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Article

Exploring the Potential Relationship between Oxidative Stress in Fertile Individuals and Secondary Infertility: A Comparative Analysis of Proteomic Profiles in Seminal Plasma

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Abstract: Oxidative stress is increasingly implicated in male secondary infertility, where the seminal plasma's altered proteome composition plays a critical role. This study aims to explore proteomic changes in the seminal plasma of fertile individuals with oxidative stress and secondary infertility patients, hypothesizing oxidative stress as a key factor in secondary infertility. Pooled semen samples from both groups underwent quantitative proteomics analysis using advanced mass spectrometry, with subsequent bioinformatic analysis using tools like DAVID, STRING, and IPA for identifying differentially expressed proteins (DEPs).The study identified 377 DEPs in the secondary infertile group and 523 DEPs in healthy controls, revealing seven mutual pathways including acute phase response signalling and free radical scavenging. Significant alterations were noted in proteins like C3 and SERPINA3, associated with sperm function and fertility. The findings suggest that oxidative stress-induced proteomic alterations in seminal plasma are linked to secondary infertility, highlighting potential biomarkers and therapeutic targets. This research underscores the importance of understanding the interplay between oxidative stress and seminal plasma proteins, potentially guiding personalized treatment strategies to improve fertility outcomes in affected individuals.

Keywords: sperm proteome; oxidative stress; secondary male infertility; bioinformatics

1. Introduction

Male infertility is estimated to be responsible for 50-60% of infertile couples, as a primary or contributing cause (1). Male infertility is defined as either primary, in which male patients have never fathered a child, or secondary, in which a male patient had achieved pregnancy in the past but is unable to achieve a new one (2).

Secondary infertility is believed to constitute a large proportion of male infertility cases and causes a considerable amount stress and economic burden on coupler. Multiple factors have been implicated in secondary male infertility, which include varicocele, infection, smoking, obesity, exposure to high temperature and hazardous materials, and oxidative stress (3).

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds ability of the cell to scavenge the ROS. Oxidative stress negatively impacts sperm cells causes DNA damage, mitochondrial dysfunction, and apoptosis, all of which are related to male infertility (4).

Sperm cells are highly vulnerable to ROS production as they contain high levels of polyunsaturated fatty acids in both the membrane and cytoplasm and have a low ability to scavenge ROS compared to other cell types. As a result, sperm cells are dependent on the seminal plasma for

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oxidative stress protection, which is crucial for maintaining proper sperm function and its fertility potential (5).

The seminal plasma is a complex composition rich in proteins as well as other molecules such as sugars and lipids. This protein rich composition makes it a promising biological fluid for biomarker discovery and a target for proteomics and bioinformatic analysis(6). Multiple proteomic studies have revealed that the protein components of the seminal plasma play a pivotal role in maintaining proper fertility potential and that differential seminal plasma protein expression is associated with oxidative stress and infertility (7).

In this study we aimed to elucidate proteomic modifications occurring within the seminal plasma of individuals afflicted by concomitant oxidative stress and secondary infertility. Our underlying hypothesis posits that oxidative stress increases susceptibility toward secondary infertility. Results of our study could impact clinical care and suggest ways to treat secondary infertility by targeting oxidative stress, which would help to overcome infertility in the future.

2. Materials and Methods

2.1. Ethical Statement

Samples used for proteomic analysis originated from patients and donors after informed consent, pursuant of IRB protocols #11–451 and# 14-235, as previously described (8,9).

2.2. Sample and Data Collection

Semen samples were obtained from patients diagnosed with secondary male infertility, characterized by the inability to achieve conception following one year of unprotected intercourse. Additionally, samples were drawn from fertile donors who have successfully fathered a child within the preceding two years and exhibited elevated semen oxidative markers (Reactive Oxygen Species—ROS >93RLU/sec and Total Antioxidant Capacity—TAC < 1790 MTE). The control group comprised healthy fertile donors who have fathered a child within the past two years and with normal semen characteristics and exhibited no signs of oxidative stress in their semen (Reactive Oxygen Species—ROS <93RLU/sec and Total Antioxidant Capacity—TAC > 1790 MTE). Infertility patients were excluded from the study if they presented with varicocele, azoospermia, severe oligospermia, or when there was an identifiable female contributory factor.

2.3. Semen Analysis and Protein Extraction

Semen specimens were collected and processed in alignment with the World Health Organization (WHO) 2010 fifth edition guideline (World Health Organization., 2010). A chemiluminescence assay was employed to determine ROS levels, while sperm DNA fragmentation (SDF) was ascertained using the terminal deoxynucleotidyl transferase-mediated fluorescein end labeling (TUNEL) assay (citations). Following centrifugation of the samples at 13,000g for 20 minutes, seminal plasma was separated and subsequently preserved at −80 °C for proteomic evaluation.

2.4. Protein Identification and Global Proteomics Analysis

For the proteomic analysis, seminal plasma samples were equilibrated to room temperature and subsequently centrifuged at 3,000g for 30 minutes at 4 °C to remove residual sperm and other biological contaminants. Subsequently, samples were treated with a protease inhibitor cocktail to inhibit proteolysis. After protein quantification, five pooled samples from each group were selected for further evaluation. Seminal plasma concentrations were normalized to ensure equal protein concentrations for each specimen. Pooling of samples was employed to minimize biological variation (11,12). Subsequently, specimens were combined with SDS-PAGE buffer and subjected to 1D-PAGE, conducted in triplicate runs to mitigate technical variation, resulting in a final volume of 30 µL (13).

The extracts were concentrated using a Speedvac and resuspended in 1% acetic acid to reach a 30 µL volume for LC-MS. Finnigan LTQ-Orbitrap Elite hybrid mass spectrometer system was utilized

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with a Dionex 15 cm × 75 μm Acclaim Pepmap C18 column. Extracts (5 μL) were injected, and peptides eluted using an acetonitrile/0.1% formic acid gradient at 0.25 μL/min, introduced directly into the mass spectrometer. The ion source operated at 2.0 kV, and digestions were analysed using the instrument's data-dependent multitask capability, acquiring both full scan spectra for peptide weights and MS/MS for amino acid sequences.

