Glycosylation analysis of immune-related molecules

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Chapter 6

Seminal plasma N-glycome as a new biomarker of environmental exposure associated with semen quality

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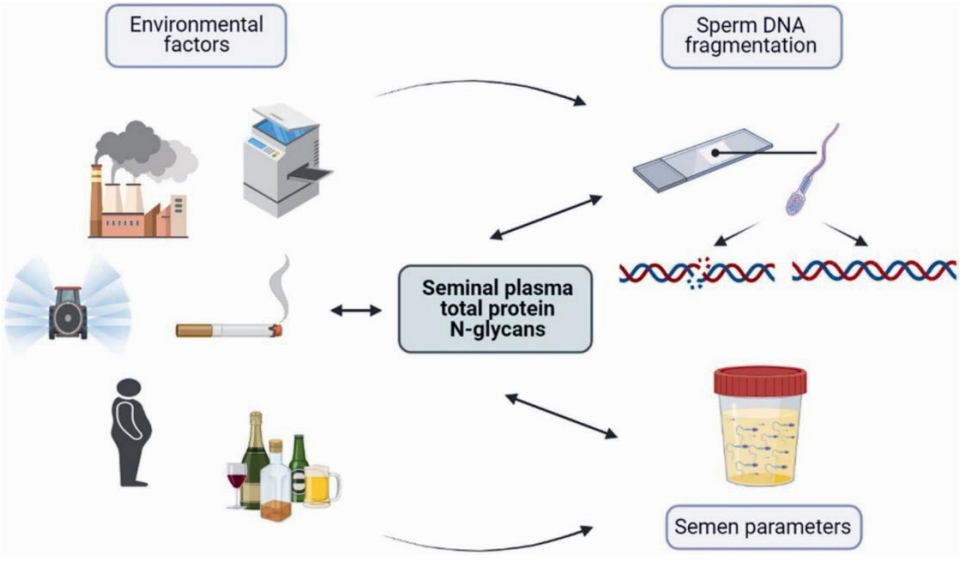
6.1 ABSTRACT

Male infertility, a condition that has during the last decade raised significant concern, is a diagnostically demanding and socially sensitive topic. The number of unsolved issues on infertility etiology, especially potential environmental causes, in couples, demonstrates the need for further investigations into infertility biomarkers. Semen parameters are often insufficient for reliable profiling of male infertility. Thus, this study aims to evaluate for the first time seminal plasma N-glycosylation as a biomarker of environmental exposure in semen samples from 82 normozoospermic men and 84 men with abnormal semen parameters and compare it with genome damage measured by DNA fragmentation. We obtained information about chronic exposure to environmental factors from the self-reported questionnaire, and determined sperm DNA fragmentation by sperm chromatin dispersion, while N-glycans were characterized with liquid chromatography-mass spectrometry (LC-MS). Based on previously published results, ten N-glycans were selected. Results show that the selected seminal plasma N-glycans were significantly associated with smoking, exposure to pesticides, air pollution, agents emitted during photocopying, alcohol consumption, and obesity. Some N-glycans showed a simultaneous association with DNA fragmentation, semen parameters, and environmental stressors. These subgroups of N-glycans are new potential candidates for biomonitoring of exposure to different environmental factors in men with semen abnormalities.

GRAPHICAL ABSTRACT



6.2 INTRODUCTION

Reproduction is one of the most sensitive components of human social and personal life and it has been challenged during the last decades due to the significant rise of reproductive disorders in both sexes.(1) Male infertility, a condition characterized by failure to achieve pregnancy with a healthy female, may have a hormonal or genetic background, but can also be associated with lifestyle habits such as smoking, alcohol consumption, or excess caloric intake leading to overweight and obesity.(2-4) Moreover, exposure to environmental and occupational factors including pesticides, bisphenols, perfluoroalkyl chemicals, phthalates, heavy metals, etc can also negatively affect fertility.(5-8) Many of these factors act as endocrine disruptors (EDs), compounds with the ability to interfere with hormones, which may lead to disrupted hormonal balance and fertility.(9)

