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Quantitative analysis of the sHLA-G protein in seminal plasma

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Abstract

Background: Recent studies revealed that maternal and embryonic contributions impact on HLA-G protein expression and might contribute to pregnancy success or failure. The main objective of this study was to examine the paternal levels of the immunoregulatory soluble human leukocyte antigen-G (sHLA-G) protein in seminal plasma and testicular biopsy samples during artificial reproductive technique (ART) treatment and to investigate possible correlations with other semen parameters, age, and pregnancy outcome of the female partner.

Methods: Soluble HLA-G levels of 106 seminal plasma samples and eight testicular biopsy samples were determined using a commercial sHLA-G Enzyme-linked immunosorbent assay (ELISA) kit.

Results: We observed a significant negative correlation of male age with total sHLA-G amount (P 0.023, R −0.221) and semen volume (P = 0.047, R −0.193). Testicular biopsy samples were analyzed and tested positively with sHLA-G ELISA. Levels of sHLA-G in seminal plasma samples from men with normozoospermia did not deviate significantly from those with reduced semen quality. No significant difference of sHLA-G levels in seminal plasma and pregnancy outcome of the female partner was detected. Our data showed that age of men with normozoospermia was significantly lower when the female partner conceived after ART treatment (P = 0.016, Mann-Whitney U test).

Conclusion: High sHLA-G levels in seminal plasma of the male partner appear not to be required for pregnancy but might contribute among other factors to the success of establishing and maintaining pregnancy through long-term priming of the female uterine milieu.

KEYWORDS
fertility, HLA-G, in vitro fertilisation, pregnancy, semen

1 | INTRODUCTION

Successful in vitro fertilization (IVF) treatment depends on many factors, for example, oocyte and sperm quality. Therefore, up to this point the primary focus has been on the quality of parental gametes in artificial reproductive techniques (ART). For the last decades, seminal plasma has been seen primarily as a transport vector for the male gametes.

However, growing evidence demonstrates that seminal plasma has additional important functions that might impact on fertility.[1] Seminal plasma consists of secretions from different accessory glands, such as epididymis, seminal vesicle, prostate, and

bulbourethral gland [2,3] containing a heterogeneous mixture of various cytokines, chemokines, and growth factors.[4]

There is emerging evidence that seminal plasma induces an inflammatory response and contributes to transforming the female uterine immunologic milieu.[1] So far, only few studies revealed that seminal fluid contains varying amounts of soluble HLA-G (sHLA-G) protein,[5,6] and studies from different fields, such as cancer research, transplantation surgery, and IVF, have suggested HLA-G to play a role not only in tumour escape,[7,8] but also in immunomodulation at the fetal-maternal niche.[9-11]

The HLA-G gene is differentially spliced and translated as 7 different isoforms,[12,13], consisting of soluble [G5, G6, G7] and membrane-bound protein structures [G1, G2, G3, G4].[14] The latter can be shed via matrix metalloproteinases.[15] The HLA-G gene promotor contains progesterone and hypoxia response elements,[13] indicating a hormone and oxygen-based control of expression, and in cancer cells regulation via hypoxia-inducible factor 1 (HIF-1) is assumed,[7,16]. HLA-G interacts with different receptors on immune cells [eg, ILT2, ILT4 and KIR2L4],[17] with partially increased avidity compared to classical MHC I.[18] Hypermethylation of the HLA-G promotor [19] and decreased sHLA-G levels in maternal blood have been suggested to play a role in pre-eclampsia,[20] and several studies indicated that sHLA-G contributions from the developing embryo have an impact on pregnancy success.[9,21-23]

On the other hand, also in seminal fluid, different levels of sHLA-G have been detected [5]—the author suggested that successful pregnancy is a complex interplay of maternal, embryonic, and paternal factors within a certain window of time. Also, Bromfield et al[24] demonstrated that in mice, after removal of the paternal seminal vesicle, successful maternal conception was hampered. When reproduction succeeded, the offspring was suffering from various maladies, for example, obesity and hypertension syndrome.

