Potential testicular aging biomarkers identified in seminal plasma by proteomic analysis

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Introduction. This research aims to identify biomarkers for the early detection of testicular aging by comparing protein expression levels in seminal plasma from young and older individuals.

Methods. Seminal plasma samples from five young and five older men were analyzed using Data Independent Acquisition (DIA) proteomics to identify proteins with differential expression. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were applied to these proteins. Further validation of selected proteins was conducted in new samples of seminal plasma and serum using enzyme-linked immunosorbent assay (ELISA).

Results. Using the DIA method, 73 proteins with altered expression levels were detected in seminal plasma—46 exhibited increased expression, while 27 showed decreased expression. GO and KEGG analyses linked these proteins to lipid metabolism, inflammation, and cellular aging processes. ELISA tests confirmed that the proteins GNA13, SAA4, and CYCS were prominently expressed in older seminal plasma samples. Serum analysis using ELISA indicated consistent levels of SAA4 and CYCS compared to seminal plasma but revealed a delayed expression of GNA13.

Conclusion. The elevated levels of GNA13 in seminal plasma, which are observed before its detection in serum, correlate with the aging process. This indicates that GNA13 could serve as a promising early indicator for diagnosing testicular aging.

Keywords. Testicular Aging; Biomarkers; Seminal Plasma; Differential Proteins; Proteomics

1.INTRODUCTION

With the development of population aging, people are paying more attention to aging and the series of functional degradations caused by it[1]. Studies have proven that male testicular aging not only leads to fertility decline and sexual dysfunction, but may also manifest as sleep disorders, osteoporosis, and central obesity[2–4]. However, the diagnosis of testicular aging still lags far behind. In clinical practice, testosterone level, sperm concentration and morphology are frequently used as diagnostic criteria[5,6] . Nevertheless, the outcomes are not very predictive due to the wide individual variations. Therefore, there is a critical clinical need for early warning biomarkers that can indicate potential risks to the male reproductive system based on the overall body's condition at an early stage, enabling timely intervention.

Proteomics technology has become more widely used in recent years, which has increased our understanding of human biology and disease. Data-independent acquisition (DIA) proteomics is a high-throughput proteomics technique based on mass spectrometry technology that can simultaneously identify multiple proteins[7]. By analyzing a large number of samples, DIA can identify proteins associated with specific diseases, thereby offering crucial insights for early disease diagnosis and treatment. Rose et al. extracted reproducible proteins from mouse bones for DIA proteomic analysis and identified age-related bone-specific proteins, such as extracellular matrix (ECM) proteins and collagen-specific post-translational modifications (PTMs) [8]. Yet, studies on testicular aging do not utilize DIA proteomics techniques.

This study aimed to utilize Data Independent Acquisition (DIA) methods to investigate the differential proteins in the seminal plasma of older and younger men. Additionally, the study aimed to elucidate the developmental process and pathogenesis of testicular senescence through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Lastly, it provides a novel therapeutic target and treatment concept for testicular aging.

2.MATERIALS AND METHODS

2.1Study subjects

45 participants who visited the Peking Union Medical College Hospital from March 2019 to October 2022 were recruited, and seminal plasma and serum were collected separately. Forty-five participants were selected for the seminal plasma group, divided into two age groups: the young group and the middle-aged group. The average age of the young group was 27 years, while the middle-aged group had an average age of 44 years. Twenty-four participants were selected for the serum group and divided into three age groups: young, middle-aged, and elderly. The average age was 27 years for the young group, 47 years for the middle-aged group, and 60 years for the elderly group. This study was conducted after approval by the hospital's Medical Ethics Committee (Approval Number: JS-3112).

Inclusion criteria: Males with a percentage of A-grade sperm greater than 16% on seminal plasma biochemistry report and no obvious abnormalities on routine semen examination.

Exclusion criteria: Males with underlying diseases.

Participants refrained from sexual activity for 3 to 5 days before semen collection. Semen specimens were left to liquefy at 37°C, then centrifuged, and stored at -80°C for cryopreservation.

