Received: 7 October 2022 | Revised: 23 November 2022 | Accepted: 12 December 2022

DOI: 10.1002/hsr2.1038

ORIGINAL RESEARCH

Health Science Reports Open Access WILEY

A cross-sectional study comparing the inflammatory profile of menstrual effluent vs. peripheral blood

Sara Naseri[1,2] | Yael Rosenberg-Hasson[3] | Holden T. Maecker[3] | Maria I. Avrutsky[2] | Paul D. Blumenthal[1]

[1]Department of Obstetrics and Gynecology, Stanford University, Stanford, California, USA

[2]Qurasense Inc., Menlo Park, California, USA

[3]Institute for Immunity, Transplantation, and Infection, Stanford University School of Medicine, Stanford, California, USA

Correspondence
Paul D. Blumenthal, 300 Pasteur Dr, Stanford, CA 94305, USA.
Email: pblumen@stanford.edu

Funding information
Stanford University

Abstract

Background and Aims: Cytokine profiles of peripheral blood and other bodily fluids provide diagnostic indicators for assessing inflammatory processes. Menstrual effluent may provide a noninvasive source of biological material for monitoring cytokine levels in blood and in endometrial tissues. This pilot study investigated the potential of measuring cytokines in menstrual effluent, and compared the cytokine profiles of menstrual versus peripheral blood.

Methods: Seven healthy donors (aged ≥18 and ≤45 years) collected menstrual effluent on day 2 of menses. Matched peripheral blood samples were collected by venous blood draw on the same day. Levels of 62 cytokines were measured in all samples by 62-plex Luminex assay.

Results: Peripheral blood and menstrual effluent cytokine profiles were tenuously correlated ($r^2 = 0.26$, $p < 0.0001$), with higher levels detected in menstrual effluent for 48/62 cytokines. Thirty five cytokines were significantly elevated in menstrual effluent compared to peripheral blood samples (IL-8, CCL2, CCL4, LIF, IL-1RA, IL-6, IL-1β, HGF, CCL3, FGF-2, TNF-α, VEGF-A, IL-1α, CXCL1, IL-9, IL-10, EGF, CXCL5, CSF3, EOTAXIN, TGF-α, TRAIL, CXCL10, VEGF-D, IL-12P40, CXCL9, IL-18 RESISTIN, IL-22, IL-21, CSF1, IFN-γ, IL-17A, CXCL12, IL-12p70). Two cytokines (LEPTIN, CSF2) were expressed at significantly lower levels in menstrual effluent compared to peripheral blood. Linear regression of individual cytokines found low predictive power (linear regression $p > 0.05$) for 53/62 cytokines in menstrual effluent versus peripheral blood. Levels of TGF-β ($r^2 = 0.87$, $p = 0.002$) and CCL7 ($r^2 = 0.63$, $p = 0.033$) were significantly positively correlated between matched menstrual and peripheral blood samples.

Conclusion: In this group of study participants, the cytokine profile of menstrual effluent was quantitatively distinct from peripheral blood, and also characterized by higher levels of inflammatory signaling. This pattern of comparative menstrual blood cytokine profiles points to a need for further studies to evaluate the relationship

Abbreviations: CCL7, CC motif chemokine ligand 7; TGF-β, transforming growth factor beta.

between peripheral and menstrual blood cytokines in broader populations including both healthy and diseased states.

KEYWORDS
chemokine, cytokine, endometriosis, endometrium, inflammation, menstrual blood, TGF-β

1 | INTRODUCTION

Cytokines are a broad category of small, nonstructural proteins that participate in regulation of inflammatory and immune responses. These proteins are pleiotropic intercellular messengers, and have diverse proinflammatory, anti-inflammatory, chemo-attractive, or hematopoietic colony-stimulating functions.[1] Cytokine secretion patterns provide insight into the biology of tissue homeostasis in health and disease, and multiplexed cytokine assays are widely used for quantification and analysis of diagnostic biomarkers in clinical research and medical practice. While peripheral blood is the most commonly studied fluid for cytokine analysis across a range of health conditions, cytokine profiles in other fluid samples, such as cerebral spinal fluid, saliva, urine, semen, or tears provide a unique perspective into the cellular signaling specific to these tissues.[2]

Menstrual effluent is a complex fluid comprised of blood, vaginal secretions, and endometrial cells. Absence of implantation triggers menstruation via hormonal signaling to induce ischemia, apoptosis, necrosis, and shedding of cellular debris from the uterine wall as menstrual effluent.[3] In both healthy and endometriotic women, laparoscopic and endometrial sampling from peritoneal and endometrial tissues identified clinically relevant alterations in inflammatory signaling compared to healthy controls.[4] However, utility of these approaches is limited by the surgically invasive specimen sampling methods. Menstrual effluent is a readily accessible source of endometrial cytokines, which can be passively collected and does not require invasive sampling procedures. However, to date, only limited evaluations of cytokines in menstrual blood have been reported in healthy women.[5,6]

Recent studies have demonstrated that menstrual blood may be a comparable alternative to systemic blood for monitoring common biomarkers such as HbA1c, cholesterol, follicle stimulating hormone, lipoproteins, and high sensitivity C-reactive protein.[7,8] N-acetyl-b-D-glucosamine and myeloperoxidase activities in menstrual effluent from patients with endometriosis show positive correlation with peripheral blood measurements, and are significantly elevated compared to control subjects.[9] In women with menorrhagia, menstrual effluent has increased levels of tumor necrosis factor alpha (TNF-α) and reduced levels of vascular endothelial growth factor A (VEGF-A), matrix metalloproteinase-2 (MMP-2) and metalloproteinase-9 (MMP-9).[10] Meanwhile, combined interleukin-6 (IL-6)/interleukin-1 beta (IL-1β) or TNF-α/IL-6 measurements in menstrual effluent demonstrate high sensitivity or specificity for detection of chronic endometritis when both cytokines exceed cutoff values.[11] Thus, while menstrual effluent is rich in inflammatory markers specific to endometrial tissues and provides insights into menstrual cycle physiology, our understanding of cytokine profiles of menstrual effluent in healthy women remains limited.

In the present study, we characterized the peripheral and menstrual effluent profile of 62 cytokines in 7 women via a Luminex Magnetic assay. The primary goal of our study was to compare the cytokine profiles of peripheral and menstrual effluent samples, and to evaluate potential correlates between these samples.

