Original Article

Proteomic approach towards identification of seminal fluid biomarkers from individuals with severe oligozoospermia, cryptozoospermia and non-obstructive azoospermia: a pilot study

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Background: Infertility becomes a global problem that affects to the same extent females and males. As reasons of male infertility can differ among individuals, the accurate diagnostics is essential for effective treatment. The most problematic both in diagnostics and in treatment are disturbances of spermatogenesis. Seminal fluid is rich in proteins that potentially can serve as markers for male infertility and among them, markers of spermatogenesis which are highly desired.

Methods: To find biomarkers of spermatogenesis, we applied comparative proteomics using nano ultra performance liquid chromatography and tandem mass spectrometry (nanoUPLC-MS/MS) followed by single-sample Western blotting (WB) using seminal fluid samples from males with different types of infertility including non-obstructive azoospermia (NOA), cryptozoospermia (C) and severe oligozoospermia (SO). Then, the extensive survey on the identified proteins and their function in male reproductive system has been done.

Results: The proteomic approach has enabled to identified five seminal fluid proteins being potential markers of spermatogenesis disorders: ADGRG2, RAB3B, LTF, SLC2A3 and spermine synthase (SMS). Among them ADGRG2 seems to be strongly involved in male infertility. In addition, WB indicated that the distribution of LTF, SLC2A3 and SMS was not coherent among the individuals, especially in a group with NOA. Functional annotation analysis and search in proteomics databases revealed that vast majority of the proteins originated from extracellular environment.

Conclusions: The presented data point out several proteins that potentially can become biomarkers of male infertility. The data suggest, however, different mechanisms behind the male infertility indicating that the etiology is more complex. We assume that recognition of these mechanisms may lead to the creation of specific protein panel helpful in the management of male infertility and therefore, further studies are required.

Keywords: Spermatogenesis; proteomics; male infertility; pathological spermiogram; biomarkers

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Introduction

In recent years, male infertility has become a real medical and sociological problem of a modern world. It is mainly due to deteriorating semen parameters like reduced number of spermatozoa, their motility or morphology. Among these, total lack of spermatozoa in the semen is the most difficult one for successful therapy. The reduced number or absence of spermatozoa in an ejaculate can be a result of either anatomical blockage within male reproductive tract (obstruction at the level of vas deference, epididymis or ejaculatory ducts) (1) or spermatogenesis disruption. The later one may occur due to genetic mutations, hormonal imbalance, incomplete testis development or varicocele. These factors may lead to the total lack of spermatozoa in the testes or arrested spermatogenesis (2). Nevertheless, not all the factors of reduced spermatogenesis have been identified yet.

Seminal plasma is a mixture of secretions released during activity of the testis and accessory sex glands, within male reproductive tract. The fluid comprises 80–90% of the ejaculate and its role is to support spermatozoa with an environment enabling final fertilization success (3). As seminal plasma is abundant in proteins, a number of studies aimed to find reliable biomarkers reflecting the conditions of male reproductive tract and sperm quality in different animal species (4-6), including humans (7-9). The studies on seminal biomarkers concerned stallion sperm motility (10), human spermatogenesis (11) and/or azoospermia (12). The proteomic approach was also applied regarding the prediction of in vitro fertilization (IVF) success (13). Among these all, the most attractive option is the possible use of seminal fluid as convenient for handling of biological material containing biomarkers being helpful for diagnosis of spermatogenesis and possible therapy monitoring.

Non-obstructive azoospermia (NOA), cryptozoospermia (C) and severe oligozoospermia (SO) are diseases very often linked to genomic aberrations (14-17). However, such diagnostic attempt usually requires invasive testicular open biopsy which is rather a controversial issue. Thus, seminal plasma proteins are still of high interest for clinicians and infertility researchers. The optimal seminal fluid biomarker should distinguish among different types of male infertility and/or indicate their etiology. Despite of many studies on infertility biomarkers, still the successful protein candidates have not been selected. Taking into account sperm cells, proAKAP4 has become a commercial biomarker of sperm quality predicting successful fertilization (18-20). Unfortunately, yet no seminal fluid protein has become an universal and/or commercial marker for male infertility. One of the prospective seminal plasma biomarkers is Heat shock-related 70 kDa protein 2 (HSPA2) which is a testis-enriched molecule involved in spermatogenesis (11). According to our previous data, HSPA2 is a biomarker of azoospermia and in some cases of C (11). Yao *et al.* (12) indicated seminal fluid sodium-coupled monocarboxylate transporter 2 (SLC5A12) as a protein diversifying NOA and obstructive azoospermia (OA) while Histone H2B type 1-A (H2BC1) as a predictive factor for efficient sperm retrieval from NOA patients. Légaré *et al.* (21) found cysteine-rich secretory protein 1 (CRISP1) as a marker of OA whereas Davalieva *et al.* (8) described fibronectin (FN1), prostatic acid phosphatase (PAP), proteasome subunit alpha type-3 (PSMA3), beta-2-microglobulin (B2M), galectin-3-binding protein (LGALS3BP), prolactin-inducible protein (PIP) and cytosolic nonspecific dipeptidase (CNDP2) as protein biomarkers of azoospermia.

