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Oxidative stress and endogenous DNA damage in blood mononuclear cells may predict anti-SARS-CoV-2 antibody titers after vaccination in older adults

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ABSTRACT

Immune senescence in the elderly has been associated with chronic oxidative stress and DNA damage accumulation. Herein we tested the hypothesis that increased endogenous DNA damage and oxidative stress in peripheral blood mononuclear cells of older adults associate with diminished humoral immune response to SARS-CoV-2 vaccination. Increased oxidative stress and DNA double-strand breaks (DSBs) were detected in 9 non-immunocompromised individuals aged 80–96 years compared to 11 adults aged 27–44 years, before, as well as on days 1 and 14 after the first dose, and on day 14 after the second dose of the BNT162B2-mRNA vaccine (all $p < 0.05$). SARS-CoV-2 vaccination induced a resolvable increase in oxidative stress and DNA damage, but individual DSB-repair efficiency was unaffected by vaccination irrespective of age, confirming vaccination safety. Individual titers of anti-Spike-Receptor Binding Domain (S-RBD)-IgG antibodies, and the neutralizing capacity of circulating anti-SARS-CoV-2 antibodies, measured on day 14 after the second dose in all participants, correlated inversely with the corresponding pre-vaccination endogenous oxidative stress and DSB levels (all $p < 0.05$). In particular, a strong inverse correlation of individual pre-vaccination DSB levels with both the respective anti-S-RBD-IgG antibodies titers ($r = -0.867$) and neutralizing capacity of circulating anti-SARS-CoV-2 antibodies ($r = -0.983$) among the 9 older adults was evident. These findings suggest that humoral responses to SARS-CoV-2 vaccination may be weaker when immune cells are under oxidative and/or genomic stress. Whether such measurements may serve as biomarkers of vaccine efficacy in older adults warrants further studies.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has put great pressure on healthcare systems worldwide [1,2]. Following an impressive multidisciplinary international scientific effort over 250 vaccine candidates are currently under clinical or pre-clinical development and 4 vaccine platforms gained regular licensure or emergency use authorization by the FDA and the EMA, consisting of two mRNA-based vaccine platforms (Pfizer/BioNTech and Moderna) and two non-replicating viral vector-based vaccine platforms (Astra-Zeneca/Oxford and Janssen), in less than a year [3–5]. Apart from the unprecedented vaccine development speed, vaccines based on the mRNA vaccine-technology, exhibit more than 90% effectiveness on the general population [6], in contrast to the yearly influenza vaccination effectiveness, which does not exceed 60% [7]. Therefore, anti-SARS-CoV-2 vaccination provides a promising control strategy not requiring repeated lockdowns [8], as exemplified by the cases of Israel and UK, in which rapid vaccination of the general population resulted in

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substantially reduced cases of COVID-19 and the withdrawal of quarantine measures.

Individuals of advanced age constitute a population highly vulnerable to severe and life-threatening COVID-19 [9]. Specifically, older age is associated with increased mortality rates after SARS-CoV-2 infection [10], emphasized by the fact that frailty, an indicator of biological aging [11], is shown to be associated with increased mortality [12,13]. Taken together with the observed blunted antibody response in older individuals [14], these facts underline the exceptional threat that SARS-CoV-2 poses to this population. Recent studies examining the efficacy of SARS-CoV-2 vaccination in older individuals, reveal a sufficient yet decreased antibody response, which may undermine long term immunity [15–17]. Age-associated immune dysregulation because of impaired humoral and cellular responses advances in tandem with organismal aging and results in less efficient antibody responses after vaccination amidst persistent inflammation; these phenotypes are in general described under the terms “immunosenescence” and “inflammaging” [18–23].

Oxidative stress, a term denoting an imbalance between the oxidant and antioxidant cellular systems after exposure to noxious stimuli, is shown to play a central role in the progression of aging [24]. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), produced by several endogenous and exogenous processes, can significantly affect major cellular macromolecules if not neutralized by the designated antioxidant systems [25]. Particularly, the induction of oxidative stress may lead to the formation of oxidative DNA damage which then triggers the activation of the DNA damage response (DDR) network [26,27].

