8-4-2021

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Placenta 112 (2021) 185–188

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Technical note

A new pipeline for clinico-pathological and molecular placental research utilizing FFPE tissues

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ARTICLE INFO

Keywords:
Formalin fixed paraffin embedded placenta
FFPE placenta
FFPE RNA
Pathology database
Placenta gene expression
Placenta pathology
Archived placenta

ABSTRACT

The placenta is at the core of many pregnancy pathologies, but we have limited knowledge about placental function because of two key research barriers: 1) lack of guidelines for sample collection and pathologic diagnosis; and 2) limited tools are available for molecular analysis of stored placental samples. We aimed to create a searchable, population-based placental database of pathologic diagnoses, and to validate molecular methods for gene expression studies of matching formalin fixed paraffin embedded (FFPE) placental blocks. Our database has over 1000 pregnancies coded for clinical diagnosis with corresponding FFPE blocks that are available for gene expression studies. RNA harvested from FFPE tissues is of sufficient quality for downstream applications. We successfully used this pipeline to identify FFPE placenta from term and preterm pregnancies, and compared their gene expression. The establishment of this platform, which links clinicopathological data and molecular gene expression, will increase our understanding of obstetrical diseases.

1. Introduction

Archival formalin fixed paraffin embedded (FFPE) tissues are a stable and inexpensive source of genetic material. Archived pathology samples have been successfully used for advanced gene expression research in many organs, but not in the human placenta. The George Washington University (GWU) has a collection of placental FFPE blocks from every pregnancy between 2012 and 2017, but their utility for molecular studies has not been systematically validated. We describe a new pipeline for clinicopathological and molecular assessment of existing, archived FFPE placental samples which 1) employs a new database of clinicopathological electronic medical records (EMRs) and 2) utilizes FFPE placenta for downstream gene expression analyses. We exploit this pipeline to query the gene expression differences between term and preterm placenta using RNA-sequencing.

2. Materials and methods

Per hospital policy between 2012 and 2017, the placenta from each delivery was sent for pathological examination. GWU's placental archive of >15,000 samples contains the following sections from these deliveries: one block with 2 umbilical cord sections and amniotic membrane roll, 2 blocks with full sections (maternal and fetal aspects) of the placental disc (central), and additional sections if there were gross abnormalities. GWU keeps FFPE blocks for 10 years.

Clinical data from pregnancies (maternal and fetal EMR) were extracted from the GWU's Cerner EMR and entered into an IRB approved REDCap database. Placental pathology was re-scored by an expert pathologist and entered into the database following the "Amsterdam Consensus Guidelines" [1].

To acquire matched frozen and FFPE tissues, women were prospectively screened for uncomplicated pregnancy and delivery. Upon delivery, full thickness, central tissue cores of the placental disc were snap-frozen and stored at −80 °C; the remaining placenta was processed as standard FFPE blocks for pathological assessment. Placenta were kept at room temperature and underwent two stages of formalin fixation – first in the delivery room, within 30–60 min of placenta delivery, and then again in the gross room. Length of fixation was variable, ranging from 1 to 3 days, depending on whether delivery occurred on a weekday or weekend. Only placenta without gross lesions or significant pathologic abnormalities were included in the study and matched with their frozen specimens.

After validation of our methods for FFPE tissues, 10 term and 10 preterm cases, defined as delivery before 34 weeks, were identified

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https://doi.org/10.1016/j.placenta.2021.07.301
Received 27 March 2021; Received in revised form 26 May 2021; Accepted 27 July 2021
Available online 4 August 2021

using our database.

We used standard kits and reagents for RNA isolation, cDNA synthesis, qRT-PCR and immunostaining, and followed manufacturers' protocols. A detailed list of reagents, equipment, statistics, and primers can be found in Supplementary data.

RNA-sequencing was done by LC Sciences (Houston, TX). Details can be found in Supplementary data.