Different approaches can be used for identification of protein markers, ranging from biochemical assays (22) to high throughput proteomics (23). The biochemical (or immunological) assays are usually limited to a small number of proteins whereas proteomics ensures the analysis of large number of proteins simultaneously however, it can also generate false positive results (24). In our study, we have used nanoUPLC-MS/MS followed by a robust

Highlight box

Key findings

- The presented study has indicated ADGRG2, SLC2A3 and SMS protein as potential seminal plasma biomarkers of diminished spermatogenesis.

What is known and what is new?

- Seminal plasma proteins may reflect the condition of male reproductive health, including spermatogenesis status.
- Here, we report on the seminal plasma ADGRG2 protein as an attractive biomarker for severe oligozoospermia, cryptozoospermia and azoospermia and on SLC2A3 and SMS proteins as potential biomarkers of non-obstructive azoospermia however, with different molecular background.

What is the implication, and what should change now?

- The reported proteins may be included in potential diagnostic panels dedicated for the assessment of reasons of failing spermatogenesis however, they may not fully explain mechanisms of infertility. The potential diagnostic panel should be enriched in other biomarkers enabling correct diagnosis and application of targeted therapy.

peptide quantification analysis (25,26) and an extensive Western blotting (WB) validation to examine proteins from seminal fluid of patients with NOA, C, and SO. We present this article in accordance with the MDAR reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-23-130/rc).

Methods

Reagents

The reagents were purchased from Sigma-Aldrich ChemieGmbh (Munich, Germany), unless stated otherwise.

Seminal plasma

The samples of seminal plasma were received from healthy, normozoospermic volunteers and from males having problems with fertility. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the National Bioethical Committee, Ministry of Health, Warsaw, Poland; code: OkB-5-2/15 (No. OKB.078.6.2015) and informed consent was obtained from all individual participants. The samples were collected by masturbation following 3–5 days of sexual abstinence. The routine seminal analysis was performed according to World Health Organization (27) guidelines. The samples were classified into four groups under study: the control group (normozoospermic samples), patients with SO, C and NOA. SO and C were also non-obstructive cases. Seminal plasma was prepared for analysis as already described (11).

Mass spectrometry protein identification

Seminal plasma samples from normozoospermic males (n=10) and from patients with SO (n=10), C (n=7) and NOA (n=17) were subjected to comparative proteomic analysis. Protein identification and quantification were performed in Mass Spectrometry Lab at the Institute of Biochemistry and Biophysics (IBB), Polish Academy of Sciences.

Sample preparation

After being reduced with 0.5 M (5 mM f.c.) Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 1 h, 60 ℃) and blocked with 200 mM S-methyl methanethiosulfonate (MMTS, 10 mM f.c.; 10 min, RT), the protein mixtures were trypsinized overnight with 10 µL of 0.1 µg/uL trypsin. Peptide mixtures were separated with RP-18 pre-column (Waters, Milford, MA, USA) using 0.1% formic acid (FA) and then transferred to a nano-HPLC RP-18 column (internal diameter 75 µM, Waters, Milford, MA, USA) using a gradient of solvent B (0–35% can, 160 min) with solvent A being 0.1 % FA at a flow rate of 250 nL/min. The column setup was coupled to the Q Exactive mass spectrometer (Thermo Electron Corp., San Jose, CA, USA). For quantification, the samples were evaluated twice, once in data-dependent acquisition mode and once as a profile of LC-MS spectrum.

Analysis of mass spectrometry data

The recorded fragmentation spectra were analyzed with Mascot Distiller software (v. 2.6, MatrixScience, London, UK) and searched with the Mascot Search Engine (MatrixScience, London, UK, Mascot Server 2.5) against the human proteins deposited in Uniprot database (version 20170927, 71,579 sequences; 24,126,051 residues). The peptide and fragment mass tolerance settings were established as already described (26). The following research parameters were applied: enzyme: Trypsin, missed cleavages: 1; fixed modifications: Methylthio (C); variable modifications: Oxidation (M); instrument: higher-energy collisional dissociation (HCD). The target/decoy database search approach was applied for the peptide assignments (28) followed by the mass calibration and data filtering using MScan software—a developed in-house software (http://proteom.ibb.waw.pl/mscan/) already described elsewhere (29).

Quantification

The quantification was performed in the same way as already described by Banaś *et al.* (30) using an in-house quantification platform of IBB (25) and Diffprot software (26) with the following settings: number of random peptide sets: 10^6; clustering of peptide sets: only when 90% identical; normalization: LOWESS.

Bioinformatic analysis

An enriched functional analysis of proteomes for each tested group of samples was done using David software and the protein products were annotated to the following Gene Onthology (GO) terms: biological process, cellular compartmentalization and molecular function.

Western blot validation

The validation of MS results was performed for

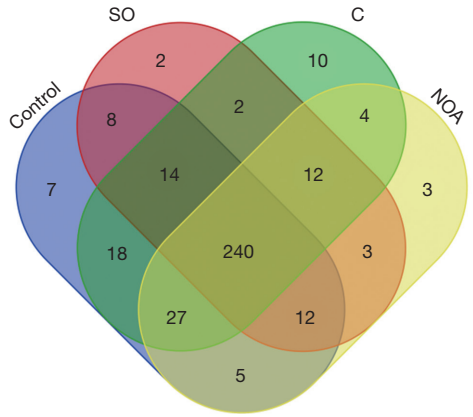

Figure 1 Venn diagram presenting the distribution of identified proteins within the tested groups. Normozoospermia (Control). SO, severe oligozoospermia; C, cryptozoospermia; NOA, non-obstructive azoospermia.