Since male infertility is a very complex condition with multiple molecular pathways involved, its diagnostics is highly demanding and novel biomarkers are constantly investigated. Currently, the golden standard in male infertility diagnostics is an assessment of basic semen parameters including sperm count, morphology, motility, and semen volume.(10) Besides semen analysis, additional sperm quality assay may improve infertility diagnostics.(11) Due to the highly condensed packaging of sperm DNA,(12) the assessment of sperm chromatin integrity through sperm DNA fragmentation (SDF) with a relatively simple sperm chromatin dispersion (SCD) assay, provides useful insight into sperm quality.(6, 13) Previous studies have shown the association of increased sperm DNA fragmentation with exposure to different environmental factors and obesity.(14-17)

N-linked protein glycosylation is a post-translational mechanism that can influence the structure and activity of secretory, transmembrane, and intracellular proteins.(18) Various N-glycosylated proteins are found in sperm and seminal plasma, with the proposed role in spermatogenesis, sperm maturation, capacitation, and sperm-egg interaction.(19) Precisely, seminal plasma N-glycoproteins facilitate sperm transportation in the female reproductive tract and maintain the spermatozoa in a decapacitated state until they reach the egg.(20) Several studies showed that the N-glycosylation pattern of seminal plasma can be altered in men with disrupted fertility.(18, 21-23) Hence, glycosylation patterns have been proposed as potential biomarkers of different semen abnormalities, although the current knowledge is still very limited.(24-26)

Currently, there are no data on the association of N-glycosylation changes in seminal plasma total proteins in men with disrupted semen parameters with DNA damage and environmental exposure. Our previous study(27) has shown the significant association of ten seminal plasma N-glycan peaks (SPGPs) with sperm quality parameters which are further investigated herein. Because there is a constant demand for the development of novel biomonitoring and diagnostic tools for male infertility, the current study aims to investigate for the first time (a) the association between the specific seminal plasma N-glycans, DNA damage, and the exposure to environmental factors in men with

normozoospermic (N) and abnormal (Ab) semen parameters, and (b) potential of seminal plasma N-glycans as a biomarker of environmental exposure in infertile men.

6.3 EXPERIMENTAL SECTION

6.3.1 Subjects

All procedures were conducted according to the regulations of the Declaration of Helsinki. The study was approved by the Ethics Committee of the University Hospital Zagreb, Croatia under reference number 021–1/41–18. The study cohort and the sampling of study subjects were described in our previous study.(27) Briefly explained, the study participants were recruited at the University Hospital Zagreb, Croatia. Informed consent for the use of patients' clinical data for scientific purposes was signed voluntarily by all of the study participants. After 2–7 days of abstinence, samples were obtained by masturbation in a sterile container, liquefied for 30–60 min, and analyzed by computer-aided sperm analysis (CASA). According to World Health Organization (WHO) recommendations and criteria for semen parameters, the semen analysis was conducted.(11) Exclusion criteria were the absence of sperm in the neat ejaculate, cryptozoospermia, leukocytospermia, and previous chemo- or radiation therapy. Sperm parameters including volume, pH, concentration, motility and morphology were determined and study participants were grouped as normozoospermic (N = 82), oligozoospermic (N = 21), asthenozoospermic (N = 29) and oligoasthenozoospermic (N = 34).

6.3.2 Assessment of exposure to environmental factors

All of the participants were offered a self-reported questionnaire for the assessment of lifestyle habits and the frequent environmental and occupational factors they are exposed to. The questionnaire was used in previously published studies and adapted for the infertility population.(28-30) The questionnaire included questions that assessed chronic exposures to various environmental factors containing endocrine-disrupting agents, such as pesticides, solvents, dyes, and plastics in the last year (yes/no). Information about the lifestyle habits of participants including smoking (yes/no), alcohol consumption (yes/no), red meat (n ≥ 3 per week/less than 3/no), poultry (n ≥ 3 per week/less than 3/no), vegetable (everyday/less/no), fruit (everyday/less/no), fish (n ≥ 1 per week /no), and dairy consumption (yes/no) were also obtained. Moreover, the participants self-reported whether they live near the industry (distance ≤ 500 m) or highway (distance ≤ 500 m). Obesity was characterized as BMI ≥ 30. The questionnaire served as an initial robust screening of the most common environmental factors and lifestyle habits that participants were exposed to in the last year.

