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Effect of Uterine Flushing Leukemia Inhibitory Factor on Reproductive Outcome of Infertile Couple

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Abstract

Aim: To determine the role of Leukemia Inhibitory Factor (LIF) in predicting the reproductive outcome in infertile couple.

Method of study: Uterine flushing and endometrial samples were collected from infertile women during implantation window with stage I/II endometriosis (*n*=17), patients with idiopathic infertility (*n*=24), luteal phase defect (*n*=16), and fertile control (*n*=26). LIF was assessed in uterine flushings in all patients by ELISA. In endometrium, semiquantitative RT-PCR was performed for LIF mRNA expression. Reproductive outcome of all infertile women were recorderd.

Results: 34.6% of patients included in the study got pregnant. LIF concentration had 96.5% sensitivity and 86.1% specificity at a cut-off point of 2.45 pg/ml for predicting the reproductive outcome.

Conclusion: LIF in uterine flushing could be used as a predictor of successful reproductive outcome in infertile women.

Keywords: Endometrium; Infertility; Leukemia inhibitory factor; Idiopathic infertility; Receptivity

Introduction

Eighty million couples worldwide are diagnosed with infertility [1]. The main factors contributing to infertility are: poor semen parameters, blocked tubes, lack of ovulation and endometriosis [2]. However, some patients despite accurate diagnosis and proper treatment still fail to achieve pregnancy. In addition, it is estimated that some 15% of the diagnosed couples have so called idiopathic infertility [3]. Currently the age of a patient, the ovarian reserve and the duration of infertility are the only factors that seem predictive of pregnancy probability [4].

Basically, for successful implantation, there is a need for a healthy embryo and a receptive endometrium. The endometrium exhibits a unique receptivity period from the seventh until the ninth day after ovulation during which the implantation is possible as noticed in *in vivo* and *in vitro* studies [5-8]. Implantation before or after this period is either impossible or results in very high miscarriage rates [9]. Many physicians ordered an endometrial biopsy relied on the morphological criteria of Noyes and Hertig to detect the so called 'luteal phase defect' [10,11]. When there were more than 2 days lag between the ovulation cycle and the endometrial cycle, it was considered that the reason for infertility was endometrial maldevelopment, and this prompted progestin supplementation. However, in recent years it has been stated that over 50% of women with proven fertility could be diagnosed with the luteal phase defect [12]. Also ultrasound assessment of endometrial development with measurement of the endometrial thickness, volume or Doppler studies still fail to correctly identify receptive endometrium [13,14].

A numerous attempts has been made to discover various substances involved in the creation of the 'implantation window', that is, the period of maximal endometrial receptivity [15-18]. Recently advances in the genetic technologies and the use of gene matrix chips have enabled scientists to study at one time the expression of many thousands of genes in infertile women during the implantation window [19].

One of the factors that is believed to play a major role in the endometrial receptivity is the Leukemia Inhibitory Factor (LIF).The presence of LIF in the uterine lumen has been shown as an absolute requirement for implantation to take place in gene knock-out studies on mice [20]. It has also been shown that LIF plays a similar role in humans, with many infertile patients exhibiting low or absent LIF, both at the mRNA and protein level [21-23]. To date there has been no prospective study, regarding the true impact of LIF levels during the implantation window on human fertility. The only study that has made such an attempt has been performed in an IVF setting, thus potentially biased by ovulation induction [24,25]. In addition, the measurement of LIF concentration took place past the period of maximal receptivity, so the conclusions might not reflect the natural cycle scenario.

The aim of our study was to assess the impact of the leukemia inhibitory factor concentration in uterine flushing on the reproductive potential of women with different causes of infertility.

Material and Methods

A total of 230 consecutive infertile women were recruited to this study. Patients who were attendants of infertility outpatient clinic of Royal Commission hospitals, El Jubail, Saudia arabia and who were admitted for diagnostic hysteroscopy and laparoscopy during infertility workup were included in this study. The mean duration of infertility was 5.2 years (3-14 years). The mean age of the patients was 28 years (20-41 years). The patients underwent ovulation folliculometry, semen analysis and tubal patency tests. Any anomaly found within those tests excluded the patient from the study. Patients underwent laparoscopy and hysteroscopy 7-9 days after ovulation that was assessed by serial ultrasound folliculometry.

