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Exploiting the molecular basis of age and gender differences in outcomes of SARS-CoV-2 infections

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ABSTRACT

Motivation: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (coronavirus disease, 2019; COVID-19) is associated with adverse outcomes in patients. It has been observed that lethality seems to be related to the age of patients. While ageing has been extensively demonstrated to be accompanied by some modifications at the gene expression level, a possible link with COVID-19 manifestation still need to be investigated at the molecular level.

Objectives: This study aims to shed out light on a possible link between the increased COVID-19 lethality and the molecular changes that occur in elderly people.

Methods: We considered public datasets of ageing-related genes and their expression at the tissue level. We selected human proteins interacting with viral ones that are known to be related to the ageing process. Finally, we investigated changes in the expression level of coding genes at the tissue, gender and age level.

Results: We observed a significant intersection between some SARS-CoV-2 interactors and ageing-related genes, suggesting that those genes are particularly affected by COVID-19 infection. Our analysis evidenced that virus infection particularly involves ageing molecular mechanisms centred around proteins EEF2, NPM1, HMGA1, HMGA2, APEX1, CHEK1, PRKDC, and GPX4. We found that HMGA1 and NPM1 have different expressions in the lung of males, while HMGA1, APEX1, CHEK1, EEF2, and NPM1 present changes in expression in males due to ageing effects.

Conclusion: Our study generated a mechanistic framework to clarify the correlation between COVID-19 incidence in elderly patients and molecular mechanisms of ageing. We also provide testable hypotheses for future investigation and pharmacological solutions tailored to specific age ranges.

1. Introduction

At the end of 2019 in Wuhan (China), medical facilities reported acute pneumonia cases with an unknown origin. Further analysis revealed that a novel coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was responsible for that disease, subsequently called coronavirus disease 2019 (COVID-19) [1,2]. The clinical manifestations spanned from asymptomatic infection to severe pneumonia and a severe state of inflammation (molecularly characterised by a cytokine storm) leading to a fatal outcome [3–8].

Starting from China, the virus spread in almost all other countries globally, causing infections and deaths. On 11th March 2020, the World Health Organisation (WHO) declared SARS-CoV-2 as a pandemic. Current data revealed that the impact of COVID-19 presents certain peculiar aspects in different nations that have been deeply investigated [9,10]. Some authors hypothesised that virus mutations were responsible for these differences [11–14]. Nevertheless, many independent studies agreed that the mutations might not have a primary role in explaining these differences [15–17].

Despite the lack of the individuation of the causes, there was a substantial agreement on the fact that the variation of the observed case fatality rate (CFR), i.e. the fraction of confirmed cases leading to fatal outcomes, ranging from 0 to 20% and beyond at

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country level, needs to be deeply investigated [18–20]. Among the other differences, we focused on observing that the infection is significantly more lethal in older people [21–25]. This consideration has also guided the optimisation of vaccination strategy [26].

Some studies have focused on the possible link between increased mortality rate and some characteristics of older people [27,28]. In addition, these studies suggested the potential effect of the virus as a trigger activating the decompensation of other chronic conditions [29–32]. Akbar et al., [33], discussed a possible link between the increased chronic inflammatory status occurring during ageing (termed "inflammaging" [34,35]), and COVID-19 manifestation that causes the rise of inflammation.

Previous studies have also shown that the understanding of modification of molecular mechanisms related to the ageing process (i.e. modification of gene expression and modulation of regulatory mechanisms) may reveal important insights about ageing [36]. Many studies contributed to identifying such ageing-related diseases despite the lack of having experimental data [37–39,35,40]. Computational predictions have also been made in [36,41] giving both candidate genes and networks [42,43].

Consequently, the study of the intersection between SARS-CoV-2 and ageing-related molecular alterations could augment the understanding of COVID-19, thus improving treatment options [44]. Bhattacharyya et al. presented a first analysis based on some preliminary public data reinforcing the rationale that such a possible link exists [45]. The expression of the two human receptors TMPRSS2 and ACE2, which are recognised by the SARS-CoV-2 protein Spike, increases with age in mammals [46], further suggesting a molecular cause for the more severe COVID-19 symptoms with age.

Six functional open reading frames (ORFs) in the SARS-CoV-2 genome encodes for the four main structural proteins, the Spike (S), Envelope (E), Membrane (M), and the Nucleocapsid (N), and ORF1a/ORF1b, which contain information for the replicase–transcriptase complex formed by 16 non-structural proteins (NSP1–NSP16). The SARS-CoV-2 genome also contains 9 accessory factors from sub-genomic ORFs (Orf3a, 3b, 6, 7a, 7b, 8, 9b, 9c and 10) [47]. We investigated the relationships and interactions between these viral components and age-related factors and observed a significant overlap between SARS-CoV-2 and ageing group genes' interactors, considering possible regulatory mechanisms that may be altered [48,43,49].