In this study, we analyzed sHLA-G levels in paternal seminal plasma of 106 men during artificial reproductive technique (ART) treatment and correlated these data with sperm parameters, age, and pregnancy outcome of the female partner. To gain more insights about the origin of sHLA-G in seminal plasma, we also studied testicular sperm extraction (TESE) samples using the same methodology.

2 | MATERIAL AND METHODS

2.1 | Ethical approval

This study was approved by the ethics committees of the University Hospital of Frankfurt, Germany.

2.2 | Semen samples and testicular sperm extraction samples

A total of 106 semen and eight TESE samples from male donors were obtained between March and October 2018. Semen was separated from seminal plasma using a density gradient consisting of a 90% lower layer and a 45% upper layer. Centrifugation was performed at 300 g for 15 minutes, followed by different washing steps. Seminal plasma samples were frozen immediately at −30°C until sHLA-G Enzyme-linked immunosorbent assay (ELISA), SDS-PAGE, and Western blot were performed. The pregnancy outcome of the ART treatment cycles was documented. Eight testicular tissue samples (sized 5 × 5 mm) were retrieved via surgery, and after cryopreservation and thawing, TESE samples were processed in the IVF laboratory. Using a pestle, samples were grinded and further processed via a density gradient consisting of a 45% single main layer. The liquid phase was extracted and frozen immediately at −30°C until sHLA-G ELISA was performed.

2.3 | Assessment of semen samples

Semen analysis and assessment was performed according to the WHO laboratory manual for the examination of human semen for spermiogram analysis of the male partner.[25] For sperm samples used for following IVF and intracytoplasmic sperm injection (ICSI) treatment, a simplified analysis was used renouncing peroxidase test and staining techniques to determine vitality. Normal semen quality was defined through a sperm volume of 1.5 mL or more and sperm concentration of 15 Mio/mL or higher. Normal progressive motility was specified through 32% or more progressive motile sperm or 40% or more progressive and local motile sperm cells. Normozoospermia was furthermore defined through 4% or more morphological normal sperm cells. Reduced semen quality was defined through non-reaching normal parameters for volume, concentration, motility, and morphology and included diagnostic findings of hypospermia (reduced semen volume), oligozoospermia (reduced sperm count), asthenozoospermia (reduced motility), OAT syndrome I-III (combination of reduced sperm count, reduced motility, and morphological abnormality), and teratozoospermia (morphological abnormality).

2.4 | In vitro fertilization, intracytoplasmic sperm injection, and embryo culture

Cumulus oocyte complexes (COC) were obtained via egg retrieval procedure and further processed in the IVF laboratory. Cumulus cells were removed via enzymatic treatment before ICSI procedure. IVF treatment was performed by adding at least 100 000 motile sperm cells to up to three COC complexes. Pronuclear (PN) scoring was performed 16-18 hours after the IVF/ICSI procedure. A sufficient number of two PN stages were cultured until day 5 for embryo transfer. When obtaining two or >2 PN stages from the IVF/ICSI procedure, cell culture was performed until day 3. Cells were cultured in single droplets with 20µl of cell culture media in humidified IVF incubators at 37°C, with 6% CO_2, 5% O_2, and 89% N_2 atmospheric composition.

2.5 | Assessment of embryo quality and embryo transfer

Cleavage stage embryos were scored concerning cell number and degree of fragmentation and classified into quality stages from A

TABLE 1 Soluble HLA-G levels in seminal plasma and testicular tissue samples from men receiving ART treatment

Parameter	Seminal plasma samples median	Interval SD	n	Testicular tissue samples median	Interval SD	n	[a]*P*-value
sHLA-G (U/mL)	275.63	5.92-580.02 172.70	106	232.06	16.40-530.37 163.18	8	0.513

Note: n = 114 samples.
*Mann-Whitney *U* test.

