

Original Research

The Relationship between Intra-Amniotic Sludge and the Amniotic Fluid Proteome in a Cohort of Pregnant Women with Acute Cervical Insufficiency: A Pilot Study

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Abstract

Background: We aim to determine the relationship between intra-amniotic sludge and the amniotic fluid proteome in cases of acute cervical insufficiency. **Methods:** This was a secondary analysis of data from an existing prospective cohort of women with acute cervical insufficiency. Amniotic fluid had previously been analyzed by Gram stain, culture and proteomics perioperatively. Post-processing review of stored ultrasound images to evaluate for the presence of intra-amniotic sludge (IAS) was performed by two blinded and independent observers. Results, lab, clinical, proteomic and outcome data were compared between groups with and without sludge. **Results:** Ten participants with acute cervical insufficiency from the initial cohort were included [IAS group (n = 4) and non-IAS group (n = 6)]. 75% of participants with sludge had negative Gram stain and culture compared to 17% amongst cases without sludge. 18 proteins (3.3%) were differentially abundant between the 2 groups ($p < 0.07$): the non-IAS group demonstrated increased immune, stress and inflammatory signatures with decreased signatures of keratinization relative to the IAS group ($p < 0.05$). **Conclusions:** These preliminary findings suggest that proteomic profiles of amniotic fluid with and without sludge tend to differ. Sludge is associated with an abundance of keratinizing proteins while inflammatory proteins predominate in the absence of sludge. The exploratory nature of this study illustrates the need for future large-scale prospective studies to determine the utility of intra-amniotic sludge and proteomics as markers of intra-amniotic infection in the management of acute cervical insufficiency.

Keywords: amniotic fluid; cervical insufficiency; proteome; preterm birth

1. Introduction

Preterm birth remains the leading cause of perinatal mortality and morbidity worldwide [1]. Cervical insufficiency (CI) is an important cause of second trimester loss and periviable preterm birth [2]. Cervical insufficiency is defined as painless shortening and premature dilatation of the cervix in the second trimester and without intervention, is invariably a precursor to delivery at a time when neonatal outcomes are poor [3,4]. Mechanical treatments for cervical insufficiency include surgical cerclage and cervical pessary [5,6]. Surgical placement of a cervical cerclage aims to close the cervix using suture with the intent of prolonging the pregnancy and improving fetal outcome [2,3]. However, emergency cerclage placement is potentially dangerous if there is underlying infection of the amniotic fluid or introduction of bacteria when the exposed membranes are pushed back into the uterus [3,7–10]. In either case, emergency cervical cerclage is contraindicated in the presence of intra-amniotic infection as it carries a significant risk of

maternal sepsis and associated sequelae (including death), as well as poor neonatal outcome [11–16]. It is therefore essential to rule out infection before consideration of surgical intervention [10].

Traditionally, amniocentesis with Gram stain and bacterial culture of the amniotic fluid is performed prior to placement of emergency cerclage to rule out infection; however, these methods lack sensitivity and as an invasive procedure, can itself be associated with risk [17,18]. In an attempt to improve our ability to detect subclinical intra-amniotic infection, multiple biomarkers in the amniotic fluid, cervicovaginal secretions and maternal serum have been extensively studied in the literature. Although correlation with intra-amniotic infection has been demonstrated, widespread use in the clinical setting has been limited by their sensitivity and specificity [18–20]. Mass spectrometry-based proteomics of the amniotic fluid and cervicovaginal secretions have identified differential protein expression in cases of preterm labour and prema-



ture rupture of membranes. These biomarkers and/or immune signatures may be indicative of intra-amniotic infection [21–25]. Mass spectrometry-based proteomics is used to measure numerous protein biomarkers simultaneously, offering a discovery platform to identify mechanisms of pathogenesis [26–29]. We have recently demonstrated that in women with cervical insufficiency, proteomic profiles tend to differ in women who deliver more or less than one week after their diagnosis of cervical insufficiency [30]. A recent retrospective cohort of women with cervical insufficiency and subsequent placement of emergency cerclage identified 68 proteins that were differentially expressed in women who subsequently had a spontaneous preterm birth less than 34 weeks [21]. Although this technology is in its discovery phase, proteomic analysis of amniotic fluid may provide additional insight into the complex mechanism of intra-uterine inflammation and infection.

Whether for traditional bacterial culture or proteomic analysis, sampling of the amniotic fluid via amniocentesis continues to carry inherent risks of invasive testing including pregnancy loss, preterm birth and iatrogenic infection. An ideal test prior to placing a cervical cerclage would be both sensitive and non-invasive. As such, researchers are actively searching for non-invasive markers of intra-amniotic infection, including the sonographic detection of intra-amniotic sludge. Intra-amniotic sludge (IAS) is a sonographic finding of free-floating, hyperechoic material in the amniotic fluid, typically present by the internal os of the cervix on transvaginal ultrasound (Fig. 1) [31]. IAS has been associated with intra-amniotic infection, histological chorioamnionitis and preterm birth [32–37]. Bacteria and fungi have been isolated from IAS collected both transvaginally and transabdominally and the presence of biofilms within those samples has been demonstrated [19,31,36,38]. Historically, the presence of IAS has been largely unrecognized as a potential predictor of adverse perinatal outcomes. The existing literature is inconclusive regarding the ability of IAS to reliably predict the development of overt chorioamnionitis and other pregnancy-related complications [39].

Herein, we present the first study interrogating the relationship between the amniotic fluid proteome and sonographic appearance of IAS. The objective of this study was to determine whether there are associations between the presence of IAS and the amniotic fluid proteomic profile that influence clinical outcomes amongst women with acute cervical insufficiency.