Starting from these considerations, we hypothesised that SARS-CoV-2 interacting proteins (and genes) might show an overlap with human ageing-related genes higher than chance. Therefore, the infection may affects these mechanisms that can be already impaired in older adults, causing severe outcomes. We downloaded public available interaction data from Guzzi et al. [50] and Gordon et al. [51]. Then we considered the interacting partners that were annotated as *ageing* genes in MSigDB database [52] ad we also considered the expression at tissue and sex levels extracting data from the GTEx database [53]. We identified a significant fraction of interacting partners of SARS-CoV-2 involved in ageing. These genes are also expressed in the lung, and their expression is modulated by age and sex, (while we also observed that these genes are expressed in adipose tissue as reported in Supplementary Material). The workflow of the experiment is depicted in Fig. 1.

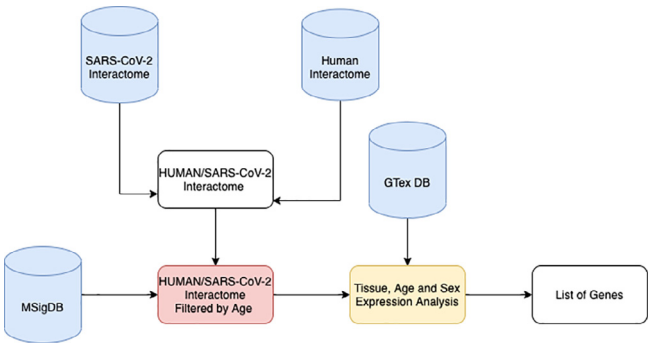

Fig. 1. Workflow of the experiment. We downloaded public available interaction data from previous studies. We built the integrated human/SARS-CoV-2 interactome. In parallel, we downloaded the list of genes annotated with *ageing* keywords as in MSigDB database. Then, for each SARS-CoV-2 protein, we calculated the probability that it contains human interactors annotated with *ageing* keyword. We obtained a list of SARS-CoV-2 proteins containing a significant number of interactors related to ageing. Then we calculated the intersection of these sets (*core interactors*) obtaining a list of eight human proteins. For each core interactor, we also considered the expression at tissue level extracting data from GTEx database. We verified that there exist a significant fraction of interacting partners of SARS-CoV-2 that are involved in ageing and that are particularly expressed in lung and in adipose tissue.

2. Methods

SARS-CoV-2 Interaction Map. We considered the SARS-CoV-2 protein interaction map provided by Gordon et al., [51], and by Guzzi et al., [50]. Both works provided data about 26 of the 29 SARS-CoV-2 proteins behaviour in human cells by identifying the human proteins that are physically associated with each of the SARS-CoV-2 proteins using affinity-purification mass spectrometry. They found high-confidence protein–protein interactions between SARS-CoV-2 and human proteins; they also provided data about possible interactions with an associated reliability score. We considered both high and low confidence interactions.

Databases. We first defined and labelled genes related to the ageing process as *ageing*. Then, we considered data provided from the GTEx dataset containing genes positively and negatively correlated with human age [53]. We gathered data from the GenAge dataset that derived human genes by projecting sequence orthologs in model organisms. We also considered the MSigDB gene set collections, which summarised gene information associated with *ageing* collected from 70 different studies. We selected datasets reporting experiments from *Homo sapiens* since orthologs' projection may produce not reliable results for ageing as described in [36].

We used the Search Tool for the Retrieval of Interacting Genes Proteins database (STRING) [54] that is a freely available repository storing both physical and functional association among proteins. Users may search the database through a web interface by specifying a protein identifier or inserting the primary sequence. We queried the database using the identifiers of the nodes of each subnetwork. We used medium confidence as the minimum confidence score for each interaction and *all* for the sources of interactions. We searched the GTEx Portal [55] using the previously described list of gens. We obtained the expression of those genes in a heat map that shows expression across all GTEx tissues. Gene Ontology analysis was performed by using Gene Ontology web portal [56] while using Reactome Database for identifying related pathways [57].

Bioinformatic and Network Analysis. We selected all known SARS-CoV-2 interacting partners. We used the Gordon dataset [51] to obtain all the partners. Then, for each SARS-COV-2 protein, we retrieved the list of its interactors. We determined the intersection between the list of human interactors and the ageing-related genes for each viral protein. We estimated the probability that this intersection is higher than chance by Fisher's exact test. In Supplementary Material, we show the sub-networks induced in human interactome by each SARS-COV-2 protein. For each subnetwork, we report the main topological parameters: number of nodes, number of edges, average node degree, average local clustering coefficient, the expected number of edges. For each sub-network,

we performed a Gene Ontology enrichment analysis. Network analysis and visualisation were performed in Cytoscape 3.7.0 [58]. We also tested the significance of the difference in the expression of EEF2, NPM1, HMGA1, HMGA2, APEX1, CHEK1, PRKDC, and GPX4 due to age (we considered six different classes), sex, and tissue. All the p-values of the tests were corrected for multiple testing using Bonferroni correction. We used a Wilcoxon Test for testing difference in the expression among classes (since the expression of genes is not gaussian as reported by a Shapiro test). In addition, the difference among age classes is evaluated using a Kruskal Wallis test.

3. Results

3.1. Network analysis

We selected human interactors for each viral protein. The analysis revealed that only ten viral proteins (M, NSP2, NSP4, NSP6, NSP11, NSP13, Orf3a, Orf7a, Orf8, and Orf9c) have interactors with a significant overlap with respect to ageing-related proteins, as summarised in Table 1 (p-values have been corrected using Bonferroni correction). Then, we considered those that are enriched for ageing in a significant way. Finally, we intersected all these sets, and we obtain a core set of eight proteins: EEF2, NPM1, HMGA1, HMGA2, APEX1, CHEK1, PRKDC and GPX2 (indicated as *core interactors* hereafter) as reported in Fig. 2 (see supplementary for the list of interactors for each viral protein, integrated with the topological characteristics of the induced subnetwork in the human interactome).

