Role of chemokines in early pregnancy loss

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Abstract. The present study aimed to compare decidual protein levels and gene expression levels of chemokines between patients with early pregnancy loss and those with voluntary abortion. A total of 15 patients between 6 and 10 gestational weeks, who presented with negative fetal heartbeat to the obstetrics and gynecology outpatient clinics of Gaziosmanpasa Hospital (Yeni Yuzyil University, Istanbul, Turkey) and who had no additional systemic disease and 13 patients between 6 and 10 gestational weeks, who presented with positive fetal heartbeat for voluntary abortion were included in the present study. CX3CL1, CCL17, CXCR4, chemokine ligand 12 (CXCL12) and intercellular adhesion molecule (ICAM)5 protein expression levels were determined by ELISA and gene expression levels by reverse transcription-quantitative PCR in fresh materials recovered after therapeutic curettage. CX3CL1, CCL17, CXCR4, CXCL12 protein levels were significantly higher and ICAM protein level was significantly lower in pregrant women with missed abortion compared with those with voluntary abortion. While the amount of increase in mean CX3CL1, CCL17, CXCR4 and CXCL12 gene expression levels in the tissues of pregnant women with missed abortion was statistically higher than the pregnant women who underwent voluntary abortion, the amount of increase in ICAM5 gene expression was found to be lower ($P<0.001$) in those with missed abortion. In conclusion, the findings of the present study suggested that CCL17, CX3CL1, CXCL12, CXCR4 and ICAM5 may be associated with missed abortion and may play an important role in placental invasion and the continuation of pregnancy.

Introduction

Early pregnancy loss or missed abortion observed in the first trimester of pregnancy is a situation that can be noticed late since the miscarriage event does not occur, despite the embryo has lost its vitality in the uterus. Missed abortion is the most common complication of all pregnancies (1,2). Impairment of the implantation process of an active trophoblast with placental cells at 5-6 weeks of pregnancy can lead to numerous serious pregnancy complications such as abortion in the first trimester, rupture of prenatal membranes, preterm labor, fetal growth restriction and preeclampsia in the third trimester (1-4).

While chemokines are generally involved in homeostasis of the immune system, inducible chemokines may also play a role mainly in inflammatory processes. Chemokines are divided into four subfamilies based on the number and spacing of the first two cysteine residues in a conserved cysteine structure (4). These four subfamilies are named C, CC, CXC and CX3C with C being a cysteine and X an amino acid residue. Among the proteins in the CX3C subclass, CX3CL1 (also called fractalkine) is synthesized as a transmembrane molecule that can be released into the circulation as a soluble isoform by metalloprotease-dependent shedding (5,6). Among the chemokines, CX3CL1, CCL17, CXCR4, chemokine ligand 12 (CXCL12) and intercellular adhesion molecule (ICAM) 5 are proteins that can be released from tissues and mediate the migration of immune system cells to tissues (5-7). Further research is needed to show that not all other markers from the cytokine family are equally affected. The thymus and activation-regulated chemokine CCL17 is one of the inducible chemokines and is produced by endometrial gland cells during pregnancy. CXCL12 and its receptors CXCR4 are widely produced at the maternal-fetal interface and play a regulatory role in materno-fetal dialogue and immune tolerance during early pregnancy. It has been reported that CX3CL1 is increased in the first trimester of pregnancy and in term pregnancy complicated with preeclampsia (6-11).

In the present study, it was aimed to examine the levels of CX3CL1, CCL17, CXCR4, CXCL12 and ICAM5 in decidual tissue and to show their relationship with missed abortion and their utility as a marker for pregnancy outcomes.

Materials and methods

Patients. The present prospectively designed study was approved (approval no. 06) by the Local Ethics Committee of Yeni Yuzyil University (Istanbul, Turkey). Written informed consent was obtained from all patients. The study was performed with patients admitted to the Department

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of Obstetrics and Gynecology of İstanbul Gaziosmanpasa Hospital (Yeni Yuzyil University). A total of 15 patients aged 18-40 years, who presented with a negative fetal heartbeat between 6 and 10 gestational weeks to the obstetrics and gynecology outpatient clinics of the hospital and who had no additional systemic disease were included. The control group consisted of 13 patients between 6 and 10 gestational weeks, who presented with a positive fetal heartbeat and requested a voluntary abortion. Women <18 years of age, with multiple pregnancies, hypertension, diabetes, active maternal infection, chronic renal failure, retroplacental hematoma or systemic lupus erythematosus were excluded from the study.

