UNIVERSITY OF PARDUBICE

FACULTY OF CHEMICAL TECHNOLOGY

Department of Biological and Biochemical Sciences

Petra Zedníková

Identification of new potential biomarkers of preterm birth in amniotic fluid

Theses of the Doctoral Dissertation

Pardubice 2020

Study program: **Analytical chemistry**

Study field: **Analytical chemistry**

Author: **Mgr. Petra Zedníková**

Supervisor: **prof. Ing. Alexander Čegan, CSc.**

Supervisor - Specialist: **PharmDr. Vojtěch Tambor, Ph.D.**

Specialist training workplace**: Biomedical Research Centre of the University Hospital Hradec Králové**

Year of dissertation defence: 2020

References

ZEDNÍKOVÁ, Petra. *Identification of new potential biomarkers of preterm birth in amniotic fluid*. Pardubice, 2020. 135 pages. Dissertation thesis (Ph.D.). The University of Pardubice, Faculty of Chemical Technology, Department of Biological and Biochemical Sciences. Supervisor prof. Ing. Alexander Čegan, CSc., Supervisor – specialist PharmDr. Vojtěch Tambor, Ph.D.

Abstract

Preterm birth is the birth of a baby less than 37 weeks of gestational age. It accounts for the main reason for perinatal mortality and long-term morbidity. A reliable screening tool that could reduce the incidence by early diagnosis has not been found yet. The aim of this project is to analyse samples of amniotic fluid using the proteomic shotgun accesses and show the list of potential predictive markers suitable for further investigation. The results of this study showed 18 significantly dysregulated proteins.

Keywords

preterm birth, amniotic fluid, biomarkers, proteomics

Abstrakt

Předčasný porod, tedy porod před ukončeným 37. týdnem těhotenství, je hlavním důvodem novorozenecké úmrtnosti a nemocnosti. V současné době nejsou dostupné vhodné predikční markery pro včasné odhalení rizika předčasného porodu. Cílem této práce je analyzovat vzorky plodových vod pomocí proteomických metod a poskytnout možný panel dysregulovaných proteinů vhodných pro další studium. V této studii bylo odhaleno 18 signifikantně dysregulovaných proteinů.

Klíčová slova

předčasný porod, plodová voda, biomarkery, proteomika

Table of Contents

Introduction

Preterm birth (PB), defined as a delivery of a baby before 37 weeks of gestation, accounts for 70 - 80 % of all perinatal mortality and more than half of the long-term morbidity in neonates. It increases the risk of neurodevelopmental, respiratory, and gastrointestinal complications as well as other serious perinatal complications.

The impact of PB consequences is not only on the individuals, families, and society but it also has a significant cost impact on whole perinatal healthcare systems.

Preterm birth could be systematically divided into two main groups: induced preterm delivery and spontaneous preterm birth. This study focuses on the second group, the spontaneous preterm births.

The precise mechanisms of PB are still unknown despite advancing knowledge of related risk factors and mechanisms. PB is thought to be a multifactorial syndrome caused by many factors. Current studies suggest the main initiator could be an inflammatory response.

The early identification of women with increased risk of PB is crucial for the optimization of prenatal management. Despite the intense research of predictive markers of PB using demographic risk factors, biochemical markers, and physiological markers, the predictive values are insufficient or ineffective, and precise screening tools are still to be found.

In this regard, proteomics can be very beneficial with its unbiased view on the protein change associated with the disease. Proteomics allows identifying a number of proteins with the ability to quantify changes in their abundance. The possibility of running the analysis across multiple samples makes proteomics a very promising tool for biomarker research.

1. The problematic of preterm birth

Preterm birth, defined as a delivery before 37 weeks of gestation, accounts for 70 - 80 % of all perinatal mortality and more than half of the long-term morbidity in neonates. PB increases the risk of neurodevelopmental, respiratory, and gastrointestinal complications as well as other serious perinatal complications. Around 10 % of neonates worldwide are born preterm, and 1,5 % of these children die each year due to the consequences of PB (Goldenberg et al. 2008). The PB rate in the Czech Republic in 2017 was at 7,8 % (Marešová 2014; Dudasova et al. 2019).

The impact of PB consequences is not only on the individuals, families, and society but it also has a significant cost impact on whole perinatal healthcare systems (Keelan and Newnham 2017).

Preterm birth could be systematically divided into two main groups. The first group, responsible for approximately 25 % of all PB, is induced preterm delivery due to maternal and/or fetal complications. The main reason for pregnancy termination (most commonly via caesarean section) are preeclampsia or intrauterine fetal growth restriction. The second group is the spontaneous preterm birth group, which includes preterm premature rupture of the membranes (PPROM; 25-30 %) and preterm delivery with intact membranes (40 - 45 %)(Kacerovský and Musilová 2013).

The precise mechanisms of PB are still unknown despite advancing knowledge of related risk factors and mechanisms. PB is thought to be a multifactorial syndrome caused by many factors including:

- genetic predisposition,
- racial or ethnic background,
- low BMI (less than 19, more than 30 can have a protective effect on fetus),
- low socioeconomic status and education,
- age of the mother (under 20 or over 35),
- smoking or drug abuse,
- long term stress, some vaginal infections or microbial colonisation of birth canal (usually *Ureaplasma urealyticum, Mycoplasma hominis, Streptococcus agalactiae, Trichomonas vaginalis* and *Chlamydia trachomatis*),
- some other infections (pyelonephritis, asymptomatic bacteriuria, pneumonia, appendicitis),
- preterm birth (spontaneous PB or PPROM) in a previous pregnancy,
- uterus abnormalities,
- multiple pregnancy,
- vaginal bleeding during current pregnancy,
- pregnancy resulting from *in vitro* fertilization,
- previous uterus or cervix operation,
- previous abortion after 16th week of gestation

and many other still unknown factors (Musilová et al. 2011; Goldenberg et al. 2008; Koucký et al. 2014; Marešová 2014; Dudasova et al. 2019).