6.3.3 DNA fragmentation test

According to the manufacturer's instructions, the percentage of sperm with fragmented DNA was determined by sperm chromatin dispersion (SCD) assay (GoldCyto Sperm® Kit, Goldcyto Biotech corp., China). Briefly, samples were diluted with a concentration of 5 million per ml with PBS, mixed with agarose, and 30 µl of the mixture was added to the slide and cooled for 10 min at 4 °C. Next, slides were incubated with the acidic denaturation solution for 7 min, for 25 min with lysis solution, washed in dH_2O for 5 min, and fixed in 70%, 90%, and 100% ethanol for 2 min each at RT. Slides were then air-dried, and incubated with commercial staining solution A for 1 min, then solution B was added and altogether was incubated for 10 min. Then, slides were washed with tap water, air-dried, and analyzed with the CASA system in the *SCA evolution* software's *DNA fragmentation* module.

6.3.4 Isolation of N-glycans from total seminal plasma proteins

Semen samples were aliquoted in 1.5 ml tubes and centrifuged for 10 min at RT 650 rpm. Seminal plasma was separated from the pellet, centrifuged once more at 3550 rpm, and again separated from the pellet. All samples were stored at − 80 °C until the subsequent N-glycan analysis. Throughout the entire analysis, in-house seminal plasma standards were used as a control and were prepared by pooling 5 µl of each sample. Samples were then randomized on a 96-well plate, using 5 µl of each sample. For isolation of N-glycans from seminal plasma total proteins, 200 µl of 50 mg/ml Chromabond C-18 beads (Marcherey-Nagel, Germany) suspension in acetonitrile (ACN, Carlo Erba, Italy)/0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, USA) (80:20) was added to each well of an Orochem plate (Orochem, USA). Using a vacuum manifold (Pall Corporation, USA), the beads were washed three times with 200 µl ACN/0.1% TFA (80:20), and three times with 200 µl ACN/0.1% TFA (5:95). Samples were diluted with 45 µl of ACN/0.1% TFA (5:95), transferred to the preconditioned C-18 beads, and resuspended with 150 µl of ACN/0.1% TFA (5:95), and incubated for 2 min. Samples were centrifuged (centrifuge 5804 with a rotor A-2-DWP, Eppendorf, Germany) for 5 min, at increasing speeds from 300 to 800 rpm for the removal of free seminal plasma glycans. Next, 200 µl of 80% ACN was added to each well of the Orochem plate, samples were incubated for 2 min and the centrifugation step was repeated. Glycoproteins were then eluted from the C-18 beads, collected in a 96-well plate (Thermo Fisher Scientific, USA), and incubated overnight at 37 °C. Glycoproteins were denatured with 30 µl of 1.33% SDS (Carl Roth, Germany) and incubated, for 10 min at 65 °C. Then 10 µl of 4% non-denaturing detergent Igepal CA-630 (Sigma-Aldrich) was added to each well. After incubation for 30 min, 1.25 mU PNGase F (Promega, USA) in 10 µl of 5 x PBS was added to each well and incubated for 18 h at 37 °C.

6.3.5 Fluorescent labeling of N-glycans from seminal plasma total proteins

Fluorescent labeling of N-glycans released from seminal plasma total proteins was performed using procainamide. Briefly, 4.32 mg of procainamide (Sigma-Aldrich) was dissolved in glacial acetic acid (Honeywell, USA)/dimethylsulfoxide (Sigma-Aldrich) (30:70),

and 25 µl of the mixture was added to each sample. The plate was sealed and incubated for 1 h at 65 °C. The solution containing the reducing agent was prepared by dissolving 4.48 mg of 2-picoline borane (J&K Scientific, China) in 25 µl of glacial acetic acid /dimethylsulfoxide (30:70) per sample. The reducing agent was then added to each well and incubated for 1.5 h at 65 °C. The samples were then cooled for 30 min at RT and the labeled N-glycans were cleaned up as previously described.(31) The only modification in the protocol was the usage of a 0.2 µm Supor AcroPrep filter plate (Pall Corporation) as a stationary phase.