Fresh materials recovered after therapeutic curettage were stored at -20˚C until the analysis. The materials were sent to Adnan Menderes University Faculty of Medicine Biochemistry Research Laboratory for the analysis of markers. CX3CL1, CCL17, CXCR4, CXCL12 and ICAM5 protein levels were determined by ELISA and gene expression levels by reverse transcription-quantitative (RT-q)PCR in fresh materials.

Gene expression of CX3CL1, CCL17, CXCR4, CXCL12, ICAM5 by RT-qPCR. A total of 5 tissue sections (each 10-μm thick) were collected from each material and put into 1.5-ml microfuge tubes. RNA isolation from tissues was performed in duplicate through the use of a commercially available FFPE RNA isolation kit (cat. no. K156002; Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. A total of 1 μg RNA was reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The amplification was carried out using ready-to-order primers. The following primer sequences were used for amplification: CX3CL1 forward, 5'-CTTCCC AGGAAGCACAGAGG-3' and reverse, 5'-CCTCCATCC TGAGCCTTTGG-3'; CCL17 forward, 5'-ACTTCAAGGGAG CCATTCCC-3' and reverse, 5'-CATCCCTGGAGCAGTCCT CA-3'; CXCR4 forward, 5'-TGACGGACAAGTACAGGC TGC-3' and reverse 5'-CCAGAAGGGAAGCGTGATGA-3'; CXCL12 forward, 5'-TGCCAGAGCCAACGTCAAG-3' and reverse, 5'-CAGCCGGGCTACAATCTGAA-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-AGG GCTGCTTTTAACTCTGGT-3', and reverse, 5'-CCCCAC TTGATTTTGGAGGGA-3, and ICAM5 forward, 5'-AGA TCGCGTAGAGCTGATGC-3' and reverse, 5'-ACCCTACAG CTCAGGGTGAA-3'.

A total of 100 ng of cDNA were amplified using SYBRGreen PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the ABI StepOne Plus detection system. The thermocycling conditions for qPCR were as follows: 95˚C for 10 min, then 40 cycles of: 95˚C for 15 sec and 60˚C for 1 min. The results were analyzed using StepOne Software v2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.), using the $2^{-\Delta\Delta Cq}$ method (12) and normalized to GAPDH mRNA results. Data are expressed as fold induction relative to the controls.

Protein levels of CX3CL1, CCL17, CXCR4, CXCL12 and ICAM5 by ELISA. A total of 4 tissue sections (each 10-15 μm thick) were collected from each material and put into a 1.5 ml centrifuge tube. The samples were incubated in a 250 μl buffer (pH 7.5, 0.05 M Tris, 1 mM EDTA, and 0.5% Tween-20). Tissue samples were homogenized thoroughly and kept samples on ice for 30 min. Samples were Centrifuged at 10,000 x g for 20 min at 4˚C and the supernatant was used for measurement. Protein concentrations were measured with Bradford method (13). The levels of CX3CL1, CCL17, CXCR4, CXCL12 and ICAM5 were determined with the sandwich ELISA method in accordance with the manufacturer's protocols (cat. no. EH0255; Wuhan Fine Biotech Co., Ltd.) with inter-assay cv: <12% and intra-assay cv: <10%, respectively. IFN-γ and IRF5 values were presented as ng/μg protein. All ELISA measurements were performed using a microplate reader (BioTek Epoch). Results were presented as ml/mg of protein.

Statistical analysis. The sample size of the study was calculated using G*Power software (version 3.1.9.7; http://www.gpower.hhu.de/). While calculating the sample size, the effect size was taken as 0.2, the type1 error as 0.05, and the power as 0.8. Statistical analyses were performed using SPSS 25.0 software (IBM Corp.). Descriptive data are expressed as numbers and percentages. Normality of continuous variables was verified with the Kolmogorov-Smirnov Test. Differences between groups in terms of normally-distributed continuous variables were evaluated with an independent samples t-test. The differences between the groups in terms of median values of the non-normally distributed variables were analyzed with the Mann-Whitney U test. Box-and-whisker plots were used to show to compare the medians and quartiles of CCL17, ICAM5, CX3CL1, CXCL12 and CXCR4 between the groups (Fig. 1). Receiver operating characteristic (ROC) analysis was conducted to determine a threshold value for CXCL12 for discrimination of missed and voluntary abortion. The results were evaluated within the 95% confidence interval and P<0.05 was considered to indicate a statistically significant difference.