2.5. Database Searching and Protein Identification

Proteins were identified using Proteome Discoverer version 1.4.1.288, Mascot (Matrix Science, London, UK; version 2.3.02), Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.0.288), and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). For validation, Scaffold (version 4.0.6.1, Proteome Software Inc., Portland, OR) was employed. Peptide identification utilized a confidence threshold of 95.0% (14), while protein identification adhered to stringent criteria with a 99.0% probability threshold, ensuring a false detection rate (FDR) below 1.0% and requiring at least two identified peptides per protein. The Protein Prophet algorithm (15) was used to assess protein probabilities. Furthermore, gene ontology (GO) terms from the National Centre for Biotechnology Information (NCBI), retrieved on October 21, 2013, facilitated protein identification.

2.6. Quantitative Proteomics

In the proteomic analysis, protein quantities were determined using spectral counts (SpCs), which represent the total mass spectra matching peptides for specific proteins. To normalize these counts, the NSAF approach was used(16), which adjusts for variations in sample replicates and considers that longer proteins typically have more peptide identifications (17). Differentially expressed proteins (DEPs) were identified using criteria such as significance tests and fold change thresholds based on the average SpC. Given the higher error rate for low-abundance proteins, different constraints were applied to SpC levels, categorizing proteins as High, Medium, Low, or Very Low based on their spectral counts. Each category had specific significance and fold change constraints to ensure accurate protein quantification and genuine biological change detection. These criteria were validated using control experiments with the NSAF method.

2.7. Bioinformatics Analysis

For bioinformatic analysis, multiple software applications were employed. Initially, we utilized Go Term Finder and UniProt for protein annotation. Subsequently, we incorporated complimentary software such as DAVID (Database for Annotation, Visualization, and Integrated Discovery), STRING (Search Tool for the Retrieval of Interacting Genes/Proteins). The DAVID software facilitated the analysis of differentially expressed proteins in terms of biological processes, cellular components, and molecular functions. The STRING software was instrumental in examining protein-protein interaction networks and conducting functional enrichment analysis. After these preliminary analyses, we employed the IPA software (Ingenuity Pathway Analysis) from Ingenuity® Systems. This software enabled a comprehensive analysis of the functional pathways of the differentially expressed proteins, encompassing top canonical pathways, disease and biological pathways, causal networks, and upstream regulators.

2.8. Statistical Analysis

Statistical analyses were performed using MedCalc Statistical Software (version 17.8; MedCalc Software, Ostend, Belgium). For semen analysis, various tests were employed based on variable types, including Analysis of Variance (ANOVA), two-sample T-tests, and the Wilcoxon rank-sum test. A p-value less than 0.05 was considered statistically significant. Quantitative protein expression comparisons between the two groups (fertile donors with high oxidative stress and secondary infertile patients) were also undertaken using two-sample T-test.

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3. Results

3.1. Semen Analysis

Table 1 presents the characteristics of the semen samples, including the percentage of sperm DNA fragmentation SDF and the concentration of reactive oxygen species ROS for intracellular oxidative stress assessment.

Table 1. Results from semen analysis, sperm DNA fragmentation (SDF) and intracellular reactive oxygen species (ROS) testing for both groups.

Semen Parameters	Fertile Donors ROS Positive (n=5)	Patients with Secondary Infertility (n=5)	P value
Sperm concentration (10^6/mL)	124 (99.20–138.90)	74.5 (61.60–84.00)	< 0.05
Sperm motility (%)	53 (22- 62.5)	39 (34.7–54.2)	< 0.05
Normal sperm morphology (%)	2 (1–4)	9 (7–9)	< 0.05
ROS levels (RLU/sec/10^6 sperm)	128.55 (61.6–195.5)	185.32 (177.24–193.4)	< 0.05
Sperm DNA fragmentation (%)	11.3 (4.0–16.4)	23.9 (15.3–36.5)	< 0.05

Results are presented as median and (25th, 75th percentile). For all values, $P < 0.05$ indicate a significant difference based on the Mann-Whitney test. RLU: relative light units.

3.2. Proteomic Profiling

In the pooled samples from both groups, distinct proteomic analyses were conducted. Figure 1 summarizes the distribution of proteins. Within the cohort of fertile donors with high oxidative stress in the semen, 377 proteins were identified. Of these, 44 proteins exhibited differential expression with the control group: 29 were overexpressed and 15 were underexpressed. In terms of protein abundance, 18 proteins were classified as highly abundant, 60 as moderately abundant, 84 as low in abundance, and 216 as very low in abundance. In contrast, for the group of patients with secondary infertility and high oxidative stress in the semen, analysis revealed 523 proteins. Among these, 53 proteins were differentially compared to the control group, with 51 being overexpressed and 2 underexpressed. The abundance categorization for this group was as follows: 25 proteins were highly abundant, 110 were of moderate abundance, 143 had low abundance, and 245 were very low in abundance. Supplementary Table S1.