Herein, we sought to investigate the hypothesis that augmented oxidative stress and/or increased DNA double-strand breaks (DSBs) in circulating immune cells may predict lower antibody titers after SARS-CoV-2 vaccination. To this end, we examined critical oxidative stress and DDR parameters in peripheral blood mononuclear cells (PBMCs) from aged individuals undergoing vaccination against SARS-CoV-2, while younger adults were studied in parallel.

2. Material and methods

2.1. Subjects

Nine non-immunocompromised older individuals (median age: 83 years; range: 80–96 years; Table 1) and eleven young, apparently healthy, members of the Laiko Hospital personnel (median age: 32 years; range: 27–44 years), who received the Pfizer/BioNTech mRNA-vaccine (Comirnaty/BNT162B2), were recruited between January–April 2021 and January 2022. Exclusion criteria from participating in the study included cancer, pregnancy, personal or family history of autoimmunity, and active or recent (last 2 weeks) infection. Peripheral blood samples were collected immediately before, 24 h after the first dose of the mRNA SARS-CoV-2 vaccine, and 14 days after both the first and second dose. All volunteering individuals provided written informed consent according to the declaration of Helsinki. The study was approved by Laiko Hospital Ethics Committee (Protocol Nr.1110).

Table 1
Demographics and clinical characteristics of the older adults population.

Characteristic	Older adults
Age, median (range)	83 years (80–96)
Smoking ever (%)	2/9 (22%)
Diabetes melitus (%)	3/9 (33%)
Arterial hypertension (%)	7/9 (78%)
Coronary artery disease (%)	2/9 (22%)
Chronic heart failure (%)	4/9 (44%)
Chronic obstructive pulmonary disease (%)	2/9 (22%)
Dementia (%)	6/9 (66%)
Charlson comorbidity index (median, range)	7 (range: 5–10)
Clinical frailty scale index (median, range)	5 (range: 3–7)

2.2. Cells isolation

Peripheral blood mononuclear cells (PBMCs) were isolated and purified using the Ficoll gradient centrifugation, as previously described [28]. Cells were resuspended in Freezing Medium [90% Fetal Bovine Serum (FBS), 10% Dimethyl sulfoxide (DMSO)] or lysed in TRITidy G (AppliChem, Germany) and stored at −80 °C until further processing.

2.3. Oxidative stress quantification and detection of abasic sites

Endogenous oxidative stress levels were measured using a luminescence-based system that quantifies total glutathione (GSH), oxidized glutathione (GSSG) and the GSH/GSSG ratio, according to manufacturer's experimental protocol (GSH/GSSG-Glo™ Assay, Promega, UK) [29]. ROS are capable of inducing various types of DNA damage, including oxidized purines and pyrimidines, single-strand breaks (SSBs), double-strand breaks (DSBs) and abasic (AP; apurinic/apyrimidinic) sites [30,31]. Therefore, the endogenous abasic site levels (Cell Signaling Inc., UK) were also measured as previously described [29].

2.4. Measurement of DSBs and DSB-R efficiency

DSB levels in PBMCs were assessed using immunofluorescence quantification of γH2AX (H2AX phosphorylated at Ser139; #9718T, Cell Signaling Technology) as previously described [32]. For the evaluation of DSB-R efficiency, PBMCs were treated with 100 μg/ml melphalan for 5 min at 37 °C in complete RPMI medium. Subsequently PBMCs were incubated in drug-free medium for 0, 8 and 24 h, adhered to coverslip, fixed and analyzed as previously described [28].

2.5. Measurement of anti-spike (S)-receptor binding domain (RBD) IgGs against SARS-CoV-2

Serum and plasma were isolated, following venipuncture, and stored at −80 °C until further processing. We measured SARS-CoV-2 antibodies using the FDA-approved Elecsys©anti-SARS-CoV-2 electrochemiluminescence immunoassay in human serum 14 days after the second dose of the SARS-CoV-2 vaccine, according to the manufacturer's protocol (Roche Diagnostics GmbH, Germany) as previously described [33]. The Elecsys© assay is a SARS-CoV-2 antibody assay primarily detecting IgG antibodies. In this assay we used a modified double-antigen sandwich immunoassay using a recombinant SARS-CoV-2 Spike (S) Receptor Binding Domain (RBD) polypeptide, to specifically detect late, mature, high affinity antibodies targeting the S-RBD.

