Study protocol:

All women admitted to The Center for Recurrent Pregnancy Loss of Western Denmark from January 2016 to March 2020 were identified in this combined case-control and cohort study and screened for study eligibility. Only patients with a history of ≥3 consecutive spontaneous pregnancy losses were included. Both biochemical (confirmed by a positive human chorionic gonadotropin (hCG) test) and clinical losses (confirmed by an ultrasound examination and/or histology of aspirated tissue from the uterus) documented in hospital records were accepted. Verified extrauterine pregnancy losses, complete molar pregnancies, and induced abortions of social and genetic reasons were not included in the total number of pregnancy losses. Women with significant uterine malformations, significant parental chromosomal abnormalities, irregular and/or abnormal menstrual cycle length (<22 and >35 days interval), <3 consecutive pregnancy losses (n=26), and/or no p-MBL measurement were excluded. 267 women fulfilled all study criteria. The RPL patients were followed until birth of a liveborn child after RPL or until March 2021, whichever came first.

At the first consultation in The Center for Recurrent Pregnancy Loss of Western Denmark, all women underwent a routine diagnostic work-up including collection of an obstetric and gynecologic history, a routine blood analysis with plasma mannose binding lectin (p-MBL)
measurement, a uterine hydrosoneography, hysteroscopy or hysterosalpingography, and in most cases also a parental chromosomal analysis.

According to the individual health profile, the obstetric history, and the results from blood sample, treatment options such as vaginal progesterone, oral prednisolone, intravenous immunoglobulin (IVIG) infusion, and/or subcutaneous heparin were considered. In patients with an estimated good prognosis, only psychological support and close ultrasound scan monitoring (tender loving care) was offered.

Retrospectively, data was retrieved from the RPL clinical database of The RPL Center in Aalborg University Hospital, Denmark (Data Protection Agency of Region Nordjylland Approval Number 2018-5) in which relevant data is collected on patients from their first consultation and until their contact to the clinic is terminated. Data on perinatal outcomes of all pregnancies before and after RPL was collected at the first consultation, from hospital records, and, when needed, completed by telephone or e-mail correspondence. This included information about mode of conception and delivery, gestational age (GA), birth weight (BW), gender of offspring, volume of peripartum hemorrhage, occurrence of preeclampsia, and perinatal complications. GA was divided into preterm and very preterm birth defined as birth <37 weeks and <32 weeks of gestation, respectively. BW was divided into low and very low BW defined as <2500 g and <1500 g, respectively. Peripartum hemorrhage was divided into moderate hemorrhage of 500-999 ml and severe hemorrhage of ≥1000 ml lost within 24h after birth including both peripartum hemorrhage during vaginal delivery and caesarean section. Peripartum hemorrhage was routinely estimated and noted by the midwifes in the delivery records generally by weighing absorbent bed sheets.
Live births refer to children born alive and who were still alive after 1 week. Miscarriages were divided into early and late miscarriages defined as <12 weeks and 12-21+6 weeks of gestation, respectively. Stillbirth was defined as fetal death ≥22 weeks of gestation and within 1 week after delivery. pRPL was defined as ≥3 consecutive pregnancy losses while secondary RPL (sRPL) was defined as ≥3 consecutive pregnancy losses after the birth of a viable child. Whenever the gender of the fetus was known, it was recorded.

**Blood samples**

Routine blood analysis comprised the following plasma and genetic biomarkers: plasma level of MBL, autoantibodies (anti-nuclear antibodies [ANA], anti-ds-DNA-antibodies and anti-thyroid peroxidase [TPO] antibodies, lupus anticoagulant [LAC], anti-cardiolipin antibodies [IgG/IgM ACA] and anti-β2-glycoprotein-I antibodies [IgG/IgM aGPL-I]), thyroid stimulating hormone (TSH), homocysteine, the HLA-DRB1 genotype and hereditary thrombophilic factors (the prothrombin- and factor-V Leiden genetic variants, protein S and C, and antithrombin III).