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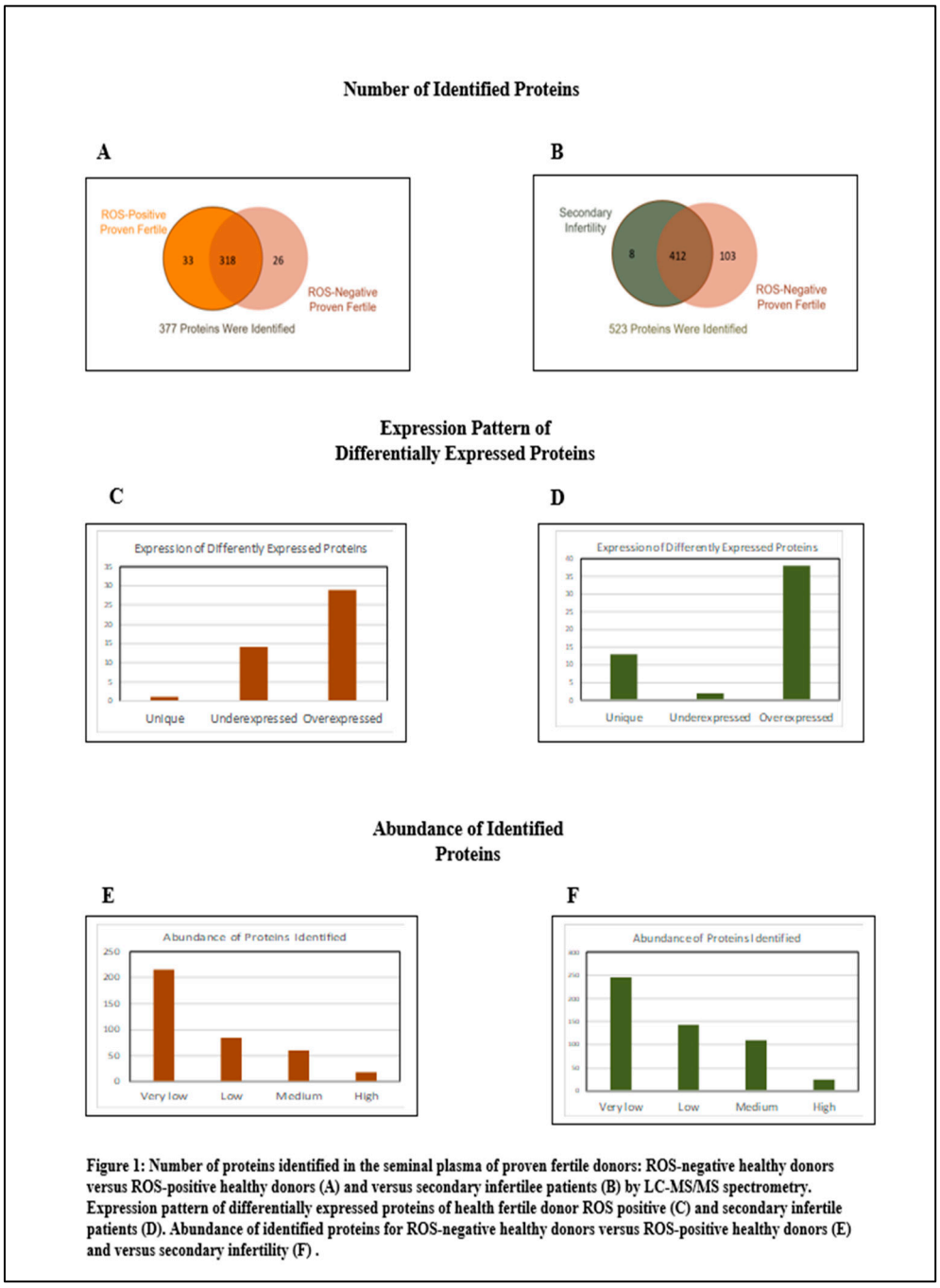

Figure 1: Number of proteins identified in the seminal plasma of proven fertile donors: ROS-negative healthy donors versus ROS-positive healthy donors (A) and versus secondary infertilee patients (B) by LC-MS/MS spectrometry. Expression pattern of differentially expressed proteins of health fertile donor ROS positive (C) and secondary infertile patients (D). Abundance of identified proteins for ROS-negative healthy donors versus ROS-positive healthy donors (E) and versus secondary infertility (F) .

Figure 1. Number of Identified proteins in fertile donors ROS positive and patients with secondary infertility.

3.3. Bioinformatic Analysis Results

Tables 2 and 4 present the raw analysis conducted using DAVID software for both the group of fertile donors ROS positive and the group of secondary infertile patients. We had a further analysis and restructured the data to have a deep overview of the involved proteins. Tables 3 and 5 summarize the proteins by their expression trend. Figures 2–5 show the protein expression frequency and proportion of protein expression status of each group.

For fertile donors experiencing high oxidative stress, the overexpressed proteins are associated with antioxidant activity and immune response. Conversely, the underexpressed proteins pertain to protein folding, stabilization, binding, and energy metabolism.

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For patients with secondary infertility overexpressed proteins are associated with inflammatory and immune response, energy metabolism and cell stability. Underexpressed proteins are related to sperm cell motility and capacitation. Unique proteins are related to protein binding, cell adhesion and extracellular matrix organization.

Table 2. DAVID raw analysis for the group of Fertile donors ROS positive.

Fertile Donors ROS Positive	**Biological Processes**	
	Overexpressed Proteins	**Negatively regulation of endopeptidase activity** (SERPINA1,SERPINA3,SERPINB6,SERPING1), **Acute-phase response**(A2M,APCS,HP,SERPINA1,SERPINA3),**Cellular oxidant detoxification**(S100A9 ALB,HP,PRDX4), **Innate immunity response**(S100A9,C3, LCN2, SERPING1)
	Underexpressed Proteins	**Protein folding**(APCS,CCT4,HSP90AA1,HSP90AB1,HSP90B1, PPIA) **Positive regulation of telomerase activity** (CCT4, HSP90AA1, HSP90AB1), **Response to cold**(HSP90AA1, HSPA2 LPL), **Response to unfold protein**(HSP90AA1, HSP90AB1, HSPA2),**Protein stabilization** (CCT4, HSP90AA1, HSP90AB1, TSPAN1),**Response to xenobiotic stimulus**(HSP90AA1, HSP90AB1, LPL).
	Molecular Functions	
	Overexpressed Proteins	**Serine protease inhibitor** (APP,SPINT1,SERPINA3,SERPINA4),**Anti-oxidant activity** (S100A9,ALB,HP), **Protease binding**(CTSD,DPP4,KLK3,PGC), **Antioxidant** (S100A9,S100A9,ALB,HP), **Zinc ion binding**(S100A9,ALB,QPCT,GLO1)
	Underexpressed Proteins	**Unfolded protein binding** (APCS, CCT4,HSP90AA1,HSP90AB1,HSP90B1, HSPA2, PPIA, TUBB4B), **Protein binding involved in protein folding, Chaperone**(CCT4,HSP90AA1, HSP90AB1, HSP90B1, HSPA2), **ATP'as activity**(CCT4,HSP90AA1,HSP90AB1,HSP90B1,HSPA2),**Disordered domain specific binding**(HSP90AA1,HSP90AB1,HSPA2),**Tau protein binding**(HSP90AA1,HSP90AB1,HSPA2), **RNA binding**(CCT4, HSP90AA1, HSP90AB1, HSP90B1, PPIA, YWHAZ),**Ion channel binding**(HSP90AA1,HSP90AB1,YWHAZ),**ATP binding**(CCT4, HSP90AA1,HSP90AB1,HSP90B1,HSPA2), **GTP binding**(HSP90AA1,HSP90AB1,TUBB4B), **Identical protein binding(3), Protein homodimerization activity**(DPP4,HSP90AA1,HSP90AB1,LPL)

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Table 3. Expression trend of the proteins involved in DAVID analysis for fertile donors ROS positive.