The Gene Ontology analysis revealed that the whole network is enriched with the following terms: (GO:0090402) oncogene-induced cell senescence, (GO:0035986) senescence-associated heterochromatin focus assembly, (GO:2000774) positive regulation of cellular senescence, (GO:2000773) negative regulation of cellular senescence, (GO:2000772) regulation of cellular senescence. The analysis of Reactome DB reveals that the subnetwork is associated with the following pathways: Formation of Senescence Associated Heterochromatin Foci (HSA2559584), Host interactions of HIV factors (HSA162909).

3.2. Expression analysis

We searched the GTEx database for the expression of *core interactors* as reported in Fig. 2 expressed as TPM (Transcripts Per Million). We found that all the interactors are expressed in the lung as well as in other human tissues (see supplementary materials for more details). To assess the different outcomes between males and females we focused on lung tissue and we compared the expression of these core interactors in males and females as reported in 3. Since data were not normally distributed (as given by Shapiro Test), we applied a Wilcoxon Test to evaluate significance of the difference in expression between male/female classes.

We evidenced a significant difference for NPM1 and HMGA1 which are significantly downregulated in males, without considering age as reported in Fig. 3.

We also explored the trend of the core interactors focusing on lung tissue and six different classes of age (20–29, 30–39, 40–49, 50–59, 60–69, 70–79). We found a significant difference considering age groups for HMGA1, APEX, CHEK1, EEF2, and NPM1 ($p \leqslant 0.05$ as evidenced by a Kruskal Wallis test). Fig. 4 reports this trend.

Table 1
P-Values of the enrichment. For each protein, we report the significance of the enrichment after correction. A p-value lower than 0.01 means that the interactors are significantly related to ageing (NS stands for not significant).

Viral Protein	P-Value	Viral Protein	P-Value
Spike	NS	E	NS
M	**6.84E−03**	N	NS
NSP1	NS	**NSP2**	**1.8E−03**
NSP3	NS	**NSP4**	**8.32E−03**
NSP5	NS	**NSP6**	**2.6E−03**
NSP7	NS	NSP8	**3.4E−03**
NSP9	NS	NSP10	NS
NSP11	**1.8E−04**	NSP12	NS
NSP13	**2.5E−03**	NSP14	NS
NSP15	NS	NSP16	NS
Orf3a	**5.06E−03**	Orf3b	NS
Orf6	NS	**Orf7a**	**1.8E−04**
Orf7b	NS	**Orf8**	**6.9E−04**
Orf9b	NS	**Orf9c**	**1.50E−02**
Orf10	NS		

4. Discussion

Deaths from COVID-19 occur predominantly among older adults. COVID-19 also appears to be more lethal for men rather than women [23,9,10,24]. This feature has been found in China, as well as in Europe and in the United States of America [59].

Starting from this observation, we investigated the molecular basis of this phenomenon. Next, we recall that ageing is a heterogeneous process that presents differences among individuals. In particular, age-related changes impact many organs producing possible multi-organ failures, even showing many inter-individual differences. Beyond these differences, we tried to explain how the age-related changes at the molecular level can be relevant to COVID-19 pathology.

To achieve this goal, we integrated interactomics and expression data related to COVID-19, age and sex. We started from SARS-CoV-2 interactors, and we isolated age-related from those. Then we considered the expression value of these genes, and we further investigated the trend of changes of these genes in age and sex groups. We identified a set of statistically significant interactors for the ageing process: EEF2, NPM1, HMGA1, HMGA2, APEX1, CHEK1, PRKDC, and GPX4. As reported in Fig. 7, we found some interesting changes of these genes considering tissue, age and sex groups. We also found that NPM1 and HMGA1 are downregulated in males (statistically significant regulation), while HMGA2 is slightly downregulated in males (not significantly) (Fig. 3).

We also found some statistically relevant changes in age for EEF2, NPM1, HMGA1, APEX1, and CHEK1 for males (Fig. 5), and for APEX1 in Females (Fig. 6). With the only exception of HMGA2, all these genes show a decreased expression with ageing in lung tissues.

As investigated in [60], ageing is characterised by the decline of the immune function. Older adults are not immuno-deficient, but the immune system's response is often not sufficient to be effective against antigens. This effect is particularly evident when they are subject to novel antigens. For example, it is known that both responses to influenza and vaccination are not efficient in the elderly [61,62]. Moreover, the elderly accumulate inflammatory mediators in tissues (inflammageing process), which may occur by the accumulation of DNA lesions that, in turn, triggers the increased production of inflammatory mediators [63]. In parallel, the link between COVID-19 and the suppression of the immune system has been observed in [64]. Authors found that many proteins related to the immune response were modulated, causing the possible suppression of such a system.

