Quantifying CD138+ cells in the endometrium to assess chronic endometritis in women at risk of recurrent pregnancy loss: a prospective cohort study and rapid review.

Michael P. Rimmer [1], Kathrine Fishwick [2], Ian Henderson [2,3], David Chinn [4], Bassel H.Al Wattar [2,3,5], Siobhan Quenby [2,3]

Addresses:

[1]MRC Centre for Reproductive Health, Queens Medical Research Institute, Edinburgh BioQuarter, University of Edinburgh, Edinburgh, UK

[2]Warwick Medical School, University of Warwick, Coventry, CV4 7AL, UK

[3]University Hospital Coventry and Warwickshire NHS trust, CV2 2DX, UK

[4]Research & Development Office, NHS Fife, Queen Margaret Hospital, Whitefield Road, Dunfermline, Fife, UK

[5]Women's Health Research Unit, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, UK

Running Head: CD138+ cells predict pregnancy loss

Corresponding author: Dr.Bassel H.Al Wattar - Warwick Medical School, University of Warwick, Coventry, UK, CV4 7AL - E-Mail: dr.basselwa@gmail.com

Abstract

Objective: To determine the value of uterine CD138+ cells, as a marker of chronic endometritis, in predicting subsequent reproductive outcome in women with history of recurrent pregnancy loss.

Design: A prospective longitudinal study.

Setting: Tertiary specialised clinic.

Patients: Women with history of recurrent pregnancy loss or implantation failure over a 12-months follow-up period.

Intervention: We quantified the CD138+ cells/high powered field (hpf) using immunohistochemistry and image analysis of endometrial biopsies obtained during the secretory stage post ovulation.

Main Outcome Measures: live birth and subsequent pregnancy loss. We calculated the receiver operator curve for predicting subsequent pregnancy loss and reported using relative risk (RR) and 95% confidence intervals (CI).

Results: We enrolled 344 women of whom eighty-eight became pregnant (88/344, 25.5%). Half of them had a subsequent live birth (47/88, 53%) and the rest lost their pregnancy (41/88, 46%). The median CD138+ score was significantly lower in the live birth group ($p<0.005$) and women with a CD138+ score$\geq$16/hpf had a higher risk of subsequent miscarriage (RR 10.0, 95%CI 2.78-36.02). CD138+ cells count showed a good prediction for subsequent pregnancy loss in high-risk women with an area under the curve of 0.75 (95%CI 0.59-0.82, $p=0.01$). A cut-off value of 4-6 cells/hpf offered the best predictive accuracy with higher scores predicting worse reproductive outcome. Our findings are limited by the small event rate and the sample size of our cohort.

Conclusion: Quantifying CD138+ cells by immunohistochemistry in women with a history of recurrent pregnancy loss is helpful to diagnose chronic endometritis and predict subsequent reproductive outcome.

Keywords: CD138+, Chronic endometritis, endometritis, recurrent pregnancy loss, Syndecan-1

Introduction

Chronic endometritis (CE) is a common condition leading to long-term inflammation of the endometrial cells affecting 15 to 50% of women (1-7) and often associated with a high risk of recurrent pregnancy loss (RPL) and implantation failure (1, 3, 8-11). The majority of affected women remain asymptomatic or present with subtle and non-specific manifestations such as pelvic pain, dysfunctional uterine bleeding, dyspareunia and leukorrhea (9) making its diagnosis very challenging in practice. Accurate and effective screening methods are therefore, needed to detect affected women and facilitate treatment provision.

Several diagnostic methods have been suggested to detect CE, including video hysteroscopy (12) and histopathological examination of endometrial biopsies to identifying inflammatory plasma cells infiltration (13). More recently, the use of immunohistochemistry with specific cell markers for CD138+ cells has been suggested as a more accurate test for CE (14). However, only a handful of small studies have evaluated its role in screening and mitigating the risks of RPL in subsequent pregnancy (15-17). We aimed to determine the value of uterine CD138+ cells, as a marker to diagnose chronic endometritis and to predict subsequent reproductive outcome in women with history of recurrent pregnancy loss.

Methods

Study Design

We undertook an observational prospective longitudinal study at a tertiary referral centre with a dedicated ‘implantation clinic’ caring for women with a history of

recurrent pregnancy loss and implantation failure. The study ran from January 2014 until December 2016 and was approved by the NHS National Research Ethics Committee (Ref 1997/5065).

