Prism 6 for Mac OS X (GraphPad Software, San Diego, CA) or the SPSS 19.0 statistical package for Mac OS X (SPSS Inc., Chicago, IL).

Results

A total of 161 women with PPROM at gestational ages between 24 + 0 and 36 + 6 weeks were recruited. Seven women were excluded from the study due to fetal growth restriction ($n = 3$), pregestational diabetes mellitus ($n = 2$), and preeclampsia ($n = 2$). The remaining 154 women were included in the study. MIAC and IAI were identified in 31% (47/154) and 19% (30/154) of the women, respectively. The women demographics and clinical data are shown in Table 1. *Ureaplasma* species was the most common microorganism and was detected in 68% (32/47) of women with MIAC. Polymicrobial findings were identified in 21% (10/47) of women with MIAC. All microbial findings are listed in Table 2. Gestational ages at admission at less than 28 + 0 weeks were found in 3% (4/154) of women, gestational ages between 28 + 0 and 31 + 6 weeks in 28% (43/154) of women, gestational ages between 32 + 0 and 33 + 6 in 22% (34/154) of women, and gestational ages between 34 + 0 and 36 + 6 in 47% (73/154) of women. All women self-reported as Caucasians.

Amniotic fluid cathepsin-G concentrations according to the presence of MIAC

The amniotic fluid cathepsin-G concentration was higher in women with MIAC than in women without MIAC upon crude analysis (with MIAC: median 82.7 ng/mL, IQR 67.1–105.6, versus without MIAC: median 64.7 ng/mL, IQR 53.2–88.4; $p = 0.0003$; Figure 1), and after adjustment for gestational age at sampling ($p < 0.0001$). Women with microbial-associated IAI had higher amniotic fluid cathepsin-G concentrations than women with colonization in crude analysis (microbial-associated IAI: median 103.0 ng/mL, IQR 87.4–393.0 versus colonization: median 71.6 ng/mL, IQR 60.5–79.9; $p < 0.0001$), as well as after adjustment for gestational age at sampling ($p < 0.0001$).

Amniotic fluid cathepsin-G concentrations according to the presence of IAI

The amniotic fluid cathepsin-G concentrations were higher in women with IAI than in women without IAI in crude analysis (with IAI: median 103.0 ng/mL, IQR 85.5–178.7 versus without IAI: median 66.2 ng/mL, IQR 53.4–82.2; $p < 0.0001$; Figure 2), and as well as after adjustment for gestational age ($p < 0.0001$). No difference in amniotic fluid cathepsin-G concentrations was found between women with microbial-associated and sterile IAI (microbial-associated IAI: median 103.0 ng/mL, IQR 87.4–393.0 versus sterile: median 105.4 ng/mL, IQR 64.0–134.4; $p = 0.47$). A weak positive correlation between amniotic fluid cathepsin-G and IL-6 concentrations was found (rho = 0.36; $p < 0.0001$).

An amniotic fluid cathepsin-G concentration of 105 ng/mL was found to be the best cutoff point in the identification of women with PPROM with the presence of IAI. The sensitivity of this cutoff point was 50% (95% confidence interval [CI] 31%–69%), its specificity was 92% (95% CI 86%–96%), its positive predictive value was 63% (95% CI 40%–81%), its negative predictive value was 88% (95% CI 82%–93%), its likelihood ratio was 6.3, and

Table 1. Maternal and neonatal characteristics of the subgroups of PPROM pregnancies.

Characteristic	Microbial-associated intra-amniotic inflammation ($n=22$)	Sterile intra-amniotic inflammation ($n=8$)	Colonization ($n=25$)	Negative ($n=99$)	p values
Maternal age [years, median (range)]	30.5 (17.2–39.4)	28.0 (21.4–35.0)	31.0 (18.5–42.0)	31.0 (21.0–40.0)	0.17
Primiparous [number (%)]	8 (36%)	6 (75%)	10 (40%)	45 (45%)	0.16
Prepregnancy body mass index [kg/m^2, median (range)]	24.6 (17.2–33.5)	24.1 (19.4–38.6)	22.3 (16.3–34.0)	22.7 (16.9–37.3)	0.45
Smoking [number (%)]	11 (50%)	1 (13%)	7 (30%)	6 (6%)	**<0.0001**
Gestational age at admission [weeks, median (range)]	32 + 0 (24 + 2–36 + 5)	33 + 6 (25 + 1–36 + 6)	33 + 6 (25 + 3–36 + 6)	34 + 0 (28 + 2–36 + 5)	0.06
Gestational age at delivery [weeks, median (range)]	32 + 1 (24 + 5–36 + 5)	34 + 1 (25 + 1–37 + 0)	34 + 0 (26 + 5–37 + 0)	34 + 5 (28 + 6–37 + 0)	**0.03**
Interval from PPROM to amniocentesis [hours, median (range)]	7 (1–97)	8 (3–43)	5 (1–35)	5 (1–68)	0.15
Latency from amniocentesis to delivery [hours, median (range)]	34 (3–106)	45 (17–768)	29 (7–390)	34 (4–452)	0.68
CRP levels at admission [mg/L, median (range)]	15.6 (2.0–106.0)	8.4 (2.2 –59.6)	4.0 (0–23.3)	5.0 (0–47.0)	**0.001**
WBC count at admission [x10^9 L, median (range)]	13.7 (9.0–24.2)	15.3 (9.4–20.3)	11.8 (7.7–22.6)	11.4 (6.7–24.5)	**0.001**
Amniotic fluid IL-6 at admission [pg/mL, median (range)]	10000 (831–10000)	996 (804–1446)	193 (50–673)	210 (50–678)	**<0.0001**
Administration of antibiotics [number (%)]	22 (100%)	7 (88%)	24 (96%)	99 (100%)	0.29
Administration of corticosteroids [number (%)]	20 (91%)	7 (88%)	18 (72%)	79 (80%)	0.27
Spontaneous vaginal delivery [number (%)]	17 (77%)	7 (88%)	20 (80%)	71 (71%)	0.38
Birth weight [grams, median (range)]	1790 (550–2290)	2215 (990–3320)	2180 (780–3250)	2280 (1100–3400)	**0.008**
Histological chorioamnionitis [number (%)]	20 (91%)	7 (88%)	17 (68%)	47 (47%)	**<0.0001**
Funisitis [number (%)]	13 (52%)	2 (25%)	9 (36%)	19 (19%)	**0.0002**
Apgar score <7; 5 minutes [number (%)]	1 (5%)	1 (13%)	0 (0%)	1 (1%)	0.12
Apgar score <7; 10 minutes [number (%)]	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0.23

PPROM: Preterm prelabor rupture of membranes; CRP: C-reactive protein; WBC: White blood cells; IL: Interleukin.
Continuous variables were compared using a nonparametric Jonckheere–Terpstra test. Categorical variables were compared using Cochran–Armitage test for trend. Statistically significant results are marked in bold. Continuous variables are presented as median (range) and categorical as number (%).

the area under the receiver-operating characteristic curve was 82% ($p<0.0001$; Figure 2).

Amniotic fluid cathepsin-G concentrations according to the presence of MIAC and/or IAI

When the women were split into four subgroups based on the presence or absence of MIAC and/or IAI, the difference in the amniotic fluid cathepsin-G concentrations was found in the crude analysis ($p<0.0001$; Figure 3) as well as after adjustment for gestational age at sampling ($p<0.0001$). The women with microbial-associated and sterile IAI exhibited higher median amniotic fluid cathepsin-G concentrations than women with colonization and women without both MIAC and IAI (median 64.5 ng/mL, IQR 52.7–84.8).

Discussion

Cathepsin-G, a serine protease stored in the primary granules of neutrophils, is thought to be important in maintaining the fragile balance between tissue destruction and protection during an inflammatory response [21]. However, its role in the IAI response has yet to be explained. The principal findings of this study are as follows: i) women with MIAC have higher amniotic fluid cathepsin-G concentrations than women without MIAC; ii) women with IAI have higher amniotic fluid cathepsin-G concentrations than women without this complication; iii) the amniotic fluid cathepsin-G concentration weakly positively correlates with the IL-6 concentration in amniotic fluid; iv) amniotic fluid cathepsin-G concentration of 105 ng/mL is the optimal cutoff point for identifying PPROM pregnancies complicated by the presence of IAI; and v) the presence of microbial-associated and sterile IAI is associated with higher amniotic fluid cathepsin-G concentrations than colonization and the absence of both MIAC and IAI.

Cathepsin-G plays a complex role in the defense against the spreading of microorganisms [17,20,21]. It participates, in combination with myeloperoxidase and reactive oxygen species, in direct intracellular killing of phagocytosed microorganisms [17,20,21]. In addition, cathepsin-G is an essential part of neutrophil extracellular traps, which serves as an extracellular physical barrier that kills microorganisms extracellularly [17,20,21]. In this study, we found that amniotic fluid cathepsin-G concentrations were higher when MIAC was present. This finding was consistent with the study by Helming et al. that showed an elevation of neutrophil elastase (serine protease) in amniotic fluid from PPROM complicated by MIAC [22].

As previously shown, MIAC is a heterogeneous group covering two subgroups of women: women with microbial-associated IAI and women with colonization [7,9,31]. In this study, women with microbial-associated IAI had higher amniotic fluid cathepsin-G concentrations than women with colonization. In addition, no difference in the amniotic fluid cathepsin-G concentrations was found when women with colonization and women without both MIAC and IAI were compared ($p=0.42$; data not shown). This indicated that amniotic fluid cathepsin-G concentrations were elevated when the presence of microorganisms in the amniotic fluid led to the development of IAI response.

Table 2. Microorganism identified in the amniotic fluid of women with preterm prelabor rupture of membranes.

The presence of microbial-associated intra-amniotic inflammation ($n = 22$)	The presence of colonization ($n = 25$)
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Ureaplasma species	*Ureaplasma* species
Streptococcus species	*Ureaplasma* species
Fusobacterium nucleatum	*Ureaplasma* species
Chlamydia trachomatis	*Ureaplasma* species
Haemophilus influenza	*Ureaplasma* species
Streptococcus agalactiae	*Ureaplasma* species
Streptococcus anginosus	*Ureaplasma* species
Ureaplasma species; *Mycoplasma hominis*	*Mycoplasma hominis*
Ureaplasma species; *Mycoplasma hominis*	*Mycoplasma hominis*
Ureaplasma species; *Lactobacillus species*	*Mycoplasma hominis*
Ureaplasma species; *Chlamydia trachomatis*	*Mycoplasma hominis*
Ureaplasma species; *Sneathia sanguinegens*	*Mycoplasma hominis*
Ureaplasma species; *Veillonela species*	*Streptococcus pneumonie*
Peptococcus species; *Propionibacterium* species; *Bacteroides* species; *Mycoplasma hominis*	*Bifidobacterium* species
	Chlamydia trachomatis; Enterococcus faecium
	Ureaplasma species; *Chlamydia trachomatis*
	Ureaplasma species; *Chlamydia trachomatis*

Cathepsin-G seems to be an important regulator of the local inflammatory response in addition to its antimicrobial properties [17,19,32]. The exposure of neutrophils to cytokines and chemokines resulted in the rapid mobilization of granules containing cathepsin-G and other proteins to their surface and their release from the neutrophils [17,19,33]. That means that the presence of local inflammation stimulates the release of cathepsin-G from the neutrophils. Our findings are in line with the abovementioned since in this study women with IAI had higher amniotic fluid cathepsin-G concentrations than women without IAI. Moreover, we found a weak positive correlation between amniotic fluid cathepsin-G and IL-6 concentrations.

The mechanisms leading to the induction of IAI are triggered through the system of pattern recognition receptors [34]. The presence of either microorganisms or endogenous molecules called alarmins (e.g. high mobility group box 1 protein) in amniotic fluid may engage these receptors and stimulate the development of IAI response [7,35]. In this study, we found the highest amniotic fluid cathepsin-G concentrations in women with IAI independently whether bacteria had been present in the amniotic fluid or not. The mechanism beyond the elevation of amniotic fluid cathepsin-G concentrations in women with sterile inflammation remains to be elucidated. It was previously shown that women with bacterial invasion in the fetal membranes had higher amniotic fluid IL-6 and IL-8 concentrations [36]. We can only speculate whether the presence of bacterial invasion in the fetal membranes or inflammation in the choriodecidual space

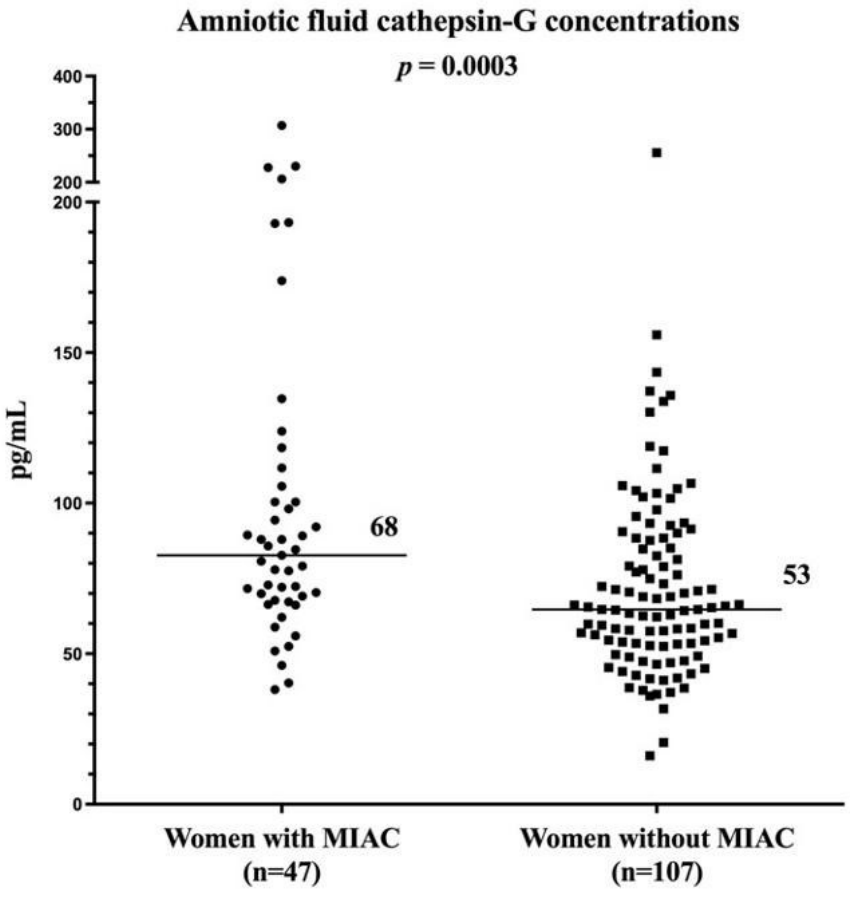


Figure 1. Amniotic fluid cathepsin-G concentrations with respect to the presence or absence of MIAC. Abbreviation: MIAC: microbial invasion of the amniotic cavity.

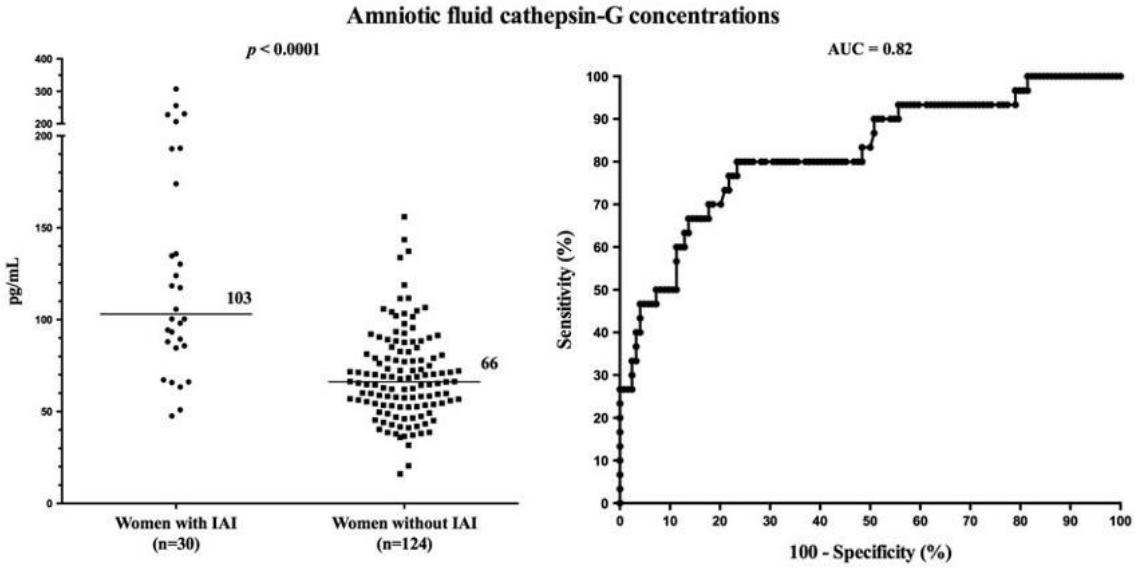
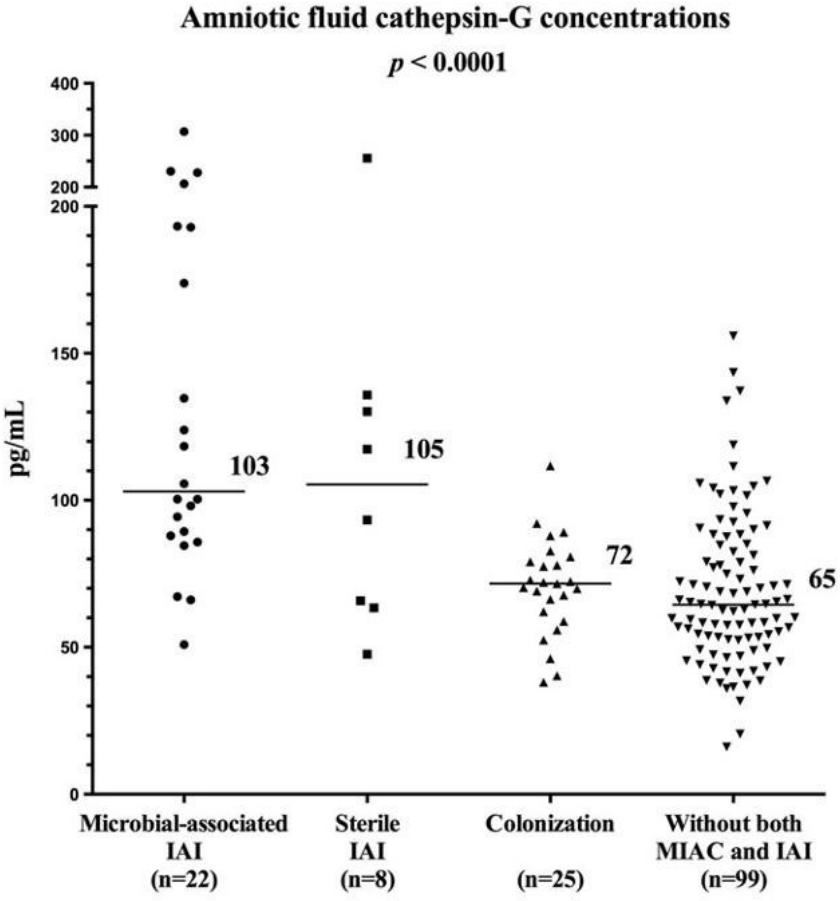


Figure 2. Amniotic fluid cathepsin-G concentrations with respect to the presence or absence of IAI. Receiver operating characteristic curve for the presence of IAI (area under curve for cathepsin-G cutoff point > 105 ng/mL: 82%; $p < 0.0001$). Abbreviation: IAI: intra-amniotic inflammation.

Figure 3. Amniotic fluid cathepsin-G concentrations in the subgroups of PPROM based on the presence of microbial-associated IAI, sterile IAI, colonization, and the absence of both MIAC and IAI. Horizontal bars represent medians. Abbreviations: PPROM: preterm prelabor rupture of membranes; MIAC: microbial invasion of the amniotic cavity; IAI: intra-amniotic inflammation.



in PPROM can lead to the development of sterile inflammation in the amniotic fluid associated with higher amniotic fluid cathepsin-G concentrations.

Our study provides new information regarding the amniotic fluid cathepsin-G concentrations in PPROM pregnancies. Our recent study has shown that fetal inflammatory response, which is determined by umbilical cord blood IL-6 concentrations, was the highest in pregnancies complicated by IAI independently on the presence or absence of MIAC [37]. From this point of view, the amniotic fluid cathepsin-G

concentration cutoff identified in this study for the prediction of IAI in women with PPROM may have important clinical implications. However, this interesting finding needs to be validated on an independent cohort of women with PPROM.

A strength of this study is the fact that MIAC was identified based on the combination of two non-cultivation approaches: (1) specific PCR for *Ureaplasma* species, *Mycoplasma hominis* and *Chlamydia trachomatis* and (2) nonspecific PCR (16S rRNA). Second, only women with a well-defined specific phenotype of spontaneous preterm delivery (PPROM) were included in this study. This study was also subject to some limitations. In this study, we specifically evaluated only one out of three neutral serine proteases stored in the primary granules of neutrophils. A complex evaluation of all of them may have provided a deeper insight in the pathophysiology of IAI in PPROM. Next, the exact source of cathepsin-G in amniotic fluid remains unclear, yet it was beyond the scope of this study. We can only speculate on the origin of cathepsin-G in the amniotic fluid, but it is likely to be released from neutrophils in both amniotic fluid and the placenta and fetal membranes. Third, the conservative management of the majority of women included in this study prevented this study from evaluating the association between the amniotic fluid cathepsin-G concentration and the presence of histological chorioamnionitis in PPROM because of a long latency between amniotic fluid sampling and delivery. Fourth, the fact that the white blood cell count in amniotic fluid was not evaluated prevented us from analyzing whether there was an association between the cathepsin-G concentrations and white blood cell count in amniotic fluid. Last, the amniotic fluid cultivations were not performed, therefore viability of the microorganisms could not be taken into consideration.

In conclusion, the presence of IAI, independently whether it was microbial-associated or sterile, was associated with the highest amniotic fluid cathepsin-G concentrations. Cathepsin-G seems to be a potential marker for the early identification of PPROM complicated by IAI.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper. This work was supported by Charles University in Prague; Faculty of Medicine in Hradec Kralove, Czech Republic; project ''PRVOUK'' P37/10; and Faculty Hospital in Hradec Kralove (long-term organization development plan).

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Příloha č. 3: Cervical fluid calreticulin and cathepsin-G in pregnancies complicated by preterm prelabor rupture of membranes

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ORIGINAL ARTICLE

Cervical fluid calreticulin and cathepsin-G in pregnancies complicated by preterm prelabor rupture of membranes

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ABSTRACT

Objective: The study aimed to determine the cervical calreticulin and cathepsin-G concentrations in pregnancies complicated by preterm prelabor rupture of membranes (PPROM) with respect to the presence of microbial invasion of the amniotic cavity (MIAC) and intra-amniotic inflammation (IAI).

Methods: Eighty women with singleton pregnancies complicated by PPROM were included in this study. Cervical and amniotic fluids were obtained at the time of admission, and concentrations of calreticulin and cathepsin-G in cervical fluid were determined using ELISA. The MIAC was defined as a positive PCR analysis for *Ureaplasma* species, *Mycoplasma hominis,* and/or *Chlamydia trachomatis* and/or by positivity for the 16S rRNA gene. IAI was defined as amniotic fluid bedside IL-6 concentrations $\geq$745 pg/mL

Result: Neither women with MIAC nor with IAI had different cervical fluid concentrations of calreticulin (with MIAC: median 18.9 pg/mL vs. without MIAC: median 14.7 pg/mL, $p = 0.28$; with IAI: median 14.3 pg/mL vs. without IAI: median 15.6 pg/mL, $p = 0.57$;) or of cathepsin-G (with MIAC: median 30.7 pg/mL vs. without MIAC: median 24.7 pg/mL, $p = 0.28$; with IAI: median 27.3 pg/mL vs. without IAI: median 25.1 pg/mL, $p = 0.80$) than women without those complications. No associations between amniotic fluid IL-6 concentrations, gestational age at sampling, and cervical fluid calreticulin and cathepsin-G concentrations were found.

Conclusions: Cervical fluid calreticulin and cathepsin-G concentrations did not reflect the presence of MIAC or IAI in women with PPROM.

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Preterm delivery; non-invasive sample; amniotic fluid; marker

Introduction

Preterm prelabor rupture of membranes (PPROM), characterized by the rupture of fetal membranes with leakage of the amniotic fluid before spontaneous onset of regular uterine contraction at less than gestational age 37 weeks, is still considered to be a major challenge of current perinatology owing to its unpredictability and non-preventability [1,2]. PPROM represents a condition that is commonly accompanied by the specific intra-amniotic complications, such as microbial invasion of the amniotic cavity (MIAC) and/or intra-amniotic inflammation (IAI) [3,4]. Despite the fact that the presence of these complications does not appear to affect the short-term neonatal outcome, knowledge about the presence or absence of these complications might be useful for obstetricians and neonatologists for the personalized management of PPROM [4,5].

So far, the evaluation of amniotic fluid obtained by transabdominal amniocentesis has been considered to be a traditional approach for the evaluation of MIAC and IAI [3,6–8]. However, the invasive nature of amniocentesis along with its difficulty in situations with a low residual amount of amniotic fluid makes clinicians reluctant to perform this procedure in women with PPROM [9,10]. Therefore, there is an urgent need for

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some noninvasive markers in cervical or vaginal fluid that can be used as a surrogate for amniotic fluid markers.

Recently, amniotic fluid calreticulin, a multifunctional and multicompartmental protein involved in many cellular functions, and cathepsin-G, a member of the neutral serine protease family, have been shown to have a diagnostic value for IAI and MIAC in PPROM [11,12]. Since the results of some valuable amniotic fluid inflammatory mediators for prediction of MIAC and IAI were successfully translated to their noninvasive forms (markers from cervical or vaginal fluid), we hypothesized that cervical fluid calreticulin and/or cathepsin-G may be useful in the prediction of the PPROM subgroups complicated by the presence of MIAC and IAI [13–19].

The main aim of this study was to evaluate the cervical fluid calreticulin and cathepsin-G concentrations with respect to the presence and absence of MIAC and IAI in pregnancies complicated by PPROM.

Materials and methods

Between May 2015 and June 2016, a prospective cohort study was conducted. Women with pregnancies complicated by PPROM between gestational age 24 + 0 and 36 + 6 weeks, who were admitted to the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic were recruited. Only women aged 18 years and above and having a singleton pregnancy were eligible for the study. Women with any medical complications (i.e., hypertension, preeclampsia, diabetes mellitus, and thyroid disease), fetal growth restriction, gross vaginal bleeding, signs of fetal hypoxia, and structural malformations or chromosomal abnormalities of the fetus were excluded from the study. Gestational age was established for all pregnancies based on the first trimester ultrasound evaluation.

PPROM was defined as the leakage of the amniotic fluid before the onset of labor. This condition was diagnosed visually using a sterile speculum examination to confirm the pooling of amniotic fluid in the vagina. In case of clinical doubt, PPROM was confirmed by the presence of insulin-like growth factor–binding protein (ACTIM PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

Women with PPROM at less than 34 weeks of gestation were treated with tocolytics for 48 h, antibiotics, and corticosteroids to accelerate lung maturation. Women with a proven microbial-associated IAI (amniotic fluid IL-6 $\geq$ 745 pg/mL and the presence of MIAC) beyond 28 gestational weeks were actively managed. Actively managed women had finished a tocolytic therapy when microbial-associated IAI was proven and were treated only with corticosteroids and antibiotics. Furthermore, labor was induced or an elective cesarean section was performed after finalizing corticosteroid treatment within 72 h of the membranes rupturing. The remaining women with PPROM at less than 34 weeks were conservatively managed, and labor was induced or an elective cesarean section was performed after gestational age 34 + 0 weeks. Women with PPROM beyond 34 weeks were treated only with antibiotics, and labor was induced or the elective cesarean section was performed after 24 h of the membrane rupturing.

This study was approved by the Institutional Review Board committee (July 2014; No 201407 S14P), and informed consent was obtained from all participants. Amniotic fluid samples and vaginal fluid data from this cohort of women have been used in our previously published papers [12,20].

Cervical and amniotic fluid sampling

Amniotic fluid and the cervical fluid samples were collected at the same time in all women on admission (cervical fluid as first, amniotic fluid second) before the administration of corticosteroids, antibiotics, or tocolytics. Cervical fluid was obtained using Dacron polyester swab, which was placed into the cervical canal for 20 s to achieve saturation. On collection, a Dacron polyester swab was inserted into polypropylene tubes containing 1.5 mL of phosphate buffered saline. The tube was shaken for 20 min followed by centrifugation for 15 min at 300 $\times$ *g* at room temperature. The supernatant was aliquoted and stored at $-70\,^{\circ}C$ until further analysis. Ultrasound-guided transabdominal amniocentesis was performed, and $\sim$5 mL of amniotic fluid was aspirated. Tubes with uncentrifuged amniotic fluid were transported to the laboratory for DNA isolation and PCR detection of *Ureaplasma* spp., *Mycoplasma hominis*, *Chlamydia trachomatis*, and 16S rRNA gene sequencing.

