〈Regular Article〉

Preliminary study for predictive indicators focused on semen analysis of inflammation for male infertility

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ABSTRACT **Introduction**: Male-infertility-associated factor is found in 50% of couples with infertility, and usually together with abnormal semen parameters. Spermatogenic dysfunction accounts for 82.4% of male infertility. In 30-40% of cases, male-associated factor is not found to explain the underlying impairment of semen parameters were referred to as idiopathic spermatogenic dysfunction. Semen analysis plays the most important role in routine evaluation of idiopathic spermatogenic dysfunction. Recent reports showed a relationship between impairment of semen parameters and inflammatory changes via various cytokines, assessment of inflammatory changes may be a predictive indicator for impairment of semen parameters. Therefore, we investigated the correlation between semen parameters and inflammatory analysis of semen using proteome assay, and extracted predictive indicators focused on semen inflammation for male infertility in this study.

Materials and Methods: Eighty-nine couples were enrolled at the Miyake Clinic (Okayama, Japan). Semen analysis findings (seminal volume, concentration, motility rate, and malformation rate) and patient's (male) age, partner's (female) age, height, weight, and body mass index (BMI) were obtained from the medical information. The mean sperm concentration (SD) was 83.57 (64.77) × 10^6/mL, mean sperm motility rate (SD) was 37.82% (16.50%) in 89 patients. From the results of the semen analysis, four patients with the highest sperm motility rate were classified as normal sperm motility group, and four patients with the lowest sperm motility rate were classified as low sperm motility group. Four patients with the highest sperm concentration were classified as normal sperm concentration group, and four patients with the lowest sperm concentration were classified as low sperm concentration group. Proteome assay was performed on 16 patients from the four groups. Quantitative assay of extracted indicators from the proteome assay were performed in 89 patients to investigate the validation of predictive indicators for male infertility in the semen using enzyme-linked immunosorbent assay (ELISA) methods.

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Results: From the results of the proteome assay, Growth differentiation factor-15 (GDF-15), Kallikrein 3 (KLK3) and Trefoli factor 3 (TFF3) were extracted as indicators related to male infertility. Quantitative assay showed a negative correlation between GDF-15, KLK3 and sperm motility. TFF3 negatively correlated not only with sperm motility but also with sperm concentration and malformation.

Conclusions: Extracted three indicators (GDF-15, KLK3 and TFF3) in the semen containing sperm showed statistically significant difference with semen parameters, and negative correlation with semen parameters (sperm motility, concentration and malformation). GDF-15, KLK3 and TFF3 levels in the semen containing sperm may be novel predictive indicators focused on semen analysis of inflammation for male infertility.

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Key words：Male infertility, Predictive indicator, Semen analysis, Proteome assay, Inflammation

INTRODUCTION

Male-infertility-associated factor is found in 50% of couples with infertility, and usually together with abnormal semen parameters[1]. Although male infertility is not clearly defined, male infertility is classified into spermatogenic dysfunction, sexual dysfunction, sperm duct obstruction, and other[2]. Spermatogenic dysfunction accounts for 82.4% of male infertility[2]. In 30-40% of cases, male-associated factor is not found to explain the underlying impairment of semen parameters were referred to as idiopathic spermatogenic dysfunction[3]. These men present with no previous history of diseases affecting fertility and have normal findings on physical examination and endocrine, genetic and biochemical laboratory testing, although semen analysis may reveal pathological findings[3]. Idiopathic spermatogenic dysfunction is not a male-associated factor that explains the underlying impairment of semen parameters[3]. Therefore, semen analysis plays the most important role in routine evaluation of idiopathic spermatogenic dysfunction.

It is now believed that idiopathic spermatogenic dysfunction may be associated with several previously unidentified pathological factors, which include endocrine disruption caused by environmental change, sperm DNA damage caused by overproduction of oxidative stress, and genetic and epigenetic abnormalities[4]. Recent reports showed a relationship between impairment of semen parameters and inflammatory changes via various cytokines[5, 6], and assessment of inflammatory changes may be a predictive indicator for impairment of semen parameters. Therefore, we investigated the correlation between semen parameters and inflammatory analysis of semen using proteome assay, and extracted predictive indicators focused on semen inflammation for male infertility in this study.

MATERIALS AND METHODS

Ethical considerations of this study

The study was conducted after obtaining approval from the ethical committee of Kawasaki Medical School / Kawasaki Medical School Hospital (approval no.5519), and was performed in accordance with the Helsinki Declaration (Fortaleza revised version, 2013), with Ethical Guidelines for Medical and Biological Research Involving Human Subjects of the Ministry of Education, Culture, Sports, Science, and Technology, Ministry of Health, Labor, and Welfare and Ministry of Economy, Trade, and Industry (enforced on June 30, 2021; revised on April 17, 2023), and with the study protocol. Written informed consent was obtained

from participants after they were provided with an explanation of the study using documentation. Data was closely managed using identification codes after deletion of medical record numbers, names, and birth dates to protect privacy.

Participants and study design

Eighty-nine couples were enrolled at the Miyake Clinic (Okayama, Japan). Semen analysis findings (semem volume, sperm concentration, sperm motility rate, and sperm malformation rate) and patient's (male) age, partner's (female) age, height, weight, and body mass index (BMI) were obtained from the medical information. Background of the participants is shown in Table 1. Semen samples were collected after 2-7 days of abstinence periods and immediately examined using BX-40 microscope (Olympus, Tokyo Japan) after semen collection. Semen analyses were duplicated at least. The mean sperm concentration (SD) was 83.57 (64.77) × 10^6/mL, mean sperm motility rate (SD) was 37.82% (16.50%) in 89 patients (Table 1). Residual semen samples were cryopreserved after injection into double-cylinder centrifuge tubes (Rapitz Lock, JMS, Tokyo, Japan). Additive solutions of HTF medium (Japan Medical Instruments Manufacturing, Osaka, Japan) were mixed as a washing liquid, and Extra Sperm Selection 90% & 45% gradient (Medi-Con International, Osaka, Japan) were used as density gradient liquid. The cryopreserved samples containing sperm were thawed and used in the following experiments.