Category	Expression	Proteins
Biological Processes	Overexpressed	SERPINA1, SERPINA3, SERPINB6, SERPING1, A2M, APCS, HP, S100A9, ALB, PRDX4, C3, LCN2
Biological Processes	Underexpressed	APCS, CCT4, HSP90AA1, HSP90AB1, HSP90B1, PPIA, CCT4, HSP90AA1, HSP90AB1, HSPA2, LPL
Molecular Functions	Overexpressed	APP, SPINT1, SERPINA3, SERPINA4, S100A9, ALB, HP, CTSD, DPP4, KLK3, PGC, QPCT, GLO1
Molecular Functions	Underexpressed	APCS, CCT4, HSP90AA1, HSP90AB1, HSP90B1, HSPA2, PPIA, TUBB4B, YWHAZ

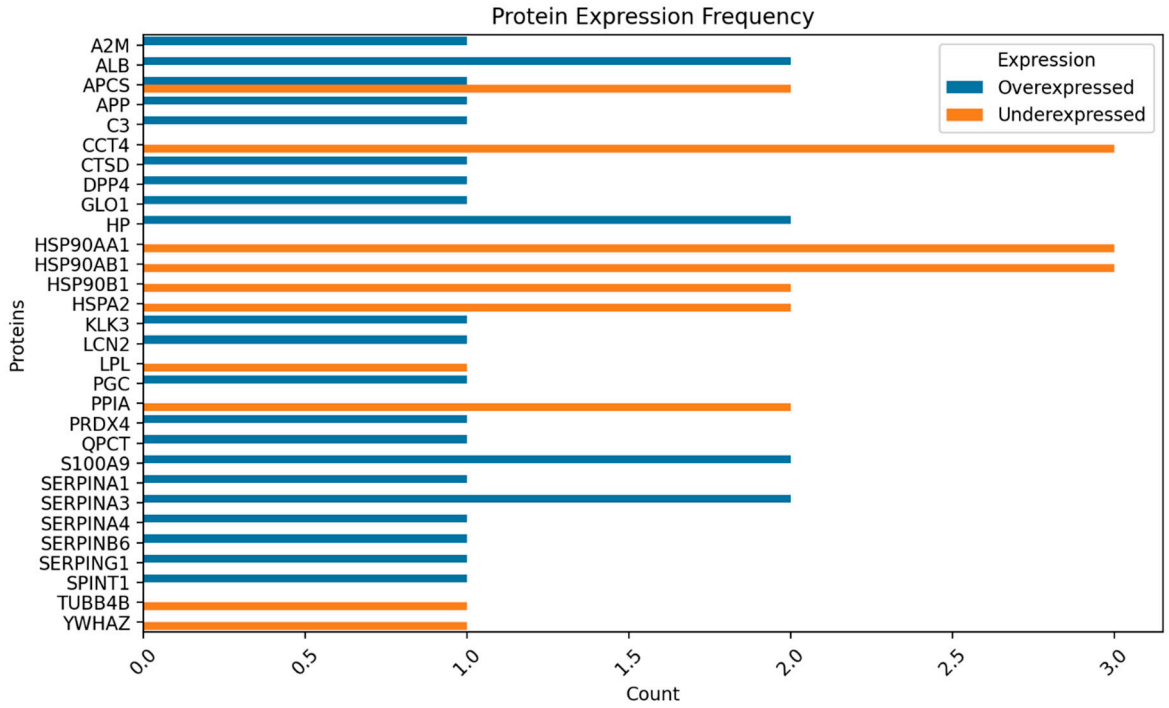

Figure 2. Protein expression frequency of proteins for fertile donors ROS positive.

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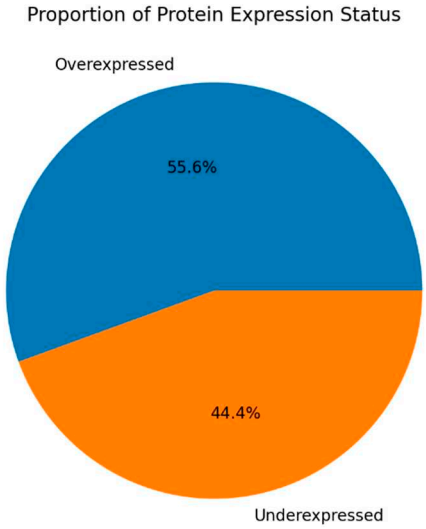

Figure 3. Proportion of protein expressed status for fertile donors ROS positive.

Table 4. DAVID raw analysis for the group of patients with secondary infertility.

Patients with Secondary Infertility	**Biological Processes**	
	Overexpressed Proteins	**Serine-type endopeptidase inhibitor activity**(APP,ANXA2,SPINT1,SERPINA3,ERPINA4), **Cell adhesion**(APP,LAMA5,MYH9,NPNT,OLFM4,PTPRS),**Actin cytoskeleton reorganization**(ANXA1,EZR,MYH9,RHOA),**Regulation of cell shape**(ANXA1,EZR,MYH9,RHOA), **proteolysis**(C3,LGMN,PRCP,TMPRSS2),**Inflammatory response**(ANXA1,C3,C4A,SERPINA3), **Innate immunity response** (ANXA1,C3,C4A)
	Underexpressed Proteins	**Coagulation**(SEMG1,SEMG2), **Positive regulation of serine type endopeptidase activity**(SEMG1,SEMG2), **Negative regulation of flagellated sperm motility**(SEMG1,SEMG2), **Sperm capacitation**(SEMG1,SEMG2), **Antibacterial humoral response**(SEMG1,SEMG2).
	Unique Proteins	**Cell adhesion**(APP,LAMA5,MYH9,NPNT,OLFM4,PTPRS), **Extracellular matrix organization** (APP,NPNT,SPINT1)
	Molecular Functions	
	Overexpressed Proteins	**Serine type endopeptidase inhibitor activity**(APP,SPINT1,SERPINA3,SERPINA4), **Protease** (APP,LGMN,PRCP,TMPRSS2), **Integrin binding**(APP,LAMA5,LCP1,MYH9,NPNT) , **Cadherin binding**(EZR, HSPA5,LDHA,MYH9,OLFM4),**Actin filament binding**(EZR,LCP1,MYH9, **GTP binding**(RAB27A,EEF1A1,RHOA,TUBA1C,TUBB4B),**GTPase**

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	activity(RAB27A,EEF1A1,RHOA,TUBA1C,TUBB4B), **Calcium ion binding**(ANXA1,ANXA2,HSPA5,LCP1, NPNT)
Underexpressed Proteins	**Zinc ion binding**(SEMG1,SEMG2)
Unique Proteins	**Integrin binding**(APP,MYH9,NPNT), **Protein binding**(RAB27A,EZR,HSPA5,MYH9,RHOA),**Identical protein binding**(APP,FTH1,FBP1 MYH9)

Table 5. Expression trend of the proteins involved in DAVID analysis for patients with secondary infertility.