2. Materials and Methods

2.1 Study Population & Ethics Statement

This was a pilot study involving a secondary analysis of a prospective cohort of women presenting with acute cervical insufficiency [30]. From the original cohort of 40 women, patients diagnosed with acute cervical insufficiency considering emergency cerclage were eligi-

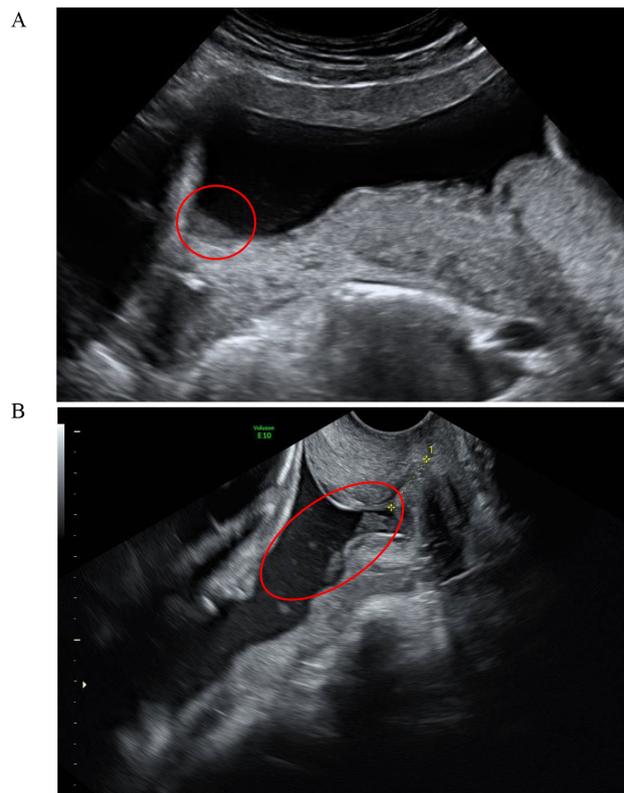


Fig. 1. 2D greyscale ultrasound images of intra-amniotic sludge (IAS). IAS (circled) as seen on transabdominal (A) and transvaginal (B) ultrasound.

ble and included in this analysis. Acute cervical insufficiency was defined as interval cervical shortening on serial ultrasound of either: (i) less than 1.5 cm intact length; or (ii) less than 2.5 cm with funneling, dilation and/or membrane prolapse. Cases were further subclassified according to presence/absence of membrane prolapse (high versus low grade). High grade cervical insufficiency was defined as evidence of an open cervix with visible fetal membranes at or below the external os. Low grade cervical insufficiency was defined as evidence of cervical shortening with/without membrane funneling above the level of external os. All those included had amniotic fluid samples collected prospectively via perioperative amniocentesis. Samples were collected from November 2014 to August 2016 at the Women’s Hospital Fetal Assessment Unit in Winnipeg, Manitoba, Canada. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of University of Manitoba (approval number: H2019:481 (HS23469), linked to H2014:328). Written patient consent was also obtained for inclusion of the ultrasound images included in this manuscript.

Table 1. Clinical details and outcomes of women with cervical insufficiency with and without intra-amniotic sludge detected.

Variable	Category	Amniotic sludge	
		No (n = 6)	Yes (n = 4)
Median mother's age (range)		29 (26–35)	35 (22–40)
Median gestational age in days (range)		147 (134–164)	154 (147–159)
Median gravidity (range)		4 (1–10)	3 (2–8)
Median parity (range)		3 (0–6)	1 (0–3)
Incompetency grade, n (%)	High	2 (33%)	2 (50%)
	Low	2 (33%)	1 (25%)
	Not specified	3 (33%)	1 (25%)
Gram stain, n (%)	Positive	3 (50%)	0 (%)
	Negative	2 (34%)	3 (75%)
	Not specified	1 (18%)	1 (25%)
Latency, n (%)	Yes	1 (17%)	3 (75%)
	No	5 (83%)	1 (25%)
Fetal outcome, n (%)	Viable	1 (17%)	3 (75%)
	Non-viable	5 (83%)	1 (25%)

2.2 Amniotic Fluid Sample Collection and Proteomics

Sample collection has been previously described [30]. All transabdominal amniocenteses were performed following the diagnosis of cervical insufficiency under sterile technique and ultrasound guidance by a single maternal-fetal medicine specialist. Three additional millilitres of amniotic fluid was withdrawn from the amniotic sac with a 22–25 gauge needle for research purposes. Gram stain, aerobic and anaerobic bacterial cultures were performed by the hospital's clinical microbiology laboratory. Proteomic analysis has been previously described [30]. Briefly, 75 μ g of protein from each sample was trypsin digested and labeled with iTRAQ 4-plex isobaric tags (SCIEX). Each multiplexed experiment was fractionated using offline reverse phase liquid chromatography, and the fractions were analyzed by tandem mass spectrometry using an Orbitrap Velos mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA) [28]. Proteins were identified using Mascot v2.4.0 (Matrix Science, London, UK) by searching against the SwissProt Human database (2015). Data analysis was performed using Scaffold Proteome Software v4.4.1.1 (Proteome Software, Portland, OR, USA).

2.3 Intra-Amniotic Sludge Detection

Post-processing review of stored ultrasound images taken at the time of diagnosis of cervical insufficiency was performed by two blinded observers to evaluate for the presence or absence of IAS. Sludge was considered present when dense particulate matter was visualized in proximity to the internal cervical os on transvaginal ultrasound, consistent with previously published studies (Fig. 1A,B). Inter-observer discordance was resolved by consensus opinion.

