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INVESTIGATION OF SEMINAL PLASMA CHITOTRIOSIDASE-1 AND LEUKOCYTE ELASTASE AS POTENTIAL MARKERS FOR 'SILENT' INFLAMMATION OF THE REPRODUCTIVE TRACT OF THE INFERTILE MALE – A PILOT STUDY

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Inflammatory mediators – chitotriosidase-1 (CHIT1) and leukocyte elastase (LE) – were analyzed in human seminal plasma in relation to total antioxidative status (TAS) and pro-inflammatory markers IL-1β and IL-6. Samples collected from 34 males who were part of infertile couples were divided into normozoospermic (N; n = 12, without symptoms of inflammation), oligozoospermic (O; n = 11) and teratozoospermic (T; n = 11) groups. Significant differences were observed only in CHIT1 concentration between N and O samples. However, a higher mean LE concentration was also observed in O and T patients (3.7-times and 900-times, respectively) compared with the N group. In IL-1β and IL-6 concentrations, an upward trend was observed from N, through O, up to the T group. The positive correlation between the concentration of IL-1β and the activity and specific activity of CHIT1 as well as the moderate negative correlation between concentrations of IL-1β and CHIT1 may suggest that elevated CHIT1 levels appeared in early stages of inflammation before the increase in IL-1β concentrations, or remained stable even after the levels of cytokine decreased. The above seem to confirm the role of CHIT1 in the manifestation of 'silent' inflammation at a very early stage. To conclude, CHIT1 concentration appears to be an interesting biomarker that signals the presence of possible 'silent' inflammation accompanying oligozoospermia. We cannot draw such conclusions regarding LE concentration, because, although we observed differences in the mean values and medians between analyzed groups, they were not significant. The utility of CHIT1 in the follow-up of oligozoospermia-associated 'silent' subclinical inflammation is promising, but further studies on a larger patient test set are required.

Key words: *human seminal plasma chitotriosidase-1, human seminal plasma leukocyte elastase, cytokines, total antioxidative status, inflammation, normozoospermia, oligozoospermia*

INTRODUCTION

Nowadays, about 15% of couples of childbearing age experience fertility problems. In almost 50% of cases, the identified cause is a male factor, as demonstrated by abnormal values of semen parameters (1-3). According to WHO guidance, the main tool for the evaluation of male factor infertility is standard semen analysis, which describes the sperm count, its motility and morphology (4). However, the values recommended by WHO do not provide a complete explanation of what causes and influences male reproductive potential (5-8). One of the factors that influence male fertility is inflammation in the male reproductive tract (9, 10).

The inflammation can induce important biochemical changes in seminal plasma and alterations in the functioning of spermatozoa, thus reducing their fertilizing potential (11-13). Interleukin-1 (IL-1) and interleukin-6 (IL-6) are well-known pro-inflammatory cytokines with a complicated interrelationship (14, 15) that may be of interest in the context of male fertility. Additionally, oxidative stress (OS) may contribute to male infertility in different ways, for instance, as an additional potential cause of male reproductive tract inflammation and oxidative damage of human ejaculate proteins (6, 7).

The identification of additional markers or mediators of inflammation may be helpful in understanding male infertility aetiology and useful in the context of patients' preparation for the procedure of artificial insemination or *in vitro* fertilisation. The measurement of at least several cytokines is recommended for a better determination of the inflammatory process, including the inflammation of male reproductive organs (11, 15). We focused on non-standard inflammatory mediators – chitotriosidase-1 (also referred to as chitinase 1, CHIT1) and leukocyte elastase (LE) – in relation to well-known inflammatory parameters, including pro-inflammatory cytokines such as IL-1 and IL-6 as well as total antioxidative status (TAS), which reflects the ability to maintain the oxidative-antioxidant balance in semen. CHIT1, a