2.2 Sample Preparation

The samples were sonicated (this step can be omitted for protein solutions) and then boiled for 15 minutes. Post-boiling, the mixture was centrifuged at 14000g for 40 minutes, and the supernatant was quantified using a BCA Protein Assay Kit (P0012, Beyotime). Samples were then preserved at -20°C.

2.3 SDS-PAGE Separation

Each sample, containing 20 μg of proteins, was combined with 6X loading buffer and boiled for 5 minutes. Proteins were separated on a 12.5% SDS-PAGE gel, and the protein bands were stained with Coomassie Blue R-250.

2.4 Filter-Aided Sample Preparation (FASP Digestion)

involves using a UA buffer (8 M Urea, 150 mM Tris-HCl, pH 8.5) to remove

detergents, DTT, and other low-molecular-weight molecules through ultrafiltration (Sartorius, 30 kDa). Subsequently, 100 µL of iodoacetamide (100 mM IAA in UA buffer) was added to neutralize reduced cysteine residues and incubated for 30 minutes in the dark. The filters were cleansed three times with 100 µL of UA buffer, followed by two 100 µL washes with 0.1M TEAB buffer. Protein suspensions were then digested with 4 µg of trypsin (Promega) in 40 µL of 0.1M TEAB buffer overnight at 37°C. Peptides were collected through filtration and quantified using UV spectral density at 280 nm, taking into account an extinction coefficient of 1.1 for a 0.1% (g/L) solution.

2.5 Peptide Fractionation with Reversed Phase (RP) Chromatography

Peptides were fractionated by reversed-phase (RP) chromatography using an Agilent 1260 Infinity II HPLC system. After diluting with buffer A (10 mM HCOONH4, 5% ACN, pH 10.0), peptides were loaded onto an XBridge Peptide BEH C18 Column (130Å, 5 µm, 4.6 mm x 100 mm). A gradient from 0% to 7% buffer B (10 mM HCOONH4, 85% ACN, pH 10.0) over 5 minutes, increasing to 40% over 35 minutes, and then to 100% from 45 to 50 minutes, was used. Fractions were collected every minute between 5 and 50 minutes and dried using vacuum centrifugation at 45°C.

2.6 Nano-HPLC-MS/MS Analysis

Peptides were resuspended in solvent A (0.1% formic acid in water) and analyzed using an Orbitrap Exploris 480 system connected to an EASY-nLC system (Thermo Fisher Scientific, MA, USA). Approximately 1 µg of peptides were loaded onto a Nano Technology Column (18 cm C18, 1.9 µm C18 Resin, Catalog Number: 26350-3) and separated over a 120-minute gradient from 2% to 35% B (0.1% formic acid, 80% ACN). The flow rate was maintained at 300 nL/min with an electrospray voltage of 2056 V applied at the inlet.

2.7 Gene Ontology (GO) Annotation

Initially, protein sequences were aligned against the Homo sapiens database from NCBI (ncbi-blast-2.2.28+-win32.exe). Top 10 sequences with an E-value of ≤1e-3 were retained. The highest-scoring sequence was selected for Gene Ontology (GO) annotation using the database version go_201504.obo in Blast2GO. Post initial

annotation, InterProScan searched the EBI database for motifs, enhancing the protein annotations with functional information derived from the motifs. Further enhancements in linking and annotating Gene Ontology (GO) terms were pursued by ANNEX. This was achieved by employing Fisher's Exact Test to enrich Gene Ontology (GO) terms by comparing the differential and total protein expressions associated with those terms.

2.8 KEGG Pathway Annotation

Pathway analysis was conducted using the KEGG database. Fisher's Exact Test was employed to identify significantly enriched pathways by comparing differentially expressed proteins to the total pathway-associated proteins.