Results

The mean age of the pregnant women was 29.7±4.7 years (Table I). There was no difference for age between groups. Patient characteristics were shown in Table I. The median CCL17 (274.9 vs. 133.8 pg/mg protein; P=0.008), CX3CL1 (765.2 vs. 284.8 pg/mg protein; P=0.001), CXCL12 (1.6 vs. 0.2 ng/mg protein; P<0.001) and CXCR4 (473.8 vs. 405.6 pg/mg protein; P=0.018) protein levels were statistically significantly higher in the pregnant women with missed abortion compared with those with voluntary abortion (Fig. 1). The median ICAM5 protein level was significantly lower in the missed abortion group than in the voluntary abortion group (1.0 vs. 1.6 ng/mg protein; P<0.001) (Table II) (Fig. 1).

The median amount of increase in gene expression levels of CCL17/GAPDH (2.9 vs. 1.2 fold increase; P<0.001), CX3CL1/GAPDH (2.9 vs. 1.1 fold increase; P<0.001), CXCL12/GAPDH (2.7 vs. 1.1 fold increase; P<0.001) and the mean CXCR4/GAPDH (1.3 vs. 0.9 fold increase; P=0.013) was found to be significantly higher in tissues of pregnant women with missed abortion than that of the pregnant women

Table I. Demographic characteristics of the studied population.

Characteristics	Median (IQR)	Missed abortion (n=15)		Voluntary abortion (n=13)	
		Mean ± SD	Median (IQR)	Mean ± SD	P-value
Age, years	30 (7)	29.5±4.1	31 (8)	30.2±6	0.749[a]
Gravida	2 (1)	1.8±0.7	1 (2)	1.8±1	0.65[b]
Parity	1 (1)	0.6±0.6	1 (2)	0.8±0.8	0.683[b]
Weight (kg)	60 (10)	60.8±10.9	63 (17)	64.8±9.8	0.325[a]
Height (cm)	160 (2)	159.4±5.2	161 (6)	162.5±4	0.095[b]
Gestational week	8 (1)	8.1±0.7	6 (0)	6±0.6	<0.001[b]
BMI (kg/m^2)	22.7 (3.2)	23.9±3.9	23.4 (5.5)	24.6±4.4	0.504[b]

[a]Independent samples t-test was used for comparison due to the normal distribution of the variables verified with the Kolmogorov-Smirnov test. [b]Mann-Whitney U test was used for comparison. IQR, inter-quartile range; SD, standard deviation; BMI, body mass index.

