Seminal significance: the forgotten father in recurrent pregnancy loss

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CHAPTER 6

Identification of distinct seminal plasma cytokine profiles associated with male age and lifestyle characteristics in unexplained recurrent pregnancy loss

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ABSTRACT

Background

Seminal plasma contains a wide range of cytokines, chemokines and growth factors. Part of these signalling molecules assist in inducing a state of active maternal immune tolerance towards the fetus. Disbalances in seminal plasma content may contribute to pregnancy loss. This study investigated cytokine expression profiles in seminal plasma of male partners of couples with unexplained recurrent pregnancy loss (RPL) and the association with clinical and lifestyle characteristics, including smoking, alcohol consumption and body mass index (BMI).

Methods

In the seminal plasma of 52 men who visited a specialised RPL clinic the levels of 25 pre-selected cytokines, chemokines and growth factors were measured by Bio-Plex assay or ELISA. Two-way hierarchical cluster analysis was performed. Identified patient clusters were compared on clinical and lifestyle characteristics.

Results

Two distinct cytokine expression profiles in the seminal plasma were revealed by cluster analysis. Patient cluster I showed relatively higher levels of pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-8, IL-12, IL-18 and TNF-α, compared to Patient cluster II. Men belonging to Patient cluster I were significantly older and had significantly more lifestyle risk factors compared to men in Patient cluster II.

Conclusion

Cluster analysis suggested the existence of a less favourable pro-inflammatory cytokine expression profile, being present in part of men affected by RPL and associated with advanced male age and lifestyle risk factors. These findings may serve as a starting point for further research into underlying mechanisms and ultimately lead to novel diagnostic and therapeutic approaches for couples with RPL.

INTRODUCTION

Previous research indicated that seminal plasma is not only a transporter medium that protects and nourishes the spermatozoa. It also primes the maternal immune system by carrying a multitude of cytokines, chemokines, paternal antigens and other immunological factors.(1-3) These signalling molecules have direct effects on the cervix and endometrium and help to induce a state of active maternal immune tolerance, important for normal development of human pregnancy.(4-6) A balance between pro-inflammatory- and immunoregulatory factors in the seminal plasma seems necessary to optimally support the female reproductive tract in developing tolerance and enabling implantation.(7) Disbalances in seminal plasma composites may contribute to pregnancy loss or complications later in gestation.(8, 9)

Recurrent pregnancy loss (RPL) is defined as a minimum of two pregnancy losses before the fetus reaches viability. Multiple risk factors for RPL have been established, including parental chromosomal translocations, uterine anomalies, maternal thrombophilia and thyroid auto-immunity)(10). Despite this, RPL remains unexplained in 60-70% of cases.(11) Advanced maternal age, smoking and obesity are established risk factors for pregnancy loss.(12-14) More recently, a number of studies showed that advanced paternal age and lifestyle factors may contribute to pregnancy loss as well.(15-17) This implicates that the male role in achieving a successful pregnancy involves more than just the conception. We hypothesise that advanced paternal age and lifestyle factors such as smoking or obesity, result in disbalances in seminal plasma components that may contribute to pregnancy loss.

Prior studies reported reference values for several immunological factors in the seminal plasma of healthy fertile men, to provide a foundation for further studies in pathologic conditions.(2, 3) The current exploratory study is the first to investigate the levels of pre-selected seminal plasma cytokines and other signalling molecules in male partners of couples with unexplained RPL. Further insight in the seminal plasma content of these men may lead to a better understanding of the complex aetiology of RPL. We used cluster analysis to analyse multiplex cytokine measurements in seminal plasma and to identify different subgroups of patients. In addition, we evaluated associations between cytokine expression profiles in the seminal plasma and clinical and lifestyle factors.

MATERIALS AND METHODS

Ethics approval

This research was approved by the Medical Research Ethics Committee of the Leiden University Medical Center (reference number P11.196, year of approval 2012). All participants provided informed consent for participation.