2.9 ELISA Validation

Procedures were followed precisely as per the ELISA kit instructions. 50 µL of standards or samples were added and incubated at 37°C for 30 minutes. After five washes, the enzyme-labeled reagent was added and incubated for another 30 minutes at 37°C. After another five washes, chromogenic solutions A and B were added, and the mixture was incubated at 37°C for 10 minutes to allow color development. The reaction was stopped, and the optical density (OD) values were read within 15 minutes for analysis.

2.10 Data Analysis

Data were statistically analyzed using GraphPad Prism (version 8.0.2, California, USA). Data sets involving more than two groups were analyzed with one-way ANOVA and Tukey's multiple comparison test, while two-group comparisons were made using the Student's t-test. Results were expressed as mean ± SD, and a p-value of < 0.05 was considered statistically significant.

3. RESULTS

3.1 73 proteins were differentially expressed in the DIA proteomics technique

Proteomics screening using DIA identified 2523 proteins in the seminal plasma. Two groups of five samples each (young and aged) were analyzed. Within these samples, 73 proteins exhibited significant differences in expression between the young and

aged groups ($P < 0.05$), with 46 proteins being up-regulated and 27 down-regulated, as shown in Figure 1. For each protein, we have listed gene symbols, accession numbers, detailed descriptions, statistical data, and fold changes. (Table 1).

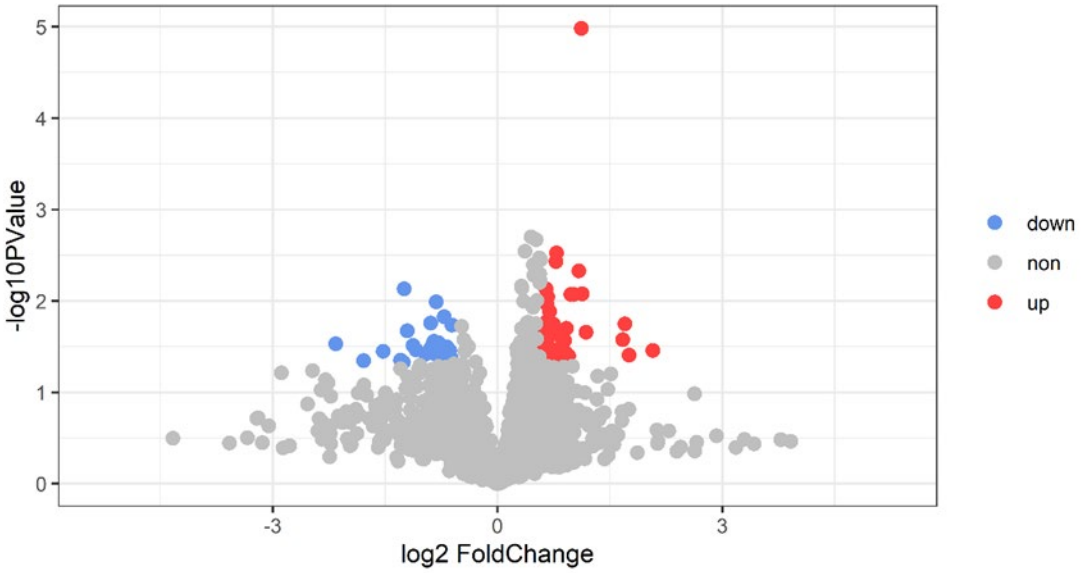

Figure 1. Proteins in the seminal plasma were detected using DIA analysis. Red indicates strongly up-regulated proteins, blue indicates significantly down-regulated proteins, and gray indicates no significant change in protein expression. The horizontal coordinate represents log2, and the vertical coordinate represents the negative logarithm of the t-test P value (- log10).

Table 1. Proteins detected using DIA show differential expression when comparing young and senescent seminal plasma.