normozoospermic controls (n=10), patients with SO (n=10), C (n=6) and NOA (n=13). The preparation of seminal plasma samples and immunoblotting were performed as in our previous studies (11,31). The following primary antibodies were selected for immunodetection: anti-ADGRG2 (1:600, HPA001478), anti-SEMG2 (Abcam, Cambridge, Great Britain; 1:500, ab108085), anti-SLC2A3 (Abcam; 1:500, ab191071), anti-spermine synthase (SMS) (Abcam; 1:1,000, ab156879), anti-Ras-related protein Rab-3B (RAB3B) (Abcam; 1:1,000, ab177949) and anti-lactotransferrin (LTF) (Millipore; 1:2,000, 07-685). The secondary antibody, horseradish peroxidase-conjugated Goat anti-rabbit IgG H&L (ab97051, Abcam) was added: in 1:40,000 (for anti-LTF, anti-SEMG2) or 1:10,000 (for anti-RAB3B, anti-SLC2A3 and anti-ADGRG2) dilutions. A representative (most often K11) control seminal plasma sample was set as an internal reference in the respective tested groups for Western blot analyses. The images were captured with the ChemiDoc™ MP System (Bio-Rad Laboratories) and were analyzed with Image Lab 6.0.1 software (Bio-Rad Laboratories) applying total protein normalization.

Statistical analysis

Statistical analysis of WB signals was performed with GraphPad Prism 7 software. The calculations were done as grouped Mann-Whitney analyses.

Results

Mass spectrometry

Altogether, the nanoUPLC-MS/MS analysis enabled the identification of 368 different proteins (see https://cdn.amegroups.cn/static/public/tau-23-130-1.xlsx) from seminal plasma. The distribution among the studied groups was as follows: 331 proteins in a group with normozoospermia, 293 in males with SO, 327 in males with C and 306 in males with NOA (*Figure 1*). The quantitative analysis revealed differentially expressed proteins (DEPs) in seminal plasma from infertile patients and there were identified 6, 3 and 2 DEPs among patients with SO, C and NOA, respectively (*Table 1* and *Figure 2*).

Bioinformatic analysis

The investigated GO terms revealed that the seminal plasma proteomes of the all tested groups were similar regarding their involvement in biological processes, cellular compartmentalization and molecular function (Figure S1).

As there was a limited number of identified DEPs among three groups of patients under study, the enriched functional analysis by means of bioinformatics tools was possible only in the group of males with SO (*Table 2*).

Western blot analysis

The results of WB validation differed slightly from quantitative analysis of MS data however, immunoblotting is much more sensitive technique and therefore it provides more precise protein identification. In *Figure 3*, there are presented WB images of proteins of which differences in their signal intensity were of statistical importance; in *Figure 4*, there are presented images of proteins of which differences in their signal intensity were out of significant importance.

Discussion

The aim of the present study was to identify seminal plasma proteins having a potential to serve as biomarkers for spermatogenesis disorders. For this purpose, a quantitative proteomic approach combined with immunological assessment was applied.

For the quantitative analysis of proteomic data, the

Table 1 The list of seminal plasma DEPs among three groups of patients compared with the males with normozoospermia. The statistical analysis was done with Diffprot (24)

Groups	Protein name	Protein ID	Peptide number	q value	Ratio infertile/controls
Severe oligozoospermia	Hypoxia up-regulated protein 1 (HYOU1)	A0A087X054 Q9Y4L1	2	NA	Only in control
	Spermine synthase (SMS)	P52788	2	NA	Only in control
	Solute carrier family 2, facilitated glucose transporter member 3 (SLC2A3)	P11169	2	NA	Only in control
	Semenogelin-1 (SEMG1)	P04279	109	0.00029	1.72
	Semenogelin-2 (SEMG2)	Q02383	151	0.00013	1.47
	Adhesion G-protein coupled receptor G2 (ADGRG2)	Q8IZP9	4	0.04313	0.51
Cryptozoospermia	Ras-related protein Rab-3B (RAB3B)	P20337	2	NA	Only in control
	Plasma alpha-L-fucosidase (FUCA2)	Q9BTY2	2	NA	Only in control
	Lactotransferrin (LTF)	E7EQB2 E7ER44 P02788	58	0.06560	1.32
Non-obstructive azoospermia	Solute carrier family 2, facilitated glucose transporter member 3 (SLC2A3)	P11169	2	NA	Only in control
	Cadherin-1 (CDH1)	A0A087WX17 A0A087WU43 P12830 A0A087WXI5	3	0.06188	2.08

The listed proteins are of q<0.01, DEPs, differentially expressed proteins; NA, not available.

application of statistical Diffprot analysis was applied reducing possible false positive outcomes (26). As a result, in the analysis was selected relatively small number of proteins defined as DEPs among patients under study and these were HYOU, SMS, SLC2A3, SEMG1, SEMG2, and ADGRG2 for patients with SO; RAB3B, FUCA2 and LTF for C patients; and SLC2A3 and CDH1 for NOA patients (*Table 1*). The functional annotation analysis was possible only for patients with SO due to too little amount of proteins identified in the seminal plasma samples from the remaining infertile patients (C and NOA). According to this analysis, most of DEPs in SO patients was of exosomal origin (*Table 2*) what is in line with general knowledge of seminal fluid composition (30,31). The immunological assessment was performed with SMS, SLC2A3, ADGRG2, RAB3B and LTF protein products. After WB identification, only two DEPs (ADGRG2 and RAB3) were of statistical significance (*Figure 3*). The remaining DEPs was out of significance among all the groups of patients analyzed however, there was strong heterogeneity among samples from the NOA patients cohort forcing us to discuss all the DEPs identified.