6.3.6 Detection and measurement of N-Glycans

Separation of labeled N-glycans was conducted with hydrophilic interaction liquid chromatography (HILIC) on a Waters Acquity ultra-high-performance liquid chromatography (UHPLC) H-class UPLC instrument (Milford, MA, USA). The procedure was previously described.(27)

6.3.7 Structural characterization of N-Glycans

The compositional characterization was conducted for glycans eluting in peaks SPGP2, SPGP4, SPGP6, SPGP14, SPGP17, SPGP18, SPGP26, SPGP32, and SPGP35 as described previously in a sister study(27) from the seminal plasma standard representing the average of a cohort (prepared as described above). Briefly explained, N-glycan compositions were identified manually by averaging the MS scans over the chromatographic retention time in which the glycan peaks of interest were eluted. The detected glycan ions inferred from their *m/z* values were characterized with the aid of the glycobioinformatics tools GlycoMod (https://web.expasy.org/glycomod/). Determinations of N-glycan compositions were done based on their accurate mass. The putative glycan structures were assigned from compositional information and the recently published reference library of seminal plasma N-glycans.(32) Mass accuracy was set within 10 ppm for assigning the glycan compositions. Putative glycan structures were visualized using the GlycoWorkbench 2.1 software.(33)

6.3.8 Statistical analysis

Statistical analysis was performed using STATA software (StataCorp. 2021. *Stata Statistical Software*: Release 17. College Station, TX: StataCorp LLC.). As for the descriptive analysis, the non-parametric Mann-Whitney test was employed.

Multivariate analysis was performed using the log-normal regression model. This statistical model allows for the estimation of the percentage change in the mean frequency of the studied variable in each group, compared to the reference group. To select the environmental factors related to the outcomes of interest a stepwise backward procedure was employed. To verify if the assumptions of the log-normal model were violated, the residual-versus-fitted plot and the test for heteroskedasticity were performed. Bonferroni's correction for multiple comparisons was applied.

6.4 RESULTS

The study included men whose semen parameters were within WHO's recommended normal range, normozoospermic (N) and abnormal (Ab) range. Abnormal sub-groups were defined according to the WHO limits for basic sperm parameters. The univariate statistical comparison between these two groups regarding age, BMI, semen parameters, and sperm DNA fragmentation is presented in **Table 1**. A comparative statistical analysis between N and Ab sub-groups – asthenozoospermic (A), oligozoospermic (O), and oligoasthenozoospermic (OA) was presented in our previous paper.(27) Subjects' age and semen volume did not significantly differ between the N and Ab or between the sub-groups.

To explore whether the N-glycan features identified in our previous study(27) showed an association between self-reported exposure to environmental factors and acted differentially between N and Ab subjects, a log-normal regression model stratified by diagnosis was applied. In N subjects SPGP5, SPGP17, and SPGP26 significantly decreased as sperm genome damage increased, and in infertile men only SPGP18 significantly decreased as sperm genome damage increased (**Table 2a and 2b**). Exposure to a mixture of chemicals for photocopying, industrial dyes, motors oils, insecticides, fish consumption, neoplastic disease in the family, residence near factories and highways, and smoking were significantly associated with SPGPs in the N group. In normozoospermic men, when the DNA fragmentation increased by the mean ratio (MR) of 10 units, SPGP5 decreased by 16% and was negatively associated with smoking and positively with industrial dyes. Similarly, SPGP17 and SPGP26 were negatively correlated with DNA fragmentation and positively with residence near industry, photocopying, while SPGP26 was also positively correlated with motor oils, and smoking. In subjects with abnormal semen parameters, only SPGP18 was negatively associated with DNA fragmentation and positively with smoking (**Table 2b**).

To compare the SPGP between normozoospermic and the three abnormal sub-groups, we refitted the log-normal regression model by adjusting for selected environmental factors (**Table 3**). It was observed that SPGP2 and SPGP4 were negatively associated with the OA group and smoking, while positively associated with pesticides. SPGP5 was associated with the O group using latex dyes and photocopying and was negatively associated with smoking. SPGP6 was negatively associated with the OA group. SPGP14 was negatively associated with the OA and O groups and with inguinal hernia. SPGP18 was positively associated with the OA group. SPGP26 was associated with the A group and with photocopying and smoking. SPGP32 was negatively associated with the OA group and pesticides and positively with fish consumption.