Cervical fluid calreticulin and cathepsin-G concentrations

The concentrations of calreticulin and cathepsin-G in cervical fluid were determined in duplicate by a sandwich enzyme-linked immunosorbent assay (ELISA) technique using an ELISA kit for calreticulin (Cloud-Clone Corp., Houston, TX) and a cathepsin-G (CtSG) ELISA kit (Cloud-Clone Corp., Houston, TX), respectively, according to the manufacturer's instructions.

The detection range of the calreticulin and cathepsin-G kits were 3.12–400 ng/mL and 1.56–400 ng/mL, respectively. Samples of cervical fluid for calreticulin and for cathepsin-G measurements were diluted 1:5 and 1:20, respectively. The absorbance values were read at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA).

Amniotic fluid IL-6 concentrations

The IL-6 concentrations were assessed with a lateral flow immunoassay Milenia QuickLine IL-6 using the Milenia POCScan Reader (Milenia Biotec, GmbH, Giessen, Germany). The measurement range was 50–10 000 pg/mL. The intra-assay and inter-assay variations were 12.1% and 15.5%, respectively [21].

Detection of Ureaplasma *species,* Mycoplasma hominis, *and* Chlamydia trachomatis

DNA was isolated from the amniotic fluid with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (using the protocol for isolating bacterial DNA from biological fluids). Real-time PCR was performed on a Rotor-Gene 6000 instrument (QIAGEN) 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 the DNA from *Ureaplasma* species, *Mycoplasma hominis*, and *Chlamydia trachomatis* in a common PCR tube. As a control, we included a PCR run for beta-actin, a housekeeping gene, to examine the presence of inhibitors of PCR.

Detection of other bacteria in the amniotic fluid

Bacterial DNA was identified by PCR targeting the 16S rRNA gene with the following primers: 5′-CCAGAC TCCTACGGGAGGCAG-3′ (V3 region), 5′-ACATTTCACAA CACGAGCTGACGA-3′ (V6 region) [22,23]. Each individual reaction contained 3 μL of target DNA, 500 nM of forward and reverse primers, and Q5 High-Fidelity DNA polymerase (NEB, Ipswich, MA) in a total volume of 25 μL. The amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The products were visualized on an agarose gel. Positive reactions yielded products of 950 bp, which were subsequently analyzed by sequencing. The 16S PCR products were cleaned and used in sequencing PCR reactions using the aforementioned primers and the BigDye Terminator kit, v3.1 (Thermo Fisher Scientific). The bacteria were then typed using the sequences obtained in BLAST® and SepsiTest™ BLAST.

Diagnosis of MIAC and definition of IAI

The MIAC was determined based on a positive PCR analysis for *Ureaplasma* species, *Mycoplasma hominis,* and/or *Chlamydia trachomatis* and/or by positivity for the 16S rRNA gene. IAI in PPROM pregnancies was defined as amniotic fluid bedside IL-6 concentrations $\geq$745 pg/mL [6,24].

Statistical analyses

The demographic and clinical characteristics were compared using a nonparametric Mann–Whitney *U*-test for continuous variables and are presented as median values (range). Categorical variables were compared using a Fisher's exact test and are presented as numbers (%). The normality of the data was tested using the D'Agostino-Pearson omnibus normality test and the Shapiro–Wilk test. Because the cervical fluid clusterin and cathepsin-G concentrations were not normally distributed, the nonparametric Mann–Whitney *U*-test was used for analyses and presented as median values (interquartile range [IQR]). Spearman's correlations were performed. Differences were considered significant at $p < 0.05$. All p values were obtained from two-sided tests, and all statistical analyses were performed using GraphPad Prism 6 for Mac OS X (GraphPad Software, San Diego, CA) or the SPSS 19.0 statistical package for Mac OS X (SPSS Inc., Chicago, IL).

Results

Demographic and clinical characteristics of the study population

In total, 84 women with singleton pregnancy complicated by PPROM were included in the study. Two women were excluded due to signs of fetal growth restriction, one woman for gestational diabetes mellitus, and one woman for preeclampsia. Therefore, the remaining 80 women were included in the analyses.

The presence of MIAC and IAI was found in 28% (22/80) and 18% (14/80), respectively. The demographic and clinical characteristics of women with respect to the presence of MIAC and IAI are shown in Table 1. The most common bacteria identified in the amniotic fluid was *Ureaplasma* spp., which were identified in 59% (13/22) of the women with MIAC. In the remaining nine women these bacterial findings were

Table 1. Maternal and neonatal characteristics of the subgroups of PPROM pregnancies.

Characteristic	With MIAC ($n = 22$)	Without MIAC ($n = 58$)	p value[a]	With IAI ($n = 14$)	Without IAI ($n = 66$)	p value[b]
Maternal age [years, median (range)]	30 (20–40)	32 (22–42)	0.05	31 (23–37)	31 (20–42)	0.27
Primiparous [number (%)]	13 (59%)	35 (60%)	1.00	7 (50%)	41 (62%)	0.55
Prepregnancy body mass index [kg/m^2, median (range)]	21.9 (16.7–36.0)	22.3 (16.6–35.5)	0.41	23.6 (16.7–36.0)	22.0 (16.6–35.5)	0.52
Smoking [number (%)]	6 (27%)	5 (9%)	0.06	3 (21%)	8 (12%)	**0.40**
Gestational age at admission [weeks, median (range)]	33 + 3 (24 + 0–36 + 5)	33 + 6 (24 + 0–36 + 5)	0.41	26 + 5 (24 + 0–35 + 0)	34 + 3 (24 + 0–36 + 5)	**<0.0001**
Gestational age at delivery [weeks, median (range)]	33 + 5 (25 + 1–36 + 5)	34 + 2 (24 + 0–37 + 1)	0.37	28 + 1 (25 + 0–35 + 0)	34 + 4 (24 + 0–37 + 1)	**<0.0001**
Interval from PPROM to amniocentesis [hours, median (range)]	4 (2–23)	5 (1–356)	0.32	4 (2–10)	5 (1–356)	0.39
Latency from amniocentesis to delivery [hours, median (range)]	63 (4–172)	36 (5–624)	0.26	103 (6–443)	34 (6–624)	**0.01**
CRP levels at admission [mg/L, median (range)]	5.1 (0.4–72.1)	4.1 (1.2–43.5)	0.51	8.5 (1.8–72.4)	3.9 (0.4–43.5)	**0.02**
WBC count at admission [$\times 10^9$ L, median (range)]	12.8 (6.3–22.1)	12.4 (7.8–25.9)	0.99	13.6 (9.2–22.1)	12.3 (6.3–25.9)	0.22
Amniotic fluid IL-6 at admission [pg/mL, median (range)]	252 (50–10000)	222 (50–10000)	0.17	3946 (747–10000)	183 (50–10000)	**<0.0001**
Administration of antibiotics [number (%)]	22 (100%)	56 (97%)	1.00	14 (100%)	64 (97%)	1.00
Administration of corticosteroids [number (%)]	16 (73%)	39 (67%)	0.79	14 (100%)	41 (62%)	**0.004**
Spontaneous vaginal delivery [number (%)]	17 (77%)	35 (60%)	0.20	9 (64%)	43 (65%)	1.00
Forceps delivery [number (%)]	1 (5%)	1 (2%)	0.48	0 (0%)	2 (3%)	1.00
Cesarean delivery [number (%)]	4 (18%)	22 (38%)	0.11	5 (36%)	21 (32%)	0.76
Birth weight [grams, median (range)]	2080 (660–3150)	2130 (560–3670)	0.47	1065 (560–2540)	2310 (560–3670)	**<0.0001**
Apgar score <7; 5 min [number (%)]	2 (9%)	2 (3%)	0.30	2 (14%)	2 (3%)	0.14
Apgar score <7; 10 min [number (%)]	0 (0%)	2 (3%)	1.00	0 (0%)	2 (3%)	1.00

PPROM: preterm prelabor rupture of membranes; MIAC: microbial invasion of the amniotic cavity; IAI: intraamniotic inflammation; CRP: C-reactive protein; WBC: white blood cells; IL: interleukin.
Continuous variables were compared using a nonparametric Mann–Whitney *U*-test. Categorical variables were compared using Fisher's exact test. Statistically significant results are marked in bold. Continuous variables are presented as median (range) and categorical as number (%).
[a]A comparison between women with and without microbial invasion of the amniotic cavity.
[b]A comparison between women with and without intraamniotic inflammation.

found: *Lactobacillus crispa*tus + *Enterococcus faecalis* + *Streptococcus salivarius* 1×, *Chlamydia trachomatis* 1×, *Streptococcus intermedius* 2×, *Peptoniphilus species* 1×, *Gardnerella vaginalis* 1×, *Sneathia sanguinegens* 1×, *Staphylococcus warneri* 1×, and non-identifiable bacteria by sequencing 1×. All the women were self-reported as Caucasians.

Cervical calreticulin and cathepsin-G concentrations according to the presence of MIAC

No differences in cervical fluid calreticulin nor in cervical fluid cathepsin-G concentrations between women with and without MIAC were found (calreticulin: with MIAC: median 18.9 ng/mL, IQR 9.4–32.0 vs. without MIAC: median 14.7 ng/mL IQR 8.6–21.4, $p = 0.25$, Figure 1(a); cathepsin-G: with MIAC: median 30.7 ng/mL, IQR 16.4–44.2 vs. without MIAC: median 24.7 ng/mL, IQR 15.0–36.6, $p = 0.28$, Figure 1(b)).

Cervical calreticulin and cathepsin-G concentrations according to the presence of IAI

No differences in cervical fluid calreticulin nor in cervical fluid cathepsin-G concentrations between women with and without IAI were found (calreticulin: with IAI: median 14.3 ng/mL, IQR 8.4–21.6 vs. without IAI: median 15.6 ng/mL, IQR 9.2–22.8, $p = 0.57$, Figure 2(a); cathepsin-G: with IAI: median 27.3 ng/mL, IQR 14.5–40.2 vs. without IAI: median 25.1 ng/mL, IQR 15.6–40.8, $p = 0.80$, Figure 2(b)).

Correlation between cervical fluid calreticulin and cathepsin-G concentrations and amniotic fluid IL-6 concentrations

Neither cervical fluid calreticulin nor cervical fluid cathepsin-G concentrations correlated with amniotic fluid IL-6 concentrations (calreticulin: rho$= -0.12$, $p = 0.22$; cathepsin-G: rho $= -0.05$, $p = 0.64$).

Correlation between cervical calreticulin and cathepsin-G concentrations and gestational age at sampling

Neither cervical fluid calreticulin nor cervical fluid cathepsin-G concentrations correlated with gestational age at sampling (calreticulin: rho $= -0.10$, $p = 0.39$; cathepsin-G: rho $= -0.06$, $p = 0.58$).

Discussion

Recently, amniotic fluid calreticulin and cathepsin-G have been found to be potential markers for an

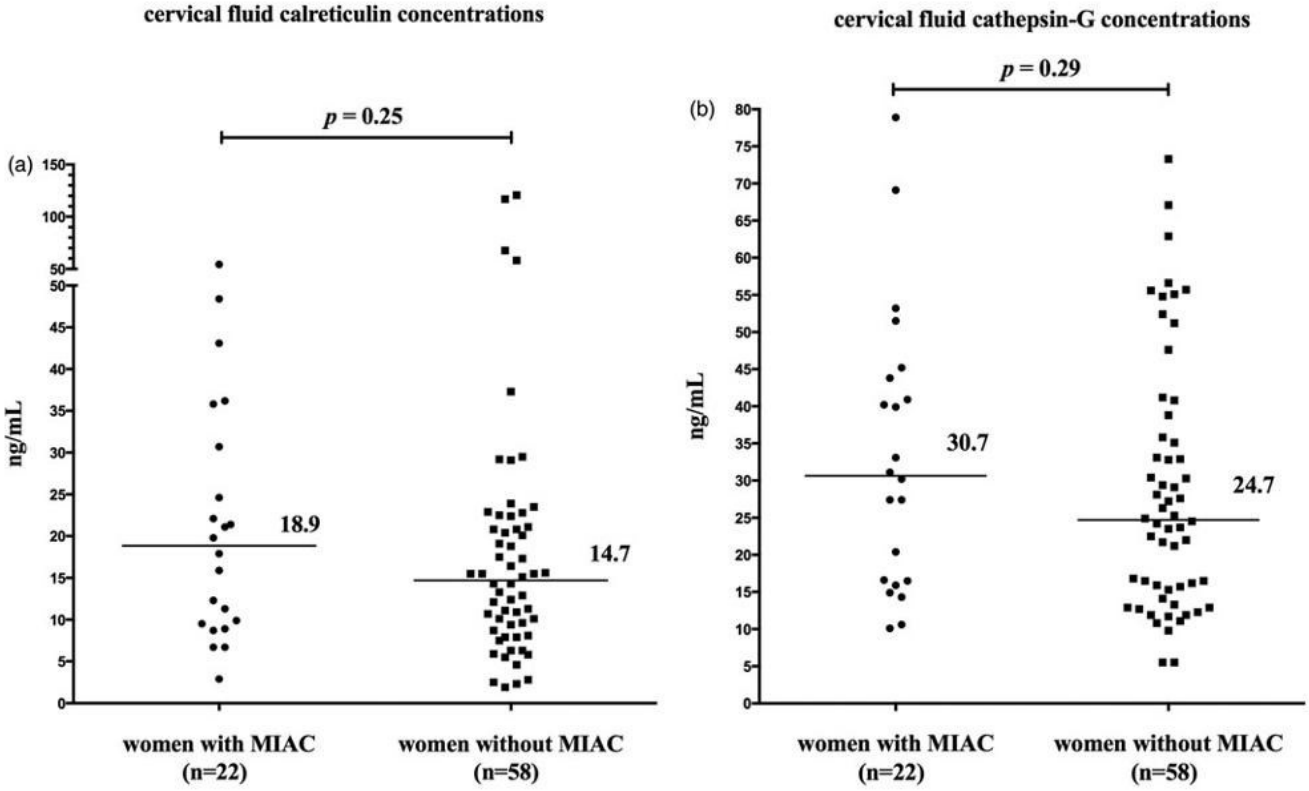

Figure 1. The cervical fluid calreticulin (a) and cathepsin-G (b) concentrations in PPROM pregnancies complicated by MIAC. Women with MIAC did not have different medians of cervical fluid calreticulin and cathepsin-G concentrations than women without MIAC. Abbreviations: MIAC: microbial invasion of the amniotic cavity.

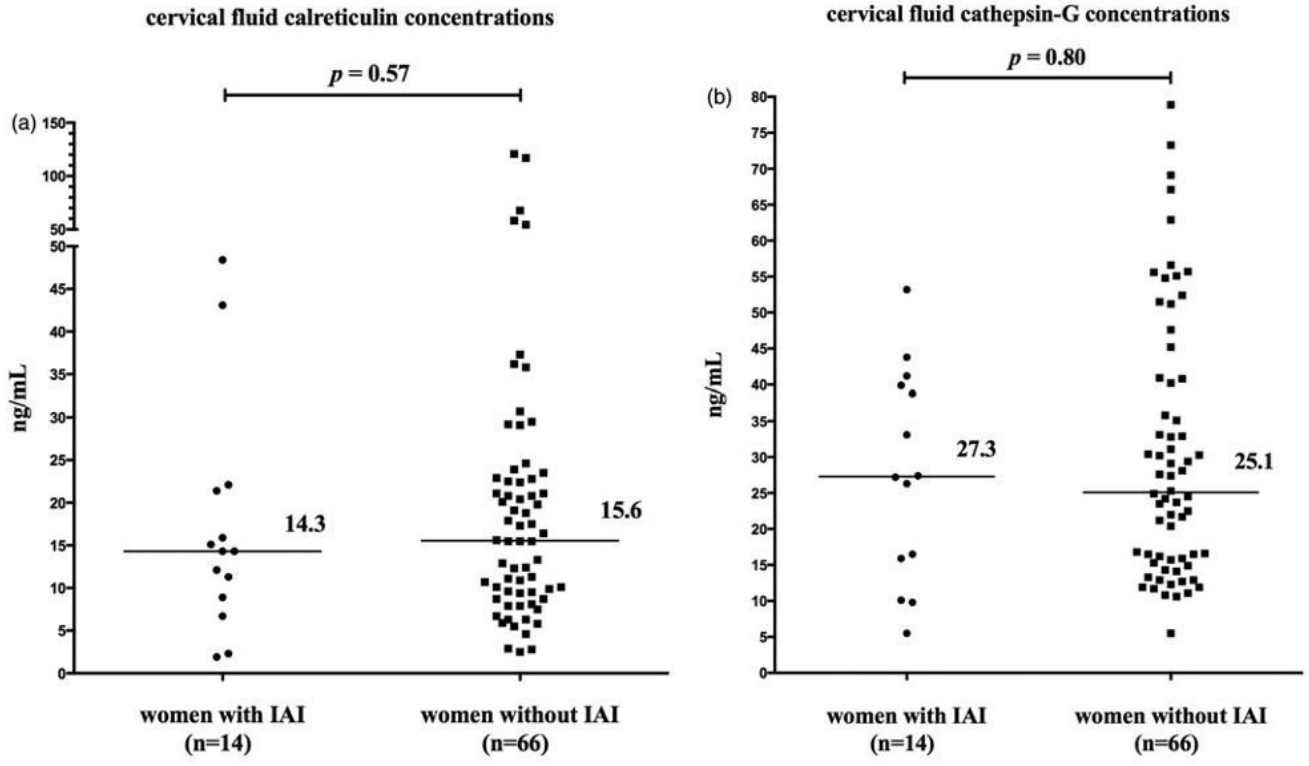

Figure 2. The cervical fluid calreticulin (a) and cathepsin-G (b) concentrations in PPROM pregnancies complicated by IAI. Women with IAI did not have different medians of cervical fluid calreticulin and cathepsin-G concentrations than women without IAI. Abbreviations: IAI: intra-amniotic inflammation.

identification of MIAC and IAI in women with PPROM [11,12]. Nevertheless, the question of whether cervical fluid calreticulin and cathepsin-G would be of value for identification of these PPROM complications has not yet been characterized. In this study, we tried to translate our promising amniotic fluid calreticulin and cathepsin-G results from invasively obtained amniotic fluid to noninvasively obtained cervical fluid samples.

The following are the key findings of the study: (a) MIAC was not associated with higher cervical fluid calreticulin and cathepsin-G concentrations; (b) IAI was not related to higher cervical fluid calreticulin and cathepsin-G concentrations; (c) neither amniotic fluid IL-6 concentrations nor gestational age at sampling correlated with cervical fluid calreticulin and cathepsin-G concentrations.

The presence of MIAC has traditionally been considered to be a major pathophysiologic condition triggering an intra-amniotic inflammatory response and subsequently leading to the development of IAI in PPROM [25–27]. A solid body of evidence has shown that the presence of MIAC is reflected on the elevation of inflammatory mediators in the cervical fluid [17–19,28]. However, the results regarding an association between MIAC and the specific cervical fluid cytokines and chemokines may be conflicting [15,17,18,29,30]. In this study, we did show that calreticulin and cathepsin-G would not be of value to identify MIAC, since no associations between their cervical fluid concentrations and MIAC were found.

At the present time, amniotic fluid IL-6 is considered to be one in a group of gold standard markers of IAI in women with PPROM, independent of the methods used for IL-6 evaluation [6,25]. In addition, IL-6 has not been shown to be inferior to modern proteomic markers of IAI [31]. Nevertheless, the process of searching for "an ideal marker" of IAI has not yet been concluded. In our previous publications, we have shown that amniotic fluid calreticulin and cathepsin-G appear to be promising candidates to be the markers of IAI in PPROM [11,12]. In this study, we failed to replicate their amniotic fluid value for IAI identification on noninvasive cervical fluid samples since no difference in their cervical fluid concentrations between women with and without IAI were found. Accordingly, we did not identify any associations between amniotic fluid IL-6 and cervical fluid calreticulin and cathepsin-G concentrations.

The cervical fluid calreticulin and cathepsin-G findings, despite their negativity, appear to be clinically relevant, since these results confirm the facts that the compositions of cervical fluid and amniotic fluid are different in women with PPROM and that concentrations of some inflammatory mediators in cervical fluid are driven by different processes than MIAC and IAI [32]. At this stage, we can only hypothesize that these negatives findings might be explained by a high production of calreticulin and cathepsin-G by endocervical cells. Cervical calreticulin and cathepsin-G seem to be responsible for a vast majority of total amount of calreticulin and cathepsin-G contained in cervical fluid. Therefore, a high cervical production of these proteins may mask slight changes in their cervical concentrations driven by MIAC and IAI. This hypothesis is strongly supported by the fact that calreticulin and cathepsin-G concentrations in the cervical fluid samples were just 2.5-fold lower than in the amniotic fluid samples (as shown in our previous publications), despite the fact that the cervical fluid samples represent diluted samples (cervical fluid was obtained from a Dacron swab placed in the cervical canal for 20 s to achieve a saturation by cervical fluid and subsequently diluted in 1.5 mL of buffer) [11,12]. Therefore, it might be anticipated that calreticulin and cathepsin-G concentrations in undiluted cervical fluid samples should be higher than in amniotic fluid samples.

An important strength of this study is the characterization of MIAC completely based on the non-cultivation approaches (combination of both a nonspecific PCR evaluation of the presence of 16S rRNA gene + a specific PCR for *Ureaplasma* species, *Mycoplasma hominis*, and *Chlamydia trachomatis*). A second strength of this study is that amniotic fluid and cervical fluid samples were obtained simultaneously at the time of admission. The main limitation of this study is that the analyses were based on a relatively small sample size, and a type II error might affect our results and could not be ruled out. The next limitation of this study is the fact that we are not able to evaluate the proportion of cervical fluid calreticulin and cathepsin-G that comes from amniotic fluid and from the cervix. If would be of interest to add an evaluation of calreticulin and cathepsin concentrations in undiluted vaginal fluid obtained from the posterior fornix by syringe, to identify the difference in these proteins' concentrations.

To conclude, cervical fluid calreticulin and cathepsin-G do not have a potential to serve as the noninvasive markers of intra-amniotic complications in PPROM because their cervical fluid concentrations, in contrast with amniotic fluid concentrations, do not reflect the presence of MIAC and IAI.

Disclosure statement

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Příloha č. 4: Amniotic fluid clusterin in pregnancies complicated by the preterm prelabor rupture of membranes

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ORIGINAL ARTICLE

Amniotic fluid clusterin in pregnancies complicated by the preterm prelabor rupture of membranes

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Abstract

Objective: The aim of this study was to evaluate clusterin concentrations in amniotic fluid in pregnancies complicated by preterm prelabor rupture of membranes (PPROM) with respect to the presence of the microbial invasion of the amniotic cavity (MIAC), intra-amniotic inflammation (IAI) and microbial-associated IAI.

Methods: One hundred thirty-six women with singleton pregnancies complicated by PPROM were included in this study. Amniotic fluid samples were obtained by transabdominal amniocentesis. Amniotic fluid clusterin concentrations were assessed by enzyme-linked immunosorbent assay. MIAC was determined by a non-cultivation approach. IAI was defined as an amniotic fluid bedside interleukin-6 concentration $\geq$745 pg/mL. Microbial-associated IAI was characterized as the presence of both MIAC and IAI.

Result: Women with MIAC, IAI and microbial-associated IAI had lower amniotic fluid clusterin concentrations than women without these complications (with MIAC: median 1314 ng/mL versus without MIAC: median 1633 ng/mL, $p = 0.003$; with IAI: median 1281 ng/mL versus without IAI: median 1575 ng/mL, $p = 0.04$; with microbial associated-IAI: median 1220 ng/mL versus without microbial-associated IAI: median 1575 pg/mL; $p = 0.008$). A week negative correlation between amniotic fluid clusterin concentrations and gestational age at sampling was revealed (rho= -0.30; $p = 0.0005$).

Conclusions: The presence of MIAC, IAI and microbial-associated IAI was characterized by lower amniotic fluid clusterin concentrations in pregnancies complicated by PPROM.

Keywords

Preterm delivery, inflammation, apolipoprotein, complement

History

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Introduction

Preterm prelabor rupture of membranes (PPROM), defined as a leakage of amniotic fluid before onset of regular activity prior to gestational age 37 weeks, represents an important public health issue, because PPROM complicates about 3% of all pregnancies [1,2]. Although knowledge about the pathophysiology of PPROM and its infection-related and inflammatory complications has improved during the last decade, PPROM still forms a threat for obstetricians and their patients in all countries of the world [2–7].

Since PPROM is often associated with infection-related and inflammatory conditions, a plethora of invasive and non-invasive markers alone or in combination has been suggested to predict or identify these complications [8–11]. From a purely clinical point of view, the presence of infection or inflammation is generally confirmed by the elevation of selected markers [e.g. white blood cells count, C-reactive protein, interleukin (IL)-6] in different body fluids. Not surprisingly, the identification of intra-amniotic complications is more or less expected based on the elevation of amniotic fluid markers, with the exception of amniotic fluid glucose [12–15]. Amniotic fluid glucose belongs in a smaller group of markers having lower amniotic fluid concentrations when intra-amniotic inflammation (IAI) is present [12]. Nevertheless, our comparative proteomic study in amniotic fluid has revealed other proteins presenting lower amniotic fluid concentrations when both microbial invasion of the amniotic cavity (MIAC) and histological chorioamnionitis were proven in PPROM [16]. One of these downregulated

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proteins, having different biological functions, was clusterin [16].

Clusterin is multifunctional protein named for its ability to induce cellular clustering [17–20]. Clusterin has been reported to have different functions – as a chaperone, an inductor of aggregation of variety of cells, a sensor of oxidative stress, an inhibitor of complement, a preventer of cytolysis and many others [17,20,21]. Clusterin plays a role in the pathophysiology of renal and neurodegenerative diseases, atherosclerosis, myocardial infarction and some cancers [17,20–22]. Recently, an association between clusterin and preeclampsia has been shown in both unbiased proteomic and hypothesis-driven studies [23–27]. However, there is a paucity of information regarding how the presence of infection-related and inflammatory complications affect amniotic fluid clusterin concentrations in women with PPROM.

Therefore, the aim of this study was to determine the amniotic fluid concentrations of clusterin in pregnancies complicated by PPROM based on the presence of MIAC, IAI and microbial-associated IAI.

Materials and methods

A retrospective cohort study of women with pregnancies complicated by PPROM between gestational age 24 + 0 and 36 + 6 weeks admitted to the Department of Obstetrics and Gynecology of the University Hospital Hradec Kralove in the Czech Republic was conducted between November 2014 and March 2016. Only women with singleton pregnancies and with a maternal age $\geq$ 18 years were recruited to the study. Women with pregnancy complications such as preeclampsia, gestational hypertension, gestational or pre-gestational diabetes, fetal growth restrictions, the presence of either chromosomal or structural fetal abnormalities, signs of fetal hypoxia, or significant vaginal bleeding were excluded from the study.

Gestational ages were established by first-trimester fetal biometry. Women with PPROM at less than 34 weeks of gestation were treated with tocolytics for 48 h, antibiotics and corticosteroids to accelerate lung maturation, whereas no treatment except antibiotics was initiated to delay delivery after 34 weeks. Women with proven microbial-associated IAI beyond 28 gestational weeks did not receive tocolytics and were treated only with corticosteroids and antibiotics. Furthermore, labor was induced or a cesarean section was performed 24 h after finalizing corticosteroid treatment. The remaining women were managed conservatively.

PPROM was diagnosed by examination with a sterile speculum to verify the pooling of amniotic fluid in the vagina and PPROM was confirmed by the presence of insulin-like growth factor binding proteins (ACTIM PROM test; MedixBiochemica, Kauniainen, Finland) in the vaginal fluid when necessary.

Ultrasound-guided transabdominal amniocentesis to obtain a sample of amniotic fluid (2–3 mL) was performed at admission but before the administration of antibiotics, corticosteroids and/or tocolytics. The bedside assessment of amniotic fluid IL-6 concentrations was performed from 100 µL of non-processed amniotic fluid immediately after sampling at the labor room. The remaining amniotic fluid was immediately divided into two aliquots. The first aliquot was immediately transported to the laboratory for polymerase chain reaction (PCR) testing for *Ureaplasma* species, *Mycoplasma hominis* and *Chlamydia trachomatis*, as well as for evaluating the 16S rRNA content. The second aliquot was centrifuged for 15 min at 2000*g* to remove cells and debris, divided into aliquots and stored at −70 °C until analysis.

This study was approved by the Institutional Review Board (July 2014; No. 201407 S14P) and informed consent was obtained from all participants. Most of the amniotic fluid samples also have been used in our previous studies [28,29].

Amniotic fluid clusterin concentrations

The amniotic fluid clusterin concentrations were determined in duplicate by a sandwich enzyme-linked immunosorbent assay technique (ELISA) using ELISA kit for Human Clusterin (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. The limit of detection of the clusterin was 0.189 ng/mL. Intra-assay and inter-assay variability for ELISA kit for Human Clusterin were 3.6% and 7.5%, respectively. Samples of amniotic fluid were diluted 1:25 and the absorbance values were read at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA).

Amniotic fluid IL-6 concentrations

The IL-6 concentrations were assessed with a lateral flow immunoassay Milenia® QuickLine IL-6 using the Milenia POCScan Reader (Milenia Biotec, GmbH, Giessen, Germany). The measurement range was 50–10 000 pg/mL. The intra-assay and inter-assay variations were 12.1% and 15.5%, respectively [30].