3. Results and discussion

Placental dysfunction is at the core of many pregnancy complications, yet our understanding of obstetrical diseases and their impact on child health is limited by a lack of tools to study the placenta. Since FFPE placenta are a potential goldmine of genetic material, that can be linked to EMRs, we sought to create a pipeline whereby clinicopathological questions could be investigated.

Our database of >1000 cases, is founded on a unique, population-based dataset that includes placenta and EMRs from both complicated and normal term pregnancies. Cases are coded for patient demographic data; gross (macroscopic) and microscopic placental pathology findings; gestational age; antenatal conditions and onset; pre- peri- and post-pregnancy status; and fetal outcomes (Table 1). Since GWU is located in the urban metropolis of Washington, D.C., this database is representative of the diverse populations of the community. Matching of maternal and fetal EMRs with systematic placental pathology reports into a searchable database now allows for interrogation of obstetric clinical questions, by enabling easy sample identification of the corresponding FFPE placental blocks.

It was previously unclear whether FFPE human placental blocks were useful for downstream genetic analysis and comparable to fresh frozen tissues since placental core sections are particularly thick and fibrous, limiting their solubility and requiring prolonged formalin fixation that can potentially degrade RNA [2–4]. Approximately 6 μg of RNA was obtained per 20 μm slice of FFPE placenta tissue. 260/280 absorbance ratios were ~2 for all samples, indicative of pure RNA. Quality of FFPE RNA (RNA integrity number, RIN, 2.38 ± 0.04, $n = 20$) was significantly lower ($t(1,38) = 74.12$, $p < 0.0001$) than matched frozen RNA frozen tissues (9.68 ± 0.09, $n = 20$) and published values from fresh or RNA-later stored placenta [5,6]. Comparing RNA from FFPE placenta to RNA-later stored tissues is an important line of future work since RNA-later is superior to snap-freezing [5,6], and both methods of RNA stabilization likely provide higher RNA quality than FFPE extraction can. However, clinical samples continue to be stored in FFPE. Although lower RINs can result in higher qRT-PCR cycle threshhold (Ct) values [5,6], the expression level of only 8% of placental genes are dependent on RIN [7] and there are methods to correct for differences in sample degradation [8]. Notably, RIN values are similar in FFPE tissues stored over time [9]; thus, samples identified using our pipeline likely undergo similar degrees of degradation and are suitable for comparison to one another. Moreover, RINs of ~2.4 are similar to that of long-term stored, snap-frozen placenta where Cts were detectable [5,6], and are par with RNA harvested from other FFPE tissues types, where RINS above 1.4 were successfully used in gene expression assays [10,11]. To our knowledge, no other method for FFPE tissues consistently returns higher RINs [12].

Confirming the quality of RNA, known placental housekeeping genes *Ywhaz*, *Cyc1*, and *Top1* [13,14] were detected in every FFPE sample, demonstrating that RNA degradation did not preclude qRT-PCR (Fig. 1A). Variability in Ct increased in proportion to gene length, possibly due to reduced transcript stability [7] and increased mRNA fragmentation. However, comparable Cts between fresh frozen and FFPE RNA suggest that expression in FFPE placenta tissues is representative of placental expression, and is consistent with findings from other tissues where snap frozen and FFPE processed samples' gene expression correlate strongly ($r = \sim 0.9$ [11,15,16]). Identification of these housekeeping genes in FFPE placenta allow for normalizing inter-sample loading variability [17,18] and make qRT-PCR analysis feasible.

Table 1
Database demographic information and common pregnancy complications. Cohort information for the term and preterm samples included in the RNA-seq.