HMGA1 and HMGA2 genes encode four proteins (HMGA1a, HMGA1b, HMGA1c, and HMGA2) belonging to the High-mobility group A (HMGA) protein family [65]. All the proteins bind AT-rich regions in DNA and modulate gene expression by

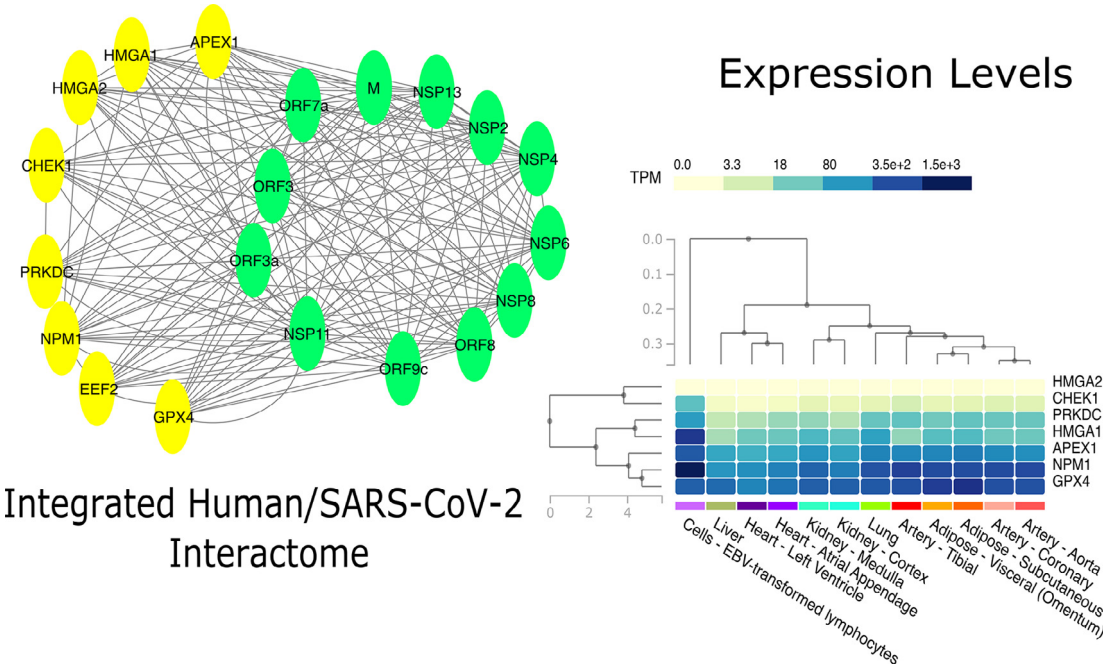

Fig. 2. Figure shows tissue level analysis of this work. The Network analysis contributed to find a set of human proteins (yellow nodes) related to aging that interact with many SARS-CoV-2 proteins (green nodes). The analysis of the expression of the related genes at tissue level revealed that all these genes are expressed in the lung, as well as in other human tissues. Expression levels are presented as TPMs. (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