Gene symbol	Accession	Protein name	p-value	Foldchange
Less expressed in aging samples				
CES5A	Q6NT32	Carboxylesterase 5A	0.035	4.201
SERPINB7	O75635	Serpin B7	0.039	3.380
GNA13	Q14344	Guanine nucleotide-binding protein subunit alpha-13	0.018	3.246
CNN3	Q15417	Calponin-3	0.027	3.185
CNNM3	Q8NE01	Metal transporter CNNM3	0.022	2.269
more expressed in aging samples				
CYCS	P99999	Cytochrome c	0.047	0.419
WISP2	O76076	WNT1-inducible-signaling pathway protein 2	0.045	0.408
IL1RL1	Q01638	Interleukin-1 receptor-like 1	0.036	0.347
SAA4	P35542	Serum amyloid A-4 protein	0.045	0.290
MYH6	P13533	Myosin-6	0.029	0.224

3.2 GO enrichment analysis of the differential proteins

The Gene Ontology (GO) functional enrichment analysis was categorized into three key areas: biological processes (BP), cellular components (CC), and molecular functions (MF). The analysis revealed that biological processes mainly involved cell differentiation, vesicle localization, T cell migration, isopentenyl diphosphate biosynthetic process, and zinc ion response. In terms of cellular components, the emphasis was placed on the apical sections of the cell, including elements such as spectrin, the photoreceptor inner segment, filamentous actin, and cardiac myofibril. In terms of molecular functions, the primary activities were associated with binding interactions with spectrin, Rab GTPase, phosphatidylinositol-3,4,5-trisphosphate, phosphatidylinositol-4,5-bisphosphate, and zinc ions (Figure 2).

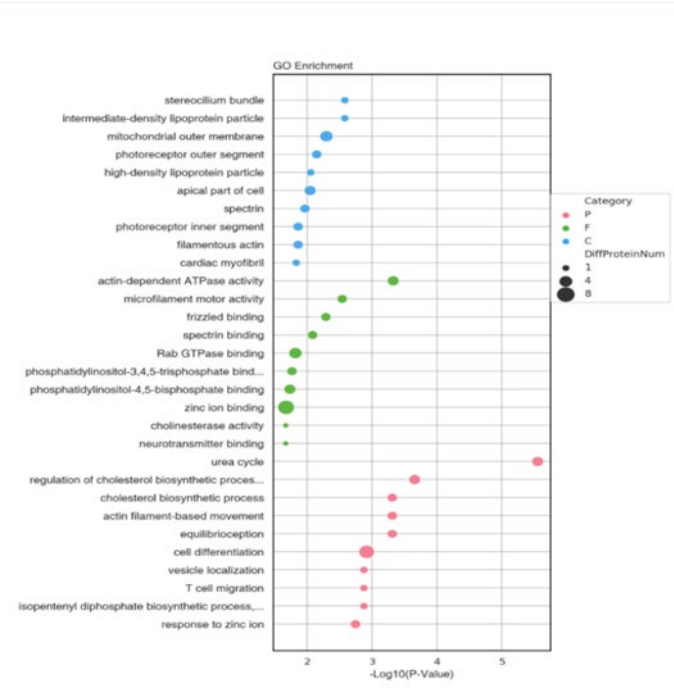

Figure 2. GO enrichment analysis of differentially expressed proteins in older and younger men. The vertical axis represents the GO function names, while the horizontal c axis represents the significance of enrichment indicated by the P-value. In the bubble diagram, the circle color indicates the GO function classification (P: Biological Process, F: Molecular Function, C: Cellular Component), and the circle size indicates the number of differential proteins associated with the function.

3.3 KEGG enrichment analysis of the differential proteins

KEGG enrichment analysis was conducted on the differentially expressed genes using the DAVID tool, identifying 139 signaling pathways. The top eight pathways, selected based on their p-values, were analyzed further. The analysis showed that the enriched differential proteins primarily participated in metabolic pathways, including alanine, aspartate, and glutamate metabolism, as well as inflammatory responses. Significant enrichment was also observed in pathways related to apoptosis and the P53 signaling pathway, as well as in pathways associated with tumorigenesis (Figure 3).

