



# Identification of intrinsically disorder regions in non-structural proteins of SARS-CoV-2: New insights into drug and vaccine resistance

Farah Anjum<sup>1</sup> · Taj Mohammad<sup>2</sup> · Purva Asrani<sup>3</sup> · Alaa Shafie<sup>1</sup> · Shailza Singh<sup>4</sup> · Dharmendra Kumar Yadav<sup>5</sup> · Vladimir N. Uversky<sup>6</sup> · Md Imtaiyaz Hassan<sup>2</sup>

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## Abstract

The outbreak of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) emerged in December 2019 and caused coronavirus disease 2019 (COVID-19), which causes pneumonia and severe acute respiratory distress syndrome. It is a highly infectious pathogen that promptly spread. Like other beta coronaviruses, SARS-CoV-2 encodes some non-structural proteins (NSPs), playing crucial roles in viral transcription and replication. NSPs likely have essential roles in viral pathogenesis by manipulating many cellular processes. We performed a sequence-based analysis of NSPs to get insights into their intrinsic disorders, and their functions in viral replication were annotated and discussed in detail. Here, we provide newer insights into the structurally disordered regions of SARS-CoV-2 NSPs. Our analysis reveals that the SARS-CoV-2 proteome has a chunk of the disordered region that might be responsible for increasing its virulence. In addition, mutations in these regions are presumably responsible for drug and vaccine resistance. These findings suggested that the structurally disordered regions of SARS-CoV-2 NSPs might be invulnerable in COVID-19.

**Keywords** SARS-CoV-2 · COVID-19 · Intrinsically disordered proteins · Vaccine development · Molecular pathogenesis · COVID-19 therapeutics

## Introduction

Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) causing coronavirus disease 2019 (COVID-2019) has been the leading cause of deaths [1]. Various ups and downs in the SARS-CoV-2 infection pattern have been accredited to the mutations in the structural proteins of the virus, especially spike (S) protein [2]. Presently, the total number of confirmed cases across the globe stands at > 350 million, whereas 5.6 million people have died (assessed on 25th January 2022) [3]. Many nations have countersigned the rapid spread of SARS-CoV-2 infection in different waves after certain time intervals [4–6]. Scientists have cautioned in contradiction of the forthcoming peaks of the present waves and the coming of new waves, which are yet to come in many countries [7, 8]. The severity of SARS-CoV-2 infection has led to the thorough lockdown in most parts of the world, leading to the physical and psychological impact on people [9–13].

SARS-CoV-2 has four capsid-forming structural proteins: spike protein (S) that assists in attaching the virus to the ACE receptor of host cells [14]; membrane protein (M)

✉ Dharmendra Kumar Yadav  
dharmendra30oct@gmail.com

✉ Md Imtaiyaz Hassan  
mihassan@jmi.ac.in

<sup>1</sup> Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

<sup>2</sup> Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi 110025, India

<sup>3</sup> Department of Microbiology, University of Delhi, New Delhi 110021, India

<sup>4</sup> National Centre for Cell Science, NCCS Complex, Ganeshkhind, SP, Pune University Campus, Pune 411007, India

<sup>5</sup> College of Pharmacy, Gachon University of Medicine and Science, Hambakmoeiro, Yeonsu-gu, Incheon City 21924, South Korea

<sup>6</sup> Department of Molecular Medicine and USF Health Byrd Alzheimer's Institute, Morsani College of Medicine, University of South Florida, Tampa, FL 33612, USA

that forms viral membrane for encircling the mature viral particles [15]; nucleocapsid protein (N) that creates a viral protein coat, i.e., nucleocapsid for surroundings the genetic material [16]; and envelope protein (E) that forms the envelope for assembling the virions [17]. The SARS-CoV-2 genome is more than 80% similar to a previous SARS-CoV strain that triggered an outbreak in 2003 [18, 19]. Consequently, it exhibits a similar replication process as witnessed in the earlier cases.

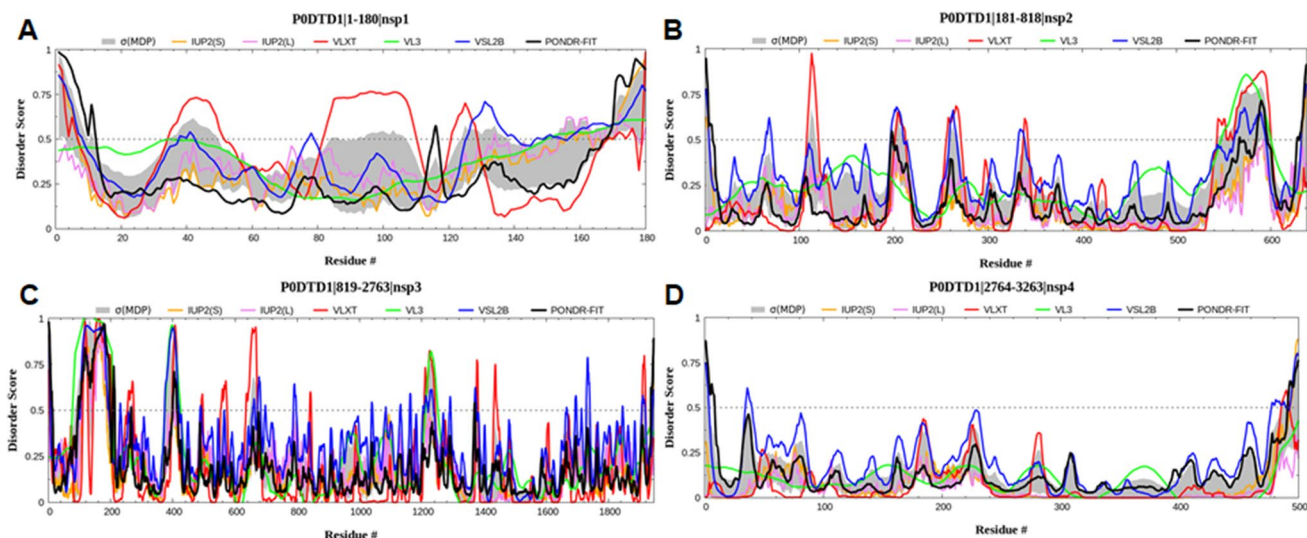
Apart from the capsid-forming structural proteins, the SARS-CoV-2 genome encodes many non-structural proteins (NSPs), playing significant roles in the replication and virus assembly [20, 21]. NSPs participate in SARS-CoV-2 pathogenesis by controlling early transcription regulation, transactivation, helicase activity, immunomodulation, and disputing the antiviral response [22–24]. A few essential functions of the SARS-CoV-2 NSPs are RNA-binding, transferase activity, ATP-binding, zinc-binding, endopeptidase activity, RNA-dependent RNA polymerase activity, exoribonuclease activity, and methyltransferase activity. They also participate in numerous biological processes such as transcription, replication, protein processing, and proteolysis [25].