Participants

We enrolled women with a history of reproductive failure (recurrent pregnancy loss defined as two or more consecutive miscarriages and/or implantation failure (no pregnancy after three or more embryo transfers) and women with a history of one pregnancy losses after a period of subfertility). There were no restrictions on the participants' age, body mass index (BMI) or medical comorbidities at the time of inclusion. All participants provided informed written informed consent before enrolment in accordance with the Declaration of Helsinki.

Procedures

Following consent, we asked participants to monitor their ovulation using home luteinising hormone (LH) surge detection strips. When positive, all participants attended a transvaginal ultrasound scan between days 4 -14 post-LH surge to ensure that endometrial biopsies were taken during the secretory stage. All samples were obtained using the Wallach Endocell® endometrial sampler and then were fixed in 10% neutral buffered formalin and labelled with unique participant identifiers. Samples were stored overnight at 4°C and then were wax embedded in Surgipath® Formula 'R'™ paraffin using the Shandon Excelsior ES Tissue Processor (ThermoFisher). Sampled tissues were sliced into 3 μM sections on a microtome and adhered to coverslips by overnight incubation at 60 °C. Deparaffinization, antigen retrieval (pH 6), antibody staining,

hematoxylin counterstain and DAB colour development were fully automated in a Leica BondMax autostainer (Leica BioSystems). Tissue sections were stained for Syndecan-1/CD138 (a plasma cell-specific cell surface antigen) using a 1:300 dilution of concentrated CD138 antibody (ab34164, Abcam, Cambridge, UK). Stained slides were dehydrated, cleared and cover-slipped in a Tissue-Tek® Prisma® Automated Slide Stainer, model 6134 (Sakura Finetek Inc. CA, USA) using DPX coverslip mountant. Bright-field images were obtained on a Mirax Midi slide scanner using a 20x objective lens and opened in Panoramic Viewer v1.15.4 (3DHISTECH Ltd, Budapest, Hungary) for analysis.

A trained assessor (MPR) reviewed each image containing the entire stained sample. The most heavily stained area was selected. The number of CD138+ stromal cells were counted in a set area of tissue measured using a panoramic viewer. Epithelial staining, on the surface of the endometrium and glands was not assessed. A total area of 1 high powered field (hpf) equating to 0.125 mm^2 was counted for each patient and a score was calculated as the number of CD138+ cells/hpf of the stroma. Where doubts arose, two senior assessors reassessed the count to reach consensus (KF and SQ).

Data collection

We collected the baseline characteristics of included women from their electronic hospital records and input data onto a dedicated anonymised secure electronic dataset. We collected data on the women's age, BMI, ethnicity, obstetric history and reproductive outcome at 12-month post-biopsy. Women self-reported pregnancies after the biopsy and those who reported pregnancy within 12 months of the biopsy were

followed up until delivery. The women and clinicians caring for them were blinded to the CD138+ score.

Statistical Analysis

We included all enrolled women in our analysis including those with no detectable CD138+ cells in their endometrial biopsies. We tabulated the CD138+ score per pregnancy outcome and categorised women into four categories (0-5, 6-10, 11-15 and ≥16/hpf). We evaluated the distribution of the CD138+ score/hpf outcome using the Chi-Square test and reported the relative risk (RR) of subsequent miscarriage in each score category with 95% confidence intervals (CI). We compared the medians of multiple groups using the Kruskal-Wallis test for non-parametric data and the t-test comparing means for parametric data.

We generated a receiver operating characteristic (ROC) curve and reported on the Area Under the Curve (AUC) with 95% confidence intervals (CI) for the accuracy of CD138+ count to predict the risk of subsequent pregnancy loss and evaluated potential thresholds, reporting on their sensitivity and specificity. All analyses were conducted in Prism (V8, GraphPad Software, La Jolla California USA) and SPSS (v22, SPSS Inc. Chicago, USA).