Table 1. Background of participants

	n	Mean (SD)
Patient's (male) age (years old)	89	36.40 (6.10)
Partner's (female) age (years old)	89	35.10 (4.40)
Height (cm)	84	171.30 (5.30)
Body weight (kg)	84	69.90 (11.50)
BMI (kg/m^2)	84	23.79 (3.38)
Semen volume (mL)	89	3.64 (1.38)
Sperm concentration (×10^6/mL)	89	83.57 (64.77)
Sperm motility (%)	89	37.82 (16.50)
Sperm malformation rate (%)	89	85.46 (8.21)

SD, standard deviation; BMI, Body mass index

Proteome assay in the semen

From the results of the semen analysis, four patients with the highest sperm motility rate were classified as normal sperm motility group, and four patients with the lowest sperm motility rate were classified as low sperm motility group (Fig. 1). Four patients with the highest sperm concentration were classified as normal sperm concentration group, and four patients with the lowest sperm concentration were classified as low sperm concentration group (Fig. 1).

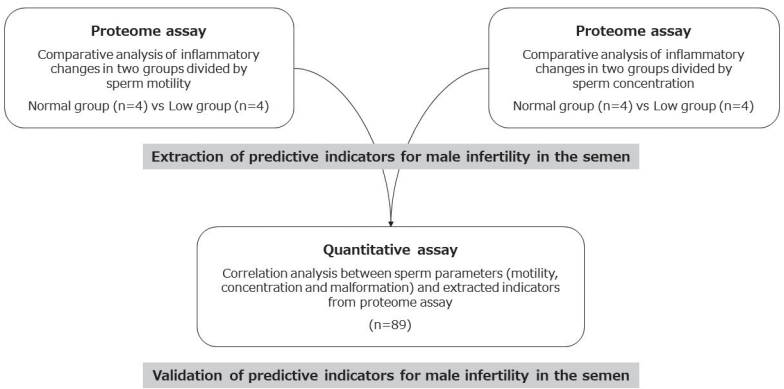

Fig. 1. Study design of this study
In this study, we investigated predictive indicators focused on inflammation for male infertility in two stage, extraction of predictive indicators and validation of extracted indicators for male infertility using residual semen samples.

Table 2. Background of low and normal sperm motility groups

	Low motility group n = 4 mean (SD)	Normal motility group n = 4 mean (SD)	t-test p-value
Patient age (years old)	39.50 (7.50)	31.00 (2.20)	0.072
Partner age (years old)	39.30 (3.50)	34.30 (4.60)	0.136
Height (cm)	172.00 (4.70)	171.50 (3.40)	0.869
Body weight (kg)	73.00 (11.90)	68.80 (12.10)	0.635
BMI (kg/m^2)	24.64 (3.55)	23.33 (3.81)	0.631
Semen volume (mL)	3.05 (0.88)	3.75 (0.13)	0.167
Sperm concentration ($\times 10^6$/mL)	28.98 (24.12)	163.33 (66.45)	0.009
Sperm motility (%)	6.30 (1.41)	71.33 (3.51)	0.000
Sperm malformation rate (%)	93.780 (2.150)	79.080 (7.420)	0.009

SD, standard deviation; BMI, body mass index

Table 3. Background of low and norp-valuemal sperm concentration groups

	Low concentration group n = 4 mean (SD)	Normal concentration group n = 4 mean (SD)	t-test p-value
Patient age (years old)	43.30 (4.30)	43.30 (6.40)	1.000
Partner age (years old)	38.00 (4.50)	38.50 (4.40)	0.879
Height (cm)	171.50 (4.40)	178.30 (7.10)	0.156
Body weight (kg)	71.80 (12.30)	74.50 (2.40)	0.676
BMI (kg/m^2)	24.38 (3.79)	23.55 (2.36)	0.725
Semen volume (mL)	2.90 (0.82)	3.35 (1.65)	0.642
Sperm concentration ($\times 10^6$/mL)	5.73 (2.48)	282.33 (57.92)	0.000
Sperm motility (%)	18.13 (7.57)	29.00 (8.75)	0.109
Sperm malformation rate (%)	94.83 (5.11)	79.40 (9.87)	0.032

SD, standard deviation; BMI, body mass index

Proteome assay was performed on 16 patients from the four groups (Fig. 1). Details of the four groups are shown in Table 2 and Table 3. The semen samples were homogenized using a homogenizer. Homogenized samples were collected after centrifugation (15,000 × g for 5 min at 5℃) and removal of tissue fragments. A proteome assay using 105 inflammation-related proteome types were performed using Proteome Profiler™ Array Human XL Cytokine Array Kit (ARY022B, R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Protein concentration in each sample was 300 μg/mL. Pixel density (PD) was calculated using an image analyzer (Image Quant LAS4000 mini, GE Healthcare Japan, Tokyo, Japan) and image analysis software (Image Quant TL, GE Healthcare Japan, Tokyo, Japan). Calculated PD was corrected using endogenous control, while negative and positive controls were set at PD values of 0 and 100, respectively. Pixel density ratio of the low and normal sperm motility groups was calculated by PD for the normal sperm motility group divided by the PD for the low sperm motility group. PD ratio of the low and normal sperm concentration groups was calculated by PD for the normal sperm concentration group divided by the PD for the low sperm concentration group. Proteome assay was also duplicated at least.