Similar to other viral proteomes, the SARS-CoV-2 proteome has chunks of intrinsically disordered regions. It is important to explore the disorder status in NSPs to better understand the roles of these proteins in the virulence. We performed a sequence-based analysis on all NSPs specific to SARS-CoV-2 to get insights into their intrinsic disorder status to look for functions of the disordered regions and their roles in viral replication. Here, we provide a computational structural disorder landscape

of the SARS-CoV-2 NSPs. The available sequence data for SARS-CoV-2 NSPs were analyzed to evaluate their intrinsic disorder in light of their biological activity relationships.

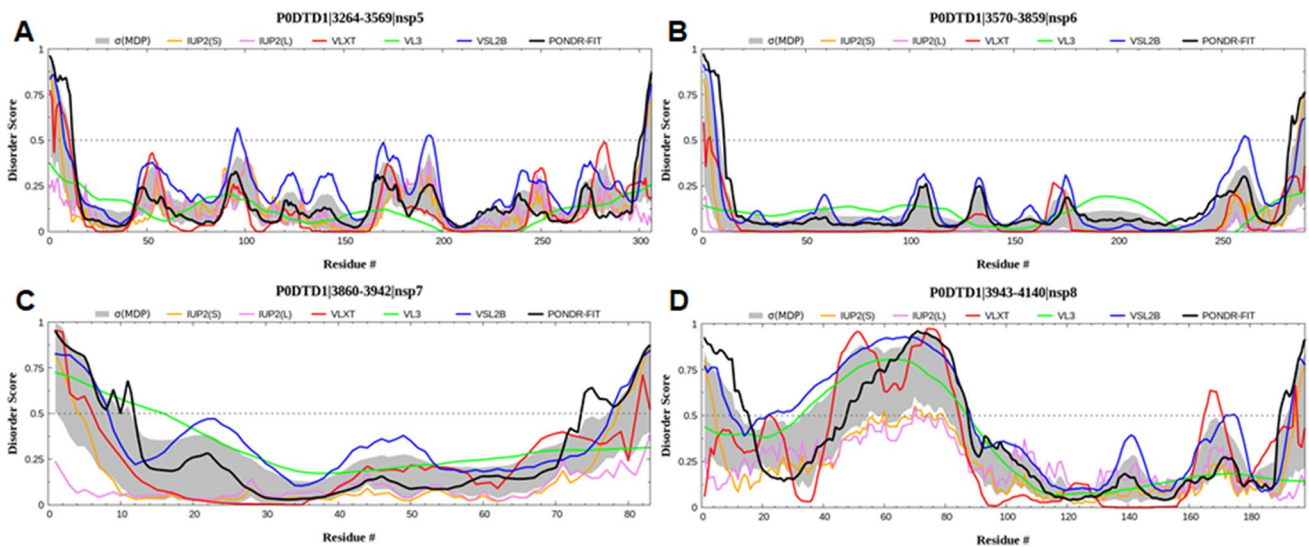
## Material and methods

The sequence data of the SARS-CoV-2 NSPs were taken from the UniProt [26] (UniProtKB: P0DTD1) and GenBank® [27]. To explore the intrinsically disordered regions in SARS-CoV-2 NSPs, multiple bioinformatics tools such as PONDR-FIT® (Predictor of Natural Disordered Regions), VLXT, VL3, VSL2B [28] and IUPred2A web servers (IUP2(S) and IUP2(L)) [29] along with  $\sigma$ (MDP). These predictors classify intrinsically disordered regions in a protein by predicting the residues which do not show the tendency to form a tertiary structure in the native conditions. The predictors consider a residue intrinsically disordered when it scores  $> 0.5$  and flexible to the residue with a score of  $0.2–0.5$ . During the analyses, the residues in each NSP were renumbered from 1. However, their original positions are shown in the titles of the figures (Figs. 1, 2, 3, 4). The analysis provides an insight into the intrinsically disordered regions in SARS-CoV-2 NSPs that may be valuable for understanding the SARS-CoV-2 virulence [25, 30].