TABLE 2 Correlation of sHLA-G levels in seminal plasma with male age and semen parameters

	Male age (y)	Concentration (10^6/mL)	Abstinence (d)	Volume (mL)	Motility (%)
sHLA-G (U/mL)					
R[a]	−0.134	−0.078	0.126	0.060	−0.118
P-value[a]	0.171	0.429	0.199	0.539	0.229
sHLA-G (U)					
R[a]	−0.221	−0.097	0.123		−0.020
P-value[a]	0.023	0.323	0.208		0.842

Note: n = 106 seminal plasma samples.
*Spearman correlation.

to C.[26] To evaluate blastocyst quality, a well-proven scoring system was used.[27] Briefly, trophectoderm (TE) and inner cell mass (ICM) were assessed concerning density and number of cells and divided from A to C, depending on quality. Grade A and B cells were classified as "ideal," grade C cells were assessed as "not ideal." Embryo transfer procedures were performed predominantly on day 5; due to embryo quality, single embryo transfers (SETs) were aimed to perform.

2.6 | Pregnancy test

Pregnancy test was conducted two weeks after embryo transfer at the Department of Gynecological Endocrinology and Reproductive Medicine, University Hospital of Frankfurt, Germany. Biochemical pregnancies were excluded from the pregnancy group.

2.7 | Enzyme-linked immunosorbent assay

Soluble HLA-G levels (sHLA-G) were measured with a sHLA-G ELISA Kit (Exbio Praha). Wells were precoated with MEM-G/9 antibody that detects HLA-G1 and HLA-G5.[28] Bound antibody-antigen complexes were detected with monoclonal anti-ß2M antibody, linked with horseradish peroxidase (HRP). Seminal plasma samples were thawed, vortexed, and diluted accordingly. 100 µL of diluted samples were loaded in triplicates into the wells of the microtiter plate. A master calibrator (HLA-G standard) from the sHLA-G ELISA Kit (Exbio) was used to calculate a calibrator curve; dilution buffer and cell culture media were used as negative controls. The microtiter plate was incubated for 20 hours, followed by five washing steps. After removal of the supernatant, 100 µL of conjugate solution was added to each well. The plates were incubated for 1 hour on a microplate shaker, followed by five washing steps. The supernatant was removed, 100 µL of substrate solution was added, and the microtiter plates were shielded from light using aluminum foil and incubated for 25 minutes, followed by adding of 100 µL stop solution. The absorbance was measured using a microplate reader, and the amount of sHLA-G protein in the samples was calculated using the calibrator curve.

TABLE 3 Correlation of sHLA-G levels in seminal plasma and TESE samples with sperm morphology

	Normal sperm morphology (%)
sHLA-G (U/mL)	
R[a]	0.040
P-Value[a]	0.603

Note: n = 114 samples.
*Spearman correlation.

2.8 | SDS-PAGE/Western blot

Samples were diluted with sample buffer [2x Laemmli buffer containing Tris-HCl, glycerol, sodium dodecyl sulfate, bromophenol blue, and β-mercaptoethanol) and denaturized at 95°C for 5 minutes, and a final volume of 10 µL was loaded on a 12% polyacrylamide gel. Dilution buffer and cell culture media were used as negative controls. After performing electrophoresis, proteins were transferred on PVDF membranes to process Western blot. Membranes were blocked with 5% bovine serum albumin (BSA in PBS, 0.1% Tween-20) for 30 minutes and incubated with mouse monoclonal antibody to HLA-G [MEM-G1/Mouse/IgG, 10 µg/mL, Exbio] for

TABLE 4 Soluble HLA-G levels in seminal plasma and age of men with normal and reduced semen quality

Parameter	Normal semen quality median	Interval SD	n	Reduced semen quality median	Interval SD	n	[a]*P*-value
sHLA-G (U/mL)	250.26	7.77-577.30 184.97	32	286.59	5.92-580.02 167.23	74	0.221
sHLA-G (U)	930.08	12.43-3302.20 867.87		890.29	23.68-3144.06 744.23		0.885
Male age (y)	37.5	28-49 4.57		38.51	26-53 5.89		0.510

Note: n = 106 seminal plasma samples.
*Mann-Whitney *U* test.