The initiation of preterm birth could be induced according to current knowledge by inflammation (Koucký et al. 2014; Dudasova et al. 2019), which can be a result of several factors:

- infection,
- uteroplacental ischemia,
- disorder of the fetus' immunological tolerance,
- allergies,
- excessive uterine span,
- cervix incompetence or gestagen and CRH (corticotrophin-releasing hormone) metabolism disorder (Koucky et al. 2009).

However, these risk factors are only a preliminary indicator and there is still a large group of asymptomatic preterm births with unknown initiators (Marešová 2014).

Despite the intense research of predictive markers of PB using demographic risk factors, biochemical markers, and physiological markers such as cervical length and prior spontaneous PB (which are currently the most reliable markers), the results are insufficient or ineffective, and precise screening tools are still to be found.

Currently, a combination of biomarkers and other predictive factors is used in clinical practice (anamnesis, **cervicovaginal cervical length screening**, **ultrasound** and analysis of the biomarkers **fFN** (fetal fibronectin), **IGFBP-1** (insulin like growth factor binding protein or its phosphorylated part) and **PAMG-1** (placental alpha macroglobulin)). It is necessary to consider the results of the analysis in the overall anamnesis. The decisive factor is still the length of the cervix, often in combination with the cultivation of a cervical smear (Dudasova et al. 2019).

Although the current criteria are able to partially detect the risk in a certain group of patients, they still have a low specificity. Biomarkers such as fFN, IGFBP-1 and PAMG-1 can be used in specific situations and are still not fully conclusive. Ultrasound measurement of the cervix is a well-established method for predicting preterm birth. However, the sensitivity and positive predictive value of ultrasound cervicometry remains relatively unsatisfactory. How to detect the risk of asymptomatic PB remains a problem.

Despite all the efforts of many research groups from various scientific disciplines, a panel of dysregulated markers has not been set up yet. However, the findings of these studies contribute to understanding the complexity of preterm birth and continue to move us forward.

The early identification of women with increased risk of PB is crucial for the optimization of prenatal management. In this regard, proteomics can be very beneficial with its unbiased view on the protein change associated with the disease. Proteomics allows identifying a large number of proteins with the ability to quantify changes in their abundance. The possibility of running the analysis across multiple samples makes proteomics a very promising tool for biomarker research (Tambor et al. 2013).

2. The aims of this study

- Developing a strategy for amniotic fluid analysis
- Developing a method using multidimensional technology for lowering sample complexity
- Optimizing the methods for lowering sample complexity – immunodepletion, high pH fractionation
- Preparing samples for proteomic analysis using multidimensional strategies and iTRAQ based quantification
- Analysing the samples using the LC-MS/MS approach
- Optimizing a method for data analysis
- Analysing the obtained data

3. The experimental part: Proteomic analysis of amniotic fluid samples

This part is only a brief overview of the experimental section of the study. Further specific details regarding the sample preparation, analysis setups, and data analysis can be found in the thesis. The experimental part workflow is shown in the scheme below (Figure 1).

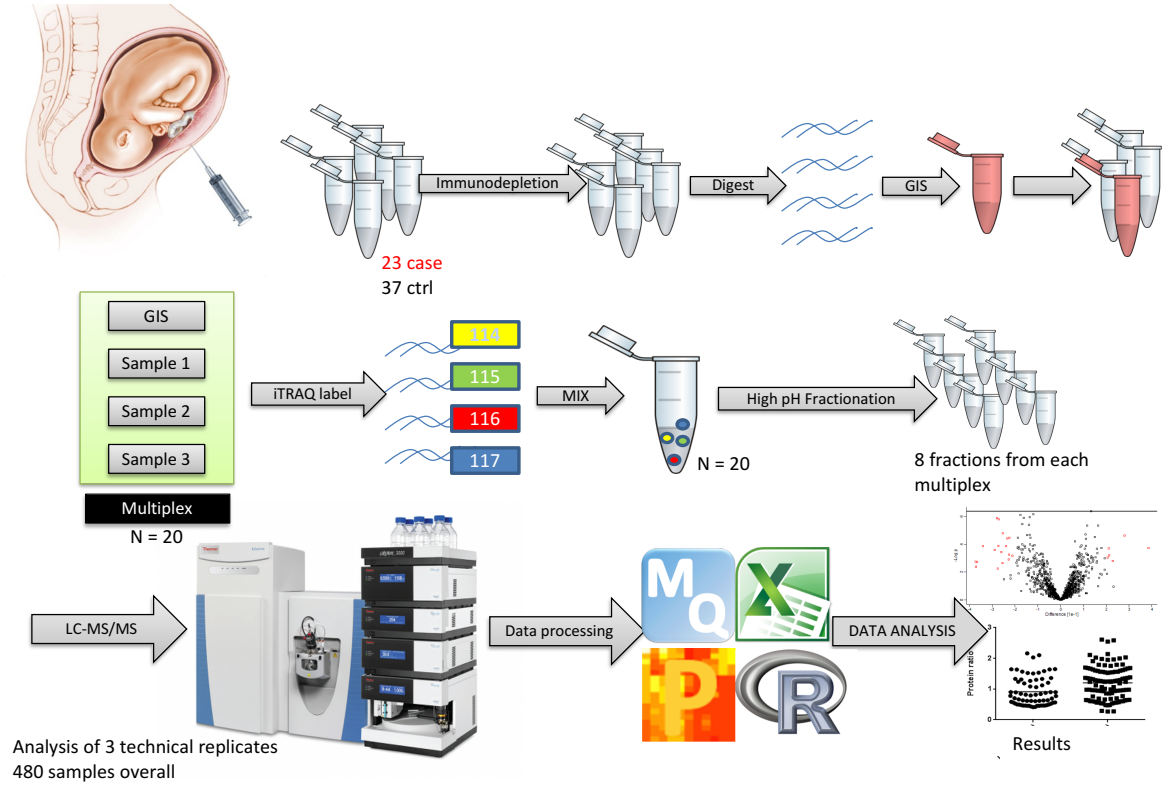

Figure 1: The experimental workflow of the proteomic LC-MS/MS analysis of amniotic fluid

3.1 Sample collection and primary processing

Ultrasound-guided mid-trimester genetic transabdominal amniocentesis was performed in all participants at the 14th -17th week of gestation. Based on the pregnancy outcome, women were retrospectively categorized into two groups; women with subsequent spontaneous PB (cases) and women with delivery at term (controls). The samples were centrifuged, and the supernatant was divided into aliquots and frozen at -80°C. These steps were not performed by the author of this doctoral thesis.