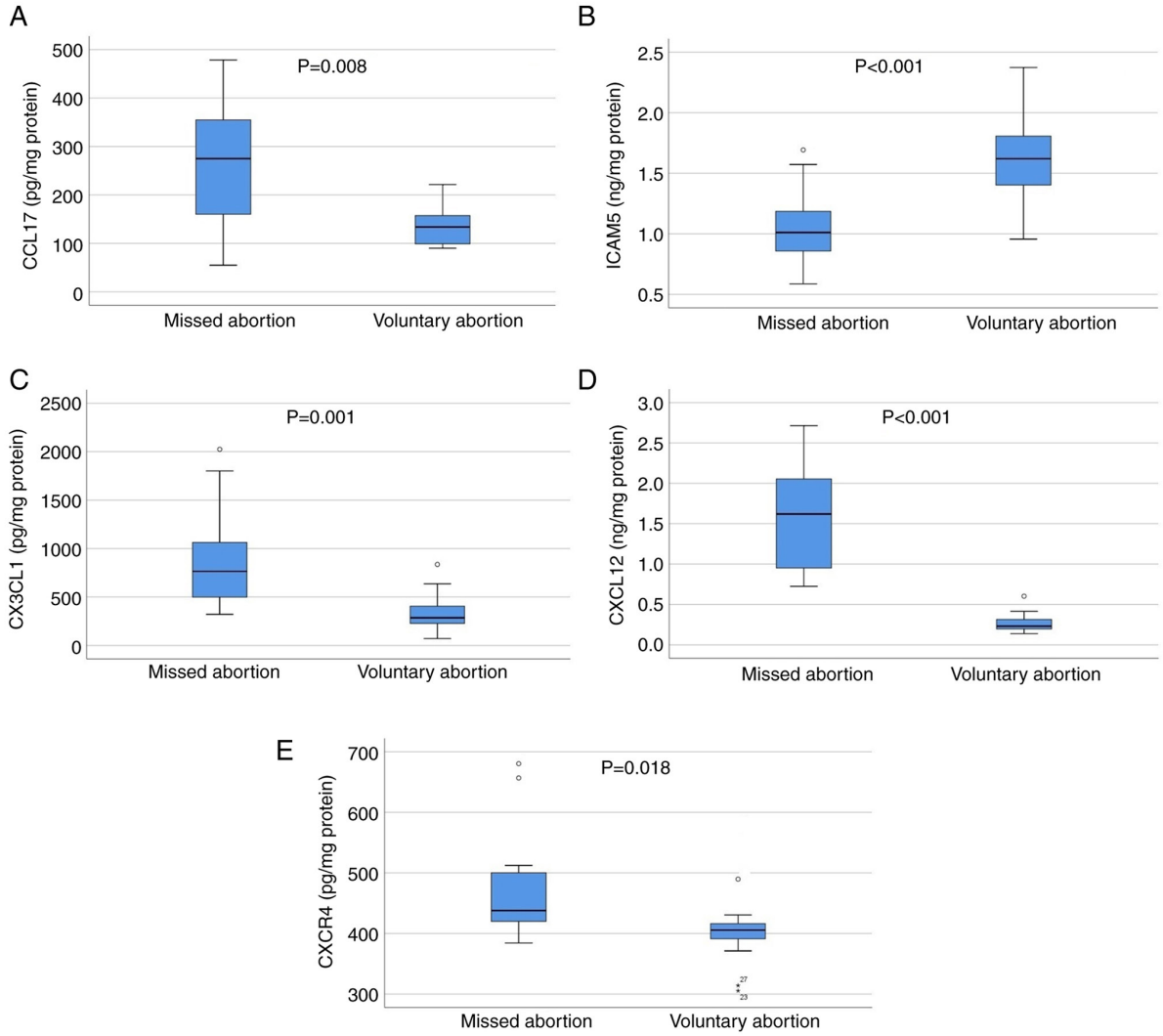

Figure 1. Box-and-whisker plots comparing the medians and quartiles of (A) CCL17 (P=0.008), (B) ICAM5 (P<0.001), (C) CX3CL1 (P=0.001), (D) CXCL12 (P<0.001) and (E) CXCR4 (P=0.018) protein levels between the two groups. Small circles show the outliers. CXCL12, chemokine ligand 12; ICAM5, intercellular adhesion molecule 5.

with voluntary abortion. The mean amount of increase in ICAM5/GAPDH tissue gene expression level was significantly lower in the missed abortion group than in the voluntary abortion group (0.9 vs. 1.3 fold increase; P<0.001) (Table II) (Fig. 2). ROC analysis revealed that a threshold value of 0.66 ng/mg protein for CXCL12 had a sensitivity and specificity

Table II. Comparison of mean gene and protein expression levels of chemokines in decidual tissues of patients between groups.

		Missed abortion (n=15)		Voluntary abortion (n=13)	
Characteristics	Median (IQR)	Mean ± SD	Median (IQR)	Mean ± SD	P-value
CCL17 (pg/mg protein)	274.9 (239)	264.9±130.3	133.8 (75)	140.3±45.7	0.008[a]
ICAM5 (ng/mg protein)	1.0 (0)	1.03±0.3	1.6 (1)	1.6±0.4	<0.001[b]
CX3CL1 (pg/mg protein)	765.2 (726)	874.1±521.7	284.8 (199)	349.1±202.2	0.001[a]
CXCL12 (ng/mg protein)	1.6 [1]	1.57±0.68	0.2 (0)	0.3±0.1	<0.001[a]
CXCR4 (pg/mg protein)	473.8 (93)	469.7±89.5	405.6 (42)	408.6±68.8	0.018[a]
CCL17/GAPDH	2.9 (0)	2.9±0.3	1.2 (0)	1.2±0.2	<0.001[a]
ICAM5/GAPDH	0.8 (0)	0.9±0.2	1.3 (0)	1.3±0.23	<0.001[b]
CX3CL1/GAPDH	2.9 (1)	3.0±0.8	1.1 (0)	1.2±0.2	<0.001[a]
CXCL12/GAPDH	2.7 (2)	2.8±1	1.1 (0)	1.1±0.2	<0.001[a]
CXCR4/GAPDH	1.2 (0)	1.3±0.4	1.0 (0)	0.9±0.3	0.013[b]