On the day of the operation, before laparoscopy, a uterine flushing was performed according to a protocol described elsewhere [26]. Briefly, it involved placing a sterile speculum in the vagina, visualizing the cervical os, and positioning insemination catheter into the uterine

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lumen. The catheter was connected to 10 mL syringe filled with 3.5 mL of sterile normal saline. The saline was slowly infused into the uterine cavity, aspirated and the procedure was repeated a few times to achieve turbulent flow and homogenic distribution of substances within the fluid. The fluid was centrifuged at 300 g for 3 min, immediately frozen and kept till further research. In addition, an endometrial biopsy was performed with Pipelle or during hysteroscopy for LIF mRNA assessment and histological evaluation.

Only patients with minimal grade endometriosis, luteal phase defect and idiopathic infertility were included in further study. The diagnosis of endometriosis was based on visualization of endometrial lesions found during laparoscopy. In 60% of the cases, with uncertain initial diagnosis, histopathologist was also asked to confirm the diagnosis. Only patients with stage I and II endometriosis according to the revised American Fertility Society endometriosis staging were considered for further studies [26,27]. Finally, a total of 14 women with stage I (n=10) and stage II (n=7) endometriosis were analyzed. Also 16 women with luteal phase deficiency (defined as a lag of more than 2 days according to the Noyes & Hertig criteria) and 24 with idiopathic infertility were included in the study. The women from the latter group had no anomalies in the tests mentioned above. The reason for inclusion in the study of patients with luteal deficiency was to assess whether the morphological changes were accompanied by molecular disturbances within the endometrium. Patients with idiopathic infertility were chosen as, in this group, a potential for yet undiscovered defects is obviously the greatest.

Twenty-six healthy fertile women aged 24-35 years (mean 24) constituted the control group, none of them experienced a miscarriage. The indication for laparoscopy was suspicion of endometriosis or pelvic pain of unknown origin. For all women an endometrial biopsy was taken at the time of laparoscopy. Only those without endometriosis and inflammation of the pelvis at the time of diagnostic laparoscopy (sterile fluid samples from cul-de-sac) were considered as control. None of the women (both in control and infertility group) have used any form of hormonal treatment or ovulation inducing drugs at least 3 months prior to the study. All the patients signed an informed consent and local ethical committee approved the design of the study.

Enzyme-Linked Immunosorbent Assay for LIF

The assessment of LIF concentration was performed with Bender Med Systems GmbH kit (Vienna, Austria).The detection of LIF was made according to the manufacturer's instructions. All samples and controls were performed in duplicate. A 100 µL of standard dilutions were added in wells covered with anti-LIF antibody, ranging from 3.13 to 200 pg/mL (including negative control) and 100 µL of the uterine fluid diluted 1:1. To each well, we added 50 µL of biotinylated secondary antibody (anti-LIF). This mix was incubated for 2 hr in room temperature. After incubation, the wells were washed three times in the buffer supplied by Bender Med Systems. Finally, the wells were filled with 100 µL of streptavidin-peroxidase conjugate (Bender Med Systems, Vienna, Austria) and incubated for 1 hr. The plates were then washed, and 100 µL of Tetra Methyl Benzidine (TMB) staining substance was added. After 10 min, the stop solution was added, and the wells were read at 450 nm wavelength in BioTek Instruments spectrophotometer (Winooski, VT, USA). The negative controls were fluids without the primary antibody.

The semiquantitive RT-PCR assay of LIF mRNA

The endometrial sample (<0.5 cm^3) was immediately placed after collection in adequate amount (10 µL/mg tissue) of RNA later Stabilization Reagent from Qiagen (Doncaster, Australia). The tissue samples were kept in -20°C till extraction of RNA. RNA was extracted from endometrial cells with RNAeasy Protect Mini Kit (Qiagen) according to the manufacturer's instructions. The reverse transcription reaction was performed with Qiagen One Step RT-PCR kit. The following oligonucleotide primers were designed from published nucleotide sequences: for LIF (forward: GATGAGTGGAAGATAGAGAGG, reverse: CGTCTTGAATCCCAGTCC). The amplification protocol was as followed: 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 1 min at 72°C (extension). This protocol was repeated in 35 cycles. Each investigation was performed in duplicate. PCR products were visualized by ethidium bromide in a 1.5% agarose gel. The quantification of PCR products was performed with the Image Quant TL software from Amersham (Piscataway, NJ, USA) with corrections for background staining. The house-keeping gene was Glyceraldehydes-3-Phosphate Dehydrogenase (GAPDH). The results are presented as LIF/GAPDH expression ratios.

