Comprehensive immunovirological and environmental screening reveals risk factors for fatal COVID-19 during post-vaccination nursing home outbreaks

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Abstract

COVID-19 vaccination has resulted in excellent protection against fatal disease, including in the elderly. However, risk factors for post-vaccination fatal COVID-19 are largely unknown. We comprehensively studied three large nursing home outbreaks (20-35% fatal cases) by combining SARS-CoV-2 aerosol monitoring, whole-genome phylogenetic analysis, and immunovirological profiling by digital nCounter transcriptomics. Phylogenetic investigations indicated each outbreak stemmed from a single introduction event, though with different variants (Delta, Gamma, and Mu). SARS-CoV-2 was detected in aerosol samples up to 52 days after the initial infection. Combining demographic, immune and viral parameters, the best predictive models for mortality comprised IFNB1 or age, viral ORF7a and ACE2 receptor transcripts. Comparison with published pre-vaccine fatal COVID-19 signatures and reanalysis of single-cell RNAseq data highlights the unique immune signature in post-vaccine fatal COVID-19 outbreaks. A multi-layered strategy including environmental sampling, immunomonitoring, and early antiviral therapy should be considered to prevent post-vaccination COVID-19 mortality in nursing homes.

Introduction

SARS-CoV-2 outbreaks affecting nursing homes have been a major public health concern since the start of the COVID-19 pandemic. During the first epidemic wave, it has been estimated that COVID-19 mortality in Belgium has been up to 130 times higher inside than outside nursing homes, due to the combined effects of age, sex, frailty, and infection risks among residents.[1] Spatial analyses also indicated an association between the hospitalization incidence and the local density of nursing home residents, thus confirming the important impact of COVID-19 outbreaks in those facilities.[2] With one of the highest documented COVID-19 mortality rates in the world[2], more than half of all COVID-19 related deaths in 2020 in Belgium were linked to nursing homes.[3] A meta-analysis of the first COVID-19 wave in Spain found that mortality at the facility level was significantly associated with a higher percentage of patients with complex diseases, lower scores on pandemic preparedness measures and higher population incidence of COVID-19 in the surrounding population.[4]

To protect this highly vulnerable population, the roll out of the vaccination campaign was initially targeted towards elderly persons and healthcare workers. Nursing home residents are usually characterized by advanced age, a wide arsenal of co-morbidities and associated polypharmacy, and a decreased function of the immune system, potentially resulting in a higher risk of breakthrough infections.[4–8] Vaccination in Belgian nursing homes began in the second half of December 2020, employing mainly the mRNA vaccine BNT162b2. The BNT162b2 vaccine is highly effective at protecting against COVID-19 hospitalization and death, with efficacies of 90–95% reported in phase 3 clinical trials,[9] and confirmed in large-scale real life studies.[10] By March 2021, vaccination coverage (two-dose scheme) among residents of nursing homes had reached 89.4% on a national scale. Starting from September 2021 on, a third or booster dose was administered in nursing homes. Reduction in hospital admissions and mortality among residents of nursing homes on account of vaccination has been reported

throughout Europe, such as for a Spanish study which included over 25,000 residents and reported a fatality rate of only 1.6% in the post-vaccination era.[11] A recent study of 10 European countries, analyzing 240 COVID-19 outbreaks in the post-vaccination era (July-October 2021), identified an average case fatality rate of 5.5% for Belgium, almost half of the European average of 10.2%.[12] While the same study identified vaccination status as significantly associated to COVID-19 hospitalization, no association was found with COVID-19 mortality. Strong variability in case fatality ratios has been observed[13–15], with no major risk factors of fatal post-vaccination COVD-19 identified so far, other than age and co-morbidities, mostly due to the limited statistical power in small outbreaks.

To date, only three high fatality rate (> 10%) post-vaccination outbreaks have been observed in Belgian nursing homes. Here, we describe a multidisciplinary investigation of these three post-vaccination outbreaks in a collaboration involving the nursing home staff, health inspectors of the respective regional agencies, the national institute for public health (Sciensano), political, academic, and governmental stakeholders, as well as the National Reference Center (NRC) of respiratory pathogens at the University Hospital and University of Leuven.

Methods

Data collection

Demographic and clinical characteristics, including comorbidities, were compiled from health records provided by the individual nursing homes. The primary outcome was COVID-19-related death, as defined by WHO criteria.[16] All residents, as well as the large majority of staff members, received the BNT162b2 (Comirnaty®, Pfizer) vaccine. This work was framed within the role of the NRC respiratory pathogens UZ/KU Leuven (as defined by the Royal Decree of 09/02/2011), as approved by the UZ/KU Leuven Ethical committee for research (S66037).

Quantification of viral loads

Consecutive screening events were organized in all three nursing homes, first testing symptomatic persons, followed by collective and repeated testing after the identification of a positive case. Next to nasopharyngeal swabs of residents and staff, aerosol samples were collected using the AerosolSense instrument (Thermo Fisher Scientific). Following RNA extraction, samples were tested by the TaqPath COVID-19 CE-IVD RT-PCR kit (Thermo Fisher Scientific). More details can be found in Supplementary Methods.

Whole-genome sequencing and phylogenetic analyses

Samples with a sufficiently high viral load (>1000 copies/ml) were subjected to whole-genome sequencing (WGS) using the ARTIC Network protocol v3.17 [17] or as described by Freed *et al.*[18] and sequenced with Oxford Nanopore Technologies ARTIC library preparation. Complete sequences were recovered using the ARTIC analysis pipeline and were typed using Pangolin and NextClade. Specifically and in order to investigate if those outbreaks could have been induced by multiple introduction events in the nursing home, we aimed to contextualize the position of those infectious cases in a more global phylogenetic tree built from the analysis of an alignment made of (i) the viral genomes collected in the considered nursing home and sequenced in the context of the present study, as well as (ii) the genomic sequences of the same variant available for Belgium at the time of the outbreak, and (iii) a subtree of the European Nextstrain build containing all the genomic sequences of that variant at the time of the outbreak. A time-calibrated maximum likelihood phylogenetic tree was constructed using IQ-TREE v2.0.3.19 [19] (GTR model) [20] and TreeTime v0.8.4.22.[21] Extended protocols are available in Supplementary Methods.