mammalian chitinase that belongs to family 18 of glycoside hydrolases, is expressed mainly by macrophages and, to a lesser extent, on neutrophils. It plays a role in innate immunity against chitin-coated pathogens. As a marker of macrophage activation, CHIT1 activity is used in the diagnosis and monitoring of Gaucher disease (16, 17). A substantial increase in the activity of this enzyme has also been observed in other diseases with accompanying inflammation (18-22). Studies also indicate a synergistic effect of CHIT1 with some proteases, *e.g.* (matrix metalloproteinase 9, (MMP-9) but not with LE (23). However, the role of CHIT1 in the pathomechanisms of these diseases is not yet understood in detail. Due to the lack of endogenous substrates for this enzyme in the human body, it is uncertain which features are necessary to perform its biological functions and whether chitinolytic activity is crucial here. The participation of CHIT1 in the activation and differentiation of macrophages affects other cells of the immune system (24). This may suggest the potential involvement of CHIT1 in inducing inflammatory response, but there is no information available about the participation of this enzyme in inflammation associated with male infertility.

LE, a proteolytic hydrolase and serine protease with a broad substrate spectrum, is present in azurophilic granules of neutrophils (25). This enzyme is one of the most important components of the phagocytic system. It is involved in immune responses to various pathogens, has the ability to degrade extracellular matrix components (hydrolyzes elastin, collagen, fibronectin, proteoglycans), and is co-expressed with some pro-inflammatory cytokines (*inter alia* IL-2 and IL-6) (26-28). Increased LE activity is observed in many inflammatory diseases, *e.g.* in diabetic angiopathy (26). The results of studies on its utility in the diagnostics of male infertility are ambiguous and relate primarily to male genital tract inflammation. Some studies indicate its importance as a marker in 'silent' inflammation, whereas others show no clinical utility of this enzyme in the diagnostics of infertility with an inflammatory etiology (29-31).

Particular attention was paid to the selection of markers for the identification of patients with 'silent' subclinical inflammation within the male reproductive system. Proper diagnosis of the etiological agent in this group of patients is particularly important when preparing them for artificial insemination. Another area of research involves developing a more detailed understanding of molecular mechanisms involved in male infertility with accompanying inflammation. In the absence of any data in the scientific literature, there is potential for innovation through determining whether CHIT1, together with LE present in human seminal plasma, may be used as an additional marker of inflammation. The aim of our study was to evaluate the concentrations and activities of CHIT1 and LE in the seminal plasma of normozoospermic, oligozoospermic, and teratozoospermic males as well as to examine their association with well-known inflammatory markers (IL-1β and IL-6) and total antioxidant status (TAS) in order to provide a deeper understanding of inflammation etiology and of the molecular pathomechanisms of its development.

METERIALS AND METHODS

Seminal plasma samples

Seminal ejaculates were collected from 34 males (25 – 45 years old) who were part of an infertile couple and analyzed in the Semen Analysis Laboratory InviMed- Fertility Clinic in Warsaw. The ejaculates were collected by masturbation into sterile containers after 3 – 5 days of sexual abstinence. The ejaculates were allowed to stand at 37°C until liquefaction was completed and standard semen analysis (ejaculate volume, pH, total number of sperm in ejaculate, sperm concentration, total motility, progressive motility, viability and morphology) was carried out manually according to WHO (4) directives. Semen samples were centrifuged at 3500 × g for 10 minutes at room temperature to obtain plasma. Seminal plasma was split into small aliquots and frozen at –76°C until use. The samples were divided into normozoospermic, without any detected symptoms of inflammation (normal values of ejaculate parameters, n = 12), oligozoospermic (sperm count lower than 15×10^6 mL^{-1}, n = 11) and teratozoospermic (lower than 4% of spermatozoa having normal morphology, n = 11) groups. In the normozoospermic ejaculates, the concentration of spermatozoa was higher than 15×10^6 mL^{-1}, and > 4% of spermatozoa exhibited normal morphology, with a total motility of ≥ 40% or progressive motility ≥ 32% (1h after ejaculation). None of the seminal plasma samples were leukospermic. All samples were collected with the informed consent of the patients.

The study has been approved by the Bioethics Committee of Wroclaw Medical University (KB-765/2018).