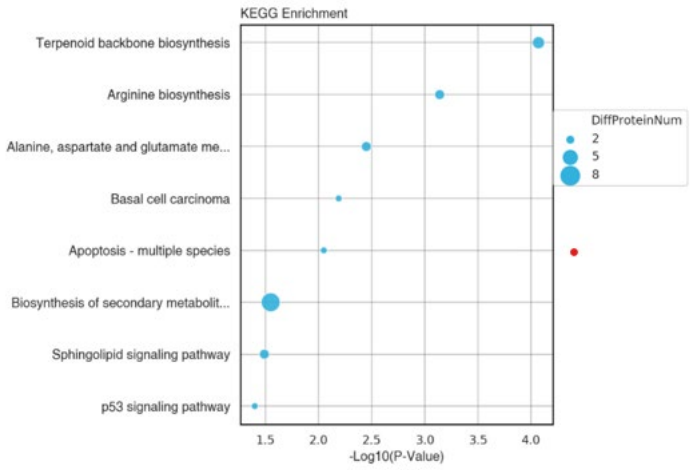

Figure 3. KEGG enrichment analysis of differentially expressed proteins in older and younger men. The vertical axis represents the KEGG pathway name, while the horizontal axis represents the enrichment level. In the bubble plot, the size of the circle indicates the number of differential proteins present in the pathway.

3.4 Validation of related differentially expressed proteins

In seminal plasma samples, the concentrations of GNA13, SAA4, and CYCS were significantly higher in middle-aged male participants compared to younger males, indicating a statistically significant difference ($P < 0.05$)（Figure 4）。

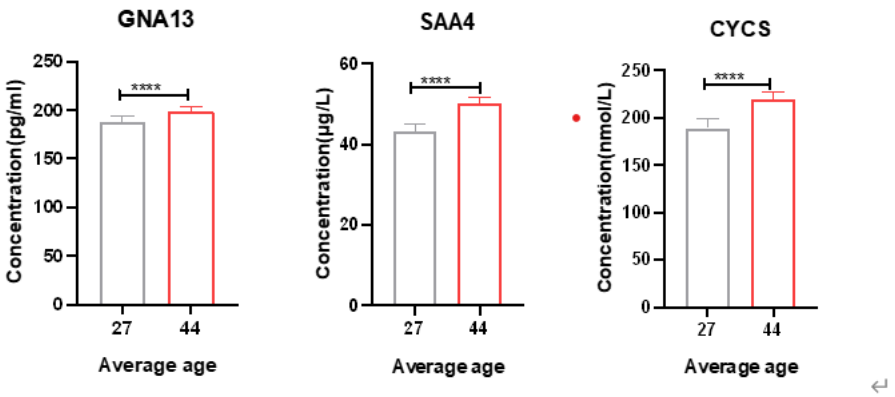

Figure 4. ELISA was used to confirm the expression of the relevant proteins in seminal plasma.

3.5 GNA13 expression in serum lags behind that in seminal plasma

In serum samples, concentrations of age-related proteins such as IL-6 and TGF-β were found to increase with age ($P < 0.05$). Similar patterns were observed for SAA4 and CYCS, with protein expression levels significantly higher in the middle-aged group compared to the young group ($P < 0.05$). However, no significant difference was detected in protein levels between the middle-aged and elderly groups. In contrast, the expression of GNA13 differed; there was no notable difference in protein levels between the young and middle-aged groups, but the expression of GNA13 was significantly elevated in the elderly group compared to the middle-aged group ($P < 0.05$) (Figure 5).