Study participants

Patients with RPL

Subjects were 52 male partners of couples with RPL who visited the RPL outpatient clinic of the Leiden University Medical Center between 2012 and 2019. Inclusion criteria were (i) a minimum of three consecutive pregnancy losses before 20 weeks of gestation (definition for RPL used at our centre when the study was initiated) and (ii) unexplained RPL (i.e. no evidence of antiphospholipid syndrome (APS), uterus anomalies, anti-thyroid peroxidase (TPO) antibodies or parental chromosomal translocations, following the ESHRE guideline for RPL(10)). Exclusion criteria were (i) symptoms of urinary or genital tract infection and (ii) use of antibiotics or immune-modifying medications. Couples received no treatment other than supportive care (including frequent ultrasound monitoring during early pregnancy). Two out of 52 included men had one or more children from a previous relationship. One participant had a brother who also suffered from RPL with his partner.

Control group

To provide an indication of normal ranges of cytokines and other immunological factors in the seminal plasma, 11 proven fertile men were included as a control group. They had at least one live birth with their partner and no history of pregnancy loss. They were non-smoking, had a BMI <25 kg/m^2 and were aged ≤40 years.

Clinical data collection

Baseline characteristics were collected during intake consultation at the RPL outpatient clinic. The following data were extracted from medical records: male age, height, weight, smoking behaviour and alcohol consumption; detailed obstetric history of the couple; date of semen collection; outcome of the subsequent pregnancy after semen collection.

Semen collection and storage

Semen samples were collected by masturbation, following 48-72 h of sexual abstinence. Complete ejaculates were collected directly in a plastic-free container and processed within 120 min after collection. Samples were centrifuged at 600 g for 10 min to remove sperm and cellular debris and the supernatant (seminal plasma) was immediately aliquoted into 100 µL volumes and stored at -20°C until analysis. Median time between last pregnancy loss and semen collection was 5 months (interquartile range 3-7).

Detection of cytokines and other immunological factors in the seminal plasma

Key signalling molecules in the seminal plasma were selected based on previous literature (shown in Supplementary Table 1). The levels of the following cytokines, chemokines, growth factors and regulatory factors present in the seminal plasma were assessed by the Bio-Plex Luminex™ system assay (Bio-Rad, Veenendaal, the Netherlands): IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-16, IL-18, TNF-α, IFN-γ, SDF-1α, MCP-1, FGF, VEGF, GM-CSF, M-CSF, G-CSF, sHLA class I and TGF-β1, TGF-β2, TGF-β3 in latent and active form. Bio-plex Luminex™ system (Bio-Rad) was used for read-outs. Methods for detection of cytokines were similar as those earlier described by Meuleman et al.(18) To measure total TGF-β1, TGF-β2 and TGF-β3, tests were performed with and without prior acid activation of the seminal plasma to release biologically active protein from the latent precursor form. An enzyme-linked immunosorbent assay (ELISA) was performed to detect Prostaglandin E2 (PGE2) and soluble HLA-G (sHLA-G). Characteristics of assays used for detection of cytokines, with individual limits of detection and lower and upper limits of quantification for each measured factor are shown in Supplementary Table 2. The manufacturer's instructions were followed. To measure sHLA class I, monoclonal antibody to HLA-class I purified antibody W6/32 (Department of Immunology, LUMC, the Netherlands) was coupled via carboxyl groups on the surface of polystyrene beads (COOH bead: Bio-Rad) according to the procedure of the Bio-Plex Amine Coupling kit (Bio-Rad). Concentrations were expressed in picograms per millilitre (pg/mL), or nanograms per millilitre (ng/mL) when indicated.

Data analysis and statistics

Analyses were performed in SPSS version 25 (IBM SPSS Statistics, IBM Corp., Armonk, NY, USA) and in R studio version 1.3.9.50 (R Foundation for Statistical Computing, Vienna, Austria). For calculations, measurement values below the detection limit were set to one half the detection limit and measurement values exceeding the upper limit of quantification were replaced by the highest concentration measured for that particular factor (similar to the study in fertile men of Politch et al.(3).

To explore correlations between individual cytokines, Spearman's rank correlation coefficients (ρ) were calculated . Since advanced age and obesity have been associated with a tendency toward a more pro-inflammatory systemic cytokine phenotype(19, 20), we assessed whether this is also reflected in the seminal plasma by calculating Spearman's rank correlations between pro-inflammatory cytokines and male age and BMI.