[a]Mann-Whitney U test was used for comparison. [b]Independent samples t-test was used for comparison due to the normal distribution of the variables verified with the Kolmogorov-Smirnov test. IQR, inter-quartile range; SD, standard deviation; GADPH, glyceraldehyde-3-phosphate dehydrogenase; CXCL12, chemokine ligand 12; ICAM5, intercellular adhesion molecule 5.

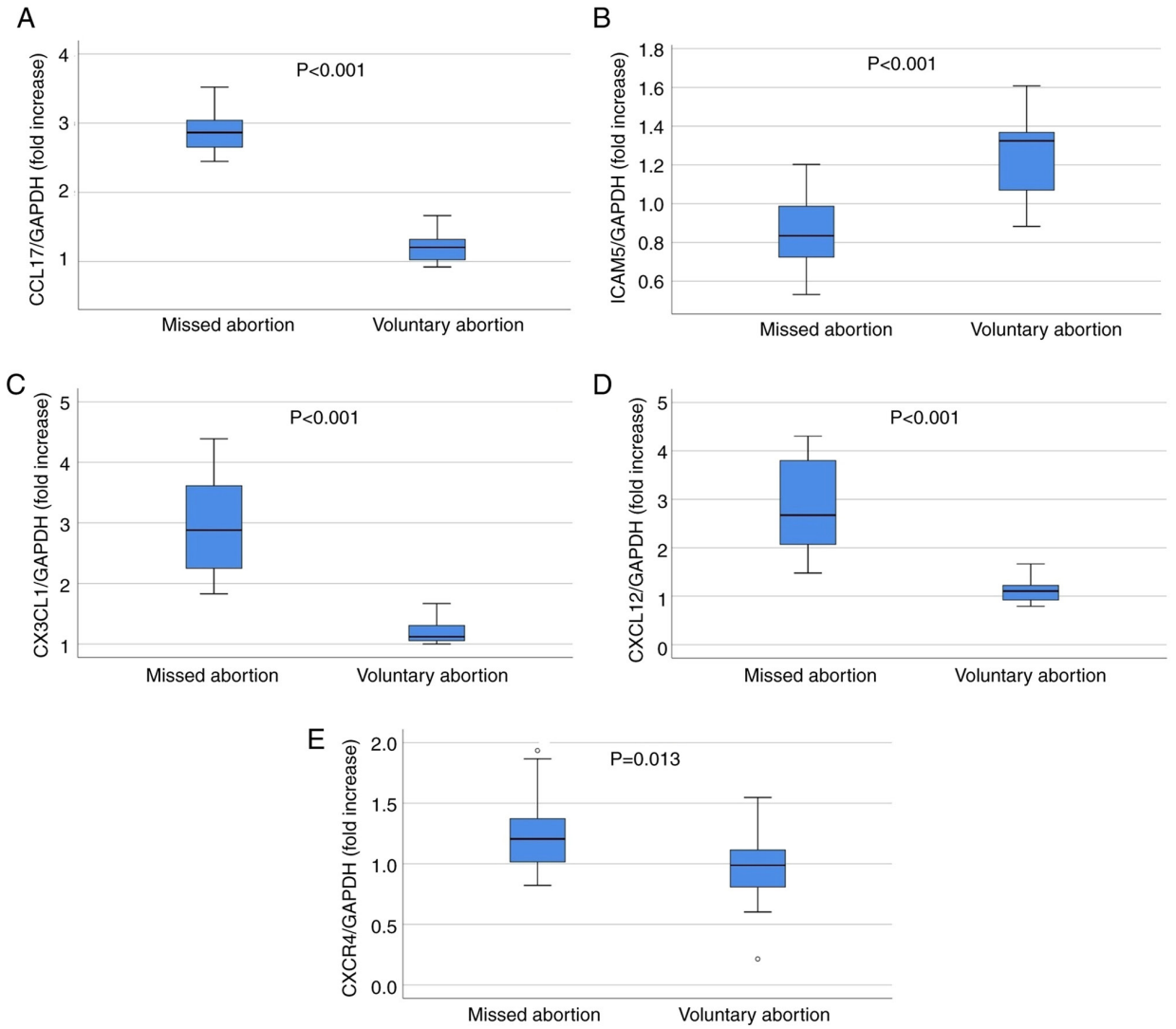

Figure 2. Box-and-whisker plots comparing the medians and quartiles of fold-increase values of (A) CCL17/GADPH (P<0.001), (B) ICAM5/GADPH (P<0.001), (C) CX3CL1/GADPH (P<0.001), (D) CXCL12/GADPH (P<0.001) and (E) CXCR4/GADPH (P=0.013) between the groups. Small circles show the outliners. GADPH, glyceraldehyde-3-phosphate dehydrogenase; CXCL12, chemokine ligand 12; ICAM5, intercellular adhesion molecule 5.

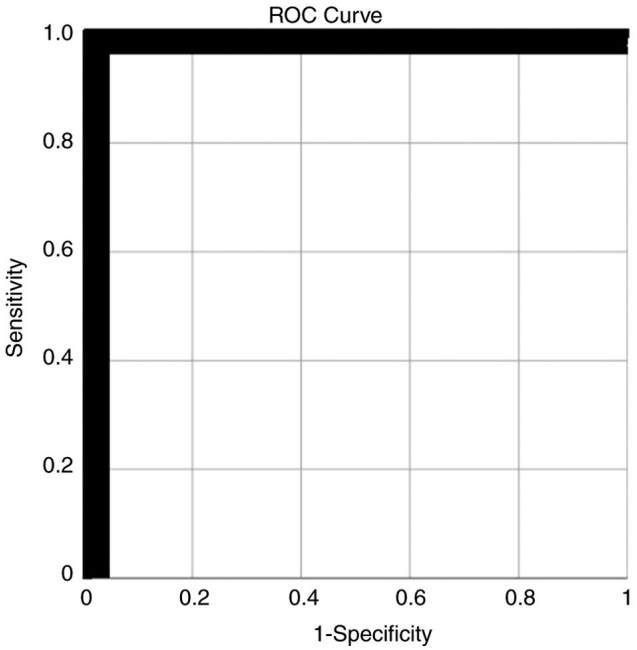

Figure 3. ROC analysis revealed that a threshold value of 0.66 ng/mg protein for CXCL12 had a sensitivity and specificity of 100% for determination of missed abortion (AUC: 1; lower bound: 1.0; upper bound: 1.0; P<0.001). ROC, receiver operating characteristic; CXCL12, chemokine ligand 12.

of 100% for determination of missed abortion (AUC: 1; lower bound: 1.0; upper bound: 1.0; P<0.001) (Fig. 3).

Discussion

