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Semen Proteomics of COVID-19 Convalescent Men Reveals Disruption of Key Biological Pathways Relevant to Male Reproductive Function

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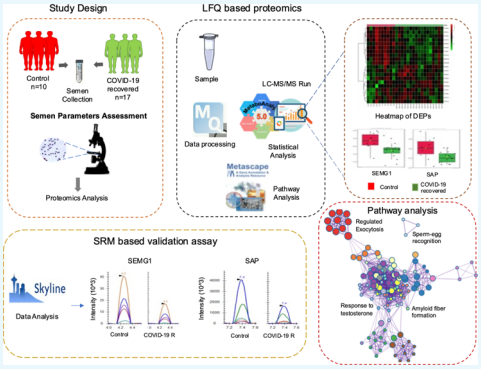
ABSTRACT: A considerable section of males suffered from COVID-19, with many experiencing long-term repercussions. Recovered males have been documented to have compromised fertility, albeit the mechanisms remain unclear. We investigated the impact of COVID-19 on semen proteome following complete clinical recovery using mass spectrometry. A label-free quantitative proteomics study involved 10 healthy fertile subjects and 17 COVID-19-recovered men. With 1% false discovery rate and >1 unique peptide stringency, MaxQuant analysis found 1099 proteins and 8503 peptides. Of the 48 differentially expressed proteins between the healthy and COVID-19-recovered groups, 21 proteins were downregulated and 27 were upregulated in COVID-19-recovered males. The major pathways involved in reproductive functions, such as sperm−oocyte recognition, testosterone response, cell motility regulation, adhesion regulation, extracellular matrix adhesion, and endopeptidase activity, were downregulated in COVID-19-recovered patients according to bioinformatics analysis. Furthermore, the targeted approach revealed significant downregulation of semenogelin 1 and prosaposin, two proteins related to male fertility. Therefore, we demonstrate the alteration of semen proteome in response to COVID-19, thus disrupting the male reproductive function despite the patient's clinical remission. Hence, to understand fertility-related biological processes triggered by this infection, a protracted evaluation of the consequences of COVID-19 in recovered men is warranted.



INTRODUCTION

Viral infections are known to affect the reproductive system of the host, either directly via infection or due to the cataclysms of the virus on other organs of the body.[1−3]

Although SARS-CoV-2 infection causes Coronavirus Disease-2019 (COVID-19) mainly in the respiratory tract, other tissues have been reported to be damaged,[4] either directly or indirectly, during the host−pathogen interaction. However, the impact of COVID-19 on the patient's reproductive organs may well be the least known facet.

Recent epidemiological investigations have revealed that males are more affected by this disease than females.[5,6] Molecular evidence indicates that angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) are critical for cell invasion and proliferation of the virus.[7] The ACE2 is strongly expressed both in the seminiferous duct and Leydig cells of testis but is low in seminal vesicles and glandular cells. Moreover, TMPRSS2 expression has been reported in the prostate, epididymis, and seminal vesicle.[8,9] Since the testis and seminal vesicles exhibit co-expression of the receptor and the protease, suggesting that they are predisposed to SARS-CoV-2 infection, TMPRSS2 is highly expressed in the prostate. As a result, the male reproductive organs serve as an ideal reservoir for the virus.[10,11]

Reports have indicated the presence of SARS-CoV-2 in the semen of affected males[12] and the testis of patients deceased due to COVID-19.[13] However, this is in conflict with reports that discount SARS-CoV-2[14−18] in the semen of patients. Yang et al. observed pathological changes in the testes of men who died from COVID-19. Further, they showed the presence of SARS-CoV-2 virus in 10% of the cases studied; a significant injury to Sertoli cells and seminiferous tubules, a reduction in Leydig cells, and a moderate inflammatory infiltrate in the interstitium have been reported.[13] Further, in another study of the testis proteome of patients who succumbed to COVID-19, it was

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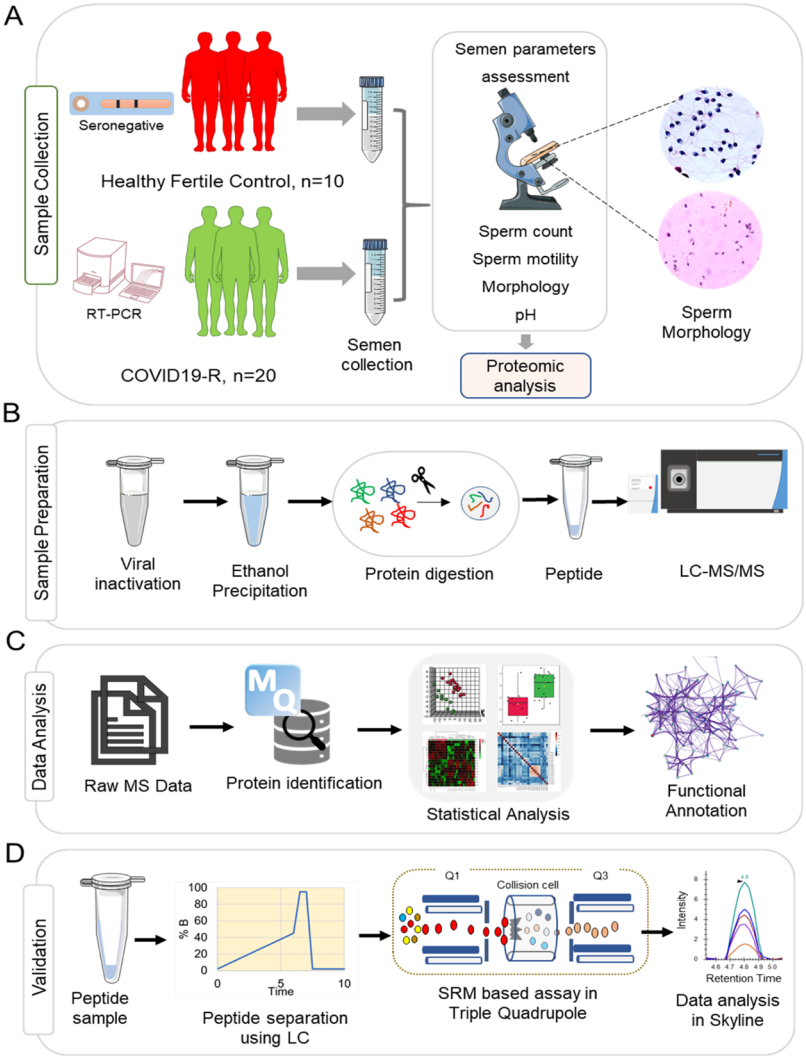