ADGRG2

Quantitative proteomics identified ADGRG2 as DEP exclusively significant for seminal plasma of SO patients (*Table 1*) but the immunoblotting indicated this protein to be significantly deregulated also in seminal plasma of C and NOA patients (*Figure 3*). The Western blot analysis showed that ADGRG2 was expressed heterogeneously among control samples as well as in SO and NOA individuals and there was no identifiable signal for this protein in C patients. ADGRG2 is an extracellular and transmembrane protein involved in G protein-coupled receptor signaling pathway. The protein is widely expressed in human epididymis [it is also known as human epididymis-specific protein 6 (He6)], however, it was also identified in efferent ductuli (32) and other tissues of human body (33-35). Its localization within a cell is apical and in efferent ductuli and proximal epididymis is limited to non-ciliated cells (32,33). Adgrg2 knockout (KO) mice were shown to exhibit simultaneous downregulation of epididymal transcripts for proteins (36) that were later proven to be involved

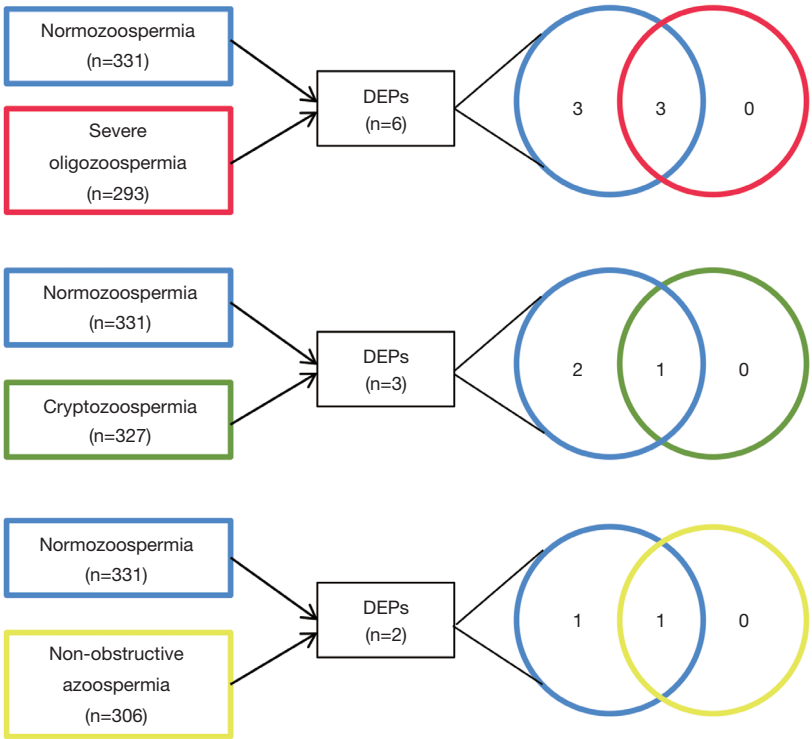

Figure 2 A scheme representing numbers of DEPs in cohorts of infertile males after quantification and statistical analysis using Diffprot (23,24). DEPs, differentially expressed proteins.

Table 2 Functional annotation analysis of DEPs from seminal plasma of patients with SO

Category	Term	Genes	Benjamini correction
GOTERM_CC_DIRECT	Extracellular exosome	*SMS, HYOU1, SLC2A3, SEMG1, SEMG2, ADGRG2*	1.6E–3
GOTERM_BP_DIRECT	Coagulation	*SEMG1, SEMG2*	1.2E–2
GOTERM_BP_DIRECT	Positive regulation of serine-type endopeptidase activity	*SEMG1, SEMG2*	1.2E–2
GOTERM_BP_DIRECT	Negative regulation of sperm motility	*SEMG1, SEMG2*	1.3E–2
GOTERM_BP_DIRECT	Antibacterial humoral response	*SEMG1, SEMG2*	8.8E–2
GOTERM_BP_DIRECT	Protein heterooligomerization	*SEMG1, SEMG2*	1.1E–1
UP_KEYWORDS	Signal	*HYOU1, SEMG1, SEMG2, ADGRG2*	9.8E–1
GOTERM_CC_DIRECT	Extracellular region	*HYOU1, SEMG1, SEMG2*	5.9E–1
UP_KEYWORDS	Glycoprotein	*HYOU1, SLC2A3, SEMG2, ADGRG2*	9.8E–1

DEPs, differentially expressed proteins; SO, severe oligozoospermia.

in processes regulating sperm maturation, e.g., lipid raft movement, $Ca2^{+}$ homeostasis, acquisition of the capability of fertilization (37-39). An advanced set of experiments performed by Zhang *et al.* (40) established the presence of a multimeric complex governing the regulation of ionic and pH homeostasis in efferent ductuli and proximal epididymis. This complex was created by the interaction between ADGRG2, Gq, beta-arrestin 1 and cystic fibrosis