Agglomerative hierarchical cluster analysis was performed using R studio packages gplots and heatmap.2. Before clustering, all values were log transformed and scaled using the mean centering with standard deviation (Z-scores), so that each cytokine would contribute in similar manner to the final classification. Four agglomerative clustering

methods were assessed by calculating the agglomerative coefficient, which measures the amount of clustering structure found in the data.(21) As Ward's minimum variance clustering (with Euclidean distance) showed the highest agglomerative coefficient (0.80), dendrograms were generated using this method.

Population characteristics were described using means or medians and percentages. Independent Samples T tests were used to compare means between clusters and Chi-square tests or Fisher's Exact Tests were used to compare categorical data. Correction for multiple comparisons was performed when indicated using the step-up Benjamini-Hochberg procedure(22), with adjusted *P*-values reported. Statistical significance was inferred when (adjusted) $P < 0.05$.

Since previous studies showed that the risk of (recurrent) pregnancy loss significantly increases with advanced paternal age and when multiple lifestyle risk factors are present simultaneously (15, 17), we developed a score to combine these factors: the Age Lifestyle Index. The Age Lifestyle Index was calculated for each patient based on the following factors: age, BMI, smoking behaviour and alcohol consumption. Cut-off points for the scoring method were based on previous studies and established classification systems: male age <40 years: score 0, 40-44: score 1, ≥45: score 2(23); BMI <25 kg/m^2: score 0, 25-29: score 1, ≥30: score 2(24); non-smoking: score 0, smoking occasionally: score 1, smoking daily: score 2(25); alcohol consumption <2 units per day: score 1 and alcohol consumption ≥2 units per day: score 1(26). Median total scores were compared between the clusters with a Mann-Whitney *U* test.

RESULTS

Concentrations of cytokines, chemokines and growth factors in the seminal plasma

Seminal plasma samples of 52 male partner of women with RPL were analysed. As the data did not satisfy the assumptions of normal distribution, concentrations of seminal plasma components were described in medians with interquartile ranges. Descriptive statistics for all measured factors are shown in Table 1.

Table 1. Descriptive statistics of cytokines, chemokines and growth factors in the seminal plasma of male partners of women with RPL

Factor	% Detectable	Percentiles			Range
		25th	Median	75th	
IL-1α	94	15.8	25.4	30.1	ND – 176.7
IL-1β	98	0.46	0.63	1.1	ND – 16.3
IL-6	98	3.2	5.5	12.3	ND – 300.3
IL-8	100	298.4	496.2	697.9	136.8 – 5770.7
IL-12 (p70)	100	2.5	3.0	3.5	0.8 – 6.9
IL-18	98	1.6	2.5	3.8	ND – 22.5
MCP-1	100	532.9	915.7	1172	143.5 – 3331
SDF-1α	98	3773	4577	5340	ND – 6278
TNF-α	100	23.3	28.0	50.0	4.8 – 275.2
IL-10	98	8.9	12.0	15.0	ND – 56.4
PGE2*	100	2374	5186	10029	559 – 35167
TGF-β1 active	100	759.7	1486	2214	88.9 – 8999
TGF-β1 latent	100	89574	157763	225494	290.4 – 396013
TGF-β2 active	100	285.2	523.7	818.8	156.1 – 1871
TGF-β2 latent	98	9204	11096	12747	ND – 17030
TGF-β3 active	100	1575	3357	4927	138.0 – 9346
TGF-β3 latent	100	49741	106504	188443	123.5 – 518739
sHLA-G*	100	29.6	50.3	107.6	4.7 – 1593
sHLA class I*	100	275.2	374.9	523.0	116.3 – 2999
IL-7	100	710.8	1267	1995	202.3 – 3542
IL-2	98	2.1	2.7	3.7	ND – 8.5
IL-16	98	4.8	8.0	15.6	ND – 174.2
IFN-γ	98	25.5	43.2	91.2	ND – 293.1
VEGF*	100	18.6	7.7	121.0	5.4 – 326.1
FGF	100	9.1	11.5	14.0	4.1 – 25.1
G-CSF	98	39.3	55.1	76.6	27.1 – 576.1
GM-CSF	79	0.23	0.53	0.88	0.10 – 3.31
M-CSF	100	157.7	182.1	226.5	35.2 – 854.6