Twelve months after ending the diagnostic tests on the last patient in the study, a questionnaire was sent out to all the patients taking part in the study. The questions referred to the present obstetrics history, whether there were any pregnancies, the outcome of the pregnancies and whether there was any additional treatment involved (like ovulation induction or intrauterine inseminations). From the original 54 infertile patients, 26 (48.1%) women were qualified for prospective assessment. The criteria for qualification for the prospective study were as follows: none of those women has had any additional treatment for infertility; all pregnancies were achieved as a result of spontaneous conception. Women with treated with ovulation induction, intrauterine inseminations, GnRH analog treatment for endometriosis were excluded, as these interventions clearly influence the fecundability. The goal of our analysis was to estimate the impact of LIF on natural fertility. As the first patient in the initial part of the study was recruited in 2009, the duration for attempts to get pregnant lasted from 1.5 years to almost 3.5 years.

A successful pregnancy outcome was defined as a 'taken home baby'.

Statistical analysis

The normality of distribution was assessed using Shapiro-Wilk's test. As results for the LIF levels in uterine flushing did not conform to normal distribution the differences between the groups were assessed using the non-parametric Mann–Whitney test, and analysis of correlations was performed with the Spearman test. Statistical analysis was performed using Sigma Stat 3.1 software (Systat Software, Inc., San Jose, CA, USA). A P<0.05 was considered statistically significant.

Results

Table 1 shows the concentration of LIF in uterine flushings in patients from all the studied groups.

The highest percentage of patients with undetectable LIF levels in uterine flushing was noted in patients with idiopathic infertility (25.9%). Both the infertile patients with endometriosis and patients with the luteal phase defect had a similar percentage of patients who exhibited undetectable (<1 pg/mL) LIF concentrations (15.4% and 14.3%, respectively).

The LIF mRNA Expression

Utilizing the Spearman test a positive, statistically significant correlation was stated between the LIF concentration in uterine flushing and expression of LIF/GAPDH mRNA in the endometrium

(rs=0.459356; *P*<0.00005). High concentrations of LIF in uterine flushings were accompanied by high levels of LIF mRNA expression Table 2.

Using the Receiver Operating Characteristic (ROC) curves, it was stated that at a cut-off value of 8.63 pg/mL LIF in uterine flushing differentiated fertile patients from women with idiopathic infertility with 70.4% sensitivity and 95.2% specificity Figure 1.

A significant positive correlation was found between LIF concentration in uterine flushing and expression of LIF/GAPDH mRNA in the endometriumr=-0.991(*P*<0.00005).

The ROC curve for patients with idiopathic infertility compared to control was illustrated in Figure 2. Also at a cut-off value 2.31 pg/mL LIF in uterine flushing differentiated patients with idiopathic compared to control with sensitivity - 95.7%; specificity - 81.8%; AUC - 0.875).

In all of the remaining groups, the LIF concentration in uterine flushing lacked the sensitivity and specificity to differentiate them from the control group. Also, when examining the whole infertile population, we were unable to differentiate them from control, based on the LIF assessment (data not shown).

Because only 26 patients qualified for prospective analysis (which represents 48% of the initial group), the effect of LIF concentration on our questionnaire was assessed for all the patients together, without splitting them into smaller subgroups, as the statistical analysis would then be impossible of those patients who qualified, nine of 26 (34.6%) did not achieve pregnancy. The remaining 17 of 26 patients (65.4%) did get pregnant and a majority of them carried the pregnancy to term. Only one patient has had an ectopic pregnancy. There was no difference between the mean time of trying to achieve pregnancy between patients who eventually did, and did not get pregnant (1.5 years *versus* 1.4 years).

We have re-examined the median values of LIF in uterine flushing regarding their influence on the reproductive outcome. There was statistically a higher median concentration of LIF in patients with successful attempt at pregnancy compared to those patients who failed to get pregnant (22.07 pg/mL *versus* 1.1 pg/mL; *P* <0.008). Furthermore, we have again used the ROC curves to determine the cut-off point that best predicts chances for a successful pregnancy, based on the LIF concentration measurement in uterine flushing, and the results are given in Figure 2.