2.4 Statistical Analysis

Descriptive statistics were used to evaluate maternal characteristics and pregnancy outcomes. Continuous variables were presented as medians with ranges while dichotomous and categorical variables were described as proportions. Clinical data was analysed using Mann-Whitney U-tests and Fisher's Exact or Chi-squared tests using GraphPad Prism v7.02 (GraphPad Software Inc., La Jolla, CA, USA), and any missingness in the data formally explored using sensitivity analyses when indicated. We chose to evaluate proteins that differed between IAS and non-IAS groups with a p -value below 0.07 using a Mann-Whitney U-test. Analyses were conducted in R v3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). Principal component analysis was conducted using proteins differentially abundant between study groups in MatLab (MathWorks, R2015b, Natick, MA, USA) and EigenVector software (EigenVector Research, Inc., Wenatchee, WA, USA). Hierarchical clustering was performed on proteins found to be differentially abundant between study groups using complete linkage and Pearson correlation as the distance metric via the NMF package (v0.21.0) available in R (v3.6.2). Protein functional and pathway analysis was conducted using ConsensusPathDB (Max Planck Institute for Molecular Genetics) (<http://cpdb.molgen.mpg.de/>) and Ingenuity Pathway Analysis (Qiagen). For the proteomic analyses, p values were calculated using hypergeometric tests and right-tailed Fisher's Exact tests.

3. Results

3.1 Demographics, IAS Detection, and Clinical Characteristics

From the original cohort of 40 women, 10 women met inclusion criteria and were included in the analysis. The median age was 32 years (range 22–40), median gravidity

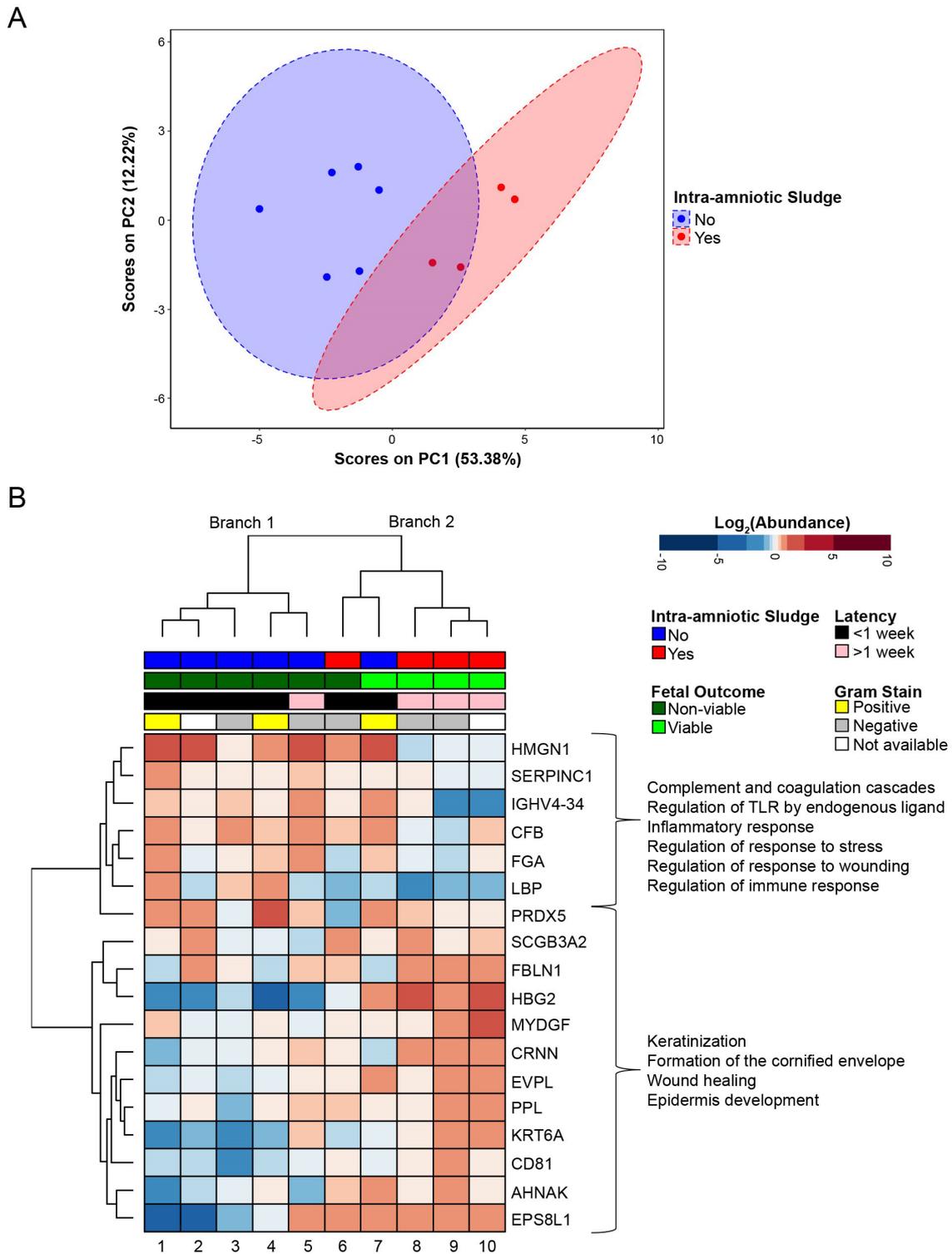


Fig. 2. Amniotic fluid protein differences between participants with and without intra-amniotic sludge (IAS). (A) Principal component analysis of proteins differentially abundant between IAS and non-IAS groups ($p < 0.07$, Mann-Whitney) demonstrates IAS samples group separately based on the variance measured in these protein signatures. (B) Hierarchical clustering of proteins differentially abundant between IAS and non-IAS groups highlights a pattern of increased keratinization, epidermis development and wound healing proteins among IAS samples (branch 2) and increased inflammation, stress and immune response proteins among non-IAS samples (branch 1) ($p < 0.05$, ConsensusPathDB).

of 3 (1–10), median parity of 1 (0–6), and a median gestational age of 21 + 6 weeks (19 + 1 to 23 + 3) at enrolment. Four women experienced greater than one week of latency (period of time between diagnosis of CI and delivery) while six had a latency period of less than one week. Four of the ten women delivered beyond viability (greater than 24 weeks gestation).