Figure 1. Workflow for proteomics analysis of human semen samples. (A) Sample cohort consisting fertile control n = 10 and COVID19-R (recovered) patients n = 20 and the semen parameters assessed for determining the sperm quality. (B) Basic methodological steps involved in LFQ-based proteomics at the discovery phase. (C) Outline of statistical data analysis. (D) Pipeline of targeted proteomics for validation. This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

reported that a Leydig cell biomarker insulin-like 3 (INSL3) and five cholesterol biosynthesis-linked proteins were reduced significantly, indicating an involvement of the Leydig cells and their steroidogenic functions. They also reported the decline of two critical sperm proteins, E3 ubiquitin-protein ligase (RNF216), and dynein regulatory complex subunit 7 (DRC7). The reduction in DRC might point to impaired sperm motility.[4] A recent study has confirmed the infection of the testes by SARS-CoV-2.[19] In contrast, another comprehensive study to evaluate the fallout of COVID-19 on the urine, semen, and expressed prostatic secretions of convalescent patients found no evidence of the virus. However, a dysregulated semen and hormonal profile was observed.[20]

Semen helps sperms survive and plays an essential part in their development and acquisition of fertilizing ability.[21] Although the testes are primarily responsible for spermatogenesis, most seminal plasma components come from the seminal vesicles, prostate, and epididymis secretions and contribute around 70, 20, and 10% of the semen volume, respectively.[21] Also, some amounts come from the accessory reproductive organs such as Cowper's glands and Littre's glands.[22] COVID-19 has been reported to change the semen characteristics of men of reproductive age,[15] although the virus has not been observed conclusively in seminal plasma. Therefore, with the surmounting evidence of changes in the biochemical and other parameters of the ejaculated semen, it was pertinent to ask whether we could detect the proteomic fingerprint of viral infection or perhaps the virus in the semen using a sensitive methodology such as mass spectrometry. Thus, analyzing the seminal fluid proteome can facilitate understanding the pathological aberrations due to COVID-19. To our best knowledge, this is the first comprehensive semen proteome study on men recovering from COVID-19 to get insight into the long-term impact of SARS-CoV-2 infection on the male reproduction mechanism.

RESULTS

Evaluation of Semen Quality. Semen characteristics, including semen volume, sperm count (mill/mL), motility (%), pH, and sperm morphology, were assessed for both the control and COVID-19-recovered groups. The median sperm

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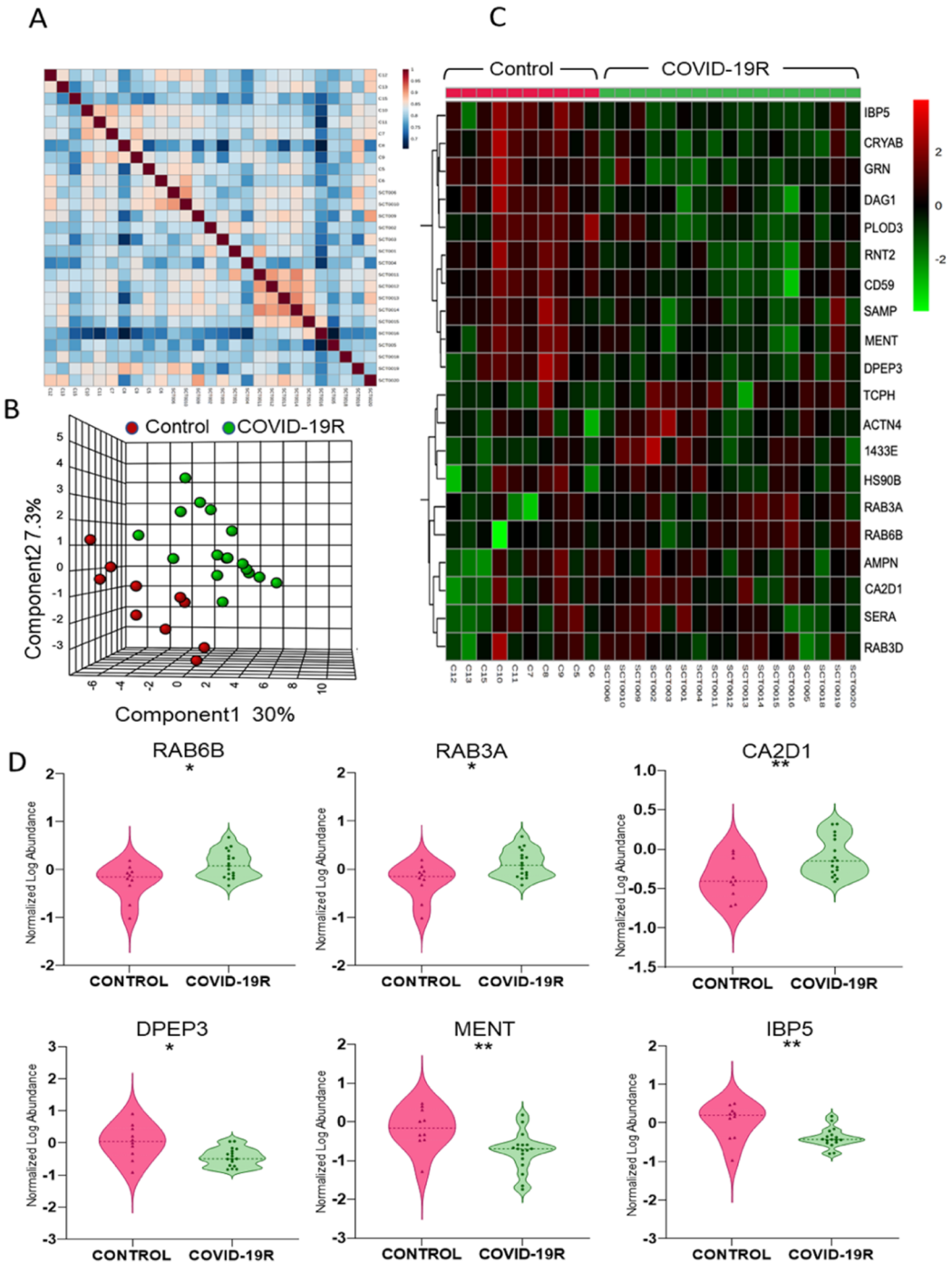