Concentrations in pg/ml, except for * (ng/ml); ND = Non detectable

Correlations between cytokines, chemokines and growth factors

Spearman's rank correlation coefficients between individual seminal plasma components are shown in Fig. 1. Significant positive correlations were mainly found between pro-inflammatory cytokines: IL-6, IL-8, IL-1β, TNF-α and IL-16 were all highly correlated

(ρ >0.57; adjusted *P*-values <0.05). Strong negative correlations were found between IL-7 and sHLA-G (ρ =-0.57), IL-7 and TGF-β1 (ρ =-0.49), VEGF and PGE2 (ρ =-0.43), IL-16 and TGF-β3 (ρ =-0.39) and IL-1α and TGF-β1 (ρ =-0.38), with adjusted *P*-values all being < 0.05.

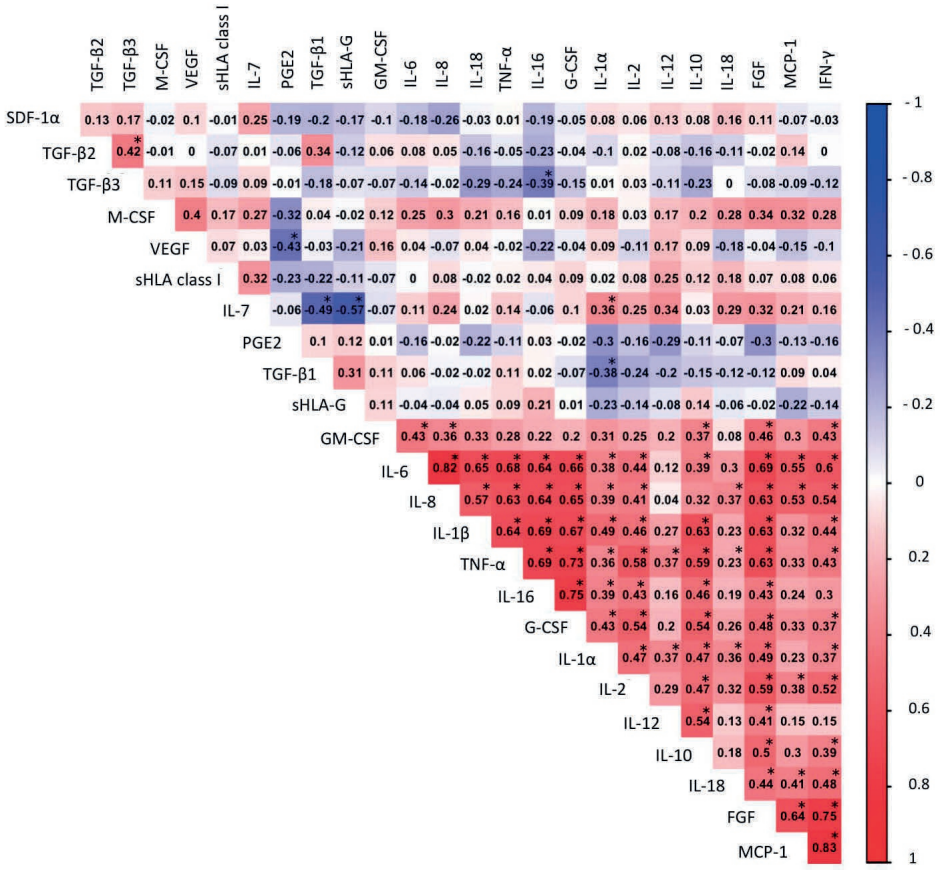

Figure 1. Correlation matrix

Spearman's rank correlations (ρ) between individual seminal plasma signalling molecules. Red colour indicates a positive correlation, white colour indicates no correlation and blue colour indicates a negative correlation. *Benjamini-Hochberg adjusted P-value <0.05 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Correlations between pro-inflammatory cytokines and age and BMI

Significant positive correlations were found between male age and IL-6 (ρ = 0.339) , IL-8 (ρ = 0.384) and IL-16 (ρ = 0.333), with adjusted *P*-values all <0.05. Correlations between BMI and pro-inflammatory cytokines did not reach significance after Benjamini-Hochberg adjustment.