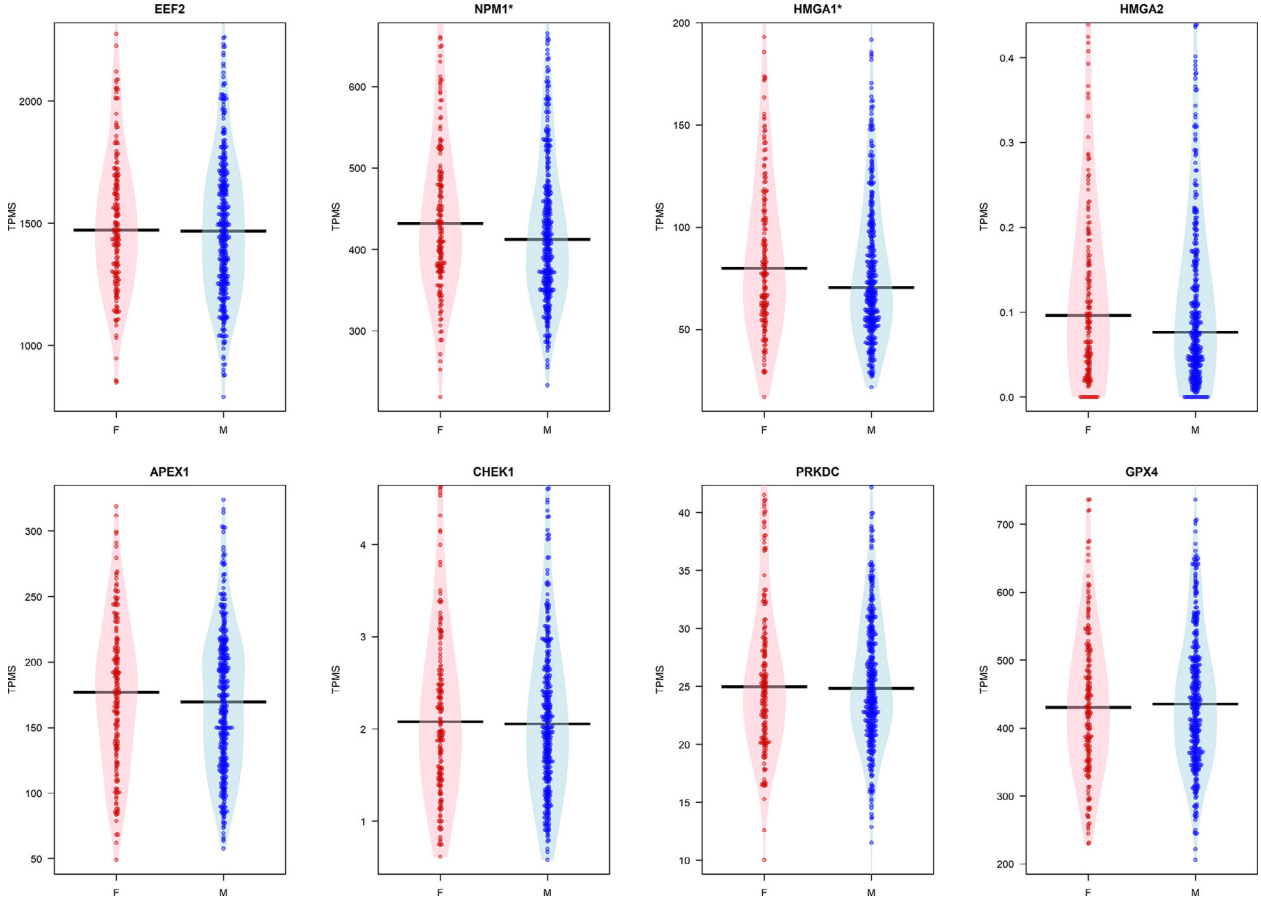

Fig. 3. Figure reports box plot of the expression of the eight core genes grouped by sex in the lung tissue. The evidences a significant difference tested by using a Wilcoxon Test for NPM1 and HMGA1 genes.

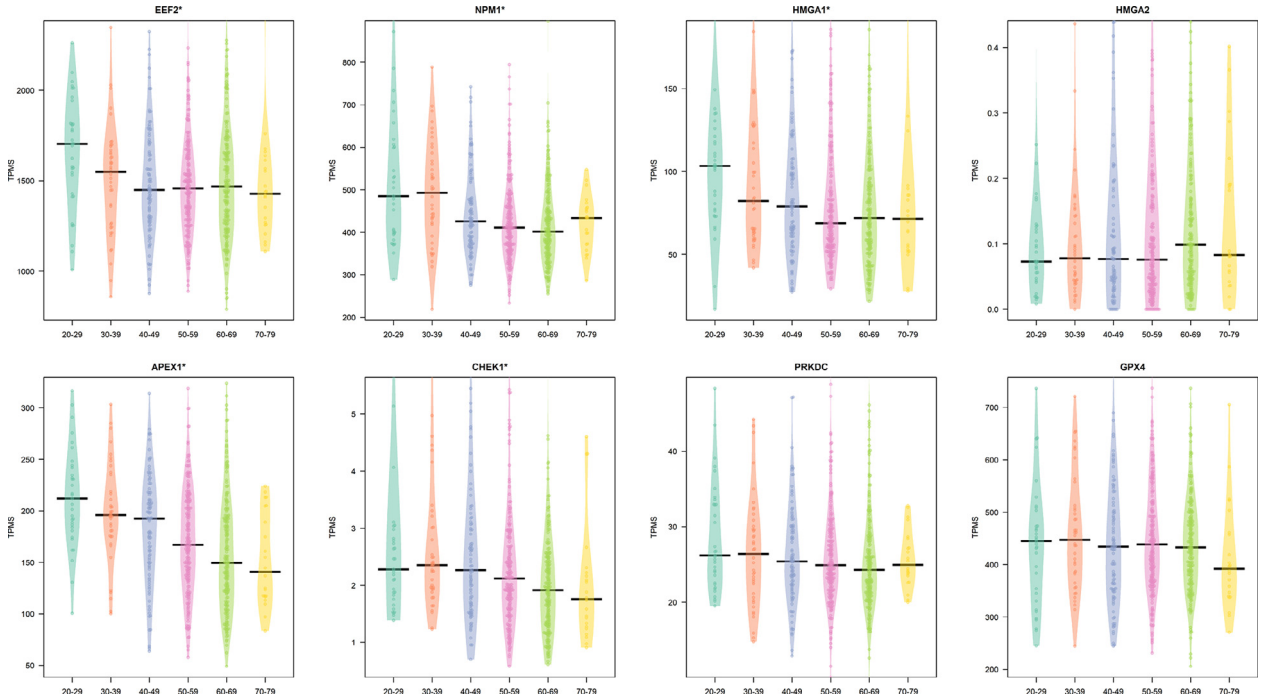

Fig. 4. Figure reports the difference of the expression of the core genes in lung tissue in different age classes. A $*$ on top of the plot means a significant difference ($p \leq 0.05$ as evidenced by a Kruskal Wallis test).