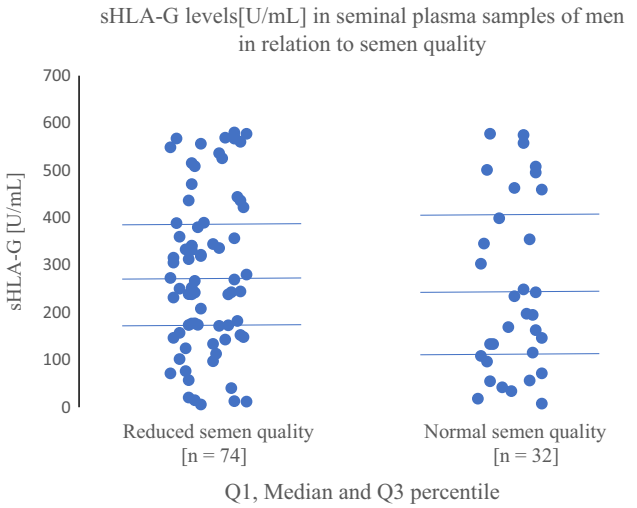

FIGURE 1 Soluble HLA-G levels (U/mL) in seminal plasma samples of men in relation to semen quality

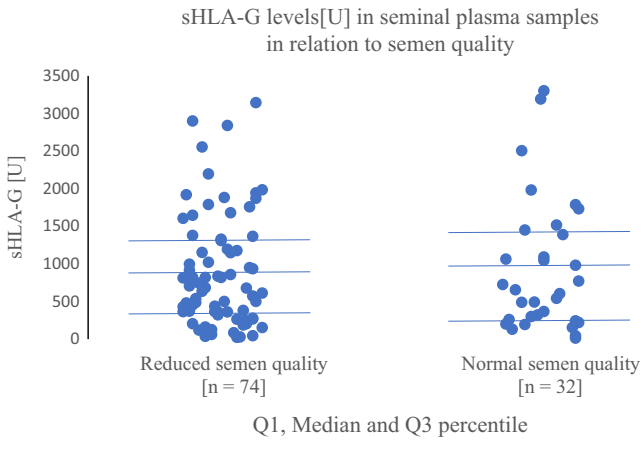

FIGURE 2 Soluble HLA-G levels (U) in seminal plasma samples in relation to semen quality

60 minutes. After three washing steps, the PVDF membranes were incubated with rabbit polyclonal secondary antibody to mouse IgG (H&L; Rabbit, IgG, Abcam) linked to horseradish peroxidase [HRP]), followed by enhanced chemiluminescence (ECL) detection.

2.9 | Statistics

Evaluation of sHLA-G ELISA values was performed for concentration (U/mL) and total volume (U) per semen donation (volume × concentration). Data were analyzed with SPSS version 25 (IBM) statistical software. Mann-Whitney *U* test was used to compare continuous parameters between groups and Spearman correlation to analyze correlations between sHLA-G, semen volume, sperm parameters, abstinence, and age. *P*-values < 0.05 were considered as significant. We also tested male and female age, semen quality, and sHLA-G as predictors for pregnancy outcome in logistic regression models among the 106 samples.

3 | RESULTS

3.1 | Soluble HLA-G levels in seminal plasma and testicular tissue

Soluble HLA-G (sHLA-G) was quantified by ELISA in 106 seminal plasma samples and in eight testicular tissue samples from male donors receiving artificial reproductive technique (ART) treatment. As shown in Table 1, we observed a broad range from 5.92 to 580.02 (U/mL) with a median of 275.63 and standard deviation (SD) of 172.70 for the plasma samples, indicating high fluctuations of sHLA-G concentrations between male donors receiving ART. In addition, sHLA-G levels from eight testicular biopsies similarly showed a spread from 16.40 to 530.37 (U/mL), a median of 232.06, and SD 163.18. We detected no significant difference in sHLA-G levels between seminal plasma samples and testicular tissue samples.

3.2 | Soluble HLA-G levels of the cohort, male age, and semen parameters

Data shown in Table 2 indicate that male age was correlated negatively with total amount (U) of sHLA-G (*P* = 0.023, *R* −0.221) and semen volume (*P* 0.047, *R* −0.193, Pearson), data not shown. We observed a significant positive correlation between abstinence and sperm concentration (*P* 0.008, *R* 0.257, Pearson). No statistical significance was observed when correlating sHLA-G levels with sperm concentration and abstinence. As shown in Table 3, we did not find

TABLE 5 Soluble HLA-G levels in seminal plasma, age, and embryo transfer parameters in relation to pregnancy outcome of the female partner