Figure 2. Overview of semen proteomics analysis of control and COVID-19R (recovered) patients. (A) Correlation matrix of a total of 27 subjects, including 10 control and 17 COVID-19R patients. (B) PLS-DA of 27 samples shows the discrimination between two groups—control and COVID-19R-recovered patients. (C) Heat map of top 20 significantly dysregulated proteins in COVID-19R patients compared to control. (D) Violin plot of six DEPs such as RAB6B, RAB3A, CA2D1, DPEP3, MENT, and IBP5 in COVID-19R. *p-value < 0.05 and **p value < 0.0005.

concentration in control was 42.5 (17−78) million/mL, while it was significantly reduced to 24 (1−72) million/mL in the recovered cohort with a p-value of 0.013. A similar trend was observed in sperm motility where controls had 50% (40−80) motile sperms, but it was significantly lower (p-value 1.130×10^{-5}) at 10% (0−65) in the recovered group. The data also shows a significant difference (p-value 0.005) in sperm morphology between the control group (8.5%) and the recovered group (2.5%). The pH and semen volume did not show any appreciable difference between groups. The detailed information on semen characteristics is presented in Table S2.

Proteome Profiling of Semen in the Sample Cohort. Discovery-based proteomics data were obtained using high-resolution mass spectrometry analysis for 30 samples (details in the Materials and Methodssection), and a label-free quantitation (LFQ) technique was used to detect and quantify the semen proteome. The semen proteome profiling workflow, including sample collection, processing, and discovered and targeted mass spectrometry data analysis, is summarized in Figure 1A−D.

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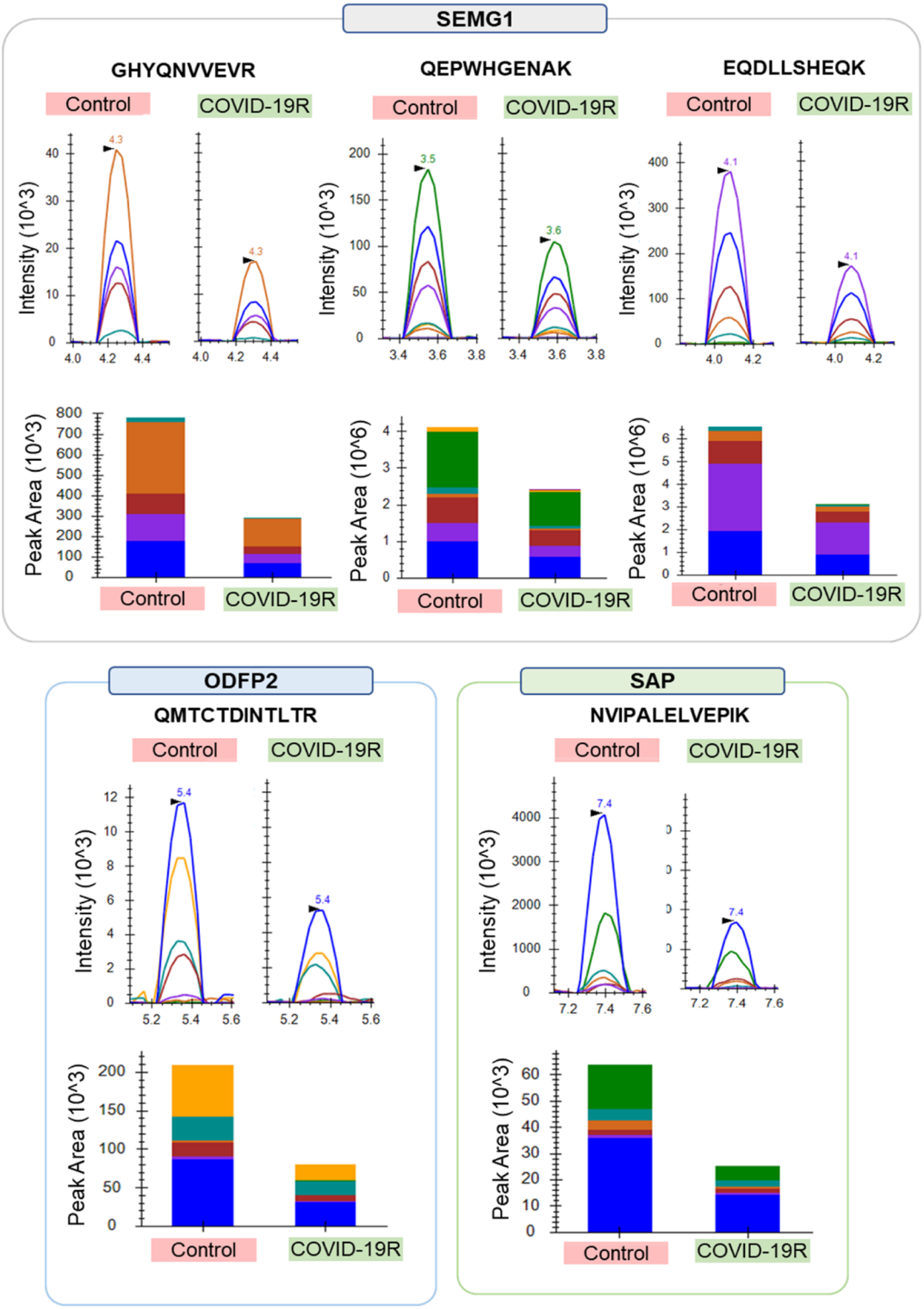

Figure 3. MRM-based validation of proteins selected based on discovery proteomics data. Semenogelin 1 (SEMG1), outer defense fiber protein 2 (ODF2), and prosaposin (PSAP) were downregulated in COVID-19R patients as compared to control. A representative diagram for peak areas of three peptides of SEMG1 and one peptide of each of ODF2 and PSAP is shown. The bar plot represents the difference in the peak areas of these peptides between COVID-19R and control.