2 | MATERIALS AND METHODS

2.1 | Subjects and sample collection

This was a prospective observational study of 7 self-reported healthy reproductive-aged women randomly selected from a cohort previously described in Naseri et al.[8] The sample size for this pilot was based on the number of matching menstrual and venous blood samples available for analysis at the time of the study. Exclusion criteria included being younger than 18 years of age, older than 45 years, postmenopausal, not menstruating regularly, and uncomfortable with or clinically unable to use a menstrual cup for menstrual effluent collection. Participants were given a study kit containing a menstrual cup (Diva International Inc.) and two blood collection tubes. Serum separator tubes and ethylenediaminetetraacetic acid (EDTA) plasma collection tubes were used for sample preparation. Participants were instructed to contact study staff on the first day of their period, being the first day with actual flow. That day, participants were instructed to stop intake of food after midnight. On the second day of their period participants were instructed to use the menstrual cup for 3 h, starting at the time they woke up in the morning, and then immediately pour the collected menstrual effluent into the designated blood collection tubes. The second day was chosen due to convenience and because the second day of menstruation for most participants was found to be the day with the heaviest flow of menstrual effluent. Participants were instructed to present at the study site on the same day of menstrual sample collection to provide a corresponding venous blood draw. Both samples were spun down and frozen at −80°C.

2.2 | Luminex cytokine analysis

All assays were performed by the Human Immune Monitoring Center at Stanford University. Human 62-plex Procarta kits (Thermo Fisher

Scientific) were used according to the manufacturer's recommendations with modifications as described below. Beads were added to a 96 well plate and washed in a Biotek ELx405 washer. Samples were added to the plate containing the mixed antibody-linked beads and incubated at room temperature for 1 h followed by overnight incubation at 4°C with shaking at 500–600 rpm. Following the overnight incubation plates were washed in a Biotek ELx405 washer and then biotinylated detection antibody was added for 75 min at room temperature with shaking. Plate was washed as above, and streptavidin-PE was added. After incubation for 30 min at room temperature, washes were performed as described above. Reading buffer was added to the wells. Each sample was measured in duplicate. Plates were read using a Luminex 200 or a FM3D FlexMap instrument with a lower bound of 50 beads per sample per cytokine. Custom Assay Chex control beads by Radix BioSolutions were added to all wells for assay quality control (Radix BioSolutions). To reduce potential variability, all laboratory experimental work was carried out on the same sample plate, and cytokine measurements for each sample were interpolated against a standard curve.

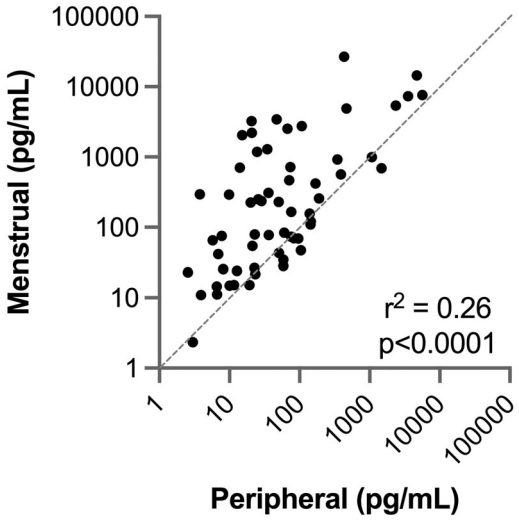

FIGURE 1 Correlation of mean peripheral and menstrual effluent cytokine profiles. Mean levels of 62 cytokines in systemic versus menstrual effluent samples from $n = 7$ subjects shown on a log-log scale. Linear regression values $r^2 = 0.26$, $p < 0.0001$. Gray dashed line indicates the equality line for a 1:1 correlation. For 48/62 cytokines, mean menstrual effluent levels are above the equality line

2.3 | Statistical analysis

Luminex data was acquired with Xponent software and reports were generated using Masterplex QT-4/Mirai Bio/Hitachi software and analyzed with Excel and Prism. Statistical tests, *n*- and *p*-values are all located in the figures and/or legends. Significance was defined as $p < 0.05$. No statistical methods were used to predetermine sample size. Nonparametric comparison between menstrual and peripheral blood samples was performed by Wilcoxon matched-pairs signed rank test. Multiple comparisons method: Two-stage linear step-up procedure of Benjamini, Kireger and Yekutieli, at desired false discovery rate (FDR) (Q) = 5%. Linear regression among the 62 cytokines was used to identify cytokines that are nominally correlated between peripheral and menstrual samples (linear regression $p < 0.05$). Robustness of linear correlation was tested by measuring if correlations remained significant after removal of any single datapoint.

3 | RESULTS

Menstrual and peripheral blood samples from 7 subjects were analyzed via 62-plex human immuno-assay to investigate the relative cytokine levels of both samples. Overall, menstrual samples contained a higher inflammatory profile compared to peripheral blood, and the cytokine profiles of peripheral and menstrual effluent were significantly positively correlated ($r^2 = 0.26$, $p < 0.001$), Figure 1. The highest levels of cytokines for both samples were interleukin-1 receptor antagonist (IL1-RA), resistin, soluble vascular cell adhesion molecule 1 (S-VCAM-1), plasminogen activator inhibitor-1 (PAI-1), soluble intercellular adhesion molecule-1 (siCAM-1), and C-X-C motif chemokine 5 (CXCL5) (Table 1). A total of 77% (48/62) of cytokines measured had higher mean levels detected in menstrual compared to peripheral blood.

To further evaluate differences between the two types of fluid samples, menstrual effluent cytokine levels were normalized against peripheral blood levels from each subject. Thirty five cytokines were significantly elevated in menstrual effluent compared to peripheral samples (IL-8, CCL2, CCL4, LIF, IL-1RA, IL-6, IL-1β, HGF, CCL3, FGF-2, TNF-α, VEGF-A, IL-1α, CXCL1, IL-9, IL-10, EGF, CXCL5, CSF3, EOTAXIN, TGF-α, TRAIL, CXCL10, VEGF-D, IL-12P40, CXCL9, IL-18 RESISTIN, IL-22, IL-21, CSF1, IFN-γ, IL-17A, CXCL12, IL-12p70) (Figure 2 and Table 2). Il-8, CCL2, and CCL4 had approximately 100-fold higher expression levels in menstrual samples, likely reflecting the recruitment of monocytes, neutrophils and various lymphocytes to endometrial tissues during induction of menstrual flow. Two cytokines (LEPTIN, CSF2) were significantly lower in menstrual effluent samples compared to serum.

To further investigate the relationship between peripheral and menstrual effluent cytokine profiles, we performed linear regression for peripheral versus menstrual effluent measurements of each cytokine. Nine cytokines (Table 3) were nominally correlated between peripheral and menstrual samples (linear regression $p < 0.05$). To reduce the likelihood of false positive results due to low sample size, we performed outlier analysis to test if linear correlations were still significant after removal of any single datapoint. Following this screen, two cytokines, TGF-β and CCL7, remained significantly correlated between menstrual and peripheral blood samples (Figure 3). For the other 53/62 cytokines analyzed, there was no correlation between menstrual and peripheral blood levels, indicating that menstrual effluent contains a distinct and elevated cytokine profile that cannot be readily predicted from peripheral blood measurements in healthy subjects.