Discussion

This study, to our knowledge, represents the first attempt in literature to assess prospectively the value of the Leukemia Inhibitory

	Number	Median	Range 25-75% confidence interval	*P**
Infertile women (the whole group)	57	13.64	0-320 2.6-27.33	<0.05
Idiopathic infertility	24	4.21	0-70.87 0.25-14.63	<0.01
Luteal phase defect	16	11.33	0-186.8 4.69-49.49	NS
Infertile women with endometriosis	17	25.53	0-379.14 12.63-43.32	NS
Control group	26	38.46	0-324.6 13.95-60.47	–

*The *P*-value corresponds to statistical significance compared with control.

Table 1: Median, Range and 25-75% Confidence Intervals for the LIF Concentration (in pg/mL) in the Uterine Flushing.

	Number	Median (25%-75%	Confidence interval) mRNA LIF/GAPDH expression	*P**
Infertile patients	57	0.914486-2.65790	(0.753495-1.157888)	<0.01
Control group	10	1.426640-1.8987654	(1.094208-1.667347)	–

*The *P*-value corresponds to statistical significance compared to control.

Table 2: The Expression Results for LIF/GAPDH mRNA, Median and 25-75% Confidence Interval for the Whole Group of Patients with Infertility and Control.

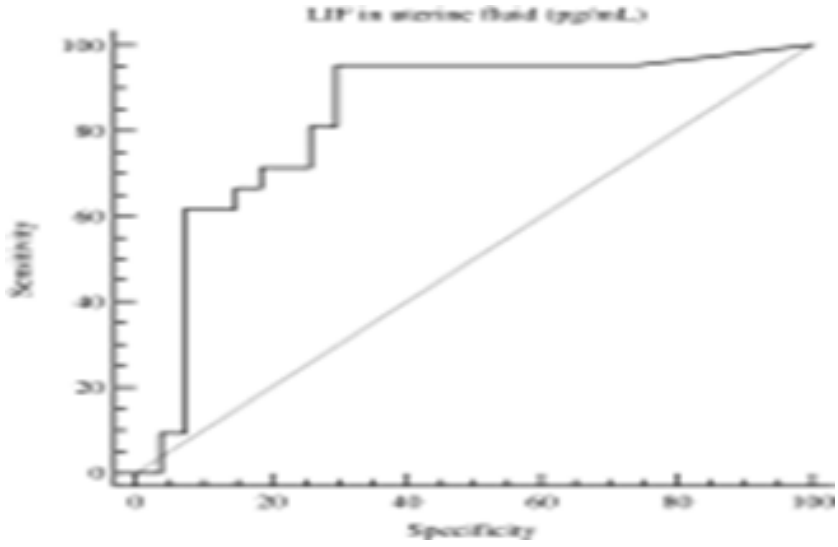

Figure 1: The ROC curve for patients with idiopathic infertility compared to control (cut-off value 8.63 pg/mL; sensitivity - 70.4%; specificity - 95.2%; AUC - 0.833).

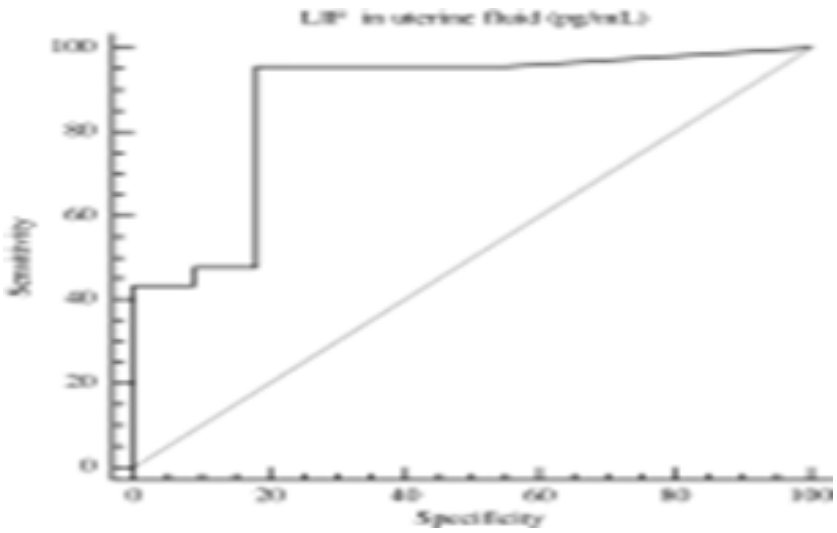

Figure 2: The ROC curve for patients with idiopathic infertility compared to control (cut-off value 2.31 pg/mL; sensitivity - 95.7%; specificity - 81.8%; AUC - 0.875).