Database Information			
Criteria			% in population
Race			
Black			40%
White			47%
Other			13%
Ethnicity			
Hispanic			9.2%
Non-Hispanic			90.8%
Body Habitus (body mass index, BMI)			
Normal, BMI less than 25			37%
Overweight, BMI within 25 and 30			35%
Obese, BMI greater than 30			28%
Gestational Age			
Term, 37 weeks or more			88%
Preterm, less than 37 weeks			12%
Pathology condition			
Chorioamnionitis			30.5%
Fetal inflammatory response			19.0%
Maternal stromal vascular lesions			18.0%
Menconium-Related changes			18.0%
Intervillous thrombi			12.0%
Gestational hypertensive disorders			10.0%
Gestational diabetes			3.4%
Summary of term and preterm cohorts			
Criteria	Term	Preterm	p-value
Gestational age	~39.8.5 ± 0.3	~26.5 ± 1.3	<**0.0001**
Sex of fetus (M/F)	3/7	6/4	0.178
Delivery type (VD/CS)	5/5	7/3	0.361
Obese	2	2	>0.999
Chronic HTN, PEC	0	2	0.136
Other delivery indications	Breech (2)		
		Cervical insufficiency or short cervix (5) h/o LEEP (1) Intrauterine fetal demise (1)	
	Non-reassuring fetal heartrate (1)	Non-reassuring fetal heartrate (1) Placental abruption (2) PPROM 3) Precipitous delivery (1)	

VD: vaginal delivery.
CS: caesarian section.
Chronic HTN, PEC: Chronic hypertension and preeclampsia with severe features.
PPROM: preterm premature rupture of membranes.
h/o LEEP: history of loop electrosurgical excision procedure.
Data analyzed by Chi-squared test and student t-test for categorical and parametric data, respectively.

We identified term and preterm FFPE placentas using our database (Table 1) and successfully performed gene expression analysis by RNA-sequencing, which was then validated by qRT-PCR (Fig. 1B–E). There were 1394 upregulated and 2235 downregulated genes in preterm placenta compared to term (Fig. 1F). Hierarchical clustering reveals distinct expression signatures between term and preterm placenta, that cluster closely within their groups (Fig. 1G). Notably, our findings agree with microarray data from second trimester versus term, fresh, frozen, human placenta. For genes that are significantly dysregulated in both datasets, there is an ~87% concurrence in the fold change direction [19]. High gene overlap, despite methodological differences, indicates the validity of placental FFPE for RNA-seq.