Immunovirological profiling by digital transcriptomics (nCounter)

To identify immunovirological risk factors, 600-plex target profiling was performed by digital nCounter transcriptomics (Nanostring) in a subset of residents (n=60). RNA was extracted from nasopharyngeal swabs as described above and used for hybridization to pre-specified Human Immunology V2 and customized SARS-CoV-2 panels, as described elsewhere.[22-24]

Analysis of publicly available bulk and single-cell RNAseq data

Bulk RNAseq data from both nasopharyngeal and blood samples, as well as corresponding gene signatures of fatal vs. non-fatal COVID-19 in hospitalized patients were obtained from Lee et al.[25] Single-cell RNA-Seq data[26] of nasopharyngeal samples of 19 COVID-19 patients (8 moderate and 11 critical according to WHO classification) and five healthy controls were obtained from https://doi.org/10.6084/m9.figshare.12436517, single-cell RNAseq data from PBMCs of COVID-19 patients were obtained from www.covidcellatlas.com[27]. Transcript levels of selected genes across cell types in healthy donors and moderate or critical COVID-19 patients were plotted proportional to the percentage of cells expressing the reported genes at a normalized expression level higher than one. and color-coded by cell types/sample/severity in nasopharyngeal samples. from healthy donors and COVID-19 patients. Normalized expression of selected genes was overlaid on the UMAP (Uniform Manifold Approximation and Projection (UMAP) space.

Statistical Analyses

Demographic and clinical data (COVID symptoms, pre-existing comorbidities, clinical outcome from all residents and detailed pre-COVID pharmacological data; the level of detail differed per nursing home) were collected from health records provided by the nursing homes and hospitals. Missing data were not

imputed, only individuals with all available parameters respective to the specific model were included. Stepwise logistic regression was used to identify risk factors for fatal COVID-19 and the best model was selected using the corrected Akaike's Information Criterion (AICc). Kaplan-Meier estimates of survival were calculated up to 60 days after the first SARS-CoV-2 PCR-positive case in each nursing home outbreak. Selected predictors were confirmed by Cox proportional hazard regression, defining survival in days since PCR diagnosis. In sensitivity analyses, only fully vaccinated (defined as two BNT162b2 doses received at least 14 days prior to the start of the outbreak) and PCR-positive residents were included, and Delta and non-Delta outbreaks were analyzed separately.

Results

Epidemiological characterization of SARS-CoV-2 outbreaks in three nursing homes

For the largest of the three outbreaks (nursing home A), the first infection was documented in the dementia ward on May 17, 2021, for an 89-year-old woman who developed COVID-19-related symptoms, was subsequently hospitalized and succumbed after two weeks of hospitalization. An additional 101 cases were documented related to this outbreak between May 18 and June 24, of which 75 were residents, 25 staff members and 2 family members of staff. All departments of the nursing home were involved, and consecutive screening moments were scheduled. Among 120 residents, 75 were SARS-CoV-2 positive by PCR (62.5%, Table 1), whereas only 25 out of 146 (17.1%) staff members tested positive (Supplementary Table 1). Timing of diagnosis by a positive PCR result and longitudinal follow-up is illustrated in Figure 1A, which clearly illustrates delayed PCR-positivity for a large subset of residents who tested PCR-negative at the start of the outbreak. This "second wave" of delayed infections was corroborated by the continuous detection of SARS-CoV-2 by qPCR in aerosol samples taken from the common areas of both staff and residents (Figure 1B). For 59.2% of positive cases, WGS information was available, identifying the Delta variant (Pangolin lineage B.1.617.2) for all of them. Phylogenetic analysis indicates that all samples from the nursing home cluster within the same clade, hence suggesting a single introduction event (Figure 1C). Among the 102 PCR positive cases, 15 residents died (case fatality ratio of 14.7%). Considering all individuals for which vaccination status was known (Table 1), 96% of residents but only 66% of staff members were fully vaccinated. One resident and five staff members were partially vaccinated at the time of the outbreak, while one resident and 28.7% of staff members were not vaccinated.

The first documented PCR-positive case for nursing home B dates from May 20, 2021, while the presumed index case developed COVID-19 symptoms the day before. Overall, 19 out of 29 residents (65.5%) tested positive for SARS-CoV-2, but none of the 17 staff members tested positive on the repetitive screening moments organized between May 20 and June 24. WGS classified the circulating virus as the Gamma variant (Pangolin lineage P.1). Our phylogenetic analysis highlights that all samples cluster together within the more global Gamma phylogeny inferred in our study, again pointing towards the hypothesis of a single introduction event (Supplementary Figure 1). Overall, 7 fatal cases were reported in this outbreak, of which one resident tested negative by PCR. However, this death was classified as COVID-

19-related due to severe respiratory symptoms and recent close contact with positive residents. For this nursing home, the vaccination rate was high among residents (86.2%), while only 52.9% of the staff members were fully vaccinated at the time of the outbreak. Nevertheless, none of them tested positive for SARS-CoV-2.

The post-vaccination outbreak in nursing home C was initially alerted by two cases (related resident and staff) infected with the Delta variant a few days prior to the large testing initiative for the other residents and staff members planned on July 20, 2021. Twenty-five additional SARS-CoV-2 positive cases were identified during the outbreak. WGS determined the presence of the variant of interest Mu (Pangolin lineage B.1.621), complemented with the mutation K417N in the spike protein, while for one isolated case (staff member without resident contact), an additional Delta infection was identified. The three Delta cases are therefore not considered for the description of the outbreak (Table 1). The Mu variant saw relatively limited circulation in Belgium, resulting in a restricted sampling of related genomic sequences in the local community. Our phylogenetic analysis however indicates that infectious cases in this nursing home related to that variant clearly cluster within the overall phylogeny inferred for that variant (Supplementary Figure 2), again advocating for a single introduction event. Among the 24 PCR-positive cases infected with variant Mu, 20 residents and four staff members were involved, all linked to the dementia unit of the nursing home. Overall, seven infected residents died, while one additional resident died of a COVID-19-unrelated cause. The final SARS-CoV-2 infection was diagnosed on August 10, 2021. Considering the 229 residents and staff members with known vaccination status, the overall vaccination rate was 98.3%. For the group of PCR-positive residents, 100% were fully vaccinated.