Factor (LIF) concentration in uterine flushing for the prediction of the reproductive outcome. Despite many advances in the field of fertility, even with the use of modern IVF techniques, the pregnancy rate per transfer remains at a disappointing 30-40% [28]. Even after bypassing the problems with tubal patency, ovulation problems, and use of ovulation inducing drugs and embryo/blastocyst transfer techniques, the true rate of implantation remains very low [29-31]. As we do have ways of assessing the embryo, it has become increasingly clear that the problem lies largely in the endometrium itself [5,6]. Until recently, the endometrium was deemed fit for implantation once its development followed the strict Noyes and Hertig criteria [10]. For some years now, the focus of scientists has been brought on the functional status of the endometrium, including various cytokines, proteins and receptors [17,18]. Most of these tests have been based on anecdotal evidence coming from animal or *in vitro* studies. Recently, a more systematic approach has been offered including study of many hundreds of genes at the same time [19]. This has allowed for a broader perspective on the function of the endometrium on the genetic and molecular level.

The leukemia inhibitory factor has been studied rather extensively in animals, humans and *in vitro* endometrial cultures [21,32,33]. Various studies have assessed the mRNA that encoded this glycoprotein, some have focused on immunohistochemistry, and some on the level of LIF in the uterine cavity [23,34,35]. All of the studies have confirmed the role of the LIF in human reproduction. Our results are in accordance with previously mentioned studies. We have found the highest number of LIF deficient patients in the idiopathic infertility group. Also in this subgroup of women, the overall mean concentration of LIF was statistically lower compared to patients from the control group. Patients with idiopathic infertility are a particular group of patients that has been studied extensively regarding the LIF expression and secretion, and all the studies point to lower LIF levels in those patients [26,34]. Also in other infertile subgroups, namely, patients with minimal endometriosis and luteal phase defects have shown lower concentrations of LIF in uterine flushing compared to controls; however, it failed to reach a statistical significance. Some of the authors have found that patients with endometriosis have significantly lower staining intensities to LIF in endometrium compared with controls [36]. We do agree that some patients with infertility might have aberrant LIF expression; however, this is not a common feature of endometriosis. We have evaluated only patients with minimal endometriosis, as, with higher stages of endometriosis, the cause of infertility appears obvious. In addition, the rates of implantation in IVF cycles in patients with all grades of endometriosis are invariably lower compared to patients without this disease [28].

We do know that endometrial implants, contrary to eutopic endometrium, contain the aromatase enzyme [37]. Therefore, as the production of LIF is highly dependent on the concentration of estrogens and progesterone, massive lesions can induce suppression of the expression of LIF mRNA [38]. In addition, during our study, contrary to Dimitriadis et al., we have used methods of assessment by an unbiased observer [36]. The results of uterine flushing are given in pg/ml and the mRNA expression is depicted as a ratio of LIF/GAPDH. As assessment of endometrial staining intensities for any given substance (even with the use of H-score) is still rather subjective, this, in part, might explain the differences between the results of our study and of the aforementioned authors.

With respect to patients diagnosed with luteal phase deficiency, we wanted to see whether the changes observed under the microscope were accompanied by changes on the molecular and gene levels. As more than 50% of women with proven fertility exhibit lagging endometrial development, it is clear that these changes do not interfere with fecundability [12]. Therefore, we were not surprised to find lower LIF concentrations in this group of patients compared to fertile control. The results were also lower compared to patients with endometriosis. It indicates that in some patients with the luteal phase deficiency, there might be a genetic defect, which either results in lag of development of the endometrium or is a result of such a lag. However, as in patients with endometriosis, it is not a very common feature, since only 15.4% of women with luteal defect had undetectable levels of LIF in uterine flushing compared to almost 26% in patients with idiopathic infertility. It is possible that other factors play a role in the delayed development of the secretory phase of the endometrium.