Four participants had evidence of IAS detected on transvaginal ultrasound at the time of diagnosis of cervical insufficiency (IAS group) while six participants had no evidence of IAS on ultrasound (non-IAS group). There were no major differences in maternal age, gravidity, parity, gestational age at time of diagnosis, grade of cervical insufficiency, Gram stain results, period of latency and fetal outcomes between IAS and non-IAS groups (Table 1). Two participants did not have targeted transvaginal images available, but review of available images did not detect features of particulate matter in the amniotic fluid or evidence of IAS on specific lower segment views of the internal cervical os on transabdominal ultrasound and they were included in the non-IAS group. The third case did not have available images to accompany the pre-cerclage ultrasound report. Consensus for including this case in the non-IAS group was reached as evidence of sludge would likely have been documented would it have been present. Following sensitivity analysis which showed no difference amongst these cases, a total of six participants were therefore included in the non-IAS group. Overall, only three participants had positive Gram stain results upon sampling with amniocentesis. Three of four participants with IAS (75%) had negative Gram stains, in contrast with only two patients (33%) in the non-IAS group. In the IAS group, three of the four (75%) reached viability to compared to only one of the six (17%) participants without IAS (Table 1).

3.2 Amniotic Fluid Proteomic Signature Related to the Presence or Absence of Intra-Amniotic Sludge Detection

A secondary analysis of previously acquired mass spectrometry data was conducted to determine possible protein relationships amongst the groups with and without IAS. Of the 551 proteins commonly measured amongst the ten amniotic fluid samples, 18 (3.3%) proteins differed (Mann-Whitney, $p < 0.07$) between the IAS and non-IAS groups (Table 2). Principal component analysis and hierarchical clustering of these differentially abundant proteins was performed. IAS samples grouped together and were positively loaded along principal component 1 (PC1) while non-IAS samples grouped together and were negatively loaded along PC1 (Fig. 2A). Indeed, all four samples with IAS clustered together on the right (branch 2) along with one non-IAS sample, while the remaining five samples without IAS clustered to the left (branch 1) (Fig. 2B).

Of the five samples that clustered to the right (branch 2), three participants had greater than 1 week of latency. All four participants that had viable fetal outcomes also clus-

tered to the right (branch 2). Proteins increased in the right (branch 2) cluster included keratinization ($n = 3$, $p = 8.45 \times 10^{-5}$), formation of the cornified envelope ($n = 2$, $p = 1.23 \times 10^{-3}$), wound healing ($n = 5$, $p = 7.39 \times 10^{-6}$), and epidermis development ($n = 3$, $p = 2.0 \times 10^{-3}$) proteins. On the left (branch 1), five of the six participants from the non-IAS group clustered together. All five samples did not have viable fetal outcomes. Proteins increased in the left cluster have known functions including complement and coagulation cascade ($n = 3$, $p = 4.7 \times 10^{-6}$), regulation of toll-like receptor (TLR) by endogenous ligand ($n = 2$, $p = 2.27 \times 10^{-5}$), inflammatory response ($n = 4$, $p = 4.2 \times 10^{-5}$), regulation of response to stress ($n = 4$, $p = 4.38 \times 10^{-4}$), regulation of response to wounding ($n = 2$, $p = 9.39 \times 10^{-4}$), and regulation of immune response ($n = 3$, $p = 2.90 \times 10^{-3}$). Ingenuity pathway analysis identified the cell proliferation of T lymphocytes pathway to be increased among IAS samples ($z = 1.96$, $p = 3.44 \times 10^{-3}$, 4 proteins), whereas cytolysis was increased among non-IAS samples ($z = -1.109$, $p = 1.19 \times 10^{-4}$, 4 proteins).

4. Discussion

This exploratory analysis of the relationship of IAS and the amniotic fluid proteome in women diagnosed with acute cervical insufficiency suggests novel differences exist between those with or without ultrasound evidence of sludge. Keratinization, epidermis development and wound healing proteins were increased amongst cases with IAS. When IAS was not identified, inflammatory, stress and immune response signatures were increased while keratinization proteins were decreased. Other studies have identified biomarkers in women with acute cervical insufficiency associated with latency after emergency cervical cerclage such as increased proinflammatory cytokines, increased neutrophil-lymphocyte ratios, and increased neutrophil protein levels (elastase, defensins-1 and -2, S100-A8 S100A8, S100A9 and S100A12) [22,40–43]. Is it important to distinguish that these studies analyzed pre-selected biomarkers which have never been proven to be sufficiently specific or sensitive for routine use in clinical practice. To our knowledge, this is the first study to compare intra-amniotic sludge to the amniotic fluid proteome.