Quantitative assay in the semen

Quantitative assay of extracted indicators from the proteome assay were performed in 89 patients to investigate the validation of predictive indicators for male infertility in the semen using enzyme-linked immunosorbent assay (ELISA) methods, according to the manufacturer's instructions (Fig. 1). Absorbance was measured at 495 nm using Varioskan Flash® (Thermo Fisher Scientific,

Waltham, MA, USA). Quantitative assay was also duplicated at least. Based on the results of quantitative assay, we investigated the correlation between quantitative result of extracted indicators and semen parameters (sperm motility, concentration and malformation).

Statistical analysis

Statistical analysis was performed using Stat Flex ver. 7 (Artec, Osaka, Japan). Data was expressed as mean (standard deviation: SD). Differences between the background of the low and normal sperm motility groups were analyzed using t-test with $p < 0.01$ considered to indicate a significant difference according to data distribution, and background of the low and normal sperm concentration group were analyzed using t-test with $p < 0.05$ considered to indicate a significant difference. Correlation between the results of quantitative assay and semen parameters (sperm motility, concentration and malformation) were analyzed using Spearman's correlation coefficient by rank test, with $p < 0.05$ indicating significant difference.

RESULTS

Extraction of predictive indicators for male infertility using proteome assay

Proteome assay showed statistically significant differences ($p < 0.01$) between the PD of low and normal sperm motility groups were observed in 14 candidates (Adiponectin, Apolipoprotein A-I, BAFF, BDNF, Chitinase 3-like 1, EGF, EMMPRIN, GDF-15, KLK3, Lipocalin-2, MMP-9, TFF3, VEGF, and Vitamin D BP), and significant changes in PD ratio (PD ratio < 0.020 or > 2.000) were observed in seven participants (GDF-15, IL-3, KLK3, PDGF-AB/BB, RANTES, TFF3, and Thrombospondin-1) (Table 4). Proteome assay showed statistically significant differences ($p < 0.05$) between the PD of low and normal sperm concentration groups were observed in 10 candidates (CD30, CD40 ligand, EGF, EMMPRIN, GM-CSF, IL-3, IL-18Bpa, I-TAC, KLK3, and MMP-9), and significant changes in PD ratio (PD ratio < 0.100 or > 2.000) were observed in three candidates (GDF-15, TFF3, and TGF-alfa) (Table 5). Based on the results, we focused on GDF-15, KLK3, and TFF3, which showed differences in both PD Ratio and PD Difference for sperm motility. For concentration, we focused on GDF-15 and TFF3, which showed differences in both PD Ratio and PD Difference, as well as KLK3, which showed a difference in PD Difference. Correlation analysis was performed using these three proteins across all cases.

Correlation between semen parameters and predictive indicators for male infertility

Quantitative assay using Quantikine™ ELISA Human GDF-15 (QK957, R&D Systems, Minneapolis, MN, USA), KLK3 (DKK300, R&D Systems, Minneapolis, MN, USA), and TFF3 (DTFF30, R&D Systems, Minneapolis, MN, USA) immunoassay showed a negative correlation between GDF-15 and sperm motility (Fig. 2), KLK3 and sperm motility (Fig. 3), TFF3 and sperm motility (Fig. 4), TFF3 and sperm concentration (Fig. 5), TFF3 and sperm malformation (Fig. 6). However, the correlation was not shown between GDF-15 and sperm concentration (Spearman rank correlation coefficient test, $n = 89$, $p = 0.450$, $rS = -0.081$. not shown in figure), KLK3 and sperm concentration (Spearman rank correlation coefficient test, $n = 89$, $p = 0.245$, $rS = -0.124$. not shown in figure), GDF-15 and sperm malformation (Spearman rank correlation coefficient test, $n = 87$, $p = 0.061$, $rS = -0.202$. not shown in figure), KLK3 and sperm concentration (Spearman rank correlation coefficient test, $n = 87$, $p = 0.723$, $rS = -0.039$. not shown in figure).

DISCUSSION

The WHO recommends evaluating inflammatory

Table 4. Proteome assay classified according to low and normal sperm motility groups