TABLE 1 Expression levels of cytokines in peripheral and menstrual effluent

Type	Cytokine	Peripheral Blood (pg/mL)							Menstrual Effluent (pg/mL)							Peripheral (mean ± SD)	Menstrual (mean ± SD)
Interleukin	IL-1RA	708	597	102	316	513	322	473	56662	24302	18409	19737	21194	38193	7926	433 ± 202.6	26631.8 ± 15997.7
Hormone	RESISTIN	4911	5633	7078	4500	3352	3876	3564	25502	13306	12822	9013	15832	17638	6888	4702.1 ± 1316.2	14428.8 ± 6122.6
Cell adhesion molecule	sVCAM-1	6831	7575	6686	4287	4060	3829	6438	28487	3026	2455	3878	5166	6294	3794	5672.4 ± 1554.1	7585.7 ± 9306.1
Serpin	PAI-1	4707	6129	1927	1998	2897	2009	5016	11760	4956	4724	5747	9880	9703	4489	3526.2 ± 1731.0	7322.6 ± 3021.1
Cell adhesion molecule	sICAM-1	3898	1127	2230	1510	1038	5222	1451	18510	2138	5181	2286	924	7038	1780	2353.8 ± 1601.7	5408.2 ± 6165.7
Chemokine	ENA78 / CXCL5	1187	200	77	426	661	390	316	9635	7492	8112	2255	3421	899	2367	465.1 ± 367.4	4883.0 ± 3441.2
Interleukin	IL-6	59	52	19	45	60	43	53	10327	2168	4211	2190	666	2865	1545	47.1 ± 14.0	3424.7 ± 3235.8
Interleukin	IL-8	27	18	10	20	29	19	22	6949	2099	1099	736	4770	6520	495	20.7 ± 6.2	3238.1 ± 2785.1
Growth factor	VEGF-A	154	130	45	96	118	95	111	7624	2616	3210	1385	784	2828	819	107.1 ± 34.2	2752.1 ± 2361.1
Growth factor	HGF	88	86	31	53	74	75	64	3943	2391	2440	1948	2007	4015	945	67.3 ± 20.1	2527.2 ± 1107.1
Chemokine	MIP-1b/ CCL4	31	32	3	18	19	13	29	668	4245	2013	1530	1672	4570	711	20.9 ± 10.9	2201.3 ± 1587.8
Chemokine	MCP-1/ CCL2	34	26	1	2	24	11	8	3540	1311	933	2805	544	4364	790	15.1 ± 12.9	2041.1 ± 1516.3
Tumor necrosis factor	TNF-α	42	51	15	29	32	33	40	180	3185	1798	627	664	2400	134	34.6 ± 11.5	1284.1 ± 1188.9
Growth factor	FGF-2	39	18	6	24	37	26	23	4074	269	1400	1718	47	172	625	24.9 ± 11.3	1186.4 ± 1423.2
Growth factor	PDGF-BB	2799	1452	78	215	941	793	1267	977	1921	114	176	1701	1157	898	1078.0 ± 911.2	991.9 ± 687.8
Interleukin	IL-22	419	363	158	330	421	357	388	1540	990	1012	771	845	696	584	347.9 ± 90.1	919.7 ± 313.2
Interleukin	IL-9	125	71	23	59	101	66	75	1106	793	712	504	751	837	302	74.2 ± 32.2	714.9 ± 255.0
Interleukin	IL-1β	19	19	6	10	18	12	16	820	1766	1442	549	133	134	107	14.1 ± 5.0	707.1 ± 673.1
Hormone	LEPTIN	1156	3597	1066	1436	1162	901	1047	759	2012	275	289	579	263	671	1481.0 ± 947.4	692.7 ± 616.5
Chemokine	SDF-1a/ CXCL12	489	409	265	345	405	353	449	919	593	518	443	661	404	417	387.7 ± 73.9	565.0 ± 182.6
Chemokine	MIG/ CXCL9	85	99	45	59	76	54	79	200	289	1831	332	169	316	116	71.0 ± 19.1	464.6 ± 607.9
Interleukin	IL-21	187	152	72	146	228	153	237	720	505	458	328	361	284	272	168.0 ± 56.1	418.2 ± 158.4
Interleukin	IL-18	62	36	16	42	44	26	26	1378	246	231	102	93	58	48	36.1 ± 15.1	308.1 ± 478.5
Interleukin	LIF	5	4	1	3	5	3	4	442	283	236	278	335	358	119	3.8 ± 1.2	292.9 ± 101.7
Chemokine	MIP-1a/ CCL3	11	10	4	18	9	10	8	209	398	357	239	299	420	118	9.9 ± 4.0	291.3 ± 109.3
Interleukin	IL-27	260	228	84	151	212	181	203	465	256	253	191	213	235	183	188.5 ± 57.4	256.3 ± 96.1
Chemokine	GROa/ CXCL1	63	19	5	21	28	22	23	591	248	162	197	315	163	79	25.7 ± 17.7	250.9 ± 167.4
Colony stimulating factor	G-CSF/ CSF3	46	30	10	24	35	26	27	509	200	283	252	150	111	140	28.5 ± 11.0	235.0 ± 135.8

TABLE 1 (Continued)