2.6. Measurement of neutralizing antibodies against SARS-CoV-2

Neutralizing antibodies (NAbs) against SARS-CoV-2 were assayed using the surrogate virus neutralization assay cPass™ SARS-CoV-2 Neutralizing Antibody detection kit (GenScript, USA), as previously described [29,33].

2.7. Statistical analysis

Variable distribution was examined by D'Agostino-Pearson and Shapiro-Wilk tests. Continuous variables are presented as mean ± SD. Paired comparisons were performed with the use of Wilcoxon signed-rank test and independent comparisons were performed with the use of Mann-Whiney *U* test. Correlations were examined with the use of the non-parametric Spearman's test. The level of statistical significance was set at $p = 0.05$. Statistical analysis was performed in SPSS v.26(IBM, USA) and GraphPad Prism v.8.02 (GraphPad, USA).

3. Results

3.1. Increased oxidative stress induction in PBMCs of older individuals

First, we investigated whether SARS-CoV-2 vaccination, acting as an acute immune stimulant in the PBMCs of older and younger individuals, induces an increase in intracellular oxidative stress levels. Increased pre-vaccination oxidative stress levels, as indicated by the reduction of total glutathione (GSH) to oxidized glutathione (GSSG) ratio ($p < 0.001$), and the increased formation of abasic sites ($p < 0.001$), were observed in the aged compared to younger individuals, as was previously reported [28]. Additionally, we found that SARS-CoV-2 vaccination elicited a transient increase in oxidative stress among the older individuals 24 h after vaccination, as depicted by both the reduction of GSH to GSSG ratio ($p = 0.008$ vs baseline; Fig. 1a) and the increase of AP-site formation ($p = 0.007$ vs baseline; Fig. 1b), which were subsequently restored to pre-vaccination levels. Of note, SARS-CoV-2 vaccination also induced a similar transient increase of oxidative stress in young adults (glutathione oxidation: $p = 0.05$ vs baseline; AP-site formation: $p = 0.003$ vs baseline), as was shown in detail previously [28].