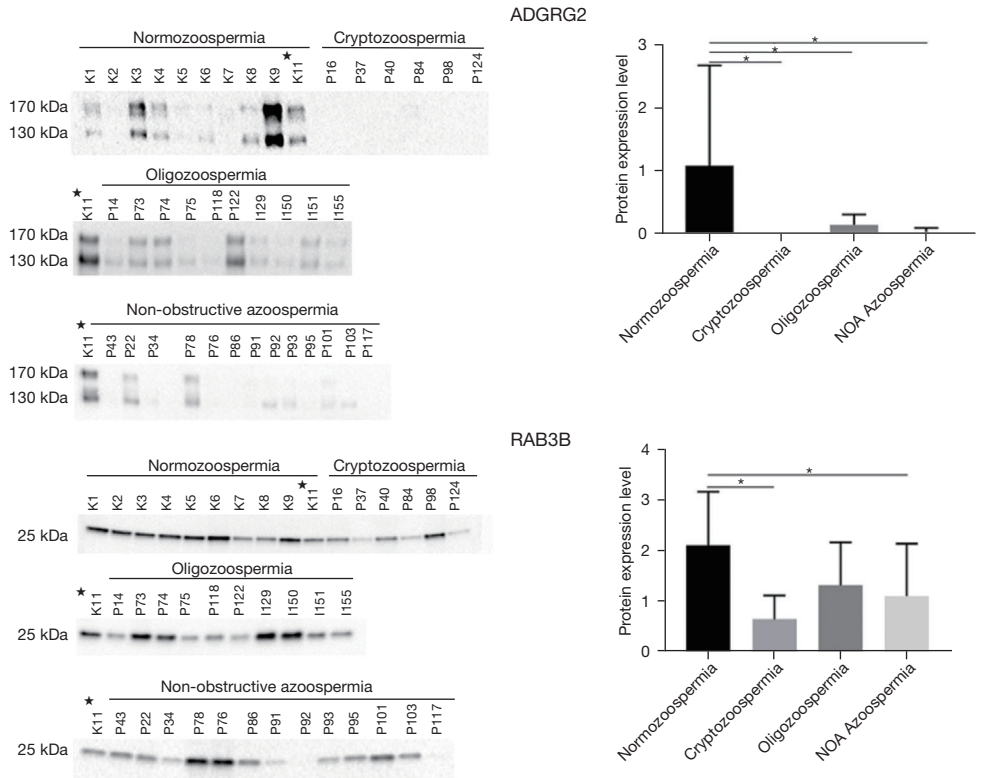

Figure 3 Western blot analysis of seminal plasma samples for ADGRG2 and RAB3B proteins. ADGRG2 is the only protein of which expression was significantly decreased in samples from all groups of patients (C, SO and NOA). For RAB3B, the expression level was significantly decreased in samples of NOA patients compared to controls. All signals were normalized against total protein level. A star refers to the internal reference sample. The error bars indicate standard deviation. * indicates P<0.01. C, cryptozoospermia; SO, severe oligozoospermia; NOA, non-obstructive azoospermia.

transmembrane conductance regulator (CFTR) ion channel among which beta-arrestin 1 was shown to act as a scaffold for ADGRG2/CFTR complex in the apical membranes of non-ciliated cells. According to the study (40), the coupling between proteins Gq and ADGRG2 enables the regulation of Cl^- currents *via* CFTR as the reduction in either ADGRG2, beta-arrestin1 or Gq protein finally resulting in decreased constitutive CFTR currents and imbalance in pH homeostasis. Thus, ADGRG2 is claimed to be involved in fluid resorption in efferent ductuli and initial segment of epididymis (40). Indeed, a targeted degradation of one of the transmembrane domains of ADGRG2 in male mice resulted in disregulation of fluid resorption and consequently, accumulation of fluid in testes and stasis of spermatozoa within efferent ducts (41). In the literature, there were reported different types of *ADGRG2* mutations that are linked to congenital absence of the vas deferens (CAVD) and thus, obstructive azoospermia (42-46). It is estimated that *ADGRG2* mutations comprise 2% of all CAVD cases (47) however, they used to accompany mutations in CFTR gene which is assessed to be the main pathogenic gene in CAVD (42). In our study, we showed that ADGRG2 is present also in human seminal plasma (secretory ADGRG2, sADGRG2) (*Figure 3*) and that all the C patients under study had no ADGRG2 in their seminal plasma samples. It is very probable that C in these patients was a cause of inhibited ADGRG2 expression due to mutation in this gene. It can be assumed that spermatogenesis in the C patients was active but the problems with pH homeostasis and fluid resorption in efferent ductuli and epididymis (place of sperm maturation) resulted in obstructive fluid stasis and drastic reduction in sperm count. To prove this hypothesis there would be required testis and/or epididymis biopsy from all the C patients but, on the other hand, it can be supported by the research by Wang *et al.* (45) in which CAVD patients had active spermatogenesis (successful