**Fig. 1** Graph showing the disordering tendency of each residue in SARS-CoV-2 **a** NSP1, **b** NSP2, **c** NSP3, and **d** NSP4. The middle line is the threshold value of the PONDR score, i.e., 0.5. The residues

in each NSP were renumbered from 1; however, their original positions are shown in the titles of each panel



**Fig. 2** Graph showing the disordering tendency of each residue in SARS-CoV-2 **a** NSP5, **b** NSP6, **c** NSP7, and **d** NSP8. The middle line is the threshold value of the POND-R-FIT score, i.e., 0.5

## Results and discussion

Two-thirds of the SARS-CoV-2 genome comprises ORF 1a/b genes, leading to the production of two different replicase polyproteins, pp1a and pp1b, respectively. These polyproteins undergo further processing by proteases to produce 16 NSPs that assist in replication, transcription, assembly, and packaging of the virion particles [31]. These proteins regulate virus functions, and targeting these proteins is therefore ideal for devising treatment against the coronavirus.

### Non-structural protein 1 (NSP1)

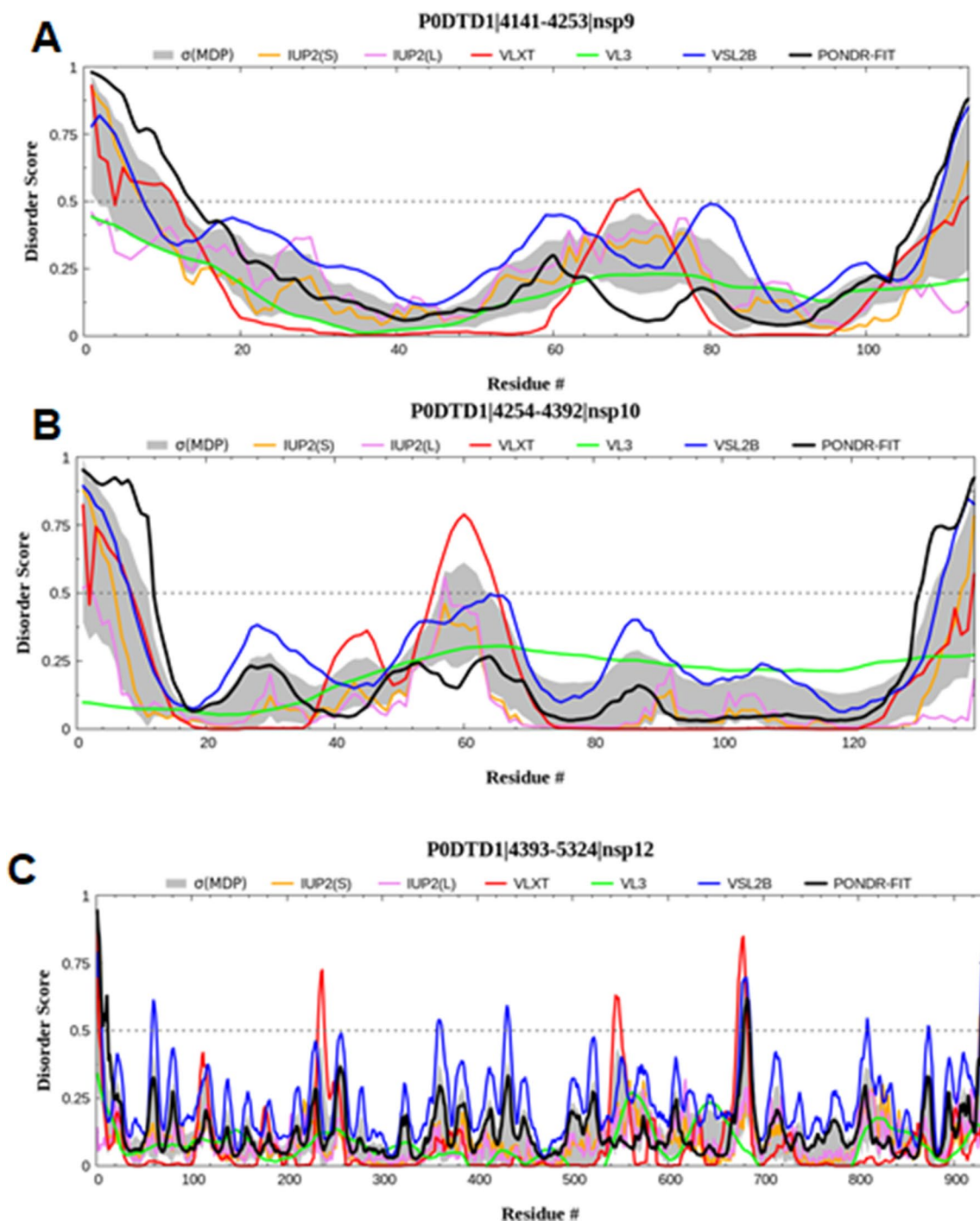
A papain-like proteinase (PLpro) cleaves replicase polyprotein of coronaviruses to yield an N-terminal product called NSP1 [32]. The main functions associated with the NSP1 include degradation of viral mRNA, blocking of host cell translation process, and inhibition of the host's innate immune response to initiate a successful viral replication [33–35]. The viral evasion successfully suppresses the host genes, allowing the virus to control its immune system [36, 37]. NSP1 blocks translation by binding to the 40S subunit of rRNA, thus preventing the entry of host mRNA for subsequent translation [38]. This binding is initiated by the NSP1 carboxyl-terminal domain of SARS-CoV-2 [39]. Earlier studies have demonstrated that the deletion of the *NSP1* gene in infectious virus particles resulted in the inability of the virus to infect the culture cells [40]. Mutations in the ORF1a polyprotein that prevented the release of NSP1 resulted in the limited viability of the virus [41].