In the present study, it was found that median CCL17, CX3CL1, CXCL12 and CXCR4 protein levels were significantly higher in pregnant women with missed abortion than in those with voluntary abortion. The mean ICAM5 protein level was identified to be significantly lower in the missed abortion group compared with the voluntary abortion group. When chemokines were examined in terms of gene expression levels, the median amounts of increase in gene expression levels of CCL17, CX3CL, CXCL12 and CXCR4 were significantly higher in the missed abortion group compared with the voluntary abortion group. The mean amount of increase in gene expression levels of ICAM5 protein was significantly lower in the missed abortion group compared with the voluntary abortion group. The maternal component of the placenta was used as the material. The maternal component of the placenta is known as the decidua basalis. Oxygen and nutrients from maternal blood diffuse through the villus walls in the intervillous spaces and enter the fetal capillaries. Also decidua secretes hormones, growth factors and cytokines. It has receptors for estrogen, progesterone, growth hormone, and others. If missed abortion is not diagnosed, it may be accompanied by maternal complications such as septic abortion or excessive bleeding (4). In addition, the obtained data will shed light on recurrent spontaneous pregnancy losses. Certain studies have shown that these 5 chemokines (CX3CL1, CCL17, CXCR4, CXCL12 and ICAM5) that were studied play a role in the implantation process of the embryo (9,8,14-16).