Parameter	Pregnancy group median	Interval SD	n	Non-pregnancy group median	Interval SD	n	[a]*P*-value
sHLA-G (U/mL)	290.69	7.77-577.30 175.68	37	267.54	5.92-580.02 171.82	69	0.484
sHLA-G (U)	933.67	12.43-3302.20 873.63		885.49	23.68-3191.92 735.37		0.835
Female age (y)	34.65	24-42 3.83		36.67	25-45 4.46		0.010
Male age (y)	36.92	28-52 5.15		38.94	26-53 5.62		0.064
Embryos transferred (n)	1.35	1-2 0.48		1.39	1-2 0.49		0.687
Ideal embryos transferred (n)	1.08	0-2 0.54		0.93	0-2 0.73		0.256
Non-ideal embryos transferred (n)	0.27	0-2 0.56		0.46	0-2 0.58		0.053

Note: n = 106 samples.
*Mann-Whitney *U* test.

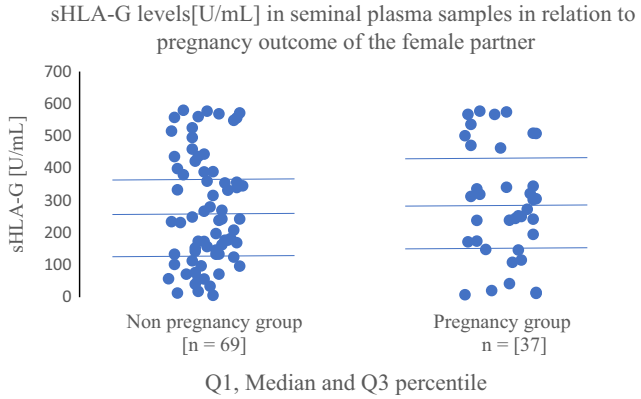

FIGURE 3 Soluble HLA-G levels (U/mL) in seminal plasma samples in relation to pregnancy outcome of the female partner

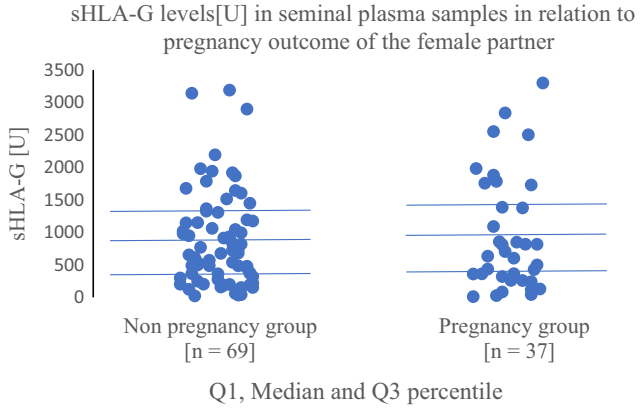

FIGURE 4 Soluble HLA-G levels (U) in seminal plasma samples in relation to pregnancy outcome of the female partner

TABLE 6 Univariate logistic regression of pregnancy outcome

	Odds ratio	95% confidence interval	*P*-value
Male age (per y)	0.932	0.86-1.01	0.075
Female age (per y)	0.896	0.81-0.99	0.026
Semen quality	0.967	0.40-2.31	0.940
sHLA-G (U)	1.000	1.00-1.001	0.762
sHLA-G (U/mL)	1.001	0.998-1.003	0.506

TABLE 7 Multivariate logistic regression of pregnancy outcome

	Odds ratio	95% confidence interval	*P*-value
Male age (per y)	0.968	0.88-1.06	0.502
Female age (per y)	0.911	0.81-1.03	0.123
Semen quality	0.991	0.40-2.46	0.985
sHLA-G (U)	1.000	1.00-1.00	0.671

significant differences in sHLA-G levels in samples of male individuals with normal sperm morphology.

3.3 | Soluble HLA-G levels in seminal plasma, semen quality, and male age

We detected no significant difference in sHLA-G levels between seminal plasma samples from men with normozoospermia and men with reduced semen quality, data shown in Table 4, Figures 1 and 2.