Table 1. *Descriptive analysis of men diagnosed with normozoospermic (N) and abnormal (Ab) semen parameters. P-value was achieved using the Mann-Whitney test.*

	N (*N*=82)		Ab (*N*=84)		P-value
	Mean	SD	Mean	SD	
Age	34.5	6.4	36.0	6.8	0.178
Sperm concentration (10^6/ml)	59.1	29.0	33.4	92.9	< 0.001
Total sperm count (10^6/sample)	188.3	116.1	72.4	115.4	< 0.001
Volume (ml)	3.3	1.3	3.2	1.8	0.269
Rapid progressive (%)	15.7	7.2	8.6	7.7	< 0.001
Slow progressive (%)	35.7	11.1	17.1	11.0	< 0.001
Non-progressive (%)	16.1	4.9	13.1	7.4	0.004
Motile (%)	67.4	10.8	37.9	16.8	< 0.001
Immotile (%)	32.7	11.0	62.1	16.8	< 0.001
Abnormal morphology (%)	90.2	4.0	93.4	4.2	< 0.001
Dead (%)	25.6	8.7	47.6	16.3	< 0.001
Non-fragmented sperm (%)	82.2	9.2	69.3	17.5	< 0.001
Fragmented sperm (%)	17.8	9.2	30.7	17.5	< 0.001
Big halo sperm (%)	58.1	18.7	42.1	20.3	< 0.001
Medium halo sperm (%)	24.1	15.4	27.4	12.3	0.010
Small halo sperm (%)	3.6	2.1	5.4	5.3	0.019
Sperm without halo (%)	13.6	8.4	24.7	14.2	< 0.001
Degraded sperm (%)	0.6	0.8	0.6	0.8	0.421
BMI	26.7	4.1	27.1	4.5	0.754

Table 3a. *Association of SPGP, DNA fragmentation and exposure to environmental factors in normozoospermic subjects: log-normal regression model adjusted for the selected confounders.*

SPGP	DNA fragmentation		Significantly associated factors: (+ / -) positive / negative association
	MR[a]	p-value	
2	1.08	0.082	Residence near industry (+), Photocopying (+), Smoking (-)
4	1.00	0.907	Smoking (-), Photocopying (+)
5	0.84	**0.006**	Smoking (-), Industrial Dyes (+), Photocopying (+)
6	1.11	0.071	Motor oils (-),
14	1.03	0.262	Residence near highway (+),
17	0.89	**0.025**	Residence near industry (+), Photocopying (+)
18	0.99	0.861	none
26	0.89	**0.038**	Motor oils (+), Smoking (+), Photocopying (+)
32	1.03	0.515	Smoking (+), Insecticides (-), Fish consumption (+)
35	1.12	0.108	Pesticides (-), Industrial Dyes (-), Smoking (+), neoplastic diseases in the family (+)

[a]: MR when the variable increased by 10 units

Table 2b. *Association of SPGP, DNA fragmentation, and exposure to environmental factors in subjects with abnormal semen parameters: log-normal regression model adjusted for the selected confounders.*

SPGP	DNA fragmentation		Significantly associated factors: (+ / -) positive / negative association
	MR[a]	p-value	
2	1.02	0.319	Lubricating oil (+)
4	1.05	0.068	Residence Near Factories (+),
5	0.99	0.655	Disinfection (-)
6	1.02	0.412	Alcohol consumption (+),Obesity (-), Drug consumption (-)
14	1.02	0.240	Obesity(-)
17	0.98	0.323	Lacquers (+)
18	0.91	**0.001**	Smoking (+)
26	0.97	0.316	Glues (-), Lacquers (+)
32	0.99	0.867	Smoking (+)
35	1.04	0.259	Residence near highway (+)

[a]: MR when the variable increased by 10 units

Table 3. *Mean Ratio of SPGPs in subgroups of subjects with abnormal semen parameters. A – asthenozoospermia, O – oligozoospermia, OA – oligoasthenozoospermia.*

SPGP	A		O		OA		Significantly associated factors: (+ / -) positive/negative association
	MR*	p-value	MR*	p-value	MR*	p-value	
2	0.85	0.060	1.01	0.909	0.78	**0.006**	Pesticides (+), smoking (-)
4	0.96	0.625	1.03	0.696	0.78	**0.003**	Pesticides (+), smoking(-)
5	1.11	0.415	1.41	**0.011**	1.11	0.412	Latex dyes (+), photocopying (+), smoking (-)
6	0.92	0.464	0.83	0.108	0.70	**0.002**	
14	0.97	0.683	0.85	**0.021**	0.76	**< 0.001**	
17	1.19	0.066	1.15	0.169	0.87	0.176	Industrial dyes (+), neoplastic diseases in the family(-)
18	0.93	0.444	1.03	0.754	1.27	**0.018**	
26	1.42	**0.001**	1.23	0.076	1.02	0.882	Photocopying (+), smoking (+)
32	1.02	0.873	0.95	0.642	0.80	**0.036**	Pesticides (-), fish (+)
35	0.86	0.266	1.13	0.376	0.79	0.083	Industrial dyes (-)