	Low motility group n = 4 mean（SD）	Normal motility group n = 4 mean（SD）	t-test p-value	PD ratio
Adiponectin	2.38（0.70）	0.27（0.17）	0.001	0.112
Apolipoprotein A-I	2.75（0.43）	0.47（0.25）	0.000	0.169
Angiogenin	4.48（2.395）	0.57（0.27）	0.018	0.127
Angiopoietin-1	0.75（0.45）	0.75（0.50）	0.983	0.983
Angiopoietin-2	3.78（1.60）	1.52（0.50）	0.035	0.402
BAFF	2.12（0.83）	0.43（0.39）	0.010	0.201
BDNF	3.12（1.15）	0.73（0.46）	0.008	0.232
Complement Component C5/C5a	1.44（0.67）	0.28（0.35）	0.022	0.195
CD14	1.11（0.57）	0.43（0.48）	0.117	0.387
CD30	3.29（1.47）	0.82（0.36）	0.017	0.248
CD40 ligand	2.34（1.31）	0.16（0.09）	0.016	0.065
Chitinase 3-like 1	1.62（0.42）	0.13（0.15）	0.001	0.082
Complement Factor D	1.15（0.70）	0.45（0.36）	0.125	0.392
C-Reactive Protein	1.17（0.78）	0.60（0.52）	0.269	0.510
Cripto-1	0.63（0.64）	0.58（0.52）	0.898	0.912
Cystatin C	2.56（1.45）	0.32（0.42）	0.025	0.124
Dkk-1	2.69（1.68）	0.28（0.32）	0.031	0.102
DPP4	1.05（0.71）	0.12（0.18）	0.045	0.115
EGF	2.46（0.79）	0.18（0.28）	0.002	0.073
EMMPRIN	4.24（1.38）	0.21（0.23）	0.001	0.049
ENA-78	0.17（0.18）	0.15（0.10）	0.852	0.866
Endoglin	0.95（0.69）	0.25（0.23）	0.099	0.258
Fas Ligand	0.54（0.61）	0.39（0.20）	0.653	0.720
FGF basic	1.20（0.80）	0.49（0.29）	0.147	0.406
FGF-7	0.74（0.78）	0.58（0.75）	0.778	0.786
FGF-19	3.85（3.39）	0.42（0.63）	0.093	0.108
Flt-3 Ligand	0.45（0.51）	0.15（0.22）	0.316	0.324
G-CSF	1.31（1.05）	0.04（0.07）	0.052	0.029
GDF-15	10.23（2.62）	0.20（0.27）	0.000	0.019
GM-CSF	1.63（0.90）	0.04（0.06）	0.012	0.021
GRO alfa	0.37（0.22）	0.08（0.10）	0.057	0.207
Growth Hormone	0.21（0.18）	0.18（0.18）	0.795	0.825
HGF	0.44（0.38）	0.44（0.23）	1.000	0.997
ICAM-1	0.36（0.49）	0.43（0.18）	0.797	1.199
IFN-gamma	1.73（0.86）	1.03（0.18）	0.164	0.597
IGFBP-2	0.63（0.69）	0.53（0.53）	0.838	0.850
IGFBP-3	0.60（0.68）	0.51（0.71）	0.853	0.843
IL-1alfa	1.41（1.26）	0.79（0.72）	0.427	0.561
IL-1beta	0.98（0.57）	0.09（0.15）	0.023	0.094
IL-1ra	1.07（0.64）	0.20（0.35）	0.053	0.182
IL-2	0.48（0.61）	0.03（0.06）	0.199	0.069
IL-3	0.32（0.50）	0.01（0.01）	0.261	0.020
IL-4	0.79（0.50）	0.11（0.21）	0.047	0.136
IL-5	1.57（1.09）	0.85（0.32）	0.253	0.541
IL-6	0.80（0.57）	0.43（0.28）	0.280	0.528
IL-8	1.07（0.35）	0.42（0.20）	0.019	0.392
IL-10	1.22（0.70）	0.46（0.24）	0.084	0.373
IL-11	1.67（1.31）	1.03（0.59）	0.411	0.617
IL-12p70	0.89（0.73）	0.53（0.73）	0.511	0.590
IL-13	0.71（0.45）	0.32（0.50）	0.288	0.451
IL-15	1.08（0.45）	0.21（0.33）	0.020	0.194
IL-16	1.33（0.61）	0.29（0.51）	0.040	0.218
IL-17A	1.71（1.04）	0.19（0.17）	0.028	0.107

IL-18Bpa	1.92 (1.01)	0.06 (0.11)	0.011	0.030
IL-19	0.31 (0.27)	0.14 (0.25)	0.402	0.457
IL-22	0.64 (0.58)	0.27 (0.34)	0.315	0.414
IL-23	1.10 (0.57)	0.19 (0.25)	0.026	0.168
IL-24	0.95 (0.36)	0.49 (0.33)	0.108	0.516
IL-27	2.18 (1.56)	0.38 (0.26)	0.064	0.175
IL-31	1.25 (0.97)	0.71 (0.53)	0.370	0.571
IL-32	3.13 (2.70)	0.54 (0.74)	0.113	0.170
IL-33	0.99 (0.67)	0.41 (0.64)	0.261	0.418
IL-34	1.36 (0.63)	0.32 (0.62)	0.055	0.232
IP-10	1.24 (0.70)	0.35 (0.65)	0.111	0.277
I-TAC	1.87 (1.09)	0.14 (0.25)	0.021	0.073
KLK3	76.06 (7.43)	1.67 (0.38)	0.000	0.020
Leptin	0.92 (0.50)	0.12 (0.25)	0.029	0.133
LIF	0.26 (0.21)	0.22 (0.43)	0.873	0.846
Lipocalin-2	3.90 (1.37)	0.40 (0.48)	0.003	0.102
MCP-1	0.63 (0.17)	0.50 (0.45)	0.622	0.798
MCP-3	2.11 (0.77)	0.33 (0.35)	0.006	0.153
M-CSF	1.60 (1.31)	0.92 (0.70)	0.391	0.571
MIF	2.00 (1.27)	0.58 (0.76)	0.103	0.287
MIG	1.34 (0.81)	0.53 (0.81)	0.207	0.393
MIP-1alfa/MIP-1beta	1.51 (0.76)	0.42 (0.79)	0.094	0.278
MIP-3alfa	1.83 (1.09)	0.43 (0.64)	0.067	0.230
MIP-3beta	4.93 (2.61)	0.18 (0.28)	0.011	0.037
MMP-9	4.56 (1.83)	0.14 (0.17)	0.003	0.029
Myeloperoxidase	0.78 (0.88)	0.26 (0.28)	0.300	0.323
Osteopontin	0.95 (1.04)	0.45 (0.44)	0.407	0.470
PDGF-AA	0.31 (0.07)	0.35 (0.58)	0.902	1.126
PDGF-AB/BB	0.10 (0.10)	0.36 (0.61)	0.442	3.427
Pentraxin3	1.64 (0.63)	0.95 (0.82)	0.236	0.581
PF4	0.56 (0.34)	0.54 (0.58)	0.955	0.968
RAGE	0.60 (0.49)	0.68 (0.69)	0.856	1.132
RANTES	0.15 (0.15)	0.67 (0.88)	0.284	4.661
RBP-4	0.33 (0.28)	0.51 (0.73)	0.665	1.536
Relaxin-2	0.71 (0.74)	0.42 (0.58)	0.550	0.580
Resistin	0.84 (0.69)	0.71 (0.43)	0.751	0.838
SDF-1alfa	0.94 (0.79)	0.30 (0.21)	0.169	0.318
Serpin E1	3.64 (2.75)	0.44 (0.27)	0.060	0.119
SHBG	1.31 (0.81)	0.37 (0.46)	0.090	0.279
ST2	1.32 (0.65)	0.32 (0.61)	0.064	0.238
TARC	0.96 (0.64)	0.46 (0.72)	0.337	0.473
TFF3	39.06 (22.14)	0.44 (0.74)	0.010	0.011
TfR	2.23 (1.04)	0.84 (0.78)	0.075	0.373
TGF-alfa	1.42 (1.12)	0.31 (0.43)	0.114	0.218
Thrombospondin-1	0.25 (0.22)	0.63 (0.76)	0.371	2.558
TNF-alfa	0.40 (0.19)	0.30 (0.53)	0.728	0.739
uPAR	1.05 (0.54)	0.21 (0.31)	0.036	0.200
VEGF	1.14 (0.14)	0.12 (0.20)	0.000	0.103
Vitamin D BP	3.31 (1.46)	0.37 (0.58)	0.010	0.112
CD31	1.18 (0.86)	0.38 (0.64)	0.184	0.318
TIM-3	1.78 (1.02)	0.28 (0.50)	0.039	0.156
VCAM-1	2.23 (1.34)	0.20 (0.28)	0.025	0.090