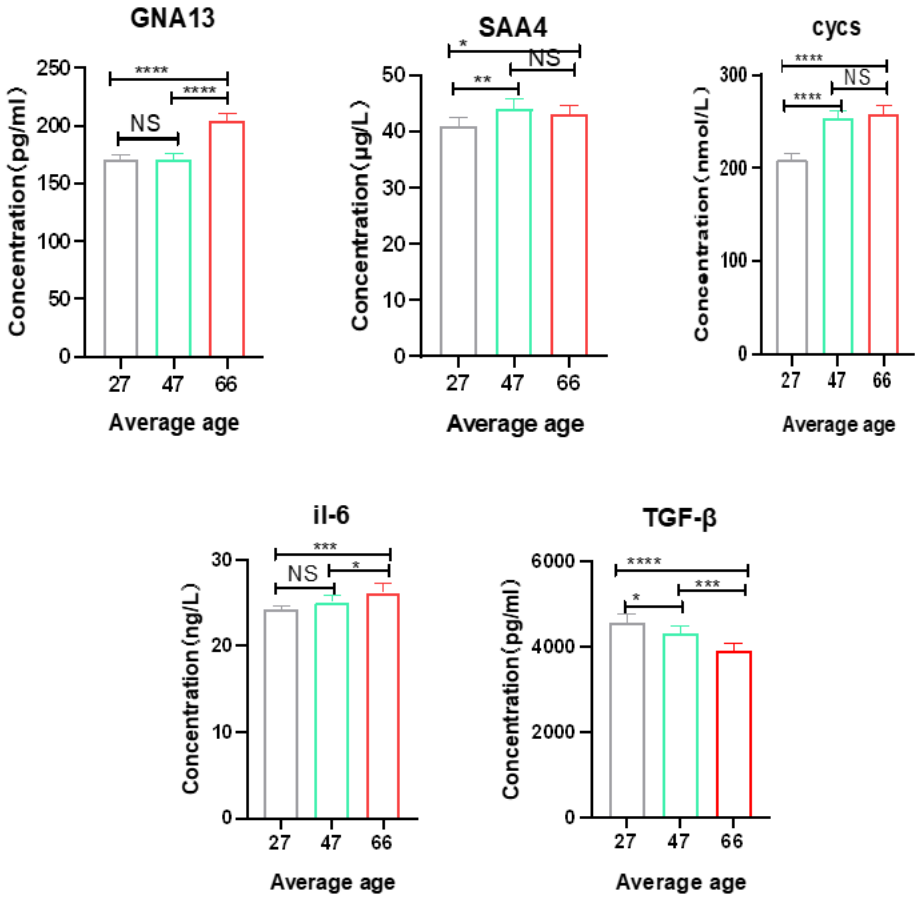

Figure 5. ELISA was used to confirm the expression of the relevant proteins in serum.

4.DISCUSSION

Nowadays, the world's population is getting older. As a result, age-related illnesses are growing more prevalent, which necessitates further investigation[9]. A decline in gonadal function is closely associated with aging. It can lead to significant reductions in fertility and endocrine changes that upset the balance of several systems[10]. While numerous studies have reported the causes of female ovarian aging, less is known about the aging of male testicles[11]. As reported by Stone et al., men's total semen counts start to decline around the age of 34. And the data indicated a significant downward trend after the age of 40[12]. Testosterone concentration is the most commonly used clinical diagnostic criterion to assess testicular age in males. In general, the typical range of serum total testosterone concentrations in adult males is

300-800 ng/dL. Testosterone levels only begin to significantly decline after the age of 60. Therefore, testosterone concentration as a diagnostic criterion has a significant delay and does not accurately identify testicular aging. Our research results also show that, when using the ELISA method to detect age-related proteins IL-6 and TGF-β in serum, these two proteins were observed to significantly increase only after the age of 47. Hence, serum is not suitable as a bodily fluid for the early identification of testicular aging.

DIA is an increasingly mature proteomics technology that can enhance the reproducibility and throughput of proteomic studies. In our research, we compared seminal plasma samples from young and aging males using Data-Independent Acquisition (DIA). We observed significant differences in the expression levels of numerous proteins in the seminal plasma. This suggests the potential use of this fluid to discover new biomarkers for the early diagnosis of testicular aging. Using DIA technology, we screened a total of 73 differential proteins related to testicular aging. After reviewing the literature, we selected the proteins GAN13, SAA4, and CYCS, which exhibited significant differences, as the focus of our study.