Results

We enrolled 344 women into our study, of whom 88 became pregnant within 12 months from biopsy (88/344, 25.5%). Women included in the study had a history of recurrent miscarriage (194/344, 56.4%), implantation failure (87/344, 25.3%) or a mixed history

of both recurrent miscarriage and implantation failure (63/344, 18.3%). The median CD138+ score was similar in those who had a history of miscarriage after embryo transfer compared to those who had a history of implantation failure (13 vs 15, p=~~0.6~~ *ns*). The CD138+ score was also similar in those with a severe history of recurrent pregnancy loss (>5 losses) compared to those with 1-3 losses (Figure 1). Of those who were pregnant at 12-months follow-up, 75% (66/88) conceived spontaneously and a 25% (22/88) conceived following embryo transfer. Of those pregnancies, almost half resulted in live birth (47/88, 53%) and 46% resulted in miscarriage (41/88, 46%) (Table 1).

The median CD138+ score in women with live birth was significantly lower compared to those with miscarriage or no pregnancy (5 vs 13 vs 14, p<0.0001) (Figure 2). There was no statistically significant difference in CD138+ score between women with a miscarriage and no pregnancy, p=0.9. Women with a CD138+ score ≥16/hpf had a significantly higher risk of a miscarriage compared to those with a score 0-5 (RR 10.0, 95%CI 2.78, 36.02). Women with lower CD138+ scores showed levels of relative risk which were not statistically significant at a P-value <0.05 but were suggestive of increased risk with P-values <0.10 (Table 2), in our analysis we identified a significant test for trend between rising CD138+ count and miscarriage (p<0.001).

Our ROC curve demonstrated a good performance of CD138+ score for the prediction subsequent pregnancy loss in high-risk women with an AUC of 0.75 (95%CI 0.59-0.82, p=0.01) (Figure 3). A cut-off value between 4-6 CD138+/hpf provided the optimal sensitivity and specificity to predict the risk of subsequent pregnancy loss. (Supplementary Table 1)

Discussion

Summary of findings

Our findings suggest an association between elevated CD138+ scores, as an objective marker for CE, and the risk of subsequent pregnancy loss following both assisted and spontaneous conception. Our ROC analysis suggests an overall good performance for $CD138^{+}$ as a diagnostic test with scores above a cut-off value of 4 to 6 cells/hpf suggesting higher risk for future RPL. Given the lack of a validated diagnostic criteria, the proposed threshold could help clinicians caring for women with history of RPL to offer guidance and mitigate their future risk. This however, should be interpreted this with caution due to the relatively small sample size of our cohort.

Strengths and limitations

Our study was conducted prospectively within a specialist clinic offering optimal care for women with a history of RPL. We adopted a well-established methodology to obtain samples prospectively and evaluate the results in the laboratory within a specific time frame of the menstrual cycle, thus reducing the risk of bias in our patient selection, sample acquisition and outcome assessment. Both the clinicians and the women involved in the study were blinded to the results with no antibiotics treatment to reduce performance bias. We included a large number of women compared to similar published studies (15-17) and followed them over 12 months to reduce detection bias. We were unable to complete the CD138+ scoring in duplicate due to funding limitations, however, we mitigated any perceived uncertainty by employing a priori regular quality assurance measures. Longer follow-up was not feasible which could have revealed recurrent pregnancy loss in the remainder of the cohort. In contrast

to other studies (16, 18, 19), we did not adopt a set cut-off to diagnose chronic endometritis or treat women with antibiotics. We also reported the outcomes of all included women regardless of their CD138+ score to reduce performance and reporting bias. There was a modest event rate of live births and miscarriage in our cohort at 12 months, which may limit the generalisability of our findings.

The pathophysiology of miscarriage is multifactorial often persisting as a continuum causing further miscarriages and morbidity in future pregnancies (20, 21). Histopathological testing may help to identify reversible infectious causes, however, clinicians should consider the whole spectrum of contributing factors to recurrent miscarriage such anatomical and chromosomal abnormalities. Our study did not include a chromosomal analysis or other microarray investigations for failed pregnancies which may limit the generalisability of the findings to all groups of women at risk of recurrent miscarriage. We also did not carry out any assessment of the endometrial microbiome in our study. Assessment of known causes of miscarriage, such as chromosome abnormalities would have enabled us to define with greater certainty, the role of CD138+ cells in miscarriage.

Wider Implications

Our findings shed more light on two important questions: how to diagnose CE in affected women and what are its implications on their future reproductive outcomes if left untreated.

The cause of CE is poorly understood however a typical appearance of the endometrium has been reported. This was first published Greenwood and Moran (1981) who identified stromal oedema and inflammatory infiltrate dominated by lymphocytes

and plasma cells and has been reported in numerous subsequent studies (9, 22, 23). More recently numerous organisms have been identified within the endometrium, associated with poor pregnancy outcomes (24, 25).