In general, NSP1 consists of an  $\alpha$ -helix located at either side of seven stranded  $\beta$ -barrel with two  $3_{10}$  helices positioned across one side of  $\beta$ -barrel. Clark et al. reported the sequence and structural similarities of NSP1 from SARS-CoV-1 and SARS-CoV-2. However, minor differences were observed, which may be responsible for the difference in their viral pathogenic life cycles [42]. Firstly, an extra  $\beta$ -strand and  $3_{10}$  helices were found in SARS-CoV-2. Secondly, an increased polarity between the amino acids and the globular domain resulted in alternative conformations of major loops in SARS-CoV-2. Thirdly, the differences in the amino acids showed different electrostatic surface potentials. These differences might have defined altered SARS-CoV-2 behaviour in terms of pathogenicity and infectivity. Very few structural studies have been done on NSP1, but it seems like a potential area to be explored in the coming time to identify its ability to be used as an antiviral drug treatment [42–47].

In the analysis of the intrinsic disorder's predisposition, the generated graph showed the disordering tendency of each residue in NSP1, where higher values correspond to a higher probability of being disordered (Fig. 1a). The graph indicated that NSP1 has many intrinsically disordered regions lacking well-defined structures in native circumstances. The region amino acid residues 25–50, 80–110 and 120–140 of NSP1 showed a higher tendency of disorder as predicted by one or more predictors.

### Non-structural protein 2 (NSP2)

NSP2 plays a significant role in modifying the host's environment, making it more suitable for viral needs. This protein is also involved in misbalancing the host's intracellular

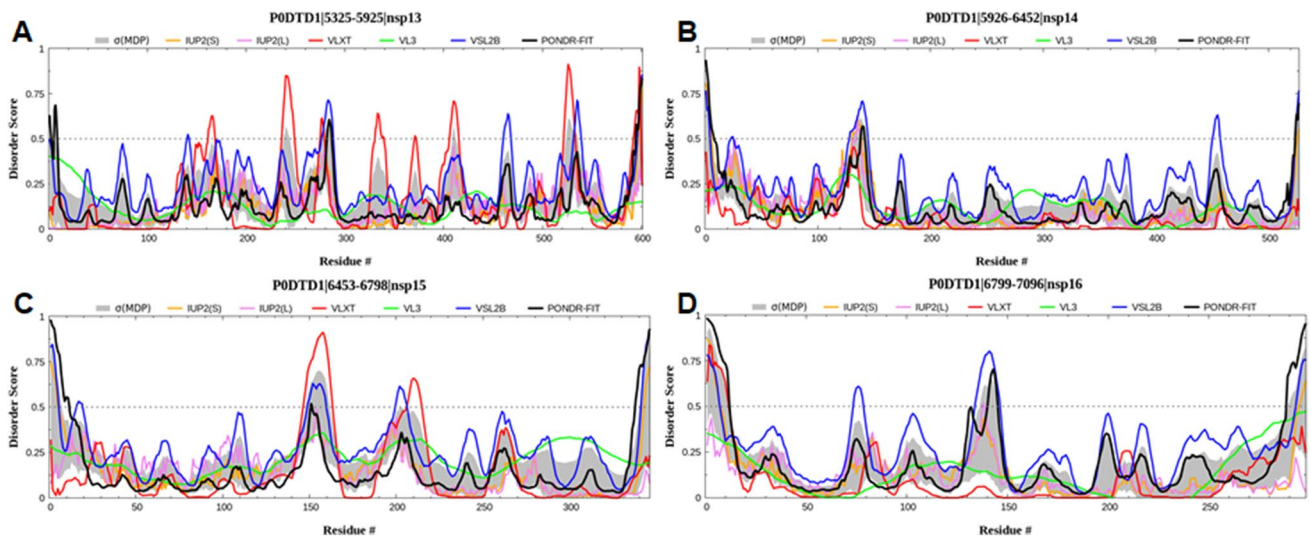


**Fig. 3** Graph showing the disordering tendency of each residue in SARS-CoV-2 **a** NSP9, **b** NSP10, and **c** NSP12. The middle line is the threshold value of the PONDR score, i.e., 0.5

signaling pathways, though the precise mechanism of this function is not yet known. NSP2 possesses variability among different strains of coronaviruses [48]. Compared to bat-SARS like coronavirus, a polar amino acid was found at position 321 of the NSP2 protein compared to SARS-CoV-2, which contained a polar glutamine residue. The stability of

this protein may be due to this change in polarity, allowing it to make hydrogen bonds, and due to the changes in the side chain length and interactions. A stabilizing mutation within the endosome-associated protein-like domain of NSP-2 was found in SARS-CoV-2, suggesting their highly contagious nature [49].