A greater proportion of down-regulated differentially expressed

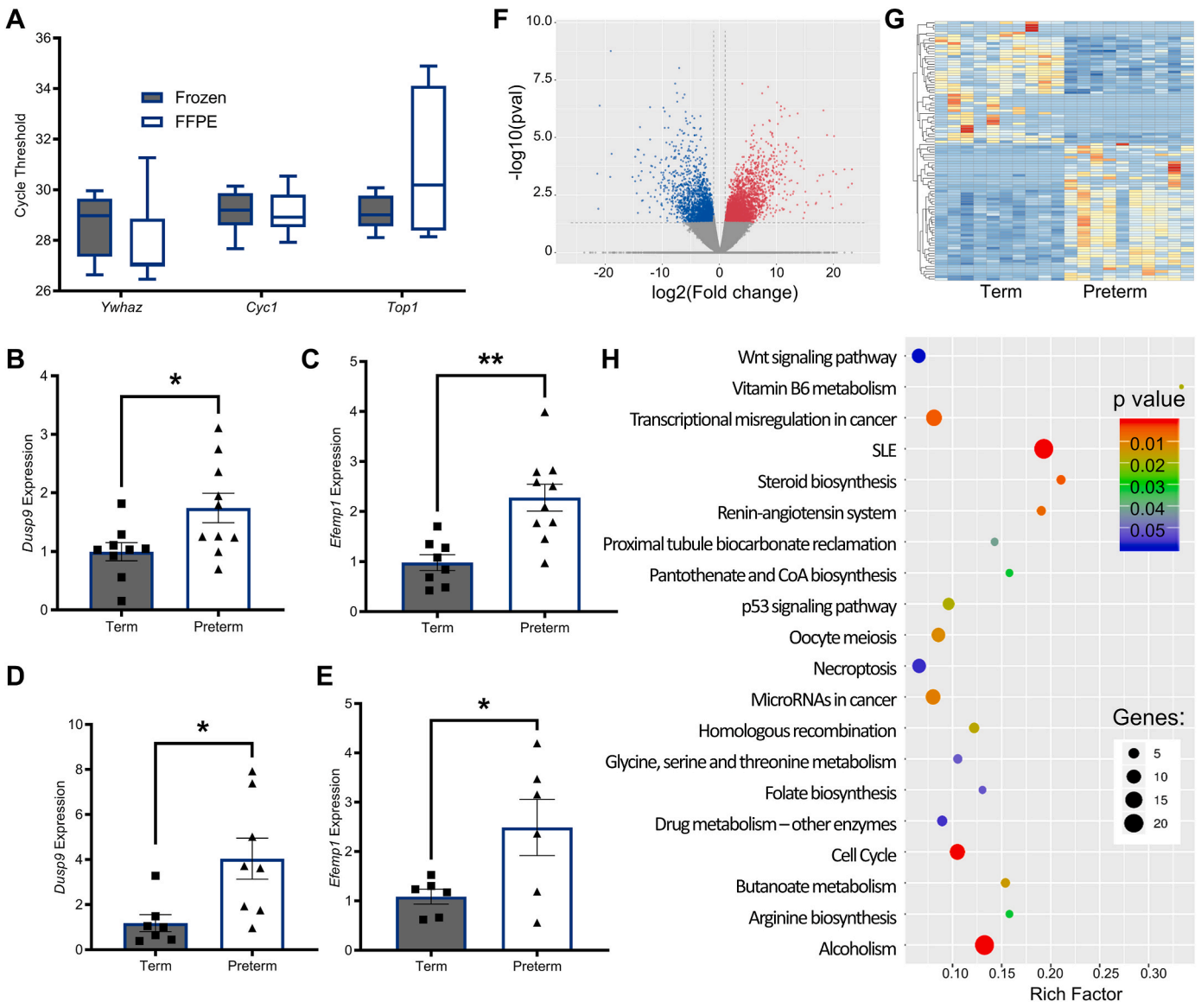

Fig. 1. FFPE RNA is suitable for downstream gene expression analysis
(A) Comparison of matched fresh frozen and FFPE placenta by qRT-PCR. FFPE tissues express known placental housekeeping genes, *Ywhaz*, *Top1*, and *Cyc1* at levels comparable to fresh frozen (F (1,55) = 0.7326, p = 0.396). There was a main effect of gene (F (2,55) = 9.314, p = 0.003) due to higher *TOP1* expression than *CYC1* and *YWHAZ* in FFPE tissues (p = 0.048 and $p < 0.0001$, respectively, Sidak's). **(B–C)** Validation of select DEGs identified in the RNA-seq by qRT-PCR. Consistent with the RNA-seq, *Dusp9* and *Efemp1* were significantly upregulated preterm placenta (t (17) = 2.465, p = 0.025 and t (16) = 3.871, p = 0.001, respectively). **(D-E)** In a second cohort of preterm and term placenta, both genes were significantly upregulated (t (13) = 2.739, p = 0.017 for *Dusp9* and t (10) = 2.382, p = 0.039 for *Efemp1*). **(F)** Volcano plot of 1394 upregulated genes (red) and 2235 downregulated genes (blue) in preterm placenta. **(G)** Hierarchical clustering of the top 100 DEGs. **(H)** Significant KEEG pathways, with p value represented by color, and the number of genes represented by the circle size. Rich factor is the ratio of the DEG number to the background number for a specific KEGG pathway.

genes (DEGs) may reflect premature loss of late-gestation placental factors, exhibited by disruptions to cell cycling and steroid biosynthesis (Fig. 1H), which are gestationally regulated processes [20,21]. Placenta steroid disruption is one pathway significantly disrupted in preterm birth that deserves further investigation [22]. Alcoholism and systemic lupus erythematosus (SLE) were the most represented pathways and are known risk factors for preterm delivery [23,24]. Likely the DEGs in alcoholism and SLE overlap with genes related to inflammation and maternal immune activation, which are comorbidities for some of the clinical conditions indicated in our preterm cohort.