TABLE 8 sHLA-G levels in seminal plasma in relation to normal semen quality and pregnancy outcome of the female partner

	Parameter	Pregnancy group median	Interval SD	n	Non-pregnancy group median	Interval SD	n	[a]P-value
Normal semen quality	sHLA-G (U/mL)	308.70	7.77-577.30 222.59	11	219.66	18.20-557.79 159.37	21	0.312
	sHLA-G (U)	1187.05	12.43-3302.20 1072.58		795.48	43.68-3191.92 749.17		0.439
	Male age (y)	34.91	28-41 4.06		39.00	31-49 4.26		0.016
	Embryos transferred (n)	1.45	1-2 0.52		1.43	1-2 0.50		0.890
	Ideal embryos transferred (n)	1.27	1-2 0.46		0.86	0-2 0.72		0.097
	Non-ideal embryos transferred (n)	0.18	0-1 0.40		0.57	0-2 0.59		0.062

Note: n = 32 seminal plasma samples.
*Mann-Whitney *U* test.

3.4 | Soluble HLA-G levels in seminal plasma, age, and embryo transfer parameters in relation to pregnancy outcome of the female partner

We could not find significant differences in sHLA-G levels in seminal plasma samples of male individuals and pregnancy outcome of the female partner, data shown in Table 5, Figures 3 and 4. We observed a statistically significant difference between female age [*P* = 0.010, Mann-Whitney *U* test] and pregnancy outcome. As expected, more ideal embryos and less non-ideal embryos were transferred in the pregnancy group. In univariate regression shown in Table 6, only female age significantly predicted pregnancy outcome (OR 0.896, 95% CI 0.81-0.99, *P* = 0.026), while male age showed a trend to significance (*P* = 0.075). Semen quality and sHLA-G were not significant. In multivariate logistic regression including all parameters, a trend for an independent predictive value of female age was still detected (OR 0.911, 95% CI 0.81-1.03, *P* = 0.123), data shown in Table 7.

3.5 | Soluble HLA-G levels in seminal plasma in relation to semen quality and pregnancy outcome of the female partner

Age of men with normozoospermia was significantly lower in case of pregnancy of the female partner (*P* = 0.016, Mann-Whitney *U* test), data shown in Table 8. Interestingly, we observed the highest sHLA-G values in seminal plasma samples from men with normal semen quality when the female partner conceived after ART treatment, data shown in Figures 5 and 6. However, this finding did not reach statistical significance. We did not find significant differences in sHLA-G levels in seminal plasma samples of male individuals with

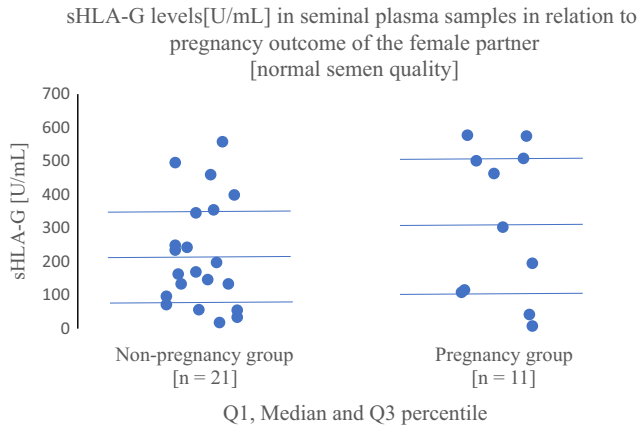

FIGURE 5 Soluble HLA-G levels (U/mL) in seminal plasma samples in relation to pregnancy outcome of the female partner (normal semen quality)

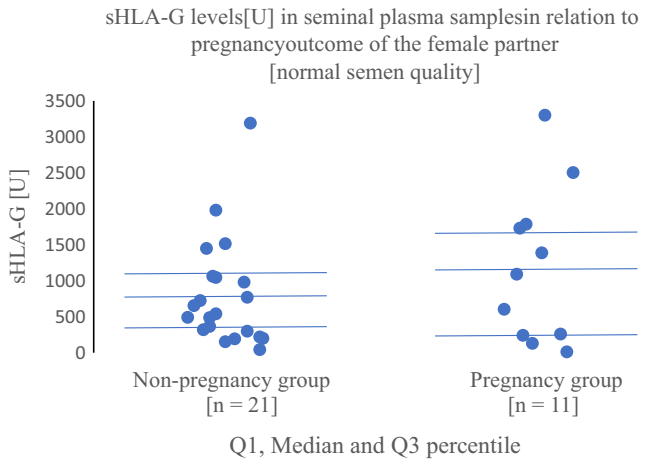

FIGURE 6 Soluble HLA-G levels (U) in seminal plasma samples in relation to pregnancy outcome of the female partner (normal semen quality)