According to the investigation for correlations between semen characteristics and SPGPs, the most frequently associated N-glycans with sperm quality parameters were those with significant associations with exposure to environmental factors (**Table 4**).

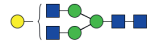
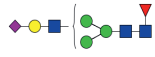
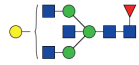
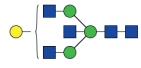
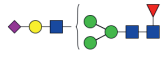
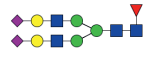
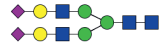
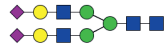
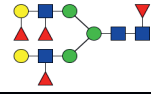
Table 4. *Association between sperm quality parameters and selected SPGPs. N – normozoospermic and Ab - abnormal semen parameters.*

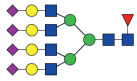
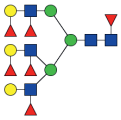
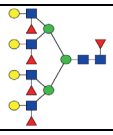
Empty Cell	Associated N-glycan peaks Total	Associated N-glycan peaks (N)	Associated N-glycan peaks (Ab)
M/ml (≥ 15 M/ml)	none	6 (+)	none
M/sample (≥ 39 M/sample)	6 (+)	none	6 (+)
Volume (ml)	2 (-), 4 (-), 14 (-), 17 (-), 18 (+)	5 (-),	4 (-), 14 (-), 17 (-), 18 (+)
Rapid progressive (≥32%)	none	none	5 (+), 32 (-)
Slow progressive (%)	2 (+), 5 (+)	none	2 (+), 4 (+), 5 (+)
Non-progressive (%)	32 (+)	none	14 (+), 18 (-), 32 (+)
Immotile (%)	35 (-)	none	2 (-), 4 (-), 5 (-), 35 (-)
Abnormal (%)	none	none	none
Dead (%)	none	none	5 (-)

Associations with a nominal p-value < 0.05; positive or negative (+/-).

Moreover, we structurally characterized selected N-glycan peaks that showed significant associations with environmental factors by liquid chromatography-mass spectrometry (LC-MS). Proposed structures are described and presented in **Table 5**.

Table 5. *Compositions and proposed structures of N-glycans identified in the glycan peaks SPGP2, SPGP4, SPGP5, SPGP6, SPGP14, SPGP17, SPGP18, SPGP26, SPGP32, and SPGP35 of HILIC-UHPLC profile of seminal plasma N-glycome. Blue square: N-acetylglucosamine, red triangle: fucose, green circle: mannose, yellow circle: galactose, pink diamond: N-acetylneuraminic acid (sialic acid).*

Glycan peak	Observed *m/z*	z	Glycan composition	Proposed structure	Description
SPGP2	1641.70	1	H4N3F1		Complex type glycan, monoantennary monogalactosylated glycan with core fucose or Hybrid type glycan, monoantennary agalactosylated glycan with core fucose and one terminal mannose
	1698.71	1	H4N4		Complex type glycan, diantennary monogalactosylated glycan without fucose
SPGP4	922.90 1844.77	2 1	H4N4F1		Complex type glycan, diantennary monogalactosylated glycan with core fucose
SPGP5	966.89 1932.78	2 1	H4N3F1S1		Complex type glycan, monoantennary monogalactosylated monosialylated glycan with core fucose
	1024.42	2	H4N5F1		Complex type glycan, diantennary monogalactosylated with bisected N-acetylglucosamine and core fucose
	951.41	2	H4N5		Complex type glycan, diantennary monogalactosylated with bisected N-acetylglucosamine
SPGP6	966.89 1932.78	2 1	H4N3F1S1		Complex type glycan, monoantennary monogalactosylated monosialylated glycan with core fucose
SPGP14	1295.01	2	H5N4F1S2		Complex type glycan, diantennary digalactosylated disialylated glycan with core fucose
	1221.98	2	H5N4S2		Complex type glycan, diantennary digalactosylated disialylated glycan without fucose
SPGP17	1221.98	2	H5N4S2		Complex type glycan, diantennary digalactosylated disialylated glycan without fucose
SPGP18	1223.02	2	H5N4F4		Complex type glycan, diantennary digalactosylated glycan with core and antennary fucose