Category	Expression	Proteins
Biological Processes	Overexpressed	APP, ANXA2, SPINT1, SERPINA3, ERPINA4, LAMA5, MYH9, NPNT, OLFM4, PTPRS, ANXA1, EZR, RHOA, C3, LGMN, PRCP, TMPRSS2
Biological Processes	Underexpressed	SEMG1, SEMG2
Biological Processes	Unique	APP, LAMA5, MYH9, NPNT, OLFM4, PTPRS, APP, NPNT, SPINT1
Molecular Functions	Overexpressed	APP, SPINT1, SERPINA3, SERPINA4, LGMN, PRCP, TMPRSS2, LAMA5, LCP1, MYH9, NPNT, EZR, HSPA5, LDHA, MYH9, OLFM4, RAB27A, EEF1A1, RHOA, TUBA1C, TUBB4B
Molecular Functions	Underexpressed	SEMG1, SEMG2

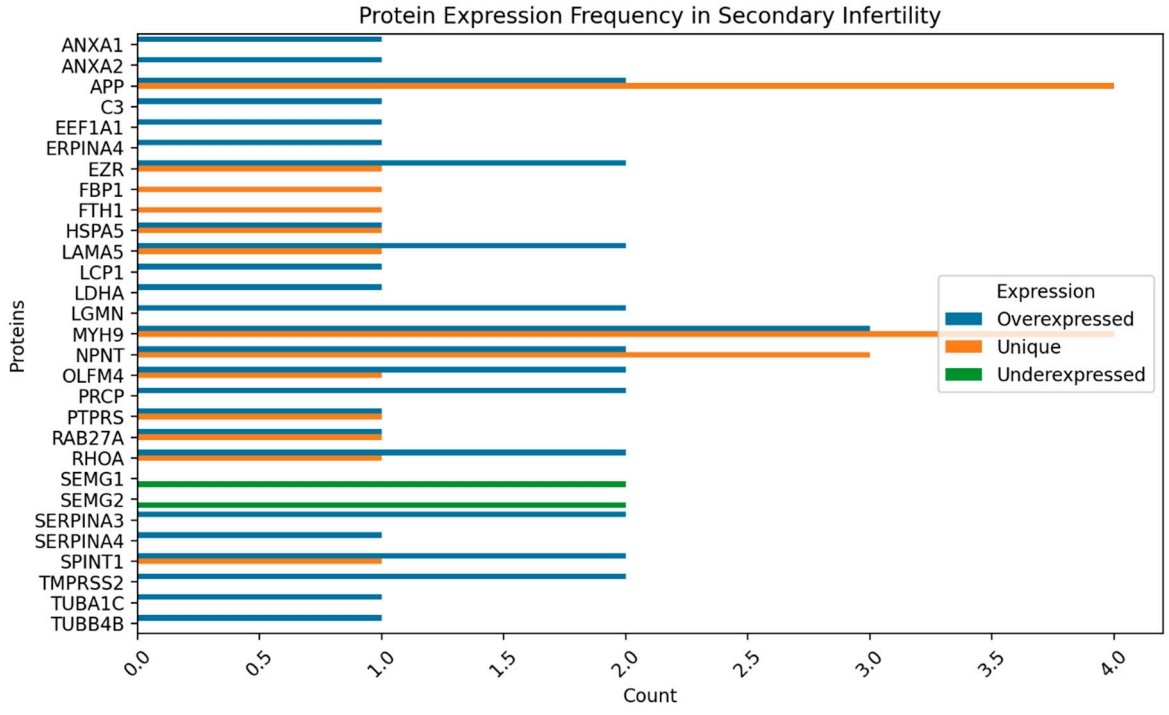

Figure 4. Protein expression frequency of proteins for patients with secondary infertility.

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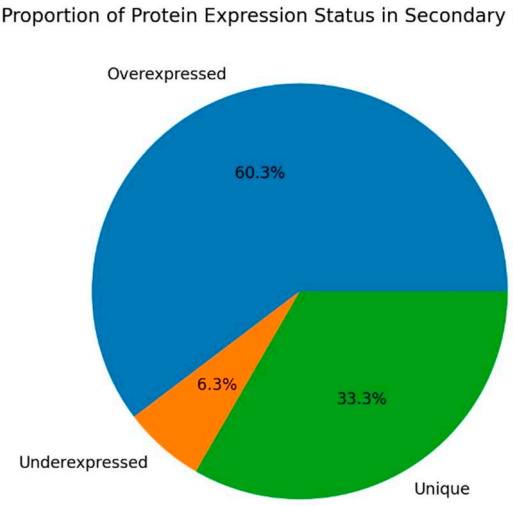
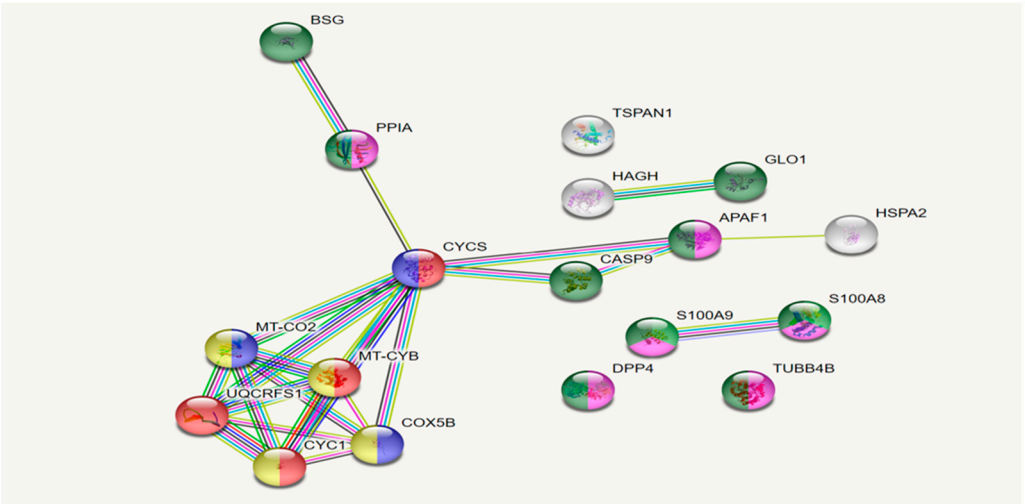