Chitotriosidase-1 concentration

In all seminal plasma samples, the concentration of CHIT1 was determined using the ELISA Kit for human Chitinase-1 (Cloud-Clone Corp., Huston, TX, USA). The test was carried out in accordance with the manufacturer's instructions. The absorbance values were measured using the Stat Fax 200 microplate reader (Awareness Technology Inc., Palm City, FL, USA), and the concentrations of CHIT1 were calculated based on the calibration curve (0.312 – 20.0 ng/mL) and expressed in ng/mL. The minimum detectable concentration of CHIT1 is typically less than 0.114 ng/ml.

Determination of enzymatic activities of chitotriosidase-1

The activity of CHIT1 was measured in each sample using a 22 μM 4-methylumbelliferyl-β-N-N′-N″-triacetylchitotriose (Sigma Chemical Co, St Louis, MO, USA) fluorogenic substrate in McIlvain's buffer (pH 5.3) according to the method described by Hollak *et al.* (16) after being adapted to the microplate reader. A 5 μl sample of seminal plasma sample was incubated with a 100 μl substrate solution for 30 minutes at 37°C. The reaction was terminated with 100 μl 0.3 M glycine-NaOH buffer (pH 10.5), and fluorescence was measured with the Synergy HTC multi-mode reader (BioTek Instruments, Inc., USA) at excitation λ = 365 nm and emission λ = 445 nm. CHIT1 activity was calculated using a standard calibration curve for 4-methylumbelliferone (Sigma Chemical Co, St Louis, MO, USA) (0.25 – 5.0 nM/ml) and expressed as the nanomoles of converted substrate per 1h at the reaction conditions per 1 ml of the seminal plasma.

Leukocyte elastase concentration

The concentrations of LE in seminal plasmas were determined using a Human PMN Elastase ELISA Kit (BioVendor-Laboratorni medicina, a.s., Brno, Czech Republic). The test was carried out in accordance with the manufacturer's instructions. The absorbance values were measured using the Stat Fax 200 microplate reader (Awareness Technology Inc., Palm City, FL, USA) and the concentrations of LE were calculated based on the calibration curve (15.6 – 1000 ng/ml) and expressed in ng/ml with a detection limit of 0.2 ng/ml.

Determination of enzymatic activities of leukocyte elastase

The activity of LE in each sample was measured using 1 mM N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma

Chemical Co, St Louis, MO, USA) colorimetric substrate in 0.2 M Tris-HCl buffer (pH 8.0), as described previously (32). A 10 µl seminal plasma sample was incubated on a microplate with 190 µl of a substrate solution for 60 minutes at 37°C. The reaction was terminated with 50 µl of glacial acetic acid and the absorbance was measured at 405 nm (Synergy HTC multi-mode reader, BioTek Instruments, Inc., USA). The activities of LE were calculated using the standard calibration curve for p-nitroaniline (Sigma Chemical Co, St Louis, MO, USA) (0.1 – 4.5 µM/ml) and expressed as the micromoles of converted substrate per 1 h at the reaction conditions per 1 ml of the seminal plasma.

Specific activities of chitotriosidase-1 and leukocyte elastase

The specific activities of CHIT1 and LE were then calculated as the ratio of CHIT1 or LE activity to enzyme concentration and expressed as the nanomoles or micromoles of converted substrate per 1h at the reaction conditions per 1 ng of CHIT1 or LE, respectively.

Cytokine concentrations

Concentrations of cytokines IL-1β and IL-6 were determined in all seminal plasma samples using commercially available immunochemical ELISA tests: Human IL-1β Elisa^{PRO} Kit (MABTECH, Nacka Strand, Sweden) and Human IL-6 HS Elisa Kit (pre-coated) (Covalab R&D in Biotechnology, Villeurbanne, France), respectively. The tests were carried out in accordance with the manufacturer's instructions. Concentrations of IL-1β and IL-6 were calculated using the MR-96A Microplate reader (Shenzhen Mindray Bio-Medical Electronics Co., China) based on calibration curves for IL-1β: 1 – 316 pg/mL and Il-6: 1.56 - 50 pg/ml, respectively. The sensitivity was 0.316 pg/mL for Il-1β and 0.81 pg/ml for IL-6.