The uterine flushing technique has been validated in many studies. To validate our uterine fluid collection technique, we have also assessed the LIF mRNA content in the endometrium. Since the LIF mRNA follows the same expression curve as the LIF in uterine flushing, we have decided to try and correlate the mRNA expression with uterine flushing to assess the accuracy of our flushing method. A positive, statistically significant correlation achieved in our studies between the

LIF concentration in uterine flushing and the LIF mRNA expression in endometrium confirms the accuracy of the flushing method used. These observations confirm the results of all the previous papers regarding the role of LIF in the implantation processes in humans.

In our current study, we have decided to check whether there is a cut-off point for the concentration of LIF in uterine flushing that would enable us to differentiate between fertile patients and patients with infertility. Therefore, we have used a Receiver Operating Characteristic (ROC) curves. However only in a group of patients with idiopathic infertility were we able to distinguish fertile from infertile population based on LIF concentration values at a cutoff point of <8.63 pg/mL with sensitivity of 70.4% and a specificity of 95.2%. Based on retrospective analysis, we have concluded that the LIF concentration might play a role in certain types of infertility, and contribute to a defect in the implantation window.

On taking reproductive history after the initial evaluation of LIF in uterine flushing. We found that the shortest duration for attempting to get pregnant was 1 years. Only 26 48.1% (patients) from the original 54 patients enrolled in the study, were followed up. The remaining patients were lost to follow-up due to address changes; some have failed to correctly fill out the questionnaire. A 65.4% (17/26) of the responders have achieved a spontaneous pregnancy. All of them (except one case of ectopic) have carried their pregnancies past the 34th week of gestation and have taken home a healthy baby.

Based on the reproductive outcome, we have divided the patients; into those who have achieved pregnancies and those who have not. As the number of patients in each group was quite small, we decided to treat the LIF concentration as an independent infertility factor, regardless of the initial allocation of the patient to one of the three infertility groups: the luteal phase defect, idiopathic infertility and endometriosis. Then, we have again utilized the ROC curves in order to test, whether a certain LIF concentration predicted the future reproductive potential. Much to our surprise, contrary to our previous results, it turned out that virtually all women with a LIF level in excess of 2.45 pg/mL achieved pregnancy. This value has allowed us to detect almost 97% of fertile women (sensitivity- 96.5%) with specificity for the test of 86.1%. The results of our prospective study on the impact of LIF concentrations in uterine flushing on expected reproductive potential suggest that low level, are necessary for the proper function and, in turn, successful implantation. Contrary to our retrospective study, when we have compared the LIF concentrations in infertile patients with those of a healthy control, much lower LIF levels are needed for implantation to occur (8.63 pg/mL *versus* 2.45 pg/mL).

In a previous prospective study that assessed the role of LIF in reproduction [25]. They have concluded that the higher the LIF value, the less likely is the implantation. However, there are certain differences in their study that can account for such discordant results. First of all, they have used a different collection technique for gathering the uterine fluid, which, in itself, can be a source of differing results. Second, their patients undergone an IVF protocols, with ovulation induction, and their LIF production might have been disturbed, as LIF responds to stimulation by high doses of estrogens. Finally, we have decided to test the LIF concentration during the implantation window, 7-9 days after ovulation, which is the period of the maximal receptivity of the endometrium. On the contrary, in their study they tested LIF concentration closer to an expected menses, that is, at 26th day of the cycle.

We believe that our study performed in natural cycles, within a period of endometrial receptivity, yields results closer to those occurring naturally. In conclusion, we believe that the low LIF concentration around the time of maximal endometrial receptivity interferes with proper implantation in humans. A potential limitation of this study was that too few subjects (48% of the initial group) were qualified for prospective analysis.

Based on the time it took patients with LIF levels above 2.45pg/mL to get pregnant (1years) we believe that LIF is only one of the several hundred of factors to effect implantation. This study has some obvious limitations. First, the number of patients studied prospectively is rather low, and larger studies are needed to confirm the results of our findings, which could possibly lead to a treatment for patients with low LIF levels, as suggested previously [24].

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