A representative total ion chromatogram obtained from liquid chromatography-tandem mass spectrometry (LC−MS/MS) run among all the 30 samples is given in Figure S1A. In addition, the LC−MS parameters used for the study are given in Figure S1B,C. On an average, more than 640 proteins were identified in each sample. Three samples from the COVID-19-recovered group were excluded from further analysis as they provided poor mass spectrometry data.

A sample-wise correlation between the remaining 27 samples showed a correlation score of more than 0.65 on an average (Table S3 and Figure 2A). Thus, the LFQ proteomics data of 27 samples identified 1099 proteins after removing the contami-

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nants and reverse proteins and 8503 peptides (Table S4). These were then subjected to data filtering based on missing values. A list of proteins having less than or equal to 30% missing value was imputed in a group-wise manner by kNN imputation (feature-wise). Totally, 475 missing value imputed proteins were taken forward for supervised clustering using PLS-DA model that showed group segregation between control and COVID-19-recovered patients (Figure 2B).

No significant group separation was observed in the unsupervised clustering, that is, principal component analysis (PCA)-based clustering (Figure S2). Of the 475 proteins, 48 passed the Welch *t*-test (p-value < 0.05) with the absolute fold change equal to or more than 1.5. The analysis revealed 27 proteins to be upregulated and 21 proteins to be downregulated in COVID-19-recovered patients (Table S5). Figure 2C depicts the differential expression of the top 20 statistically significant (both *t*-test and fold change passed) proteins. The violin plots of the proteins Ras-related protein Rab-6B (RAB6B), Ras-related protein Rab-3A (RAB3A), voltage-dependent calcium channel subunit alpha-2/delta-1 (CACNA2D1), dipeptidase 3 (DEPE3), protein MENT (MENT), and insulin-like growth factor-binding protein 5 (IGFBP5) depict the differential expression in COVID-19-recovered patients as compared to control (Figure 2D). A total of 54 and 53 proteins were abundant in COVID-19-recovered and control groups, respectively (Table S4). Therefore, together with the differentially expressed proteins (DEP), these proteins were taken forward for pathway enrichment analysis.

Functional Annotation and Biological Pathway Analysis of Significantly Dysregulated Proteins. In order to identify the biological association of the dysregulated proteins, functional annotation was performed in the PANTHER classification system. Briefly, 105 and 167 gene hits were assigned to molecular function and cellular components, respectively. Most dysregulated proteins have catalytic activity (49%), followed by binding activity (39%), and less than 5% of these proteins were assigned to transduction, translation regulation, and molecular transportation. Moreover, under the "cellular component", the total hits were assigned to three terms: cellular, anatomical entity (52%), intracellular (35%), and protein-containing complex (13%) (Table S6 and Figure S3). The Metascape pathway analysis[23] revealed several significantly up- or downregulated pathways in COVID-19-recovered patients (Table S7 and Figure S4). For example, vesicle-mediated transport, response to wounding, HSP 90 chaperone cycle, and antigen processing and presentation were shown to be upregulated.

Of all the significantly downregulated pathways, eight were directly or indirectly related to the reproductive functions inside the body. Among those, sperm−egg recognition, response to testosterone, regulation of cell motility, and localization correlate with the reproductive function directly. Individual proteins of these pathways were further taken to check their protein−protein interactions using STRING software (Figure S5). Proteins related to sperm−egg recognition and regulated exocytosis showed a strong interaction among themselves. In contrast, proteins related to extracellular matrix (ECM) organization and cell motility pathway were found interacting with each other—the strong interaction between these dysregulated proteins attributed to the significant alteration of their associated pathways.

Validation of Significant Proteins by Targeted Proteomics. Selected reaction monitoring (SRM) aims at targeted monitoring of only the selected list of peptides, hence validating the presence of targeted proteins in a given sample. Based on the shotgun (DDA) data, a total of 10 proteins (downregulated in COVID-19-recovered patients compared to controls) were considered for the validation experiment using the SRM approach. The initial optimization was done in pooled samples for which the transition list was generated in Skyline. Around 399 transitions corresponding to 1 synthetic peptide and 54 peptides of 7 host proteins were selected for the final run based on their consistency in optimization runs (Table S8).

Peptides from a total of 27 samples (10 controls and 17 COVID-19−recovered patients) were subjected to SRM using a triple quadrupole mass spectrometer to validate the selected proteins having known biological significance to male reproductive functions. The instrument quality was monitored with the profile of quality control standards, BSA and MCF-7, run with each set of samples. In addition, the consistency between the experimental runs was confirmed with the presence of heavy labeled synthetic peptide (THCLYTHVCDAIK) spiked equally in all samples (Figure S6A). After data acquisition, MSstats tool inbuilt in Skyline software was used for the statistical analysis.

The targeted proteomics data analysis qualitatively confirmed the trends of the proteins similar to LFQ data. Further, three peptides of semenogelin-1 (SEMG-1) and one peptide each of outer dense fiber protein 2 (ODF2) and prosaposin (PSAP) proteins showed significant downregulation in COVID-19-recovered patients with a p-value at or less than 0.05. In addition, these peptides exhibited more than 2-fold decreased abundance in COVID-19-recovered patients (Table S9 and Figure 3). The intensity of these peptides in each sample is given in Figure S6B−F. The validation of these proteins confirms the accuracy of our proteomic findings and indicates a change in the proteins linked with reproductive functions.

DISCUSSION

Global studies put forward unequivocally that males are getting more affected by COVID-19 than females in severity and fatality.[5] Also, the reproductive age group was more susceptible to SARS-CoV-2 infection.[6,24] Thus, the gender predilection of COVID-19 makes the male reproductive system more susceptible. The presence of ACE2 in the testis[10,11] suggested that the virus may localize within the male gonad, thus provoking an interest in investigating the consequences of SARS-CoV-2 infection on the male reproductive function. Moreover, COVID-19 is a disease characterized by a hyper-inflammatory host response. Inflammatory molecules such as cytokines, interferons, and interleukins are substantially higher in COVID-19 patients.[25] Previous studies have shown that chronic inflammation due to obesity can cause sperm dysfunctions which might be due to reduction in sperm concentration and motility.[26] Also, a pro-inflammatory state induced by chronic infection, smoking, and in response to toxins may lead to impaired male reproduction.[27] Recently, Maleiki et al. have reported the correlation of semen parameter impairment with the higher expression levels of inflammatory markers such as IL-6, IL-8, and TNF-⟨ in the semen of COVID-19-recovered patient.[28] Thus, it is apparent that infection may perturb biological pathways related to the male reproductive system. Our study aimed to dissect the molecular interactors that may lead to an impaired fertility status, which could help clinicians assess male fertility in COVID-19-recovered patients.