**Fig. 4** Graph showing the disordering tendency of each residue in SARS-CoV-2 **a** NSP13, **b** NSP14, **c** NSP15 and **d** NSP16. The middle line is the threshold value of the POND-R-FIT score, i.e., 0.5

The interactors of coronavirus NSP2 proteins were identified, such as prohibitin 1 and prohibitin 2 having a profound role in mitochondrial biogenesis [50]. Evaluation of various other interactors by Davies et al. revealed their presence in the endoplasmic reticulum (ER) and mitochondrial membrane, suggesting the involvement of NSP2 in the regulation of ER  $\text{Ca}^{2+}$  ion signaling [48]. NSP2 interacts with NSP3 and NSP4 to mediate different functions in promoting the viral infectious life cycle [51]. The complex ERLIN1/2 was found to interact with NSP2 and NSP4 present in both SARS-CoV and SARS-CoV-2 but absent in bat-like coronavirus. The interaction of these two NSP proteins with this complex might regulate ER  $\text{Ca}^{2+}$  signaling and the associated host responses related to these signaling cascades. NSP2 interaction with NSP3 is involved in the formation of proteases which cleaves ORF1ab [52]. More significant studies are required in this direction to identify their role in imparting viral pathogenesis fully.

The intrinsic disorder profile indicated that the NSP2 has several intrinsically disordered regions that lack a stable structure under physiologic conditions. The regions spanning residues 260–300, 370–410, 430–470, and 730–780 of NSP2 showed a higher tendency of disorder as predicted by all four predictors (Fig. 1b).

### Non-structural protein 3 (NSP3)

NSP3 is a multi-pass transmembrane protein consisting of ~1945 amino acid residues. It is primarily associated with translating viral mRNA transcripts and inhibiting protein synthesis in the host [53, 54]. Apart from this, it is also involved in delegating and deubiquitinating activities [55].

An interaction of NSP2 and NSP3 mediates cleavage of ORF1ab by coding for viral proteases. Such processing of polyprotein releases NSP1, NSP2, and NSP3. NSP3 also interacts with NSP4 where they, together in complex with other proteins, are involved in structural membrane rearrangement, facilitating viral replication [56].

The NSP3 structure consists of different functional domains. These domains include a SARS unique domain (SUD) having N-terminal, middle, and C-terminal sub-domain [57, 58]. In addition to this, an RNA binding domain facilitates the viral protein interaction with hosts rRNA [59], and the papain-like protease (PL-PRO) domain allows to code for proteases that regulate the full viral activity [60]. The intrinsic disorder graph showed that NSP3 has multiple intrinsically disordered regions distributed throughout the protein (Fig. 1c).

### Non-structural protein 4 (NSP4)

NSP4 plays a key role in replicative structural assembly for coronavirus replication associated with the NSP3 protein [61]. The loss of the NSP3-NSP4 complex was associated with abolished viral replication [56]. NSP4 maintains interactions with many other proteins and cofactors to bring about other functions. These interactions are unique to different members of coronaviruses, such as interaction with members of the E3 ubiquitin ligase family in SARS-CoV and interactions with factors associated with ER homeostasis in SARS-CoV-2 [48]. Other commonly observed interactions of NSP4 were observed with proteins related to unfolded protein response (UPR) (TMEM33), ER-phagy (CCPG1), and machinery associated with *N*-linked glycosylation

(STT3B, MAGT1, CANX, DDOST). These signaling factors mediate an antiviral innate immune response (RNF5, an E3 ubiquitin ligase present in ER) [62, 63] and Vitamin K reducing complex (VKORC1), which is required by coagulation, causing proteins as a cofactor [64]. Like NSP2, these interactors are localized in the membranes of mitochondria and ER, regulating their role in mediating major functions related to the contact site like calcium homeostasis, biogenesis and cell organization, transport, and various other metabolic processes. Some of the NSP4 interactors were also found to have a role in cell communication, cell differentiation, and cell death [48]. The intrinsic disorder analysis suggested that the NSP4 doesn't have many disordered regions (Fig. 1d).

### Non-structural protein 5 (NSP5)/3C-like proteinase (3CLpro)

NSP5, a ~305 amino acid long protein, is responsible for the maturation of other NSPs in the coronaviruses [65]. NSP5 is a cysteine protease that is automatically cleaved from the polyprotein to produce a mature enzyme, cleaving the polyprotein at 11 different locations to yield NSP4-NSP16 [66]. NSP5 is also referred to as 3CL<sup>pro</sup>, which has three distinct domains I-III, where domain II and domain III are connected via a loop consisting of a long amino acid stretch from 185 to 200 residues. The active site of this protein is in the form of a catalytic dyad (CysHis) located within domain I and domain II [67]. The catalytic residues (Cys145 and His41) and 3-domain structure are conserved among the NSP5, providing them the ability to mediate the viral activity by acting as a protease in viral maturation [68]. The intrinsic disorders graph indicated that the NSP5 does not have many disordered regions except at around residues 3360–3370 and 3530–3550 (Fig. 2a).

### Non-structural protein 6 (NSP6)

NSP6 is a ~290 amino acid long, a multi-pass membrane protein that induces the formation of double-membrane vesicles (DMV) in the host [69]. NSP6, along with NSP3 and NSP4, plays an important role in the coronavirus replication by forming a part of the replication/transcription complex (RTC) [70]. Several other NSPs are also involved in this complex, each assigned specific roles. Once NSP6 is inserted into the ER [71], it complexes with NSP3 and NSP4 for the formation of DMV during assembly of RTC [69].