Demographic and clinical characterization of SARS-CoV-2 outbreaks in three nursing homes

Demographic and clinical risk factors for fatal COVID-19 were identified by multivariable logistic regression models (Table 2), with the best model including age, male sex, non-Delta SARS-CoV-2 variants (Gamma/Mu), and later timing of infection (PCR-positivity >7 days after start of the outbreak). In the sensitivity analysis, only fully vaccinated and PCR-positive residents (n=107) were included. The results remained statistically significant, with a similar effect size (Supplementary Table 2). The importance of these four factors as predictors of mortality was confirmed by Kaplan-Meier survival estimates (Supplementary Figure 3) and time-to-event analysis (Cox Proportional Hazard regression, Supplementary Table 3). Of interest, dementia or peak viral load (nadir Cq value) were not predictive of fatal cases in the joint analysis of the three outbreaks (Table 2) but were significant predictors in single nursing homes (Supplementary Table 3).

Virological and immunological characterization of SARS-CoV-2 outbreaks in three nursing homes

In search of candidate biomarkers for post-vaccine fatal COVID-19, as well as possible novel therapeutic targets, we opted for nCounter digital transcriptomics for immunovirological profiling of the nasal mucosa, encouraged by previous results[22-24]. For 20 out of 28 fatal cases, a sufficient volume of diagnostic nasopharyngeal swabs was available for nCounter analysis, to explore immunological (600 genes representative of the major immune cell types) and virological parameters (SARS-CoV-2 transcripts and ACE2/TMPRSS2 receptors) as possible risk factors for fatal post-vaccine COVID-19. Thus, we carefully matched (age, sex, outbreak) 20 fatal cases (all those with available nasopharyngeal swabs) with 30 PCR-positive non-fatal cases, with similar timing of infection, as well as 10 PCR-negative but SARS-CoV-2-exposed residents.

As shown in Figure 2 (Volcano plot), a total of 193 human and 7 viral gene transcripts were significantly up- or down-regulated ($p<0.05$) when comparing fatal vs. non-fatal cases. In addition to the antiviral cytokines *IL28A* (also known as *IFNL2,* interferon-λ2) and *IFNB1* (the gene encoding interferon-beta, IFN-β), the most upregulated genes were predominantly expressed by innate immune cells: monocytes/macrophages (*CX3CR1, TNFSF15, CLEC6A, ITLN1, LILRB5*), Natural Killer (NK) cells (*THY1, CDH5, KIR3DL3, CD160, B3GAT1, NCAM1, CCL3*) and conventional dendritic cells (*XCR1*). Thus, the predominant immunopathogenic signature of fatal COVID-19 in vaccinated residents represents exacerbated innate immune activation, rather than a failed adaptive (B and T-cell) vaccine response. Surprisingly, a large subset of B-cell genes (*CD19, CR2, CD79A, CD79B, PAX5, CD70),* regulatory T-cell (Treg) genes *(FOXP3, PTGER4)* and cytotoxic CD8 T-cell genes (*EOMES, PTGER4*) were also significantly up-regulated in fatal cases, arguing against a curtailed B- or T-cell response or a failure of B or T-cells to migrate to the nasal mucosa. Since the top down-regulated genes were most representative of mucosal epithelial cells (*PIGR, CD9, MUC1*), the observed exacerbated innate response might represent enhanced migration of innate immune cells but also virus-mediated destruction of the mucosal epithelial cells.

In favor of the latter hypothesis, fatal cases were characterized by significantly higher viral transcript levels, when measured by nCounter. Transcript levels for Spike, Envelope, Nucleoprotein, ORF1ab, ORF3a and ORF7a genes (Figure 3A and data not shown, all $p<0.05$ with False Discovery Rate (FDR) correction), were higher in fatal cases compared to non-fatal PCR-positive residents. In addition, antisense SARS-CoV-2 was selectively increased in 8 out of 20 fatal cases (Figure 3A) versus PCR-positive cases, indicating heightened intracellular viral replication. Of note, peak viral load (nadir Cq values) or viral load of the first PCR-positive sample, measured by qPCR, were not significantly different between fatal cases and PCR-positive controls (Figure 3A), underscoring the sensitivity of nCounter digital transcriptomics. Exacerbated viral replication in fatal cases was paralleled by a marked eightfold increase in viral receptor *ACE2* transcript levels ($p<0.001$), as well as an unexpected two-fold decrease ($p<0.01$) in viral co-receptor *TMPRSS2* expression (Figure 3B).

Among all immune genes, *IFNB1* transcripts displayed the strongest negative correlation to survival time (starting from the date of PCR-positive diagnosis, Spearman's $\rho=-0.24$, $p=0.0024$). Corroborating our previous findings in a Belgian cohort of ICU patients,[22] we found that increased *IFNB1* transcript levels significantly predicted a fatal outcome (Figure 3C-D, AUROC 0.76 (95%CI 0.63-0.89), $p=0.0013$), which

was only slightly increased by adding age and sex to the model (Figure 3D, AUROC 0.80 (95%CI 0.69-0.92), p=0.0002). *IFNB1* remained a significant predictor in multivariable logistic regression, independent of age, sex and peak viral load (nadir Cq value), which was also confirmed by time-to-event analysis (Cox Proportional Hazard models, Table 3), and was replicated when Delta and non-Delta (Gamma/Mu) outbreaks were analyzed separately (Supplementary Table 3).

Lastly, when combining all available demographic, immune and viral parameters, the best predictive model for mortality, according to the corrected Akaike's Information criterion (AICc), included age (OR 1.07, 95%CI 0.98-1.19), increased viral *ORF7a* (OR 1.67 95%CI 0.98-3.46) and viral receptor *ACE2* (15.43 95%CI 2.54-165.9) transcript levels, resulting in correct classification of 18 out of 20 (90%) fatal cases (AUROC 0.87, 95%CI 077-0.97, p<0.0001), as visualized in Figure 3D.

Comparison with published pre-vaccine fatal COVID-19 signatures and reanalysis of single-cell RNAseq data highlights the unique immune signature in post-vaccination fatal COVID-19 outbreaks

To our knowledge, this is the first well-powered study of immune signatures in post-vaccination fatal COVID-19 in the elderly. Thus, no public datasets are currently available for independent validation of our newly derived immune signature in a comparable epidemiological setting. Therefore, we compared published gene signatures of pre-vaccination fatal COVID-19 in nasal mucosa and matched whole blood samples[25]. In addition, we reanalyzed publicly available single-cell RNAseq data from nasal mucosa of patients with moderate and critical COVID-19[26] and PBMCs from critical and fatal COVID-19 cases[27], all from the pre-vaccination era.