Identification of *Ureaplasma* species, *M. hominis*, and *C. trachomatis* in amniotic fluid

DNA was isolated from the amniotic fluid with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (using the protocol for the isolation of bacterial DNA from biological fluids). Real-time PCR was performed on a Rotor-Gene 6000 instrument (QIAGEN) with the commercial kit AmpliSens *C. trachomatis*/*Ureaplasma*/*M. hominis*-MULTIPROME-FRT (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect the DNA from *Ureaplasma* species, *M. hominis* and *C. trachomatis* in a common PCR tube. As a control, we included a PCR run for beta-actin, a housekeeping gene, to examine the presence of inhibitors of the PCR.

Identification of other bacteria in the amniotic fluid

Bacterial DNA was identified by PCR targeting the 16S rRNA gene with the following primers: 5′-CCAGACTCCTAC GGGAGGCAG-3′ (V3 region), 5′-ACATTTCACAACACG AGCTGACGA-3′ (V6 region) [31,32]. Each individual reaction contained 3 µL of target DNA, 500 nM of forward and reverse primers, and Q5 High Fidelity DNA polymerase (NEB, Ipswich, MA) in a total volume of 25 µL. The amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The products were

Table 1. Maternal and neonatal characteristics of pregnancies complicated by PPROM according to the presence or absence of MIAC and/or IAI.

Characteristic	With MIAC ($n = 39$)	Without MIAC ($n = 97$)	p value*	With IAI ($n = 24$)	Without IAI ($n = 112$)	p value†
Maternal age [years, median (range)]	30.1 ± 6.3	30.8 ± 4.7	0.53	29.1 ± 5.3	30.9 ± 5.1	0.11
Primiparous [number (%)]	16 (41%)	45 (46%)	0.70	11 (45%)	50 (45%)	1.00
Prepregnancy body mass index [kg/m^2, median (range)]	23.4 (16.2–33.7)	22.9 (16.4–38.6)	0.62	24.8 (19.6–38.6)	22.7 (16.4–37.4)	**0.03**
Smoking [number (%)]	15 (38%)	7 (7%)	**<0.0001**	10 (42%)	12 (11%)	**0.0001**
Gestational age at admission [weeks, median (range)]	32 + 5 (24 + 2–36 + 6)	34 + 2 (25 + 1–36 + 5)	0.13	32 + 0 (24 + 2–36 + 5)	34 + 2 (25 + 3–36 + 6)	0.002
Gestational age at delivery [weeks, median (range)]	33 + 0 (24 + 5–36 + 6)	34 + 5 (25 + 1–36 + 6)	0.07	32 + 1 (24 + 5–36 + 5)	34 + 5 (26 + 5–36 + 6)	**0.001**
Interval from PPROM to amniocentesis [hours, median (range)]	5 (1–72)	5 (1–432)	0.99	6 (1–432)	5 (1–68)	0.31
Latency from amniocentesis to delivery [hours, median (range)]	29 (3–390)	31 (4–768)	0.30	40 (3–768)	29 (4–452)	0.86
CRP levels at admission [mg/L, median (range)]	7.8 (0–105.7)	5.4 (1.0–39.5)	**0.04**	12.0 (2.4–106.8)	4.9 (0–47.7)	**<0.0001**
WBC count at admission [$\times 10^9$ L, median (range)]	12.6 (7.3–22.2)	11.4 (6.3–24.8)	0.07	14.7 (9.9–20.5)	11.4 (6.3–24.8)	**<0.0001**
Amniotic fluid IL-6 at admission [pg/mL, median (range)]	632 (50–10 000)	220 (50–1446)	**<0.0001**	2668 (831–10 000)	205 (50–678)	**<0.0001**
Administration of antibiotics [number (%)]	38 (97%)	93 (96%)	1.00	24 (100%)	107 (96%)	0.59
Administration of corticosteroids [number (%)]	31 (79%)	77 (79%)	1.00	21 (88%)	87 (78%)	0.41
Vaginal delivery [number (%)]	31 (79%)	74 (76%)	0.82	20 (83%)	85 (76%)	0.59
Cesarean delivery [number (%)]	8 (21%)	23 (24%)	0.82	4 (17%)	27 (24%)	0.59
Birth weight [g, median (range)]	2016 ± 597	2253 ± 519	**0.02**	1893 ± 685	2248 ± 499	**0.02**
Histological chorioamnionitis [number (%)]	32 (82%)	49 (51%)	**0.001**	22 (92%)	59 (53%)	**<0.0001**
Funisitis [number (%)]	19 (49%)	18 (19%)	**0.001**	12 (50%)	25 (22%)	**0.01**
Apgar score <7; 5 min [number (%)]	1 (3%)	1 (1%)	0.49	1 (4%)	1 (1%)	0.32
Apgar score <7; 10 min [number (%)]	0 (0%)	1 (1%)	1.00	1 (4%)	0 (0%)	0.18

PPROM, preterm prelabor rupture of membranes; MIAC, microbial invasion of the amniotic cavity; IAI, intra-amniotic inflammation; CRP, C-reactive protein; WBC, white blood cells; IL, interleukin. Continuous variables were compared using a nonparametric Mann–Whitney U test. Categorical variables were compared using Fisher's exact test. Statistically significant results are marked in bold. Continuous variables are presented as median (range) and categorical as number (%).

*p value – a comparison between women with and without MIAC.

†p-value – a comparison between women with and without intra-amniotic inflammation.

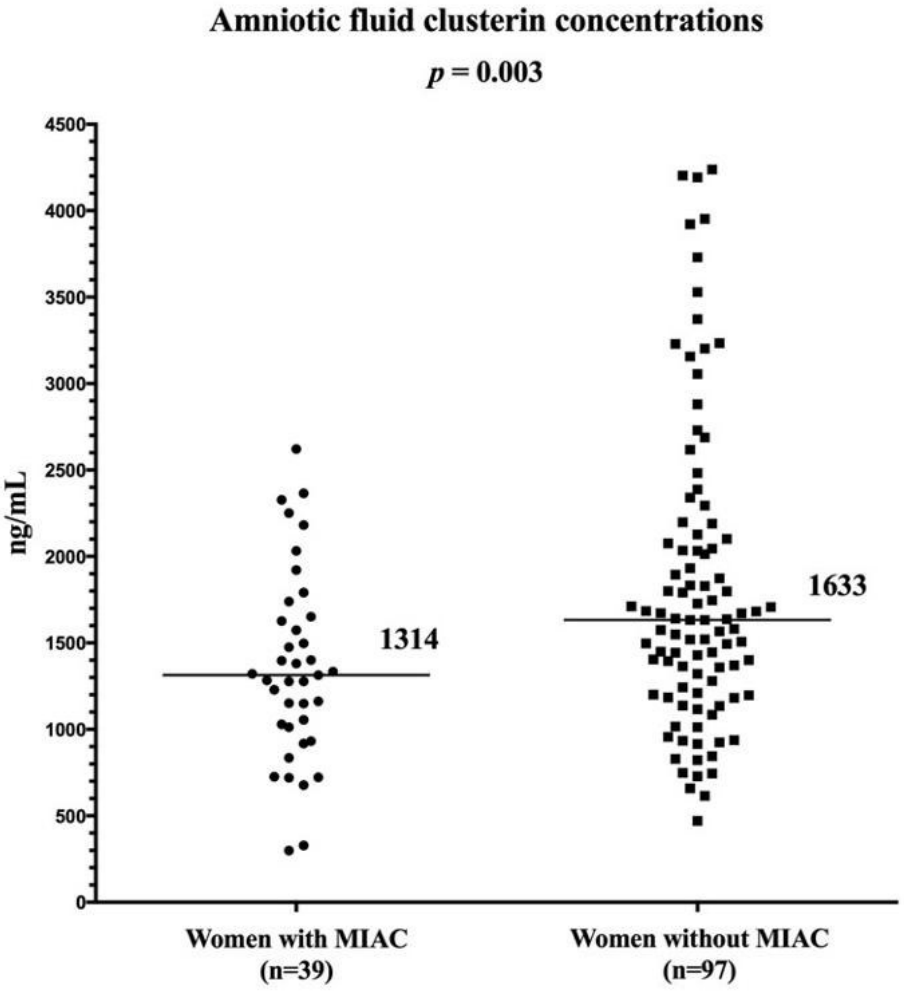

Figure 1. Amniotic fluid clusterin concentrations with respect to the presence or absence of MIAC. Abbreviation: MIAC, microbial invasion of the amniotic cavity.

visualized on an agarose gel. Positive reactions yielded products of 950 bp, which were subsequently analyzed by sequencing. The 16S PCR products were cleaned and used in sequencing PCR reactions utilizing the above primers and the BigDye Terminator kit, version 3.1 (Thermo Fisher Scientific, Waltham, MA). The bacteria were then typed using the sequences obtained in BLAST and SepsiTest BLAST.

Definition of IAI

IAI in PPROM pregnancies was determined as amniotic fluid bedside IL-6 concentrations $\geq$745 pg/mL [33,34].

Diagnosis of MIAC

MIAC was defined based on a positive PCR analysis for *Ureaplasma* species, *M. hominis* and/or for *C. trachomatis* and/or by positivity for the 16S rRNA gene.

Diagnosis of microbial-associated IAI

Microbial-associated IAI was defined as the presence of both MIAC and IAI.

Statistical analyses

The demographic and clinical characteristics were compared using a nonparametric Mann–Whitney *U* test for continuous variables and are presented as median values (range). Categorical variables were compared using Fisher's exact test and are presented as numbers (%). The normality of the data was tested using the D'Agostino–Pearson omnibus normality test and the Shapiro–Wilk test. Because the amniotic fluid concentrations of clusterin were not normally distributed, non-parametric test (Mann–Whitney *U*-test) was used for analyses and presented as median values [interquartile range (IQR)]. A Spearman partial correlation was performed to adjust for gestational age at sampling. A Spearman correlation was used to assess an association between amniotic fluid clusterin concentrations and gestational age at sampling in women with PPROM. Differences were considered significant at $p<0.05$. All p values were obtained from two-sided tests and all statistical analyses were performed using GraphPad Prism 6 for Mac OS X (GraphPad Software, San Diego, CA) or the SPSS 19.0 statistical package for Mac OS X (SPSS Inc., Chicago, IL).

Results

A total of 141 women with pregnancies complicated by PPROM at gestational ages between 24 + 0 and 36 + 6 weeks were recruited. Five women were excluded from the study owing to pregestational diabetes mellitus ($n=1$), preeclampsia ($n=1$), gestational hypertension ($n=1$) and fetal growth restriction ($n=2$). Finally, 136 women were included in the study. The presence of MIAC and IAI were found in 29%

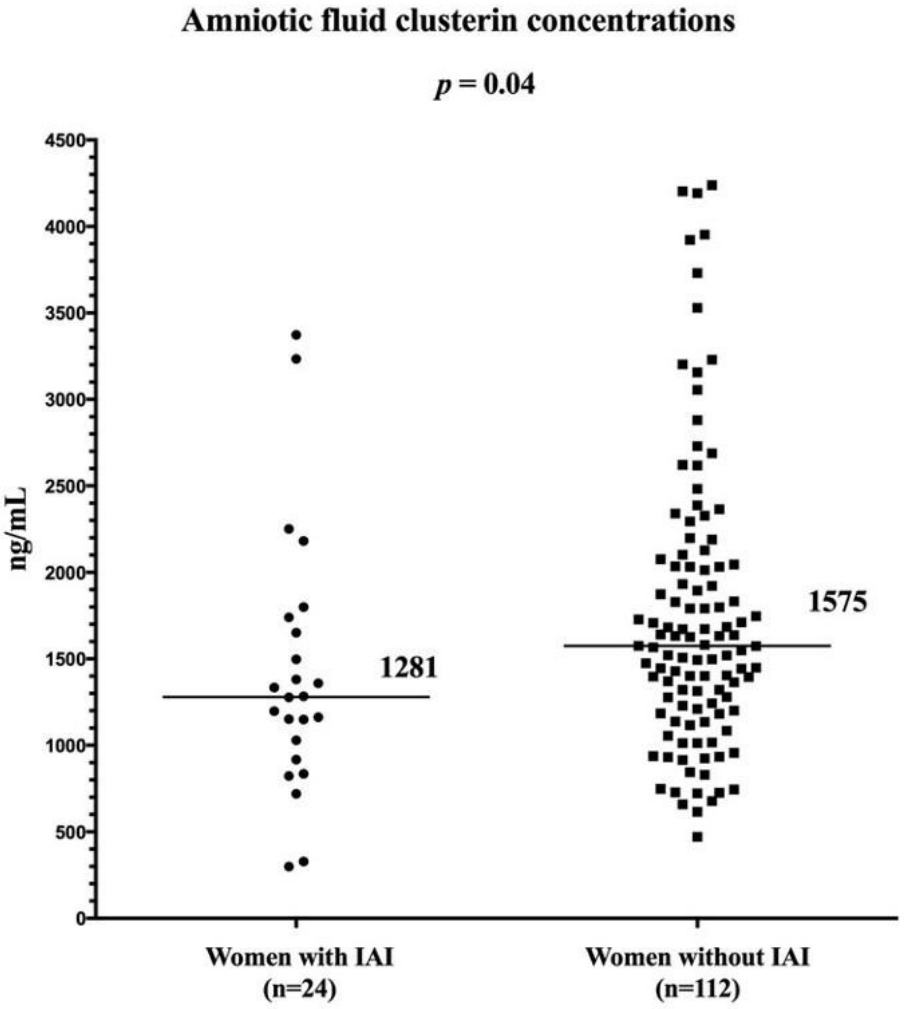

Figure 2. Amniotic fluid clusterin concentrations with respect to the presence or absence of IAI. Abbreviation: IAI, intra-amniotic inflammation.

(39/136) and 18% (24/136) of women, respectively. The women's demographics and clinical data are presented in Table 1. The presence of genital mycoplasmas (*Ureaplasma* species and/or *M. hominis*) was the most common cause of MIAC ($n = 32$). Polymicrobial finding was found in 18% (7/39) of women with MIAC (*Peptostreptococcus* species + *Propionibacterium* species + *Bacteroides* species 1×; *Ureaplasma* species + *M. hominis* 2×; *Ureaplasma* species + *C. trachomatis* 2×; *Ureaplasma* species + *Sneathia sanguinegens* 1× and *Ureaplasma* species + *Leptotrichia amnionii* 1×). All women were self-reported as Caucasians.

Amniotic fluid clusterin concentrations according to the presence of MIAC

The concentration of clusterin in the amniotic fluid was lower in women with MIAC than in women without MIAC in crude analysis (with MIAC: median 1314 ng/mL, IQR 1013–1651 versus without MIAC: median 1633 ng/mL, IQR 1206–2115; $p = 0.003$; Figure 1) and in adjusted analysis for gestational age at sampling ($p < 0.0001$).

Amniotic fluid clusterin concentrations according to the presence of IAI

Clusterin concentrations in the amniotic fluid were lower in women with IAI than in women without IAI in crude analysis (with IAI: median 1281 ng/mL, IQR 946–1718 versus without IAI: median 1575 ng/mL, IQR 1204–2043; $p = 0.04$; Figure 2), as well as after the adjustment for gestational age at sampling ($p = 0.001$).

Amniotic fluid clusterin concentrations according to the presence of microbial-associated IAI

The women with microbial-associated IAI had lower clusterin concentrations in amniotic fluid than women without microbial-associated IAI (with microbial-associated IAI: median 1220 ng/mL, IQR 898–1536 versus microbial-associated IAI: median 1575 ng/mL, IQR 1200–2053; $p = 0.008$), as well as after the adjustment for gestational age at sampling ($p < 0.0001$) (Figure 3).

Amniotic fluid clusterin concentrations according to gestational age at sampling

A weak negative correlation between amniotic fluid clusterin concentrations and gestational age at sampling was identified (rho= –0.30; $p = 0.0005$; Figure 4), as well as after adjustment for the presence of MIAC, IAI and microbial-associated IAI (rho= –0.38; $p < 0.0001$).

Discussion

Clusterin has been considered an enigmatic protein. Since clusterin was discovered more than 30 years ago, as a protein

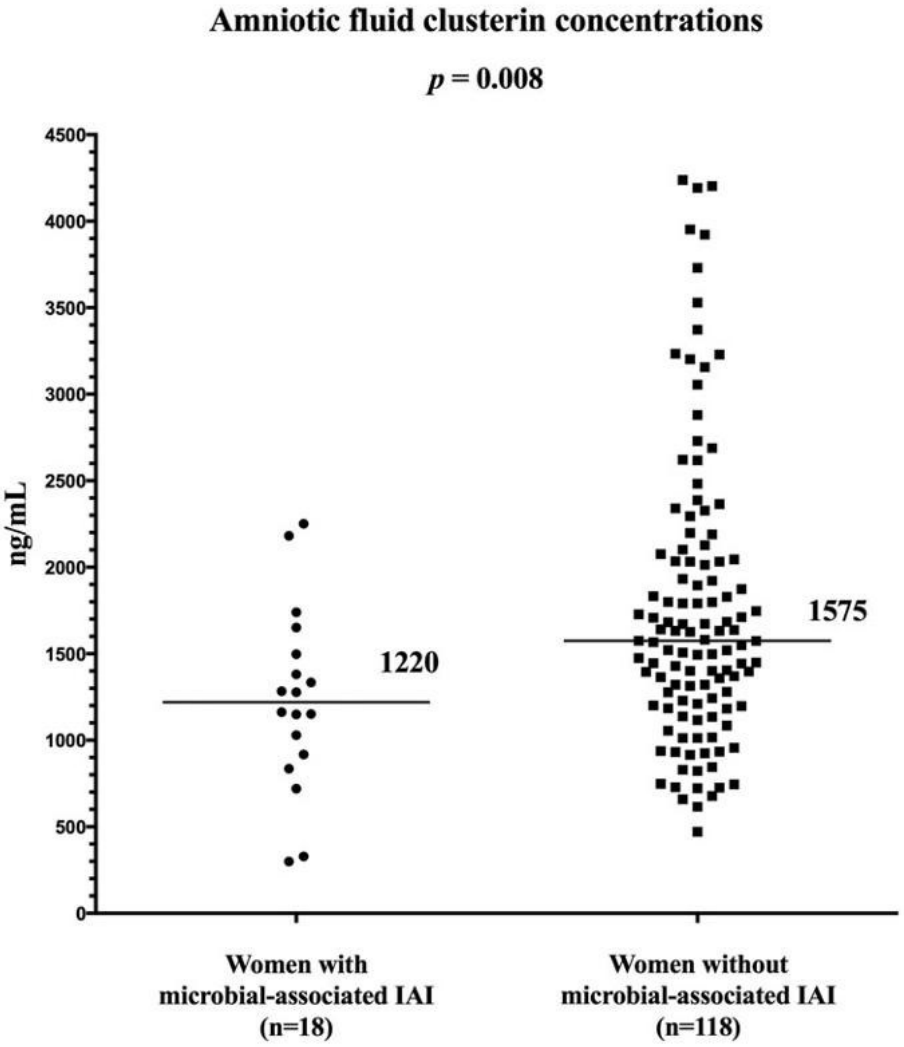

Figure 3. Amniotic fluid clusterin concentrations with respect to the presence of microbial-associated IAI. Abbreviation: IAI, intra-amniotic inflammation.

causing clustering of erythrocytes, the exact clarification of its biological roles has yet to be revealed [17,35]. However, insight into amniotic fluid clusterin may have the potential to increase our understanding of infectious-related and inflammatory complications of PPROM. The principal findings of this study are as follows: (i) clusterin is a constituent of amniotic fluid from PPROM pregnancies; (ii) women with MIAC have lower amniotic fluid clusterin concentrations than women without MIAC; (iii) women with IAI have lower amniotic fluid clusterin concentrations than women without IAI; (iv) women with microbial-associated IAI have lower amniotic fluid clusterin concentrations than women without microbial-associated IAI.

Clusterin is present in almost all human tissues, in most organs, with the highest concentrations in the testis, epididymis, liver, stomach and brain [21,22,36]. In addition, clusterin is shown to be a major protein in physiologic body fluid including plasma, urine, breast milk, semen and cerebrospinal fluid [35,36]. In this study, we found relatively high concentrations of clusterin (compared to previously evaluated concentrations of different mediators in amniotic fluid) in all samples from women with PPROM. Amniotic fluid clusterin concentrations were about 100-fold lower than reported plasma concentrations from healthy uncomplicated pregnancies [25].

As expected, based on the result from our previous proteomic study, women with MIAC had lower clusterin concentrations in the amniotic fluid than women without MIAC [16]. Reasons for the lower amniotic fluid clusterin concentrations in women with MIAC are unclear but some may be speculated because of clusterin's role as a complement inhibitor. Complement system, a part of the innate immune system, enhances the ability of antibodies and phagocytic cells to clear microorganisms and damaged cells from an organism. Complement is a complex system of functionally related proteins that are sequentially activated in a tightly orchestrated cascade. When complement activity is not properly controlled, the complement cascade is further amplified and, in consequence, toxic effectors are generated [37,38]. Clusterin inhibits complement with an ultimate goal of protecting cells or tissue from the terminal complement complex damage [18,19,39]. The terminal complement complex is a structure formed on the surface of bacterial cells or targeted cells as a result of the activation of the complement system. The terminal complement complex forms transmembrane channels and leads to cytolysis and cell death. Clusterin inhibits the disruption of the cell membranes by binding to complement proteins C5b and C6 to prevent formation of the terminal complement complex [18,19,39,40]. In many ways, clusterin's complement inhibition activity resembles another

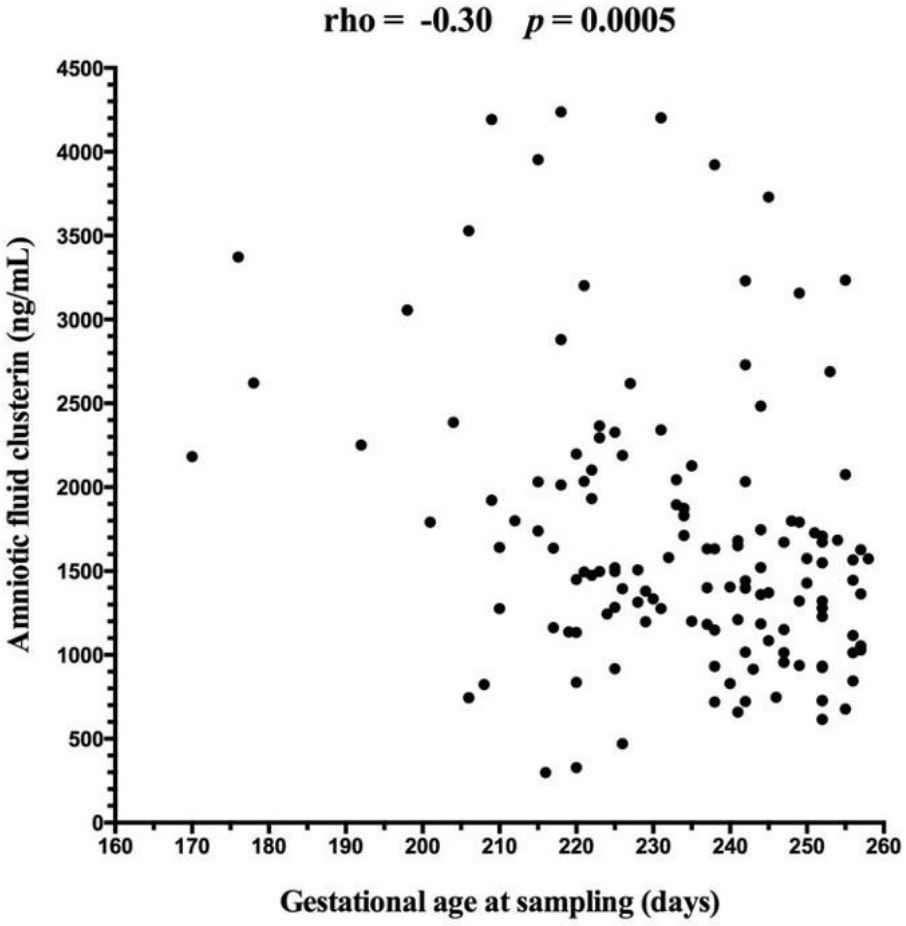

Figure 4. Correlation between gestational age at sampling and amniotic fluid clusterin concentrations.

protein named vitronectin [37,41]. Interestingly, in our previous proteomic study, we found lower amniotic fluid vitronectin concentrations in women with both MIAC and histological chorioamnionitis compared to women without these complications [16]. In addition, as every microorganism is recognized and attacked by immune system, successful microorganisms have developed strategies how to survive in immunocompetent human organisms and how to become ''immunologically invisible'' [42,43]. One of these strategies is that many pathogenic microorganisms bind the specific proteins (e.g. clusterin, vitronectin) on their surfaces and subsequently exploit these proteins for immune escape [37,41]. Therefore, we hypothesize that lower amniotic fluid clusterin concentrations in women with MIAC seem to be a result of clusterin depletion due to its role in the inhibition of the complement activity. Nevertheless, further studies of the role of amniotic fluid clusterin in MIAC may shed some light on the pathophysiology of these PPROM complications.

Since the majority of women with IAI in this study had a microbial-associated IAI (75% [18/24]), not surprisingly, the presence of IAI and microbial-associated IAI was related to lower amniotic fluid clusterin concentrations. This finding suggests that the role of clusterin is more pronounced when the presence of bacteria in the amniotic fluid leads to development of intra-amniotic inflammatory response.

The amniotic fluid concentrations of many mediators may differ across the course of pregnancy. So far, no data about the dynamics of amniotic fluid clusterin throughout the normal uncomplicated pregnancy have been published. In this study, we found that amniotic fluid concentrations of clusterin decreased with an advanced gestational age at sampling in women with PPROM. Nevertheless, we have to take into consideration that PPROM represents a pregnancy pathology and this result may not be translated into healthy pregnancies.

One of the strengths of our study is that we evaluated amniotic fluid clusterin concentrations in a specific phenotype of spontaneous preterm delivery (PPROM). There are no previous references in the literature describing the association between amniotic clusterin concentrations with respect to MIAC and IAI in this subtype of preterm delivery. The next strength of this study is a completely non-cultivation approach to identifying MIAC combining both specific (genital mycoplasma and *C. trachomatis*) and non-specific (16S rRNA) PCRs. This study was also subject to some limitations. One of the limitations of this study is that it was performed at a single institution, which prevented the use of a larger sample size. The second limitation is that the analyses were based on a relatively small sample size and a type II error might affect our results and could not be ruled out. The third limitation is that we did not evaluate amniotic fluid clusterin concentrations in women with spontaneous preterm delivery with intact membranes or in women with uncomplicated, healthy pregnancies. The fourth limitation was that an expectant management in the majority of recruited women prevented us from the evaluation of an association between clusterin concentrations in the amniotic fluid and histological chorioamnionitis because of a long latency between amniotic fluid

sampling and delivery. Finally, the exact source of amniotic fluid clusterin remains unclear, yet it was beyond the scope of this study. We can only speculate on the origin of clusterin in the amniotic fluid, but it is likely to have been released from fetal membranes and the placenta.

In conclusion, the presence of MIAC, IAI and microbial-associated IAI is associated with lower amniotic fluid clusterin concentrations. Amniotic fluid clusterin concentrations decreased with advanced gestational age in women with PPROM independently on the presence of MIAC, IAI and microbial-associated IAI.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Příloha č. 5: Amniotic fluid CD11b levels in pregnancies complicated by preterm prelabor rupture of membranes

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ORIGINAL ARTICLE

Amniotic fluid CD11b levels in pregnancies complicated by preterm prelabor rupture of membranes

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ABSTRACT

Objective: CD11b is an integrin molecule located on the surface of leukocytes. CD11b is involved in the processes of cell adhesion and migration. Expression of CD11b increases during inflammation. Therefore, this study was aimed at the evaluation of concentrations of CD11b in the amniotic fluid from pregnancies complicated by preterm prelabor rupture of the membranes (PPROM), with respect to the presence of microbial invasion of the amniotic cavity (MIAC), intra-amniotic inflammation (IAI), and microbial-associated IAI (the presence of both MIAC and IAI).

Methods: Eighty women with singleton pregnancies complicated by PPROM were included. Amniotic fluid samples were obtained by transabdominal amniocentesis. Amniotic fluid CD11b concentrations were determined by enzyme-linked immunosorbent assay. MIAC was determined by a non-cultivation approach. IAI was defined by a bedside amniotic fluid interleukin-6 concentration $\geq$745 pg/mL.

Result: Women with MIAC or microbial-associated IAI had higher CD11b concentrations in the amniotic fluid than women without these complications (with MIAC: median 0.31 ng/mL versus without MIAC: median 0.17 ng/mL, $p = .001$; with microbial associated-IAI: median 0.35 ng/mL versus without microbial-associated IAI: median 0.16 ng/mL; $p = .02$). The presence of IAI was not associated with elevated CD11b concentrations. A weak negative correlation was found between amniotic fluid CD11b concentrations and interleukin-6 concentrations (rho = 0.26; $p = .02$).

Conclusions: MIAC and microbial-associated IAI are characterized by higher amniotic fluid CD11b concentrations in pregnancies complicated by PPROM.