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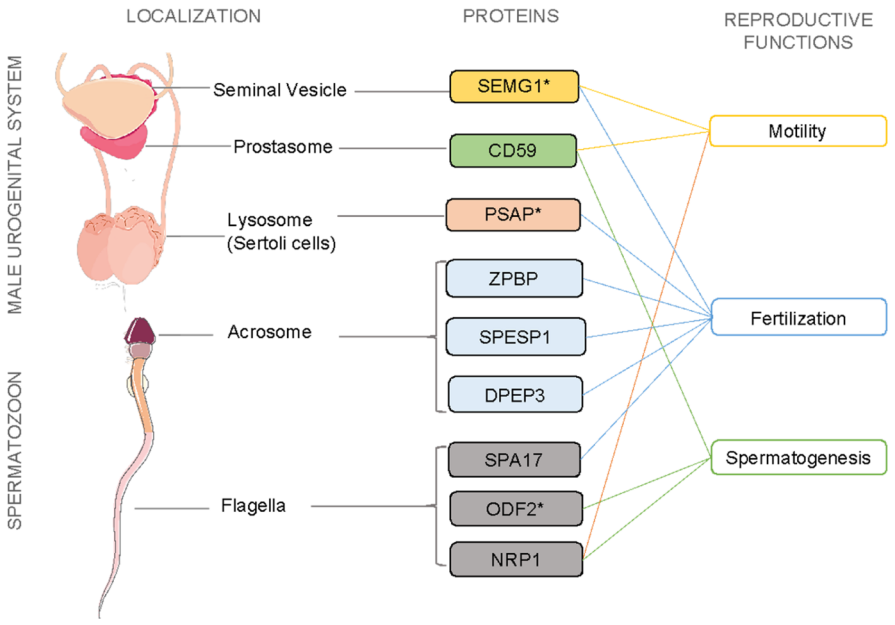

Figure 4. Schematic representation of the association of significant proteins with major male reproductive functions. Proteins shown were found significantly downregulated in COVID-19R (recovered) patients where "*" indicates the proteins validated using the SRM assay. (This figure was created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com).

From sperm production to the release of semen, various organs/structures of the male reproductive system are involved. These majorly include the testes, epididymis, vas deferens, seminal vesicles, Cowper's gland, and prostate gland.[29] Each of them has a specific role in semen formation/release, such as sperm production in testes, maturation in the epididymis, and mixing with fluid/secretions by associated downstream structures.[30] Added secretions by accessory glands may include α-glucosidase, prostaglandins, bicarbonate, and citric acid, which are essential for sperm physiology.[31] The contribution of all these organs is determinant for semen quality, such as seminal vesicles or vesicular glands producing energy-rich fructose that is very important for sperm motility.[31] Problems with any of them can affect sperm production or semen secretion. This study observed impairments in key semen parameters in COVID-19-recovered patients. Such observation might indicate a direct or indirect effect on male reproductive health by interfering with the biological processes related to sperm production or release. Here, we performed deep proteomic profiling of semen procured from 10 fertile controls and compared it with 17 COVID-19-recovered fertile men toward this goal. To the best of our knowledge, ours is the first study reporting the far-reaching effects of SARS-CoV-2 infection at the molecular level in male reproductive health. The LFQ-based proteomic profiling of 27 samples revealed a significant number of dysregulated proteins in COVID-19-recovered patients compared to healthy individuals.

Various studies have reported that the proteins upregulated in COVID-19-recovered patients in this study have roles in different reproductive events. Among those, four members of the Rab family of proteins, RAB3A, RAB3D, RAB7, and RAB6, are known to be involved in intracellular signaling and membrane trafficking and those essential for sperm differentiation were found dysregulated. The RAB3 protein localized in the acrosomal region is essential for acrosomal exocytosis and triggers the acrosomal reaction. RAB7, a known interactor for a RAB GTPase accelerating protein expressed in spermatocytes, plays a crucial role in membrane fusion during capacitation.[32] The alteration of these RAB proteins might affect the vesicular and non-vesicular trafficking and, subsequently, the sperm differentiation method. Studies[33] have shown that voltage-dependent calcium channels mediate crucial sperm functions such as acrosome reaction and capacitation. A dysregulation of voltage-dependent calcium channel subunit alpha-2 delta-1 (CACNA2D1) in our study might indicate altered sperm physiology post-COVID-19 infection.

Fructose-1,6-bisphosphatase 1 (FBP1), a critical rate-limiting enzyme involved in the gluconeogenesis pathway, was found to be upregulated in the COVID19-recovered group. Gluconeogenesis involves the synthesis of glucose from non-carbohydrate sources that, when dysregulated, may lead to hyperglycemia. Patients with COVID-19 disease were found to have hyperglycemia triggered by either infection directly or the medications used to treat the disease.[34] Furthermore, it has been reported that the absence of FBP1 suppresses gluconeogenesis,[35] leading to the inference that increased expression of FBP1 may lead to hyperglycemia that poses deleterious effects on overall male reproductive functions.[36]

GTP-binding nuclear protein (RAN/ARA24) is upregulated in COVID-19-recovered patients in this study. It is reported to act as a coactivator of the androgen receptor under normal conditions by enhancing the androgen receptor N−C (NH_2−COOH) interactions. However, its overexpression can be a risk factor for causing androgen-dependent diseases.[37]

Proteins such as proteasome subunit alpha type-3 (PSMA3) and type 1 (PSMA1) were found upregulated in COVID-19-recovered patients. These are the prostate-specific membrane antigens and are reported to be involved in cell death. A similar trend for these proteins has been observed in individuals with varicocele having low sperm count and decreased sperm quality.[38] Another upregulated protein, 14-3-3 protein epsilon, belongs to the 14-3-3 family of proteins that play vital roles in