3.2 Immunodepletion

Each sample was supplemented with a protease inhibitor and filtered using a 0.22 µm centrifugal filter to remove residual solid particles. Total protein concentration was determined using the BCA Protein Assay Kit. An equal amount of protein was taken from each sample for the immunodepletion of the 14 most abundant proteins using the Multiple Affinity Removal System (MARS) with MARS HU-14

column (MARS Hu-14, 4.6x100mm, Agilent, Palo Alto, USA) according to the manufacturer's instructions.

3.3 Digestion and labelling

Samples were supplemented with 0.1 % RapiGest, reduced with 250 mM triethylammonium bicarbonate (TEAB) 1 M and 5 mM tris-(2-carboxyethyl) phosphine (TCEP) and the thiol groups were blocked with S–methyl methanethiosulfonate (MMTS). Protein samples were digested with rLysC for 4 hours at 37°C and, finally, trypsin was added and incubated overnight at 37°C. For the isobaric labelling, the iTRAQ 4 -plex kit was used according to the manufacturer's instructions.

3.4 High pH fractionation

In total, 20 multiplexes were prepared. Each multiplex contained GIS (global internal standard, the pool of all samples) at the 114 iTRAQ channel and 3 individual amniotic fluid samples at iTRAQ channels 115, 116 and 117. For lowering the sample complexity, high pH fractionation was performed on the analytical UltiMate3000 HPLC system. Peptides were separated in a linear gradient formed by 2% ACN, 20 nM ammonium formate (mobile phase A) and 80% ACN with 20 nM ammonium formate (mobile phase B) in a 40 min gradient from 3-50 % of mobile phase B at a flow rate 0.3 ml/min on the Xterra MS C18 column (3.5 µm, 2.1x100mm; Waters) into 32 fractions. Fractions were collected between the 6^{th} and 30^{th} minutes. These separated fractions were subsequently mixed into 8 final fractions.

3.5 LC-MS/MS analysis

Fractions were analysed by the UltiMate 3000 RSLCnano system connected to a Q-Exactive Plus mass spectrometer. Samples were loaded on a PepMap100 ViperTrap (3 µm, 100 A) precolumn for desalting (by gradient of 2% ACN, 0.1% TFA (load mobile phase A) and 100% ACN (load mobile phase B) and then eluted on an analytical column Acclaim PepMap RSLC (75 µm, 50 cm, C18, 2 µm) for separation in a linear gradient formed by 2% ACN, 0,1% FA (nano mobile phase A) and 80% ACN, 0,1% FA (nano mobile phase B), from 6 to 44 % of the nano mobile phase B in 60 minutes at a flow rate of 200 nl/min. The MS analysis was performed in the Information Dependant Acquisition (IDA) mode using the full MS/Top 10 experimental setup. All samples were analysed in 3 technical replicates.

3.6 Data analysis

Peptide identification and quantification was conducted in the Max Quant software using the reverse decoy mode and the integrated false discovery rate (FDR) analysis. The data was searched against the UniProtKB/Swiss-Prot database.

Intensities of iTRAQ reporter ions were corrected using isotope correction factors supplied with the iTRAQ kit. Following the described procedure, the primary data set was obtained. The average number of identifications per multiplex is displayed in the table below (Table 1).

Table 1: The average number of identifications per multiplex after LC-MS/MS analysis

Identifications Item	Number of IDs (Coefficient of variance %)
the number of unique peptide sequences	10945 (CV 14 %)
the number of modified peptide sequences	14258 (CV 15 %)
the number of protein groups	2100 (CV 9 %)
search inputs (MS/MS submitted)	231993 (CV 4 %)
the PSM(s) (MS/MS identified)	43854 (CV 16 %)

From all the reporter ions intensities, the ration of sample to global internal standard (GIS) was calculated. The data set was further filtered from the proteins identified based on the decoy (reverse) database and from potential contaminants. After the filtering, the data set contained 3185 proteins identified overall in all three replicates.

The primary data set was further filtered based on three conditions.

1) Minimum two valid values in the three replicates: 58 % of all values were filtered in this step (as shown in Figure 2)

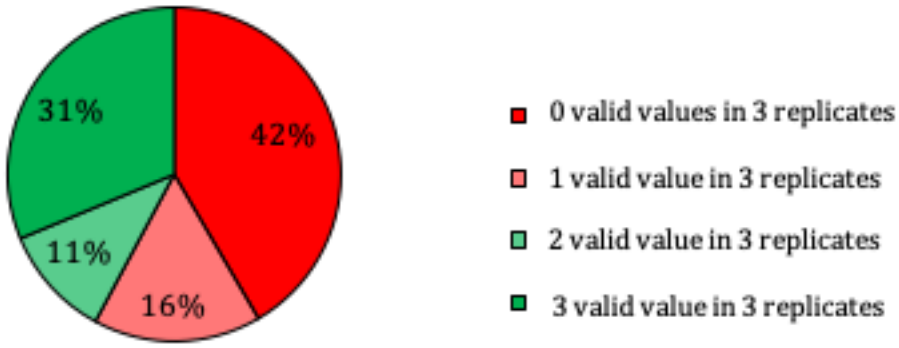

Figure 2: Filtering condition I: number of valid values across 3 replicates

2) The coefficient of variance (CV) between values in triplicates less than 20 %: Only 2 % of the primary data set were filtered in this step (as shown in Figure 3)

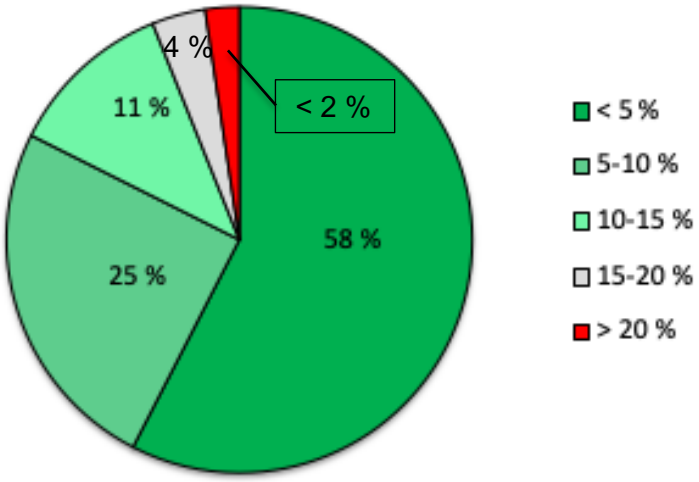

Figure 3: Filtering condition II: The distribution of the coefficient of variance across 3 replicates

3) Both previous filtering criteria must be met in at least 75 % in both groups.