Cluster analysis

The results of the cluster analysis were visualized in a heat map with two dendrograms (Fig. 2), representing the degrees of relatedness between patients (Patient cluster I and II) and between cytokines (Cytokine cluster I and II).

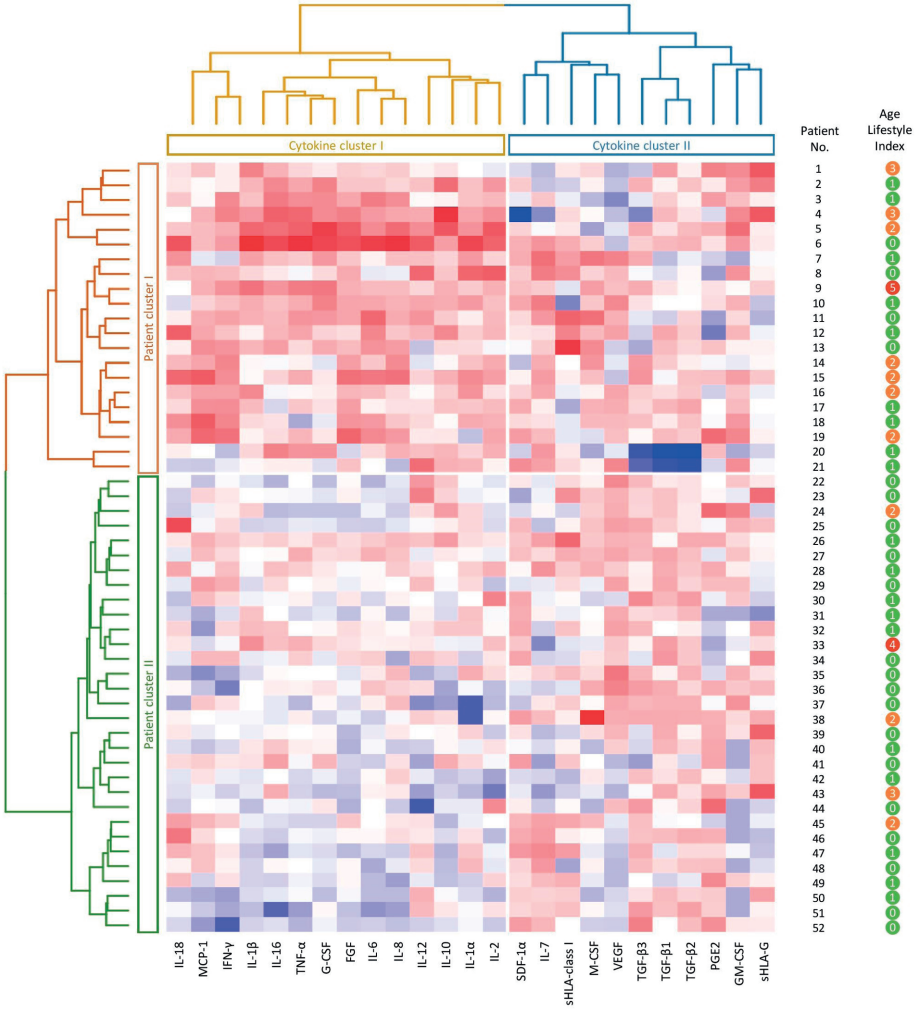

Figure 2. Cluster analysis

Two-way hierarchical cluster analysis with Euclidean distance and Ward's aggregation method. Columns represent cytokines and other immunological factors present in the seminal plasma. Rows represent 52 patient samples (seminal plasma from male partners of women with unexplained RPL). Cytokine concentrations are indicated using a colour scale, ranging from blue (low) to red (high). Both patients and cytokines were separated by the algorithm into two main clusters, indicated as Patient clusters I and II and Cytokine clusters I and II. Patient cluster I contains patients that showed higher expression of pro-inflammatory factors in the seminal plasma compared to Patient cluster II. The Age Lifestyle Index (scoring method explained in paragraph Data analysis and statistics) for each patient is shown on the right, ranging from green (low) to red (high) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Clustering of seminal plasma components relates the measured cytokines and other immunological factors to each other based on their expression in the samples. Cytokine cluster I contained mainly pro-inflammatory factors (IL-18, MCP-1, IL-1β, IL-16, TNF-α, IL-6, IL-8, IL-12, IL-1α, IL-2). Cytokine cluster II primarily contained immunoregulatory factors (TGF-β1, TGF-β2, TGF-β3, IL-7, sHLA class I, sHLA-G, PGE2, SDF-1a).