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kinase signaling, such as regulating the cell cycle and apoptosis in eukaryotic cells. Their roles in spermatogenesis, normal sperm function, and male fertility have been reported in mice. Such observations imply that dysregulation in proteins involved in apoptotic mechanisms may lead to infertility.[39] Two chaperonin-related protein-coded genes, CCT7 and CCT2, were overexpressed in COVID-19-recovered patients. These are the vital subunit proteins of the molecular chaperone complex CCT/TRiC (chaperonin containing the T-complex/TCP1-ring complex) present in haploid germ cells. In the testes, the CCT/TRiC complex plays an essential role in the differentiation of spermatids. However, some subunits of the CCT/TRiC complex, CCT2, CCT7, and CCT8, in the mammalian epididymal fluid have been reported.[40] The upregulation of this complex or its subunits may interfere with the typical secretory components of semen. One of the SPANX family of proteins, SPNXC, was upregulated in COVID-19-recovered patients. SPANX proteins are sperm proteins associated with the nucleus of the X chromosome. Although their exact role is unexplored, they have been reported to be expressed in normal males throughout the process of spermatogenesis from gamete cell precursors to ejaculated spermatozoa, indicating their link with sperm development.[41] Undoubtedly, these upregulated proteins indicate the deregulation of sperm maturation, acrosome activation, and secretory components of semen.

Among all the significant proteins found to be downregulated in COVID-19, we found a subset of proteins that play a crucial role in reproductive function (Figure 4). Semenogelin (SEMG) is one of the predominant proteins in semen secreted from the seminal vesicle. SEMG plays a crucial role in inducing sperm immobilization and inhibiting premature sperm capacitation.[42] A recent study by Martins et al. showed the under-expression of SEMG in men with primary and secondary infertility, as compared to the healthy controls.[43] The significant downregulation of SEMG1 in our study points toward the impact of infection on sperm motility and capacitation. Another vital protein required for sperm function, the Outer dense fiber protein 2 (ODF2), is also negatively regulated in the COVID-19-recovered group. ODFs constitute a significant component of the sperm tail. Scientists have shown that Odf2 haploinsufficiency in male mice led to decapitated and decaudated spermatozoa.[44] Studies on mice have revealed that a conditional knockout of the Odf2 gene led to the asthenozoospermic condition. It was exemplified by reduced sperm motility and axoneme defects in sperm flagella.[45]

Further, studies on asthenozoospermic men[45] showed that they express less ODF2. Given the inhibition of ODF2 in the proteome and reduced motility of sperms as observed in our recovered patient cohort, it seems that ODF2 inhibition after viral infection can explain the reduced motility. CD59, an inhibitor of membrane attack complex (MAC), is expressed in spermatozoa. It is also present in the extracellular organelle called "prostasome" enriched in seminal plasma. CD59 inhibits the MAC-mediated cell killing, protecting sperms from anti-sperm antibodies in male and female reproductive tracts and maintaining fertilization.[46] A study by Qin et al. confirmed the progression of male infertility in the CD59-deficient mice model.[47] Hence, our findings on the downregulation of CD59 protein in COVID-19-recovered patients insinuate the impairment of the fertilization process.

Another vital protein in semen is neuropilin (NRP1), which acts as a receptor for different growth factors to mediate signal transduction. The primary role of neuropilin in reproduction is the differentiation and self-renewal of spermatogonial stem cells to maintain their pool. Studies have shown that the loss or reduction of NRP1 in Sertoli and Leydig cells results in an increased number of undifferentiated spermatogonia and, finally, reduced fertility.[48] Thus, the alteration of sperm parameters may result from NRP1 underexpression in response to COVID-19 infection. Prosaposin (PSAP) is a precursor molecule of saposin, which mediates the sphingolipid hydrolysis in lysosomes. It has been reported that disruption of the prosaposin gene resulted in size reduction of testis, epididymis, and prostate gland.[49] In this context, the reduced level of prosaposin obtained from our proteomic findings in recovered patients suggests the deleterious effect of COVID-19 on the male reproductive organs. Dipeptidase 3 (DPEP3), a testis-specific membrane-bound protein expressed by testicular germ cells, was found to be highly downregulated in this study. The exact molecular role of DPEP3 in the fertilization or reproduction process is still unclear. Interestingly, an interaction between DPEP-3 and TEX101, a biomarker for male infertility, has been observed to modulate cellular function. Therefore, the decreased level of DPEP3 might affect the DPEP-3-TEX101 interaction and modulate the function of the testis.[50] The array of proteins downregulated in the semen proteome indicates the dysregulation and dysfunction of critical regulators of reproductive processes.

Intriguingly, alterations of all semen proteins could not be uncovered. Along with the significant proteins, we also found a subset of abundant proteins. Additionally, we found three proteins with less abundance in COVID-19-recovered patients, which are directly involved with the fertilization process. Zona pellucida binding protein 1 (ZPBP1) localized on the acrosomal membrane mediates the acrosomal reaction and sperm−oocyte binding during fertilization. Sp17 also contributes to the sperm−oocyte binding process, and studies have also pointed toward its role in a myriad of functions such as sperm maturation, capacitation, acrosomal reaction, and the fertilization process. SPESP-1 is an alloantigen that aids in sperm−egg fusion. Sperm proteomic profiling revealed that the downregulation of ZPBP-1, Sp17, and SPESP-1 is associated with male infertility.[51−54] The low abundance of these proteins in COVID-19-recovered patients corroborates the possibility of fertility impairment post-viral infection.

The functional enrichment analysis of the dysregulated proteins in recovered patients revealed their involvement in several biological processes. The primary reproductive processes found to be dysregulated in recovered patients are sperm−egg recognition and response to testosterone. Proteins such as ZPBP1, Sp17, SPESP-1, MSAP, GLCM, and TSP1 related to this pathway showed underexpression or low abundance in recovered patients, thus corroborating the negative impact of COVID-19 on male fertility. In addition, several ECM proteases, such as ADAM9 and HTRA1, glycoproteins, such as FBLN2 and DAG1, and other proteins, including TGFB1 and PEPD involved in the ECM organization, were present with low abundance in COVID-19-recovered patients. ECM has a potential role in regulating spermatogonia and Sertoli cells at different developmental stages, including blood−testis barrier dynamics.[55] Thus, the dysregulation of ECM organization in response to viral infection may alter the function of Sertoli cells and germ cells and disrupt male fertility.