4.1 Analysis of potential biomarkers for testicular aging

SAA4 is an acute-phase protein that plays a significant role in chronic inflammation[13]. Chronic inflammation can trigger and exacerbate tissue damage and cellular dysfunction, accelerating the aging process[14]. CYCS is a small heme protein loosely associated with the inner mitochondrial membrane and plays a crucial role in mitochondrial function and apoptosis. As age increases, the efficiency of the mitochondrial defense system declines, leading to increased oxidative damage, which is considered one of the key factors in the aging process[15]. In our study, both SAA4 and CYCS showed differential expression in seminal plasma and serum samples. Unfortunately, our results indicate that these two proteins are not suitable as diagnostic biomarkers for testicular aging. Although the levels of SAA4 and CYCS were significantly higher in the middle-aged male group compared to the younger group in both seminal plasma and serum samples ($P < 0.05$), a comparison between the middle-aged and elderly groups in serum samples showed that the expression of

these two proteins did not increase with age.

GNA13 is a signaling molecule downstream of G protein-coupled receptors (GPCRs) that influences the local inflammatory environment by regulating the migration of immune cells[16]. In seminal plasma and serum samples, there is a significant difference in the expression levels of GNA13, which increase with age. We found that the expression levels of GNA13 in serum samples are nearly consistent with those of aging-related protein molecules, indicating its potential as a biomarker. Notably, our research indicates that the expression of GNA13 in seminal plasma precedes its expression in serum. Previous studies have reported that the total sperm count in men starts to decline after the age of 34. Our findings indicate that disparities in serum GNA13 levels are only noticeable when comparing middle-aged and older men, which mirrors the delay observed with the commonly used clinical diagnostic criterion of testosterone levels. Studies have reported that GNA13 may be involved in tumor development by regulating the ERK signaling pathway[17]. The presence of tumors can increase inflammation levels in the body, which promotes the aging process[18]. Additionally, GNA13 can activate the ERK signaling pathway through RhoA activation, thereby playing a role in inflammation[19]. Therefore, GNA13 can regulate the inflammatory response, directly or indirectly, affecting the aging of the organism. Thus, we conclude that the specific expression of GNA13 in seminal plasma could serve as a biomarker for the early diagnosis of testicular aging.

4.2 Biological Enrichment Analysis

GO enrichment analysis involves three aspects: cellular components, molecular functions, and biological processes. The results show that the cellular localization of seminal plasma differential proteins is mainly in the cell division zone and intracellular; the molecular functions of these differential proteins are primarily related to protein binding and other molecular interactions, suggesting that these proteins mainly function through binding with other proteins. KEGG pathway analysis indicates that the differential proteins are mainly involved in the P53 signaling pathway and the sphingolipid signaling pathway. Among these, components

of the sphingolipid signaling pathway may affect cellular responses to oxidative stress[20]. Oxidative stress is a significant factor in promoting cellular and tissue aging, and oxidative damage in testicular cells may accumulate with age, thereby accelerating testicular aging[21]. Additionally, the sphingolipid metabolic pathway might also impact the synthesis and secretion of testicular hormones[22]. Therefore, it is hypothesized that the sphingolipid signaling pathway could be a crucial pathway influencing testicular aging.

5.CONCLUSION

In summary, our research finds that seminal plasma is more suitable for the early diagnosis of testicular aging. Additionally, we validated seminal plasma and serum from males of different ages using DIA technology and ELISA. Our findings indicate that GNA13 shows specific expression in seminal plasma samples. Therefore, we consider GNA13 a potential biomarker for diagnosing testicular aging. Furthermore, GO and KEGG enrichment analyses suggest that testicular aging is primarily influenced by cellular apoptosis, lipid metabolism, immune inflammation, and oxidative stress. The sphingolipid signaling pathway could be a crucial factor influencing testicular aging. These results may offer new insights for the future diagnosis and treatment of testicular aging.

ACKNOWLEDGMENTS

We appreciate all participants in this work.

CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data and materials are included in the manuscript.

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