Tumor necrosis factor	**TRAIL**	**61**	**68**	**30**	**42**	**53**	**40**	**59**	**368**	**223**	**226**	**252**	**248**	**178**	**113**	**50.3 ± 13.5**	**229.6 ± 77.7**
Chemokine	**IP-10/ CXCL10**	**24**	**22**	**37**	**16**	**16**	**11**	**15**	**26**	**83**	**1171**	**120**	**42**	**72**	**56**	**20.0 ± 8.62**	**224.4 ± 418.6**
Colony stimulating factor	**M-CSF/ CSF1**	**71**	**74**	**79**	**74**	**71**	**72**	**91**	**307**	**138**	**171**	**137**	**142**	**159**	**103**	**76.2 ± 7.1**	**165.4 ± 66.0**
Interleukin	IL-31	233	112	25	137	166	156	145	192	237	107	121	172	136	124	139.2 ± 62.7	155.9 ± 46.9
Growth factor	BDNF	366	87	12	85	187	130	147	117	121	51	112	212	115	114	144.9 ± 112.2	120.0 ± 47.3
Chemokine	MCP-3/ CCL7	236	182	33	89	165	146	147	162	134	95	58	113	94	113	142.7 ± 65.5	110.0 ± 33.1
Interleukin	IL-4	89	54	26	48	86	60	60	140	81	87	66	88	64	63	60.3 ± 21.8	84.1 ± 27.0
Chemokine	**EOTAXIN**	23	44	10	26	18	15	23	73	71	54	141	88	82	44	22.9 ± 11.0	79.1 ± 31.3
Tumor necrosis factor	sCD40L/ CD154	34	53	25	57	29	30	27	111	77	71	89	74	77	45	36.3 ± 12.9	77.8 ± 19.8
Interleukin	**IL-1α**	**11**	**10**	**4**	**6**	**8**	**7**	**9**	**154**	**102**	**78**	**62**	**60**	**37**	**34**	**7.8 ± 2.3**	**75.3 ± 41.9**
Interleukin	IL-23	122	28	41	75	104	75	86	89	81	80	74	62	54	71	75.9 ± 33.1	72.9 ± 11.9
Chemokine	RANTES/ CCL5	68	87	110	74	70	86	89	127	59	55	34	69	87	55	83.3 ± 14.8	69.6 ± 29.8
Interleukin	IL-2	123	164	26	63	81	83	128	50	127	25	31	100	74	78	95.4 ± 46.2	69.2 ± 36.7
Interleukin	**IL-10**	**6**	**9**	**2**	**5**	**6**	**5**	**6**	**54**	**79**	**133**	**91**	**35**	**41**	**22**	**5.7 ± 2.0**	**65.0 ± 38.7**
Interferon	**IFNγ**	**28**	**25**	**10**	**18**	**24**	**26**	**19**	**163**	**45**	**61**	**31**	**25**	**35**	**21**	**21.3 ± 6.3**	**54.5 ± 49.8**
Interleukin	IL-15	115	97	38	92	151	86	156	56	41	39	31	42	38	83	104.9 ± 40.7	47.3 ± 17.4
Interleukin	IL-5	78	84	6	32	49	48	59	49	77	30	25	45	41	37	50.9 ± 26.9	43.2 ± 16.9
Growth factor	**EGF**	4	22	1.4	5	9	2	5	28	63	19	39	106	17	19	6.9 ± 7.1	41.5 ± 32.9
Interleukin	IL-7	139	109	2	13	36	41	73	13	76	4	4	71	38	37	58.9 ± 50.4	34.8 ± 30.1
Colony stimulating factor	**GM-CSF/ CSF2**	**52**	**131**	**29**	**55**	**63**	**33**	**49**	**33**	**82**	**12**	**11**	**21**	**13**	**27**	**58.8 ± 33.9**	**28.3 ± 25.1**
Tumor necrosis factor	sFAS	31	18	7	21	35	21	25	38	38	21	23	25	22	19	22.6 ± 9.2	26.5 ± 8.0
Growth factor	**VEGF-D**	**9**	**9**	**11**	**6**	**7**	**6**	**10**	**17**	**39**	**57**	**22**	**22**	**7**	**14**	**8.2 ± 2.1**	**25.4 ± 17.2**
Interleukin	IL-17A	18	15	6	10	13	16	12	36	26	32	17	20	19	18	12.7 ± 4.2	24.1 ± 7.7
Growth factor	**TGF-α**	**3**	**3**	**1.1**	**3**	**3**	**2**	**3**	**23**	**22**	**61**	**18**	**12**	**14**	**12**	**2.6 ± 0.73**	**23.0 ± 17.2**
Growth factor	TGF-β	35	31	5	18	24	26	25	28	30	13	18	22	21	20	23.4 ± 9.8	21.8 ± 5.6
Growth factor	bNGF	32	33	5	17	30	23	24	44	19	20	11	21	17	20	23.3 ± 9.8	21.6 ± 10.4
Tumor necrosis factor	TNF-β	26	12	5	14	32	20	24	23	16	15	9	15	12	16	19.2 ± 9.2	15.3 ± 4.5
Interferon	IFN-α	11	12	12	10	11	10	16	23	14	17	11	14	15	13	11.7 ± 2.1	15.2 ± 3.7
Interleukin	IL-12P70	13	13	5	7	11	12	10	27	15	16	10	11	15	10	10.0 ± 3.2	14.9 ± 6.1
Interferon	IFN-β	5	7	3	15	6	5	5	17	9	9	36	7	15	7	6.6 ± 3.9	14.4 ± 10.3
Growth factor	SCF/ KITLG	8	9	4	5	7	7	7	18	11	12	11	8	11	7	6.7 ± 1.6	11.2 ± 3.3
Interleukin	IL-12P40	4	2	1.5	6	4	6	5	11	9	7	13	7	25	6	3.9 ± 1.8	11.0 ± 6.6

(Continues)

TABLE 1 (Continued)

Interleukin	IL-13	3	4	0.9	4	4	3	3	5	2	1.4	2	2	2	2	3.0 ± 1.0	2.3 ± 1.0
Interleukin	IL-17F	0.9	1.1	0.4	0.8	0.8	0.7	0.9	1.1	1.2	1.4	1.1	1.0	0.9	0.7	0.79 ± 0.19	1.04 ± 0.19
	Sample ID (sample type)	40S (EDTA)	24S (serum)	45S (EDTA)	43S (EDTA)	52S (EDTA)	48S (EDTA)	46S (EDTA)	40M (EDTA)	24M (serum)	45M (EDTA)	43M (serum)	52M (serum)	48M (serum)	46M (serum)		
	Percentile	1st			50th			99th									

Note: Mean cytokines measurements in pg/ml ±*SD* in peripheral and menstrual effluent shown for $n = 7$ subjects, on a 1–99th percentile Blue-Red color scale. Serum and plasma sample identification provided for each specimen (bottom row).