Due to the filtering steps, 8 % of data were missing and must be temporarily imputed before normalization. After the imputation, data were normalized using the loess normalization in R. Missing values were then restored in the normalized data set and the mean of the replicates were calculated and used in the statistical analysis. For the identification of dysregulated proteins, the combination of the difference between the group means (case vs. control) and the p-value was chosen. The p-value was calculated using the simple t-test for independent observation using the average value from all three technical replicates.

4. Results and Discussion

The aim of this study was to analyse samples of amniotic fluid obtained by transabdominal amniocentesis between the 14th and 17th week of gestation using proteomic approaches and try to determine if there are some potential biomarkers for preterm birth prediction in this stage of pregnancy.

Amniotic fluid represents a very promising source of potential biomarkers of a wide range of pregnancy related disorders. Unfortunately, the high complexity of this material prevents an easy and straightforward detection of high promising molecules. In order to overcome this high complexity and to identify a large number of proteins from the low abundance proteome, we have used a multidimensional approach based on immunoaffinity depletion and high pH fractionation followed by proteomic LC-MS/MS analysis based on iTRAQ quantification for 60 amniotic fluid samples (23 samples in the preterm birth group – case, and 37 samples in the control group).

All of the laboratory work (except from sample collection and primary filtering and the BCA analysis) as well as all the optimization steps, the final sample and data analysis were performed by the author of this doctoral thesis.

The proteomic LC-MS/MS analysis revealed a total number of 43 854 MS/MS spectra which correspond to 3185 protein groups (after filtering for reverse and contaminant proteins and combining data from all 3 technical replicates). Next, selection criteria were applied to identify the most likely protein candidates in mid-trimester amniotic fluid that may be correlated with spontaneous PB. The final data set after filtering consisted of 1111 quantified proteins with at least 2 valid values out of 3 technical replicates and CV < 20% in at least 75% in both groups (preterm birth group and control group). After the filtering missing data was imputed before normalization and subjected to loess normalization in R.

The data was displayed in the form of a volcano plot, which projects the connection of the p-value and the mean difference between compared groups. The data is shown in the graph below (Figure 4)

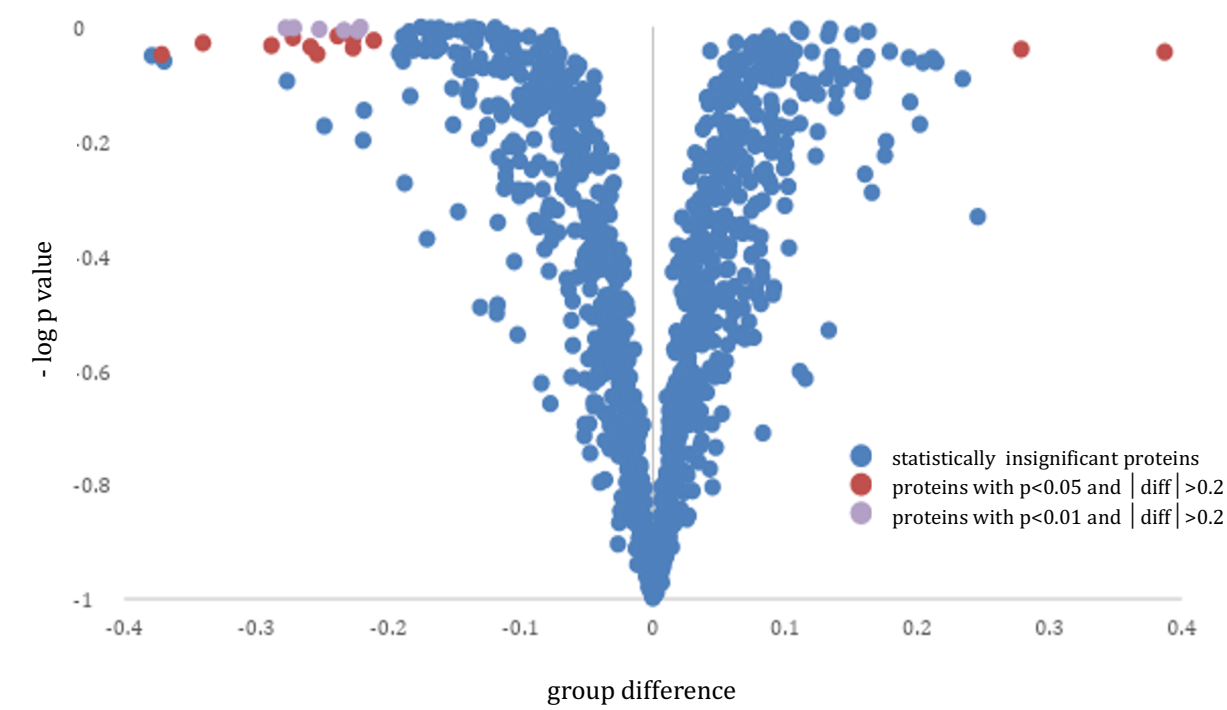

Figure 4: The final data visualisation in form of a volcano plot (blue dots represent insignificant proteins, red dots represent dysregulated proteins with p-value < 0.05 and absolute group difference (|diff|) > 0.2, violet dots represent proteins with p-value < 0.01 and absolute group difference > 0.2

In order to maximize the chances of identifying proteins correlated to spontaneous PB, the data was filtered to require a difference of intensity ratio between cases and controls of $|\Delta| \geq 0.2$ and p- value (from simple t test) $p \geq 0{,}05$. A total of 18 proteins met these filtering criteria and were significantly dysregulated between these two groups. The list of dysregulated proteins is shown in the table below (Table 2).