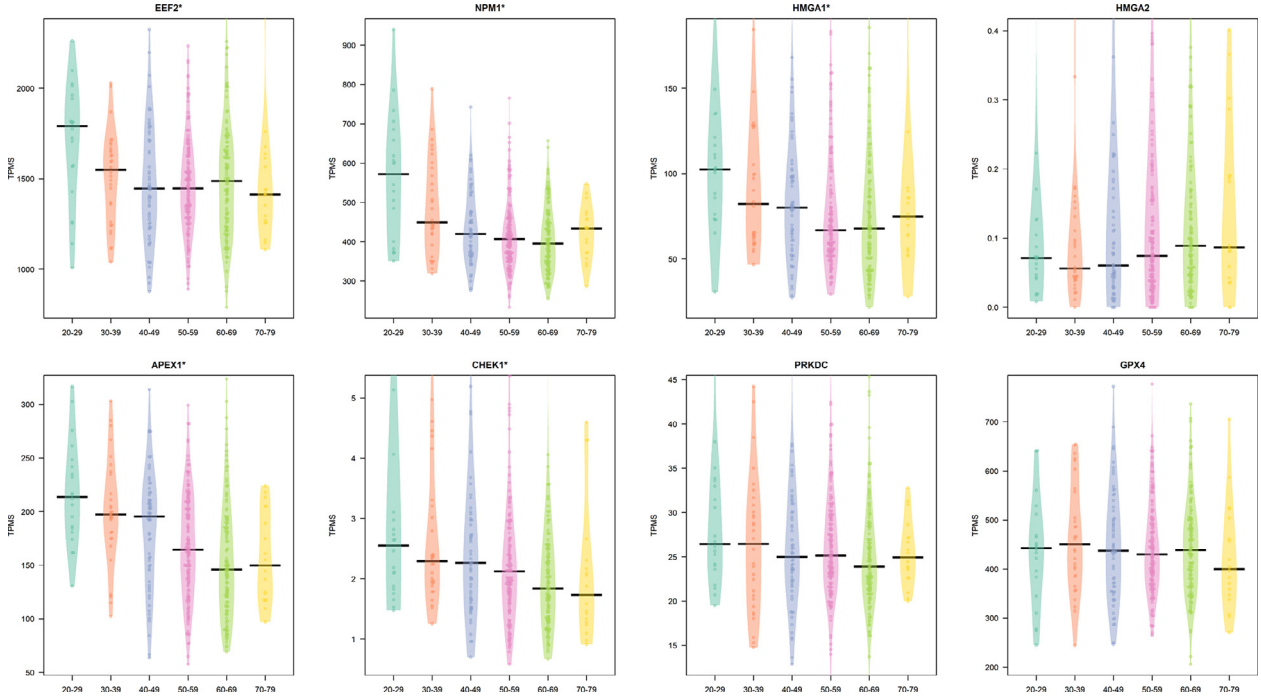

Fig. 5. Difference in the expression in lung tissue by age classes in males. Expression is reported as TPM.A $*$ on top reveals a modulation in groups.

acting as transcription factors. Literature reports that HMGA1 has critical roles in tumorigenesis and the progression of various cancers. However, the role of HMGA1 in COVID-19 has not been explored in the past. We now provide a hypothesis framework for future research in the functional interplay between ageing and SARS-CoV-2 infection. HMGA1 is significantly downregulated both in males and the elderly, and these differences may be associated with poor outcomes observed in these classes. It has been shown that HMGA1 induces inflammatory pathways in many cancers, enhance the expression of genes related to neural stmness and pathways involved cell cycle progression. HMGA1 dysregulation causes aberration in cellular development and hematopoiesis [66]. Furthermore, the involvement of HMGA1 in the transcriptional regulation of genes essential in both the inflammatory response and atherosclerosis has been established [67].