As shown in Figure 4A, there was surprisingly little overlap between our "post-vaccine" fatal COVID-19 signature (nasal swabs) and "pre-vaccine" fatal COVID-19 gene signatures (bulk RNAseq) from either nasal swabs or whole blood, the latter comparing fatal vs. non-fatal hospitalized COVID-19 patients. No differentially expressed gene was shared among the three datasets, while only six and 15 genes were shared between our "post-vaccine" fatal signature and the "pre-vaccine" nasal swab and whole blood gene fatal signatures, respectively (Figure 4A, middle panel). Moreover, directionality was opposite (up- vs. down-regulation) for 5 out of 6 nasal mucosa genes (Figure 4A left panel) and 4 out of 15 whole blood genes (Figure 4A right panel), and fold-changes were not correlated (p=0.42 and p=0.98, respectively, data not shown).

Reanalysis of publicly available single-cell RNAseq data shows cell-specific expression of the strongest up-regulated genes in fatal cases: *TNFSF15* in neutrophils (Neu) and non-resident macrophages (nrMA), *PTGER4* in neutrophils and Treg, *CX3CR1* in resident (rMa) and non-resident macrophages (nrMa), *CR2* in B-cells, *ACE2* in several epithelial cell types (Figure 4B). Of these, most findings of gene- and/or cell-specific up-regulation with disease severity were replicated between both nasal mucosa datasets (Figure 4C, upper panel), i.e. up-regulation in post-vaccine fatal cases (this study) as well as in critical vs. moderate COVID-19 (pre-vaccine era), with the exception of *CX3CR1*. In addition, increased *EOMES*

expression in cytotoxic CD8 T-cells (CTL) was also replicated in critical vs. moderate COVID-19 (Figure 4C, lower panel). However, divergent expression between post-vaccine (fatal COVID-19, this study) and pre-vaccine data sets (moderate vs critical COVID-19) was observed for *CDH5* and *THY1* in NK cells (not increased in critical COVID-19). Furthermore, *IFNB1* and *MASP2* transcripts were undetectable by single-cell RNAseq analysis of both nasal mucosa[26] (Figure 4C, lower panel) and PBMCs[27] (data not shown) from COVID-19 patients, again underscoring the sensitivity of nCounter digital transcriptomics for low-abundance transcripts. As shown in Suppl. Figure 4, top genes downregulated in fatal cases were most expressed in epithelial cell types (*PIGR, MUC1, CD9, CD46*), in agreement with our virus-mediated mucosal epithelium destruction hypothesis (Figure 2-3). On the other hand, a generalized down-regulation of MHC class I-mediated antigen presentation (*B2M, HLA-C*) was observed across all cell types, in agreement with previous reports demonstrating loss of MHC class I activity at the transcriptomic, epigenomic and functional level[25-30].

Taken together, cross-examination of published pre-vaccine fatal COVID-19 signatures and reanalysis of single-cell RNAseq data highlights the unique innate and adaptive immune signature observed in post-vaccination fatal COVID-19 in nursing home residents.

Discussion

Herein, we comprehensively studied three large outbreaks in Belgian nursing homes with high fatality ratios (20–35%), which resulted in several novel findings of both epidemiological and clinical relevance for the ongoing 'arms race' between vaccines and SARS-CoV-2 variants of concern. First, whole-genome phylogenetic analyses indicated that each outbreak stemmed from a single introduction event, though with different variants (Delta, Gamma, and Mu). Second, our study confirms previous reports of the independent relationship of older age and male sex with fatal COVID-19 yet is the first to identify Gamma/Mu variants and timing of PCR-positivity as independent risk factors for fatal post-vaccination COVID-19 among the elderly. Our findings evoke that even non-dominant variants of concern (Gamma) or of interest (Mu) can result in significantly higher mortality than the dominant strain (Delta at the time of this study, May-August 2021) in specific high-risk settings. Third, environmental sampling revealed that SARS-CoV-2 could detected in aerosol samples of common spaces (used by either residents or staff) up to 52 days after the initial infection. Fourth, gene expression profiling of nasopharyngeal swabs identified candidate immunological and virological biomarkers for early monitoring of post-vaccine breakthrough cases in high-risk elderly, which might not be limited to nursing homes.

Indeed, increased *IFNB1* transcript levels are highlighted as a significant independent predictor of fatal post-vaccination COVID-19, confirming and extending our previous findings in critical COVID-19.[22] Although IFN-β therapy was beneficial in small phase 2 clinical trials[31–32], subsequent larger trials identified no benefit[33], or even an association to a longer ICU stay[34], thus underscoring our previous findings on endogenous IFN-β expression in ICU patients[22]. As previously proposed[35–36], these apparently conflicting effects of type I IFN can be explained by a two-phase model, in which early IFN

results in antiviral protection[37], while late IFN exerts a deleterious pro-inflammatory effect. In support of this hypothesis, we found that *IFNB1* transcripts were strongly correlated (Spearman's ρ = 0.84, $p = 6.8x10^{-17}$) to IL-6 receptor (the target of tocilizumab) expression. Thus, our study suggests IL6/IL6R signaling as a plausible 'downstream' therapeutic target in *IFNB1*-overexpressing COVID-19 patients, which should be investigated in future clinical trials.

Regarding the clinical use of transcriptomic biomarkers in COVID-19, only nCounter technology was able to reliably detect *IFNB1*, as well as other low-abundant transcripts (*MASP2, THY1)*, when compared with single-cell RNAseq analysis of both nasal mucosa[26] (Fig. 4C) and blood[27] (data not shown). Moreover, 10 cytokine transcripts found to be overexpressed in fatal cases by nCounter (*IFNA1, IFNA2, IFNB1, IL2, IL3, IL17B, IL17F, IL20, IL21, IL26*), were undetectable or extremely low in several single-cell RNAseq data sets[25–27]. In addition, only a small subset of these cytokines have been reproducibly detected at the protein level as biomarkers of COVID-19 disease severity and mortality, as evidenced by a recent meta-analysis[38]. In addition, this study also found that nCounter technology outperformed conventional qPCR (Fig. 2A) for virological monitoring of nasopharyngeal swabs to instruct COVID-19 clinical management.