Our novel pipeline presents a non-bias method to examine placental functions associated with preterm birth and other obstetric complications. The creation of this database combined with validation of FFPE placenta samples as suitable genetic material provides a tool for identifying biomarkers and pathways for further investigation.

Acknowledgements

We thank our collaborators at Drs. Homa Ahmadzia, Alexis Gimovsky, and Charles Macri who were vital for prospective patient identification and subsequent tissue acquisition. This work was supported by the National Institutes of Health [R01HD092593 (to AAP), 3R01HD092593-S1 (to AAP), 1F31HD098886-01 (to JJO)] and CTSI-CN UL1TR001876 Pilot Award to SB].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.placenta.2021.07.301.

References

[1] T.Y. Khong, E.E. Mooney, I. Ariel, N.C.M. Balmus, T.K. Boyd, M.A. Brundler, H. Derricott, M.J. Evans, O.M. Faye-Petersen, J.E. Gillan, A. Heazell, D.S. Heller, S. M. Jacques, S. Keating, P. Kelehan, A. Maes, E.M. McKay, T.K. Morgan, P. G. Nikkels, W.T. Parks, R.W. Redline, I. Scheimberg, M.H. Schoots, N.J. Sebire, A. Timmer, G. Turowski, J.P. van der Voorn, I. van Lijnschoten, S.J. Gordijn, Sampling and definitions of placental lesions: amsterdam placental workshop group consensus statement, Arch. Pathol. Lab Med. 140 (7) (2016) 698–713.

[2] S.M. Hewitt, F.A. Lewis, Y. Cao, R.C. Conrad, M. Cronin, K.D. Danenberg, T. J. Goralski, J.P. Langmore, R.G. Raja, P.M. Williams, J.F. Palma, J.A. Warrington, Tissue handling and specimen preparation in surgical pathology: issues concerning the recovery of nucleic acids from formalin-fixed, paraffin-embedded tissue, Arch. Pathol. Lab Med. 132 (12) (2008) 1929–1935.

[3] J.-Y. Chung, T. Braunschweig, R. Williams, N. Guerrero, K.M. Hoffmann, M. Kwon, Y.K. Song, S.K. Libutti, S.M. Hewitt, Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue, J. Histochem. Cytochem. 56 (11) (2008) 1033–1042.

[4] F. Noack, J. Ribbat-Idel, C. Thorns, A. Chiriac, R. Axt-Fliedner, K. Diedrich, A. C. Feller, miRNA expression profiling in formalin-fixed and paraffin-embedded placental tissue samples from pregnancies with severe preeclampsia, J. Perinat. Med. 39 (3) (2011) 267–271.

[5] L.M. Wolfe, R.D. Thiagarajan, F. Boscolo, V. Tache, R.L. Coleman, J. Kim, W. K. Kwan, J.F. Loring, M. Parast, L.C. Laurent, Banking placental tissue: an optimized collection procedure for genome-wide analysis of nucleic acids, Placenta 35 (8) (2014) 645–654.

[6] N.M. Martin, K.M. Cooke, C.C. Radford, L.E. Perley, M. Silasi, C.A. Flannery, Time course analysis of RNA quality in placenta preserved by RNAlater or flash freezing, Am. J. Reprod. Immunol. 77 (4) (2017).

[7] M. Reiman, M. Laan, K. Rull, S. Sober, Effects of RNA integrity on transcript quantification by total RNA sequencing of clinically collected human placental samples, Faseb. J. 31 (8) (2017) 3298–3308.

[8] I. Gallego Romero, A.A. Pai, J. Tung, Y. Gilad, RNA-seq: impact of RNA degradation on transcript quantification, BMC Biol. 12 (2014) 42.

[9] A. Ribeiro-Silva, H. Zhang, S.S. Jeffrey, RNA extraction from ten year old formalin-fixed paraffin-embedded breast cancer samples: a comparison of column purification and magnetic bead-based technologies, BMC Mol. Biol. 8 (2007) 118.