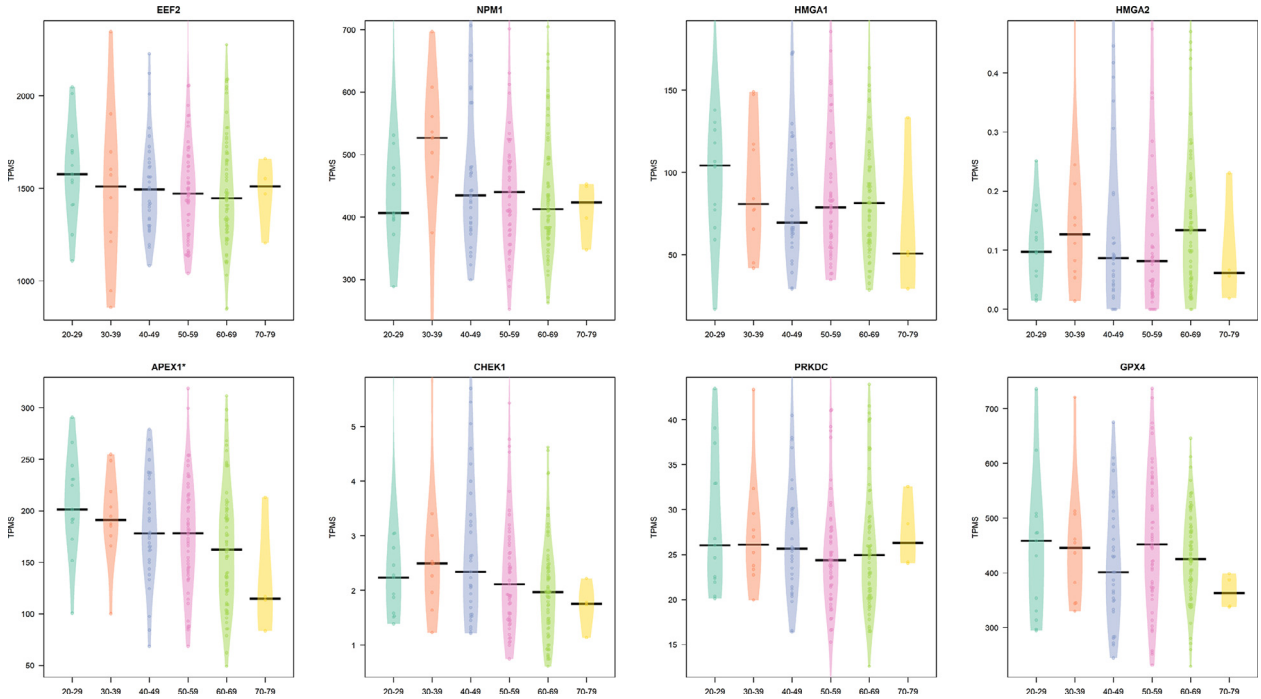

Fig. 6. Difference in the expression in lung tissue by age classes in females. Expression is reported as TPM. A ∗ on top reveals a modulation in groups.

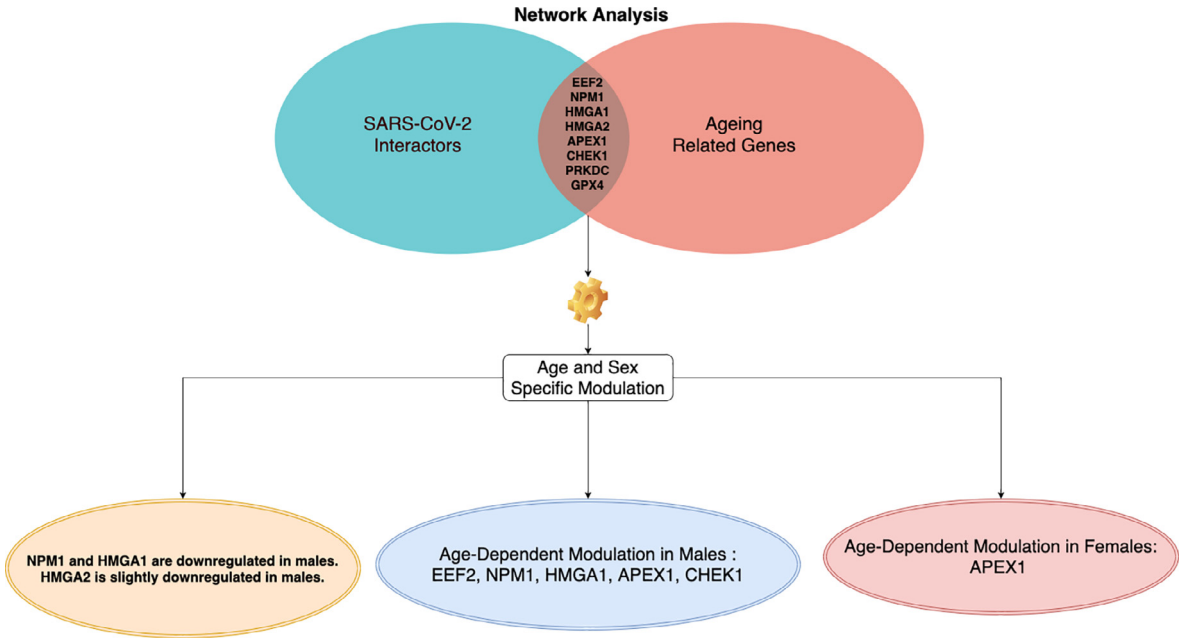

Fig. 7. Figure summarises main results of the work. Network analysis found that there exist eight proteins related to ageing that are also all targeted by ten SARS-CoV-2 proteins. The analysis of the expression of their genes revealed that there exist difference on the expression of these genes considering both age and sex.

Our results suggest that low HMGA1 levels may be a risk factor in COVID-19 patients, given the possibility that interactions between SARS-CoV-2 and HMGA1 may impair/trigger inflammatory pathways. Furthermore, it has been demonstrated that low HMGA1 levels in basal stem/progenitor cells of the human airway epithelium are associated with suppression of the expression of genes critical to normal differentiation and up-regulation of genes linked to abnormal differentiation relevant to smoking and chronic obstructive pulmonary disease [68], which have been demonstrated to be risk factors associated with COVID-19 mortality [69].

Similarly to HMGA1, the Nucleophosmin (NPM1) is also downregulated in males. NPM1 is related to DNA and cell cycle control such as ribosome biogenesis, protein chaperoning, centrosome duplication, histone assembly, and cell proliferation [70,71]. Previous studies investigated the age incidence of acute myeloid leukaemia with mutated nucleophosmin (NPM1) [72,73], while there are no studies related to these mutations and other diseases. In [74] the impact of NPM1 modification in older patients has been investigated for AML, suggesting a worse prognosis for older patients due to NPM1 changes. The interaction between NPM1 and the

nucleocapsid protein of the previous SARS-CoV is known to affect the viral particle assembly [75–77]. The role of NPM1 and Histone H2AX targeted by other viral proteins has also been reported in other viruses such as Epstein-Barr and KSHV as a common strategy to manipulate translation and to promote virus latency [78,79]. A case of SARS-CoV-2 associated sudden death in an NPM-mutated AML 50-year-old male patient was reported in [80]. Together with our findings, this suggests that further studies on interactions between SARS-CoV-2 and NPM1 are required. Moreover, for older men, the scenario is furtherly complicated by the downregulation of EEF2, APEX1 and CHEK1.