Very few studies have analyzed amniotic fluid using shotgun mass spectrometry-based proteomics from patients diagnosed with acute cervical insufficiency. In contrast to pre-selected biomarkers, this technique enables simultaneous identification of multiple proteins which more accurately captures the complex, multifactorial signatures associated with acute CI. Lee *et al.* [21] identified protein signatures that were associated with preterm birth before 34 weeks gestation in women with CI. These signatures included elevated phagocyte/neutrophil proteins (Myeloperoxidase (MPO), Neutrophil gelatinase-associated lipocalin (LCN2), Plastin-2 (LCP1), Lactotransferrin(LTF)). Interestingly, our initial analysis of women with CI included in

Table 2. Proteins differentially abundant between IAS and non-IAS groups.

Gene name	Protein name	<i>p</i> -value	Mean non-IAS group	Mean IAS group	Fold change difference (IAS-nonIAS)	Functions
<i>LBP</i>	Lipopolysaccharide-binding protein	0.00952	0.10	-0.83	-0.94	Defense response
<i>CD81</i>	CD81 antigen	0.00952	-0.17	0.24	0.74	Immunological synapse formation
<i>IGHV4-34</i>	Immunoglobulin heavy variable 4-34	0.0191	0.36	-0.62	-0.99	Defense response
<i>CRNN</i>	Cornulin	0.0191	-0.067	0.53	0.60	Cell-cell adhesion
<i>FBLN1</i>	Fibulin-1	0.0191	-0.0075	0.72	0.73	Defense response
<i>HMGN1</i>	Non-histone chromosomal protein HMG-14	0.0381	1.12	0.024	-1.09	Chromatin organization
<i>CFB</i>	Complement factor B	0.0381	0.50	0.001	-0.50	Complement activation, inflammatory response
<i>SERPINC1</i>	Antithrombin-III	0.0381	0.25	-0.02	-0.27	Acute inflammatory response
<i>EVPL</i>	Envoplakin	0.0381	-0.035	0.45	0.49	Cornification, wound healing
<i>EPS8L1</i>	Epidermal growth factor receptor kinase substrate 8-like protein 1	0.0381	-1.36	0.73	2.09	Positive regulation of ruffle assembly
<i>HBG2</i>	Hemoglobin subunit gamma-2	0.0381	-1.27	0.94	2.21	Oxygen binding
<i>PRDX5</i>	Peroxiredoxin-5, mitochondrial	0.067	0.63	-0.06	-0.69	Response to oxidative stress
<i>FGA</i>	Fibrinogen alpha chain	0.067	0.35	-0.18	-0.52	Immune response
<i>SCGB3A2</i>	Secretoglobin family 3A member 2	0.067	0.091	0.54	0.45	Anti-inflammatory
<i>PPL</i>	Periplakin	0.067	0.077	0.66	0.58	Cornification, wound healing
<i>AHNAK</i>	Neuroblast differentiation-associated protein AH-NAK	0.067	-0.26	0.35	0.61	Regulation of RNA splicing
<i>MYDGF</i>	Myeloid-derived growth factor	0.067	0.05	0.77	0.72	Angiogenesis, apoptosis
<i>KRT6A</i>	Keratin, type II cytoskeletal 6A	0.067	-0.78	0.33	1.11	Cornification, wound healing

this study found that women who experienced less than one week of latency had elevated defense response proteins including a number of the aforementioned phagocyte/neutrophil proteins (LCP1, Neutrophil defensin 3 (DEFA3), LCN2, LTF, S100A9) [30]. In our secondary analysis, proteins that were differentially abundant in women with and without IAS differed: patients with IAS and the most prolonged latency had lower levels of defense response proteins including lipopolysaccharide-binding protein (LBP), fibrinogen alpha chain (FGA), and complement factor B (CFB). This finding highlights a potential clinical role in detection of decreased inflammatory proteins for more accurately predicting prolonged latency in women with acute cervical insufficiency.

IAS has been identified as an independent risk factor for preterm prelabor rupture of membranes (PPROM), preterm delivery and histological chorioamnionitis both in cases of cervical insufficiency and in pregnancies at high risk of preterm birth without cervical insufficiency [32–37]. Although IAS has been cultured and shown to incorporate bacteria, fungi and biofilms [32,38], debate exists regarding whether all IAS seen on ultrasound truly represents intrauterine infection. Our findings suggest that IAS may represent indolent intrauterine infection or infection caused by organisms with the ability to create biofilms and thus attenuate the innate inflammatory response. This has been seen in infectious processes elsewhere in the body [44,45]. Our results echo those of a recent retrospective cohort by Yoneda *et al.* [46] that demonstrated that patients with preterm labour and intact membranes had a shorter latency period when a pathogen was identified in the amniotic fluid via molecular techniques and no evidence of IAS when compared to cases with no identified pathogen in the amniotic fluid but with evidence of IAS. Although molecular techniques have been shown to be more sensitive than traditional culture, this requires targeted testing for identifying a specific pathogen. In addition, it is possible that sampling the upper compartment of the uterus via amniocentesis may be insufficient in certain cases of cervical insufficiency, especially if IAS suggests a contained infection in the lower segment of the uterus. At a minimum, amniocentesis simply done to culture an organism, by any testing modality, will uniformly miss other signs of intraamniotic inflammation.

Placement of an emergency cerclage in the setting of negative culture does not eliminate risk for the mother and fetus. Meticulous surveillance is required to avoid maternal morbidity. It is well established that although prematurity is the most significant fetal complication in cases of cervical insufficiency, the delivery of a fetus in the setting of chorioamnionitis worsens prognosis. Intrauterine infection has been associated with multiple long-term neurodevelopmental complications including cerebral palsy.