Finally, our finding of increased viral receptor ACE2, enhanced intracellular viral replication and later stage PCR-positivity in fatal cases hints at a therapeutic window for early antiviral therapy at the start of an outbreak.[39–41] Thus, our study indicates that biomarker-guided clinical trials evaluating the role of early antiviral therapy during post-vaccination nursing home outbreaks, and conceivably also among susceptible community-dwelling elderly, are warranted.

Limitations of this study include missing demographic (8.4% out of 657), clinical (2.4% out of 620) and vaccination (26.5% out of 574) data, although no data were missing for fatal cases. Due to the unpredictable and sudden onset of these large-scale COVID-19 outbreaks in nursing homes, no baseline serum samples were available before the three outbreaks, nor from fatal cases, to compare the levels of vaccine-elicited SARS-CoV-2-neutralizing antibodies. Finally, the observational nature of the study and the heterogeneity among three outbreaks (three different variants in nursing homes with different population sizes) might result in residual confounding factors, although the demographics, major pre-existing comorbidities and vaccine rates were highly similar between the nursing homes (Supplementary Table 1). A major strength of this study is the simultaneous vaccination of residents in each nursing home (prioritized in the national vaccination campaign), and the defined onset (outbreaks) of SARS-CoV-2 infections, thus eliminating any possible bias in waning vaccine efficacy between fatal and non-fatal cases. Since this study was performed before vaccination booster doses were offered to the elderly in Belgium (start September 2021), the risk factors identified herein might not be directly applicable in (recently) boosted elderly populations but are highly relevant in the global context, in which currently only 57% of people have received an initially full vaccination protocol (2 doses)[42], as exemplified by recent high Omicron COVID-19 mortality among unvaccinated elderly in Hong Kong (ourworldindata.org)[42].

In conclusion, high case fatality ratios in susceptible elderly can be observed with non-dominant SARS-CoV-2 strains, e.g. Gamma and Mu variants during the Delta 'era'. Broad immunovirological profiling by

nCounter transcriptomics allowed prediction of fatal COVID-19, while standard qPCR viral load quantification did not. A multi-layered strategy including environmental sampling, immunomonitoring, and early antiviral therapy should be considered to prevent post-vaccination COVID-19 mortality in nursing homes.

Declarations

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Tables

Table 1: Demographic and clinical characteristics of nursing home residents involved in the three post-vaccination outbreaks

Characteristics	Nursing home A Delta	Nursing home B Gamma	Nursing home C Mu[1]
Median age (range)	87 (63 – 102)	82 (59 – 98)	87 (64 – 103)
Sex – number (%)			
Male	71 (26.5%)	11 (23.9%)	77 (25.2%)
Female	197 (73.5%)	35 (76.1%)	229 (74.8%)
Start vaccination residents	08 Jan 2021	12 Jan 2021	26 Jan 2021
Vaccination ratio (among PCR+)			
2 doses	96% (94.6%)	86.2% (89.5%)	98.0% (100%)
1 dose	1% (1.4%)	13.0% (10.5%)	0.4% (0%)
0 doses	3% (4.1%)	0%	1.3% (0%)
First documented case	17 May 2021	20 May 2021	20 July 2021
PCR positivity	62.5% (75/120)	65.5% (19/29)	12.0% (20/166) 69.0% (20/29)[4]
Case Fatality ratio	20.0% (15/75)[2]	36.8% (7/19)[3]	35.0% (7/20)[2]

[1]For nursing home C, three isolated Delta cases were observed in addition to the Mu outbreak. All residents received the Comirnaty (Pfizer) vaccine.

[2] An additional resident died, not SARS-CoV-2 PCR-positive, with death considered not-COVID-19 related.

[3] Although one fatal case was not SARS-CoV-2 PCR positive, this death was classified as COVID-19-related due to severe respiratory symptoms and recent close contact with positive residents.

[4] Considering only the 29 residents of the two affected wards, positivity rates increase up to 69.0%.

Table 2: Multivariable Logistic Regression of Demographic and Clinical Characteristics of Residents with COVID-19

	Model 1		Model 2		Model 3*		Model 4	
Variable	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**	**Odds ratio**	**95% CI**
Gender[M]	3.38	1.24-9.47	3.55	1.29-10.1	6.03	1.91-21.21	5.68	1.72-20.94
Age	**1.08**	**1.02-1.15**	**1.08**	**1.02-1.16**	**1.13**	**1.05-1.22**	**1.15**	**1.06-1.25**
SARS-CoV-2 Gamma/Mu	-	-	1.74	0.71-4.32	3.97	1.26-13.98	3.73	1.14-13.62
Late PCR+	**-**	**-**	**-**	**-**	**3.28**	**1.04-11.58**	2.96	0.92-10.72
Dementia	-	-	-	-	-	-	0.99	0.36-2.76
Diabetes	-	-	-	-	-	-	1.44	0.39-5.10
Nadir Cq value	-	-	-	-	-	-	1.01	0.95-1.06

*Model 3 was the best model, according to corrected Akaike's Information Criterion (AICc), significant variables are indicated in bold.

Table 3: Multivariable Cox Proportional Hazard Regression of immunological and virological parameters in fatal vs. non-fatal post-vaccination COVID-19 in nursing home residents

	Model 1* (50 residents)		Model 2 (50 residents)		Model 3 (Delta, n=18)		Model 4 (Gamma/Mu, n=32)	
Variable	**Hazard ratio**	**95% CI**	**Hazard ratio**	**95% CI**	**Hazard ratio**	**95% CI**	**Hazard ratio**	**95% CI**
Gender[M]	1.97	0.64-5.45	2.02	0.65-5.83	2.13	0.27-12.58	2.62	0.52-12.94
Age	**1.08**	**1.02-1.15**	**1.08**	**1.02-1.16**	**1.13**	**1.05-1.22**	**1.15**	**1.06-1.25**
IFNB1 transcript levels (log)	**2.32**	**1.26-4.48**	**2.36**	**1.26-4.63**	**13.02**	**2.41-125.2**	**2.64**	**1.10-7.86**
Nadir Cq value	-	-	1.01	0.95-1.06	-	-	-	-

*Model 1 was the best model, according to corrected Akaike's Information Criterion (AICc), significant variables are indicated in bold