Table 2: Results of the statistical analysis of proteomic data of dysregulated proteins

Protein Name	Protein Group IDs	P-value	Group Difference	Dysregulation
Lipocalin-15	Q6UWW0	0,04417	0,38700	up
Chorionic somatomammotropin hormone 2	P0DML3; P0DML2; Q14406	0,03932	0,27849	up
Plastin-2	P13796	0,02396	-0,21126	down
Semaphorin-3B	Q13214	0,00099	-0,22140	down
Urotensin-2	O95399	0,00716	-0,22344	down
Beta-galactoside alpha-2,6-sialyltransferase 1	P15907	0,02137	-0,22675	down
Insulin-like growth factor-binding protein 5	P24593	0,03701	-0,22694	down
Protein S100-A6	P06703	0,00620	-0,23385	down
Rho GDP-dissociation inhibitor 2	P52566	0,01597	-0,23855	down
FERM and PDZ domain-containing protein 1	Q5SYB0	0,00440	-0,25244	down
Cathelicidin antimicrobial peptide	P49913	0,04774	-0,25402	down
Glutathione peroxidase 3	P22352	0,03509	-0,25909	down
Plasminogen activator inhibitor 1	P05121	0,00192	-0,27168	down
Neutrophil defensin 3	P59666; P59665	0,01900	-0,27238	down
Extracellular superoxide dismutase [Cu-Zn]	P08294	0,00227	-0,27784	down
Neutrophil gelatinase-associated lipocalin	P80188	0,03269	-0,28865	down
Insulin-like growth factor-binding protein 7	Q16270	0,02842	-0,34045	down
Keratin, type II cytoskeletal 2 epidermal	P35908	0,04849	-0,37175	down

Two of these proteins (Lipocalin-15 and Chorionic somatomammotropin hormone 2) were upregulated, the rest were downregulated in the preterm birth group.

Some of these proteins were already mentioned in other studies focused on pregnancy complications, however most of these studies analysed other biological materials than amniotic fluid like cervicovaginal fluid (Plastin-2 and Rho GDP-dissociation inhibitor 2) or maternal serum or placenta (Liebler 2002). The plasminogen activator inhibitor is connected to embryo development, and there are several studies suggesting its role in early abortion, preeclampsia, intrauterine growth restriction or pregnancy *diabetes mellitus* (Gianazza et al. 2007; Chen et al. 2015). In some studies, this protein was only identified, others reported an upregulated level in comparison with the healthy control, however in the maternal serum and not in amniotic fluid (Ye et al. 2017).

Cathelicidin antimicrobial peptide and Neutrophil gelatinase-associated lipocalin were reported in studies focusing on preterm rupture of the membranes. Their levels were downregulated in groups with histological chorioamnionitis and microbial invasion, which is a risk factor for preterm birth (Tambor et al. 2012; 2013).

The results of our study showed different trends. However, the comparison of these studies is complicated. There were different proteomic and statistical approaches used, the samples were collected in different stages of pregnancy.

But even though comparing the results of studies is difficult, all the results reported dysregulation of these proteins, which suggests a potential for further investigation for biomarker research.

Unfortunately, many studies still offer only a list of found peaks without further identification or quantification (Stella et al. 2009). This fact is one very important obstacle to the development of new biomarkers.

With the onset of "*omic*" approaches the reported number of potential biomarkers has risen rapidly. This could significantly help patients in treatment and also greatly reduce its overall cost. To ensure the biomarker becomes useful in clinical practice, it must go through a phase of identification, quantification and further confirmation and validation on hundreds of different samples and ideally also by different methods (which can be very complicated due to the very low repeatability and comparability of the results obtained by different methods) and, of course, through the phase of clinical testing, which is very time and finance consuming. The analysis of new potential candidates must be reproducible, and primarily highly sensitive and specific. All of these factors must be already accounted for in the planning of the study. There are lots of studies terminated after the first (discovery) phase (either intentionally or due to lack of finance, samples, time, etc.), so unfortunately most of the published potential biomarkers which have not been further quantified and / or validated and as a result, lose their relevance. However, these kinds of results could at least help with a better understanding of given pathological conditions such as pregnancy complications for example preterm birth (Drucker and Krapfenbauer 2013).

In our study we have tried to avoid all the above-mentioned pitfalls. By analysing 60 samples of amniotic fluid, this study is among the largest in the field with a focus on preterm birth and its biomarkers in early pregnancy. For comparison, similar studies, i.e. bottom-up proteomic studies focused on pregnancy complications (PPROM, preeclampsia, Down s, Turner s and Klinefelter's syndrome or PB) performed on amniotic fluid collected at an early stage, i.e. 15^{th} - 20^{th} gestational week, were based on the analysis of 10 to 54 samples (20 on average) (Vuadens et al. 2003; Vascotto et al. 2007; Anagnostopoulos et al. 2010; Mavrou et al. 2008; Park et al. 2010; Cho et al. 2010; 2011; Cheng et al. 2011; Martínez-Morillo et al. 2012). An analysis of 71 proteomic studies performed on amniotic fluid by different technologies shows that on average, these studies have analysed 31 samples (unpublished data from our upcoming review). Although there are also some extensive studies analysing 258 samples (Bujold et al. 2008).

Proteomic analysis was performed using multidimensional approaches to reduce the complexity of the samples and as many procedures as possible were subject to a standardized protocol.

The results of this study were further subjected to a validation and replication phase. 9 proteins were subsequently studied by ELISA in collaboration with a Swedish team.

The dysregulation of two of these proteins (Neutrophil gelatinase-associated lipocalin and Plasminogen activator inhibitor 1) was confirmed in a validation phase, which corresponds with the results of the proteomic exploratory analysis. Additionally, the two proteins were shown to be significantly decreased in the positive group of patients compared to the control group. The replication phase, which was performed on a different cohort of 20 positive (case) and 40 negative (control) samples, no longer revealed this trend (Hallingstrm et al. 2020) .