The presence of these organisms subsequently leads to disruption of the *Lactobacillus spp.* endometrial flora, which following antimicrobial therapy both eliminates these organisms but also restores *Lactobacillus spp.* within the endometrium (24). These findings suggest that the role of CE on pregnancy outcomes may not simply be due to the presence of infection but a perturbation of the endometrial flora and disruption of the implantation window, a period characterised by pro-inflammatory changes (26, 27).

We suggest that Immunochemistry with CD138+ cell quantification could to help accurately diagnose women affected by CE and those at risk of future RPL, however, adopting this test in clinical practice is limited by several factors. A clear validated diagnostic criteria for CE remains unclear (14), histopathological examination is cumbersome and expensive (28), and lastly, there are wide variations in the clinical pathways for caring for those women.

Certain interventions have been proposed to facilitate the diagnostic process for CE such as using analytical software to streamline the histopathological examination (14), and hysteroscopic screening of affected women to reduce the number of endometrial biopsies (16). As such, there is a need to validate and streamline the clinical pathways to establish the criteria on one a immunochemistry testing is needed.
Our cohort offers a unique insight into the effect of CE on the longterm if left untreated.

To put our findings in context, we conducted a rapid systematic review and meta-analysis (CRD42016036949) to evaluate the reported longterm effects of CE on women's reproductive outcomes with and without treatment. We searched the major electronic databases (MEDLINE, EMBASE, Web of Science, CINAHL and Cochrane databases) using no search filters and conducted the study selection and data extraction process in duplicate (MPR & IH). We conducted a pair-wise meta-analysis using a random effect model (Ref) and reported using summary risk ratio (RR) with 95% confidence intervals (CI).

Out of 177 potentially relevant citations we screened 21 articles in full and included 13 studies (7 cohort and 6 case series) in the meta-analysis. All studies diagnosed CE using histology and were either case control or cohort studies of women with a history of implantation failure following embryo transfer or >2 pregnancy losses. Four studies conducted a test of cure following antibiotics, reporting pregnancy outcomes in women with both cured and persistent CE (22, 25, 29, 30). The remaining eight studies reported pregnancy outcomes in women with CE compared to no CE (22, 24, 29-34).

There were higher live birth rates in women with resolved CE compared to those with persistent CE (RR 2.48, 95%CI 1.48-4.15, I^2= 38.9%). However, the live birth rate was lower in women with treated CE compared to women with no evidence of CE (RR 1.61, 95%CI 1.28-2.02, I^2=39.2) (Figure 4).

Therefore, our findings compare well to the literature in supporting the importance of treating CE in affected women in order to reduce the risk of future RPL. The use of antibiotics over two-weeks seems to cure CE in over 90% of cases (22, 32, 35) with some evidence suggesting long-term benefits in preventing RPL (19, 22, 32, 34, 35). Our group is currently undertaking the CERM trial (36) aiming to address this evidence gap and provide more evidence on the effective treatment for this group of women.

Quantifying CD138+ cells by immunohistochemistry in women with a history of recurrent pregnancy loss is helpful to diagnose chronic endometritis and predict subsequent reproductive outcome.

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Conflict of interest: None.

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Figure Legends

Table 1: Demographics of women included and the median/range of CD138+ score per subsequent pregnancy outcome at 12-month follow-up

Table 2: Relative risk with 95% confidence intervals of miscarriage compared to live birth per CD138+ score group.

Figure 1: Median and interquartile range for CD138+ score in women with a history of implantation failure, miscarriage after embryo transfer, 1-3 miscarriages, 4-5 miscarriages and >5 miscarriages.

Figure 2: Median and interquartile range of $CD138^{+}$ score per pregnancy outcome at 12-month follow-up

Figure 3: Receiver operating characteristic (ROC) curve for CD138+ score to predict subsequent pregnancy outcome in women with history of recurrent pregnancy loss.

Figure 4: Forest plots of random effects meta-analysis for reported pregnancy outcomes in women with resolve CE vs persistent CE and treated CE vs no CE.

Supplementary Table 1: Cut-off points and the associated sensitivity and 1- specificity for CD138+ scores to predict subsequent pregnancy loss in high risk women.