Figures

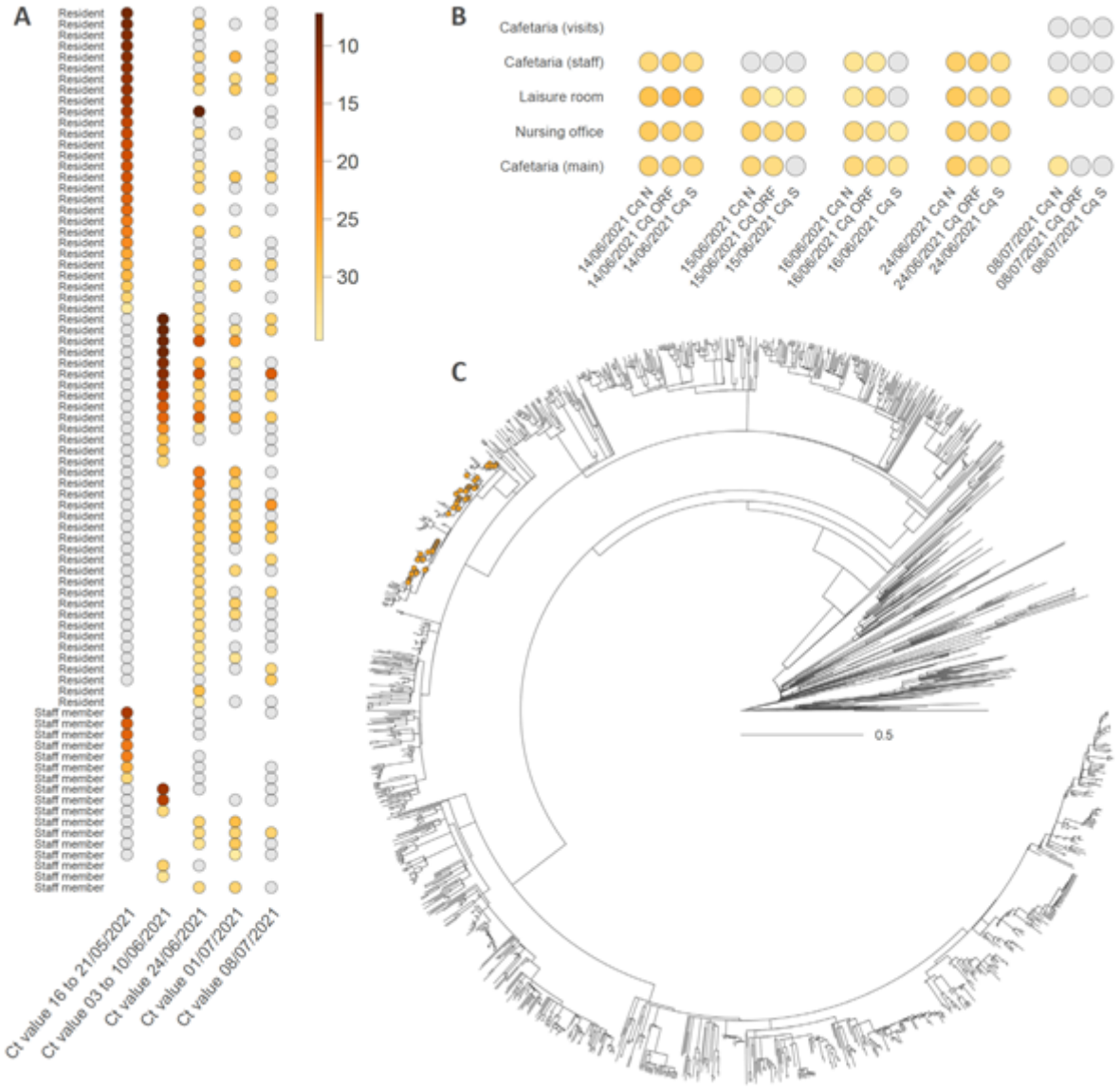

Figure 1

Overview of the outbreak in nursing home A (Delta/B.1.617.2). We report the evolution through time of Ct values measured in both infected resident/staff members (**A**) and aerosols analyzed in various sections within the nursing home (**B**). Grey dots refer to negative PCR results. (**C**) Time-scaled phylogenetic analysis involving Delta (B.1.617.2) genomes sampled and sequenced from this outbreak reveals that all 58 genomes originating from nursing home A are clearly clustered within the overall phylogenetic tree (orange dots), suggesting a single introduction event.