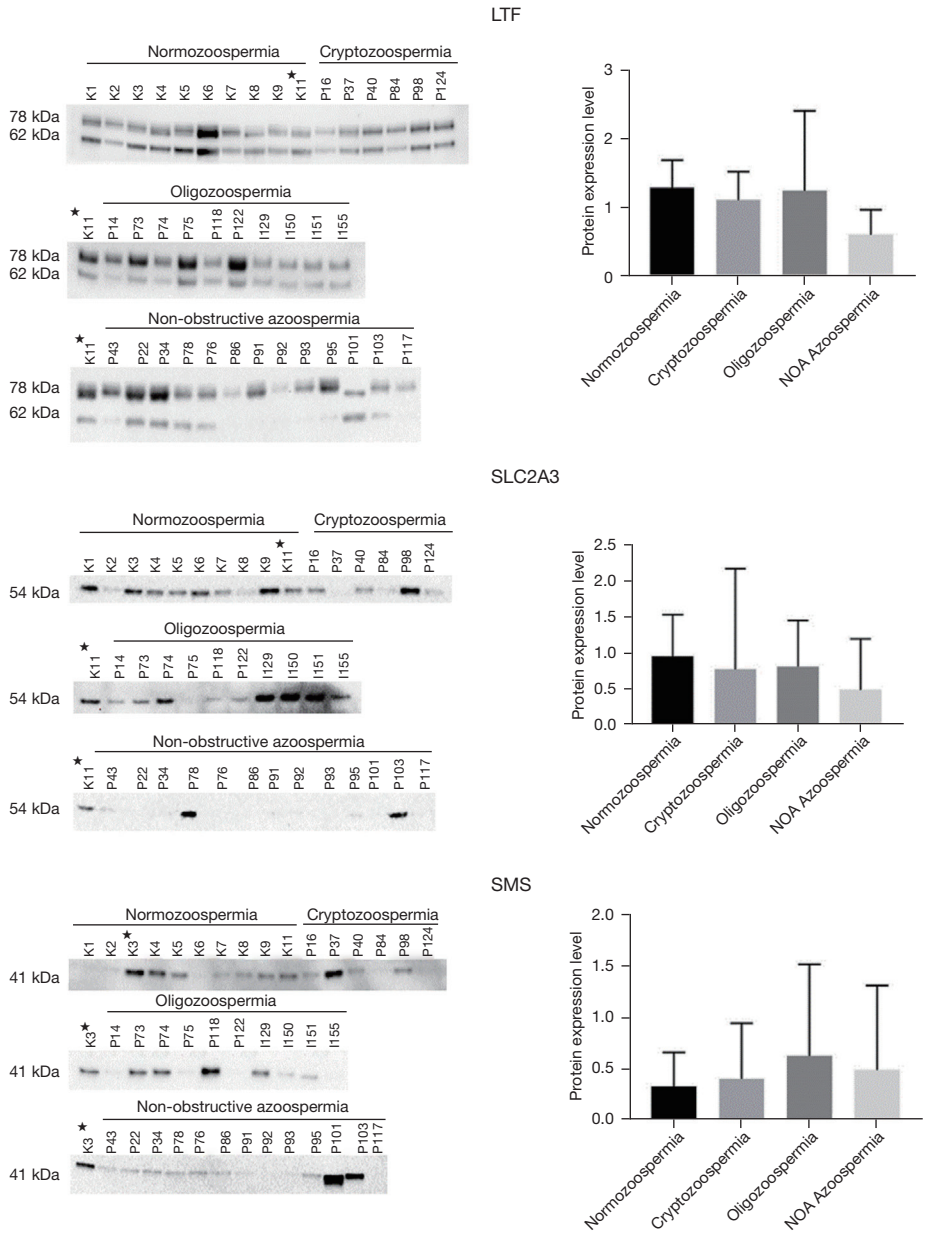

Figure 4 Western blot analysis of seminal plasma samples for LTF, SLC2A3 and SMS proteins. Although the differences in expression levels were out statistical importance, for all the three proteins there was visible strong heterogeneity among signals obtained from individual samples of NOA patients. All signals were normalized against total protein level. The error bars indicate standard deviation. *, refers to the internal reference sample. NOA, non-obstructive azoospermia.

MESA or TESE) but the extracted spermatozoa were of low quality. It is very probable that ADGRG2 may also play a role in NOA but due to its heterogeneous content in seminal plasma such statement would require a study including much bigger cohort.

RAB3B

In our MS analysis, RAB3B was found to be not present in semen plasma of C patients (*Table 1*) however, WB showed this protein to be only downregulated in this group of individuals (*Figure 3*). Reduced levels of RAB3B were also

detected by WB in patients with SO and NOA (*Figure 3*).

Rab proteins are GTP-binding proteins that are involved in exocytosis and localization of other proteins towards plasma membrane (48). Some of them were found to be involved in female and male meiosis (49). They localized close to Golgi apparatus, plasma membrane and cell junctions. At the protein level, the RAB3B isoform is mainly expressed in gastrointestinal tract, pancreas, prostate and placenta but its high mRNA content was also detected in brain tissues (according to Human Protein Atlas). Other RAB3 isoforms (RAB3A, RAB3C and RAB3D) have been reported to be involved in oncogenesis however, there is no knowledge on the oncogenic role of RAB3B (48). Currently, several reports indicated various signaling pathways and mechanisms engaging RAB3B, i.e., in human epithelial cells Rab effector Noc2 protein (NOC2) was documented to be an effector of RAB3B (50) and in human platelets RAB3B was documented to bind calmodulin in $Ca2^{+}$-dependent manner (51). Looking at the results from the present study (*Table 1*, *Figure 3*) it can be concluded that reduced sperm number in ejaculate may be also associated with disturbances in exocytosis (see *Table 2* for SO).