CX3CL1 has recently attracted attention in the field of reproductive research by regulating adhesion and migration processes in fetal-maternal interaction at different stages of human pregnancy (8). In addition, an increasing body of evidence has suggested that a number of pregnancy pathologies are associated with increased placental CX3CL1 expression, including chorioamnionitis (17), diabetic pregnancy (18), and severe early-onset preeclampsia (19,20). In a previous study by Hannan *et al* (21) investigating the CX3CL1 level in the serum of 34 first trimester abortions and 44 normal births, CXCL3 was not detected in term patients, but it was found in the aborted group. Li *et al* (22) detected significantly upregulated expression of CX3CL1 and CX3CL1 receptor in the uterus of IFN-γ-induced aborted mice. In the present study, the median CX3CL1 protein and gene expression levels were found to be significantly higher in pregnant women with missed abortion compared with pregnant women with voluntary abortion. The abortion week in the materials received was at most 10 weeks, and it was observed in only 1 patient. Brain tissue is not considered to be dominant for such a small fetus. Similarly, the brain tissue is damaged in voluntary abortion cases. The present data are consistent with previous studies showing the role of CX3CL1 in the feto-maternal interaction in the process of placental invasion and indicated that CX3CL1 may be associated with missed abortion (20,21).

Chemokines are unique proteins with the ability to control immune cell chemoattraction and participate in cell proliferation, migration and apoptosis. CXCL12 can modulate the proliferation, invasion and survival of human trophoblasts that are crucial for establishing a successful pregnancy (23-25). CXCL12 and its receptor CXCR4 are involved in uterine natural killer cell recruitment, placentation, vascular remodeling, embryogenesis, and cardiovascular and central nervous system organogenesis (23-29). Suppression of CXCL12/CXCR4 signaling during the small window of conceptus implantation reduces placental vascularization, induces autophagy, and moistens the inflammatory placental environment (30-33). In addition, recent evidence has suggested that suppression of CXCL12-induced actions at the fetal-maternal interface reduces trophoblast invasion into the maternal endometrium and delays uterine remodeling (30). Chao *et al* (34) found that the expression of CXCL12 chemokine and its receptor CXCR4 increased in the abortion material induced in mice with IFN-gamma. In another study, Dimova *et al* revealed an increased expression of CXCL12 chemokine and its receptor CXCR4 in the decidua of 30 patients with spontaneous abortion compared with 15 patients with elective abortion (35). In the present study, the median protein level and gene expression of CXCL12 and CXCR4 were found to be significantly higher in pregnant women with missed abortion than in pregnant women with voluntary abortion. In addition, the present findings revealed a highly reliable threshold value for CXCL12 (with a sensitivity and specificity of 100%) in discrimination of missed and voluntary abortions. These findings showed the association of these molecules with missed abortion.

It has been reported that CCL17 level does not change in cases of spontaneous abortion (11). In the present study, the mean protein level and gene expression of CCL17 were identified to be significantly higher in pregnant women with

missed abortion than in pregnant women with voluntary abortion. Furthermore, the mean ICAM5 protein level was found to be significantly lower in pregnant women with missed abortion than in the those with voluntary abortion. As ICAM5 is solely formed in the brain tissue, its level may be reduced in abortion cases due to lack of brain development (36). However, it was planned to discriminate the abortion whether it was missed or voluntary, thus the voluntary abortion cases were used as the control group. According to the present findings, it was observed that brain tissue is damaged in missed abortion significantly more than the voluntary abortion cases.

Since the present study was planned as a cohort study, the pregnant women were not followed up for a long time after abortion and the long-term changes in the levels of these molecules were not examined, which can be considered as the major limitation of the present study. Again, there is a need for further studies on whether the amount of gene expression in the tissue changes on the day of missed abortion diagnosis. Another limitation is the relatively small number of pregnant women included in the study, although it was sufficient for statistical analysis. In addition, other markers from the family of cytokines also need to be investigated to reveal that not all are equally affected. In future studies, broader genetic analyzes can be obtained by examining the relationship between karyotype analyses and chemokines in missed abortion.

In conclusion, the findings of the present study suggested that CCL17, CX3CL1, CXCL12, CXCR4 and ICAM5 may be associated with missed abortion and may play an important role in placental invasion and the continuation of pregnancy. However, further comprehensive studies are needed to draw more definitive conclusions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DH was involved in project development and manuscript writing. SG was responsible for data collection, management and analysis. OC and DH confirm the authenticity of all the raw data. OC and VT contributed to data analysis, manuscript writing and editing. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present prospectively designed study was approved (approval no. 06) by the local ethics committee of Yeni Yuzyil University (Istanbul, Turkey). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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