Substantial evidence indicates the role of amyloid fibers in various reproductive processes, including gametogenesis, sperm maturation, and fertilization.[56] A recent study by Sonesson et al.

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confirmed the presence of serum amyloid p component and its correlation with sperm concentration.[57] Downregulation of serum amyloid p component (SAMP) in recovered patients indicates the disruption of amyloid fiber formation and crucial reproductive processes due to SARS-CoV-2 infection. Besides, pathways such as glycosylation, regulated exocytosis, vesicle-mediated transport, and membrane protein proteolysis, which are essential biological processes for fertilization, are also dysregulated in COVID-19-recovered patients.[58] These findings indicate the systemic dysfunction of the milieu of the reproductive system in COVID-19-recovered males.

Based on our findings, two insights can be gained. First, although COVID-19 primarily precipitates into a respiratory disorder in severe cases and leads to multi-organ failure and death, there is an impact on other organ systems even in mild and moderate COVID-19.[59,60] These effects transcend the boundaries of the respiratory system. Whether this is an impact of the virus or the "butterfly effect" of the infection it causes remains to be understood, but the effect is profound in COVID-19 survivors. Hence, there is a need to assess fertility-related issues in recovered patients carefully. The second significant observation is that even though the patients of COVID-19 appear to have recovered clinically, biological processes such as reproduction may be compromised. To further understand these processes, it would be intriguing to validate more of these dysregulated proteins in a more significant cohort split into grades of disease severity and long-term recovered patients longitudinally to confirm the implications of COVID-19.

CONCLUSIONS

The comprehensive proteomics analysis of the semen of individuals recovering from COVID-19 provides an invaluable proteomic resource to the scientific community to decipher the implications of COVID-19 on male reproductive health. It sheds light on the significant alteration of reproductive function at the molecular level and identifies several dysregulated proteins and pathways associated with the reproductive process. The current study on proteomics analysis of semen from COVID-19-recovered patients can enlighten the physicians and reproductive biologists about the sequelae of COVID-19 on male reproduction and help formulate strategies to avoid or minimize bearing of COVID-19 on male reproduction.

MATERIALS AND METHODS

Selection of Participants for the Study. The Institutional Review Board of Jaslok Hospital approved this pilot study vide letter no. EC/10509/2020 dated 12 November 2020. All individuals provided written consent for the study. Semen samples were collected from 10 healthy individuals (control group) and 20 COVID-19-recovered individuals. The control group included fertile men who were biological fathers and were seronegative for the antibody test against the SARS-CoV-2 antigen (Meril COVID-19 IgG/IgM rapid test, Product code-NCVRPD-02). The COVID-19-recovered group included men who had earlier tested positive for SARS-CoV-2 by qRT-PCR by oral and nasopharyngeal swab (Trupcr SARS COVID-2 RT qPCR kit, catalog no. 3B308), had mild to moderate symptoms during their infection, did not require any antiviral therapy or steroids, and were subsequently in clinical remission. All COVID-19-recovered men had fathered at least one child by natural conception, and they had no history of infertility. Sample collection from the recovered patients was done after their quarantine period of 17 days (the quarantine period started from the day of testing positive). Control individuals had typical semen parameters as per the WHO guidelines of 2010. The detailed clinical information of these individuals included is given in Table S1A,B.

Inclusion and Exclusion Criteria. Males between 20 and 45 years of age were included in the study. All individuals enrolled in the study were non-diabetic, non-smokers, and did not consume alcohol. These men did not have any history of prior exposure to harmful radiation, chemicals, or external trauma that may otherwise be detrimental to their reproductive organs. Men with a demonstrated history of azoospermia, oligozoospermia, leukocytospermia, asthenozoospermia, asthenoteratozoospermia, oligoasthenozoospermia, and oligoasthenoteratozoospermia were excluded. Also, men under supportive medication, such as steroids, chemotherapy, antiviral treatment, or other medications affecting the reproductive system, were excluded. Additionally, those with sexually transmitted diseases and diseases causing either systemic or reproductive tract inflammation were excluded from the study.

Sample Collection, Preparation, and Processing for Proteomics Analysis. The sample collection, storage, assessment of semen parameters, and protein extraction were performed following the applicable guidelines and regulations of the WHO laboratory manual (2010). Semen samples were obtained by masturbation into sterile containers after 3 days of abstinence from sexual activity. All COVID-19 appropriate protocols were followed. After collection, the samples were kept at 37 °C for 30 min for liquefaction before analysis. A dedicated room and laminar flow were assigned for this purpose. Semen viscosity, pH, and volume were measured. Qwik Check Diff Quik was used to assess sperm morphology, whereas sperm motility was determined with a phase contrast microscope (Carl Zeiss Trinocular Microscope). Before processing for proteomics, the samples were subjected to heat inactivation of the virus[61] at 56 °C for 30 min as a precautionary step and then stored at −80 °C. From the semen samples, the proteins were precipitated with ethanol, followed by sonication in urea lysis buffer. Then, centrifugation was done at 8000*g* for 15 min, and the clear supernatant was collected (Figure 1).

The protein extracts were sent for proteomics analysis at MASSFIITB (Mass Spectrometry Facility at IIT Bombay). LFQ-based discovery proteomics was employed to investigate the persistent effect of viral infection on the male reproductive system. The protein amount in each sample was estimated using Bradford assay, and 30 μg of the protein was taken forward for digestion. Before tryptic digestion, the protein was reduced with Tris carboxyethyl phosphine with a final concentration of 20 mM for 1 h at 37 °C and alkylated iodoacetamide with a final concentration of 40 mM for 15 min in the dark. Next, the digested peptide was desalted to remove contaminants using a C-18 stage tip. The clean-up peptides were vacuum-dried, reconstituted in Milli-Q water with 0.1% formic acid, and quantified using the Scopes method. Finally, 1 μg of the peptide was injected for the LC−MS/MS run. A high-resolution Orbitrap Fusion Tribrid Mass Spectrometer coupled to an easy nano-liquid chromatography (LC) system was used in this study to acquire the proteomic data in a data-dependent manner. The settings used for the LC−MS/MS runs were as described earlier.[62]

Quantitative Proteomics Analysis by MaxQuant. The mass spectrometric raw data sets were processed using the LFQ-based parameter in MaxQuant[63] using the default parameters

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against the UniProt human database (downloaded on March 11, 2021) release date 10.02.2021 (first release of 2021). Furthermore, using the built-in search engine, Andromeda, only proteins having a maximum of 1% false discovery rate and unique peptides greater than 1 were selected to increase the reliability of the data obtained. The detailed parameters of MaxQuant are given in File S1.