The patient clustering showed a separation into two main clusters, containing 21 and 31 patients, respectively. The two patient clusters mostly differed with respect to the expression of seminal plasma signalling molecules belonging to Cytokine cluster I. Patient cluster I contained patients that showed high expression of pro-inflammatory factors in the seminal plasma. Less pronounced differences were observed between the patient clusters with regard to Cytokine cluster II (TGF-β, SDF-1α, IL-7, sHLA class I, M-CSF, VEGF, PGE2, sHLA-G). Median concentrations of cytokines and other immunological factors were compared between the two Patient clusters and the control group (shown in Supplementary Figure 1). For pro-inflammatory cytokines IFN-γ, IL-1β, IL-6, IL-8, IL-18 and MCP-1 the seminal plasma concentrations were markedly higher in Patient cluster I compared to the control group, while for these factors the concentrations of Patient cluster II overlapped with concentrations of the control group.

In Table 2, clinical parameters and characteristics of patients belonging to Patient cluster I and II are shown. The 21 patients in Patient cluster I were significantly older compared to Patient cluster II (mean ages of 37.8 and 34.4, respectively, P = 0.002). In Patient cluster I, 33% of men was beyond 40 years of age, compared to 6% in Patient cluster II (P = 0.012). The proportion of alcohol consumers (≥2 units per day) was also significantly higher in Patient cluster I (19% versus 0%, P = 0.022). No significant difference was found in mean BMI between the clusters, Patient cluster I comprised significantly more men with BMI ≥25 (52% versus 29%, P = 0.043). Male age, BMI, smoking behaviour and alcohol consumption were also evaluated as a combined variable, the Age Lifestyle Index. Individual scores are shown in Fig. 2. The median Age Lifestyle Index was significantly higher for Patient cluster I compared to Patient cluster II: medians and interquartile ranges 1 (1-2) and 0 (0-1), P = 0.010.

The live birth rate (for the first pregnancy after semen collection) was 71% in Patient cluster I, compared to 81% in Patient cluster II (P = 0.355). The miscarriage rate was 24% in Patient cluster I and 13% in Patient cluster II (P = 0.231).

Table 2. Clinical parameters and male age and lifestyle characteristics compared between Patient clusters I and II

	Patient cluster I *n* = 21	Patient cluster II *n* = 31	*P*-value
Reproductive details of couples:			
Number of pregnancy losses *median (interquartile range)*	3 (3-4)	3 (3-4)	
History of fertility treatment	4 (19)	1 (3)	0.079
IVF	2 (10)	0 (0)	
IUI only	2 (10)	1 (3)	
Outcome of subsequent pregnancy			
Live birth	15 (71)	25 (81)	0.355
Miscarriage	5 (24)	4 (13)	0.231
Termination of pregnancy	0 (0)	1 (3)	
missing	1 (5)	1 (3)	
Male characteristics:			
Age *mean (SD)*	37.76 (3.91)	34.36 (3.66)	0.002*
Age ≥40 years *n* (%)	7 (33)	2 (6)	0.012*
BMI *mean (SD)*	26.17 (3.82)	24.7 (3.02)	0.133
BMI ≥25 *n* (%)	11 (52)	9 (29)	0.043*
Smoking			
Occasionally	-	4 (13)	0.138
Daily	3 (14)	3 (10)	0.675
missing	1 (5)	0 (0)	
Alcohol consumption ≥ 2 units per day *n* (%)	4 (19)	0 (0)	0.022*
missing	2 (10)	3 (10)	
Age Lifestyle Index *median (interquartile range)*	1 (1-2)	0 (0-1)	0.015*

IVF = in vitro fertilisation; IUI = intrauterine insemination; BMI = body mass index
*$P < 0.05$