[10] M. Doleshal, A.A. Magotra, B. Choudhury, B.D. Cannon, E. Labourier, A. E. Szafranska, Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues, J. Mol. Diagn. 10 (3) (2008) 203–211.

[11] I. Wimmer, A.R. Troscher, F. Brunner, S.J. Rubino, C.G. Bien, H.L. Weiner, H. Lassmann, J. Bauer, Systematic evaluation of RNA quality, microarray data reliability and pathway analysis in fresh, fresh frozen and formalin-fixed paraffin-embedded tissue samples, Sci. Rep. 8 (1) (2018) 6351.

[12] A. Ribeiro-Silva, H. Zhang, S.S. Jeffrey, RNA extraction from ten year old formalin-fixed paraffin-embedded breast cancer samples: a comparison of column purification and magnetic bead-based technologies, BMC Mol. Biol. 8 (118) (2007).

[13] M. Meller, S. Vadachkoria, D.A. Luthy, M.A. Williams, Evaluation of housekeeping genes in placental comparative expression studies, Placenta 26 (8–9) (2005) 601–607.

[14] J.K. Cleal, P. Day, M.A. Hanson, R.M. Lewis, Measurement of housekeeping genes in human placenta, Placenta 30 (11) (2009) 1002–1003.

[15] S. Graw, R. Meier, K. Minn, C. Bloomer, A.K. Goodwin, B. Fridley, P. Beyerlein, J. Chien, Robust gene expression and mutation analyses of RNA-sequencing of formalin-fixed diagnostic tumor samples, Sci. Rep. 5 (1) (2015) 12335.

[16] C.P. Kolbert, R.M. Feddersen, F. Rakhshan, D.E. Grill, G. Simon, S. Middha, J. S. Jang, V. Simon, D.A. Schultz, M. Zschunke, W. Lingle, J.M. Carr, E.A. Thompson, A.L. Oberg, B.W. Eckloff, E.D. Wieben, P. Li, P. Yang, J. Jen, Multi-platform analysis of microRNA expression measurements in RNA from fresh frozen and FFPE tissues, PloS One 8 (1) (2013), e52517.

[17] T.J. Kokkat, M.S. Patel, D. McGarvey, V.A. LiVolsi, Z.W. Baloch, Archived formalin-fixed paraffinembedded (FFPE) blocks: a valuable underexploited resource for extraction of DNA, RNA, and protein, Biopreserv. Biobanking 11 (2) (2013) 101–106.

[18] A. Radonic, S. Thulke, I.M. Mackay, E. Landt, W. Siegert, A. Nitsche, Guideline to reference gene selection for quantitative real-time, Biochem. Biophys. Res. Commun. 313 (4) (2004) 856–862.

[19] A.M. Mikheev, T. Nabekura, A. Kaddoumi, T.K. Bammler, R. Govindarajan, M. F. Hebert, J.D. Unadkat, Profiling gene expression in human placentae of different gestational ages: an OPRU Network and UW SCOR Study, Reprod. Sci. 15 (9) (2008) 866–877.

[20] C.P. Gheorge, R. Goyal, A. Mittal, L.D. Longo, Gene expression in the placenta, Int. J. Dev. Biol. 54 (2–3) (2010) 507–523.

[21] R.S. Boroditsky, F.I. Reyes, J.S. Winter, C. Faiman, Maternal serum estrogen and progesterone concentrations preceding normal labor, Obstet. Gynecol. 51 (6) (1978) 686–691.

[22] A.M. Pasca, A.A. Penn, The placenta: the lost neuroendocrine organ, NeoReviews 11 (2) (2010).

[23] S. Wei, K. Lai, Z. Yang, K. Zeng, Systemic lupus erythematosus and risk of preterm birth: a systematic review and meta-analysis of observational studies, Lupus 26(6) (207) 563-571.

[24] K. Albertson, A.-M.N. Anderson, J. Olsen, M. Grønbæk, Alcohol consumption during pregnancy and the risk of preterm delivery, Am. J. Epidemiol. 159 (2) (2004) 155–161.