SD, standard deviation

Table 5. Proteome assay classified according to low and normal sperm concentration groups

	Low concentration group n = 4 mean (SD)	Normal concentration group n = 4 mean (SD)	t-test p-value	PD ratio
Adiponectin	1.71 (1.20)	0.77 (0.36)	0.185	0.451
Apolipoprotein A-I	0.47 (0.29)	0.70 (0.28)	0.308	1.475
Angiogenin	2.89 (2.74)	0.49 (0.25)	0.132	0.169
Angiopoietin-1	0.20 (0.36)	0.15 (0.01)	0.791	0.747
Angiopoietin-2	3.45 (2.95)	3.37 (1.57)	0.962	0.976
BAFF	0.15 (0.12)	0.21 (0.16)	0.592	1.358
BDNF	2.50 (1.81)	2.49 (1.53)	0.997	0.997
Complement Component C5/C5a	0.17 (0.14)	0.12 (0.09)	0.582	0.721
CD14	0.72 (0.33)	0.35 (0.17)	0.093	0.484
CD30	3.51 (2.02)	0.69 (0.35)	0.033	0.195
CD40 ligand	1.65 (0.61)	0.53 (0.13)	0.012	0.320
Chitinase 3-like 1	0.27 (0.25)	0.27 (0.16)	0.975	1.014
Complement Factor D	0.43 (0.33)	0.22 (0.15)	0.282	0.497
C-Reactive Protein	0.53 (0.41)	0.29 (0.14)	0.316	0.550
Cripto-1	0.25 (0.34)	0.27 (0.17)	0.919	1.087
Cystatin C	0.75 (0.57)	0.39 (0.13)	0.252	0.509
Dkk-1	1.52 (0.93)	0.58 (0.27)	0.099	0.378
DPP4	0.51 (0.30)	0.39 (0.19)	0.536	0.766
EGF	2.03 (1.19)	0.30 (0.20)	0.028	0.143
EMMPRIN	2.68 (0.56)	0.69 (0.40)	0.001	0.257
ENA-78	0.29 (0.31)	0.27 (0.17)	0.903	0.909
Endoglin	0.27 (0.34)	0.15 (0.19)	0.557	0.544
Fas Ligand	0.45 (0.48)	0.31 (0.24)	0.637	0.701
FGF basic	0.53 (0.46)	0.31 (0.25)	0.422	0.568
FGF-7	0.40 (0.39)	0.27 (0.26)	0.594	0.665
FGF-19	0.81 (0.40)	0.42 (0.30)	0.165	0.509
Flt-3 Ligand	0.47 (0.54)	0.24 (0.24)	0.467	0.508
G-CSF	0.50 (0.54)	0.21 (0.28)	0.363	0.405
GDF-15	18.94 (16.45)	1.39 (0.67)	0.077	0.073
GM-CSF	1.46 (0.41)	0.39 (0.34)	0.007	0.266
GRO alfa	0.15 (0.13)	0.12 (0.18)	0.797	0.798
Growth Hormone	0.26 (0.23)	0.09 (0.11)	0.253	0.350
HGF	0.52 (0.41)	0.34 (0.20)	0.463	0.649
ICAM-1	0.44 (0.49)	0.29 (0.30)	0.627	0.655
IFN-gamma	2.20 (1.68)	1.65 (0.77)	0.574	0.748
IGFBP-2	0.54 (0.61)	0.25 (0.23)	0.417	0.461
IGFBP-3	0.49 (0.59)	0.24 (0.21)	0.454	0.488
IL-1alfa	2.35 (1.51)	2.42 (0.95)	0.938	1.029
IL-1beta	0.62 (0.61)	0.24 (0.30)	0.317	0.391
IL-1ra	1.28 (0.99)	0.43 (0.23)	0.144	0.331
IL-2	0.80 (0.38)	0.38 (0.12)	0.082	0.474
IL-3	1.09 (0.27)	0.38 (0.16)	0.004	0.347
IL-4	0.25 (0.20)	0.17 (0.23)	0.595	0.667
IL-5	1.14 (0.81)	0.84 (0.47)	0.541	0.733
IL-6	0.76 (0.60)	0.44 (0.34)	0.384	0.573
IL-8	1.25 (1.48)	0.65 (0.40)	0.459	0.514
IL-10	0.61 (0.73)	0.36 (0.35)	0.556	0.581
IL-11	1.07 (1.36)	0.97 (0.40)	0.887	0.900
IL-12p70	0.66 (0.80)	0.25 (0.22)	0.352	0.369
IL-13	0.71 (0.86)	0.23 (0.26)	0.322	0.316
IL-15	0.63 (0.76)	0.25 (0.17)	0.361	0.387
IL-16	1.19 (0.96)	0.41 (0.10)	0.157	0.342
IL-17A	1.73 (0.60)	1.23 (0.26)	0.178	0.710