Figure 5. Proportion of protein expressed status for patients with secondary infertility.

Figures 6 and 7 show the STRING analysis for each the group of fertile donors ROS positive and Secondary infertile patients. The analysis shows the interactions between the mapped proteins (known, predicted or other interactions) and the functional enrichments of the biological process.



Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0006122	Mitochondrial electron transport, ubiquinol to cytochrome c	4 of 14	2.49	3.90e-06
GO:0006123	Mitochondrial electron transport, cytochrome c to oxygen	3 of 20	2.21	0.00092
GO:0002523	Leukocyte migration involved in inflammatory response	2 of 18	2.08	0.0394
GO:1902600	Proton transmembrane transport	4 of 150	1.46	0.0060
GO:0045321	Leukocyte activation	6 of 929	0.85	0.0368
GO:0002376	Immune system process	9 of 2481	0.6	0.0382

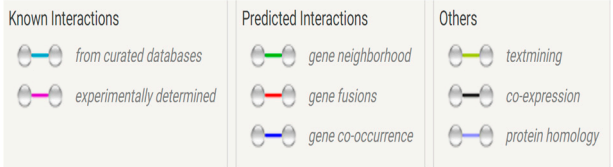


Figure 6. STRING analysis for fertile donor ROS positive.

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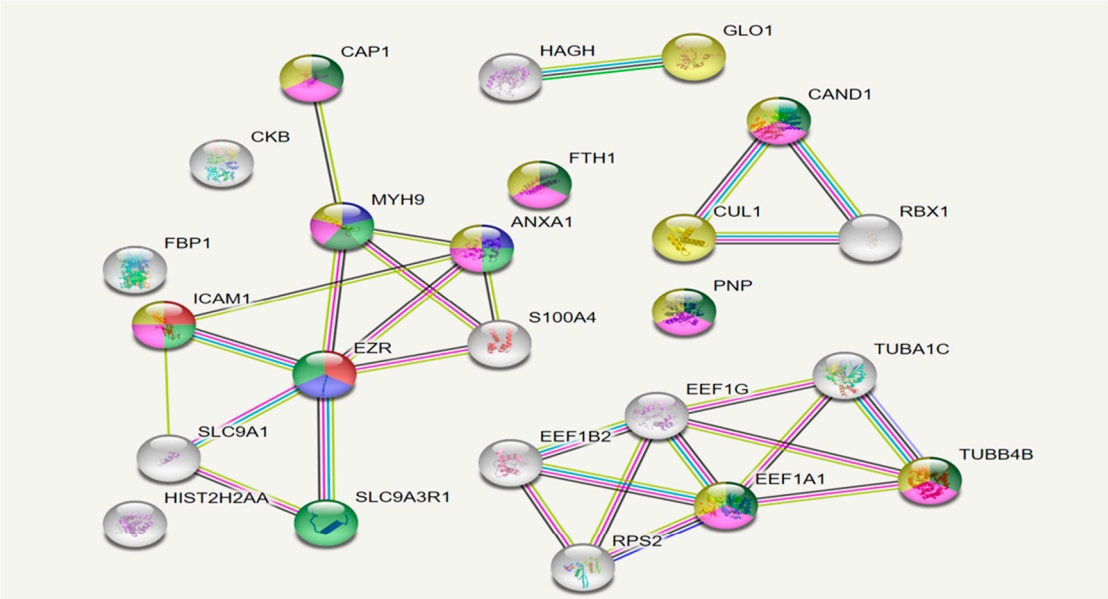

Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0008360	Regulation of cell shape	5 of 153	1.43	0.0056
GO:0045321	Leukocyte activation	9 of 929	0.9	0.0056
GO:0045055	Regulated exocytosis	7 of 697	0.91	0.0168
GO:0022614	Membrane to membrane docking	2 of 5	2.51	0.0215
GO:0002376	Immune system process	11 of 2481	0.56	0.0310
GO:0031532	Actin cytoskeleton reorganization	3 of 66	1.57	0.0333

Figure 7. STRING analysis for patients with secondary infertility.

Figures 8 and 9 show the main result of the IPA analysis for each group of fertile donors with ROS positive and patients with secondary infertility.