Glycan peak	Observed *m/z*	z	Glycan composition	Proposed structure	Description
SPGP26	1033.75	3	**H6N5S3**		Complex type glycan, triantennary trigalactosylated trisialylated glycan without core fucose
SPGP32	1301.16	3	**H7N6F1S4**		Complex type glycan, tetraantennary tetragalactosylated tetrasialylated glycan with core fucose
	1551.62	2	**H6N5F6**		Complex type glycan, triaantennary trigalactosylated glycan with core and antennary fucose
SPGP35	1107.78	3	**H7N6F5**		Complex type glycan, tetraantennary tetragalactosylated glycan with core and antennary fucose

6.5 DISCUSSION

Protein N-glycans are a highly dynamic and diverse system, and their pattern in various pathological conditions, including disorders of the male reproductive system, has strong biomarker potential.(34) Since seminal plasma is a rich source of N-linked glycoproteins, in this study we found statistically significant correlations of specific N-glycan peaks with semen parameters, sperm DNA fragmentation (SDF), and environmental exposure in men with normozoospermic and abnormal semen parameters.

Structural characterization of SPGP18 showed the presence of biantennary digalactosylated glycans with core fucose and antenna fucose, which are linked to both GlcNAc forming Lewis^{x} (Le^{x}), or both GlcNAc and Gal forming Lewis^{y} (Le^{y}) structures. These results are in contrast with the observation that seminal plasma of O, A, and OA patients is enriched with Le^{x} and Le^{y} epitopes compared to normozoospermic men,(25) and lower semen quality is associated with increased SDF.(27) It is suggested that these epitopes contribute to the low immunogenicity of seminal plasma and induce tolerance of the female adaptive immune system, hence their quantitative changes could considerably impact male fertility.(32)

In subjects with normal semen parameters, we found a significant association between highly sialylated N-glycans SPGP5 ($p = 0.006$), SPGP17 ($p = 0.025$), and SPGP26 ($p = 0.038$), increased SDF, exposure to photocopying, and smoking. This may be because working next to a photocopy machine on an everyday basis represents a mixture of several possible substances such as ozone, electro-magnetic fields, volatile organic compounds, heavy metals, particulate matter, and increased temperature, all of which may have hazardous effects on male infertility.(35) Similarly, increased genome damage in newborns was

reported to be associated with sialylated glycans in blood plasma.(28) Association of smoking with significant changes in N-glycosylation is expected since smoking was already shown as a risk factor for disrupted sperm quality, including genome damage, motility, and morphology.(36) Smoking has a dual effect as a source of genotoxic compounds and EDs, such as cotinine, an aromatase inhibitor causing a decrease in estradiol.(37) Smoking was previously described to change the pattern of N-glycosylation in blood serum proteins in lung cancer patients.(38) A positive association of SPGP5 with industrial dyes, SPGP17, and residence near industry, as well as SPGP26 and usage of motor oils, together with increased SDF indicate the potential of N-glycans as biomarkers of exposure in men with disrupted semen parameters. An association of N-glycans with residence near industry (SPGP2, SPGP17) or highways (SPGP14), an effect potentially caused by air pollution, a factor shown to negatively impact fertility is detected in the current study.(39) Similarly, the changes in N-glycosylation of immunoglobulin G were previously associated with exposure to a traffic air pollutant.(40-42)

Considering the subjects with abnormal semen parameters, a significant negative association of SPGP18 with increased SDF ($p = 0.001$) and an association with smoking is detected. These results confirm smoking as a risk factor in men with disrupted sperm quality, however, the effect was observed for different N-glycans than those in normozoospermic men.