DISCUSSION

This is the first study that evaluated the content of cytokines, chemokines and growth factors in the seminal plasma of male partners of couples with RPL. Hierarchical cluster analysis revealed two distinct patient clusters. Patient cluster I showed a trend towards more unfavourable characteristics, both with regard to cytokine expression in the seminal plasma and clinical parameters. Levels of pro-inflammatory cytokines such as IL-6, IL-8, IL-12, IL-16, IL-18 and TNF-α were relatively high. Previous studies associated abundance of these agents in the seminal plasma with silent male reproductive tract infection, inflammation and infertility.(1) A profile with high levels of pro-inflammatory cytokines might induce an inflammatory maternal immune response eventually leading to pregnancy loss.(27)

Some remarkable differences were found with respect to clinical and lifestyle parameters between the two patient clusters. Patients of cluster I were significantly older and included significantly more moderate to heavy alcohol consumers. In addition, Patient cluster I scored significantly higher on the Age Lifestyle Index, reflecting the combined factors of age, BMI, smoking and alcohol consumption. There is solid evidence that these (lifestyle) risk factors may lead to oxidative stress, caused by accumulation of reactive oxygen species (ROS).(28, 29) Therefore, what could potentially be underlying our observations, is the complex interplay between ROS and seminal plasma cytokines. Positive correlations have been observed between ROS production and pro-inflammatory cytokines in the seminal plasma, including TNF-α, IL-6, IL-8 and IL-16.(28, 30) The mechanisms behind this are not fully elucidated. Some cytokines may stimulate the generation of ROS.(31, 32) On the other hand, ROS can promote production of cytokines. (32, 33) Excessive ROS in the seminal plasma may negatively affect sperm DNA integrity. (34, 35) This suggests that pro-inflammatory cytokines in the seminal plasma may not only contribute to pregnancy loss by interfering with the maternal immune response, but also through (either directly or indirectly) damaging the DNA of the male gamete. While conventional sperm parameters including volume, morphology and motility have no clear link with pregnancy loss and are poor predictors of future pregnancy outcomes, increased levels of sperm DNA fragmentation are strongly associated with RPL.(36, 37)

When comparing seminal plasma concentrations of our RPL patients to the results in the control group and the results earlier reported in healthy fertile men by Politch et al.(3), we found remarkably higher levels of pro-inflammatory cytokines including IL-1β, IL-6, IL-8, IL-10 and IFN-γ, suggesting a potential role for these cytokines in the development of RPL. However, as cytokines do not act in isolation, but function in a network, it seems more appropriate to study these signalling molecules as a system and not as individual factors. Therefore, cluster analysis was applied to find patterns in seminal plasma

cytokine profiles. We showed that this is a helpful tool to visualize clusters that do not have to be pre-defined and can therefore be used to find subgroups of patients with potentially similar disease-related mechanisms. Another strength of this study is the well-defined population of male partners of couples with unexplained RPL. Since no female risk factors for RPL could be identified in these couples, it is plausible that male (sperm) factors may play a role here.

A next step would be to validate the identified patient clusters and their associations with clinical factors within larger datasets. This would also allow for multivariable regression analysis, to assess the effect of individual clinical factors adjusted for other potentially confounding factors. Furthermore, since cytokine networks are dynamic, it would be valuable to examine the robustness of these clusters over time. Two studies indicated variation over time in seminal plasma cytokine content, particularly for IFN-γ and to a lesser extent for IL-8. The authors mentioned lifestyle factors as potentially contributing to the regulation of cytokine fluctuations.(38, 39) It seems promising to investigate to what extent any lifestyle modifications are reflected in the content of the seminal plasma. Additionally, the link between the seminal plasma microbiome, cytokine expression profiles and RPL may be a subject of future research.(Tomaiuolo et al. 2020)

The ultimate goal is to develop a panel consisting of cytokines and other seminal biomarkers such as DNA fragmentation level, which can be used for diagnostic and prognostic purposes in clinical practice. Our results suggest that there might be a potential difference in outcome of the subsequent pregnancy based on the two identified cytokine expression profiles, however, potentially due to the moderate sample size of this exploratory study, differences in live birth and miscarriages rate did not reach statistical significance. Larger studies are needed to further investigate the potential of cytokines and other semen factors to predict future pregnancy outcome. This will contribute to providing answers to couples with unexplained RPL and may also serve as a starting point for therapeutic interventions.

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