IL-18Bpa	3.31 (0.86)	0.58 (0.09)	0.001	0.174
IL-19	0.12 (0.09)	0.23 (0.35)	0.574	1.896
IL-22	0.45 (0.51)	0.25 (0.25)	0.513	0.557
IL-23	0.18 (0.20)	0.20 (0.30)	0.925	1.104
IL-24	0.70 (0.72)	0.41 (0.43)	0.508	0.579
IL-27	0.78 (0.90)	0.64 (0.41)	0.790	0.819
IL-31	0.66 (0.89)	0.62 (0.34)	0.924	0.928
IL-32	0.71 (0.77)	0.23 (0.15)	0.270	0.322
IL-33	0.76 (0.93)	0.20 (0.14)	0.274	0.254
IL-34	0.55 (0.52)	0.14 (0.14)	0.175	0.248
IP-10	1.11 (0.85)	0.37 (0.16)	0.137	0.330
I-TAC	1.93 (0.62)	0.72 (0.18)	0.010	0.372
KLK3	85.10 (11.16)	62.01 (5.97)	0.011	0.729
Leptin	0.12 (0.10)	0.17 (0.24)	0.673	1.500
LIF	0.12 (0.18)	0.16 (0.25)	0.829	1.281
Lipocalin-2	2.38 (1.17)	1.33 (0.83)	0.191	0.556
MCP-1	0.89 (0.77)	0.57 (0.33)	0.477	0.637
MCP-3	0.81 (0.70)	0.25 (0.30)	0.191	0.301
M-CSF	1.75 (1.60)	1.70 (1.36)	0.960	0.969
MIF	0.56 (0.63)	0.14 (0.19)	0.250	0.241
MIG	0.97 (0.86)	0.20 (0.17)	0.129	0.204
MIP-1alfa/MIP-1beta	0.55 (0.45)	0.11 (0.17)	0.113	0.189
MIP-3alfa	1.44 (1.19)	0.27 (0.08)	0.098	0.185
MIP-3beta	1.72 (0.59)	0.25 (0.19)	0.003	0.141
MMP-9	5.34 (0.91)	1.13 (0.21)	0.000	0.211
Myeloperoxidase	1.03 (0.62)	0.61 (0.28)	0.271	0.594
Osteopontin	1.38 (0.99)	0.93 (0.28)	0.411	0.671
PDGF-AA	0.73 (0.62)	0.28 (0.24)	0.224	0.380
PDGF-AB/BB	0.19 (0.22)	0.23 (0.32)	0.832	1.233
Pentraxin3	2.58 (1.38)	2.34 (0.80)	0.774	0.906
PF4	0.50 (0.44)	0.24 (0.21)	0.334	0.490
RAGE	0.20 (0.18)	0.24 (0.22)	0.764	1.219
RANTES	0.30 (0.26)	0.18 (0.20)	0.506	0.607
RBP-4	0.41 (0.37)	0.22 (0.12)	0.364	0.529
Relaxin-2	0.70 (0.47)	0.35 (0.06)	0.184	0.492
Resistin	0.93 (0.46)	1.40 (0.14)	0.094	1.511
SDF-1alfa	1.20 (0.41)	0.95 (0.36)	0.386	0.786
Serpin E1	3.26 (1.69)	1.44 (0.28)	0.078	0.442
SHBG	1.03 (0.69)	0.90 (0.30)	0.732	0.868
ST2	0.38 (0.39)	0.24 (0.21)	0.551	0.632
TARC	0.42 (0.37)	0.34 (0.26)	0.711	0.789
TFF3	10.60 (8.77)	0.59 (0.32)	0.063	0.055
TfR	1.74 (1.35)	2.48 (0.97)	0.407	1.424
TGF-alfa	0.11 (0.12)	0.22 (0.29)	0.513	2.012
Thrombospondin-1	0.22 (0.18)	0.40 (0.24)	0.268	1.871
TNF-alfa	0.22 (0.17)	0.15 (0.15)	0.577	0.686
uPAR	0.79 (0.59)	0.33 (0.17)	0.190	0.419
VEGF	0.46 (0.17)	0.16 (0.22)	0.074	0.346
Vitamin D BP	5.42 (4.10)	2.20 (0.71)	0.172	0.405
CD31	0.36 (0.28)	0.32 (0.22)	0.828	0.890
TIM-3	0.23 (0.17)	0.29 (0.27)	0.710	1.287
VCAM-1	0.76 (0.44)	0.76 (0.35)	0.986	0.998

SD, standard deviation

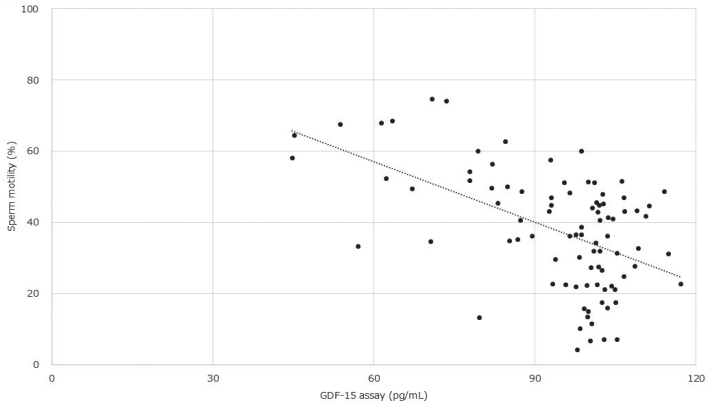

Fig. 2. Correlation between GDF-15 and sperm motility
Quantitative assay using ELISA methods showed a negative correlation between GDF-15 and sperm motility in a Spearman rank correlation coefficient test (n = 89, p = 0.000, rS = -0.438).
GDF-15, growth differentiation factor-15; ELISA, enzyme-linked immunosorbent assay