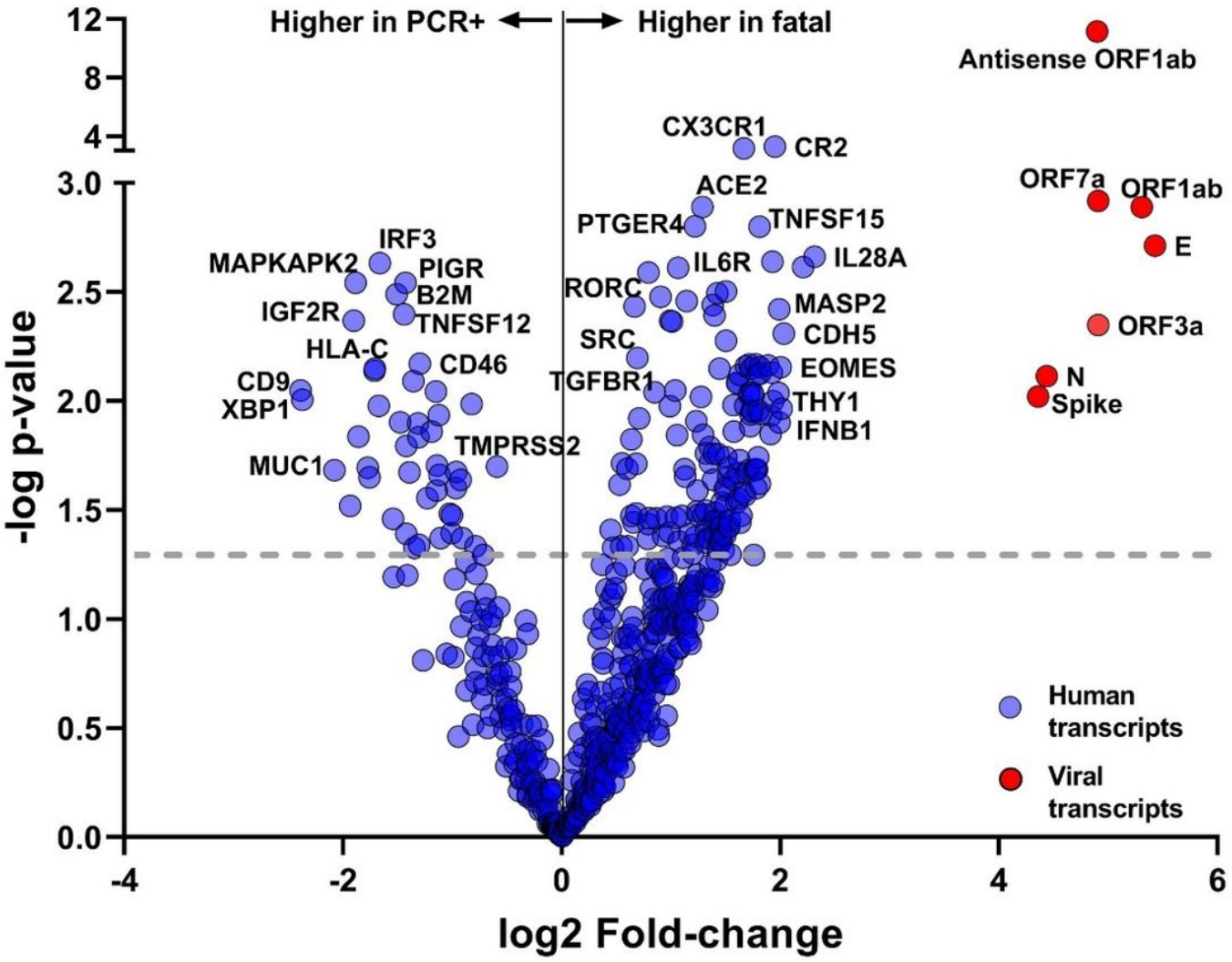

Figure 2

Differentially expressed genes in nasal mucosa of fatal COVID-19 outbreak cases, as compared to matched PCR-positive residents from three nursing homes.

Volcano plot of differentially expressed genes in nasal mucosa of fatal (n=20) vs. age-, gender-, and outbreak-matched non-fatal PCR+ cases (n=30), quantified by nCounter digital transcriptomics (p-values from linear model, negative binomial distribution, dotted line showing p<0.05 cutoff). Selected viral (red circles) and host immune transcripts (blue circles) significantly up-or down-regulated in fatal vs. non-fatal cases are highlighted with gene names. Details on immune genes are given in the Results section.

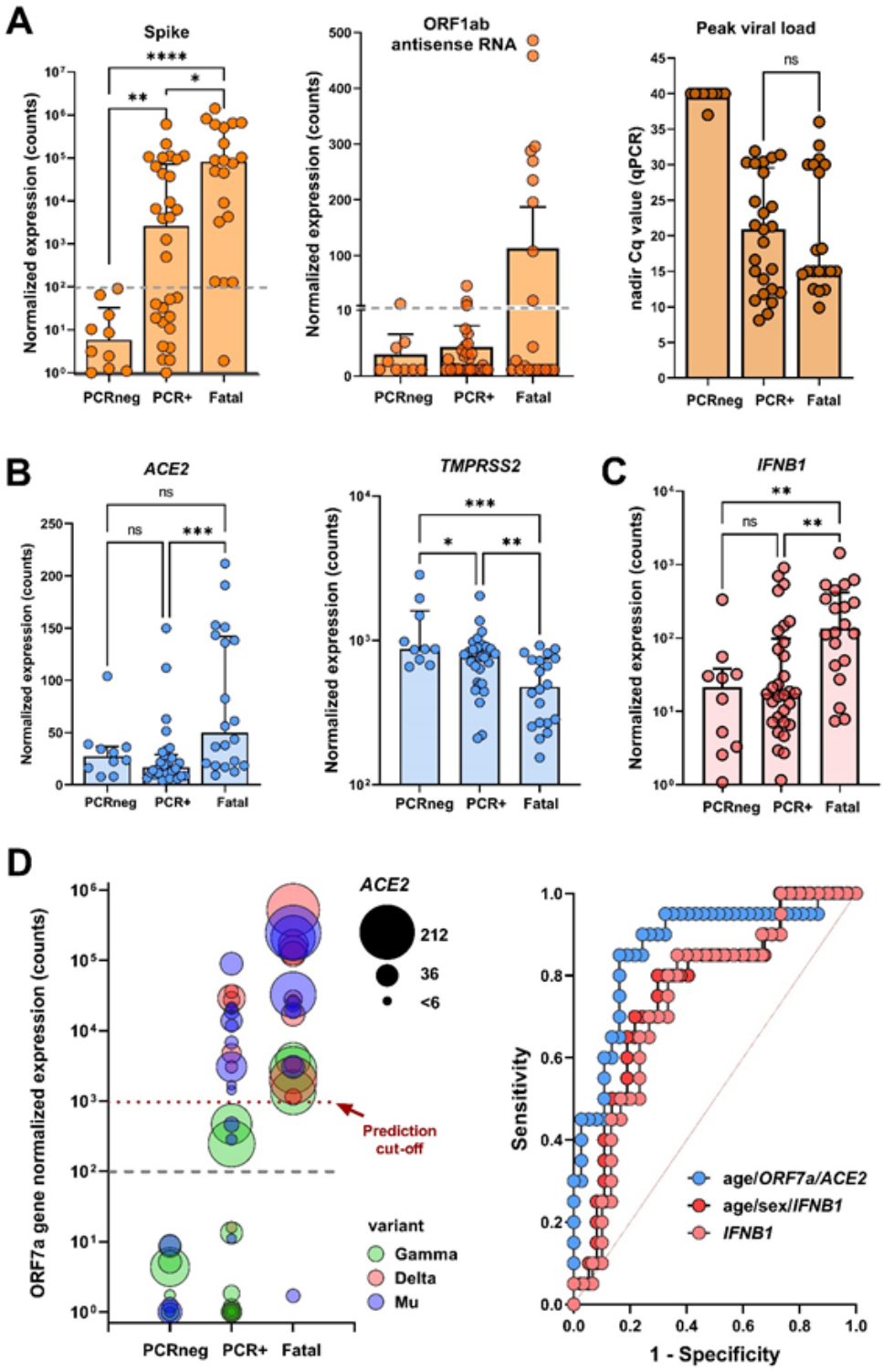

Figure 3

Immunological and virological risk factors identified in fatal COVID-19 outbreak cases among residents in three nursing homes.