Total antioxidant status measurement

The total antioxidant status (TAS) in seminal plasma samples was measured using an automated colorimetric method (Randox Laboratories Ltd., Crumlin, County Antrim, UK) in the Konelab 20i® (Thermo Scientific, Waltham, Massachusetts, USA) auto-analyser. The recommended measuring range was 0.21-2.94 mmol/l.

Statistical analysis

Statistical analysis was performed using the statistical software STATISTICA 13.0 PL (StatSoft, Inc., Tulsa, OK,

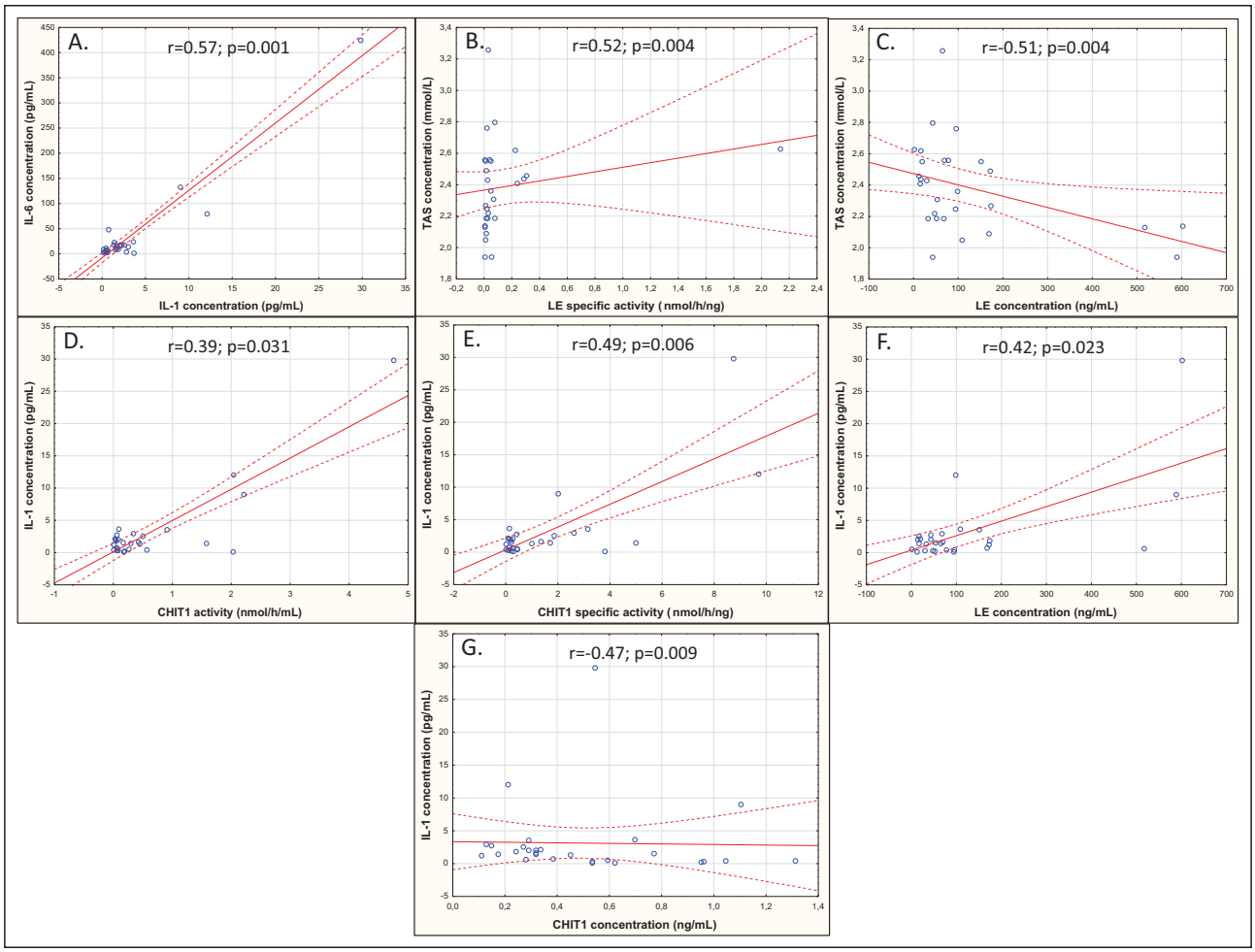

Fig. 1. The correlations between determined parameters. The correlations were estimated according to Spearman's rank correlation coefficient, and a two-tailed p-value of less than 0.05 was considered significant. 95% confidence interval is marked with dotted lines. CHIT1 – chitotriosidase 1, LE – leukocyte elastase, IL-1 – interleukin 1β, IL-6 – interleukin 6, TAS – total antioxidative status. The presented correlations are between: **A**: IL-1β and IL-6 concentrations, **B**: LE specific activities and TAS concentrations, **C**: TAS and LE concentrations, **D**: CHIT1 activities and IL-1β concentrations, **E**: CHIT1 specific activities and IL-1β concentrations, **F**: LE and IL-1β concentrations, **G**: CHIT1 and IL-1β concentrations. A two-tailed p-value of less than 0.05 was considered significant.

Table 1. The results of the determination of selected seminal plasma parameters.

Parameter / Group	CHIT1 concentration (ng/ml)	CHIT1 activity (nmol/h/ml)	CHIT1 specific activity (nmol/h/ng)	LE concentration (ng/ml)	LE activity (μmol/h/ml)	LE specific activity (nmol/h/ng)	IL-1β (pg/ml)	IL-6 (pg/ml)	TAS mmol/ll