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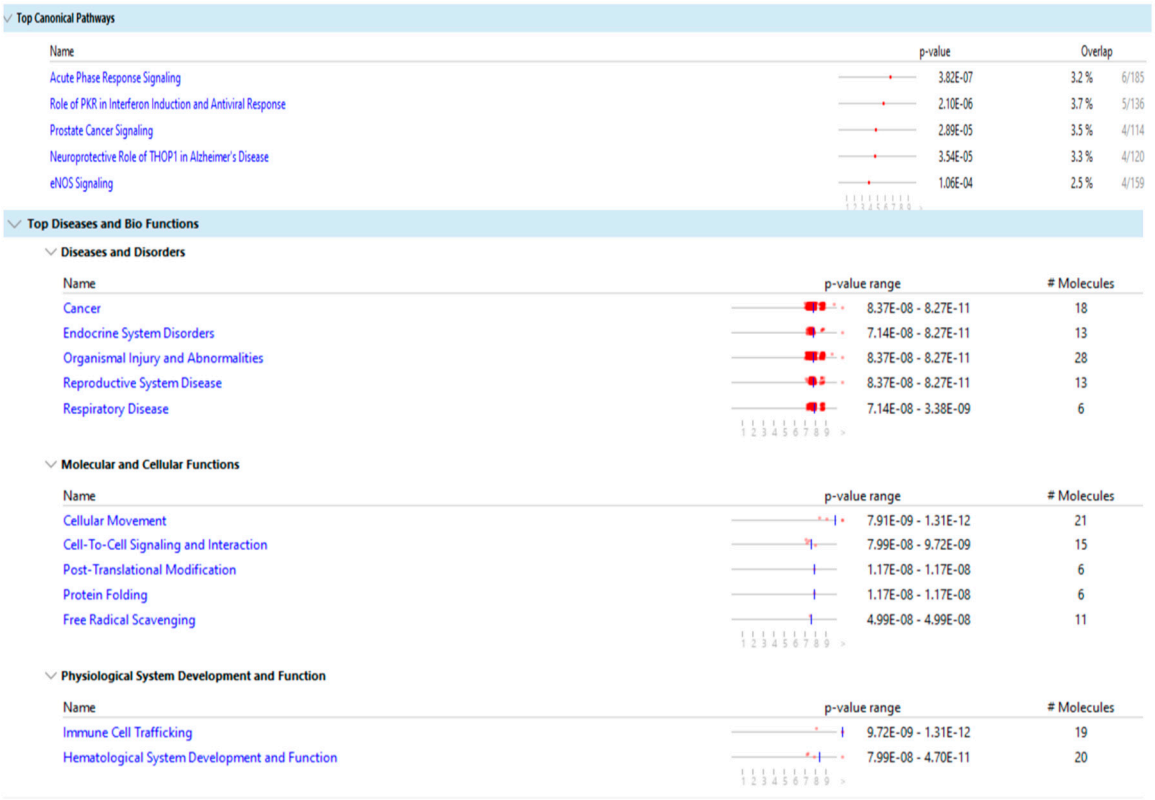


Top Canonical Pathways

Name	p-value	Overlap
Acute Phase Response Signaling	3.82E-07	3.2 % 6/185
Role of PKR in Interferon Induction and Antiviral Response	2.10E-06	3.7 % 5/136
Prostate Cancer Signaling	2.89E-05	3.5 % 4/114
Neuroprotective Role of THOP1 in Alzheimer's Disease	3.54E-05	3.3 % 4/120
eNOS Signaling	1.06E-04	2.5 % 4/159

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value range	# Molecules
Cancer	8.37E-08 - 8.27E-11	18
Endocrine System Disorders	7.14E-08 - 8.27E-11	13
Organismal Injury and Abnormalities	8.37E-08 - 8.27E-11	28
Reproductive System Disease	8.37E-08 - 8.27E-11	13
Respiratory Disease	7.14E-08 - 3.38E-09	6

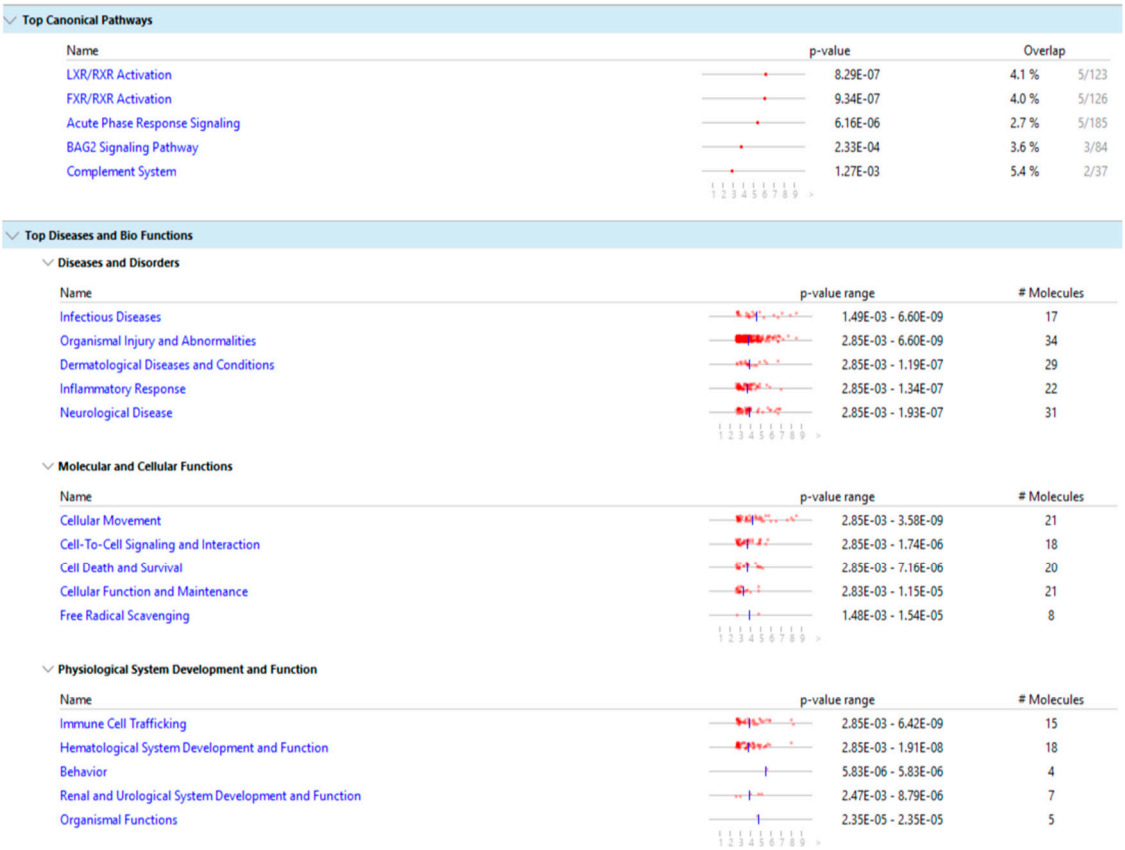
Molecular and Cellular Functions

Name	p-value range	# Molecules
Cellular Movement	7.91E-09 - 1.31E-12	21
Cell-To-Cell Signaling and Interaction	7.99E-08 - 9.72E-09	15
Post-Translational Modification	1.17E-08 - 1.17E-08	6
Protein Folding	1.17E-08 - 1.17E-08	6
Free Radical Scavenging	4.99E-08 - 4.99E-08	11

Physiological System Development and Function

Name	p-value range	# Molecules
Immune Cell Trafficking	9.72E-09 - 1.31E-12	19
Hematological System Development and Function	7.99E-08 - 4.70E-11	20

Figure 8. Fertile donors ROS positive.