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Introduction

Preterm prelabor rupture of the membranes (PPROM) is defined as rupture of fetal membranes prior to 37 weeks of gestation [1]. It complicates about 2–4% of all pregnancies and is responsible for 40% of all spontaneous preterm births [2,3]. PPROM development is driven by a complex of pathophysiological changes that lead to oxidative stress and inflammation [4]. However, social, behavioral, and demographic factors also contribute to the development of PPROM [1]. Intra-amniotic inflammation, which is involved in the etiology of PPROM, may be associated with microbial infection (non-sterile IAI), but may also occur as sterile IAI. Sterile IAI is triggered by substances released from apoptotic and damaged cells called damage-associated molecular patterns (DAMPs) or alarmins. Non-sterile IAI is caused by bacterial colonization, which is not an inflammation itself, but activates the immune system into a pro-inflammatory setting and may lead to non-sterile inflammation [5]. Identifying a suitable marker from available biological material that would quickly predict the onset of inflammatory changes leading to PPROM would facilitate care for women at risk of premature birth.

The CD11b protein, also known as integrin alpha M (ITGAM), together with the CD18 molecule creates the heterodimeric alpha-M beta-2 complex ($\alpha M\beta 2$), also known as complement receptor 3 (CR3) or macrophage-1 antigen (Mac-1). The CD11b molecule is expressed on monocytes, macrophages, granulocytes, NK cells, and subsets of dendritic cells [6] and plays a role in inflammation, especially in leukocyte adhesion and migration. CD11b alone mediates the binding of

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integrin to numerous substrates, such as fibrinogen, Factor X, IC3b, or ICAM-1 [7]. This protein also mediates phagocytosis by binding the inactivated complement component C3b [8]. However, stimulation of lymphocytes *via* antiCD3 and IL-2 also induces CD11b expression on T cells [9]. CD11b deficiency is associated with impaired adhesion to the vascular endothelium, and is also associated with neutrophil accumulation in the extravascular space and impaired apoptosis of granulocytes [10]. On the other hand, increased expression of CD11b on monocytes and neutrophils promotes their adhesion to endothelial cells, extracellular matrix, and smooth muscle cells [11]. Proper regulation of CD11b expression can therefore be considered a homeostatic mechanism controlling inflammation [10]. Decreased CD11b expression is also associated with a worsened prognosis or increased mortality in infectious diseases [12–15]. Most CD11b molecules in neutrophils are not expressed on the membrane, but are stored in specific granules [16] and their expression on the membrane and release is upregulated in response to inflammatory conditions [17–20]. The growing importance of this molecule in the inflammatory process is also demonstrated by recent efforts to therapeutically affect CD11b expression in activated platelets, monocytes, and granulocytes [11]. We previously obtained relevant data on the effects of increased levels of other neutrophil-specific molecules, such as serine protease cathepsin-G, in patients with PPROM [5].

The main aim of this study was to evaluate CD11b concentrations in the amniotic fluid in pregnancies complicated by PPROM, with respect to the presence of microbial invasion of the amniotic cavity (MIAC), intra-amniotic inflammation (IAI), and microbial-associated IAI (the presence of both MIAC and IAI).

Materials and methods

A retrospective cohort study of women with PPROM-complicated pregnancies between gestational age 24 + 0 and 36 + 6 weeks admitted to the Department of Obstetrics and Gynecology of the University Hospital Hradec Kralove in the Czech Republic was conducted between November 2014 and March 2016. This study was approved by the Institutional Review Board (July 2014; No. 201407 S14P) and informed consent was obtained from all participants. All women self-reported as Caucasians. Only women with singleton pregnancies and a maternal age $\geq$18 years were recruited to the study. Women with pregnancy complications, such as preeclampsia, gestational hypertension, gestational or pre-gestational diabetes, fetal growth restriction, the presence of either chromosomal or structural fetal abnormalities, signs of fetal hypoxia, or significant vaginal bleeding were excluded from the study. Gestational ages were established by first-trimester fetal biometry. Women with PPROM at less than 34 weeks of gestation were treated with tocolytics for 48 h, antibiotics and corticosteroids to accelerate lung maturation, whereas no treatment beyond antibiotics was initiated to delay delivery in women with PPROM after 34 weeks. Women with proven microbial-associated IAI beyond 28 gestational weeks did not receive tocolytics and were only treated with corticosteroids and antibiotics. Furthermore, labor was induced or a cesarean section was performed 24 h after the final corticosteroid treatment. The remaining women were managed conservatively. PPROM was diagnosed by examination with a sterile speculum to verify amniotic fluid pooling in the vagina, and PPROM was confirmed by the presence of insulin-like growth factor binding proteins (ACTIM PROM test; MedixBiochemica, Kauniainen, Finland) in the vaginal fluid when necessary. Ultrasound-guided transabdominal amniocentesis was used to sample amniotic fluid (2–3 ml) at the time of admission, but prior to the administration of antibiotics, corticosteroids, and/or tocolytics. The bedside assessment of amniotic fluid IL-6 concentrations was performed on 100 ml of non-processed amniotic fluid immediately after sampling at the labor room. The remaining amniotic fluid was immediately divided into two aliquots. The first aliquot was immediately transported to the laboratory for polymerase chain reaction (PCR) testing for the presence of *Ureaplasma* species, *Mycoplasma hominis*, and *Chlamydia trachomatis*, and for evaluating the 16S rRNA content. The second aliquot was centrifuged for 15 min at 2000 g to remove cells and debris, divided into aliquots, and stored at −70 °C for analysis. Amniotic fluid CD11b concentrations were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) using an ELISA kit for Human ITGAM/CD11b (LifeSpan BioSciences, Inc., Seattle, WA) according to the manufacturer's instructions. The detection range of the CD11b ELISA kit was 0.156–20 ng/mL. Intra-assay and inter-assay variability for the ELISA kit for Human CD11b were $<$5.1% and $<$10.2%, respectively. Amniotic fluid sample was not diluted and the absorbance values were read at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA).

Amniotic fluid IL-6 concentrations

The IL-6 concentrations were assessed with the Milenia® QuickLine IL-6 lateral flow immunoassay using the Milenia POCS can Reader (Milenia Biotec, GmbH, Giessen, Germany). The measurement range was 50–10,000 pg/mL. The intra-assay and inter-assay variations were 12.1% and 15.5%, respectively [21].

Identification of Ureaplasma *species,* Mycoplasma hominis, *and* Chlamydia trachomatis *in amniotic fluid*

DNA was isolated from the amniotic fluid using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (using the protocol for the isolation of bacterial DNA from biological fluids). Real-time PCR was performed on a Rotor-Gene 6000 instrument (QIAGEN) using the commercial kit AmpliSens *C. trachomatis/Ureaplasma/M. hominis*-MULTIPROME-FRT (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect DNA from *Ureaplasma* species, *Mycoplasma hominis*, and *Chlamydia trachomatis* in a common PCR tube. PCR for beta-acting, a housekeeping gene, was used as a control to examine the presence of PCR inhibitors in each sample. Other bacteria in the amniotic fluid were identified by PCR targeting the 16S rRNA gene with the following primers: 5′-CCAGACTCCTACGGGAGGCAG-3′ (V3 region), 5′-ACATTTCACAACACG AGCTGACGA-3′ (V6 region) [22,23]. Each individual reaction contained 3 μL of target DNA, 500 nM of forward and reverse primers, and Q5 High Fidelity DNA polymerase (NEB, Ipswich, MA) in a total volume of 25 μL. Amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). PCR products were visualized on an agarose gel. Positive reactions yielded products of 950 bp, which were subsequently analyzed by sequencing. The 16S PCR products were cleaned and used in sequencing PCR reactions using the above primers and the BigDye Terminator kit, version 3.1 (Thermo Fisher Scientific, Waltham, MA). Bacteria were then identified using the sequences obtained in BLAST and SepsiTest BLAST.

Definition of IAI

IAI in PPROM pregnancies was determined as having amniotic fluid IL-6 concentrations $\geq$745 pg/mL [24,25].

Diagnosis of MIAC

MIAC was defined based on a positive PCR result for *Ureaplasma* species, *Mycoplasma hominis*, and/or for *Chlamydia trachomatis*, and/or by positive 16S rRNA amplification.

Diagnosis of microbial-associated IAI

Microbial-associated IAI was defined as the presence of both MIAC and IAI.

Statistical analyses

Demographic and clinical characteristics were compared using a nonparametric Mann–Whitney *U* test for continuous variables and are presented as median values (range). The normality of the data was tested using the D'Agostino–Pearson omnibus normality test and the Shapiro Wilk test. Because the amniotic fluid concentrations of CD11b were not normally distributed, a non-parametric test (Mann–Whitney *U*-test) was used for analyses and results are presented as median values [interquartile range (IQR)]. A Spearman partial correlation was performed to adjust for gestational age at sampling. A Spearman correlation was used to assess the association between amniotic fluid CD11b concentrations and gestational age at sampling in women with PPROM. Differences were considered significant at $p < .05$. All p values were obtained from two-sided tests and all statistical analyses were performed using GraphPad Prism 8 for Windows OS (GraphPad Software, San Diego, CA).

Results

A total of 80 women with PPROM were enrolled during the study period. MIAC, IAI, and microbial-associated IAI were identified in 35% (28/80), 33% (26/80), and 21% (17/80) of women, respectively. *Ureaplasma* species were the most common microorganisms in women with MIAC and were detected in 61% of women (17/28). Polymicrobial infections were identified in 29% (8/28) of women with MIAC. Demographic and clinical data are shown in Table 1

Amniotic fluid CD11b concentrations in the presence of MIAC

CD11b concentrations in the amniotic fluid were higher in women with MIAC than in women without MIAC based on crude analysis (with MIAC: median 0.31 ng/ml, range 0.07–5.19, versus without MIAC: median 0.17 ng/ml, range 0.001–0.89; $p = .004$; Figure 1), as well as after adjusting for gestational age at sampling ($p = .001$).

Table 1. Maternal and clinical characteristics of pregnancies complicated by preterm prelabor rupture of membranes with respect to the presence and the absence of microbial invasion of the amniotic cavity and intra-amniotic inflammation.

Characteristic	Women with MIAC ($n = 28$)	Women without MIAC ($n = 52$)	*p*-value	Women with IAI ($n = 26$)	Women without IAI ($n = 54$)	*p*-value
Maternal age [years, median (IQR)]	32 (25–34)	31 (27–35)	0.94	33 (26.3–34.3)	30 (26–34.3)	0.55
Primiparous [number (%)]	13 (46%)	24 (46%)	0.98	11 (42%)	26 (48%)	0.62
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	23.1 (21.9–26.3)	23.2 (19.9–26)	0.46	23.1 (21.8–26.5)	23.2 (19.7–25.9)	0.24
Smoking [number (%)]	6 (21%)	12 (23%)	**0.03**	8 (31%)	10 (19%)	0.22
Interval between PPROM and amniocentesis [hours, median (IQR)]	6 (3–12)	7.5 (4–12.8)	0.54	6.5 (3–11.8)	7 (4–12.3)	0.58
Gestational age at admission [weeks, median (IQR)]	33 + 6 (31 + 5–35 + 4)	33 + 6 (31 + 3–35 + 1)	0.88	33 + 0 (31 + 2–34 + 4)	34 + 0 (32 + 6–35 + 2)	**0.05**
Gestational age at delivery [weeks, median (IQR)]	33 + 6 (32 + 0–35 + 4)	34 + 0 (32 + 1–35 + 2)	0.98	33 + 1 (31 + 5–34 + 5)	34 + 2 (33 + 1–35 + 3)	**0.04**
Latency between PPROM and delivery [hours, median (IQR)]	24 (0–66)	24 (6–72)	0.23	24 (0–72)	24 (0–72)	0.99
Amniotic fluid IL-6 levels at admission [pg/nL, median (IQR)]	4824 (765–8099)	793 (502.8–1955)	**0.0003**	6406 (4069–8576)	672 (408–1036)	**<0.0001**
CRP levels at admission [mg/L, median (IQR)]	8.1 (3.7–20.2)	5.8 (2.3–9.5)	0.09	12.3 (4.8–21.8)	5.3 (2.2–8.7)	**0.0009**
WBC count at admission [$\times 10^9$ L, median (IQR)]	13.8 (11.3–16.4)	11.9 (9.6–14.1)	**0.009**	13.8 (11–16.6)	11.9 (10.2–14.6)	**0.04**
Administration of antibiotics [number (%)]	28 (100%)	51 (98%)	0.46	26 (100%)	53 (98%)	0.49
Administration of corticosteroids [number (%)]	15 (54%)	29 (56%)	0.85	18 (69%)	26 (48%)	0.08
Spontaneous vaginal delivery [number (%)]	21 (75%)	37 (71%)	0.71	16 (62%)	42 (78%)	0.13
Cesarean delivery [number (%)]	7 (25%)	15 (29%)	0.71	10 (39%)	12 (22%)	0.13
Forceps delivery [number (%)]	0 (0%)	0 (0%)	0.99	0 (0%)	0 (0%)	0.99
Birth weight [grams, median (IQR)]	1950 (1735–2383)	2185 (1770–2485)	0.45	1895 (1358–2415)	2185 (1850–2483)	0.08
Apgar score <7; 5 minutes [number (%)]	1 (4%)	1 (2%)	0.65	2 (8%)	0 (0%)	**0.04**
Apgar score <7; 10 minutes [number (%)]	0 (0%)	1 (2%)	0.17	1 (4%)	0 (0%)	0.15

CRP: C-reactive protein; IQR: interquartile range; PPROM: preterm prelabor rupture of membranes; WBC: white blood cells. Continuous variables were compared using a nonparametric Mann–Whitney *U* test. Categorical variables were compared using the Fisher's exact test. Continuous variables are presented as median (IQR) and categorical as number (%). Statistically significant results are marked in bold.

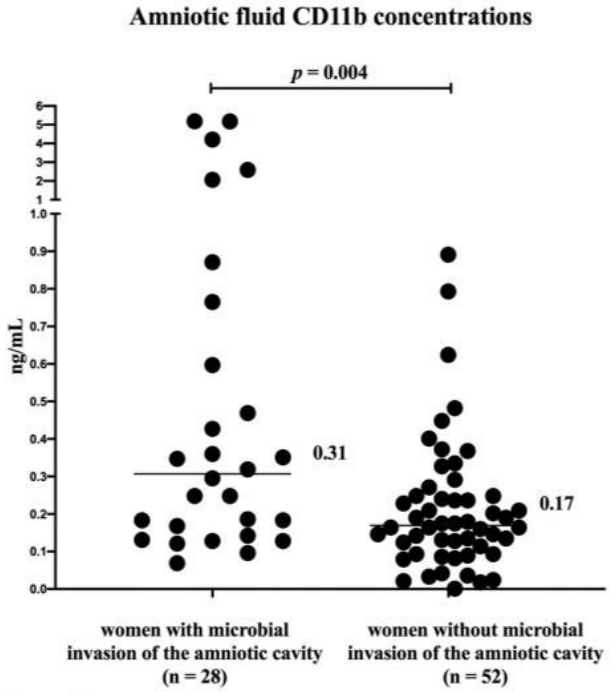

Figure 1. Amniotic fluid CD11b levels in relation to the presence or absence of microbial invasion of the amniotic cavity.

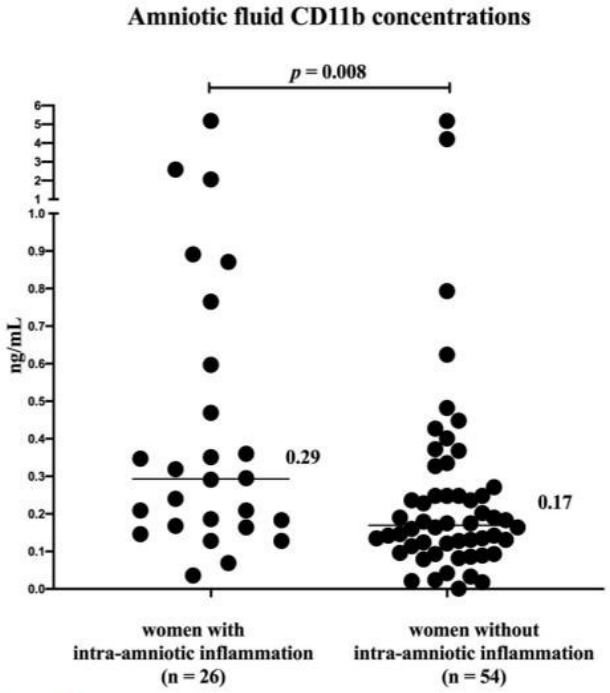

Figure 2. Amniotic fluid CD11b levels in relation to the presence or absence of intra-amniotic inflammation.

Amniotic fluid CD11b concentrations in the presence of IAI

CD11b concentrations in the amniotic fluid were higher in women with IAI than in women without IAI based on crude analysis (with IAI: median 0.29 ng/ml, range 0.04–5.19 versus without IAI: median 0.17 ng/ml, range 0.001–5.1; $p = .008$; Figure 2), but were not higher after adjusting for gestational age at sampling ($p = .37$).

Amniotic fluid CD11b concentrations in the presence of microbial-associated IAI

The presence of microbial-associated IAI was associated with higher amniotic fluid CD11b concentrations in the crude analysis (with microbial-associated IAI: median 0.35 ng/ml, range 0.07–5.19 versus without microbial-associated IAI: median 0.18 ng/ml, range 0.001–5.17; $p = .001$; Figure 3), as well as after adjusting for gestational age at sampling ($p = .04$).

Correlation between CD11b and IL-6 concentrations in the amniotic fluid

A weak positive correlation between amniotic fluid CD11b levels and IL-6 concentrations was found (rho = 0.26; $p = .02$; Figure 4).

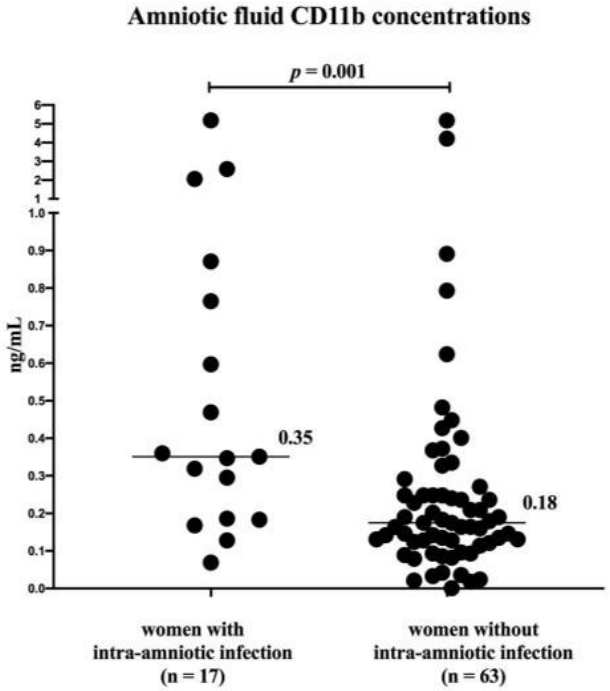

Figure 3. Amniotic fluid CD11b levels in relation to the presence or absence of microbial-associated IAI (intra-amniotic infection).

Discussion

This study aimed to detect the concentration of CD11b in the amniotic fluid of patients with PPROM. The principal findings of this study are as follows: (i)

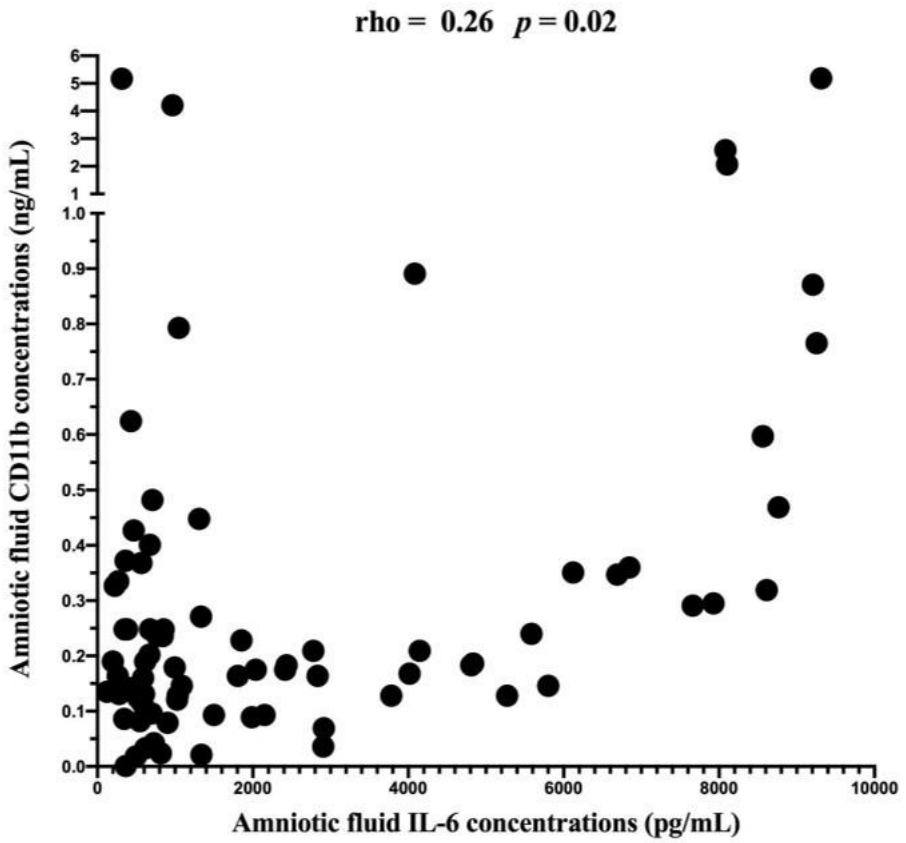

Figure 4. A correlation between CD11b and interleukin-6 levels in amniotic fluid.

women with MIAC have higher amniotic fluid CD11b concentrations than women without MIAC; (ii) women with IAI do not have higher amniotic fluid CD11b concentrations as compared to women without IAI; (iii) women with microbial-associated IAI have higher amniotic fluid CD11b concentrations than women without these complications; (iv) there is a weak correlation between amniotic fluid CD11b concentration and IL-6 level.

We consider neutrophils and monocytes the principal source of CD11b in amniotic fluid. Therefore, CD11b concentrations in the amniotic fluid may be increased in response to inflammation accompanied by neutrophils and monocytes accumulating at the site of amniotic fluid origin. MIAC occurs either in the presence of IAI, or is only colonization without signs of inflammation. The highest CD11b concentration was found in women with microbial-associated IAI.

The differences in amniotic fluid CD11b concentrations, after the adjustment for gestational age at sampling, were found just between the women with and without MIAC but not with and without IAI. It means, that the difference in the amniotic fluid concentrations CD11b between the women with and without microbial-associated IAI is mainly driven by microorganisms in amniotic fluid. Therefore, we hypothesized that IAI triggered by microbial stimulus was associated with a higher expression of CD11b on the surface of immune cells in amniotic fluid than in cases with IAI triggered by noninfectious stimulus.

This hypothesis is supported by the observation that increased CD11b expression is induced by bacterial pathogen-associated molecular patterns, such as lipopolysaccharide [26], the proinflammatory cytokine TNFα [27], granulocyte myeloperoxidase [28], platelet-activating factor, f-Met-Leu-Phe [29], granulocyte-macrophage colony-stimulating factor, IL-8, or leukotriene B4 [30]. Additionally, CD11b is considered as an effective marker for diagnosing early-onset neonatal infection [31–33]. Genel at al. also reported the effect of elevated CD11b concentrations on neutrophils and monocytes during neonate infections [34]. This finding could be consistent with the immunological observation that inflammatory pathways are unlikely to differ based on their origin or location, but that they vary according to the pattern of tissue damage. Thus, an increase in CD11b expression may be triggered by infection-associated inflammation as opposed to inflammation caused by sterile tissue damage (or damage caused by other non-bacterial stimuli) based on the mechanics of the initial noxious trigger rather than its origin. Interestingly, CD11b is further

involved in maintaining inflammation in an unprecedented manner, atypical for innate immune cells. Cross-linking of CD11b has results in the rapid expression of CD80, CD86, and HLA-DR molecules [35]. These molecules are responsible for antigen presentation and it has been assumed that their expression is limited to the antigen-presenting cells. Neutrophil RNA and proteome analyses showed that these molecules are stored in cytoplasmic granules [36]. Upon signaling through CD11b, they are fused to the cytoplasmic membrane and expressed. This model, in which the cell exposes preexisting molecules previously deposited in cytoplasmic granules on its surface, as opposed to undergoing their complete *de novo* synthesis, allows a much faster and more efficient inflammation response and was demonstrated in a study of CD64, an IgG Fc receptor, expression on neutrophils after stimulation with interferon γ [37]. CD64 expression is also increased after crosslinking of CD11b [35]. This finding is in line with our own results, which showed an increased CD64 concentration in patients with IAI (author's data, unpublished). Thus, increased CD11b expression contributes comprehensively to the activation and pro-inflammatory immune settings and promotes communication between the innate and antigen-specific components of immunity, which was previously thought to be the sole task of dendritic cells. The possible involvement of a higher level of immunity, i.e. lymphocyte-mediated defense, indicates the depth of inflammation given that different levels of immunity are involved gradually in inflammatory processes only when needed.

This is the first work evaluating CD11b in amniotic fluid obtained from women with PPROM, however, the question of how membrane-bound CD11b becomes soluble remains unanswered. Each neutrophil moves to the site of inflammation by a number of steps that involve constant binding and detachment from various substrates in the extracellular space [38]. As Zen et al. showed the detachment and shedding of CD11b from its ligands is mediated by serine proteases, including cathepsin G [39]. As mentioned above, an elevated concentration of cathepsin G has also been found in women with IAI and MIAC [5]. Therefore, it is possible that elevated levels of these granulocyte molecules under pathological conditions reflect their effort to ensure the smooth movement of these cells to the sites of infection. We also do not know the exact source of CD11b, although innate immune cells appear to be the most important producers of this protein.

In conclusion, the presence of MIAC and microbial-associated IAI is connected with increased levels of CD11b, which has a complex activating effect on inflammation. However, this was not the case for IAI. The increased presence of CD11b in the amniotic fluid points to bacterial colonization and the associated development of inflammatory changes at the maternal-fetal interface, but this does not appear to be a suitable or universal marker for IAI and PPROM prediction.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Příloha č. 6: Extracellular granzyme A in amniotic fluid is elevated in the presence of sterile intra-amniotic inflammation in preterm prelabor rupture of membranes

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ORIGINAL ARTICLE

Extracellular granzyme A in amniotic fluid is elevated in the presence of sterile intra-amniotic inflammation in preterm prelabor rupture of membranes

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ABSTRACT

Introduction: To determine the levels of granzyme A in amniotic fluid in pregnancies complicated by preterm prelabor rupture of membranes (PPROM), based on the presence of microbial invasion of the amniotic cavity (MIAC) and/or intra-amniotic inflammation (IAI).

Methods of study: A total of 166 women with singleton pregnancies complicated by PPROM were included. Amniocentesis was performed at the time of admission and assessments of MIAC (using both cultivation and non-cultivation techniques) and IAI (interleukin-6 in amniotic fluid) were performed on all subjects. Based on the presence/absence of MIAC and IAI, the women were further divided into the following subgroups: intra-amniotic infection, sterile IAI, colonization, and absence of both MIAC and IAI. Amniotic fluid granzyme A levels were assessed using ELISA.

Results: Women with MIAC had lower levels of granzyme A in the amniotic fluid than women without this condition (with MIAC: median 15.9 pg/mL vs. without MIAC: median 19.9 pg/mL, $p = .03$). Women with sterile IAI had higher amniotic fluid granzyme A levels than women with intra-amniotic infection, colonization and women with the absence of either MIAC or IAI (intra-amniotic infection: median 15.6 pg/mL; sterile IAI: median 31.8 pg/mL; colonization: median 16.9 pg/mL; absence of both MIAC and IAI: median 18.8 pg/mL; $p = .02$).

Conclusions: The presence of sterile IAI was associated with elevated levels of granzyme A in amniotic fluid.

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Adaptive immunity; intra-amniotic inflammation; microbial invasion of the amniotic cavity; preterm delivery; protease; T cells; NK cells

Introduction

Preterm prelabor rupture of membranes (PPROM), defined as leakage of amniotic fluid before the onset of regular uterine activity prior to the 37th week of pregnancy [1], is associated with approximately one-third of preterm deliveries [2]. PPROM represents a serious problem in current perinatal medicine [3,4]. The exact determination of all the underlying pathophysiological processes leading to PPROM may play the most important role in the development of optimal diagnostic and therapeutic management for women with PPROM.

There is a solid body of evidence showing that microbial invasion of the amniotic cavity (MIAC) and intra-amniotic inflammation (IAI) are conditions closely associated with PPROM [2,5,6]. In addition, the presence of these complications in PPROM pregnancies has been shown to be related to worse maternal and neonatal outcomes [7–9], regardless of the fact that gestational age at delivery is the most important confounder affecting neonatal outcomes [3].

The occurrence of IAI is typically associated with an increased number of amniotic fluid neutrophils and monocytes/macrophages, immunocompetent cells which play an important role in innate immunity in pregnancies complicated by PPROM [10] or spontaneous preterm labor [11]. Nevertheless, IAI and/or its

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microorganism-driven forms, termed intra-amniotic infections, have been shown to be associated with a higher number of B lymphocytes, T lymphocytes, and natural killer cells in the amniotic fluid [10–12]; collectively, these comprise the immunocompetent cells dealing with the adaptive immune response.