A growing body of evidence suggests that proteomic analysis provides valuable information in the setting of cer-

vical insufficiency [21,22]. We have previously shown that proteomic profiles differ in women who deliver more or less than one week after diagnosis of cervical insufficiency [30]. In our small sample, protein signatures associated with IAS were more prevalent in cases with prolonged latency >1 week and achieving latency to fetal viability. We are currently limited in our ability to optimize delivery planning in cases of cervical insufficiency managed with emergency cerclage. Removing the cerclage in the late preterm period in an otherwise uncomplicated postoperative course versus removing the cerclage in cases of overt chorioamnionitis are the most common end points. The addition of the assessment of IAS and the amniotic fluid proteomic profile may provide clinically meaningful information in managing these cases. In addition, the assessment of IAS is also readily available on standard transvaginal ultrasound images taken at the time of assessment for cervical insufficiency, requiring no additional healthcare resources. A non-inflammatory proteomic profile with absence of IAS may provide additional reassurance when contemplating expectant management. Although at this moment we cannot say that presence of IAS is an absolute contraindication to placement of emergency cerclage in acute cervical insufficiency, more intense postoperative surveillance and/or invasive testing and lower threshold for cerclage removal and delivery may be indicated. An inflammatory profile post emergency cerclage may warrant delivery prior to the development of overt chorioamnionitis and offer additional important clinical information beyond Gram stain and culture alone.

Our small study has a number of strengths. This cohort represents a retrospective secondary analysis of prospectively collected amniotic fluid samples. Our cohort included women with acute cervical insufficiency, four of which (40%) had an advanced presentation with an open cervix and amniotic membranes funnelling to or beyond the external cervical os. The findings of this study were not influenced by cases of short cervical length that may have gone to term without intervention. In addition, no cases of prophylactic cerclages were included in our study. Prophylactic cerclages and cases of short cervical length are a different population and should not be included in a study to determine the role of intrauterine infection in acute cervical insufficiency.

Our study also has limitations that warrant discussion. The main limitation of this study is its sample size as cases of cervical insufficiency are uncommon, and those with prospectively collected amniotic fluid, even fewer. Due to the small sample we were unable to control for maternal factors that may confound results (e.g., maternal age, obstetrical history, smoking history or cervical status). For the proteomic analyses, the *p*-value was relaxed to $p < 0.07$ to avoid type II error in this cohort. As this was a secondary analysis of a previously collected cohort involving, *post hoc* evaluation of IAS from available imaging,

we acknowledge the risk of sampling bias. To mitigate the chance of observer bias during the retrospective review, two blinded observers independently reviewed stored images in a standardized fashion to detect the presence or absence of intraamniotic sludge. In three cases without targeted transvaginal ultrasound images, extrapolation from other available ultrasound images or clinical assumptions were therefore required and undertaken as previously described. While this may bias our findings, it reflects the reality of many ultrasound units without transvaginal capability or in cases where transvaginal assessment is declined. We did perform a sensitivity analysis and since no difference was noted when including or excluding these three cases, the decision to include these three cases in the non-IAS group was justified.

5. Conclusions

In conclusion, we demonstrated that mass spectrometry-based proteomics of amniotic fluid and the assessment of the presence of IAS at the time of diagnosis of cervical insufficiency are feasible. Patterns of protein expression in IAS cases demonstrated a predominance in functions of keratinization, epidermis development and wound healing, while cases without IAS demonstrated a heightened inflammatory pattern with increased stress and immune response signatures. The exploratory nature of this study illustrates the urgent need for large prospective studies to determine the utility of IAS and proteomics in the risk stratification of cases of cervical insufficiency and prediction of severe maternal and fetal morbidity and mortality. Further research will be important to determine whether the assessment of IAS and proteomic analysis of amniotic fluid could optimize the management of patients with acute cervical insufficiency.

Availability of Data and Materials

Data may be available upon reasonable request.

Author Contributions

RNMG, KDB, ADB, VP, SMM were involved with recruitment, collection of amniotic fluid samples, and specimen processing in the original cohort. RNMG and CLP were involved with ultrasound data collection and data management. Statistical analysis was performed by KDB. RNMG and CLP produced the initial drafts and final version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the University of Manitoba (approval number: H2019:481 (HS23469)).

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper. Preliminary findings were presented as a poster abstract at the 2020 World Congress in Ultrasound in Obstetrics and Gynecology.

References

- [1] Kemp MW. Preterm birth, intrauterine infection, and fetal inflammation. *Frontiers in Immunology*. 2014; 5: 574.
- [2] Namouz S, Porat S, Okun N, Windrim R, Farine D. Emergency cerclage: literature review. *Obstetrical & Gynecological Survey*. 2013; 68: 379–388.
- [3] Romero R, Espinoza J, Erez O, Hassan S. The role of cervical cerclage in obstetric practice: can the patient who could benefit from this procedure be identified? *American Journal of Obstetrics and Gynecology*. 2006; 194: 1–9.
- [4] Brown R, Gagnon R, Delisle MF. No. 373-Cervical Insufficiency and Cervical Cerclage. *Journal of Obstetrics and Gynecology Canada*. 2019; 41: 233–247.
- [5] Boelig RC, Berghella V. Current options for mechanical prevention of preterm birth. *Seminars in Perinatology*. 2017; 41: 452–460.
- [6] Abdel-Aleem H, Shaaban OM, Abdel-Aleem MA, Aboelfadle Mohamed A. Cervical pessary for preventing preterm birth in singleton pregnancies. *The Cochrane Database of Systematic Reviews*. 2022; 12: CD014508.
- [7] Diago Almela VJ, Martinez-Varea A, Perales-Puchalt A, Alonso-Diaz R, Perales A. Good prognosis of cerclage in cases of cervical insufficiency when intra-amniotic inflammation/infection is ruled out. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2015; 28: 1563–1568.
- [8] Lee SE, Romero R, Park CW, Jun JK, Yoon BH. The frequency and significance of intraamniotic inflammation in patients with cervical insufficiency. *American Journal of Obstetrics and Gynecology*. 2008; 198: 633.e1–633.e8.