NSP6 is 34 kDa in size and consists of a C-terminal domain and six transmembrane helices [72]. The outer surface of NSP6 marks the presence of phenylalanine residues, providing higher binding affinity and stability between NSP6 and ER membrane [73]. This protein is commonly present in both  $\alpha$  and  $\beta$  coronaviruses, and its location is central to

ER, where it assists in forming autophagosomes [74]. This binding reduces the transfer of viral factors to lysosomes and thus promotes coronavirus replication [75]. Therefore, the expansion of the autophagosome is also compromised either by starvation or through chemical inhibition of mTOR signaling [76, 77]. NSP6 sends immunomodulatory proteins synthesized by the ER to autophagosomes for their destruction, a process that modifies the adaptive immune response of the host. In addition, NSP6 interaction with the sigma factor helps their participation in ER stress response [78]. The intrinsic disorder graph indicated that the NSP6 does not have many disordered regions, except at the end of its C-terminal region (Fig. 2b).

### Non-structural protein 7 (NSP7)

NSP7 is a small protein of 83 amino acid residues in length. In association with NSP8, this protein acts as a cofactor for the activity of NSP12, which is RNA-dependent RNA polymerase (RdRp) required for viral transcription [79]. These three NSP forms a trimeric RdRp-NSP7-NSP8 super-complex [80], the basic minimal machinery required for nucleotide polymerization [79]. Mutations in NSP7 or NSP8 are associated with mutations of NSP12, and they might therefore show altered super-complex formation affecting the viral replication, infectivity, and pathogenicity [81]. Since NSP7 has less significance when unbound, few studies have been conducted on its structure. More studies on the super-complex formation and its structure have been performed, where targeting one NSP could inhibit the process of viral replication, and transcription is majorly studied. The intrinsic disorder graph suggested that NSP7 does not have any disordered regions (Fig. 2c).

### Non-structural protein 8 (NSP8)

NSP7, NSP8 forms a hexadecamer and acts as a cofactor for NSP12 activity required for viral replication. NSP8 is made of ~198 amino acid residues. Mutations of NSP8 were linked to the altered RNA synthesis in SARS-CoV-2 [82]. This protein may possess RNA processivity or RNA primase function [83]. NSP8 also interacts with NSP9 and is involved in replicating RNA virulence and promoting the virulence properties of SARS-CoV-2 [84]. More studies are warranted in assessing the impact of mutations on NSP8 linked to the RdRp-NSP7-NSP8 super-complex. Detailed understanding of the NSP8 structure is also necessary, as fewer studies have been performed to date. The intrinsic disorder graph showed that NSP8 has a chunk of intrinsically disordered regions distributed between residues 3960–4030 and 4110–4120 (Fig. 2d).

### Non-structural protein 9 (NSP9)

NSP9 consists of ~ 113 amino acid residues in length and primarily functions as a dimeric ssRNA-binding protein involved in viral replication [82]. The sequence of NSP9 is conserved among  $\beta$ -coronaviruses, especially among SARS-CoV and SARS-CoV-2, sharing almost ~97% homology [85]. NSP9 colocalizes with various NSPs and forms an important part of the replication machinery of coronaviruses and is therefore directly involved in their growth [86]. Studies have revealed that deletions in the NSP9 gene are marked by impaired synthesis of RNA and viral infectivity [84]. Protein-nucleic acid interactions are facilitated by multimerization of NSP9 [87]. During NSP9 dimerization, the two  $\alpha$ -helices bind to the GXXXG motif, an essential step for coronavirus replication [84].

In general, the protomer of NSP9 consists of seven  $\beta$ -strands surrounded by N-terminal and C-terminal  $\alpha$ -helices. The protein core is made up of  $\beta$ -barrel comprising two orthogonally packed antiparallel  $\beta$ -sheets. One sheet consists of  $\beta_1$ – $\beta_3$  strands along with half  $\beta_7$ -strand, while the other sheet is made up of  $\beta_4$  and  $\beta_5$  strands. This  $\beta$ -barrel is extended with the help of  $\beta$ -hairpin consisting of  $\beta_6$  and  $\beta_7$ -strands adjoining  $\alpha$ -helix. The barrel sheets are linked to N-terminal  $\beta$ -strand and C-terminal  $\alpha$ -helix via elongated loops [88]. Such NSP9 protomer folding is similar to the OB-fold, a structural motif that recognizes nucleic acids [89].

The structural analysis of NSP9 in SARS-CoV-2 revealed a horseshoe-like tetramer structure, which assists in its oligomerization and binding to the virus's nucleic acid during the replication process. The stabilization of this protein occurs via the presence of two contact surfaces. The first interaction is a parallel association between  $\alpha$ -helix at the C-terminal region with N-terminal loop ( $\alpha$ -helix interface) [90]. It then involves the antiparallel interactions between two  $\beta$ -strands at both protomers of NSP9 ( $\beta$ -sheet interface) [86], bringing the two barrels together for function and stabilization. In SARS-CoV-2, the sheet interface accounts for more stability in the tetrameric structure of NSP9 than in SARS-CoV. Breaking of the dimeric structure of NSP9 affects their stability and, in turn, prevents RNA synthesis and viral growth [91]. The intrinsic disorders graph suggested that the NSP9 has no disordered regions (Fig. 3a).

### Non-structural protein 10 (NSP10)

NSP10 is a ~ 130 amino acid residues long scaffold protein involved in stimulating NSP14 and NSP16. NSP14 possesses two distinct domains that mediate different functions. N-terminal consists of 3'–5' exoribonuclease activity (ExoN), and C-terminal possesses N7-methyltransferase (N7-MTase) activity [82]. NSP10 binding to ExoN of

NSP14 causes its stimulation; however, N7-MTase remains unaffected with this binding [92]. NSP10 also stimulates the activity of 2'-O-methyltransferase (2'-O-MTase) in NSP16 [93]. Therefore, NSP10 serves as an essential protein for activating the methylation and capping machinery for viral mRNA in association with NSP14 and NSP16 [94]. This protein is exclusively found in viruses and not in prokaryotes or eukaryotes.