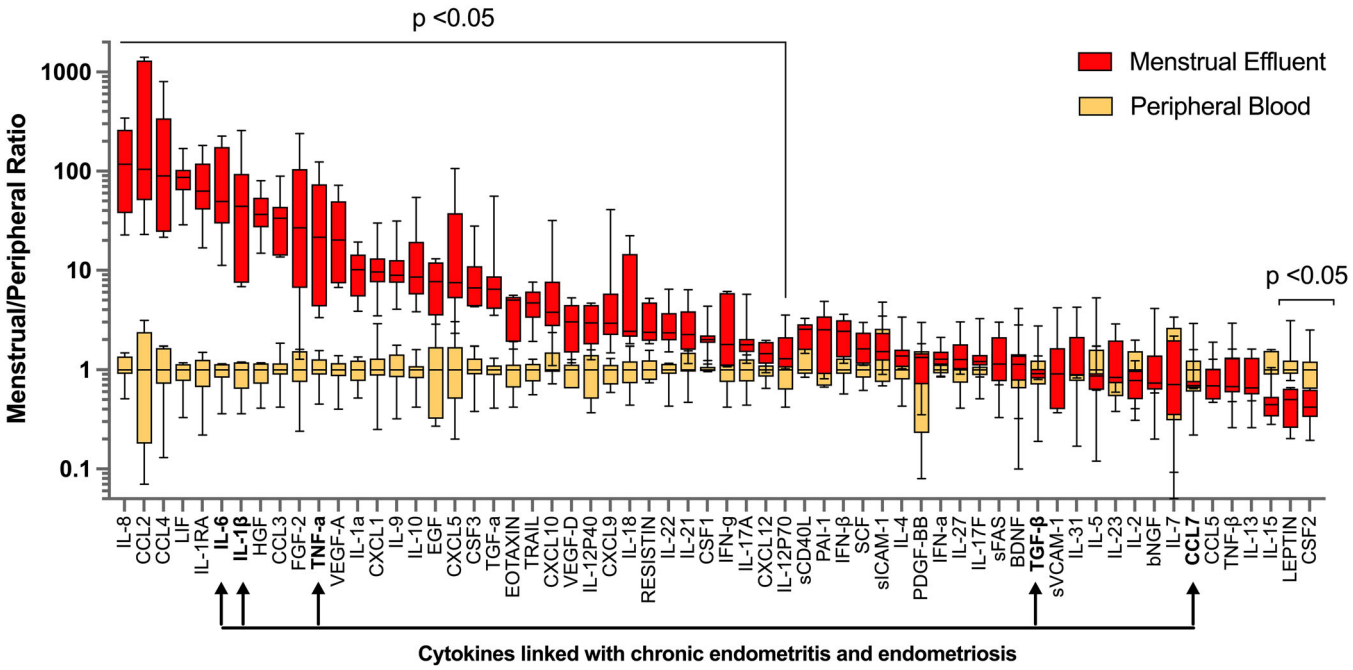

FIGURE 2 Fold change in cytokine levels in menstrual versus peripheral blood samples.
Menstrual levels of each cytokine were normalized to the systemic blood levels of each patient. Graph depicts median and min/max for normalized menstrual and systemic blood levels for each cytokine. Differences between groups were tested by Wilcoxon matched-pairs signed rank test. Multiple comparisons method: two-stage linear step-up procedure of Benjamini, Kireger and Yekutieli, at desired false discovery rate (q) = 5%. FDR-adjusted *p*-values are depicted. Arrows indicate cytokines in endometrial tissues that are associated with chronic endometritis and endometriosis. FDR, false discovery rate

4 | DISCUSSION

We measured levels of 62 clinically-relevant cytokines in menstrual and peripheral blood from 7 healthy subjects. In these healthy subjects, the cytokine profile of menstrual effluent was only moderately correlated with values that were obtained peripheral blood draws, revealing a unique, menstrual pattern of physiological inflammatory signaling. Normal menstruation is an inflammatory process, featuring intercellular signaling between endometrial cells, neutrophils and macrophages.[12] Many of the highly-expressed cytokines in menstrual effluent have distinct physiological roles in endometrial and menstrual health, including regulation of leukocyte recruitment, regulation of chronic inflammation, and promotion of endometrial repair via stimulation of angiogenic growth factor secretion (Table 4). These results demonstrate the feasibility of measuring diverse cytokines in menstrual effluent and highlight the potential clinical utility of menstrual effluent as a unique and noninvasive source of biological material for investigations into inflammatory processes relevant to female reproductive health.

Our study offers an exploratory glimpse into the comparative inflammatory profiles of peripheral blood and menstrual effluent. The results create a snapshot view of inflammation at a single timepoint during the menstrual cycle, allowing direct comparison of data from menstrual and peripheral samples. Further studies should investigate

TABLE 2 Differentially expressed cytokines in menstrual effluent

Cytokine		Mean menstrual/peripheral ratio (*SD*)	Adjusted *p*-value
Higher in menstrual effluent vs. peripheral blood			
Chemokine (C-C motif) ligand 2	CCL2	481.6 (606.5)	0.020
Chemokine (C-C motif) ligands 4	CCL4	212.1 (278.9)	0.020
Interleukin 8	IL-8	150.1 (115.7)	0.020
Leukemia inhibitory factor	LIF	87.1 (43.0)	0.020
Interleukin 6	IL-6	85.3 (81.2)	0.020
Interleukin 1 receptor antagonist	IL-1RA	77.2 (56.2)	0.020
Interleukin 1β	IL-1β	67.3 (88.6)	0.020
Fibroblast growth factor 2	FGF2	66.1 (84.8)	0.032
Tumor necrosis factor alpha	TNF-α	44.1 (44.3)	0.020
Hepatocyte growth factor	HGF	40.6 (21.5)	0.020
Chemokine (C-C motif) ligand 3	CCL3	36.2 (26.2)	0.020
Vascular endothelial growth factor A	VEGF-A	28.5 (21.1)	0.020
Chemokine (C-C motif) ligand 5	CXCL5	24.5 (37.8)	0.020
Interleukin 10	IL-10	15.3 (17.8)	0.020
Transforming growth factor alpha	TGF-α	13.2 (18.8)	0.020
chemokine (C-X-C motif) ligand 1	CXCL1	12.0 (8.4)	0.020
Interleukin 9	IL-9	12.0 (8.9)	0.020
Interleukin 1 alpha	IL-1α	10.1 (5.3)	0.020
Colony-stimulating factor 3	CSF3	9.9 (8.3)	0.020
Chemokine (C-C motif) ligand 9	CXCL9	8.7 (14.2)	0.020
Chemokine (C-C motif) ligand 10	CXCL10	8.2 (10.6)	0.032
Epidermal growth factor	EGF	8.1 (3.9)	0.032
Interleukin-18	IL-18	7.5 (8.0)	0.020
TNF-related apoptosis-inducing ligand	TRAIL	4.9 (1.9)	0.020
Eotaxin	EOTAXIN	4.0 (1.7)	0.032
RESISTIN	RESISTIN	3.2 (1.5)	0.020
Interleukin-12 subunit beta	IL-12P40	3.1 (1.4)	0.020
Vascular endothelial growth factor D	VEGF-D	3.0 (1.6)	0.020
Interleukin-22	IL-22	2.9 (1.7)	0.020
Interleukin-21	IL-21	2.9 (1.8)	0.045
Interferon gamma	IFN-γ	2.7 (2.2)	0.020
Interleukin-17A	IL-17A	2.2 (1.6)	0.032
Colony stimulating factor 1	CSF1	2.2 (1.0)	0.032
Interleukin-12	IL-12P70	1.6 (0.9)	0.045
Chemokine (C-C motif) ligand 12	CXCL12	1.5 (0.4)	0.032
Lower in menstrual effluent vs. peripheral blood			
Colony stimulating factor 2	CSF2	0.4 (0.2)	0.020
LEPTIN	LEPTIN	0.4 (0.2)	0.020

Note: Name, fold change in menstrual/peripheral blood expression levels, and FDR-adjusted *p*-value for differences between menstrual and peripheral blood expression.

Abbreviations: FDR, false discovery rates; IFN, interferons; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

TABLE 3 Correlation between cytokines in peripheral and menstrual effluent

Cytokine		Nominal correlation		Outlier analysis	
		r^2	*p*-value	r^2	*p*-value
Transforming growth factor beta	**TGF-β**	**0.87**	**0.002**	**0.82**	**0.013**
Chemokine (C-C motif) ligand 7	**CCL7**	**0.63**	**0.033**	**0.66**	**0.0497**
Interleukin 5	IL-5	0.63	0.033	0.60	0.07
Colony-stimulating factor 2	CSF2	0.87	0.002	0.20	0.37
	Leptin	0.86	0.003	0.00	0.99
Transforming growth factor alpha	TGF-α	0.81	0.005	0.21	0.36
Chemokine (C-X-C motif) ligand 1	CXCL1	0.78	0.009	0.47	0.53
C-X-C motif chemokine ligand 10	CXCL10	0.73	0.014	0.09	0.57
Interferon beta	IFN-β	0.72	0.015	0.05	0.68

Note: Name, linear regression r^2 values, and slope *p*-values for cytokine expression levels measured in peripheral versus menstrual effluent. Outlier analysis shows correlation after removal of the single most significant datapoint. Bold values are statitistically significantly different.