TABLE 9 sHLA-G levels in seminal plasma in relation to reduced semen quality and pregnancy outcome of the female partner

	Parameter	Pregnancy group median	Interval SD	n	Non-pregnancy group median	Interval SD	n	[a]*P*-value
Reduced semen quality	sHLA-G (U/mL)	283.07	11.99-567.31 156.28	26	288.49	5.92-580.02 174.75	48	0.964
	sHLA-G (U)	826.47	28.78-2840.41 773.74		924.87	23.68-3144.06 733.73		0.383
	Male age (years)	37.77	29-52 5.39		38.92	26-53 6.16		0.427
	Embryos transferred (n)	1.31	1-2 0.47		1.38	1-2 0.48		0.565
	Ideal embryos transferred (n)	1.00	0-2 0.56		0.96	0-2 0.74		0.783
	Non-ideal embryos transferred (n)	0.31	0-2 0.61		0.42	0-2 0.57		0.281

Note: n = 74 seminal plasma samples.
*Mann-Whitney *U* test.

reduced semen quality and pregnancy outcome of the female partner, data shown in Table 9, Figures 7 and 8.

3.6 | Validation of sHLA-G expression by Western blot

To confirm the results obtained by sHLA-G ELISA analysis, Western blot analysis of seminal plasma and testicular biopsy samples was performed. Dilution buffer and cell culture media were used as negative controls. Samples with low (range 18.2-54.97) and high (range 108.37-250.33) sHLA-G ELISA values (U/mL) were selected. Bands with ~ 37 kDa were considered as positive. (Figure 9).

All lanes from samples with high (5-8) sHLA-G ELISA values showed a band at ~ 37 kDa. From the samples with low (1-4) sHLA-G ELISA values three lanes showed a weak band, one lane showed a fading band at ~37 kDa. These findings correspond to the sHLA-G ELISA analysis and were considered as affirmative.

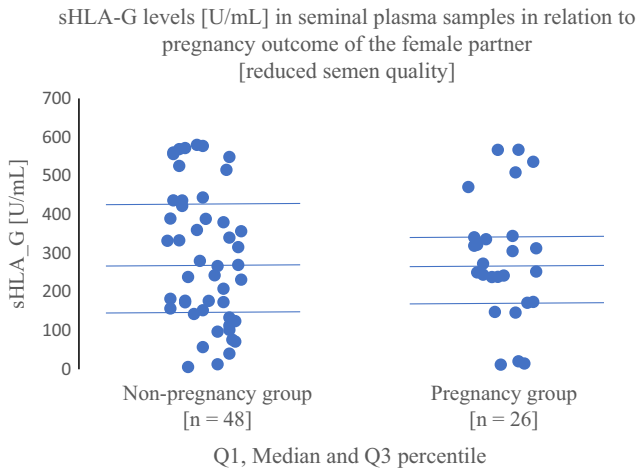

FIGURE 7 Soluble HLA-G levels (U/mL) in seminal plasma samples in relation to pregnancy outcome of the female partner (reduced semen quality)

4 | DISCUSSION

Our analysis showed a wide spread of sHLA-G protein levels in seminal plasma samples of male donors, supporting reports of previous