**MBL assay**

Plasma MBL was measured using enzyme-linked immunosorbent assay (ELISA). Patient plasma was diluted 1:100 in dilution buffer (20 mM Tris + 10 mM CaCl2 + 1 M NaCl + 0,05 % Triton X-100 + 0,1 % BSA, pH 7.4) and incubated on mannose-coated ELISA microtitre wells (Mannose from Saccharomyces cerevisiae SIGMA M-7504). After each step, wells were washed 3 times with washing buffer (Tris-buffered saline + 5 mM CaCl2 + 0,05 % Tween 20) and incubated min. 1 hour in room temperature. Biotin conjugated monoclonal anti-MBL (HYB 131-01B-0 1 mg/ml, from Antibody Shop) was added to the wells. Then, after incubation and washing, wells were incubated
in alkaline phosphatase conjugated Streptavidin (Dako code D0396) and finally CSPD substrate was added (ELISA-LIGHT CSPD/Sapphire-II T1023, from Applied Biosystem). After 20 min. the wells were analyzed by a LUMIstar ELISA reader. The analysis used a control patient plasma sample as reference, and a highly purified MBL-standard (SER101) was used for calibration, both tested in several dilutions. This method did not allow precise measurement of the exact plasma concentration of MBL when <100 µg/l and >5000 µg/l; thus, these levels were noted in the data collection as a plasma concentration of 100 µg/l and 5000 µg/l, respectively. The cut-off for low p-MBL used routinely in Danish laboratories is <500 µg/l.

*Ethical approval*

Since only data on routine investigations and interventions in the RPL Center is analyzed and reported, no approval from the ethics committee was needed.

**Statistical analysis plan:**

*Statistical Analysis*

Data was analyzed using Stata/MP 15.0 for Mac, revision 19 June 2017. Level of statistical significance was defined as p <0.05. Patients were divided into 3 subgroups according to their p-MBL levels: low (≤500 µg/l), intermediate (501-3000 µg/l), and high p-MBL group (>3000 µg/l).

Cross-sectional data on p-MBL levels were compared between RPL patients and the MBL reference group and analysed using χ² test; first comparing prevalence of ≤500 µg/l and >500 µg/l between the two groups and next the same procedure for intermediate and high levels, respectively. The
relative prevalence of low MBL was compared using prevalence proportion ratio (PPR) with 95% confidence intervals (CI).

A binominal logistic regression analysis was performed to identify the possible prognostic value of low and/or high MBL level on the reproductive outcome in first pregnancy after admission which was adjusted for confounding variables known to influence on the reproductive outcome. The reproductive outcome was defined 1= pregnancy loss and 0= pregnancy>12 weeks or birth in the first pregnancy after admission. Patients with no pregnancy after admission, pregnant at the time of admission, and pregnant after admission but <12 weeks of gestation at final follow-up were not included in the logistic regression analysis of reproductive outcome. p-MBL level was defined as 1=p-MBL level ≤500 µg/l and 0= p-MBL level >500 µg/l in the analysis. Confounders included in the analysis were measured at baseline and involved age (continuous), BMI (continuous), number of pregnancy losses before admission (continuous) and exposure to tobacco smoke (1=yes, 0=no).

A linear regression analysis was performed to analyze the effect of p-MBL level on birth weight and GA of first birth before and after RPL; all as continuous variables.

According to perinatal data on first birth before and after admission, the differences in categorical data were determined by $\chi^2$ test, but in comparisons in which less than 5 numbers were expected the Fisher’s exact test was performed. Differences in continuous variables were determined by the two-sample Mann-Whitney U test if data was non-normally distributed, and by t-test if data fitted a normal distribution. When analyzing the previous children’s sex, only women with one or more children of same sex born before the RPL diagnosis with a GA >22 weeks were included. Women with sRPL with ≥2 children of mixed gender were excluded from analyses involving sex of child(ren) born before admission. Sex ratio was reported as a male:female ratio.