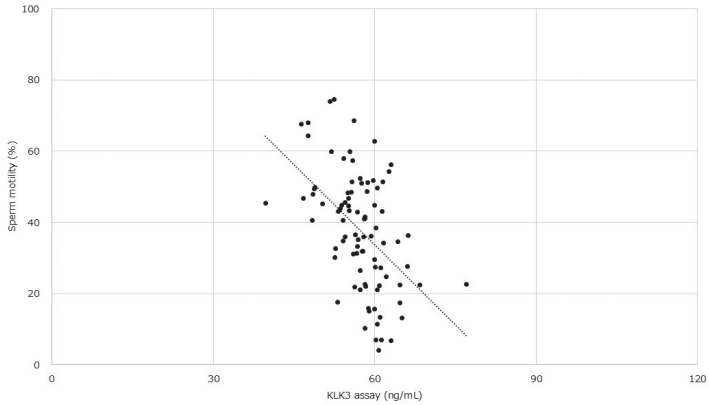

Fig. 3. Correlation between KLK3 and sperm motility
Quantitative assay using ELISA methods showed a negative correlation between KLK3 and sperm motility in a Spearman rank correlation coefficient test (n = 89, p = 0.000, rS = -0.507).
ELISA, enzyme-linked immunosorbent assay; KLK3, Kallikrein 3

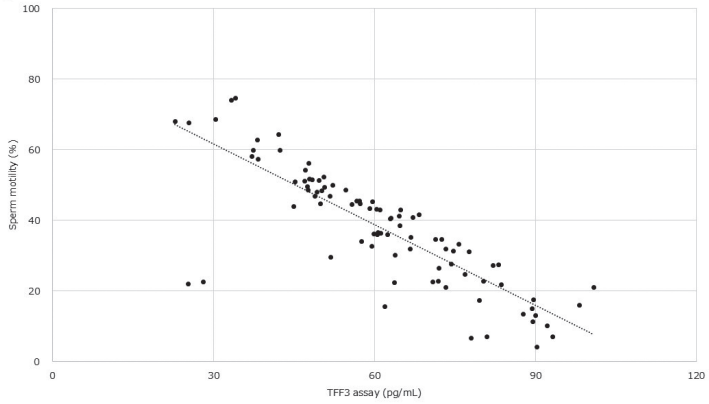

Fig. 4. Correlation between TFF3 and sperm motility
Quantitative assay using the ELISA methods showed a negative correlation between TFF3 and sperm motility in a Spearman rank correlation coefficient test (n = 89, p = 0.000, rS = -0.848).
TFF3, Trefoli factor 3; ELISA, enzyme-linked immunosorbent assay

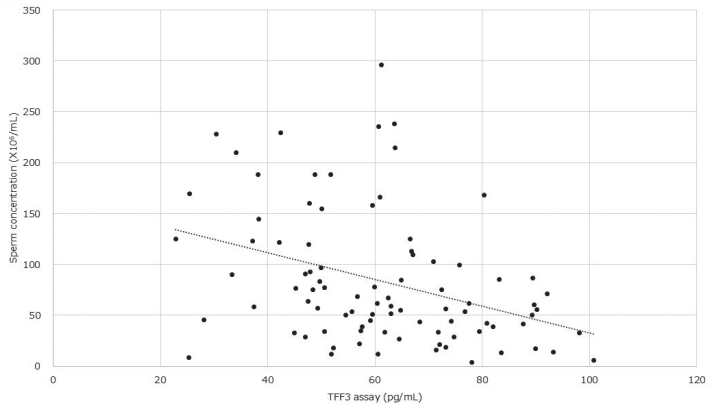

Fig. 5. Correlation between TFF3 and sperm concentration
Quantitative assay using the ELISA methods showed a negative correlation between TFF3 and sperm concentration in a Spearman rank correlation coefficient test (n = 89, p = 0.000, rS = -0.417). ELISA, enzyme-linked immunosorbent assay; TFF3, Trefoli factor 3

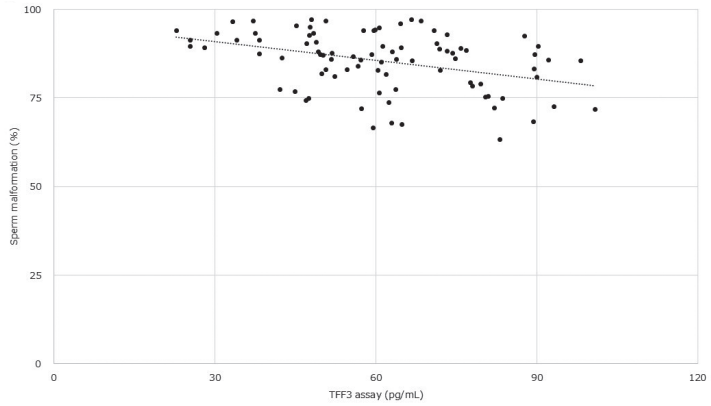

Fig. 6. Correlation between TFF3 and sperm malformation
Quantitative assay using the ELISA methods showed a negative correlation between TFF3 and sperm malformation in a Spearman rank correlation coefficient test (n = 87, p = 0.000, rS = -0.378). ELISA, enzyme-linked immunosorbent assay; TFF3, Trefoli factor 3

changes in semen as the first step in diagnostic and therapeutic management. This evaluation can be conducted using protein analysis, such as leukocyte concentration tests, oxidative stress markers, and seminal cytokine levels. These tests enable the identification of underlying inflammation contributing to male infertility, facilitating timely and appropriate interventions[7]. La Vignera S, *et al.*[7] reported that the assessment of inflammatory changes in the semen containing sperm is important for the diagnostic and therapeutic management of male infertility. Cytokine and cytokine-induced oxidative stress in the semen affect sperm parameters and associated with male infertility[8]. The seminal plasma levels of IL-1 and IL-6 are higher in infertile patients than those in fertile men[8, 9]. The seminal plasma levels of IL-6, Il-8, INF-gamma, TNF-α are suggested to have negative correlation with sperm motility[10–12]. Interferon-gamma is also suggested to have the effects of increases sperm membrane