Abbreviations: IFN, interferons; IL, interleukin; TGF, transforming growth factor.

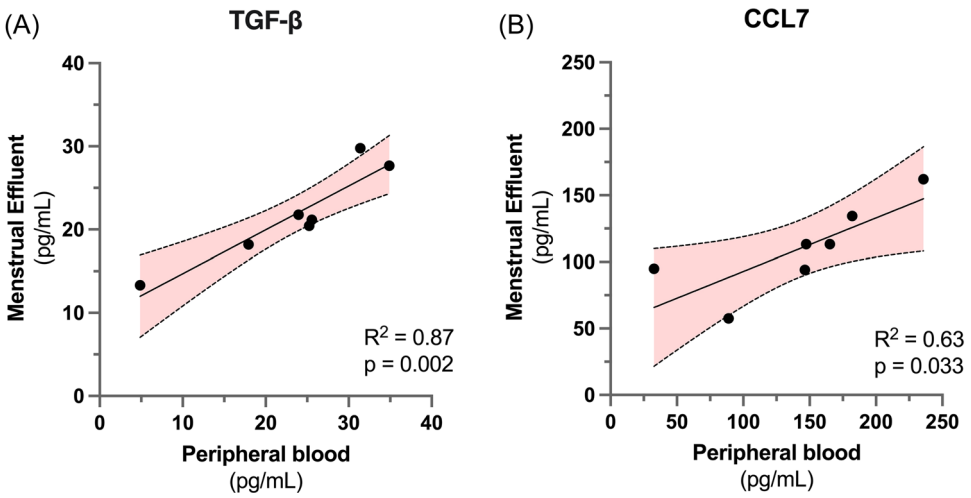

FIGURE 3 Menstrual and peripheral blood measurements of TGF-β and CCL7 levels are significantly correlated. Linear correlation and 95% confidence interval for (A) TGF-β (r^2 = 0.87, p = 0.002) and (B) CCL7 (r^2 = 0.63, p = 0.033) measurements in menstrual vs peripheral blood samples for n = 7 subjects. Elevated levels of TGF-β and CCL7 have been previously implicated in endometrial inflammatory disorders. TGF, transforming growth factor

the temporal profile of cytokines expression levels throughout the menstrual cycle, as distinct populations of cytokines may be differentially regulated in menstrual versus peripheral blood at different times during menstruation. Menstruation typically lasts 4 days, and the relationship between cytokine profiles of menstrual and peripheral blood may change over the course of the menstrual cycle.

Our small sample size is a key limitation to the interpretations facilitated by this study. Factors such as aging-related changes in fecundity may influence the cytokine profile of menstrual effluent and could not be evaluated in the present study due to the limited number of participants. However inter-individual differences in cytokine profiles are reported to be strikingly low,[6] and we observed similarly low levels of inter-sample variability in our data. We performed FDR correction on statistical analyses to account for multiple comparisons testing.

A second limitation of this study is that the health status of participants was self-reported, and medical records were not assessed to confirm absence of intrauterine conditions such as endometriosis and endometritis. Further studies on menstrual effluent may benefit from including an infectious screen to minimize potential interference from infection. A third potential limitation of this study is the use of both plasma and serum samples for cytokine analysis. Although we did not note any confounding effect on cytokine levels from blood collection in serum-separator versus EDTA-coated tubes (Table 1), the low number of subjects in this pilot study does not allow for robust evaluation of potential effects of serum versus plasma blood collection tubes on cytokine measurements. Consequent to these limiting factors, our analyses likely under-represent the number of differentially expressed cytokines, as

TABLE 4 Description of 10 most-differentially expressed cytokines in menstrual versus venous blood samples

Cytokine		Type	Functions	Refs.
Chemokine (C-C motif) ligand 2	CCL2	Chemokine	• Chemotaxis for monocytes, T lymphocytes and basophils • Follicular chemotaxis for macrophages in ovulation	1,13
Chemokine (C-C motif) ligands 4	CCL4	Chemokine	• Chemotaxis for natural killer (NK) cells and monocytes • Endometrial NK recruitment during secretory phase	14,15
Interleukin 8	IL-8	Interleukin	• Chemotaxis for neutrophils and T cells • Endometrial angiogenesis and repair	1,16
Leukemia inhibitory factor	LIF	Interleukin	• Hematopoietic differentiation • Blastocyst attachment and formation of maternal decidua • Stimulates prostaglandin synthesis	17,18
Interleukin 6	IL-6	Interleukin	• Induces synthesis of inflammatory acute phase proteins • Stimulates angiogenesis, antibody production, effector T-cells	19
Interleukin 1 receptor antagonist	IL-1RA	Interleukin	• Suppresses inflammation via inhibition of IL1a and IL1β	20
Interleukin 1β	IL-1β	Interleukin	• Induces inflammatory signaling through the inflammasome when proteolytically activated by caspase-1 • Stimulates growth factor, cytokine secretion by endometrial cells	20,21
Fibroblast growth factor 2	FGF2	Growth Factor	• Regulates cellular proliferation, migration, differentiation, and angiogenesis • Expressed by endometrial epithelial cells to regulate endometrial receptivity and blastocyst implantation	22,23
Tumor necrosis factor alpha	TNF-α	Tumor Necrosis factor	• Regulates inflammation, apoptosis and proliferation	1

well as the number of cytokines with significant correlations between peripheral and menstrual samples. Fourth, our study represents an observational profile of cytokine signaling in healthy subjects, and does not enable functional inference as to the physiological significance of differential cytokine expression. Differences in cytokine stability in peripheral versus menstrual effluent may confound physiological differences of cytokine expression in endometrial tissues versus systemic circulation. Further research should investigate these relationships among a broader population, and explore menstrual effluent cytokine profiles in both healthy and diseased states.

Our findings expand upon the initial reports of Guterstam et al, who compared peripheral and menstrual effluent profiles of 20 cytokines measured on day 1 of menstrual flow.[6] Measures of fold-change between menstrual and peripheral blood samples are highly consistent for the 13 cytokines that were measured in both studies. Our findings corroborate significantly increased levels of IL-6, IL-8, TNF-α, IFN-γ, VEGF, IL-1β, MIP-1a/CCL3, CXCL10, IL-18, IL-12p70, and IL-10, and identify an additional 24 cytokines with differential expression in menstrual effluent. Together these reports support the replicability of comparative cytokine measurements in menstrual effluent in relation to peripheral blood measures.