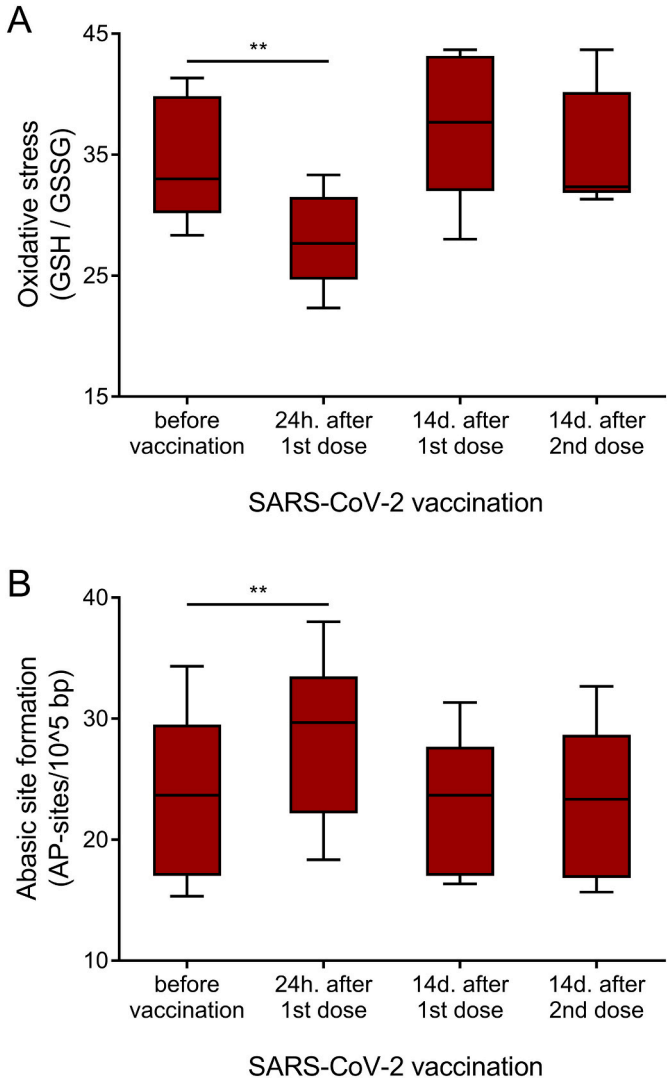

Fig. 1. Resolvable oxidative stress induction after BNT162B2vaccination in PBMCs of older individuals. Tukey boxplots representing oxidative stress levels expressed as (a) the ratio of reduced Glutathione (GSH) to oxidized glutathione (GSSG) and (b) the number of AP-sites in PBMCs of older adults ($n = 9$) before and after SARS-CoV-2 vaccination. *P*-values are derived from Wilcoxon signed-rank test. $^{**}P < 0.01$.

3.2. Oxidative stress drives DNA damage formation

Next, we investigated whether the augmented oxidative stress levels, being observed at all studied timepoints in older individuals compared to younger individuals, could lead to DDR activation, since oxidative stress is a crucial factor of DNA damage formation [26]. DNA damage was assessed by measuring the phosphorylation of histone H2AX at serine 139, an initial response induced after the formation of DNA DSBs. We found that PBMCs from older individuals exhibit increased DSB levels before ($p < 0.001$) and after every vaccination timepoint studied (1d: $p < 0.001$, 14d: $p < 0.001$, 35d: $p < 0.001$) compared to young individuals (Fig. 2). Notably, SARS-CoV-2 vaccination caused a transient DSBs accumulation in both older and young individuals ($p = 0.008$ and $p = 0.013$ respectively), which were effectively repaired 14 days after the second dose of SARS-CoV-2 vaccination (Fig. 2), showing the vaccination safety.

3.3. Unaffected DNA damage repair efficiency after SARS-CoV-2 vaccination

Next, we examined whether the observed DNA damage accumulation following vaccination may be attributed to defective repair mechanisms, apart from the increased DNA damage formation. Thus, we studied a primary DDR mechanism, namely double-strand breaks repair (DSB-R) [34]. In order to measure DSB-R capacity, PBMCs were treated with 100 μg/ml melphalan for 5 min. and, subsequently, incubated in drug-free medium for 0, 8 and 24 h; the DSB-R capacity was measured by calculating the Area Under the Curve (AUC) of γH2AX formation/removal kinetics at the aforementioned timepoints. We found that older individuals exhibit diminished DSB-R capacity compared to the younger individuals ($p < 0.001$ at each time-point; Fig. 3a), although SARS-CoV-2 vaccination did not influence DSB-R capacity in any of the two populations (young adults: $p = 0.594$ vs baseline; Fig. 3b; aged adults: $p = 0.097$ vs baseline; Fig. 3c), again confirming its safety.

3.4. Pre-vaccination oxidative stress and DNA damage levels in PBMCs inversely correlate with post-vaccination humoral response

We then measured humoral responses following BNT162B2 vaccination at 14 days after the second dose. We measured the IgG antibodies targeting the S-RBD, as well as the neutralizing capacity of the circulating anti-SARS-CoV-2 antibodies. Individual anti-S-RBD antibody titers were found to strongly associate with the neutralizing capacity of circulating antibodies ($r = 0.793$, $p < 0.001$). Interestingly, the anti-S-RBD IgG antibody titers inversely correlated with pre-vaccination oxidative stress (GSH/GSSG: $r = 0.699$, $p = 0.001$; Fig. 4a) and AP-site levels ($r = -0.713$, $p < 0.001$; Fig. 4b). Similarly, we found that post-vaccination neutralizing capacity inversely associated with pre-vaccination oxidative stress (GSH/GSSG: $r = 0.580$, $p = 0.007$; Fig. 4c) and AP-sites ($r = -0.539$, $p = 0.014$; Fig. 4d).

Finally, pre-vaccination DSB levels inversely correlated with both the anti-S-RBD IgG titers ($r = -0.869$, $p < 0.001$; Fig. 5a) and the neutralizing capacity of circulating antibodies ($r = -0.640$, $p = 0.002$; Fig. 5b). In particular, a strong inverse correlation of pre-vaccination DSB levels with both S-RBD-IgG antibodies titers ($r = -0.867$) and neutralizing capacity of circulating anti-SARS-CoV-2 antibodies ($r = -0.983$) among the 9 older adults was evident.