However, all these results need to be verified by subsequent detailed studies, which could lead to the compilation of panels of biomarkers to predict premature birth at such an early stage of pregnancy.

5. Conclusion

This study has brought to light important knowledge about the character of amniotic fluid in the middle early stage of pregnancy.

The strength of the study is its ingenious methodology, standardized protocols for sample handling, and, especially compared to other studies, a large number of analysed samples. However, there is a possible limitation of any study involving the analysis of amniotic fluid. Amniocentesis is performed only in special cases with suspected risks, therefore any group of patients cannot represent the wide population, either in terms of age or ethnicity (only women who were able to understand Swedish and were therefore able to sign an informed consent for the use of amniotic fluid in the study have been accepted for this study). This suggests an impossibility of generalizing the results. Nevertheless, at this stage there is no other option of obtaining samples of amniotic fluid.

Owing to the extensive studies that have been carried out in this field, a list of potential biomarkers for prediction of preterm birth can be assembled, although most of them still need to be verified by independent tests (ELISA or SRM and others). After verifying these results, it may be possible to identify these markers in another biological material than amniotic fluid. Even though the risk associated with amniocentesis is minimal, it remains an invasive method and an analysis of the biomarkers of preterm birth from maternal blood, or cervicovaginal fluids would certainly be more acceptable to patients.

Currently, a combination of several tests is used to estimate the risk of preterm birth such as measuring the length of the cervix, bedside tests for fetal detection of fetal fibronectin, insulin-like growth factor binding protein-1 (IGFBP-1), interleukin-6 and placental alpha-macroglobulin-1. The mothers medica l history and her general health are also taken into account, as well as her socio-economic status. However, overall progress in clearly identifying the causes of preterm birth, which would reduce the risks and incidence, is still complicated by the fact that it is a multifactorial syndrome and many factors are still unknown to us.

6. List of abbreviations

PB	Preterm Birth
PPROM	Preterm Premature Rupture Of Membranes
BMI	Body Mass Index
CRH	Corticotrophin-Releasing Hormone
fFN	Fetal Fibronectin
IGFBP-1	Insulin Like Growth Factor Binding Protein or Its Phosphorylated Part
PAMG-1	Placental Alpha Macroglobulin
iTRAQ	Isobaric Tag for Relative and Absolute Quantitation
LC-MS/MS	Liquid Chromatography coupled with Tandem Mass Spectrometry
BCA	Bicinchionic Acid Assay
MARS	Multiple Affinity Removal System
TEAB	Triethylammonium Bicarbonate
TCEP	Tris-(2-Carboxyethyl) Phosphine
MMTS	S–Methyl Methanethiosulfonate
rLysC	Recombinant Lysinase C Enzyme
ACN	Acetonitrile
TFA	Trifluoroacetic Acid
FA	Formic Acid
FDR	False Discovery Rate
CV	Coefficient of Variance
PSM	Peptide Spectrum Matches
GIS	Global Internal Standard

7. List of reference

ANAGNOSTOPOULOS, Athanasios K, Aggeliki KOLIALEXI, Ariadni MAVROU, Konstantinos VOUGAS, Nikos PAPANTONIOU, Aris ANTSAKLIS, Emmanuel KANAVAKIS, Michael FOUNTOULAKIS and George Th TSANGARIS, 2010. Proteomic analysis of amniotic fluid in pregnancies with Klinefelter syndrome foetuses. *Journal of proteomics* [online]. **73**(5), 943–50. ISSN 1876-7737. Available at: doi: 10.1016/j.jprot.2009.12.009

BUJOLD, Emmanuel, Roberto ROMERO, Juan Pedro KUSANOVIC, Offer EREZ, Francesca GOTSCH, Tinnakorn CHAIWORAPONGSA, Ricardo GOMEZ, Jimmy ESPINOZA, Edi VAISBUCH, Yeon MEE KIM, Samuel EDWIN, Mike PISANO, Beth ALLEN, Vladimir N PODUST, Enrique A DALMASSO, Jennifer RUTHERFORD, Wade ROGERS, Allan MOSER, Bo Hyun YOON and Tim BARDER, 2008. Proteomic profiling of amniotic fluid in preterm labor using two-dimensional liquid separation and mass spectrometry. *The journal of maternal-fetal & neonatal medicine: the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians* [online]. **21**(10), 697–713. ISSN 1476-4954. Available at: doi:10.1080/14767050802053289

DRUCKER, Elisabeth and Kurt KRAPFENBAUER, 2013. *Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalised medicine* [online]. 2013. ISSN 18785085. Available at: doi:10.1186/1878-5085-4-7

DUDASOVA, J, P SIMJAK, M KOUCKY and A PARIZEK, 2019. Current options of prediction of preterm labour. *Česká gynekologie*. **84**(5), 355–360. ISSN 1210-7832 (Print).

GIANAZZA, Elisabetta, Robin WAIT, Shaina BEGUM, Ivano EBERINI, Monica CAMPAGNOLI, Sara LABÒ and Monica GALLIANO, 2007. Mapping the 5-50-kDa fraction of human amniotic fluid proteins by 2-DE and ESI-MS. *Proteomics – Clinical Applications* [online]. **1**(2), 167–175. ISSN 18628346. Available at: doi:10.1002/prca.200600543

GOLDENBERG, Robert L, Jennifer F CULHANE, Jay D IAMS and Roberto ROMERO, 2008. Epidemiology and causes of preterm birth. *The Lancet* [online]. **371**(9606), 75–84. ISSN 01406736. Available at: doi:10.1016/S0140-6736(08)60074-4

HALLINGSTRÖM, Maria, Petra ZEDNÍKOVÁ, Vojtěch TAMBOR, Malin BARMAN, Marie VAJRYCHOVÁ, Juraj LENČO, Felicia VIKLUND, Linda TANCRED, Hardis RABE, Daniel JONSSON, Alisa KACHIKIS, Staffan NILSSON, Marian KACEROVSKÝ, Kristina M ADAMS WALDORF and Bo JACOBSSON, 2020. Mid-trimester amniotic fluid proteome's association with spontaneous preterm delivery and gestational duration. *PloS one* [online]. **15**(5), e0232553. ISSN 1932-6203 (Electronic). Available at: doi:10.1371/journal.pone.0232553