Other exposures to environmental stressors were also associated with specific N-glycans in men with abnormal semen parameters, although not with SDF. An interesting observation was an association of obesity with SPGP6 and SPGP14 in men with abnormal, but not in men with normal semen parameters, and in abnormal sub-groups, indicating the importance of participants sub-grouping for detection of the environmental effect. Moreover, lubricating oil, rich in petroleum (crude oil),(43) was associated with SPGP2, the N-glycan was also negatively associated with semen volume and immotile sperm, while positively with slow progressive motility in total and Ab subjects. Studies on animal models also showed that petroleum can negatively affect sperm motility, concentration, and velocity.(44, 45)

Classification of the subjects with abnormal semen parameters into A, O, and OA sub-groups yielded different environmental factors as significant. A significant association between exposure to photocopying, smoking, and the glycan peak SPGP26 in the A sub-group, the same effect that was observed in the normozoospermic group was observed. In the O group, the SPGP5 showed a significant positive association with photocopying and latex dyes and a negative with smoking, again similarly to the normozoospermic group, shown to be associated with sperm chromatin maturity,(27) which might be sperm characteristics affected by these environmental stressors. In the OA group, pesticides, known as risk factors for sperm quality,(46, 47) showed a significant association with SPGP2 and SPGP4, while a negative association with SPGP32. These glycans were also associated with decreased

motility and semen volume, parameters previously associated with pesticide exposure.(48, 49)

Seminal plasma total protein N-glycans might be potentially used as biomarkers for sperm quality affected by environmental exposure. In favor of the introduction of N-glycans is that clusterin (CLU), one of the main glycoproteins in seminal plasma and a biomarker of oxidative stress has already been investigated as a potential male infertility biomarker.(50) The large-scale proteomic analysis revealed clusterin has the same N-glycan composition as detected in SPGP14, SPGP17, and SPGP26 from our study, indicating the potential origin of these N-glycans. Moreover, seminal plasma glycoproteins fibronectin, prostatic acid phosphatase, metalloproteinase inhibitor, and semenogelin-2 were shown to have the highest number of glycoforms. SPGP2 and SPGP4 were found on fibronectin, and in the metalloproteinase inhibitors together with SPGP14 and SPGP17. Furthermore, abnormal semen parameters were associated with decreased fibronectin sialylation, lower expression of the α-2,3-sialylated fibronectin, and the existence of the asialo-fibronectin glycoforms, indicating the importance of glycosylation pattern in a specific protein, which could be reflected also in our study.(21)

Seminal plasma proteins are secretory, hence they are rich in post-translational modifications such as N-glycosylation. Previous studies have demonstrated that N-glycans and N-glycosidic bonds may differentiate between men with abnormal and normal semen parameters.(23, 26, 51) For example, lowered sialylation was demonstrated in O, A, and OA men. Moreover, the absence of glycans with sialic acid and an increase in fucosylation was detected in A men.(51) Next, sperm DNA damage can occur post-testicularly potentially from oxidative stress. That can affect not only sperm DNA integrity but also the pattern of seminal plasma protein N-glycans.(52) Since N-glycan composition can be highly dynamic also due to the environmental effects on the organism, the pattern of N-glycans from seminal plasma total proteins can change. The environmental toxicants can also induce oxidative stress, which can altogether lead to an altered N-glycan profile and sperm DNA damage.(53, 54)

Our study has certain limitations that should be addressed. Exposure to environmental factors was assessed with a questionnaire, which is a robust screening method. The more accurate recent data on exposure to different environmental stressors would be obtained by the direct measurements of xenobiotics in the participants' urine or blood samples. This also presents a potential future study direction, where precisely measured individual exposures to environmental factors in patients' samples and their correlation with seminal plasma N-glycans can be further studied. Based on the results, we suggest that the altered glycosylation in seminal plasma may reflect environmental exposures associated with sperm pathology. Further research is required on large cohorts which will enable additional profiling of N-glycans predictability of association between infertility in men and

environmental exposure which in some cases may be corrected and reflected in the improvement of sperm quality.

6.6 CONCLUSION

Seminal plasma N-glycan profile disturbances and sperm genome damage were shown to be associated with reduced semen quality and exposure to environmental factors. A significant association of these glycan features with smoking, exposure to complex mixtures such as working next to a photocopy machine, air pollution, industrial oils, and smoking is presented. The application of a battery of selected seminal plasma glycans may become a reliable predictor of infertility risk. Future investigations could also focus on the potential of N-glycans as biomarkers of assisted reproduction success.

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