LTF

Label free proteomics indicated LTF to be slightly (1.32 times) upregulated in semen plasma of C patients (*Table 1*) however, WB analysis showed no significant differences for that protein amount in any group of patients under study (*Figure 4*). Nonetheless, there was clearly visible lack of the lower LTF band in the WB image for several NOA individuals (*Figure 4*).

LTF is a glycoprotein expressed in epididymis and prostate gland and secreted to the semen in seminal vesicles (52,53). LTF can attach to the surface of spermatozoa *via* its receptor (54). Generally, LTF displays antioxidant and antibacterial properties due to its ability to bind iron. Indeed, numerous data reported altered LTF levels in semen samples exposed to oxidative stress conditions (31,55,56). In our previous study on asthenozoospermia, there was reported an increased level of LTF in low-motile spermatozoa accompanied by elevated production of ROS however, there was no significant difference in the levels of semen plasma LTF between control and asthenozoospermic samples (31). Nonetheless, a protective role of LTF to spermatozoa was clearly documented in the literature (57,58). The presence of two bands (~65 and ~78 kDa) for semen plasma LTF is in an accordance to our previous study (31). These bands can represent two LTF isoforms being a result either of different levels of protein glycosylation or the presence of an additional truncated form (~65 kDa) of LTF. It can be assumed that in NOA patients there is a tendency of the lower mass LTF isoform to disappear.

Solute carrier family 2, facilitated glucose transporter member 3 (SLC2A3)

In our study, the MS analysis has indicated the SLC2A3 not to be present in seminal fluid samples from SO an NOA patients (*Table 1*) but the WB showed this protein to be strongly reduced only in the NOA cohort (*Figure 4*). The grouped statistical analysis showed this depletion was out of significance however, the WB image clearly shows the presence of SLC2A3 only in 2 out of 13 samples from NOA patients (*Figure 4*).

According to the Human Protein Atlas, SLC2A3 is mainly expressed in cerebral cortex, lungs, testis, epididymis, placenta, bone marrow and lymphoid tissues. It is also known as Glucose transporter type 3 (GLUT3) and is an integral component of plasma membrane. In a study by Soudmand *et al.* (57) using a mouse model, animals subjected to a high-intense exercise training protocol had a diminished level of Slc2a3 in their Sertoli cells and increased in their germ cells. Based on immunohistochemical staining, the mice displayed impaired spermato- and spermiogenesis. The authors explained this phenomenon by reduced glucose uptake and inhibited export of lactate within Sertoli cells what resulted in disturbed sperm development. As the lactate is the preferred energy substrate for developing germ cells (58), it seems that diminished SLC2A3 levels in Sertoli cells disturb their nurturing of spermatozoa. Additionally, it seems that the expression of SLC2A3 in testes can be regulated at the epigenetic level. Indeed, the upregulated expression of mmu-miR-320-3p (the murine homolog of hsa-miR-320c, one of the most upregulated miRNAs in testicular biopsies from SC-only syndrome (SCOS) (59) inhibited Slc2a3 expression in murine Sertoli cells and contributed to germ cell loss driven by Sertoli cell dysfunction (58). Nonetheless, not only epigenetics is reported to have an impact on SLC2A3 level. It is very well established that sex steroid hormones, including 5α-dihydrotestosteron, have an impact on the glucose uptake and the expression of glucose transporters in Sertoli cells (60,61). It has been also documented that a deficiency in thyroid hormones in

early developmental period also affects this transport due to reduced Slc2A3 expression and finally leads to the apoptosis of male germ cells (62). As it was documented that in human males SLC2A3 level is considerably higher in testes than in epididymis (63), we may conclude that the lack of this protein in 11 (out of 13) seminal plasma NOA samples is connected with dysfunctional testes (probably due to Sertoli cells dysfunction) and may be a reason of azoospermia.

SMS

The quantitative MS approach have indicated SMS to be not present in seminal plasma from SO patients (*Table 1*) however, the validation by WB showed this protein to be present in this cohort but with a very heterologous distribution within the samples (*Figure 4*). Analogically to SLC2A3, despite of lack of significance in a group statistical analysis, WB showed a drastic reduction in the level of this protein in 11 (out of 13) seminal plasma samples from patient with NOA (*Figure 4*).

SMS is expressed in almost all human tissues but both at the mRNA and protein levels the most intense expression takes place in male and female reproductive organs (according to Human Protein Atlas). SMS catalyzes the production of spermine (from spermidine and decarboxylated S-adenosylmethionine) which is a polyamine involved in cellular metabolism in animals, plants, some fungi and bacteria (64). Experimental and clinical data clearly show that SMS is crucial for normal development in humans and other mammals; an experiment with transgenic mice showed that SMS is required for normal growth, viability and fertilization (65) whereas in humans, alterations in SMS gene have been connected with the X-linked recessive condition termed Snyder-Robinson syndrome (SRS) characterized with mild-to-moderate mental retardation, muscle and bone abnormalities, facial dysmorphism and other symptoms (66-68). Although SMS is widely expressed within eukaryotic cells, it is little known about this protein in context of male fertility. Male mice carrying a chromosomal deletion of part of the chromosome X containing most of the SMS gene had diminished numbers of meiotic and postmeiotic cells in their testes (65). Also, at the RNA level, seminal plasma SMS was found to be reduced in patients with asthenozoospermia (69). It can be concluded that local lack of spermine in testicular cells may be a cause of NOA in the patients under study although. According to the Human Protein Atlas, SMS is present in testes, epididymis and prostate but testes are the place where the protein is the most abundantly present.