The structural features of NSP10 consist of a long loop which connects two antiparallel  $\alpha$ -helices (H1 and H2) with a  $\beta_1$ -strand, followed by a sheet formation involving  $\beta_2$  and  $\beta_3$ -strands [95]. Two zinc finger motifs are present in the NSP10 structure. In the first case, the  $\beta$ -sheet is folded into two helices named H3 and H4, containing the zinc finger comprised three cysteines and one histidine to coordinate the zinc ion. Then a small helical turn is present after  $\beta_3$  adjoining  $\alpha$ -helix H6. The two strands  $\beta_4$  and  $\beta_5$  interrupt the long C-terminal region, which harbors the second zinc finger motif composed of only four cysteine side chains as required to stabilize this non-classical zinc finger protein [95, 96]. NSP10 of SARS-CoV-2 exhibited high structural similarity to NSP10 of SARS-CoV [95]. The intrinsic disorder graph indicated that NSP10 does not have many disordered regions, except at the residue range 4305–4325 (Fig. 3b).

### Non-structural protein 11 (NSP11)

The cleavage of pp1a polyprotein by 3CL<sup>pro</sup> results in the production of NSP11, a small product whose exact function has not been characterized yet. Depending on the coronavirus species, its length may vary from 13 to 23 amino acid residues [82]. In the case of SARS-CoV-2, the sequence of NSP11 is: SADAQSFLNGFAV [97]. The independent function of NSP11 is not known yet; however, NSP11 becomes the N-terminal of NSP12 during ribosomal frameshift of ORF1b required for NSP11-NSP16 translation [98]. Gadhave and colleagues suggested the helical propensity of NSP11, as studied through SDS micelle experiments [99]. The intrinsic disorder prediction was not performed for NSP11 due to the short sequence.

### Non-structural protein 12 (NSP12)/RdRp

NSP12 is one of the most important proteins required for viral growth. It is associated with both replication and transcription of coronaviruses. This protein is ~932 amino acid residues in length and is generally referred to as RNA-dependent RNA polymerase (RdRp) [82]. RdRp proteins contain multiple domains, which catalyzes the production of phosphodiester bonds between the ribonucleotides in the presence of a divalent metal ion [100]. NSP12 of SARS-CoV-2 possesses more than 95% similarity to polymerases of SARS-CoV. It is inhibited by Remdesivir, a nucleoside

analog that binds to NSP12 for bringing out this inhibition [101]. NSP12 of SARS-CoV-2 exhibited decreased thermal stability and enzyme activity compared to SARS-CoV NSP12 [102].

NSP12 in the presence of cofactors such as NSP7 and NSP8 mediates the replication and transcription of the virus with high efficiency, instead of being independent for mediating the replication function [103]. NSP12-NSP7-NSP8 thus serves as a minimal unit for carrying out the viral replication [102]. NSP7 and NSP8 initiate the primase activity, followed by the facilitation of NSP12 polymerase activity for viral replication [104]. NSP12 interacts with various other proteins or transcription factors, including CREB regulated transcription coactivator 3, ribonucleoprotein 4B, PLKHA5, E3 ubiquitin ligase, and ubiquitin associated protein 2, for transcription of the virus [105]. The intrinsic disorder graph showed that NSP12 has multiple intrinsically disordered regions distributed throughout the protein (Fig. 3c).

### Non-structural protein-13 (NSP13)/helicase

NSP13 is ~ 601 amino acid residue protein that acts as a helicase with specificity for dsDNA and dsRNA as a substrate with 5'–3' polarity [106]. NSP13 exhibits nucleoside triphosphate hydrolase (NTPase) activity for hydrolysis of different nucleosides. It is involved in unwinding the viral genome by binding to a single-stranded template extending from 5' to 3' direction using ATP hydrolysis [107]. Generally, NSP13 is more efficient in unwinding duplex DNA than duplex RNA; however, in higher concentrations of ATP, unwinding of duplex RNA is preferred to exhibit higher processivity than duplex DNA [106, 108]. Since, ATP induces a conformational change in helicase directing its affinity towards RNA. Therefore, it is believed that changing the ATP concentration and availability may alter the unwinding and translocation of helicase from its substrate. NSP13 may lead to helicase dissociation from the substrate at the replication site, thus becoming a promising candidate for developing effective SARS-CoV-2 antiviral strategies [98]. Shu and colleagues have identified inhibition of both NTPase and helicase activity of NSP13 by addition of Bismuth salts in a dose-dependent manner [109].

NSP13 is a multifunctional enzyme involved in inhibiting type1 interferon (IFN) response in addition to showing a helicase activity. It inhibits the activation of IFN- $\beta$  and IFN- $\alpha$  associated signaling in HEK293T cells [110, 111]. Moreover, NSP13 assists in replication by interacting with NSP12 along with its participation in mRNA capping. The catalytic efficiency of NSP13 is increased by twofolds in association with NSP12 [112]. The intrinsic disorder graph showed that NSP13 has several intrinsically disordered regions distributed throughout the protein (Fig. 4a).