The dysregulation of EEF2 may cause the accumulation of DNA damage [81]. The role of EEF2 in severe cases of COVID-19 has also been elucidated in [64], and the possible association of downregulation of EEF2 with COVID-19 severity is also suggested by our study. Moreover, this protein is targeted together with the Eukaryotic translation initiation factor 2 subunit 1 (EIF2S1) by Orf3a, Orf8, NSP2, NSP6, NSP11, NSP13, indicating a possible role of the virus to promote viral translation over cellular translation [82]. In [83] the synergistic downregulation of both APEX1 and NPM1 has been clearly observed in oligodendrocyte cells in relation to ageing APEX1 plays a protective role in the cellular response to oxidative stress [84], and has a major role in DNA repair and in redox regulation of transcription factors [73]. CHEK1 is targeted together with CDK1 by many SARS-CoV-2 interactors (NSP2, NSP4, NSP11, NSP13) and with CDKN2A (Orf3, NSP13), suggesting an additive effect on the disruption of pathways of apoptosis mediated by TP53 [85] yet dis-regulated by both senescence and ageing. [86].

Differently, for females we found only the age-dependent modulation of APEX1. Thus, this may suggest that females may have less risk factors than males.

In parallel, in supplementary material we report that *core interactors* are also significantly overexpressed in adipose tissue, therefore suggesting a second factor of co-morbidity. Changes in adipose tissue promote a chronic state of low-grade systemic inflammation on a phenotypic level, thus increasing the risk of age-associated diseases [35,87]. Here, we report that *core interactors* are expressed in adipose tissue, suggesting a possible role that should be further investigated. We hypothesise that the molecular relationship between SARS-CoV-2 and aging is intrinsical: on one side, SARS-CoV-2 induces a major change to the host cell's transcriptome/proteome, with hundreds of transcripts/proteins affected [51,88]; on the other side, this effect is larger in older transcriptomes [89]. Secondly, ageing modulates the expression of proteins necessary for the viral cycle of SARS-CoV-2 [46], including those included in the interactome described in this study.

5. Conclusion

We applied a bioinformatic analysis to perform a qualitative study of mechanisms of infection by SARS-CoV-2 in older people.

Several studies have shown in the past the modifications of genes and proteins that occur in older adults. Other studies have partially elucidated the mechanism of infections and the dysregulated pathways in COVID-19 patients.

We detected a statistically significant overlap between SARS-CoV-2 interacting proteins and those related to ageing, suggesting a potentially different response in older people. Our analysis showed that virus infection mainly affects ageing molecular mechanisms centred around proteins EEF2, NPM1, HMGA1, HMGA2, APEX1, CHEK1, PRKDC, and GPX4. We also found that some of these genes are differentially expressed in lung tissues of the elderly, suggesting an increased susceptibility of the elderly to COVID-19 inflammatory-related manifestations. Finally, we found that there is a significant difference in the expression considering both age and sex.

While causality is often hard to derive in high-throughput datasets such as the proteomics/transcriptomics data on which our study is based [90], we believe that the capability of SARS-CoV-2 to interact with proteins increasing in abundance with ageing may justify part of the increased severity of COVID-19 in older individuals.

These results will provide a first step for understanding the molecular basis of the mechanism of infection and will shed light on infection progression. The limitation of this study is that the dataset is correlative, and thus it should be confirmed by *in vivo experiments*.

6. Key Points

- A network-based analysis identified some molecular mechanisms that could play a role in the SARS-CoV-2 molecular aetiology and ultimately affect COVID-19 outcome.
- Our analysis evidenced that virus infection particularly affects ageing molecular mechanisms centred around proteins EEF2, NPM1, HMGA1, HMGA2, APEX1, CHEK1, PRKDC, and GPX4.
- We found an age-dependent modulation of EEF2, NPM1, HMGA1, APEX1 and CHEK1 in lung tissue of males.
- We found an age-dependent modulation of APEX1 in females.
- Our study generated a mechanistic framework aiming at clarifying the correlation between COVID-19 incidence in elderly patients and molecular mechanisms of ageing considering differences by age and sex.

Author contribution

F.M.G and P.H.G. conceived the main idea of this manuscript. D. M. performed the experimental analysis. F.M.G., D.M., and P.H.G. participated in the experimental phase and the discussion of the results. P.V participated in the design and implementation of data analysis and integration. E.P. participated in the writing of Discussion Section and also validated the clinical aspects of this work. All authors read and approved the manuscript.

CRediT authorship contribution statement

Daniele Mercatelli: Data curation, Visualization, Writing - original draft. **Elisabetta Pedace:** Writing - original draft, Conceptualization. **Pierangelo Veltri:** Supervision, Writing - review & editing. **Federico M. Giorgi:** Conceptualization, Methodology, Writing - original draft. **Pietro Hiram Guzzi:** Conceptualization, Methodology, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.csbj.2021.07.002.

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