Cytotoxic subsets of T lymphocytes and natural killer cells, the immunocompetent cells that play an important role in defense against virally infected cells and tumor cells, express a family of homologous serine proteases called granzymes [13–15]. Recently, granzymes have been shown to also be produced by other immunocompetent cells such as neutrophils [16], macrophages [17], mast cells [18], and dendritic cells [19].

The family of granzymes consists of five members (A, B, H, K, and M) with granzymes A and B being the most comprehensively studied and characterized [20]. Granzyme A is the most abundant serine protease constitutively present in the granules of cytotoxic T lymphocytes [21–23]. Granzyme A, as well as the other granzymes, is stored in granules inside the immunocompetent cells, but may be released: (i) through immunological synapse into a targeted cell, leading to cell death, or (ii) extracellularly [20]. Extracellular granzyme A has been found in different body fluids including plasma, serum, umbilical cord blood [24], synovial fluid, sputum, and bronchoalveolar lavage. An elevation in the levels of extracellular granzyme A has been identified in patients with: (i) bacterial infections [25–29]; (ii) viral infections [30–35]; (iii) parasitic infection [36]; (iv) hypersensitivity pneumonia [37,38]; (v) rheumatoid arthritis [30,31,39,40]; (vi) Behçet's disease [41]; (vii) celiac disease [42]; and (viii) experimental endotoxemia or sepsis [25,43]. Extracellular granzyme A could therefore be considered as a potential biomarker of infection or inflammation [27,44–46].

Nevertheless, there is a lack of information about the occurrence of extracellular granzyme A in amniotic fluid and whether its levels reflect the presence of inflammatory intra-amniotic conditions. To fill this gap in our knowledge, we conducted a study to assess and quantify levels of extracellular granzyme A in amniotic fluid from PPROM pregnancies with respect to the presence/absence of MIAC and IAI.

Material and methods

This retrospective cohort study was performed at the Department of Obstetrics and Gynecology of the University Hospital Hradec Králové between May 2014 and June 2017. The inclusion criteria were: (i) singleton pregnancies complicated by the presence of PPROM and (ii) age $\geq$ 18 years. The exclusion criteria were as follows: (i) fetal growth restriction; (ii) congenital or chromosomal fetal abnormalities; (iii) gestational or pregestational diabetes; (iv) gestational hypertension; (v) preeclampsia; (vi) signs of fetal hypoxia; and (vii) significant vaginal bleeding.

Gestational age was determined by first-trimester fetal biometry. Women with PPROM were treated with intravenous antibiotics. Women with IAI (a concentration of bedside IL-6 $\geq$ 745 pg/mL) received clarithromycin intravenously, 500 mg every 12 h, for 7 days, unless delivery occurred. Women without IAI (a concentration of bedside IL-6 $<$ 745 pg/mL) were treated with benzylpenicillin—initially, 5.0 million IU intravenously and, further, 2.5 million IU every 6 h intravenously for 7 days, unless delivery occurred. In case of a penicillin allergy, patients were treated with clindamycin, 900 mg intravenously every 8 h for 7 days, unless delivery occurred. Once the results of MIAC were available, the antibiotic therapy was eventually modified accordingly. Women with PPROM prior to 35 weeks gestational age received a course of corticosteroids. During the study period, women with PPROM were treated conservatively, except those with proven intra-amniotic infection (the presence of both MIAC and IAI) beyond 28 weeks of gestation. The women were induced for birth, or an elective cesarean section was performed within 72 h of admission.

All the subjects were Caucasian women, and they provided written informed consent prior to the collection of amniotic fluid. The collection of amniotic fluid samples for research was approved by the Institutional Review Board (June 2015; No. 201506 I96L).

Amniotic fluid sampling

Ultrasonography-guided transabdominal amniocentesis was performed prior to the administration of corticosteroids, antibiotics, or tocolytics. The details of amniotic fluid sampling are described in our previous publication [47].

Detection of *Ureaplasma* species, M. hominis, and C. trachomatis in amniotic fluid

DNA was isolated from the amniotic fluid using a QIAamp DNA mini kit according to the manufacturer's instructions (using the protocol for isolating bacterial DNA from biological fluids). RT-PCR was performed using a Rotor-Gene 6000 instrument using the commercial AmpliSens® *C. trachomatis/Ureaplasma/M.*

hominis-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect the DNA from *Ureaplasma* species, *M. hominis*, and *C. trachomatis* in the same PCR tube. As a control, we amplified beta-actin, a housekeeping gene, to exclude the presence of PCR inhibitors. The concentration of *Ureaplasma* spp. DNA (copies/mL) was determined using an absolute quantification technique using an external calibration curve. Plasmid DNA (pCR4; Invitrogen, Carlsbad, CA) was used to construct the calibration curve.

Detection of other bacteria in amniotic fluid

Bacterial DNA was identified by PCR targeting 16S rRNA using the following primers: 5′-CCAGACTCCTACGGG AGGCAG-3′ (V3 region) and 5′-ACATTTCACAACACGAGC-GACGA-3′ (V6 region) [48]. Each reaction contained 3 μL target DNA, 500 nM forward and reverse primers, and Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) in a total volume of 25 μL. Amplification was performed using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). The products were visualized on an agarose gel. Positive reactions yielded 950-bp products that were subsequently analyzed by sequencing. The 16S PCR products were purified and sequenced by PCR using the above primers and BigDye Terminator kit v3.1 (Thermo Fisher Scientific, Waltham, MA). The bacteria were then typed using the sequences obtained from BLAST® and SepsiTest™ BLAST.

Aerobic and anaerobic cultures of amniotic fluid

The amniotic fluid samples were cultured in Columbia agar with sheep's blood, *Gardnerella vaginalis* selective medium, MacConkey agar, *Neisseria*-selective medium (modified Thayer–Martin medium), Sabouraud agar, and Schaedler anaerobe agar. The plates were cultured for 6 days and checked daily. Species were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using MALDI Biotyper software (Bruker Daltonics, Billerica, MA).

Quantitation of extracellular granzyme a in amniotic fluid

Levels of extracellular granzyme A in amniotic fluid were assessed by enzyme-linked immunosorbent assays (ELISA) by means of the ELISA Kit for Granzyme A (GZMA) (Cloud-Clone Corp., Houston, TX), according to the manufacturer's instructions. The detection range of the kit is from 8 to 500 pg/mL. The absorbance values were read at 450 nm on a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA).

Assessment of the levels of interleukin-6 in amniotic fluid

Levels of interleukin 6 in fresh unprocessed amniotic fluid samples were assessed using a Milenia QuickLine IL-6 lateral flow immunoassay with a Milenia POCScan reader (Milenia Biotec, GmbH, Giessen, Germany) [49]. The measurement range was 50–10,000 pg/mL. The intra- and inter-assay variations were 12.1 and 15.5%, respectively [49].

Clinical diagnosis

The presence of MIAC was determined based on positive results from PCR analysis of *Ureaplasma* spp., *M. hominis*, *C trachomatis*, or a combination of these species; 16S rRNA assay; cultivation of microbes under aerobic/anaerobic from the amniotic fluid; or a combination of these parameters.

IAI in PPROM pregnancies was identified by an amniotic fluid IL-6 concentration $\geq$745 pg/mL, where IL-6 was measured using a lateral flow-based immunoassay point-of-care test [50,51].

Based on the presence/absence of MIAC and IAI, the subjects were subdivided into the following subgroups: (i) intra-amniotic infection, when both MIAC and IAI were found; (ii) sterile IAI, when IAI was observed without MIAC; (iii) colonization, when MIAC was observed without IAI; and (iv) absence of both MIAC and IAI.

Statistical analyses

The demographic characteristics were compared by a non-parametric Kruskal–Wallis test for the continuous variables and by chi-square test for the categorical variables and presented as a median (range) and numbers (%). The normality of the data was tested using the D'Agostino-Pearson omnibus normality test, the Shapiro–Wilk test, and the Kolmogorov–Smirnov test. In women with amniotic fluid granzyme A levels below the limit of detection (LOD), the value 7.9 pg/mL (99 percentage) was used for analyses. Fisher exact test and chi-square test were used to assess the differences in the proportion of the samples with granzyme A levels below LOD. Since levels of granzyme A in amniotic fluid were not normally distributed, the non-parametric Mann–Whitney *U*-test and Kruskal–Wallis test were used for analyses, as

appropriate. The Spearman partial correlation was used for the adjustment of the results for gestational age at the time of sampling. The Spearman correlation was used to assess the association between amniotic fluid granzyme A levels, interleukin-6 levels, and gestational age at the time of sampling. Differences were considered statistically significant at $p < .05$. All p-values were obtained from two-sided tests and all statistical analyses were performed using GraphPad Prism 8.4.3 for Mac (GraphPad Software, San Diego, CA).

Results

A total of 166 women with PPROM between the gestational ages of 24 + 0 and 36 + 6 weeks were included in this study. MIAC or IAI was detected in 30% (50/166) and 20% (33/166) of the women, respectively. When the women were divided into subgroups according to the presence/absence of MIAC and IAI, intra-amniotic infection, sterile IAI, colonization, and absence of both MIAC and IAI were identified in 15% (25/166), 5% (8/166), 15% (25/166), and 65% (108/166), respectively. The demographical and clinical data of the women in these four subgroups are shown in Table 1. The microbial species identified in the amniotic fluid obtained from the subjects with intra-amniotic infection and colonization are presented in Table 2.

Levels of extracellular granzyme A levels in amniotic fluid

Levels of extracellular granzyme A levels were measurable in 87% (144/166) of all amniotic fluid samples from PPROM pregnancies. No correlation between its

Table 1. Demographical and clinical characteristics of preterm prelabor rupture of membranes pregnancies according to the presence/absence of MIAC and IAI.

Characteristic	Intra-amniotic infection ($n = 25$)	Sterile intra-amniotic inflammation ($n = 8$)	Colonization ($n = 25$)	Without both MIAC and intra-amniotic inflammation ($n = 108$)	p-Value
Maternal age [years, median (range)]	30.5 (17–42)	28.0 (21–35)	32.0 (18–42)	31.0 (21–40)	.34
Prepregnancy body mass index [kg/m^2, median (range)]	24.6 (16.5–38.0)	24.1 (19.3–37.8)	22.3 (16.0–33.5)	22.7 (15.8-39.0)	.67
Gestational age at admission [weeks, median (range)]	31 + 6 (24 + 2–36 + 5)	33 + 6 (25 + 1–36 + 6)	33 + 3 (25 + 3–36 + 6)	34 + 0 (25 + 3–36 + 6)	**.03**
Gestational age at delivery [weeks, median (range)]	32 + 1 (24 + 5–36 + 5)	34 + 1 (25 + 1–36 + 6)	34 + 2 (26 + 5–36 + 6)	34 + 4 (25 + 2–36 + 6)	**.005**
Latency from PPROM to amniocentesis [hours, median (range)]	9 (1–97)	8 (3–432)	6 (1–35)	6 (1–68)	.15
Latency from PPROM to delivery [hours, median (range)]	36,5 (3–106)	45 (17–768)	28 (7–390)	32,5 (4–452)	.72
CRP levels at admission [mg/L, median (range)]	15.5 (1.5–106.3)	8.4 (2.3–59.1)	4 (0.2–13.2)	5.6 (0.1–47)	**<.0001**
WBC count at admission [$\times 10^9$ L, median (range)]	13.8 (9.2–23.5)	15.3 (8.9–19.9)	11.9 (6.5–21.6)	11.5 (5.7–24.2)	**.001**
Amniotic fluid IL-6 at admission [pg/mL, median (range)]	8478 (831–10,000)	996 (801–1446)	199 (50–673)	194 (50–678)	**<.0001**
Administration of corticosteroids [number (%)]	21 (84%)	5 (63%)	16 (64%)	77 (70%)	.40
Administration of antibiotics [number (%)]	25 (100%)	8 (100%)	24 (96%)	101 (95%)	.66
Spontaneous vaginal delivery [number (%)]	19 (76%)	7 (88%)	20 (80%)	80 (73%)	.86
Cesarean section [number (%)]	6 (24%)	1 (12%)	5 (20%)	30 (27%)	.67
Forceps delivery [number (%)]	0 (0%)	0 (0%)	0 (0%)	1 (1%)	.91
Birth weight [grams, median (range)]	1790 (550–2840)	2215 (990–3320)	2285 (780–3250)	2240 (700–3550)	**.005**
Histological chorioamnionitis [number (%)]	23 (92%)	7 (88%)	17 (68%)	57 (52%)	**.002**
Funisitis [number (%)]	15 (60%)	2 (25%)	10 (40%)	23 (21%)	**<.0001**
Apgar score <7; 5 minutes [number (%)]	2 (8%)	1 (13%)	0 (0%)	2 (2%)	.12
Apgar score <7; 10 minutes [number (%)]	1 (4%)	1 (13%)	0 (0%)	1 (1%)	.08

Continuous variables were compared using a nonparametric Kruskal–Wallis test. Categorical variables were compared using the chi-square test. Continuous variables are presented as median (IQR) and categorical as number (%). Statistically significant results are marked in bold.
CRP: C-reactive protein; IL: inteleukin; MIAC: microbial invasion of the amniotic cavity; PPROM: preterm prelabor rupture of membranes; WBC: white blood cells.

levels and gestational ages at the time of sampling was identified (rho = −0.14; $p = .07$).

Extracellular granzyme A in amniotic fluid and MIAC

Granzyme A levels were detected in 70% (35/50) and 93% (108/116) of women with and without MIAC ($p = .0003$), respectively. Women with MIAC had lower levels of extracellular granzyme A in amniotic fluid than those without this complication (with MIAC: median 15.9 pg/mL, IQR 6.9–26.1 vs. without MIAC: median 19.9 pg/mL, IQR 12.7–29.5; $p = .03$; Figure 1(a)) in crude analysis, as well as after adjustment for gestational age at the time of sampling ($p = .02$).

Extracellular granzyme A in amniotic fluid and IAI

Granzyme A levels were measurable in 82% (27/33) and 88% (117/133) of women with and without IAI ($p = .39$). No difference was observed in the levels of extracellular granzyme A in amniotic fluid between women with and without IAI (with IAI: median 16.9 pg/mL, IQR 11.4–26.9 vs. without IAI: median 18.5 pg/mL, IQR 10.9–28.7; $p = .93$; Figure 1(b)).

Extracellular granzyme A in amniotic fluid and MIAC and/or IAI

Granzyme A was detected in: (a) 76% (19/25) of the samples from women with intra-amniotic infection; (b) 100% (8/8) of the samples from women with sterile IAI; (c) 68% (17/25) of the samples from women with colonization; and (d) 93% (100/108) of the samples from women with the absence of both MIAC and IAI ($p = .002$). A difference in the levels of extracellular granzyme A in amniotic fluid was found between the women with intra-amniotic infection, sterile IAI, colonization, and absence of both MIAC and IAI (intra-amniotic infection: median 15.6 pg/mL, IQR 8.3–24.7; sterile IAI: median 31.8 pg/mL, IQR 25.5–46.0; colonization: median 16.9 pg/mL, IQR 7.9–31.1; absence of both MIAC and IAI: median 18.8 pg/mL, IQR 12.3–28.7; $p = .02$; Figure 2). Women with sterile IAI had higher

Table 2. Bacterial species identified in the amniotic fluid from women with preterm prelabor rupture of membranes.

Women with intra-amniotic infection ($n = 25$)	Women with colonization ($n = 25$)
Ureaplasma spp. ($n = 14$)	*Ureaplasma* spp. ($n = 15$)
Ureaplasma spp. + *Chlamydia trachomatis* ($n = 2$)	*Ureaplasma* spp. + *Mycoplasma hominis* ($n = 5$)
Ureaplasma spp. + *Lactobacillus* spp. ($n = 1$)	*Ureaplasma* spp. + *Chlamydia trachomatis* ($n = 2$)
Ureaplasma spp. + *Sneathia sanguinegens* ($n = 1$)	*Ureaplasma* spp. + *Leptotrichia amnionii* ($n = 1$)
Ureaplasma spp. + *Veilonella* spp. ($n = 1$)	*Enterococcus faecium* ($n = 1$)
Mycoplasma hominis +*Peptococcus* spp. + *Propionibacterium* spp. + *Bacteroides* spp. ($n = 1$)	*Streptococcus pneumonia* ($n = 1$)
Chlamydia trachomatis ($n = 1$)	
Fusobacterium nucleatum ($n = 1$)	
Streptococcus agalactiae ($n = 2$)	
Streptococcus spp. ($n = 1$)	

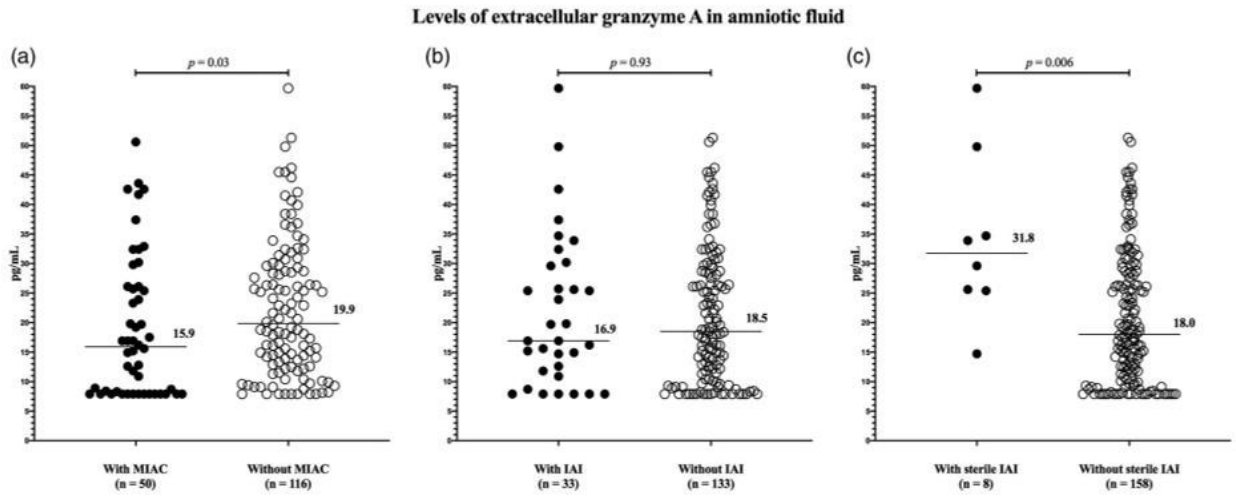

Figure 1. Comparisons of the levels of extracellular granzyme A in amniotic fluid between women with and without MIAC (a), IAI (b), and sterile IAI (c). IAI: intra-amniotic inflammation; MIAC: microbial invasion of the amniotic cavity.

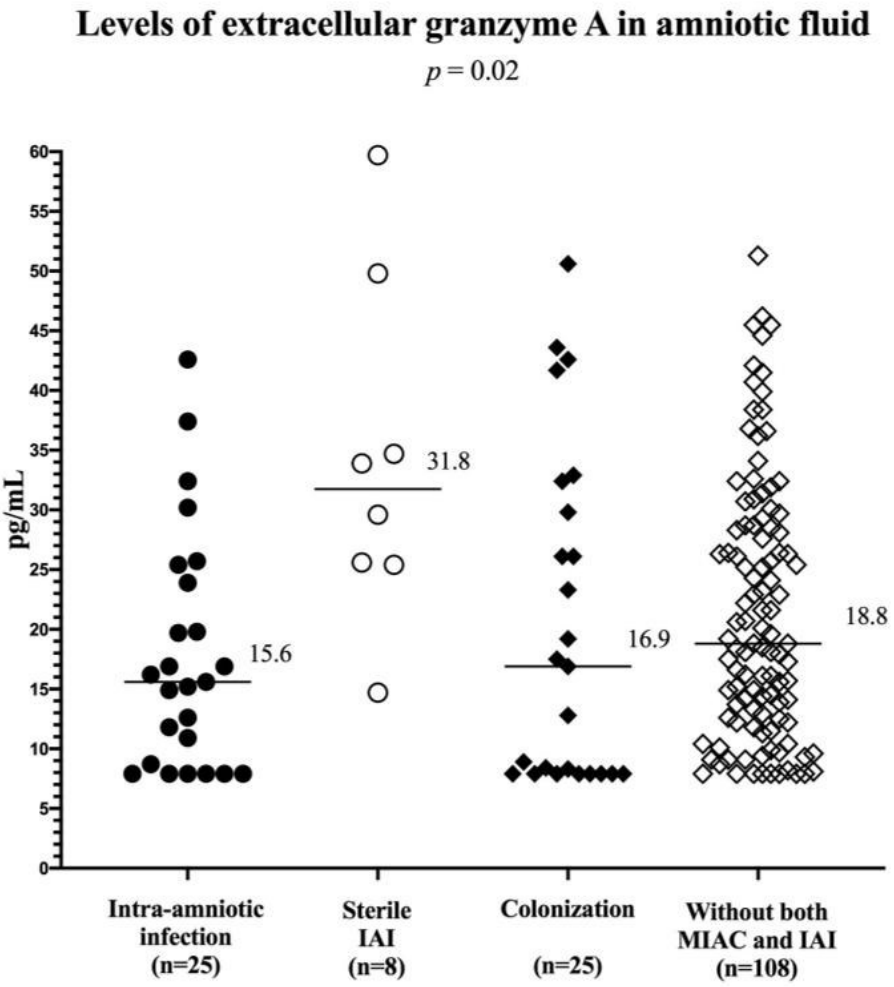

Figure 2. Comparisons of the levels of extracellular granzyme A in amniotic fluid between women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization, and without either MIAC or IAI. IAI: intra-amniotic inflammation; MIAC: microbial invasion of the amniotic cavity.

Table 3. Extracellular granzyme A in amniotic fluid—comparisons among subgroups of women based on the presence/absence of MIAC and IAI.

	Intra-amniotic infection	Sterile IAI	Colonization	Without both MIAC and IAI
Intra-amniotic infection	×	**p = .004; adj. p = .02**	p = .73; adj. p = .44	p = .09; **adj. p = .03**
Sterile IAI	**p = .004; adj. p = .02**	×	**p = .02; adj. p = .02**	**p = .01; adj. p = .004**
Colonization	p = .73; adj. p = .44	**p = .02; adj. p = .02**	×	p = .23; adj. p = .36
Without both MIAC and IAI	p = .09; **adj. p = .03**	**p = .01; adj. p = .004**	p = .23; adj. p = .36	×

IAI: intra-amniotic inflammation; MIAC: microbial invasion of the amniotic cavity.
p-Value: a comparison between two subgroups (a nonparametric Mann–Whitney U test). adj. p-value: a comparison between two subgroups after the adjustment for gestational age at sampling (a Spearman partial correlation).
Statistically significant results are represented in bold.

levels of extracellular granzyme A in amniotic fluid than those with intra-amniotic infection, colonization, and absence of both MIAC and IAI in crude and adjusted analyses (Table 3). Women with sterile IAI had higher levels of extracellular granzyme A in amniotic fluid than those without sterile IAI in crude analysis (without sterile IAI: median 18.0 pg/mL, IQR 10.4–28.4; p = .006; Figure 1(c)), as well as after adjustment for gestational age at time of sampling (p = .002).

Extracellular granzyme A and interleukin-6 in amniotic fluid

No correlation was found between the levels of extracellylar granzyme A and interleukin-6 in amniotic fluid (rho = −0.04; p = .58).

Discussion

The main findings of this study are as follows: (i) extracellular granzyme A is measurable in a majority of

amniotic fluid from PPROM pregnancies; (ii) the amount of extracellular granzyme A in amniotic fluid is diminished in the presence of MIAC; (iii) the presence of sterile inflammation is associated with elevated levels of extracellular granzyme A in the amniotic fluid; and (iv) extracellular granzyme A levels were not correlated with interleukin-6 levels in the amniotic fluid.

Granzyme A, a serine protease from the family of granzymes, plays an important role in the modulation of inflammation [20]. Extracellular granzyme A has been identified in plasma/serum circulation [25–41,43], as well as in the local body fluid [29,30,37–40,52]. In this study, extracellular granzyme A levels were assessed in samples of amniotic fluid obtained from singleton pregnancies complicated by PPROM between the gestational ages of 24 and 36 weeks. Extracellular granzyme A was measurable in 87% of all amniotic fluid samples. The exact source of extracellular granzyme A in the amniotic fluid is not clear. It is likely that immunocompetent cells in the amniotic fluid, mainly T lymphocytes and natural killer cells, are an important source of extracellular granzyme. However, we hypothesize that various sources (e.g. the placenta, the fetal membranes) contribute to the presence of extracellular granzyme A in the amniotic fluid, due to the following reasons: (i) regardless of the fact that the number of T cells and natural killers cells is at its highest between weeks 15–30 of gestation and decreases toward the completion of term [12], no correlation between extracellular granzyme A levels in the amniotic fluid and gestational age at time of sampling was found; (ii) IAI is associated with elevated numbers of all amniotic fluid immunocompetent cells (except innate lymphoid cells) [12] but women showing intra-amniotic infection did not have different levels of extracellular granzyme A in amniotic fluid than those without either MIAC or IAI; (iii) MIAC in PPROM is associated with higher numbers of total T cells, CD4+ T cells, CD8+ T cells, neutrophils, and monocytes/macrophages [10] but women with MIAC did not display higher levels of extracellular granzyme A; (iv) granzyme B- and K-positive cells were found in human placentas with and without villitis unknown etiology [53]; (v) granzyme B-positive cells were found in normal placentas from the first trimester of pregnancy [54], and (vi) effector T cells, granzyme-expressing T cells are present in the placenta and fetal membranes [55–57]. Collectively, these findings provide indirect evidence that the placenta and/or the fetal membranes should contribute to the production of extracellular granzyme A in the amniotic fluid.

Approximately one-third of PPROM pregnancies are associated with MIAC at the time of diagnosis of PPROM [2]. MIAC in PPROM represents a very heterogeneous condition due to: (i) the variety of microorganisms present in amniotic fluid [2,58]; (ii) the possibility of the concomitant presence of multiple microorganisms in amniotic fluid [2,58,59]; and (iii) the broad range of microbial loads possible in amniotic fluid [2,60,61]. Since extracellular granzyme A has been shown to be elevated in the systemic circulation of patients with tuberculosis [27], with melioidoses (caused by the gram-negative bacterium *Burkholderia pseudomallei*) [25], and with typhoid fever (caused by *Salmonella enteric*) [26], as well as in bronchoalveolar lavage from patients with pneumonia (caused by *Streptococcus pneumonia*) [29], a similar trend in the levels of extracellular granzyme A in amniotic fluid was expected in subjects with MIAC. Surprisingly, women with MIAC did not show higher levels of extracellular granzyme A in amniotic fluid than women without this complication, but showed lower levels instead. The explanation for this unexpected observation is unclear. However, when the subset of women with sterile IAI, having the highest extracellular granzyme A levels in amniotic fluid, was discounted from the cohort of women without MIAC, no difference in the levels of extracellular granzyme A levels was observed between the women with and without MIAC (data not shown). This finding supports our speculation that a source other than amniotic fluid immunocompetent cells contributes to the extracellular granzyme A level in amniotic fluid.

The employment of both cultivation and molecular biology methods to assess MIAC gave us a unique opportunity to identify a subset of the PPROM women with sterile IAI. The frequency of this condition represents between 5% and 29% of PPROM pregnancies [2,5]. The underlying pathology leading to the development of sterile IAI remains unclear, however, two main mechanisms (or a combination thereof) are considered: (i) damage to the fetal membranes that leads to the release of endogenous molecules (alarmins) into the amniotic fluid with a subsequent inflammatory response through the system of pattern recognition receptors [2,62,63]; (ii) infection in the choriodecidual space triggering the release of inflammatory mediators from the fetal membranes into the amniotic fluid [64]. Sterile IAI in pregnancies is usually associated with a milder intensity of intra-amniotic inflammatory response than intra-amniotic infection in terms of lower levels of inflammatory mediators [2,5] and lower

numbers of immunocompetent cells [11] in amniotic fluid.

In this study, a subset of women with sterile IAI showed higher levels of extracellular granzyme A in the amniotic fluid than the other women tested, even those with intra-amniotic infection. This observation, along with the absence of a correlation between the levels of interleukin-6 and granzyme A in amniotic fluid, supports the abovementioned hypothesis that the placenta or fetal membranes contribute intensively to the extracellular levels of granzyme A in amniotic fluid. In contrast, the finding that sterile IAI is related to the highest levels of extracellular granzyme A in amniotic fluid should be taken with caution owing to the small sample size of this subset of women.

The main strength of this study was the assessment of MIAC with a very extensive and thorough approach combining aerobic/anaerobic cultivation, a specific PCR for *Ureaplasma* spp., *Mycoplasma hominis*, and *Chlamydia trachomatis*, and a nonspecific PCR for the 16S rRNA gene. The study used a relatively large cohort of women with singleton pregnancies with well-defined phenotypes of spontaneous preterm delivery (PPROM), to whom an amniocentesis was performed at the time of admission. This study also suffered from some limitations that are important to mention. For instance, the results showing elevated levels of granzyme A in amniotic fluid in the subset of women with sterile IAI were not replicated in an independent cohort of women. Additionally, the presence of granzyme A-positive cells in the placenta and the fetal membranes was not evaluated.