Normozoospermia (n=12)	0.36±0.33 [0.28] *0.11-1.34*	0.19±0.19 [0.72] *0.0-0.5*	0.8±0.87 [0.34] *0.0-2.6*	51.69±46.58 [42.55] *13.62-171.65*	2.53±1.12 [2.59] *0.44-4.49*	0.093±0.10 [0.05] *0.004-0.29*	1.66±0.85 [1.57] *0.32-2.99*	13.34±6.02 [15.28] *4.04-23.48*	2.34±0.24 [2.32] *1.94-2.8*
Oligozoospermia (n=11)	0.61±0.36 [0.54] *0.2-1.1* p=0.011*	1.21±1.5 [0.26] *0.0-4.76*	2.83±3.57 [1.32] *0.0-9.7*	191.97±214.82 [95.22] *18.9-601.69*	2.1±1.32 [1.87] *0.64-4.64*	0.026±0.024 [0.021] *0.0-0.08*	5.58±8.94 [1.5] *0.39-29.83*	68.47±125.12 [17.76] *3.31-425.14*	2.42±0.4 [2.36] *1.94-3.26*
Teratozoospermia (n=11)	0.55±0.37 [0.59] *0.07-1.31*	0.95±1.61 [0.26] *0.05-5.56*	8.60±23.26 [0.43] *0.13-78.28*	$46.42\times10^3\pm104.86\times10^3$ [108.5] *$0.77\text{-}297.76\times10^3$*	2.33±1.32 [1.69] *0.67-4.67*	0.24±0.64 [0.01] *0.002-2.14*	25.71±77.58 [1.83] *0.14-259.48*	71.53±196.12 [10.74] *2.16-662.05*	2.28±0.28 [2.29] *1.79-2.63*

The parameter values are presented as mean ± SD and median (in square brackets). *significant differences versus normozoospermic group; CHIT1 – chitotriosidase 1, LE – leukocyte elastase, IL-1β – interleukin 1β, IL-6 – interleukin 6, TAS – total antioxidative status. Range of values are written in cursive.

USA). All quantitative data are presented as mean ± standard deviation (SD) as well as median and quartiles. According to a Shapiro-Wilk W test, none of the values of the determined parameters fit normal distribution, thus the differences between groups of oligo- and teratozoospermic patients and normozoospermic men were studied using the nonparametric Mann-Whitney *U* test. Correlations between the examined parameters were tested using Spearman rank analysis. A two-tailed p-value of less than 0.05 was considered significant.