- [9] Ahmadi A, Khodabandehloo M, Ramazanzadeh R, Farhadifar F, Nikkhoo B, Soofizade N, *et al.* Association between Ureaplasma urealyticum endocervical infection and spontaneous abortion. *Iranian Journal of Microbiology.* 2014; 6: 392–397.
- [10] Bujold E, Morency AM, Rallu F, Ferland S, Tétu A, Duperron L, *et al.* Bacteriology of amniotic fluid in women with suspected cervical insufficiency. *Journal of Obstetrics and Gynaecology Canada.* 2008; 30: 882–887.
- [11] Lisonkova S, Sabr Y, Joseph KS. Diagnosis of subclinical amniotic fluid infection prior to rescue cerclage using gram stain and glucose tests: an individual patient meta-analysis. *Journal of Obstetrics and Gynaecology Canada.* 2014; 36: 116–122.
- [12] Pagni L, Pietrasanta C, Acaia B, Merlo D, Ronchi A, Ossola MW, *et al.* Chorioamnionitis and neonatal outcome in preterm infants: a clinical overview. *The Journal of Maternal-fetal & Neonatal Medicine.* 2016; 29: 1525–1529.
- [13] Kim CJ, Romero R, Chaemsaitong P, Chaiyasit N, Yoon BH, Kim YM. Acute chorioamnionitis and funisitis: definition, pathological features, and clinical significance. *American Journal of Obstetrics and Gynecology.* 2015; 213: S29–S52.
- [14] Lee Y, Kim HJ, Choi SJ, Oh SY, Kim JS, Roh CR, *et al.* Is there a stepwise increase in neonatal morbidities according to histological stage (or grade) of acute chorioamnionitis and funisitis?: effect of gestational age at delivery. *Journal of Perinatal Medicine.* 2015; 43: 259–267.
- [15] Mastrolia SA, Erez O, Loverro G, Di Naro E, Weintraub AY, Tirosh D, *et al.* Ultrasonographic approach to diagnosis of fetal inflammatory response syndrome: a tool for at-risk fetuses? *American Journal of Obstetrics and Gynecology.* 2016; 215: 9–20.
- [16] Lau J, Magee F, Qiu Z, Houbé J, Von Dadelszen P, Lee SK. Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than chorioamnionitis displaying a maternal inflammatory response only. *American Journal of Obstetrics and Gynecology.* 2005; 193: 708–713.
- [17] Oh KJ, Lee KA, Sohn YK, Park CW, Hong JS, Romero R, *et al.* Intraamniotic infection with genital mycoplasmas exhibits a more intense inflammatory response than intraamniotic infection with other microorganisms in patients with preterm premature rupture of membranes. *American Journal of Obstetrics and Gynecology.* 2010; 203: 211.e1–211.e8.
- [18] Buhimschi IA, Nayeri UA, Laky CA, Razeq SA, Dulay AT, Buhimschi CS. Advances in medical diagnosis of intra-amniotic infection. *Expert Opinion on Medical Diagnostics.* 2013; 7: 5–16.
- [19] Genç MR, Ford CE. The clinical use of inflammatory markers during pregnancy. *Current Opinion in Obstetrics & Gynecology.* 2010; 22: 116–121.
- [20] Cobo T, Kacerovsky M, Holst RM, Hougaard DM, Skogstrand K, Wennerholm UB, *et al.* Intra-amniotic inflammation predicts microbial invasion of the amniotic cavity but not spontaneous preterm delivery in preterm prelabor membrane rupture. *Acta Obstetrica et Gynecologica Scandinavica.* 2012; 91: 930–935.
- [21] Lee J, Lee JE, Choi JW, Han MH, Seong SY, Park KH, *et al.* Proteomic Analysis of Amniotic Fluid Proteins for Predicting the Outcome of Emergency Cerclage in Women with Cervical Insufficiency. *Reproductive Sciences.* 2020; 27: 1318–1329.
- [22] Weiner CP, Lee KY, Buhimschi CS, Christner R, Buhimschi IA. Proteomic biomarkers that predict the clinical success of rescue cerclage. *American Journal of Obstetrics and Gynecology.* 2005; 192: 710–718.
- [23] Parry S, Leite R, Esplin MS, Bukowski R, Zhang H, Varner M, *et al.* Cervicovaginal fluid proteomic analysis to identify potential biomarkers for preterm birth. *American Journal of Obstetrics and Gynecology.* 2020; 222: 493.e1–493.e13.
- [24] Hong S, Lee JE, Kim YM, Park Y, Choi JW, Park KH. Identifying potential biomarkers related to pre-term delivery by proteomic analysis of amniotic fluid. *Scientific Reports.* 2020; 10: 19648.
- [25] Gravett MG, Novy MJ, Rosenfeld RG, Reddy AP, Jacob T, Turner M, *et al.* Diagnosis of intra-amniotic infection by proteomic profiling and identification of novel biomarkers. *The Journal of the American Medical Association.* 2004; 292: 462–469.
- [26] Zevin AS, Xie IY, Birse K, Arnold K, Romas L, Westmacott G, *et al.* Microbiome Composition and Function Drives Wound-Healing Impairment in the Female Genital Tract. *PLoS Pathogens.* 2016; 12: e1005889.
- [27] Arnold KB, Burgener A, Birse K, Romas L, Dunphy LJ, Shahabi K, *et al.* Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells. *Mucosal Immunology.* 2016; 9: 194–205.
- [28] Birse K, Arnold KB, Novak RM, McCorrister S, Shaw S, Westmacott GR, *et al.* Molecular Signatures of Immune Activation and Epithelial Barrier Remodeling Are Enhanced during the Luteal Phase of the Menstrual Cycle: Implications for HIV Susceptibility. *Journal of Virology.* 2015; 89: 8793–8805.
- [29] Birse KD, Romas LM, Guthrie BL, Nilsson P, Bosire R, Kiarie J, *et al.* Genital Injury Signatures and Microbiome Alterations Associated With Depot Medroxyprogesterone Acetate Usage and Intravaginal Drying Practices. *The Journal of Infectious Diseases.* 2017; 215: 590–598.
- [30] Govia RNM, Birse KD, Sepeshri S, Khafipour E, Menticoglou SM, Burgener AD, *et al.* Amniotic fluid proteomic signatures of cervical insufficiency and their association with length of latency. *American Journal of Reproductive Immunology.* 2018; 80: e13030.
- [31] Romero R, Kusanovic JP, Espinoza J, Gotsch F, Nhan-Chang CL, Erez O, *et al.* What is amniotic fluid ‘sludge’? *Ultrasound in Obstetrics & Gynecology.* 2007; 30: 793–798.
- [32] Kusanovic JP, Romero R, Martinovic C, Silva K, Erez O, Maymon E, *et al.* Transabdominal collection of amniotic fluid “sludge” and identification of *Candida albicans* intra-amniotic infection. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2018; 31: 1279–1284.
- [33] Bujold E, Pasquier JC, Simoneau J, Arpin MH, Duperron L, Morency AM, *et al.* Intra-amniotic sludge, short cervix, and risk of preterm delivery. *Journal of Obstetrics and Gynaecology Canada.* 2006; 28: 198–202.
- [34] Espinoza J, Gonçalves LF, Romero R, Nien JK, Stites S, Kim YM, *et al.* The prevalence and clinical significance of amniotic fluid ‘sludge’ in patients with preterm labor and intact membranes. *Ultrasound in Obstetrics & Gynecology.* 2005; 25: 346–352.
- [35] Battarbee AN, Ellis JS, Manuck TA. Beyond Cervical Length: Association between Postcerclage Transvaginal Ultrasound Parameters and Preterm Birth. *American Journal of Perinatology.* 2019; 36: 1317–1324.
- [36] Paules C, Moreno E, Gonzales A, Fabre E, González de Agüero R, Oros D. Amniotic fluid sludge as a marker of intra-amniotic infection and histological chorioamnionitis in cervical insufficiency: a report of four cases and literature review. *The Journal of Maternal-Fetal & Neonatal Medicine.* 2016; 29: 2681–2684.
- [37] Yasuda S, Tanaka M, Kyojuka H, Suzuki S, Yamaguchi A, Nomura Y, *et al.* Association of amniotic fluid sludge with preterm labor and histologic chorioamnionitis in pregnant Japanese women with intact membranes: A retrospective study. *The Journal of Obstetrics and Gynaecology Research.* 2020; 46: 87–92.
- [38] Romero R, Schaudinn C, Kusanovic JP, Gorur A, Gotsch F,