In conclusion, extracellular granzyme A is present in a majority of the amniotic fluid from singleton pregnancies with PPROM between the gestational ages of 24–36 weeks and its levels in amniotic fluid are higher in the presence of sterile IAI.

Disclosure statement

The authors report that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Příloha č. 7: IgGFc-binding protein in pregnancies complicated by spontaneous preterm delivery: a retrospective cohort study

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IgGFc-binding protein in pregnancies complicated by spontaneous preterm delivery: a retrospective cohort study

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To determine the IgGFc-binding protein (FcgammaBP) concentration in amniotic and cervical fluids in preterm prelabor rupture of membranes (PPROM) and preterm labor with intact membranes (PTL) and to assess the diagnostic indices of FcgammaBP to predict intra-amniotic infection (the presence of both microbial invasion of the amniotic cavity and intra-amniotic inflammation). In this study, we included 170 and 79 women with PPROM and PTL, respectively. Paired cervical and amniotic fluid samples were obtained using a Dacron polyester swab and transabdominal amniocentesis, respectively. The FcgammaBP concentrations in the samples were assessed using an enzyme-linked immunosorbent assay. The presence of intra-amniotic infection was associated with elevated FcgammaBP concentrations in pregnancies with PPROM and PTL [PPROM—presence: 86 ng/mL vs. absence: 13 ng/mL, $p < 0.0001$, area under receiver operating characteristic curve (AUC) = 0.94; PTL—presence: 140 ng/mL vs. absence: 22 ng/mL, $p < 0.0001$, AUC = 0.86]. In cervical fluid, the concentrations of FcgammaBP were elevated in the presence of intra-amniotic infection in pregnancies with PPROM only (presence: 345 ng/mL vs. absence: 60 ng/mL, $p < 0.0001$, AUC = 0.93). FcgammaBP in amniotic fluid might be a marker of intra-amniotic infection in women with both PPROM and PTL However, in cervical fluid, it is only observed in women with PPROM.

Preterm delivery, defined as delivery before 37 weeks of gestation, is divided into two major subgroups: (i) iatrogenic preterm delivery and (ii) spontaneous preterm delivery[1,2]. The latter form of preterm delivery is more frequent and is responsible for more than two-thirds of all preterm deliveries[1,2]. Spontaneous preterm delivery can also be divided into two clinical phenotypes: (i) preterm prelabor rupture of the membranes (PPROM) and (ii) preterm labor with intact membranes (PTL)[1,2].

Some pregnancies can be complicated by the elevation of amniotic fluid concentrations of various inflammatory mediators, such as cytokines, chemokines, and antimicrobial peptides[3–11]. This condition is termed intra-amniotic inflammation and can be identified in both PPROM[12,13] and PTL[14]. Based on the presence or absence of microorganisms and/or their nucleic acids in amniotic fluid, intra-amniotic inflammation can be further divided into two clinical subtypes: (i) intra-amniotic infection and (ii) sterile intra-amniotic inflammation[12,14,15]. Clinical

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Characteristic	The presence of intra-amniotic infection (n = 19)	The absence of intra-amniotic infection (n = 151)	*p*-value
Maternal age [years, median (IQR)]	30 (24–33)	31 (27–35)	0.19
Primiparous [number (%)]	10 (53%)	90 (60%)	0.63
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	22.8 (20.9–25.2)	24.0 (21.2–27.2)	0.25
Smoking [number (%)]	3 (16%)	25 (17%)	1.00
Interval between PPROM and amniocentesis [hours, median (IQR)]	3 (2–8)	4 (2–7)	0.57
Gestational age at admission [weeks, median (IQR)]	29 + 3 (27 + 0–33 + 4)	34 + 3 (32 + 3–35 + 3)	**0.0003**
Gestational age at delivery [weeks, median (IQR)]	29 + 6 (27 + 3–33 + 4)	34 + 5 (33 + 0–35 + 6)	**< 0.0001**
Latency between PPROM and delivery [hours, median (IQR)]	60 (17–113)	45 (13–157)	0.70
Amniotic fluid IL-6 levels at admission [pg/nL, median (IQR)]	30,932 (9427–50,000)	667 (329–50,000)	**< 0.0001**
CRP levels at admission [mg/L, median (IQR)]	19.2 (3.3–36.5)	5.2 (2.5–8.2)	**0.003**
WBC count at admission [×10^9 L, median (IQR)]	14.9 (11.2–17.6)	11.9 (9.9–14.3)	**0.03**
Administration of corticosteroids [number (%)]	15 (79%)	89 (59%)	0.13
Vaginal delivery [number (%)]	13 (68%)	108 (72%)	0.79
Birth weight [grams, median (IQR)]	1460 (1090–2220)	**2290 (1930–3620)**	**< 0.0001**
Apgar score < 7; 5 min [number (%)]	3 (16%)	5 (3%)	**0.05**
Apgar score < 7; 10 min [number (%)]	2 (11%)	2 (1%)	0.06

Table 1. Maternal and clinical characteristics of women with preterm prelabor rupture of membranes based on the presence and absence of intra-amniotic infection. Continuous variables were compared using a nonparametric Mann–Whitney *U* test. Categorical variables were compared using the Fisher's exact test. Continuous variables are presented as median (IQR) and categorical as number (%). Statistically significant results are marked in bold. CRP: C-reactive protein; IL-6: interleukin-6; IQR: interquartile range; WBC: white blood cells.

relevance of intra-amniotic inflammation, its association with adverse short- and long-term neonatal outcomes, and optimal diagnostic markers, are still a matter of intense debate[16–28].

The broad availability and recent advances in proteomics, an unbiased technology, bring it within the scope of researchers working in the field of intra-amniotic inflammatory complications[29–38]. The ability to identify hundreds of proteins and to quantify changes in their abundance across multiple amniotic fluid samples makes proteomics very appealing, particularly in the discovery phase of the biomarker search process[29–61]. Therefore, proteomic analysis of amniotic fluid may reveal new proteins involved in the complex pathogenesis of intra-amniotic inflammation[29–40].

IgGFc-binding protein (FcgammaBP) is one of the proteins identified in amniotic fluid using proteomics[36,37,40,42]. FcgammaBP is a relatively unknown protein, with limited reports in relation to conditions such as bowel inflammatory disease, autoimmune disease, or thyroid gland tumors[62–64]. Nevertheless, FcgammaBP has been identified in amniotic fluid obtained from women with uncomplicated pregnancies[40,42] as well as from women with pregnancies complicated by PPROM[36] and PTL[37]. In pregnancies with PPROM, an elevation of FcgammaBP concentration in amniotic fluid has been observed during microbial invasion of the amniotic cavity and in acute histological chorioamnionitis[36]. However, there is a paucity of information on whether concentrations of FcgammaBP in amniotic and cervical fluid reflect the presence of intra-amniotic inflammatory complications in both the clinical phenotypes of spontaneous preterm delivery.

To fill this gap in the knowledge, we conducted this study with the following goals: (i) to quantify the FcgammaBP concentration in amniotic fluid samples from pregnant women with PPROM and PTL based on the phenotype of intra-amniotic inflammation; (ii) to quantify the FcgammaBP concentration in cervical fluid samples from pregnant women with PPROM and PTL based on the phenotype of intra-amniotic inflammation; and (iii) to assess the predictive value of FcgammaBP concentrations in amniotic and cervical fluids for intra-amniotic infection in pregnant women with PPROM and PTL.

Results

In total, 170 and 79 women with PPROM and PTL, respectively were included in the study. Among women with PPROM, the presence of intra-amniotic infection, sterile intra-amniotic inflammation, colonization, and negative amniotic fluid were observed in 11% (19/170), 5% (9/170), 10% (16/170), and 74% (126/170) of women, respectively. Among women with PTL, intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid were found in 15% (12/79), 27% (21/79), and 58% (46/79) of the women, respectively. None of the women with PTL had colonization.

The demographics of all the women in this study and clinical data of the women with PPROM and PTL, based on the presence and absence of intra-amniotic infection, are shown in Tables 1 and 2, respectively. The microorganisms identified in the amniotic fluid from women with PPROM and PTL are listed in Table 3.

Amniotic fluid FcgammaBP concentrations based on the phenotype of intra-amniotic inflammation. *PPROM pregnancies.* PPROM pregnancies with intra-amniotic infection and sterile intra-amniot-

Characteristic	The presence of intra-amniotic infection (n = 12)	The absence of intra-amniotic infection (n = 67)	*p*-value
Maternal age [years, median (IQR)]	27 (25–28)	28 (23–30)	0.86
Primiparous [number (%)]	7 (58%)	47 (70%)	0.50
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	27.5 (23.1–30.6)	25.0 (23.0–27.9)	0.51
Smoking [number (%)]	1 (8%)	7 (10%)	1.00
Gestational age at admission [weeks, median (IQR)]	27 + 6 (26 + 6–31 + 2)	30 + 6 (26 + 6–32 + 3)	0.17
Gestational age at delivery [weeks, median (IQR)]	29 + 0 (27 + 1–33 + 3)	32 + 6 (29 + 0–36 + 5)	**0.04**
Interval from amniocentesis to delivery [days, median (IQR)]	2 (0–15)	5 (1–39)	0.10
Amniotic fluid IL-6 levels at admission [pg/mL, median (IQR)]	43,431 (23,597–50,000)	1495 (484–4050)	**< 0.0001**
CRP levels at admission [mg/L, median (IQR)]	42.0 (7.5–75.1)	6.0 (2.4–11.4)	**0.0007**
WBC count at admission [×10^9 L, median (IQR)]	16.3 (14.1–19.5)	13.9 (10.6–16.5)	0.05
Administration of corticosteroids [number (%)]	9 (75%)	57 (85%)	0.41
Vaginal delivery [number (%)]	9 (75%)	55 (82%)	0.69
Birth weight of the newborn [grams, median (IQR)]	1230 (936–1958)	1940 (130–2690)	**0.03**
Apgar score < 7; 5 min [number (%)]	3 (25%)	7 (10%)	0.17
Apgar score < 7; 10 min [number (%)]	2 (17%)	3 (5%)	0.16

Table 2. Maternal and clinical characteristics of women with spontaneous preterm labor with intact membranes based on the presence and absence of intra-amniotic infection. Continuous variables were compared using a nonparametric Mann–Whitney *U* test. Categorical variables were compared using the Fisher's exact test. Continuous variables are presented as median (IQR) and categorical as number (%). Statistically significant results are marked in bold. RP: C-reactive protein; IL: interleukin; IQR: interquartile range; WBC: white blood cells.

Preterm prelabor rupture of membranes	Preterm labor with intact membranes
Ureaplasma spp. (n = 18)	*Ureaplasma* spp. (n = 4)
Ureaplasma spp. + *Mycoplasma hominis* (n = 1)	*Klebsiella pneumoniae* + *Streptococcus anginosus* (n = 1)
Ureaplasma spp. + *Gardnerella vaginalis* (n = 1)	*Gardnerella vaginalis* + *Lactobacillus plantum* (n = 1)
Ureaplasma spp. + *Streptococcus mitis* (n = 1)	*Ureaplasma* spp. + *Mycoplasma hominis* (n = 1)
Ureaplasma spp. + *Escherichia coli* (n = 1)	*Haemophilus influenzae* (n = 1)
Ureaplasma spp. + *Fusobacterium nucleatum* v	*Lachnoanaerobaculum* spp. (n = 1)
Ureaplasma spp. + *Dialister micraerophilus* + *Atopobium vaginae* (n = 1)	*Lactococcus lactis* (n = 1)
Gardnerella vaginalis + *Sneathia sanguinegens* (n = 1)	*Sneathia sanguinegens* (n = 1)
Haemophilus influenzae (n = 4)	Non-identifiable bacteria by sequencing (n = 1)
Anaerococcus tetradius (n = 1)	
Chlamydia trachomatis (n = 1)	
Lactobacillus iners (n = 1)	
Lactobacillus jensenii (n = 1)	
Mycoplasma hominis (n = 1)	
Streptococcus anginosus (n = 1)	

Table 3. The microbial species identified in the amniotic fluid of women with preterm prelabor rupture of membranes and with spontaneous preterm labor with intact membranes.

ic inflammation had higher amniotic fluid FcgammaBP concentrations than did the women with colonization and with negative amniotic fluid (infection: median 85.6 ng/mL, IQR 37.3–146.0, sterile: median 41.3 ng/mL, IQR 22.5–91.4, colonization: median 12.3 ng/mL, IQR 8.0–19.8, negative 12.2 ng/mL, IQR 9.0–17.8; $p < 0.0001$; Fig. 1a). No differences in amniotic fluid FcgammaBP concentrations were found between the women with colonization and negative amniotic fluid (Table 4).

Women with intra-amniotic infection had higher amniotic fluid FcgammaBP than did those without intra-amniotic infection (with infection: median IQR 85.6 ng/mL, IQR 37.3–146.0 vs. without infection: median 12.6 ng/mL, IQR 9.1–20.3; Fig. 2a). The amniotic fluid FcgammaBP cutoff value of 60 ng/mL was optimal in the prediction of intra-amniotic infection (area under the ROC curve [AUC] = 0.94; $p < 0.0001$; Fig. 2b). The diagnostic indices of these cutoff values are in Table 5.

PTL pregnancies. Differences in the concentrations of FcgammaBP were identified among the subgroups of women with intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid (infec-

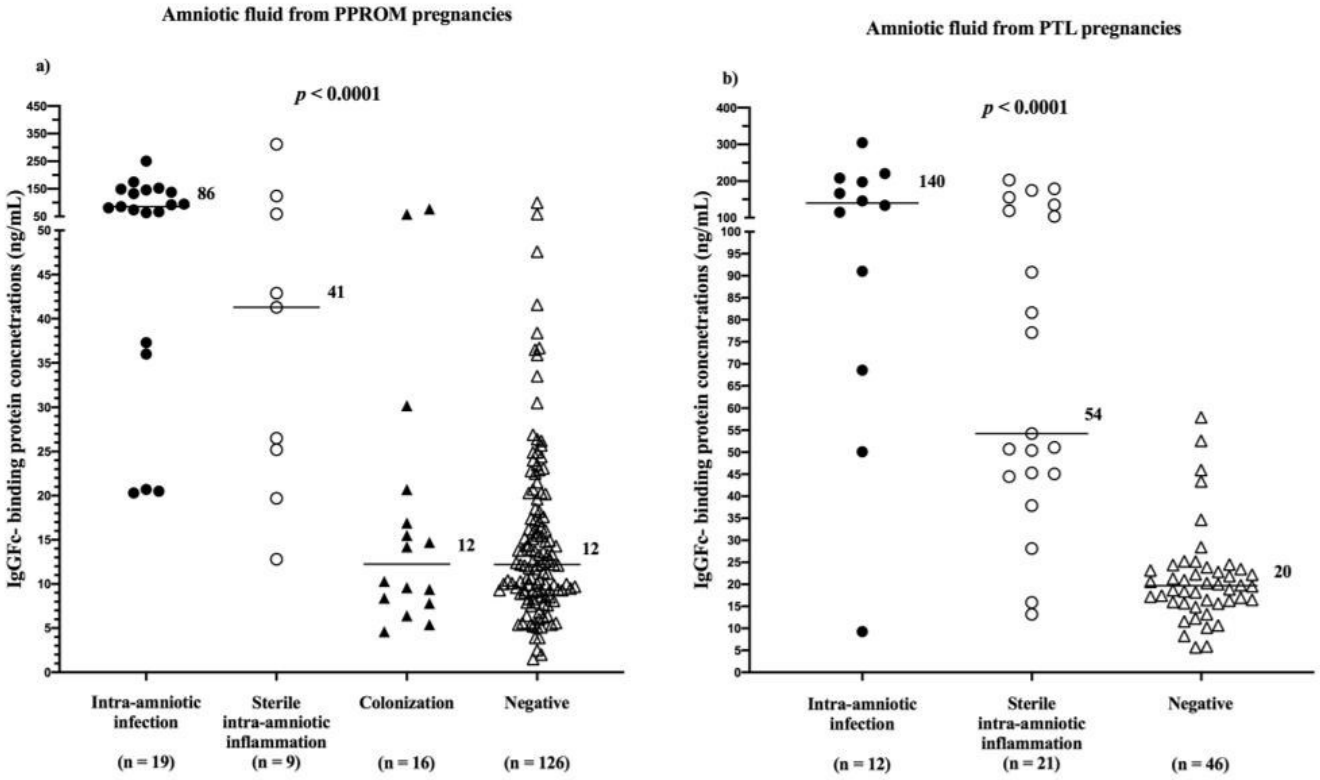

Figure 1. Amniotic fluid IgGFc-binding protein concentrations in the subgroups of the women with PPROM (**a**) and with PTL (**b**). PPROM, preterm prelabor rupture of membranes; PTL, preterm labor with intact membranes.

	Intra-amniotic infection	Sterile intra-amniotic inflammation	Colonization	Negative
Intra-amniotic infection	x	$p = 0.11$ adj. $p = 0.51$	$p < 0.0001$ **adj. p < 0.0001**	$p < 0.0001$ **adj. p < 0.0001**
Sterile intra-amniotic inflammation	$p = 0.11$ adj. $p = 0.51$	x	$p = 0.004$ **adj. p = 0.05**	$p < 0.0001$ **adj. p < 0.0001**
Colonization	$p < 0.0001$ **adj. p = 0.0001**	$p = 0.004$ **adj. p = 0.05**	x	$p = 0.98$ adj. $p = 0.22$
Negative	$p < 0.0001$ **adj. p < 0.0001**	$p < 0.0001$ **adj. p < 0.0001**	$p = 0.98$ adj. $p = 0.22$	x

Table 4. IgGFc-binding protein in amniotic fluid from preterm prelabor rupture of membranes: the comparisons among the subgroups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization, and negative amniotic fluid. *p*-value: a comparison between two subgroups (a nonparametric Mann–Whitney *U* test); Adj. *p*-value: a comparison between two subgroups after the adjustment for gestational age at sampling (a Spearman partial correlation). Statistically significant results are marked in bold.

tion: median 139.7 ng/mL, IQR 74.2–205.3; sterile: median 54.2 ng/mL, IQR: 44.8–127.0; negative: median 19.7 ng/mL, IQR: 15.9–23.6; Fig. 1b) in the crude analysis and after the adjustment for gestational age at sampling (both *p*-values < 0.0001). Women with intra-amniotic infection had higher amniotic fluid FcgammaBP concentrations than did women with sterile intra-amniotic inflammation and with negative amniotic fluid (Table 6). Women with sterile intra-amniotic inflammation had higher amniotic fluid FcgammaBP concentrations than those with negative amniotic fluid (Table 6).

Women with intra-amniotic infection had higher concentrations of amniotic fluid FcgammaBP than those without intra-amniotic infection (with infection: median 139.7 ng/mL, IQR 74.2–205.3 vs. without infection: median 22.2 ng/mL, IQR 16.5–46.0; Fig. 3a). The amniotic fluid FcgammaBP cutoff value of 120 ng/mL was found to be optimal in the prediction of intra-amniotic infection (AUC = 0.86; $p < 0.0001$; Fig. 3b). The diagnostic indices of these cutoff values are in Table 5.

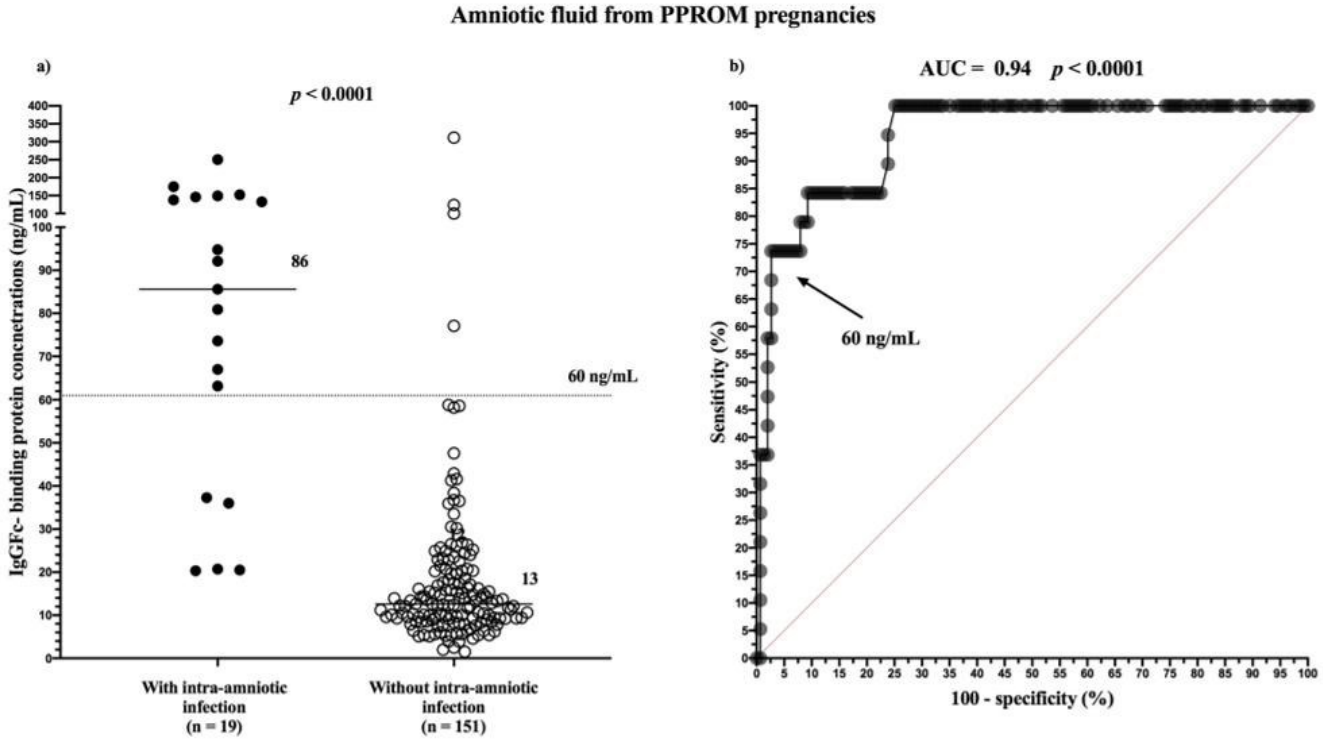

Figure 2. Amniotic fluid IgGFc-binding protein concentrations based on the presence of intra-amniotic infection in women with PPROM (**a**) and receiver operating characteristic curves for amniotic fluid IgGFc-binding protein in women with PPROM with intra-amniotic infection (**b**). PPROM, preterm prelabor rupture of membranes.

Cut-off value	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Positive likelihood ratio	Negative likelihood ratio	Odds ratio
Preterm prelabor rupture of membranes—amniotic fluid							
60 ng/mL	14/19 74% (95% CI 51–88)	147/151 97% (95% CI 93–99)	14/18 78% (95% CI 55–91)	147/152 97% (95% CI 93–99)	27.9 (95% CI 10.2–75.9)	0.27 (95% CI 0.13–0.57)	103 (95% CI 33–339)
Preterm labor with intact membranes—amniotic fluid							
120 ng/mL	7/12 58% (95% CI 32–81)	62/67 93% (95% CI 84–97)	7/12 58% (95% CI 32–81)	62/67 89% (95% CI 81–93)	7.8 (95% CI 3.0–20.6)	0.45 (95% CI 0.25–0.88)	17 (95% CI 3–64)
Preterm prelabor rupture of membranes—cervical fluid							
300 ng/mL	11/19 58% (95% CI 36–77)	145/151 96% (95% CI 92–98)	11/17 65% (95% CI 41–83)	145/153 95% (95% CI 90–97)	14.6 (95% CI 6.1–34.9)	0.44 (95% CI 0.26–74)	33 (95% CI 10–97)

Table 5. The predictive values of cut-off values of amniotic fluid IgGFcBP to identify intra-amniotic infection. CI: confidence interval.

	Intra-amniotic infection	Sterile intra-amniotic inflammation	Negative
Intra-amniotic infection	x	$p = 0.04$ **adj. p = 0.02**	$p < 0.0001$ **adj. p < 0.0001**
Sterile intra-amniotic inflammation	$p = 0.04$ **adj. p = 0.02**	x	$p < 0.0001$ **adj. p < 0.0001**
Negative	$p < 0.0001$ **adj. p < 0.0001**	$p < 0.0001$ **adj. p < 0.0001**	x

Table 6. IgGFc-binding protein in amniotic fluid from preterm labor with intact membranes: the comparisons among the subgroups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid. *p*-value: a comparison between two subgroups (a nonparametric Mann–Whitney *U* test); Adj. *p*-value: a comparison between two subgroups after the adjustment for gestational age at sampling (a Spearman partial correlation). Statistically significant results are marked in bold.

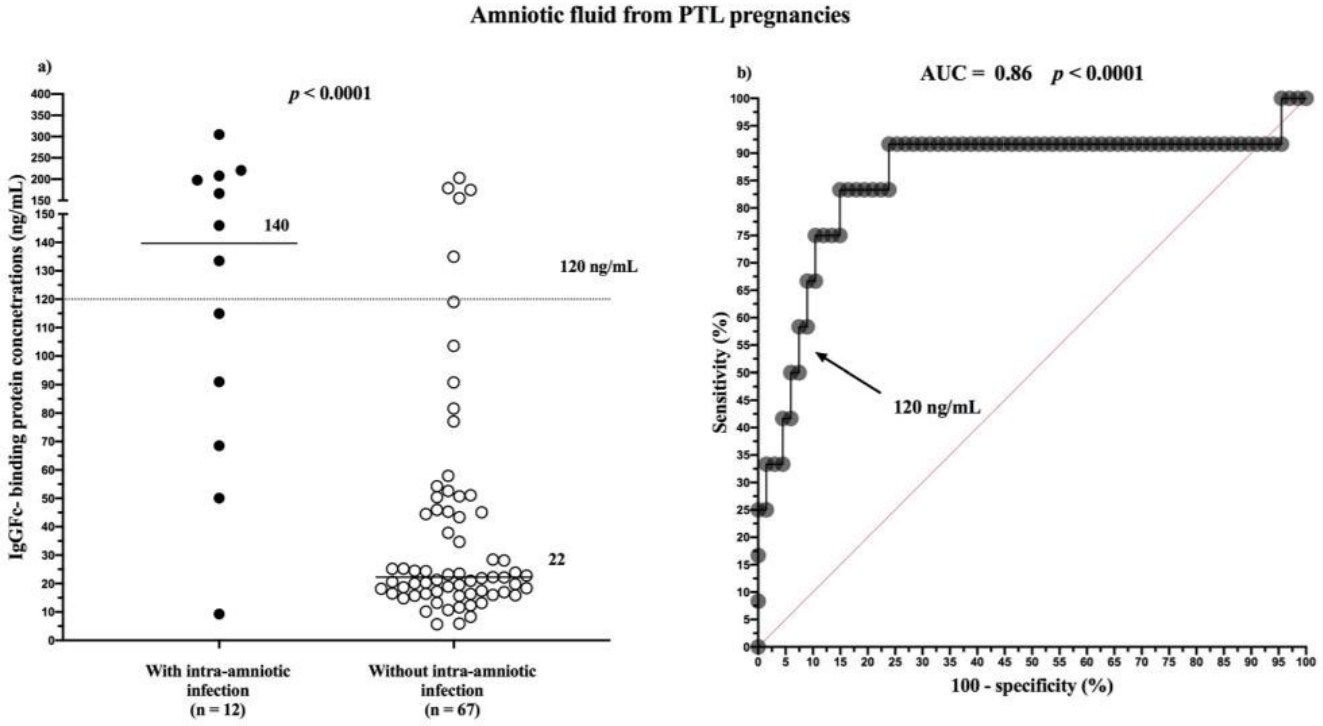

Figure 3. Amniotic fluid IgGFc-binding protein concentrations based on the presence of intra-amniotic infection in women with PTL (**a**) and receiver operating characteristic curves for amniotic fluid IgGFc-binding protein in women with PTL with intra-amniotic infection (**b**). PTL, preterm labor with intact membranes.

Concentration of FcgammaBP in cervical fluid based on the phenotype of intra-amniotic inflammation. *PPROM pregnancies.* A positive correlation was found between the concentrations of FcgammaBP in amniotic and cervical fluids ($\rho = 0.34$; $p < 0.0001$). The differences in cervical fluid FcgammaBP concentrations were revealed among the subgroups (infection: median 345.0 ng/mL, IQR 201.9–480.0; sterile: median 56.1 ng/mL, IQR 36.5–139.3; colonization: median 130.6 ng/mL, IQR 51.4–186.9; and negative: median 55.4 ng/mL, IQR 31.1–92.6; Fig. 4a) in the crude analysis as well as after the adjustment for gestational age at sampling (both $p < 0.0001$). Women with intra-amniotic infection had higher cervical fluid FcgammaBP concentrations than women with sterile intra-amniotic inflammation, colonization, and negative amniotic fluid (Table 7).