RESULTS

The results of the determination of CHIT1 and LE concentrations, their activities and specific activities, as well as concentrations of IL-1β, IL-6 and TAS, are shown in *Table 1*. Although a trend for increased concentrations of examined parameters (except TAS) is observed in pathological seminal plasmas when compared with normozoospermic patients, only the CHIT1 concentrations (0.61 ± 0.36 ng/ml) were significantly higher in the oligozoospermic group than in the normozoospermic group (0.36 ± 0.33 ng/ml, p = 0.011). However, there were visible differences between the concentration of CHIT1 in the oligozoospermic group and in teratozoospermic subjects (0.55 ± 0.37 ng/ml).

The Spearman test analysis showed correlations between some determined parameters. High positive correlations were observed between IL-1β and IL-6 concentrations (r = 0.57, p = 0.001; *Fig. 1A*) as well as between TAS concentrations and LE specific activities (r = 0.52, p = 0.004; *Fig. 1B*). Moreover, a high negative correlation was revealed between TAS concentrations and LE concentrations (r = –0.51, p = 0.004; *Fig. 1C*). Moderate positive correlations were observed between IL-1β concentrations and: CHIT-1 activities (r = 0.39, p = 0.031; *Fig. 1D*), CHIT1 specific activities (r = 0.49, p = 0.006; *Fig. 1E*), and LE concentrations (r = 0.42, p = 0.023; *Fig. 1F*), while the average negative correlation was demonstrated between IL-1β concentrations and CHIT1 concentrations (r = –0.47, p = 0.009; *Fig. 1G*).

DISCUSSION

In the present study, we examined changes in the levels of selected seminal plasma parameters that participate in the formation of inflammatory conditions in the context of male infertility. Moreover, we analysed the associations between the concentration, activity, and specific activity of CHIT1 and the levels of known markers of inflammation – LE, IL-1β, IL-6, and TAS – which reflect the presence of inflammation and the state of oxidative-antioxidant balance in the body or the ejaculate.

Significant differences were observed only in CHIT1 concentration between normozoospermic and oligozoospermic seminal plasma samples (p = 0.011), with no significant differences in the levels of other parameters analysed. As a product of non-specific macrophage activation, CHIT1 can be considered as a biomarker of systemic inflammation (33, 34). Although underestimated in clinical practice, the inflammation of male genital glands is a very common condition among infertile men. The relationship between inflammation of the male reproductive system and oligozoospermia has been frequently reported in the clinical work-up of male infertility (35, 36). Unfortunately, although it has been reported that up to 15% of male fertility disorders are related to infections or to inflammatory disorders of the accessory glands (37), inflammation at this site is often clinically silent. The impact of these conditions on male fertility may depend on many factors, such as the affected tract (epididymis, prostate, or seminal vesicles), etiology (the type of pathogen or non-infectious causes), and the course of the disease (acute or chronic) (38-40). It is well documented that adverse changes in male fertility also seem to be associated with environmental exposure to different substances, especially xenoestrogens. Among these, metalloestrogens are of particular importance (41). It has been suggested that the process of inflammation may alter fertility potential, acting through the following major mechanisms: impaired secretion of the accessory glands, obstruction of the seminal ducts, development of an inflammatory microenvironment leading to the dysregulation of spermatogenesis (38-40). Nowicka-Bauer *et al.* (42) have also studied new, non-standard parameters present in human ejaculate in the context of their impact on possible future diagnostics and their therapeutic applications in male infertility, the determination of which may be especially important for patients' preparation for assisted reproductive techniques.

It is indicated that CHIT1 plays a pivotal role in the context of the pathogenesis of chronic inflammation (18). Our observations, although of a preliminary character, are of interest in this respect. They should nevertheless be confirmed on a larger group of infertile oligozoospermic men. Only single studies concerning men, for example by Kuzgunbay *et al.* (43), evaluated serum chitotriosidase-1 levels in varicocele patients to investigate the pathophysiology of varicocele. The authors revealed that although the mean brachial and testicular

chitotriosidase levels were visibly higher in oligozoospermic than in normozoospermic patients, this difference was statistically insignificant (43). The above observations confirm the differences we observed in seminal plasma CHIT1 concentrations between the normozoospermic and the oligozoospermic group. The occurrence of inflammation within the male reproductive system can lead to oligozoospermia (44, 45). Therefore, it is very important to identify it as early as possible.