(A) Viral transcript levels for Spike protein (left panel) and ORF1ab antisense RNA (middle panel), measured by nCounter digital transcriptomics. Right panel shows peak viral load (nadir Cq values), as

quantified by qPCR. (B) Viral receptors (*ACE2/TMPRSS2*) and (C) antiviral cytokine *IFNB1* were quantified by nCounter digital transcriptomics. (D) Left panel: Visualization of best predictive model (multivariable logistic regression, selected by corrected Akaike's Information Criterion), including age (not depicted), *ORF7a* and *ACE2* transcripts. Dashed grey lines indicate the detection limit of SARS-CoV-2 transcripts. Each circle represents a resident, and the size of the circle is a measure of the *ACE2* gene expression. Right panel: Comparison of ROC curves of predictive models by univariable (*IFNB1)* or multivariable logistic regression *(IFNB1*/age/sex, and age/*ORF7a/ACE2*). ROC curves showing significant prediction of fatal vs. non-fatal COVID-19 according to *IFNB1* transcript levels (right panel), with and without age and sex as additional factors. For A-B-C, statistical results are from Kruskal-Wallis test with FDR correction for multiple testing, ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. Statistical details for D are detailed in the Results section.

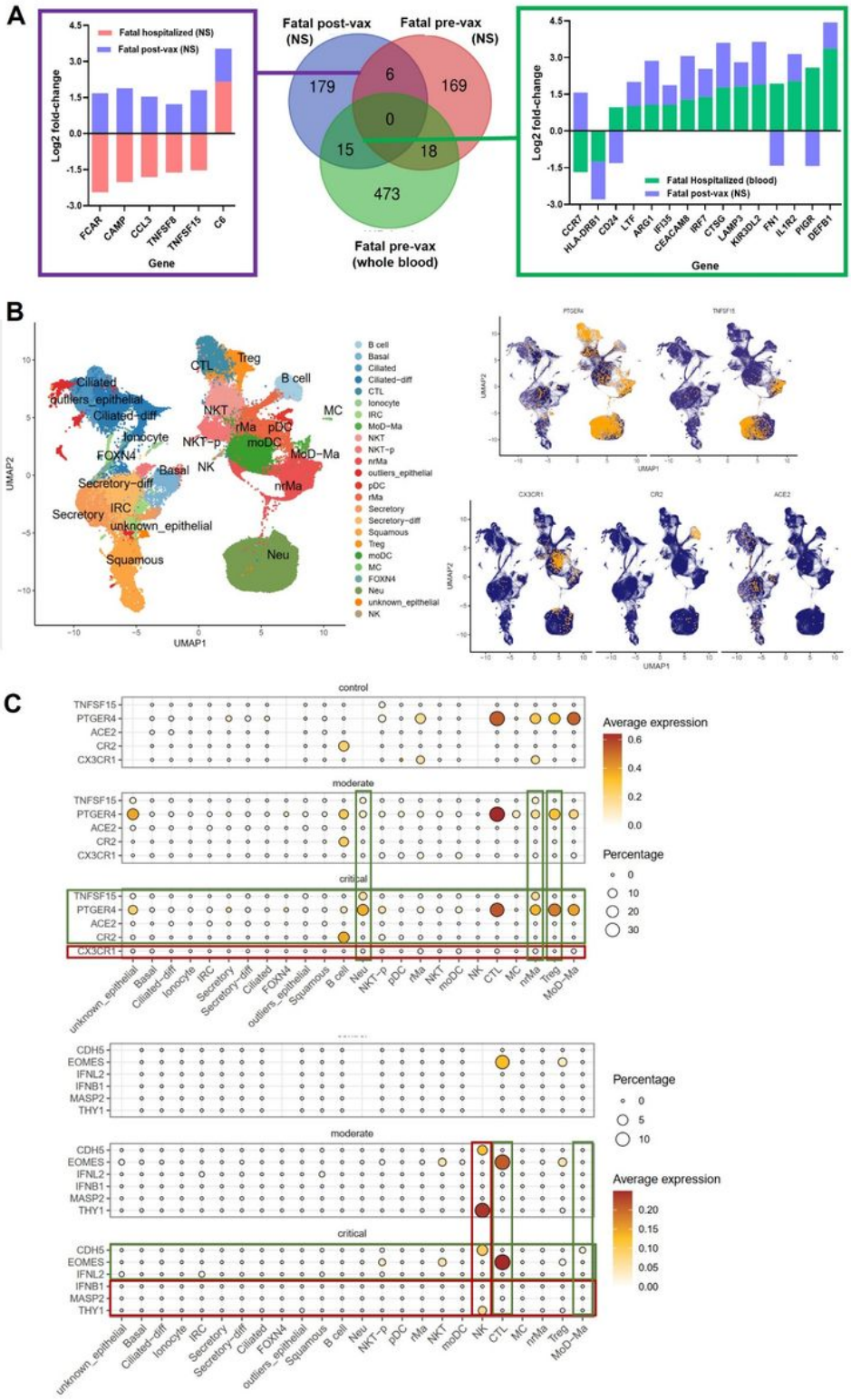

Figure 4

Comparison with published pre-vaccine fatal COVID-19 signatures and reanalysis of single-cell RNAseq data highlights the unique fatal COVID-19 immune signature in post-vaccination outbreaks

(A) Overlap between post-vaccine fatal COVID-19 gene signature determined by nCounter in nasal mucosa (this study) and published pre-vaccine fatal COVID-19 gene signature determined by bulk

RNAseq of nasal mucosa (NS) and whole blood. (B) and (C) Reanalysis of publicly available single-cell RNAseq data shows cell-specific expression of strongest up-regulated genes in fatal cases: TNFSF15 in neutrophils (Neu) and non-resident macrophages (nrMA), PTGER4 in neutrophils and Treg, CR2 in B-cells, ACE2 in several epithelial cell types. UMAP plot of single-cells from all controls and patients in (B). Green boxes show replicated findings of gene- and/or cell-specific up-regulation, red boxes show divergent expression between post-vaccine (fatal COVID-19, this study) and pre-vaccine data sets (moderate vs critical COVID-19) in (C).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation.docx