Women with intra-amniotic infection had higher cervical fluid FcgammaBP than those without intra-amniotic infection (with infection: median 345.0 ng/mL, IQR 201.9–480.0 vs. without infection: median 59.6 ng/mL, IQR 31.9–111.5; Fig. 5a). The cervical fluid FcgammaBP cutoff value of 300 ng/mL was found to be optimal in the prediction of intra-amniotic infection (AUC = 0.93; $p < 0.0001$; Fig. 5b). The diagnostic indices of these cutoff values are in Table 5.

PTL pregnancies. A weak positive correlation was observed between the concentrations of FcgammaBP in amniotic and cervical fluids ($\rho = 0.25$; $p = 0.02$). No difference in cervical fluid FcgammaBP concentrations was found among the subgroups (infection: median 341.1 ng/mL, IQR 95.2–614.8; sterile: median 341.2 ng/mL, IQR 138.1–523.4; and negative: median 200.9 ng/mL, IQR 56,7–443.8; $p = 0.18$; Fig. 4b). There was no difference in cervical fluid FcgammaBP concentrations between women with and without intra-amniotic infection (with infection: median 341.1 ng/mL, IQR 95.2–614.8 vs. without infection: median 227.0 ng/mL, IQR 95.7–455.4; $p = 0.45$).

Discussion

Principal findings of the study. (1) FcgammaBP was identified as a constituent of amniotic and cervical fluids from pregnancies complicated by PPROM and PTL; (2) the concentration of FcgammaBP in amniotic fluid was elevated in the presence of both phenotypes of intra-amniotic inflammation, being more pronounced in the presence of intra-amniotic infection in women with PTL; (3) the concentration of FcgammaBP in cervical fluid was elevated in the presence of intra-amniotic infection only in women with PPROM; (4) the FcgammaBP in amniotic fluid might be a marker of intra-amniotic infection in women with PPROM and PTL; and (5) the FcgammaBP in cervical fluid might be a non-invasive marker of intra-amniotic infection in women with PPROM.

Meaning of the study. FcgammaBP was discovered more than 30 years ago as a specific site for the fragment of crystallizable (Fc) region of the immunoglobulin (Ig) G antibody in the small intestinal and colonic epithelia[65]. This specific site differed from previously recognized receptors in the Fc region of IgG[65]. The specific

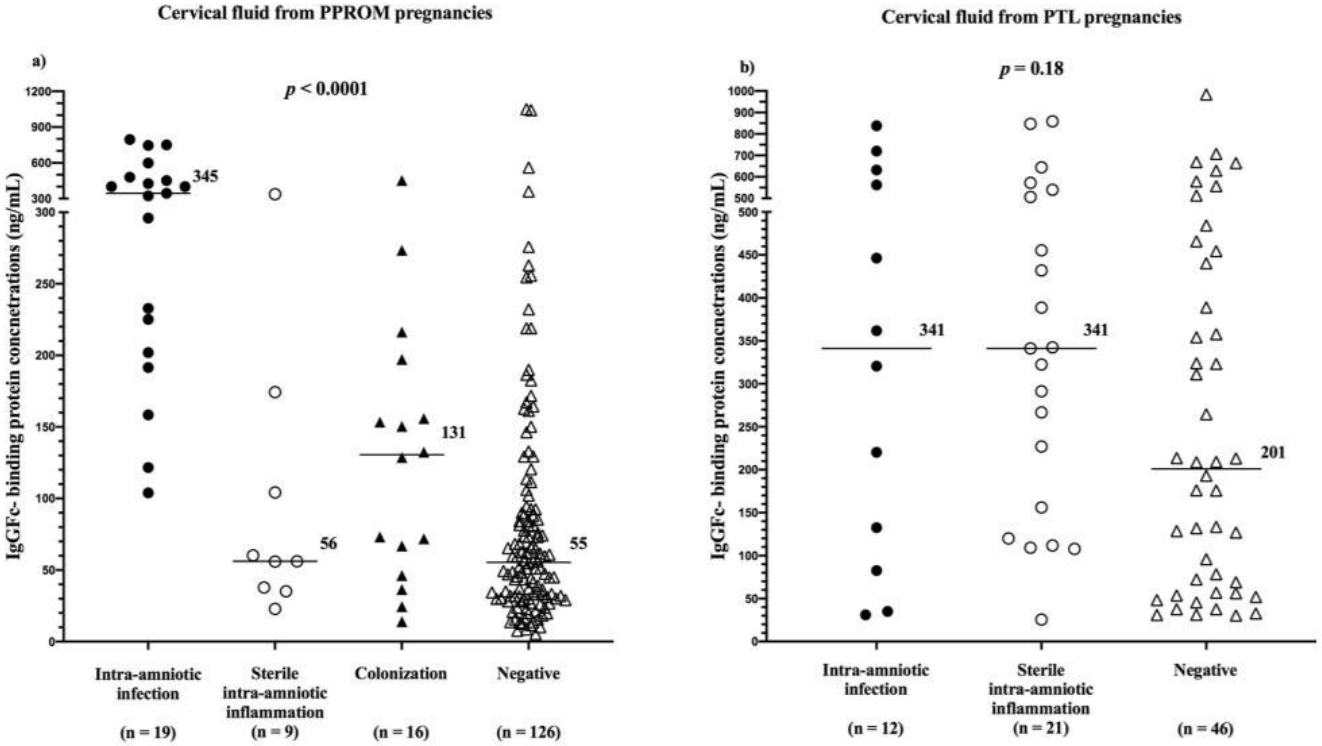

Figure 4. Cervical fluid IgGFc-binding protein concentrations in the subgroups of the women with PPROM (**a**) and with PTL (**b**). PPROM, preterm prelabor rupture of membranes; PTL, preterm labor with intact membranes.

	Intra-amniotic infection	Sterile intra-amniotic inflammation	Colonization	Negative
Intra-amniotic infection	x	$p = 0.0001$ **adj. p = 0.001**	$p < 0.0001$ **adj. p < 0.0001**	$p < 0.0001$ **adj. p < 0.0001**
Sterile intra-amniotic inflammation	$p = 0.0001$ **adj. p = 0.001**	x	$p = 0.28$ adj. $p = 0.76$	$p = 0.57$ adj. $p = 0.93$
Colonization	$p < 0.0001$ **adj. p = 0.0001**	$p = 0.28$ adj. $p = 0.76$	x	$p = 0.02$ adj. $p = 0.25$
Negative	$p < 0.0001$ **adj. p < 0.0001**	$p = 0.57$ adj. $p = 0.93$	$p = 0.02$ adj. $p = 0.25$	x

Table 7. IgGFc-binding protein in cervical fluid from preterm prelabor rupture of membranes: the comparisons among the subgroups of the women with intra-amniotic infection, sterile intra-amniotic inflammation, colonization, and negative amniotic fluid. *p*-value: a comparison between two subgroups (a nonparametric Mann–Whitney *U* test); adj. *p*-value: a comparison between two subgroups after the adjustment for gestational age at sampling (a Spearman partial correlation). Statistically significant results are marked in bold.

site for the Fc region of IgG was later termed FcgammaBP and identified as a protein primarily localized in the mucosal granules of the small intestinal and colonic epithelia that are secreted into the intestinal lumen[66]. Based on the current knowledge, FcgammaBP is considered to be a protein that provides immunologic protection of the intestinal tissue and facilitates the interaction between the intestinal mucus and potentially harmful stimuli (microorganisms, alarmins) with the ultimate goal of protecting the mucosal surface[62,65,66]. However, its exact biological function has yet to be fully elucidated.

The production of FcgammaBP has been described to occur in the intestinal epithelial cells, placenta, and thyroid tissue[62,63]. However, its expression has not been observed in the brain, heart, kidney, liver, lung, and skeletal muscles[62]. Interestingly, the ability to produce FcgammaBP was confirmed only in humans and monkeys, but not in mice, rats, rabbits, dogs, bovines, and porcines[62].

FcgammaBP has been found in low concentrations in human serum from healthy individuals[64]. However, its serum concentrations were elevated in the presence of autoimmune diseases such as Crohn's disease, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, and progressive systemic sclerosis[64]. The presence of FcgammaBP has been further proven in amniotic fluid, urine, saliva, and cerebrospinal fluid[40,42]. Liu et al. found FcgammaBP to be a constituent of amniotic fluid in the second trimester of uncomplicated pregnancies[42]. In

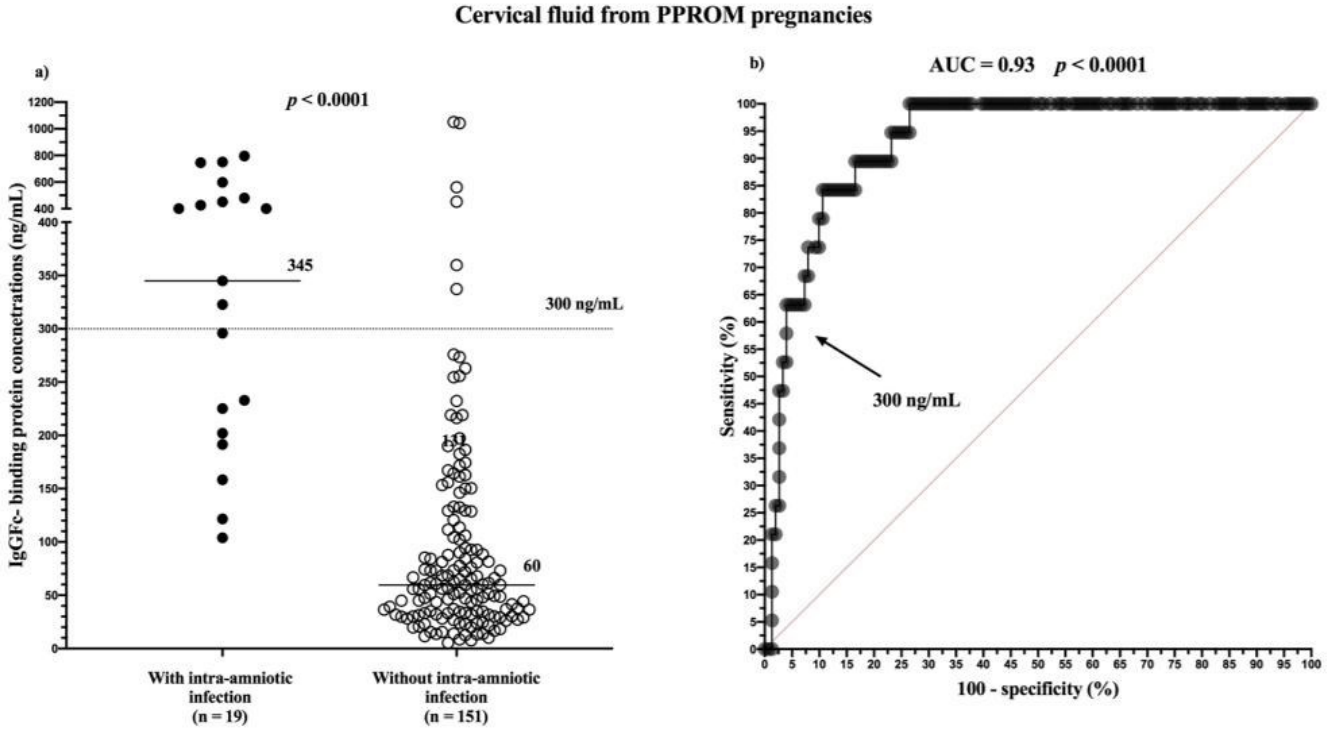

Figure 5. Cervical fluid IgGFc-binding protein concentrations based on the presence of intra-amniotic infection in women with PPROM (**a**) and receiver operating characteristic curves for cervical fluid IgGFc-binding protein in women with PPROM with intra-amniotic infection (**b**). PPROM, preterm prelabor rupture of membranes.

addition, FcgammaBP was shown to be among the most abundant (35/1624) proteins found in amniotic fluid[42]. Our group described the amniotic fluid presence of FcgammaBP in pregnancies complicated by PPROM and PTL[36,37]. The observation from this study, where we found FcgammaBP as a constituent of amniotic fluid from pregnancies with PPROM and PTL, is in line with the abovementioned findings.

Previously, concentrations of FcgammaBP in amniotic fluid have been shown to be higher in women with PPROM with microbial invasion of the amniotic cavity and acute histological chorioamnionitis than in those without these complications[36]. Interestingly, no differences in the amniotic fluid concentration of FcgammaBP between the presence and absence of the abovementioned complications were identified in women with PTL, where amniotic fluid was obtained from the forewaters at the end of the first stage of labor[37].

In this study, we found elevated amniotic fluid concentrations of FcgammaBP in the presence of both phenotypes of intra-amniotic inflammation. Interestingly, in women with PTL, the concentrations of FcgammaBP in amniotic fluid were higher in the presence of intra-amniotic infection than in the presence of sterile intra-amniotic inflammation. Collectively, the results from this study clearly show that both infectious and non-infectious stimuli might trigger the production of FcgammaBP.

In this study, the concentrations of FcgammaBP were measured in paired amniotic and cervical fluid samples obtained from women with both phenotypes of spontaneous preterm delivery. Interestingly, the FcgammaBP concentrations were higher in the cervical fluid samples than in the amniotic fluid samples, despite the fact that cervical fluid samples obtained with a swab were diluted in 1.5 mL of the buffer. These observations suggest that epithelial cells and/or immune cells in the endocervical canal are able to produce FcgammaBP. This finding supports the key role of the cervix during pregnancy, which is its immunologic protection against the ascension of microorganisms from the vagina and the cervix toward the upper genital tract[67–70].

It is obvious that the protein composition of a cervical fluid sample from pregnancies with PPROM may be substantially affected by amniotic fluid that has leaked from the amniotic cavity. Therefore, the cervical fluid samples of PPROM pregnancies may reflect such a situation in both the intra-amniotic and cervical compartments. This is a possible explanation as to why women with intra-amniotic infection had higher cervical fluid FcgammaBP concentrations than those without intra-amniotic infection in PPROM but not in PTL pregnancies. However, a weak positive correlation between amniotic and cervical fluid FcgammaBP protein concentrations was also found in PTL.

This study suggests that FcgammaBP might be a new biomarker for intra-amniotic infection in both phenotypes of spontaneous preterm delivery. This finding is clinically very relevant since confirmation of intra-amniotic infection represents a challenge for clinicians. The necessity to rule in or rule out the presence of microorganisms in amniotic fluid makes the diagnosis of intra-amniotic infection time-consuming and more expensive when the techniques used to identify either non-culturable or difficult-to-culture microorganisms are employed. Therefore, from a clinical point of view, there is an urgent need to discover a single marker of intra-amniotic infection that has reliable sensitivity and specificity. In this study, FcgammaBP in amniotic fluid was identified as a potential

marker of intra-amniotic infection in pregnancies with PPROM and PTL. Previously, Chaemsaithong et al. has described diagnostic indices of rapid matrix metalloproteinase (MMP)-8 and interleukin (IL)-6 point-of-care test (two cut-off values: 745 pg/mL and 1000 pg/mL) to identify intra-amniotic infection in pregnancies with PTL[21]. Comparing diagnostic indices among amniotic fluid FcgammaBP, MMP-8, and IL-6 to identify intra-amniotic infection in pregnancies with PTL, amniotic fluid FcgammaBP has the highest likelihood ratio [FcgammaBP (cut-off value of 120 ng/mL) 7.8, MMP-8 (cut-off value of 10 ng/mL) 3.3, IL-6 (cut-off value of 745 pg/mL) 2.6, and IL-6 (cut-off value of 1000 pg/mL) 3.0][21].

In addition, in women with PPROM, FcgammaBP in cervical fluid was also revealed as a potential marker of intra-amniotic infection. Particularly, cervical fluid FcgammaBP can be a clinically relevant marker given the non-invasive nature of cervical fluid sampling. Moreover, its diagnostic indices are better than previously published diagnostic indices of IL-6 in cervical fluid (AUC = 0.78, the positive likelihood ratio of 4.8) in PPROM pregnancies[71]. In addition, cervical fluid sampling can be safely repeated during the latency period of pregnancy complicated by PPROM. Therefore, FcgammaBP assessment in cervical fluid might be used to monitor the development of secondary intra-amniotic infection during the latency interval in women with PPROM.

Strengths and limitations of the study. The strength of this study is the relatively large cohort of paired samples of amniotic and cervical fluid. Second, the fluid samples were collected from well-defined phenotypes of spontaneous preterm delivery (PPROM and PTL). Finally, the thorough assessment of microbial invasion of the amniotic cavity, by a combination of culture and non-culture methods, provided an opportunity to precisely distinguish the subsets of women with intra-amniotic infection and sterile intra-amniotic inflammation.

This study also has some limitations that are worth mentioning. For example, there was a small number of women with intra-amniotic infection (n = 19 and n = 12). To confirm whether the concentration of FcgammaBP in amniotic fluid and cervical fluid is a reliable marker of intra-amniotic infection, the results need to be replicated in independent cohorts. Next, despite the FcgammaBP expression in the placenta that was described[62], the questions of which part of the placenta is a source of FcgammaBP and whether fetal membranes produce FcgammaBP still remain unanswered. A body of evidence has shown that intestinal epithelial cells produce FcgammaBP (97, 102, 103), but no data are available on whether amniotic epithelial cells can produce FcgammaBP. Given the importance of the amniotic epithelium as a barrier against the ascension of microorganisms into the amniotic cavity[72–74], some similarities between intestinal and amniotic epithelial cells might be identifiable such as, (1) to serve as mechanical barriers[72–76]; (2) to have spatially expressed toll-like receptors[77,78]; and (3) to indicate that the expression of toll-like receptors changes when inflammation is present[77–79]. Therefore, we hypothesize that the amniotic epithelium might be involved in FcgammaBP production.

Conclusion

The concentrations of FcgammaBP observed in amniotic and cervical fluid were elevated in women with intra-amniotic infection. Thus, after replication in an independent cohort, FcgammaBP in amniotic fluid might be a potential marker of intra-amniotic infection in pregnancies with PPROM and PTL. Moreover, FcgammaBP in cervical fluid could be a marker of intra-amniotic infection in pregnancies with PPROM.

Methods

This retrospective cohort study included pregnant women who were admitted to the Department of Obstetrics and Gynecology at the University Hospital Hradec Kralove in the Czech Republic between March 2017 and May 2020. The inclusion criteria were the following: (1) singleton pregnancy, (2) maternal age ≥ 18 years, (3) gestational age between 22 + 0 and 36 + 6 weeks, (4) PPROM or PTL, and (5) the performance of transabdominal amniocentesis at the time of admission to determine intra-amniotic inflammation. In contrast, the exclusion criteria were the following: (1) pregnancy-related and other medical complications such as fetal growth restriction, gestational or pre-gestational diabetes, gestational or chronic hypertension, and preeclampsia; (2) structural or chromosomal fetal abnormalities; (3) signs of fetal hypoxia; and (4) significant vaginal bleeding.

The gestational age was determined via the use of first-trimester fetal biometry. PPROM was diagnosed by examining the women, using a sterile speculum, for pooling of amniotic fluid in the posterior fornix of the vagina. In the case of clinical uncertainty in diagnosing PPROM, amniotic fluid leakage was confirmed by the presence of insulin-like growth factor-binding proteins (Actim PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

PTL was diagnosed as the presence of regular uterine contractions (at least two contractions every 10 min), along with cervical length, measured using transvaginal ultrasound, shorter than 15 mm or within the 15–30 mm range with a positive PartoSure test (Parsagen Diagnostics Inc., Boston, MA)[80].

Women with PPROM were treated with antibiotics. Those with intra-amniotic inflammation received first-line treatment with intravenous clarithromycin for seven days. Unless delivery occurred earlier, the antibiotic treatment was eventually modified under the condition of microbial invasion of the amniotic cavity; the women without intra-amniotic inflammation received benzylpenicillin (clindamycin was used in women allergic to penicillin). Women with PPROM below the gestational age of 35 + 0 weeks received corticosteroids (betamethasone) to accelerate fetal lung maturation and reduce neonatal mortality and morbidity. Women with PPROM were managed expectantly, except those with intra-amniotic infection beyond the gestational age 28 + 0 weeks wherein labor was induced or an elective cesarean section was performed within 72 h of admission.

Women with PTL received a course of corticosteroids (betamethasone) and tocolytic therapy with either intravenous atosiban (for gestational age ≤ 28 weeks) or with nifedipine, which was administered orally, for 48 h. Patients with proven intra-amniotic inflammation received treatment with intravenous clarithromycin for seven days, unless delivery occurred earlier. Antibiotic treatment was eventually modified under the condition of

microbial invasion of the amniotic cavity. Women with PTL that were positive for group B Streptococcus (GBS), as determined from the vaginal-rectal swab, or with an unknown GBS status received intravenous benzylpenicillin (clindamycin, in case of penicillin allergy) during an active labor.

All participants in this study provided informed written consent prior to the collection of amniotic and cervical fluid samples. Sample collection for this research was approved by the Institutional Review Board of the University Hospital Hradec Kralove (July 2014; No. 201408 S07P). All experiments were performed in accordance with relevant guidelines and regulations. All participants were Caucasian.

Cervical and amniotic fluid sampling. Paired cervical fluid and amniotic fluid samples were collected at the time of admission from all women included in this study, prior to the administration of antibiotics, tocolytics, and/or corticosteroids. Each cervical fluid sample was obtained by placing a Dacron polyester swab in the cervical canal for 20 s to achieve saturation. Once collected, the polyester swab was inserted into a polypropylene tube containing 1.5 mL of phosphate-buffered saline; the tube was then shaken for 20 min. Upon removal of the polyester swab, the tube was centrifuged at 300×*g* for 15 min at room temperature. The supernatant was divided into aliquots and stored at − 80 °**C** until further analysis.

Ultrasonography-guided transabdominal amniocentesis was performed after cervical fluid sampling. Approximately 2–3 mL of amniotic fluid was aspirated, and the amniotic fluid was immediately divided among polypropylene tubes. The samples of amniotic fluid were used for (i) the assessment of amniotic fluid interleukin (IL)-6; (ii) polymerase chain reaction (PCR) analysis of *Ureaplasma* species, *Mycoplasma hominis*, and *Chlamydia trachomatis*; (iii) sequencing of the 16S rRNA gene; and (iv) aerobic and anaerobic cultivation.

Amniotic fluid IL-6 concentrations. IL-6 concentrations were assessed using the immuno-analyzer Cobas e602, a part of the Cobas 8000 platform (Roche Diagnostics, Basel, Switzerland). The measurement range was 1.5–50,000 pg/mL. The coefficient of inter- and intra-assay precision was < 10%[22].

Detection of *Ureaplasma* species, *M. hominis*, and *C. trachomatis*. DNA was isolated from amniotic fluid using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Real-time PCR was conducted on a Rotor-Gene 6000 instrument (Qiagen) using the commercial AmpliSens *C. trachomatis*/*Ureaplasma*/*M. hominis*-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect the DNA from *Ureaplasma* species, *M. hominis*, and *C. trachomatis* in the same PCR tube (multiplex format). We included a PCR run for beta-actin, a housekeeping gene that served as the control, to examine the presence of polymerase chain reaction inhibitors.

Detection of other bacteria in amniotic fluid. Bacterial DNA was identified by PCR targeting the 16S rRNA gene with the following primers: 5′-CCAGACTCCTACGGGAGGCAG-3′ (V3 region) and 5′-ACATTT CACAACAC-GAGCTGACGA-3′ (V6 region)[81,82]. Each reaction contained 3 µL of target DNA, 500 nM forward and reverse primers, and Q5 High-Fidelity DNA polymerase (NEB, Ipswich, MA, USA) in a total volume of 25 µL. Amplification was carried out on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The products were visualized on an agarose gel. Positive reactions yielded 950 bp products that were subsequently analyzed by sequencing. The 16S rDNA PCR products were purified and subjected to sequencing with the above-mentioned primers and the BigDye Terminator kit v.3.1 (Thermo Fisher Scientific, Waltham, MA, USA). The bacteria were then typed via searches for the obtained sequences using BLAST and SepsiTest BLAST.

Aerobic and anaerobic cultures of amniotic fluid. The amniotic fluid samples were cultured on Columbia agar with sheep's blood, *Gardnerella vaginalis* selective medium, MacConkey agar, a *Neisseria*-selective medium (modified Thayer–Martin medium), Sabouraud agar, or Schaedler anaerobe agar. The plates were cultured for 6 days and checked daily. The species were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany).

Clinical definitions. *Microbial invasion of the amniotic cavity* was determined based on a positive PCR analysis of *Ureaplasma* species, *M. hominis, C. trachomatis* or a combination of these species or positivity for the 16S rRNA gene, aerobic/anaerobic cultivation of the amniotic fluid, or a combination of these parameters. *Intra-amniotic inflammation* was defined as amniotic fluid IL-6 concentrations ≥ 3000 pg/mL[22]. *Intra-amniotic infection* was defined by both microbial invasion of the amniotic cavity and intra-amniotic inflammation. *Sterile intra-amniotic inflammation* was defined as the presence of intra-amniotic inflammation without the concomitant microbial invasion of the amniotic cavity. *Colonization* was defined as the microbial invasion of the amniotic cavity without intra-amniotic inflammation. *Negative amniotic fluid* was defined as the absence of microbial invasion of the amniotic cavity and intra-amniotic inflammation.

Quantification of FcgammaBP in amniotic and cervical fluids. The concentrations of FcgammaBP were assessed in the amniotic fluid and cervical fluid samples using an enzyme-linked immunosorbent assay (ELISA), the Human FCGBP ELISA Kit (LifeSpan BioSciences, Inc., Seattle, WA, USA), according to the manufacturer's instructions. The amniotic fluid and cervical fluid samples were diluted tenfold and 50-fold, respectively. The sensitivity of the kit was 0.117 ng/mL. The absorbance values were read at 450 nm on a Multiskan RC ELISA reader (Thermo Fisher Scientific).

Statistical analyses. The women's demographic and clinical characteristics were compared using the non-parametric Mann–Whitney *U* test for continuous variables and are presented as median values (interquartile range [IQR]). Categorical variables were compared using Fisher's exact test and are presented as a number (%). The normality of the data was tested using the Anderson–Darling test. Because the FcgammaBP concentrations in the amniotic fluid were not normally distributed, the nonparametric Kruskal–Wallis and Mann–Whitney *U* tests were performed for statistical analyses, and the results are presented as a median value (IQR). Spearman's partial correlation analysis was performed to adjust the results for gestational age at sampling. Spearman's correlation was used to assess the relationship between the concentrations of amniotic fluid FcgammaBP and cervical fluid FcgammaBP and gestational age at sampling. Receiver operating characteristic (ROC) curves were constructed to assess the predictive value of amniotic fluid and cervical fluid FcgammaBP for the presence of intra-amniotic infection. Cutoff values were determined based on the highest positive likelihood ratio. Differences were considered significant at $p < 0.05$. All *p* values were obtained using two-tailed tests, and all statistical analyses were performed using GraphPad Prism, version 8.1.1. for Mac OS X (GraphPad Software, San Diego, CA, USA) or the Statistical Package for Social Sciences (SPSS), version 19.0 for Mac OS X (SPSS Inc., Chicago, IL, USA).

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Author contributions

J.S.: samples collection, investigation, formal analysis, writing-review and editing. M.K.: writing-original draft, conceptualization, methodology, samples collection, funding acquisition, project administration, formal analysis. O.S., M.K., L.P., R.B., P.B.: methodology, investigation, writing-review and editing. I.M.: methodology, investigation, formal analysis, samples collection, writing-review and editing. J.M.: methodology, formal analysis, investigation, writing-review and editing. B.J.: supervision, methodology, investigation, writing-review and editing. C.A.: conceptualization, methodology, investigation, writing-review and editing.

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Competing interests

The authors declare no competing interests.

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Příloha č. 8: Macrophage inflammatory protein-1α in amniotic and cervical fluids in spontaneous preterm labor with intact membranes with respect to intra-amniotic inflammation

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ORIGINAL ARTICLE

Macrophage inflammatory protein-1α in amniotic and cervical fluids in spontaneous preterm labor with intact membranes with respect to intra-amniotic inflammation

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ABSTRACT

Objective: Macrophage inflammatory protein 1α is a chemokine produced by various immune, epithelial, mesothelial, and fibroblast cells after exposure to bacterial lipopolysaccharide or pro-inflammatory molecules. The primary aim of this study was to determine MIP-1α concentrations in amniotic and cervical fluids from pregnancy with spontaneous preterm labor with intact membranes (PTL) with respect to the presence of intra-amniotic infection (both microbial invasion of the amniotic cavity and intra-amniotic inflammation) and sterile intra-amniotic inflammation (intra-amniotic inflammation alone). The secondary aim was to assess the diagnostic indices of MIP-1α in predicting intra-amniotic infection.

Materials and methods: Seventy-four women with PTL were included in this study. Paired amniotic and cervical fluid samples were obtained using transabdominal amniocentesis and a Dacron polyester swab, respectively. Microbial invasion of the amniotic cavity was diagnosed based on a combination of culture and molecular biology methods. The concentration of IL-6 in the amniotic and cervical fluids was measured using an automated electrochemiluminescence immunoassay method. Intra-amniotic inflammation was defined as an amniotic fluid IL-6 concentration of ≥3000 pg/mL. The MIP-1α concentrations in the samples were assessed using an enzyme-linked immunosorbent assay.