CHEN, Hui, Shuping NIE and Ming LU, 2015. Association between plasminogen activator inhibitor-1 gene polymorphisms and recurrent pregnancy loss: a systematic review and meta-analysis. *American journal of reproductive immunology (New York, N.Y. : 1989)* [online]. **73**(4), 292–300. ISSN 1600-0897 (Electronic). Available at: doi:10.1111/aji.12321

CHENG, Po-Jen, Tzu-Hao WANG, Shang-Yu HUANG, Chuan-Chi KAO, Jen-Hao LU, Ching-Hwa HSIAO and S W Steven SHAW, 2011. Differential proteomics analysis of amniotic fluid in pregnancies of increased nuchal translucency with normal karyotype. *Prenatal diagnosis* [online]. **31**(3), 274–81. ISSN 1097-0223. Available at: doi:10.1002/pd.2719

CHO, Chan-Kyung J, Andrei P DRABOVICH, Ihor BATRUCH and Eleftherios P DIAMANDIS, 2011. Verification of a biomarker discovery approach for detection of Down syndrome

in amniotic fluid via multiplex selected reaction monitoring (SRM) assay. *Journal of proteomics* [online]. **74**(10), 2052–9. ISSN 1876-7737. Available at: doi:10.1016/j.jprot.2011.05.025

CHO, Chan-Kyung J, Christopher R SMITH and Eleftherios P DIAMANDIS, 2010. Amniotic fluid proteome analysis from Down syndrome pregnancies for biomarker discovery. *Journal of proteome research* [online]. **9**(7), 3574–82. ISSN 1535-3907. Available at: doi:10.1021/pr100088k

KACEROVSKÝ, M and I MUSILOVÁ, 2013. [Management of preterm prelabor rupture of membranes with respect to the inflammatory complications - our experiences]. *Česká gynekologie* [online]. **78**(6), 509–13. ISSN 1210-7832. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24372427

KEELAN, Jeff A and John P NEWNHAM, 2017. Recent advances in the prevention of preterm birth. *F1000Research* [online]. **6**. ISSN 2046-1402 (Print). Available at: doi:10.12688/f1000research.11385.1

KOUCKY, M, A GERMANOVA, Z HAJEK, A PARIZEK, M KALOUSOVA and P KOPECKY, 2009. [News in pathophysiology and management of preterm labour]. *Česká gynekologie / Česká lékařská společnost J.Ev. Purkyně*. **74**(1), 54–63. ISSN 1210-7832.

KOUCKÝ, Michal, Jan SMÍŠEK, Antonín PAŘÍZEK and Marian KACEROVSKÝ, 2014. *Spontánní předčasný porod*. Praha: Maxdorf. ISBN 978-80-7345-416-6.

LIEBLER, Daniel C, 2002. *Introduction to proteomics tools for the new biology* [online]. Totowa, NJ: Humana Press. ISBN 0585418799. Available at: http://site.ebrary.com/id/10181181

MAREŠOVÁ, Pavlína, 2014. *Moderní postupy v gynekologii a porodnictví*. Praha: Mladá Fronta: Edice Postgraduální medicíny. ISBN 978-80-204-3153-0.

MARTÍNEZ-MORILLO, Eduardo, Chan-Kyung J CHO, Andrei P DRABOVICH, Julie L v SHAW, Antoninus SOOSAIPILLAI and Eleftherios P DIAMANDIS, 2012. Development of a multiplex selected reaction monitoring assay for quantification of biochemical markers of down syndrome in amniotic fluid samples. *Journal of proteome research* [online]. **11**(7), 3880–3887. ISSN 1535-3907 (Electronic). Available at: doi:10.1021/pr300355a

MAVROU, Ariadni, Athanasios K ANAGNOSTOPOULOS, Aggeliki KOLIALEXI, Konstantinos VOUGAS, Nikos PAPANTONIOU, Aris ANTSAKLIS, Michael FOUNTOULAKIS and George Th TSANGARIS, 2008. Proteomic analysis of amniotic fluid in pregnancies with Turner syndrome fetuses. *Journal of proteome research* [online]. **7**(5), 1862–6. ISSN 1535-3893. Available at: doi:10.1021/pr700588u

MUSILOVÁ, I, M KACEROVSKÝ, V TAMBOR and J TOSNER, 2011. [Proteomics and biomarkers for detection of preterm labor: a systematic review]. *Česká gynekologie* [online]. **76**(1), 37–45. ISSN 1210-7832. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21656999

PARK, Jisook, Dong-Hyun CHA, Jin Woo JUNG, Young Hwan KIM, Sook Hwan LEE, Youngjun KIM and Kwang Pyo KIM, 2010. Comparative proteomic analysis of human amniotic fluid supernatants with Down syndrome using mass spectrometry. *Journal of microbiology and biotechnology* [online]. **20**(6), 959–67. ISSN 1017-7825. Available at: doi:10.4014/jmb.0912.12035

STELLA, Caroline L., Michael R. BENNETT, Prasad DEVARAJAN, Kenneth GREIS, Michael WYDER, Stephen MACHA, Marepalli RAO, Cristiano JODICKE, Hind MOUSSA, Helen Y. HOW, Leslie MYATT, Rose WEBSTER and Baha M. SIBAI, 2009. Preterm labor

biomarker discovery in serum using 3 proteomic profiling methodologies. *American Journal of Obstetrics and Gynecology* [online]. **201**(4), 387.e1-387.e13. ISSN 00029378. Available at: doi: 10.1016/j.ajog.2009.06.034

TAMBOR, V, M KACEROVSKY, J LENCO, G BHAT and R MENON, 2013. Proteomics and bioinformatics analysis reveal underlying pathways of infection associated histologic chorioamnionitis in pPROM. *Placenta* [online]. **34**(2), 155–161. ISSN 0143-4004. Available at: doi: 10.1016/j.placenta.2012.11.028