### Non-structural protein 14 (NSP14) / Proofreading exoribonuclease

NSP14, a ~ 527 amino acid protein, stimulates 3'–5' exoribonuclease (proofreading) activity in interaction with NSP10 and N7-guanine methyltransferase activity for capping of viral mRNA and its prevention from degradation [82]. An exoribonuclease domain is required for maintaining the capping function [92]. The proofreading ability of NSP14 helps in the excision of any misincorporated nucleotides, thus protecting the viral genomic from mutations. Various studies were performed to judge the potential of NSP14 in viral replication. Mutations in NSP14 of coronavirus murine hepatitis virus were associated with a 15-fold increase in mutations in the viral genome, suggesting their involvement in maintaining the replication fidelity.

Similarly, the mutations in NSP14 of SARS-CoV showed a 21-fold increase in genomic mutations [113]. NSP14 of SARS-CoV also inhibits the production and signaling of INF- $\beta$  by blocking the IRF3 localization to the nucleus [111]. NSP14 is known for attenuating the therapeutic potential of several antiviral drugs that act through premature termination of viral replication. Narayanan and Nair had shown binding of ritonavir drug to the active site, i.e., exoribonuclease domain of NSP14, as a probable therapeutic agent for SARS-CoV-2 and its ability to reverse the inhibitory effects on various other drugs of therapeutic potential against coronaviruses [114].

NSP14 can interact with other NSPs, including NSP7, NSP8, NSP10, and NSP12 [115]. When associated with the NSP7-NSP8-NSP12 super-complex, exoribonuclease activity is not shown by NSP14 unless NSP10 associates with the replication complex [103]. This association of all NSPs like 7, 8, 10, 12, and 14 leads to the formation of another super-complex with proteins possessing primase, polymerase, and exoribonuclease activities. The intrinsic disorder graph showed that NSP14 has an intrinsically disordered region at the N-terminal tail spanning from 6040 to 6050 (Fig. 4b).

### Non-structural protein 15 (NSP15)/Uridylate-specific endoribonuclease

NSP15 is made up of ~ 346 amino acid residues in length. This protein possesses multifunctional roles, including an inhibitor of IFN, a binding partner for retinoblastoma protein (pRb), endoribonuclease activity, and helps prevent the virus from dsRNA sensors in the host [116]. Firstly, this protein mediates the cleavage of RNA nucleotides in the 3' direction of uridylates, hence behaving as an endoribonuclease [117]. Second, much like NSP13 and NSP14, NSP15 can also block the nuclear localization of IRF3 and could thus inhibit the IFN production during viral infection [118]. Third, NSP15 possesses a domain that has a binding affinity towards pRb.



In the Huh-7 cells, NSP15 can increase the cytoplasmic to a nuclear ratio of pRb by changing its distribution pattern. Such changes in the form of reduced expression of Rb cause a change in the cell cycle progression in NSP15-transfected cells [119]. Moreover, it has been observed that magnesium ions can alter the conformation of NSP15 [120], and mutation within the active site or outside the active site of NSP15 can result in the inhibition of viral infection rates [121]. The intrinsic disorder analysis suggested that NSP15 has a few disordered regions spanning throughout the protein (Fig. 4c).

### Non-structural protein 16 (NSP16)/2'-O-methyltransferase

NSP16 is composed of ~298 amino acid residues in length. NSP16, upon interaction with NSP10, gets activated and facilitates the viral mRNA capping as it possesses the 2'-O-MTase domain [82]. NSP16 is an S-adenosylmethionine dependent-methyltransferases required for the virus' life cycle, where NSP10 acts as an essential cofactor for regulating the enzyme activities of NSP16 [122]. Studies on different cell cultures show MTase activity is essential for the replication of coronaviruses. It may also help the virus escape the host's antiviral sensors and thus successfully initiate the viral infection [123, 124]. Many significant studies have been performed on investigating the structure of NSP16 in complex with NSP10; however, more studies on the individual structure of NSP16 are warranted in the future [125]. The intrinsic disorders analysis suggested that NSP16 has one major disordered region spanning at the C-terminal region of the protein (Fig. 4d).

## Conclusions

The viral genome encodes several proteins that are not sufficient to support viral replication. Thus, viruses dependent on the host machinery to complete their life cycles. Usually, viruses have extremely efficient genomes. If a viral protein comprises intrinsic disorders regions, it can be implicated in various functions because it can interact with different partner proteins. This mechanism is common in the SARS-CoV-2 genome that encodes 16 NSPs, which play numerous roles in virus replication and assembly. They contribute to viral pathogenesis by regulating early transcription, gene transactivation, helicase activity, and immunomodulation. Like other viral proteomes, SARS-CoV-2 has a dark proteome where all the proteins, especially NSPs, have a chunk of intrinsic disorders. Exploring the intrinsic disorders in the SARS-CoV-2 NSPs is vital to understanding the virulence of the virus and its subsequent infection.

In this study, we performed a sequence-based analysis of SARS-CoV-2 NSPs to get insights into their intrinsic

disorders and their possible functions in the viral replication. The available sequence data for SARS-CoV-2 NSPs were analyzed to evaluate their intrinsic disorders by considering their biological activity relationships. The analysis showed that SARS-CoV-2 NSPs have many intrinsic disorder regions which might lack a well-defined tertiary structure in native conditions. In conclusion, intrinsic disorders are of use to SARS-CoV-2 as they allow efficient usage of the replication machinery, enable it to tolerate the high mutation frequency and changing environments. As such, targeting the intrinsic disorders conferred to the SARS-CoV-2 NSPs to impair critical protein–protein interactions could establish a broad and tempting antiviral policy in COVID-19 research.

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**Data availability** All data generated or analyzed during this study are included to this article.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval for Human Subject** Not applicable.

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