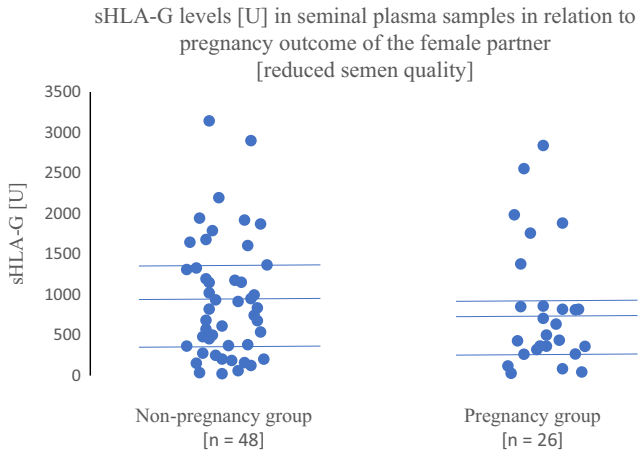

FIGURE 8 Soluble HLA-G levels (U) in seminal plasma samples in relation to pregnancy outcome of the female partner (reduced semen quality)

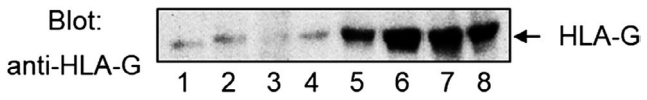

FIGURE 9 Western blot analysis of seminal plasma and TESE samples from men with ART treatment. Samples with low (1-4) and high (5-8) sHLA-G ELISA values (U/mL) were selected

studies.[5,6] We observed a significant negative correlation of male age with total amount of sHLA-G (P = 0.023, R −0.221) and semen volume (P = 0.047, R −0.193). There is evidence that semen volume decreases in aging men.[29,30] Our analysis showed a significant difference of male age in men with normal semen quality in relation to pregnancy outcome of the female partner. (P = 0.016, Mann-Whitney U test). No significant difference of sHLA-G levels in seminal plasma and pregnancy outcome of the female partner was observed. The absence of statistical significance is corresponding to the findings of Dahl et al.[5] Female age was significantly lower in the pregnancy group than in the non-pregnancy group. (P 0.010, Mann-Whitney U test).

Our data show limited comparability with results from another study analyzing seminal plasma samples for intrauterine inseminations,[5] an ART treatment where processed sperm is placed directly in the patient's uterus. In this study, we analyzed seminal plasma samples used for IVF and intracytoplasmic sperm injection (ICSI) treatment, both followed by an embryo transfer procedure. In IVF and ICSI, fertilization is performed in the IVF laboratory, followed by cell culture and an embryo transfer procedure resulting in higher pregnancy rates compared with other artificial reproductive techniques (ART). All three ART methods are addressing specific male (sperm quality) and female (endometriosis, polycystic ovary syndrome, tubal damage) preconditions with according medical indications. Differences between the observed sHLA-G interval values in our study and the findings of [5] may also reflect use of different laboratory protocols. Due to the high spread of sHLA-G values in seminal plasma and other factors influencing pregnancy outcome (female age, embryo quality, male and female preconditions), a high sample number is required to obtain statistical significance and reproducible results. To obtain more information about the origins of sHLA-G in seminal plasma, testicular biopsy samples were analyzed and tested positively with sHLA-G ELISA. Different accessory glands like epididymis, seminal vesicle, prostate, and bulbourethral gland contribute to the total volume of seminal fluid.[3]

The origins and isoform compositions of sHLA-G in seminal fluid have been discussed previously and evidence suggests that HLA-G in the male reproductive system might derive from contributions of the prostate gland.[6,31]

By immunohistochemically staining, Larsen et al[6] could detect HLA-G in paraffin-embedded tissue samples of testis and epididymis. In testicular tissue, HLA-G might play an immunosuppressive role. In Western blot experiments, it could be shown by Dahl et al[5] that HLA-G5 and potentially the shed HLA-G1 isoforms are present in seminal plasma. The determination of the origins of HLA-G in seminal fluid and the quantitative contributions from epididymis, seminal vesicle, and prostate require further detailed studies.

Today, there is growing evidence that seminal plasma is more than a simple transport vector for sperm cells, due to its rich composition of numerous factors with immunomodulatory potential like TGF-ß[32] and HLA-G.[5,6] Establishing and maintaining pregnancy is a complex process, influenced by maternal, embryonic, and paternal factors. There are many factors that affect succeeding pregnancy, one of them might be the quality of paternal seminal plasma. It will be interesting to investigate this issue in a future study with a larger sample number.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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