Statistical Analysis Using MetaboAnalyst. The MaxQuant-analyzed files of 30 samples were taken forward for sample-wise correlation analysis in MetaboAnalyst[64] (version 5.0). Unfortunately, three samples did not provide good mass spectrometric spectra and were removed as outliers. The proteomic data of the remaining 27 samples were analyzed for PCA and PLS-DA-based cluster analysis. The missing values of the features having LFQ intensities in more than 70% of each group were imputed separately by the KNN algorithm and considered for differential protein expression analyses. Log-transformed and median normalized data were taken for statistical analysis (i.e., *t*-test and fold change). The two-sample *t*-test (Welch *t*-test) was performed to identify the significant DEPs, where a *p*-value of 0.05 was set as the maximum threshold value. Among all the *t*-test passed proteins, proteins with fold change values greater than or equal to 1.5 were considered significantly DEPs. Moreover, a panel of proteins was found to be abundant in one group depending on the percentage of missing values. Finally, gene ontology and pathway analysis were performed for both DEPs and abundant proteins.

Pathway and Protein−Protein Interaction Enrichment Analysis. The gene ontology classification of 155 dysregulated proteins (DEPs and abundant proteins) in COVID-19 recovered patients obtained from the shotgun proteomic data was performed in the PANTHER classification system (version 16.0), whereas the biological process and pathway enrichment analysis were done in Metascape.[23] In the pathway enrichment analysis, features having a *p*-value less than 0.01 were considered statistically significant. In addition, the network visualization was done in Cytoscape, and the protein−protein interaction analysis for the selected pathways was done in STRING (version 11.0).

Validation by the SRM Assay. The statistically significant downregulated proteins in COVID-19-recovered patients compared with control or healthy individuals obtained from the LFQ data were selected for SRM-based targeted analysis. An overview of the targeted proteomics approach is illustrated in Figure 1D. The unique peptides of the selected proteins were screened in Skyline (Ver 20.2.1.286), and a transition list for the same was prepared using human proteome FASTA for background proteome. The missed cleavage criterion was set as 0. All y ions (from ion 3 to the last ion 1) corresponding to singly and doubly charged product ions of doubly charged precursors were monitored for selected peptides.

Pooled samples from both the groups, COVID-19 recovered and control, were run against all the generated transitions. A final list of transitions was prepared (consisting of 10 proteins) based on the consistency of peptide peaks in pooled samples. All samples were spiked in with a heavy labeled synthetic peptide (THCLYTHVCDAIK—labeled at C-terminal arginine) for monitoring the consistency of the mass spectrometry runs. In addition, the standard protein BSA and a cell line sample (MCF-7) were run before and after each batch of samples were run as instrument quality checks. The SRM data was acquired using a TSQ Altis Triple Quadrupole Mass Spectrometer (Thermo) coupled with an HPLC system described earlier.[62] Data were analyzed using Skyline software.[65]

Limitations of the Study. Being an exploratory pilot study, we could sample only a limited number of patients. The cohort size, therefore, is a primary limitation of the study. Further, due to the same reason, we could not segregate the patients based on symptoms and disease severity, and therefore, the potential impact of disease severity on the semen parameters could not be assessed. Moreover, this study lacks a disease control group of men recovering from other flu-like infections that would have enabled an understanding of whether the findings represent a response to systemic infection primarily rather than to COVID-19 specifically.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c06551.

Clinical information of COVID-19 recovered patients (Table S1); demographic representation of semen parameters in all individuals (Table S2); sample-wise correlation analysis (Table S3); label free quantification datasheet (Table S4); significant differentially expressed proteins and their fold change (Table S5); functional annotation of proteins dysregulated in COVID-19 recovered group (Table S6); pathways upregulated and downregulated in COVID-19 recovered group (Table S7); SRM peptide and transition list (Table S8); comparison analysis of significant DEPs between control and COVID-19 recovered (Table S9); total ion chromatogram and MS settings used for the study (Figure S1); principal component analysis of control and COVID19-R (Recovered) patients (Figure S2); major molecular function and cellular components for all the dysregulated proteins in COVID19-R (recovered) vs control (Figure S3); significant upregulated and downregulated pathways in COVID19-R (recovered) patients as compared to control (−log10 $P > 2$) (Figure S4); a protein-protein interaction network analysis of a few downregulated proteins associated with reproductive function (Figure S5); comparative SRM intensity of significant peptides across the sample cohort (Figure S6) (PDF)

MaxQuant parameter (PDF)

Semen proteomics of COVID-19 (XLSX)

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S.G., S.P., and M.U.N. contributed equally. F.P., S.P., S.S., S.G., and M.U.N involved in the concept and design. P.M. and Arundhati Athalye involved in sample collection. P.M., Arundhati Athalye, S.G., M.U.N., and Arup Acharjee involved in sample preparation. S.S., S.G., M.U.N., and A.B involved in mass spectrometry analysis and data acquisition. S.G., D.P., Arup Acharjee, A.S., and A.B. involved in statistical data analysis and data visualization. M.U.N. and S.G. involved in MRM-based validation experiment and analysis. S.G., M.U.N., A.A., F.P., S.S., and M.L. involved in manuscript preparation.

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Notes

The authors declare no competing financial interest.
All data relevant to this study are present in the Supporting Information files. The mass spectrometry-based shotgun proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD026703 (User name: reviewer_pxd026703@ebi.ac.uk, Password: IDUq0Ss4). The SRM-based targeted proteomics data has been submitted to the Panorama Public repository and can be accessed using the link https://panoramaweb.org/covid19semen.url (reviewer account details: panorama+reviewer40@proteinms.net, password: shgvHJNS).

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