lipoperoxidation at physiological concentrations[13], and suppresses the spontaneous acrosome reaction and acrosine activity[14]. Migration inhibitory factor is expressed all along the epididymis, with a negative correlation between the amount of sperm-associated migration inhibitory factor (MIF) and the percentage of motility[15]. The MIF and TNF-alfa are also suggested to have the effects of increasing the phosphatidylserine externalization and DNA fragmentation[12, 16]. However, these studies only reported the relationship between seminal plasma and cytokines, and there was no study to examine the relationship between sperm and cytokines. This study is a highly innovative study to investigate the relationship between semen containing sperm and cytokines, and results did not show significant difference between semen parameters (sperm motility and sperm concentration) and IL-1, IL-6, IL-8, IFN-gamma and MIF (Tables 4 & 5). On the other hand, this study revealed three more significant indicators (GDF-15, KLK3, and TFF3) as more sensitive predictive indicators for male infertility compared to the main Inflammatory markers that have been reported using proteome assay and quantitative assay.

Growth differentiation factor-15 (macrophage inhibitory cytokine-1: MIC-1, non-steroidal anti-inflammatory drugs activated gene-1: NAG-1, placental transforming growth factor-b: PTGF, or prostate-derived factor) is a divergent member of the TGF-beta family[17]. The highest expression of GDF-15 is found in prostate epithelial cells and placental trophoblast[18]. Growth differentiation factor-15 is generally considered to be a component of the antitumorigenic activity, mostly because the expression of GDF-15 is crucial for the chemo-preventive effects[19]. Additionally, GDF-15 is also abundantly present in semen[18]. However, the physiological and pathophysiological role of GDF-15 in reproductive processes remains unknown. Our results indicate that GDF-15 is present in semen, and the high presence of GDF-15 may be associated with decreased seminal motility. Kallikrein 3 is also one of the most abundant proteins in the secretion of normal human prostate epithelium and semen[20]. Kallikrein 3 is an androgen dependent 30 KDa glycoprotein with chymotrypsin like enzymatic activity and plays a key role in the fragmentation of seminal vesicle secreted proteins[21]. However, the correlation between KLK3 concentration and semen liquefaction (sperm motility) remains unknown, and the results of reports showed a positive correlation[22, 23], negative correlation[24], and no association at all[25, 26]. Our results indicate that KLK3 is present in semen, and the high presence of KLK3 may be negatively correlated with sperm motility. However, quantitative assay using the ELISA methods showed no correlation between GDF-15, KLK3 and sperm concentration. Trefoli factors are small molecular peptides secreted by the goblet and epithelial cells of various tissues in mammals (TFF1 containing 60 amino acid residues, TFF2 containing 106 residues, and TFF3 containing 59 residues)[27]. These proteins are involved in the protection and maintenance of healthy secretory epithelia via interactions with mucins and the stimulation of cell motility[28–30]. Recent studies have reported that TFF3 is widely distributed in the human body, and present in intestinal tract, brain, liver, kidney, pancreas, breast, lung, conjunctiva, spleen, and lymph nodes[31]. However, the physiological function of TFF3 is still not fully understood[32], but it may play a role in tumor development and progression in different tumor entities[33–35]. Furthermore, it is also unclear whether TFF3 is present in semen and the physiological function in semen. Our results indicate that TFF3 is present in semen, and high presence of TFF3 may be negatively correlated not only with sperm motility but also with sperm concentration. TFF3 may be strongly associated with spermatogenic dysfunction.

In this study, we did not show significant

difference between semen parameters (sperm motility and sperm concentration) and IL-1, IL-6, IL-8, IFN-gamma and MIF that have been reported the relationship between seminal plasma and cytokines. On the other hand, this study revealed three more significant three indicators (GDF-15, KLK3, and TFF3) as more sensitive predictive indicators for male infertility compared to the main Inflammatory markers that have been reported using proteome assay and quantitative assay. Three significant indicators (GDF-15, KLK3 and TFF3) levels in the semen containing sperm may be novel predictive indicators focused on semem analysis of inflammation for male infertility. However, Our study has not been adequately considered in lifestyle history (smoking, drinking, etc.), medical history (presence or absence of sexually transmitted diseases and intrascrotal surgery, etc.), physical findings (testicular volume, presence or absence of varicocele, inflammation in the scrotum, etc.), laboratory findings (urinary examination, presence or absence of white blood cells in semen, etc.) and female factors. Furthermore, although our study showed the possibility of a novel predictive indicators focused on semen analysis of inflammation for male infertility, the therapeutic effect of predictive indicators (GDF-15, KLK3 and TFF3) inhibition has not been demonstrated. Further studies are needed to investigate male and female factors, and evaluate achieving pregnancy and live births for a long time.

CONCLUSIONS

Extracted three indicators (GDF-15, KLK3 and TFF3) in the semen containing sperm showed statistically significant difference and negative correlation with semen parameters (sperm motility, concentration and malformation). The level of novel three indicators in the semen containing sperm may be predictive indicators focused on semen analysis of inflammation for male infertility.

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AUTHORSHIP STATEMENT

W.S. and K.S. designed the study. W.S and T.M. contributed to sample collection. W.S. and T.M. contributed to data collection. W.S., S.O. and T.M. contributed to sample quantification. W.S. and S.O. analyzed the data. W.S. and S.O. wrote the manuscript. All authors reviewed and approved the manuscript.

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CONFLICT OF INTEREST

None

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