There are several studies that applied proteomics to assess seminal plasma biomarkers in disrupted spermatogenesis. Some of them adapted separation and quantification of seminal plasma proteins in 2-dimentional electrophoresis (2DE) before being selected for mass-spectrometry identification (8,70) while the others used more advanced mass-spectrometry based protein quantification (71,72). Our proteomic approach was based on relatively big group of samples (7–17 samples in a group, depending on the infertility type and analysis) and a robust spectra quantification method (25,26) selecting possible protein candidates indicative of male infertility. Looking at the protein-protein interaction analysis, it can be noticed that some of the proteins identified within one infertile group do not interact with other proteins suggesting the presence of different mechanisms (*Figure 5*). The selected in our study proteins are novel when compared to other proteomic studies aiming to identify infertility biomarkers (8,72,73). However, several of the identified proteins, namely LTF, SEMG1 and SEMG2 were already identified in seminal plasma from fertile donors in other studies and were referred as fertility-connected (23). The generated in our study list of differentially expressed proteins is rather short however, this outcome stands in a line with the outcomes of other studies aiming to identify specific biomarkers for male infertility (23,71).

Conclusions

In our study, we have tried to analyze and compare seminal plasma proteomes from infertile patients having problems with very affected sperm count suggesting severe problems with spermatogenesis. The application of high-throughput nanoUPLC-MS and the quantification approach eliminating the presence of false positives (25), have resulted in the identification of relatively small number of DEPs in males with SO, C and NOA (*Table 1*). Western immunoblotting performed for single individuals within a studied cohort provided a precise insight into the distribution of DEPs abolishing the limitations of MS analysis. The obtained data showed that the protein content among the samples studied was not unique (*Figures 3,4*). The most interesting candidate for a potential biomarker of spermatogenesis status, ADGRG2, turned out to be more attractive in context of C rather than azoospermia, whereas SLC2A3 and SMS are the proteins definitely requiring more attention regarding NOA studies. The presented data

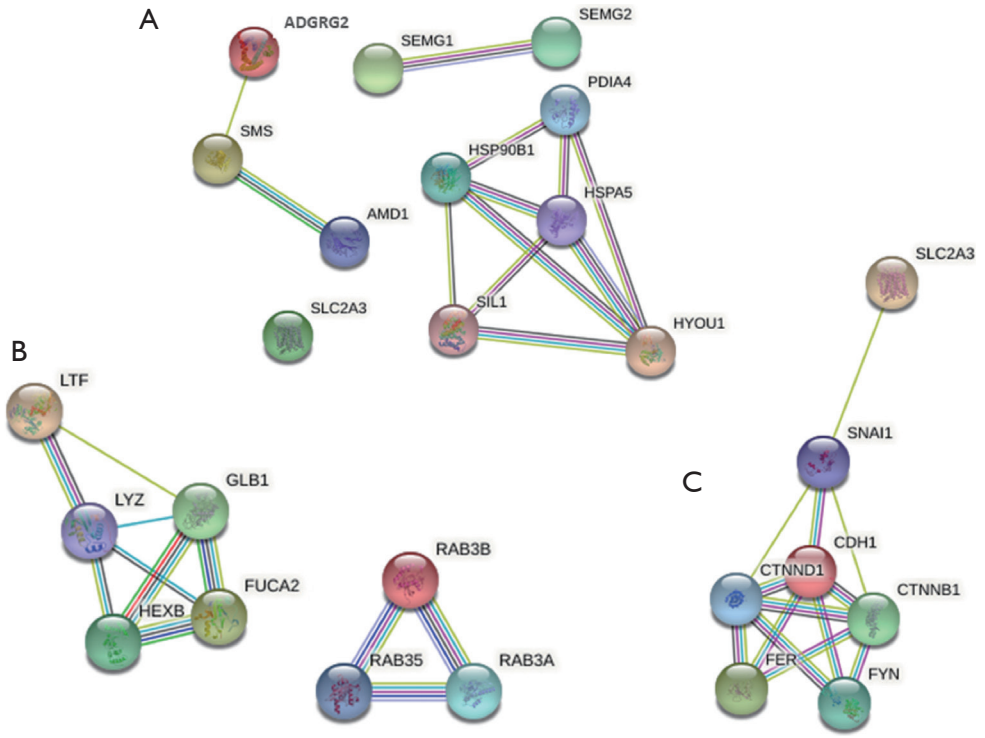

Figure 5 The enriched String analysis of PPI of DEPs from seminal plasma samples obtained from (A) severe oligozoospermia, (B), cryptozoospermia and (C) non obstructive azoospermia patients. PPI, protein-protein interaction; DEPs, differentially expressed proteins.

clearly suggest that different mechanisms can stand behind the same phenotypic entity of male infertility. It can be predicted that the knowledge on these mechanisms would lead in the future to the composition of specific protein panels useful for diagnostic and prognostic purposes in the treatment of male infertility while using non-invasive approach with seminal plasma protein content.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-23-130/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the National Bioethical Committee, Ministry of Health, Warsaw, Poland; code: OkB-5-2/15 (No.: OKB.078.6.2015) and informed consent was obtained from all individual participants.

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