Additionally, we uncovered two cytokines in menstrual effluent whose levels may be reliably predicted from peripheral blood analysis: TGF-β and MCP-3/CCL7. Testing menstrual effluent for levels of IL-1β, IL-6, TNF-α, as well as TGF-β and CCL7 may provide clinicians insights into fertility conditions associated with elevated levels of these cytokines. TGF-β is a multifunctional growth factor that controls proliferation, differentiation, and chemotaxis.[1] It is highly expressed in whole blood by multiple cell types,[24] and in human uterine tissues throughout the menstrual cycle.[25] Peripheral blood levels of TGF-β have been investigated as biomarkers of diabetic retinopathy,[26] intraperitoneal adhesions,[27,28] and lupus nephritis.[29,30] In the endometrium, TGF-β regulates vascular maturation, and increased *TGFB1* expression is associated with recurrent pregnancy loss and with decreased menstrual bleeding.[31,32] Dysregulation of TGF-β signaling is also implicated in reproductive health conditions such as polycystic ovaries,[33] and endometrial cancer.[34]

CCL7 (also known as MCP-3), is a chemokine for monocytes, and other leukocytes.[35] Elevated levels of CCL7 are present in laparoscopic aspiration samples from adults with endometriosis,[36] and uterine CCL7 production has been associated with leukocyte chemotaxis in cervical ripening,[37] and with preterm delivery.[38] Serum and plasma levels of CCL7 have been proposed as biomarkers of atherosclerosis,[39] systemic sclerosis,[40] and severity of COVID-19 infection.[41] Elevated plasma levels of both TGF-β and CCL7 have also been associated with amnestic mild cognitive impairment.[42] Larger follow-up studies should evaluate the predictive power of TGF-β and CCL7 in menstrual effluent compared to peripheral blood samples, and explore the potential utility of noninvasive menstrual effluent sampling as a diagnostic measure for different health conditions.

5 | CONCLUSION

This observational study expands upon recent endeavors to elucidate the biomarker profiles of peripheral vs menstrual effluent samples. Menstrual effluent contains a unique inflammatory profile that reflects the inflammatory state of endometrial tissues, and is distinctly different from the cytokine signature found in peripheral blood. Further study is needed to establish how various cytokines are correlated between menstrual and peripheral blood across different health conditions, such as endometriosis, endometritis, polycystic ovary syndrome, and recurrent pregnancy loss. Our preliminary results suggest that menstrual effluent can be utilized as a noninvasive option for monitoring uterine inflammatory and fertility signaling. These measures provide a baseline for further comparisons with menstrual cytokine measures in health and disease.

AUTHOR CONTRIBUTIONS

Sara Naseri: investigation; methodology; writing—review & editing. **Yael Rosenberg-Hasson**: formal analysis; investigation; writing—original draft. **Holden T. Maecker**: investigation. **Maria I. Avrutsky**: formal analysis; visualization; writing—original draft; Writing—review & editing. **Paul D. Blumenthal**: conceptualization; funding acquisition; resources; writing—review & editing.

ACKNOWLEDGMENT

This study was internally funded by the Section of Family Planning Services and Research in the Department of Obstetrics and Gynecolgy at Stanford University School of Medicine.

CONFLICTS OF INTEREST

S. Naseri and M. Avrutsky are employed by Qurasense Inc., which develops laboratory tests for analyzing menstrual blood. Funding sources and financial relationships had no role in study design, collection, analysis, or interpretation of data.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online Supporting Information Material.

ETHICS STATEMENT

The study was approved by the Stanford Institutional Review Board (IRB-35817). Interested women completed a telephone screening to assess eligibility and willingness to participate, and all participants signed consent forms.

TRANSPARENCY STATEMENT

The lead author Paul D. Blumenthal affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

ORCID

Sara Naseri http://orcid.org/0000-0002-9729-6893
Maria I. Avrutsky http://orcid.org/0000-0002-3477-0084

REFERENCES

1. Chauhan P, Nair A, Patidar A, Dandapat J, Sarkar A, Saha B. A primer on cytokines. *Cytokine*. 2021;145:155458.
2. Liu C, Chu D, Kalantar-Zadeh K, George J, Young HA, Liu G. Cytokines: from clinical significance to quantification. *Adv Sci*. 2021;8(15):2004433.
3. Yang H, Zhou B, Prinz M, Siegel D. Proteomic analysis of menstrual blood. *Molecular & Cellular Proteomics: MCP*. 2012;11(10):1024-1035.
4. Kyama CM, Overbergh L, Debrock S, et al. Increased peritoneal and endometrial gene expression of biologically relevant cytokines and growth factors during the menstrual phase in women with endometriosis. *Fertil Steril*. 2006;85(6):1667-1675.
5. Gargiulo AR, Fichorova RN, Politch JA, Hill JA, Anderson DJ. Detection of implantation-related cytokines in cervicovaginal secretions and peripheral blood of fertile women during ovulatory menstrual cycles. *Fertil Steril*. 2004;82(Suppl 3):1226-1234.
6. Crona Guterstam Y, Strunz B, Ivarsson MA, et al. The cytokine profile of menstrual blood. *Acta Obstet Gynecol Scand*. 2021;100(2):339-346.
7. Naseri S, Brewster RCL, Blumenthal PD. Novel use of menstrual blood for monitoring glycaemic control in patients with diabetes: a proof-of-concept study. *BMJ Sexual & Reproductive Health*. 2022;48(2):123-127.
8. Naseri S, Lerma K, Blumenthal P. Comparative assessment of serum versus menstrual blood for diagnostic purposes: a pilot study. *J Clin Lab Med*. 2019;4(2). doi:10.16966/2572-9578.130
9. da Silva CM, Vilaça Belo A, Passos Andrade S, et al. Identification of local angiogenic and inflammatory markers in the menstrual blood of women with endometriosis. *Biomed Pharmacother*. 2014;68(7):899-904.
10. Malik S, Day K, Perrault I, Charnock-Jones DS, Smith SK. Reduced levels of VEGF-A and MMP-2 and MMP-9 activity and increased TNF-α in menstrual endometrium and effluent in women with menorrhagia. *Hum Reprod*. 2006;21(8):2158-2166.
11. Tortorella C, Piazzolla G, Matteo M, et al. Interleukin-6, interleukin-1β, and tumor necrosis factor α in menstrual effluents as biomarkers of chronic endometritis. *Fertil Steril*. 2014;101(1):242-247.
12. Evans J, Salamonsen LA. Inflammation, leukocytes and menstruation. *Rev Endocr Metab Disord*. 2012;13(4):277-288.
13. Dahm-Kähler P, Ghahremani M, Lind AK, Sundfeldt K, Brännström M. Monocyte chemotactic protein-1 (MCP-1), its receptor, and macrophages in the perifollicular stroma during the human ovulatory process. *Fertil Steril*. 2009;91(1):231-239.
14. Lee D, Shin KJ, Kim DW, et al. CCL4 enhances preosteoclast migration and its receptor CCR5 downregulation by RANKL promotes osteoclastogenesis. *Cell Death Dis*. 2018;9(5):495.
15. Kitaya K, Nakayama T, Okubo T, Kuroboshi H, Fushiki S, Honjo H. Expression of macrophage inflammatory Protein-1β in human endometrium: its role in endometrial recruitment of natural killer cells. *J Clin Endocr*. 2003;88(4):1809-1814.
16. Maybin JA, Hirani N, Jabbour HN, Critchley HOD. Novel roles for hypoxia and prostaglandin E2 in the regulation of IL-8 during endometrial repair. *Am J Pathol*. 2011;178(3):1245-1256.
17. Nicola NA, Babon JJ. Leukemia inhibitory factor (LIF). *Cytokine Growth Factor Rev*. 2015;26(5):533-544.
18. Salleh N, Giribabu N. Leukemia inhibitory factor: roles in embryo implantation and in nonhormonal contraception. *Sci World J*. 2014;2014:1-10.

19. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harbor Perspect Biol*. 2014;6(10):a016295.
20. Dinarello CA. The IL-1 family of cytokines and receptors in rheumatic diseases. *Nat Rev Rheumatol*. 2019;15(10):612-632.
21. Gonçalves GA, Invitti AL, Parreira RM, Kopelman A, Schor E, Girão MJBC. p27(kip1) overexpression regulates IL-1β in the microenvironment of stem cells and eutopic endometriosis co-cultures. *Cytokine*. 2017;89:229-234.
22. Benington L, Rajan G, Locher C, Lim LY. Fibroblast growth factor 2-A review of stabilisation approaches for clinical applications. *Pharmaceutics*. 2020;12(6):508.
23. Paiva P, Hannan NJ, Hincks C, et al. Human chorionic gonadotrophin regulates FGF2 and other cytokines produced by human endometrial epithelial cells, providing a mechanism for enhancing endometrial receptivity. *Hum Reprod*. 2011;26(5):1153-1162.
24. Morikawa M, Derynck R, Miyazono K. TGF-β and the TGF-β family: context-dependent roles in cell and tissue physiology. *Cold Spring Harbor Perspect Biol*. 2016;8(5):a021873.
25. Chegini N, Zhao Y, Williams RS, Flanders KC. Human uterine tissue throughout the menstrual cycle expresses transforming growth factor-beta 1 (TGF beta 1), TGF beta 2, TGF beta 3, and TGF beta type II receptor messenger ribonucleic acid and protein and contains [125I]TGF beta 1-binding sites. *Endocrinology*. 1994;135(1):439-449.
26. Bonfiglio V, Platania CBM, Lazzara F, et al. TGF-β serum levels in diabetic retinopathy patients and the role of Anti-VEGF therapy. *Int J Mol Sci*. 2020;21(24):9558.
27. Torres K, Pietrzyk Ł, Plewa Z, et al. TGF-β and inflammatory blood markers in prediction of intraperitoneal adhesions. *Adv Med Sci*. 2018;63(2):220-223.
28. Eren EC, Basım P. Role of peripheral inflammatory biomarkers, transforming growth factor-beta and interleukin 6 in predicting peritoneal adhesions following repeat cesarean delivery. *Ir J Med Sci*. 2022;191(6):2697-2704.
29. Rashad NM, El-Shabrawy RM, Said D, El-Shabrawy SM, Emad G. Serum levels of transforming growth factor beta-1 (TGF-β1) as an early no invasive marker for diagnosis of lupus nephritis in systemic lupus erythematosus patients. *Egypt J Immunol*. 2019;26(1):31-42.
30. Jin T, Almehed K, Carlsten H, Forsblad-d'Elia H. Decreased serum levels of TGF-β1 are associated with renal damage in female patients with systemic lupus erythematosus. *Lupus*. 2012;21(3):310-318.
31. Lu Q, Sun D, Shivhare SB, et al. Transforming growth factor (TGF) β and endometrial vascular maturation. *Front Cell Dev Biol*. 2021;9:640065.
32. Maybin JA, Boswell L, Young VJ, Duncan WC, Critchley HOD. Reduced transforming growth Factor-β activity in the endometrium of women with heavy menstrual bleeding. *J Clin Endocr Metab*. 2017;102(4):1299-1308.
33. Kristensen SG, Kumar A, Kalra B, et al. Quantitative differences in TGF-β family members measured in small antral follicle fluids from women with or without PCO. *J Clin Endocr Metab*. 2019;104(12):6371-6384.
34. Zakrzewski PK. Canonical TGFβ signaling and its contribution to endometrial cancer development and Progression-Underestimated target of anticancer strategies. *J Clin Med*. 2021;10(17):3900.
35. Tsou CL, Peters W, Si Y, et al. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest*. 2007;117(4):902-909.
36. Bailey AP, Hill AS, Beste MT, et al. Comparison of cytokines in the peritoneal fluid and conditioned medium of adolescents and adults with and without endometriosis. *Am J Reprod Immunol*. 2021;85(3):e13347.
37. Takemura M, et al. Cyclic mechanical stretch augments both interleukin-8 and monocyte chemotactic protein-3 production in the cultured human uterine cervical fibroblast cells. *Mol Hum Reprod*. 2004;10(8):573-580.
38. Jacobsson B, Holst RM, Andersson B, Hagberg H. Monocyte chemotactic protein-2 and -3 in amniotic fluid: relationship to microbial invasion of the amniotic cavity, intra-amniotic inflammation and preterm delivery. *Acta Obstet Gynecol Scand*. 2005;84(6):566-571.
39. Waśkiel-Burnat A, Niemczyk A, Blicharz L, et al. Chemokine C-C motif ligand 7 (CCL7), a biomarker of atherosclerosis, is associated with the severity of alopecia areata: a preliminary study. *J Clin Med*. 2021;10(22):5418.
40. Yanaba K, Komura K, Kodera M, et al. Serum levels of monocyte chemotactic protein-3/CCL7 are raised in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. *Ann Rheum Dis*. 2006;65(1):124-126.
41. Yang Y, Shen C, Li J, et al. Plasma IP-10 and MCP-3 levels are highly associated with disease severity and predict the progression of COVID-19. *J Allergy Clin Immunol*. 2020;146(1):119-127.e4.
42. Lee KS, Chung JH, Lee KH, et al. Plasma levels of monocyte chemotactic protein 3 and beta-nerve growth factor increase with amnestic mild cognitive impairment. *Cell Mol Immunol*. 2009;6(2):143-147.

How to cite this article: Naseri S, Rosenberg-Hasson Y, Maecker HT, Avrutsky MI, Blumenthal PD. A cross-sectional study comparing the inflammatory profile of menstrual effluent vs. peripheral blood. *Health Sci Rep*. 2023;6:e1038. doi:10.1002/hsr2.1038