Results: A difference in amniotic fluid MIP-1α was observed among women with intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid (infection: median 1779.0 pg/mL; sterile, median 102.7 pg/mL; negative, median 19.9 pg/mL; $p < .0001$). No difference in the concentrations of MIP-1α was identified in cervical fluid after adjustment for gestational age at sampling (infection: median 77.7 pg/mL, sterile: median 152.7 pg/mL, negative: median 18.0 pg/mL; $p = .30$). The presence of intra-amniotic infection was associated with elevated MIP-1α concentrations in amniotic fluid (presence: 1779.0 pg/mL vs. absence: 26.3 pg/mL, $p < .0001$, area under receiver operating characteristic curve = 0.87).

Conclusions: In PTL pregnancies with the presence of intra-amniotic infection, the concentration of MIP-1α is elevated in amniotic fluid but not in cervical fluid. Amniotic fluid MIP-1α may provide a useful marker for intra-amniotic infection in women with PTL.

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Introduction

Spontaneous preterm labor with intact membranes (PTL) represents a clinical phenotype of spontaneous preterm delivery and is responsible for approximately one-third of all preterm deliveries [1–3]. PTL represents a syndrome of multiple etiologies; however, only intra-amniotic inflammation has been causally linked to PTL [3].

Intra-amniotic inflammation possesses two different phenotypes that include (i) intra-amniotic infection, where intra-amniotic inflammatory response is driven by the presence of microorganisms in the amniotic fluid and (ii) sterile intra-amniotic inflammation, where the intra-amniotic inflammatory response is driven by alarmins or by inflammation in the choriodecidual

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space in the absence of microorganisms or their nucleic acids in amniotic fluid [4–6].

Both phenotypes of intra-amniotic inflammation in PTL pregnancies can be characterized by (i) elevated amniotic fluid concentrations of inflammatory mediators such as cytokines, chemokines, antimicrobial peptides, and lipids [3,7–10] and (ii) increased number of immune cells in the amniotic fluid [11–15]. Nevertheless, intra-amniotic infection in pregnancies with PTL is associated with a stronger intra-amniotic inflammatory response compared to that observed during sterile intra-amniotic inflammation, as measured by the concentration of amniotic fluid IL-6 [4], amniotic fluid white blood cell count [4], and the numbers of amniotic fluid neutrophils, monocytes/macrophages, and T cells [13].

The origin of elevated amniotic fluid immune cells may be either fetal, maternal, or a mixture of both [12,14]. Collectively, amniotic fluid immune cells can be derived from (i) the fetus [16], (ii) the chorionic plate [17], and (iii) the vasculature of the chorionic membranes [18], and these cells are subsequently attracted by chemokines into the amniotic fluid.

Neutrophils, the most abundant subset of leukocytes in amniotic fluid from PTL with intra-amniotic infection [13], are predominantly attracted to inflammatory sites by C–X–C chemokines that are presented by interleukin (IL)-8 [19]. The second most abundant subset of leukocytes in amniotic fluid from PTL with intra-amniotic infection is monocytes/macrophages [13] that are predominantly attracted to and activated by C–C chemokines such as macrophage inflammatory protein-1α (MIP-1α) [19].

This protein was previously investigated in amniotic fluid obtained from healthy pregnancy (at mid-trimester and at term both in and not in labor) [20,21] and in pregnancies complicated by cervical insufficiency [22] and PLT [8,9,20,21]. In pregnancies with PTL, concentrations of MIP-1α in amniotic fluid were elevated in the presence of microbial invasion of the amniotic cavity [20], intra-amniotic infection [9], and clinical chorioamnionitis [21]. These findings appear promising; however, they were not translated on noninvasively obtained fluid samples from the cervix, a compartment with a tight proximity to the amniotic cavity from which a noninvasive sample can be obtained. Additionally, there is a shortage of information regarding the diagnostic indices of MIP-1α to predict intra-amniotic infection.

To fill these gaps, the primary objective of this study was to assess the concentrations of MIP-1α in amniotic and cervical fluid obtained from pregnancies with PTL with respect to the phenotypes of intra-amniotic inflammation. The secondary objective was to evaluate the diagnostic indices of MIP-1α in predicting intra-amniotic infection.

Materials and methods

A retrospective cohort study of women with pregnancies complicated by preterm labor between the gestational ages of 22 + 0 and 34 + 6 weeks that were admitted to the Department of Obstetrics and Gynecology of the University Hospital Hradec Kralove in the Czech Republic was conducted between March 2017 and December 2019. This study was approved by the Institutional Review Board (June 2015; No 201408 I96L), and informed consent was obtained from all participants. Inclusion criteria were as follows: (i) singleton pregnancies, (ii) maternal age ≥18 years, iii) symptoms of PTL, and (iii) transabdominal amniocentesis was performed to assess the intra-amniotic environment. The exclusion criteria were as follows: (i) the presence of pregnancy-related complications (preeclampsia, gestational hypertension, pre-gestational diabetes, and/or fetal growth restriction), (ii) the presence of chronic maternal diseases (chronic hypertension, diabetes mellitus, asthma bronchia, and others), (iii) chromosomal and/or structural abnormalities of the fetus, (iv) signs of fetal hypoxia, and (v) the presence of significant vaginal bleeding.

Gestational age was determined according to first-trimester fetal biometry. Transabdominal amniocentesis and cervical fluid sampling were both performed at the time of admission prior to the administration of corticosteroids, antibiotics, or tocolytics. All women received a course of corticosteroid and tocolytic therapy for 48 h. Those with intra-amniotic inflammation received intravenous clarithromycin for seven days, unless delivery occurred earlier. Antibiotic treatment may be individualized by the attending clinician based on the results regarding microbial invasion of the amniotic cavity.

Women delivered prior to a gestational age of 32 + 0 weeks received neuroprotection treatment with magnesium sulfate during labor. Those with the presence of group B Streptococcus in vaginal-rectal swabs or with unknown GBS status received intravenous benzylpenicillin (clindamycin in case of penicillin allergy) during active labor.

Amniotic fluid sampling

Ultrasonography-guided transabdominal amniocentesis was performed, and approximately 5 ml of amniotic

fluid was aspirated. Amniotic fluid was dispensed into polypropylene tubes and used for the assessment of interleukin-6, aerobic/anaerobic cultivation, and molecular biology analyses to identify microbial nucleic acids. The remaining amniotic fluid was centrifuged and then used for research purposes. The supernatant was aliquoted and stored at −80 °C until further analyses were performed. All cervical fluid samples used in this study were also used in our previous studies [23,24].

Cervical fluid sampling

Cervical fluid samples were collected using a Dacron polyester swab. The methods for cervical fluid sampling were described in detail in our previous publication [23].

Amniotic fluid interleukin-6 concentrations

Concentrations of IL-6 in amniotic fluid were assessed using an automated electrochemiluminescence immunoassay method. IL-6 concentrations were measured using the immuno-analyzer Cobas e602, which is a component of the Cobas 8000 platform (Roche Diagnostics, Basel, Switzerland) [25]. The basic measuring range was 1.5–5000 pg/mL, and this could be extended to 50,000 pg/mL with a 10-fold dilution of the sample. The coefficients of variation for the inter-assay and intra-assay precisions were $< 10\%$.

Identification of Ureaplasma *species,* Mycoplasma hominis, *and* Chlamydia trachomatis

Details regarding the detection of *Ureaplasma* spp., *Mycoplasma* hominis, and *Chlamydia trachomatis* in amniotic fluid using non-cultivation methods have been described in detail previously [23].

Identification of other bacteria present in the amniotic fluid

Details regarding the detection of other bacteria present in amniotic fluid with non-cultivation methods have been described in detail previously [23].

Aerobic and anaerobic cultures of amniotic fluid

Details regarding aerobic and anaerobic cultures of amniotic fluid have been described in detail previously [23].

Amniotic and cervical fluid MIP-1α concentrations

The amniotic and cervical fluid MIP-1α concentrations were determined according to a sandwich enzyme-linked immunosorbent assay (ELISA) using an ELISA kit for human MIP1α (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. The limit of detection (LOD) for human MIP-1α was 6 pg/mL. Intra-assay and inter-assay variability values for the human MIP1α ELISA kit were $< 10\%$ and $< 12\%$, respectively. Samples of amniotic and cervical fluid were not diluted, and the absorbance values were acquired at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, Waltham, MA).

Clinical definitions

PTL was defined as the presence of regular uterine contractions (at least two every 10 min) along with a cervical length (measured by transvaginal ultrasound) of shorter than 15 mm or between 15 and 15–30 mm with positive Partosure test (Parsagen Diagnostics Inc., Boston, MA, USA) [15]. **Intra-amniotic inflammation** was defined as an amniotic fluid IL-6 concentration $\geq$3000 pg/mL [19]. **Microbial invasion of the amniotic cavity** was determined based on a positive PCR analysis of *Ureaplasma* species, *M. hominis, C. trachomatis*, or a combination of these species or positivity for the 16S rRNA gene, aerobic/anaerobic cultivation of the amniotic fluid, or a combination of these parameters. **Intra-amniotic infection** was defined by both microbial invasion of the amniotic cavity and intra-amniotic inflammation. **Sterile intra-amniotic inflammation** was defined as the presence of intra-amniotic inflammation without concomitant microbial invasion of the amniotic cavity. **Negative amniotic fluid** was defined as the absence of microbial invasion of the amniotic cavity and intra-amniotic inflammation.

Statistical analyses

The demographic and clinical characteristics were compared using a nonparametric Kruskal–Wallis test for continuous variables and a chi-square test for categorical variables, and these data are presented as median values (interquartile range [IQR]) and as numbers (%), respectively. The normality of the data was assessed using the Anderson-Darling test. As the amniotic fluid and cervical fluid concentrations of MIP-1α were not normally distributed, non-parametric Kruskal–Wallis or Mann–Whitney U-tests were used for analyses, as appropriate, and the data are presented

Table 1. Maternal and clinical characteristics of women with pregnancies complicated by spontaneous preterm labor with intact membranes with respect to the presence of microbial invasion of the amniotic cavity and/or intra-amniotic inflammation.

Characteristic	With intra-amniotic infection ($n = 12$)	With sterile intra-amniotic inflammation ($n = 21$)	Women with negative amniotic fluid ($n = 41$)	p-Value
Maternal age [years, median (IQR)]	27 (24–28)	26 (22–29)	28 (24–30)	.54
Primiparous [number (%)]	7 (58%)	17 (81%)	26 (63%)	.29
Pre-pregnancy body mass index [kg/m^2, median (IQR)]	27.5 (23.1–30.6)	23.8 (22.4–25.4)	26 (23.7–28)	.11
Gestational age at admission [days, median (IQR)]	195 (174–218)	188 (170–218)	219 (199–228)	**.01**
Gestational age at delivery [days, median (IQR)]	203 (190–234)	194 (176–228)	239 (224–262)	**<.0001**
Interval from amniocentesis to delivery [days, median (IQR)]	2 (0–15)	2 (0–5)	18 (2–48)	**.005**
Delivery within 7 days from amniocentesis [number (%)]	9 (75%)	18 (86%)	17 (41%)	**.002**
Amniotic fluid IL-6 levels at admission [pg/mL, median (IQR)]	43,431 (23,597–50,000)	9464 (4279–50,000)	850 (377–1716)	**<.0001**
CRP levels at admission [mg/L, median (IQR)]	42 (7.5–75.1)	9.2 (5.5–18)	4.3 (1.9–9.7)	**<.0001**
WBC count at admission [$\times 10^9$ L, median (IQR)]	16.3 (14.1–19.5)	16.2 (12.3–19.4)	13 (10.1–15.7)	**.008**
Administration of antibiotics [number (%)]	12 (100%)	16 (76%)	15 (37%)	**<.0001**
Administration of corticosteroids [number (%)]	9 (75%)	20 (95%)	37 (90%)	.19
Spontaneous vaginal delivery [number (%)]	9 (75%)	18 (86%)	35 (85%)	.67
Cesarean delivery [number (%)]	3 (25%)	3 (14%)	5 (12%)	.55
Forceps delivery [number (%)]	0 (0%)	0 (0%)	1 (2%)	.67
Birth weight [grams, median (IQR)]	1230 (936–1958)	1180 (720–2045)	2100 (1795–2990)	**<.0001**
Apgar score <7; 5 min [number (%)]	3 (25%)	5 (24%)	2 (5%)	.053
Apgar score <7; 10 min [number (%)]	2 (17%)	3 (14%)	0 (0%)	**.03**

CRP: C-reactive protein; IL-6: interleukin-6; IQR: interquartile range; WBC: white blood cells.
Continuous variables were compared using a nonparametric Kruskal-Wallis test and presented as median (IQR).
Categorical variables were compared using the chi-square test and presented as number (%).
Statistically significant results are marked in bold.

as median values (interquartile range [IQR]). In women possessing amniotic and cervical fluid concentrations below the LOD, the value of 5.9 pg/mL (99%) was used for analyses. Spearman's partial correlation analysis was performed to adjust the results for gestational age at sampling. Receiver operating characteristic (ROC) curves were constructed to assess the predictive value of amniotic fluid MIP-1α in the presence of intra-amniotic infection. Differences were considered significant at $p < .05$. All *p-values* were obtained using two-tailed tests, and all statistical analyses were performed using GraphPad Prism 8 for Windows (GraphPad Software, San Diego, CA, USA) or the Statistical Package for Social Sciences (SPSS), version 19.0, for Mac OS X (SPSS Inc., Chicago, IL, USA).

Results

Seventy-four women with PTL were enrolled in the study. Intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid were observed in 16% (12/74), 28% (21/74), and 55% (41/74) of women, respectively. The most common microorganism present in the amniotic fluid was *Ureaplasma spp.*, and this was identified in 7% (5/74) of the women. The demographic and clinical characteristics of women according to the presence of intra-amniotic infection and sterile intra-amniotic inflammation are provided in Table 1.

Amniotic fluid MIP-1α concentrations based on the phenotype of intra-amniotic inflammation

Amniotic fluid concentrations were measurable in 100% (12/12), 100% (21/21), and 93% (38/41) of the samples with intra-amniotic infection, with sterile intra-amniotic inflammation, and with negative amniotic fluid, respectively ($p = .29$).

Differences in the concentrations of MIP-1α were identified among the subgroups of women with intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid (infection: median 1779.0 pg/mL, IQR 745.8–2952.0; sterile: median 102.7 pg/mL, IQR: 33.4–571.8; negative: median 19.9 pg/mL, IQR: 10.7–43.3; Figure 1) in the crude analysis and after adjustment for gestational age at sampling (both p-values $< .0001$). Women with intra-amniotic infection possessed higher amniotic fluid MIP-1α concentrations than did women with sterile intra-amniotic inflammation or negative amniotic

fluid (Table 2). Women with sterile intra-amniotic inflammation possessed higher amniotic fluid MIP-1α concentrations than did those with negative amniotic fluid according to crude analyses but not after adjusting for gestational age at sampling (Table 2).

Women with intra-amniotic infection possessed higher concentrations of amniotic fluid MIP-1α than did those without intra-amniotic infection (with infection: median 1779.0 pg/mL, IQR 745.8–2952.0 vs. without infection: median 26.3 pg/mL, IQR 11.4–113.9; Figure 2(a)). The amniotic fluid MIP-1α cutoff value of 1300 pg/mL was found to be optimal for the prediction of intra-amniotic infection (AUC = 0.88; $p < .0001$; Figure 2(b)). The diagnostic indices of these cutoff values included sensitivity of 75% (9/12; 95% confidence interval (CI) 47–91), specificity of 95% (59/62; 95% CI 87–98), positive predictive value of 75% (9/12; 95% CI 47–91), negative predictive value of 95% (59/62; 95% CI 87–98), positive likelihood ratio of 15.5 (95% CI 4.9–49.0), negative predictive ratio of 0.3 (95% CI 0.1–0.7), and odds ratio of 59 (95% CI 9–225).

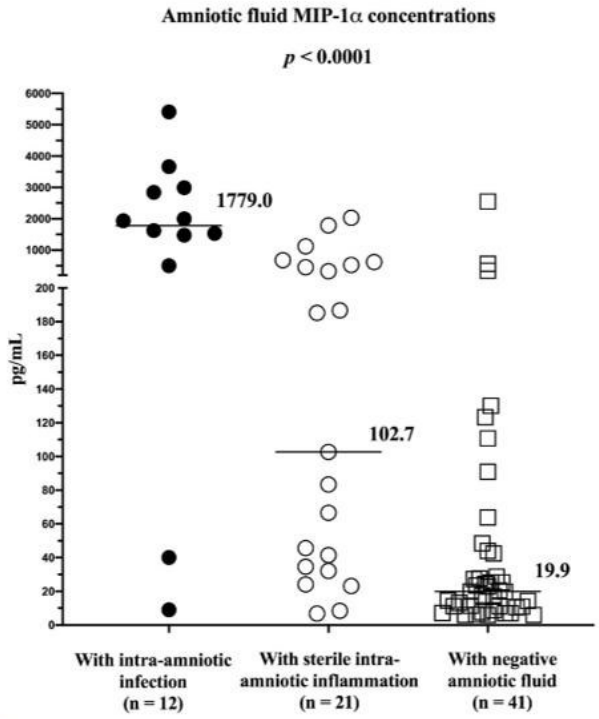

Figure 1. Amniotic fluid MIP-1α concentrations in the subgroups of the women with spontaneous preterm labor with intact membranes.

Concentration of MIP-1α in cervical fluid based on the phenotype of intra-amniotic inflammation

Concentrations of MIP-1α in cervical fluid were measurable in 75% (9/12), 90% (19/21), and 56% (23/41) of the samples with intra-amniotic infection, with sterile intra-amniotic inflammation, and with negative amniotic fluid, respectively ($p = .02$). No correlation was observed between the concentrations of MIP-1α in the amniotic and cervical fluids (rho = 0.08; $p = .49$). A difference in cervical fluid MIP-1α concentrations was observed among the subgroups (infection: median 77.7 pg/mL, IQR 8.2–211.5; sterile: median 152.7, IQR 57.8–334.2; and negative: median 18.0 pg/mL, IQR 5.9–88.5; $p = .004$; Figure 3) only in crude analysis but not after the adjustment for gestational age at the time of sampling ($p = .30$).

Discussion

MIP-1α orchestrates acute and chronic host inflammatory responses at the site of infection or injury, primarily by recruiting immune cells [26]. The main findings of this study performed on PTL pregnancies were that (i) concentrations of MIP-1α were measurable in all amniotic fluid samples from women with intra-amniotic inflammation, (ii) the concentrations of MIP-1α in amniotic fluid were elevated only in the presence of intra-amniotic infection, (iii) a concentration of MIP-1α of 1300 pg/mL in amniotic fluid was ideal for predicting intra-amniotic infection, (iv) measurable concentrations of MIP-1α in cervical fluid samples were more common in women with intra-amniotic inflammation, and (v) concentrations of MIP-1α in cervical fluid reflect neither intra-amniotic infection nor sterile intra-amniotic inflammation.

Table 2. MIP-1α in amniotic fluid in women with spontaneous preterm labor with intact membranes. Comparisons among the subgroups of women with intra-amniotic infection, sterile intra-amniotic inflammation, and negative amniotic fluid.

	Intra-amniotic infection	Sterile intra-amniotic inflammation	Negative amniotic fluid
Intra-amniotic infection	x	**$p = .003$** **adj. $p < .001$**	**$p < .0001$** **adj. $p < .001$**
Sterile intra-amniotic inflammation	**$p = .003$** **adj. $p < .001$**	x	**$p < .0001$** adj. $p = .26$
Negative amniotic fluid	**$p < .0001$** **adj. $p < .001$**	**$p < .0001$** adj. $p = .26$	x

p-Value: a comparison between two subgroups (a nonparametric Mann–Whitney *U* test); adj. *p*-value: a comparison between two subgroups after the adjustment for gestational age at sampling (a Spearman partial correlation).
Statistically significant results are marked in bold.

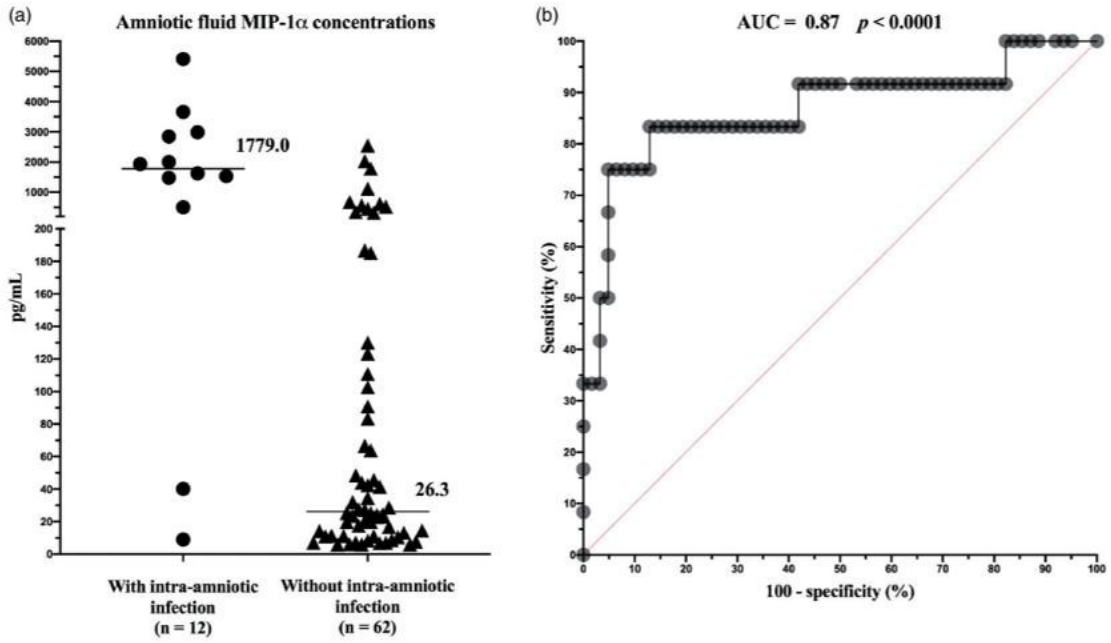

Figure 2. Amniotic fluid MIP-1α concentrations based on the presence of intra-amniotic infection in women with spontaneous preterm labor with intact membranes (a) and receiver operating characteristic curves for amniotic fluid MIP-1α in women with spontaneous preterm labor with intact membranes with intra-amniotic infection (b).

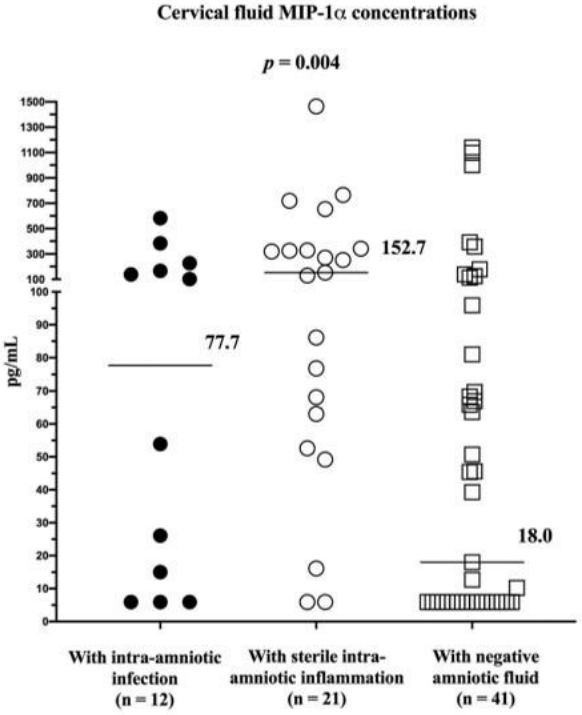

Figure 3. Cervical fluid MIP-1α concentrations in the subgroups of the women with spontaneous preterm labor with intact membranes.

MIP-1α is produced by various immune cells (macrophages, monocytes, lymphocytes, mast cells, and basophils), epithelial cells, mesothelium cells, and fibroblasts after exposure to bacterial lipopolysaccharide or pro-inflammatory molecules such as IL-1β or tumor necrosis factor-α [27,28]. MIP-1α binds to chemokine receptors on mononuclear/macrophage cells as well as on eosinophil granulocytes and acts as a chemoattractant and activator [28–30].

MIP-1α has previously been detected in various fluids of the female reproductive tract, including oviductal [31], follicular [31], and amniotic [8,9,20–22,31] fluids. Previously, concentrations of MIP-1α were determined to be undetectable in 69% of samples from women who had not been in labor (mid-trimester and term) [20]. In this study, the concentration of MIP-1α was measurable in all amniotic fluid samples from PTL complicated by intra-amniotic inflammation and in almost all (93%) amniotic fluid samples without intra-amniotic inflammation. This observation suggests that there must be another source of amniotic fluid MIP-1α than amniotic fluid immune cells, as these cells are not present or their number is very low in the absence of intra-amniotic inflammation. It is highly likely that fetal membranes and the placenta are the source of amniotic fluid MIP-1α. There is evidence from *ex vivo* studies indicating that chorion and decidual cells can produce MIP-1α in response to bacterial stimulus or inflammatory cytokines [19,32].

In this study, an elevation of amniotic fluid MIP-1α concentrations was observed only in PTL pregnancies complicated by intra-amniotic infection.

This observation is in agreement with the findings of a pioneering study examining MIP-1α in amniotic fluid from PTL that reported higher amniotic fluid concentrations of MIP-1α when microbial invasion of the amniotic cavity in PTL was present [20]. A recent observation from the same group when examining both phenotypes of intra-amniotic inflammation in PTL revealed that intra-amniotic infection is related to a higher concentration of MIP-1α compared to that of sterile intra-amniotic inflammation [9]. Additionally, this phenomenon was confirmed in three different amniotic fluid compartments, including extracellular vesicle surfaces, extracellular vesicle internal compartments, and the soluble fraction of amniotic fluid [9]. The presence of higher amniotic fluid MIP-1α concentrations in intra-amniotic infection compared to that in sterile intra-amniotic inflammation can be explained by the difference in the number of amniotic fluid immune cells between these two phenotypes of intra-amniotic inflammation.

Early and accurate identification of intra-amniotic infection in women with PTL remains a challenge for clinicians. The necessity to confirm or exclude the concurrent presence of microorganisms and/or their nucleic acids in amniotic fluid requires the use of cultivation and/or molecular biology methods, thus rendering this portion of the assessment of intra-amniotic infection time-consuming and expensive. Therefore, the availability of a single surrogate marker of intra-amniotic infection with reliable sensitivity and specificity would be extremely helpful for clinicians. In this study, amniotic fluid MIP-1α was identified as a potential marker of intra-amniotic infection in PTL pregnancies with an optimal cutoff value of 1300 pg/mL. However, to assign these cutoff values as clinically relevant, it is necessary to validate this value using a larger independent cohort. Interestingly, Romero et al. previously reported an amniotic fluid MIP-1α cutoff value of 500 pg/mL for predicting the presence of microbial invasion of the amniotic cavity [20]. The cutoff value of 500 pg/mL exhibited a sensitivity of 94% and specificity of 100% [20]. As microbial invasion of the amniotic cavity in PTL pregnancies is rarely observed without the concomitant presence intra-amniotic inflammation, it is highly likely that their cutoff value might be valid for intra-amniotic infection also. This was our rationale for attempting to apply this cutoff value to the cohort from this study. Regardless of the fact that this cutoff value was proposed 27 years ago, it still possesses great diagnostic potential (sensitivity of 83%, specificity of 87%, positive predictive value of 55%, negative predictive value of 96%, and odds ratio of 34) and is only slightly worse than the cutoff value of 1300 pg/mL.

In this study, the concentration of MIP-1α was measured in cervical fluid samples. The assessment of MIP-1α in fluid obtained from the cervical canal has not been performed prior to this study. Interestingly, women with both phenotypes of intra-amniotic inflammation (infection: 74%, sterile inflammation: 90%) exhibited a higher rate of measurable concentrations of MIP-1α in cervical fluid than did those without intra-amniotic inflammation (56%). This finding supports previous observations that the presence of intra-amniotic inflammation in PTL, irrespective of the presence or absence of microorganisms and/or their nucleic acids in amniotic fluid, is reflected by changes in the intensity of cervical inflammatory response as measured by various inflammatory mediators [23,33,34]. Nevertheless, no difference in the concentrations of MIP-1α cervical fluid among the above-mentioned groups was observed when the result was adjusted for gestational age at sampling.

The strength of this study is the availability of paired samples of amniotic and cervical fluid obtained at the time of admission. Second, the samples were collected from women with a well-defined phenotype of spontaneous preterm delivery (PTL) in whom a thorough assessment of microbial invasion of the amniotic cavity was performed using a combination of culture and non-culture methods. This approach provides an opportunity to precisely identify the subsets of women with intra-amniotic infection and sterile intra-amniotic inflammation.

This study does possess some limitations that are worth noting. First, there were a small number of women with intra-amniotic infections. Second, this study does not provide an answer to the question regarding the exact sources of amniotic and cervical fluid MIP-1α. Last, it cannot be fully excluded that a limited sample size of the cohort was the reason for failure of the statistically significant difference in the concentrations of cervical fluid MIP-1α after the adjustment for gestational age at sampling.

In conclusion, in PTL pregnancies with the presence of intra-amniotic infection, the concentration of MIP-1α is elevated in amniotic fluid but not in cervical fluid. Amniotic fluid MIP-1α may provide a useful marker for intra-amniotic infection in women with PTL.

Disclosure statement

The authors report that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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