- Webster P, *et al.* Detection of a microbial biofilm in intraamniotic infection. *American Journal of Obstetrics and Gynecology*. 2008; 198: 135.e1–135.e5.
- [39] Gorski LA, Huang WH, Iriye BK, Hancock J. Clinical implication of intra-amniotic sludge on ultrasound in patients with cervical cerclage. *Ultrasound in Obstetrics & Gynecology*. 2010; 36: 482–485.
- [40] Park JC, Kim DJ, Kwak-Kim J. Upregulated amniotic fluid cytokines and chemokines in emergency cerclage with protruding membranes. *American Journal of Reproductive Immunology*. 2011; 66: 310–319.
- [41] Son GH, You YA, Kwon EJ, Lee KY, Kim YJ. Comparative Analysis of Midtrimester Amniotic Fluid Cytokine Levels to Predict Spontaneous Very Pre-term Birth in Patients with Cervical Insufficiency. *American Journal of Reproductive Immunology*. 2016; 75: 155–161.
- [42] Jung EY, Park KH, Lee SY, Ryu A, Joo JK, Park JW. Predicting outcomes of emergency cerclage in women with cervical insufficiency using inflammatory markers in maternal blood and amniotic fluid. *International Journal of Gynaecology and Obstetrics*. 2016; 132: 165–169.
- [43] Hatakeyama Y, Miura H, Sato A, Onodera Y, Sato N, Shimizu D, *et al.* Neutrophil elastase in amniotic fluid as a predictor of preterm birth after emergent cervical cerclage. *Acta Obstetrica et Gynecologica Scandinavica*. 2016; 95: 1136–1142.
- [44] Boyle KK, Wood S, Tarity TD. Low-Virulence Organisms and Periprosthetic Joint Infection-Biofilm Considerations of These Organisms. *Current Reviews in Musculoskeletal Medicine*. 2018; 11: 409–419.
- [45] Ádám A, Pál Z, Terhes G, Szűcs M, Gabay ID, Urbán E. Culture- and PCR-based detection of BV associated microbiological profile of the removed IUDs and correlation with the time period of IUD in place and the presence of the symptoms of genital tract infection. *Annals of Clinical Microbiology and Antimicrobials*. 2018; 17: 40.
- [46] Yoneda N, Yoneda S, Niimi H, Ito M, Fukuta K, Ueno T, *et al.* Sludge reflects intra-amniotic inflammation with or without microorganisms. *American Journal of Reproductive Immunology*. 2018; 79: e12807.