Top Canonical Pathways

Name	p-value	Overlap
LXR/RXR Activation	8.29E-07	4.1 % 5/123
FXR/RXR Activation	9.34E-07	4.0 % 5/126
Acute Phase Response Signaling	6.16E-06	2.7 % 5/185
BAG2 Signaling Pathway	2.33E-04	3.6 % 3/84
Complement System	1.27E-03	5.4 % 2/37

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value range	# Molecules
Infectious Diseases	1.49E-03 - 6.60E-09	17
Organismal Injury and Abnormalities	2.85E-03 - 6.60E-09	34
Dermatological Diseases and Conditions	2.85E-03 - 1.19E-07	29
Inflammatory Response	2.85E-03 - 1.34E-07	22
Neurological Disease	2.85E-03 - 1.93E-07	31

Molecular and Cellular Functions

Name	p-value range	# Molecules
Cellular Movement	2.85E-03 - 3.58E-09	21
Cell-To-Cell Signaling and Interaction	2.85E-03 - 1.74E-06	18
Cell Death and Survival	2.85E-03 - 7.16E-06	20
Cellular Function and Maintenance	2.83E-03 - 1.15E-05	21
Free Radical Scavenging	1.48E-03 - 1.54E-05	8

Physiological System Development and Function

Name	p-value range	# Molecules
Immune Cell Trafficking	2.85E-03 - 6.42E-09	15
Hematological System Development and Function	2.85E-03 - 1.91E-08	18
Behavior	5.83E-06 - 5.83E-06	4
Renal and Urological System Development and Function	2.47E-03 - 8.79E-06	7
Organismal Functions	2.35E-05 - 2.35E-05	5

Figure 9. Patients with secondary infertility.

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4. Discussion

Seminal plasma is vital for the development of functional sperm cells. It predominantly consists of proteins secreted by the accessory glands of the male reproductive system. These proteins are responsible for the various aspects of male fertility, from ensuring sperm health and motility to facilitating successful ovum fertilization, interaction with the female reproductive system and the conveyance of genetic material.

Moreover, seminal plasma proteins also play a pivotal role in providing antioxidant protection to sperm cells. This ensures their viability, motility, and DNA integrity. As oxidative stress is a known contributing factor to male infertility, these proteins are crucial for maintaining male fertility.

In our current study, we aimed to explore whether high oxidative stress is a risk factor for secondary infertility and to validate our hypothesis, we compared the proteomic profiles of fertile donors with high oxidative stress to those of patients with secondary infertility. We used different software analysis that encompassed shared biological processes, molecular functions, predominant canonical pathways, as well as associated diseases and biological functions.

Our results show that the differentially expressed proteins of the two groups share multiple biological pathways and molecular functions such as: acute phase response signaling, organismal injury and abnormalities, cellular movement, cell to cell signaling and interaction, hematological system development and function, immune cell trafficking and free radical scavenging.

The molecular function of free radicals scavenging is activated in the group of fertile donors with ROS positive, as expected, and is also activated in the group of secondary infertile patients, which suggests that oxidative stress may be a cause of this pathology. Additionally, other shared pathways of acute phase response signaling, organismal injury and abnormalities, cellular movement and immune cell trafficking are also a result of oxidative stress, which further suggests that oxidative stress is a cause of secondary infertility.

The multiple share pathological pathways between the secondary infertile group and the fertile ROS positive group is indicative that oxidative stress is a risk factor for secondary infertility. As such, patients with high oxidative stress in semen could be advised to act and treat the oxidative stress pathology before reaching the point of infertility or potentially to correct infertility. For instance, treatment with antioxidants to reverse the infertility may be fruitful. However, more research in this area is needed.

Beside the issue of infertility, the DEPs of the group of fertile donors with ROS positive were associated with cancer, endocrine system disorders, organismal injury and abnormalities, reproductive system disease and respiratory diseases. As such, treatment of oxidative stress may have benefits beyond treating infertility.

We had a comprehensive analysis of proteins within each biological pathway and process to pinpoint potential markers for future infertility and associated comorbidities. Notably, the C3 protein was consistently present in all previously specified biological pathways and molecular functions,especially the free radical scavenging pathway, exhibiting a positive fold change in expression, indicating its overexpression.The C3 protein, is glycoprotein and an integral component of the complement immune system. It is encoded by the C3 gene situated on chromosome 19(18). Elevated C3 protein levels typically suggest inflammation(19). While increased levels of C3 have been observed in the seminal plasma of infertile patients and in abnormal semen samples (20,21), to our knowledge, this is the first documentation of its presence in the seminal plasma of fertile males exhibiting oxidative stress.

Another protein identified in the free radical scavenging pathway is SERPINA3. This protein is a serine protease inhibitor and belongs to the Serpins family (22). It is implicated in processes like inflammation and oxidative stress (23). Furthermore, SERPINA3 is associated with various pathologies, including chronic obstructive pulmonary disease, Parkinson's disease, Alzheimer's disease, and cancer (24). Past proteomic research has linked SERPINA3 with male infertility following spinal cord injuries and in patients with varicocele(25,26). However, our study is the first to demonstrate a connection between this protein and secondary male infertility, likely stemming from oxidative stress and inflammation.

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These two pivotal proteins, C3 and SERPINA3, may act as biological biomarkers for potential infertility and a spectrum of other diseases and pathologies in fertile patients experiencing oxidative stress, providing avenues for early diagnosis and pre-emptive treatment options.

5. Conclusions

Our study robustly establishes oxidative stress as a critical factor in secondary infertility. The discovery of key proteins such as C3 and SERPINA3 as overexpressed biomarkers reinforces the link between oxidative stress and inflammatory responses in male fertility and are a potential biomarker for future diagnosis and treatment. These findings not only pave the way for antioxidant-based therapeutic interventions to address fertility issues and mitigate associated health conditions, but also highlight the broader impact of oxidative stress on male health, underscoring the need for early detection and preventive measures against its potentially multifaceted consequences.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Data Availability Statement: The lists of differentially expressed proteins are available as the supplementary materials of this study.

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