IL-1β is known to be a potent pro-inflammatory cytokine crucial for host-defence responses to infection and injury (46). However, we did not observe any significant differences in IL-1β concentration between the analysed seminal plasma groups, the observed positive correlations between IL-1β concentrations, and CHIT-1 activities and specific activities. Furthermore, the average negative correlation demonstrated between IL-1β concentrations and CHIT1 concentrations seem to confirm the hypothesis about the role of CHIT1 in the manifestation of inflammation at a very early stage. In the light of these observations, it is possible that an elevated level of CHIT1 concentration is typical for early stages of inflammation development, before inflammation is manifested by an increase of IL-1β concentrations, or that otherwise, being an indicator of 'silent' subclinical chronic inflammation, it remains constant even after the concentration of cytokine is lowered. Previous research may confirm our hypothesis (47-49), as it has been documented that the inflammasome system is expected to play some role in the activation of chitotriosidase-1 expression (47, 48) and eventually to lead to the production of IL-1β (49). Chitotriosidase also mediates many inflammatory processes through the direct stimulation of various inflammatory mediators, such as IL-8 or MMP-9, thus increasing the migratory capacity of many immunological cells (50, 51). In some diseases, the levels of CHIT1 activities strongly correlate with concentrations of IL-1β and TNF-α, supporting the hypothesis of a mutual regulation cascade in the production of these inflammatory mediators (50).

The second of the examined enzymes, leukocyte elastase, is a well-known agent that participates in the inflammatory reaction to various diseases connected with chronic inflammation (52-55). We did not observe any statistically significant differences between the examined groups of men with fertility problems, although the mean concentration of LE was about 3.7-times and 900-times higher in oligozoospermic and teratozoospermic patients, respectively, when compared with normozoospermia. There were also no significant differences between the analyzed groups in other parameters – cytokines IL-1β and IL-6 – most likely due to the low number of samples, although an increasing tendency was observed in normozoospermic, oligozoospermic and teratozoospermic patients. These observations may suggest that LE concentration together with the level of IL-1β, as indicated by positive correlations between these parameters, may play a role as markers helpful in the detection of inflammatory conditions in male patients with fertility problems. However, this hypothesis needs to be confirmed by further studies on larger groups of patients.

We first evaluated the possibility of using CHIT1 as an additional biomarker that indicates the presence of possible 'silent' inflammation accompanying oligozoospermia. The main drawback of this study is related to the limited number of examined patients, which may explain the absence of statistically significant differences in LE, pro-inflammatory cytokines and TAS levels between the analyzed groups. However, this data can pave the way for new diagnostic opportunities concerning the inflammation of the male reproductive tract that accompanies decreased male fertility caused by oligozoospermia. Furthermore, the analysis of these parameters – especially CHIT1 and LE – among infertile patient groups could highlight specific outcomes that may be important for monitoring male fertility problems. Although our research is a pilot study, limited by the small number of patients and lack of information about BMI, smoking and diet, our results indicate that the evaluation of inflammatory biomarkers, such as seminal plasma chitotriosidase-1, could provide insight into aspects of oligozoospermia pathogenesis. In future studies, it will also be worth checking whether the observed differences in LE concentrations between groups with abnormal semen parameters and the normozoospermic group, although statistically insignificant, may be useful in the diagnostics of inflammatory conditions accompanying male infertility. Nonetheless, further studies, conducted on larger, well-characterized patient cohorts and including information on men's BMI, lifestyle, addictions, *etc*., are necessary to confirm these hypotheses.

To conclude the concentration of CHIT1 appears to be an interesting non-invasive biomarker that can be used in the follow-up of 'silent' subclinical inflammation associated with oligozoospermia. However, it requires further investigation on a larger group of patients.

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Conflicts of interest: None declared.

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