TAMBOR, Vojtech, Marian KACEROVSKY, Ctirad ANDRYS, Ivana MUSILOVA, Helena HORNYCHOVA, Lenka PLISKOVA, Marek LINK, Jiri STULIK and Juraj LENCO, 2012. Amniotic Fluid Cathelicidin in PPROM Pregnancies: From Proteomic Discovery to Assessing Its Potential in Inflammatory Complications Diagnosis. *PLoS ONE* [online]. **7**(7), e41164–e41164. ISSN 1932-6203. Available at: doi: 10.1371/journal.pone.0041164

VASCOTTO, Carlo, Anna Maria SALZANO, Chiara D'AMBROSIO, Arrigo FRUSCALZO, Diego MARCHESONI, Carla DI LORETO, Andrea SCALONI, Gianluca TELL and Franco QUADRIFOGLIO, 2007. Oxidized transthyretin in amniotic fluid as an early marker of preeclampsia. *Journal of proteome research* [online]. **6**(1), 160–70. ISSN 1535-3893. Available at: doi:10.1021/pr060315z

VUADENS, Françoise, Corinne BENAY, David CRETTAZ, Denis GALLOT, Vincent SAPIN, Philippe SCHNEIDER, Willy-Vincent BIENVENUT, Didier LÉMERY, Manfredo QUADRONI, Bernard DASTUGUE and Jean-Daniel TISSOT, 2003. Identification of biologic markers of the premature rupture of fetal membranes: proteomic approach. *Proteomics* [online]. **3**(8), 1521–5. ISSN 1615-9853. Available at: doi:10.1002/pmic.200300455

YE, Yao, Aurelia VATTAI, Xi ZHANG, Junyan ZHU, Christian J THALER, Sven MAHNER, Udo JESCHKE and Viktoria VON SCHÖNFELDT, 2017. Role of Plasminogen Activator Inhibitor Type 1 in Pathologies of Female Reproductive Diseases. *International Journal of Molecular Sciences* [online]. **18**(8), 1651. ISSN 1422-0067. Available at: doi:10.3390/ijms18081651

8. List of student's published work

- The results of this doctoral thesis are contained in a publication with an impact factor:

HALLINGSTRM, Maria, Petra ZEDNÍKOVÁ, Vojtěch TAMBOR, et al. Mid - trimester amniotic fluid proteome's association with spontaneous preterm delivery and gestational duration. *PLOS ONE* online. **2020**, 15(5) cit. 2020 -09-01. DOI: 10.1371/journal.pone.0232553. ISSN 1932-6203. Available at: https://dx.plos.org/10.1371/journal.pone.0232553

- The cooperation of students (Petra Zedníková – formerly Domašinská) internship with focus on data analysis used in this doctoral thesis is published in a publication with an impact factor, which is a chapter in a book:

RUPRECHT, Benjamin, Heiner KOCH, Petra DOMASINSKA, Martin FRENO, Bernhard KUSTER a Simone LEMEER. Optimized Enrichment of Phosphoproteomes by Fe-IMAC Column Chromatography. COMAI, Lucio, onathan E. KATZ a Parag MALLICK, ed. *Proteomics* online. New York, NY: Springer New York, **2017**, 2017-02-11, s. 47-60 cit. 2020 -09-01. Methods in Molecular Biology. DOI: 10.1007/978 - 1-4939-6747-65. ISBN 978 -1-4939-6745-2. Available at: http://link.springer.com/10.1007/978-1-4939-6747-65

- The author of this doctoral theses also collaborated on 2 publications with an impact factor:

TAMBOR, Vojtech, Marie VARYCHOVA, Marian KACEROVSKY, Marek LINK, Petra DOMASINSKA, Ramkumar MENON a uraj LENCO. Potential Peripartum Markers of Infectious-Inflammatory Complications in Spontaneous Preterm Birth. *BioMed Research International* online. 2015, **2015**, 1-13 cit. 2020 -09-01. DOI: 10.1155/2015/343501. ISSN 2314-6133. Available at: http://www.hindawi.com/journals/bmri/2015/343501/

currently send to redaction after second minor revision:
Marie Vajrychová, aroslav Stráník, Kristýna Pimková, Malin Barman, Rud olf Kukla, Petra Zedníková, Radka Bolehovská, Lenka Plíšková, Helena Hornychová, Ctirad Andrýs, Vojtěch Tambor, uraj Lenčo, Bo acobsson, Marian Kacerovský, *Comprehensive proteomic investigation of infectious and inflammatory changes in late preterm prelabour rupture of membranes*

- Some of the results were reported as 3 posters at international conferences:

Domašinská P., Tambor V., Kacerovský M., Lenčo ., acobsson B., Hallingstrm M., Raftery M., Vajrychová M., Čegan A., I*dentification of new potential markers of preterm birth in amniotic fluid*, HUPO Madrid 2014, Madrid, Spain, 5. – 8. 10. **2014**, p. 166

Kristýna Pimková, Marie Vajrychová, Petra Domašinská, Marian Kacerovský, uraj Lenčo, Vojtech Tambor; *A bioinformatics approach for identification and label-free quantification of endogenous peptides*; 9th European Summer School of Advanced Proteomics; 2. – 8. 8. **2015**, Brixen, Italy; p. 68-69

Maria Hallingstrm, Petra Zedníková, Vojtěch Tambor, uraj Lenco, Marie Vajrychová, Staffan Nilsson, Felicia Viklund, Linda Tancred, Teresa Cobo, Marian Kacerovský, Bo acobsson; *Proteomic Analysis In Individual Samples Of Early Mid-Trimester Amniotic Fluid In Relation To Spontaneous Preterm Delivery*, European Spontaneous Preterm Birth Congress 16-18th May **2018**, The University of Edinburgh, Edinburgh, UK

- Some of the results were presented as a lecture at a national conference:

Domašinská P., Tambor V. Vajrychová M. Kacerovský M.,Lenčo ., acobsson B., Hallingstrm M., Raftery M., Čegan A., Identifikace nových potenciálních biomarkerů předčasného porodu v plodové vodě (*Identification of new potential markers of preterm birth in amniotic fluid)*, 3. Neformální proteomické setkání (3th unformal proteomics meeting), Hradec Králové, Czech Republic 21. – 22.11. **2013**, p. 21