4. Discussion

It is generally accepted that most vaccines are less immunogenic and efficient in older adults, which are characterized by a progressive and irreversible accumulation of oxidative stress and unrepaired DNA damage, leading to immunosenescence and worse antigen-specific

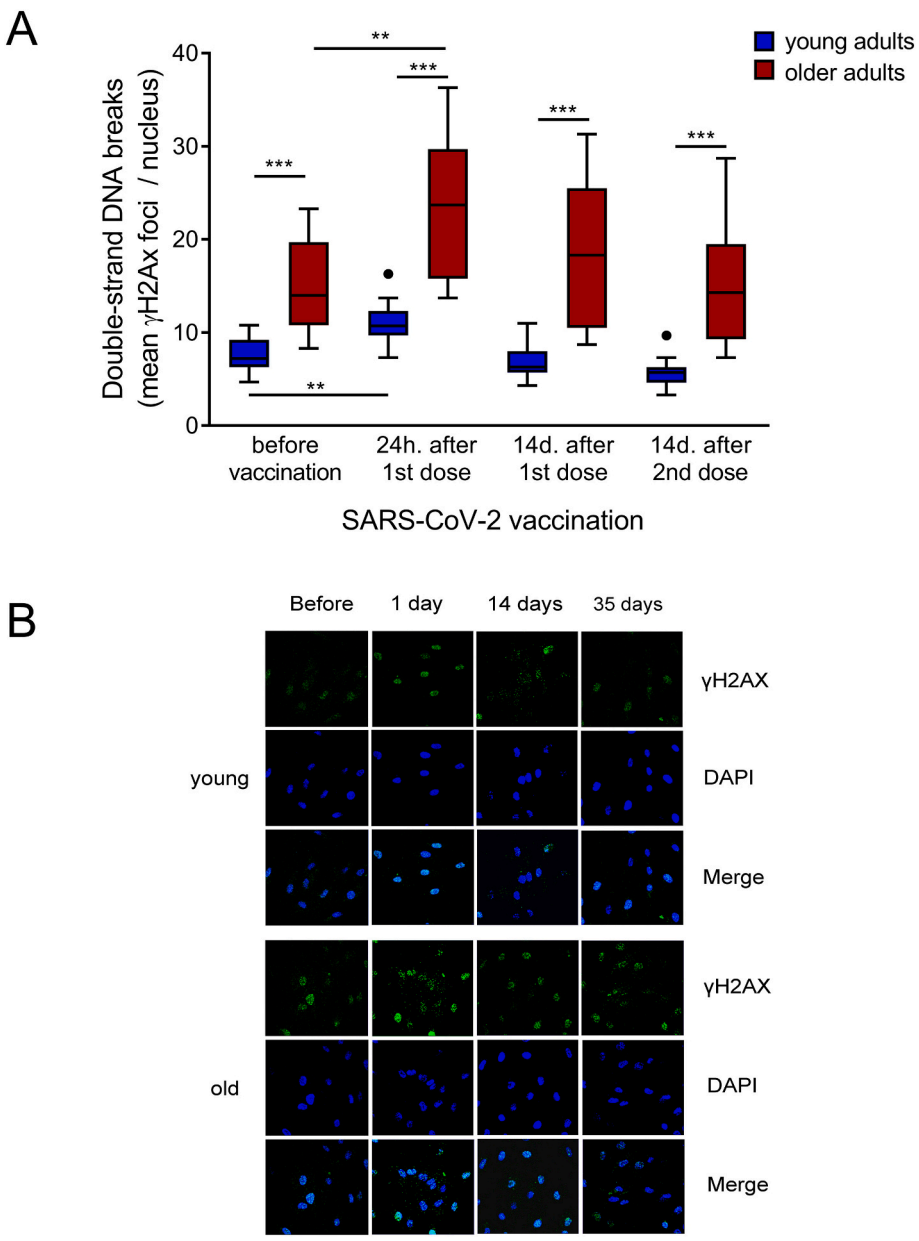

Fig. 2. DNA damage accumulation and repair following BNT162B2vaccination in young and older individuals. (a) Tukey boxplots representing the endogenous DSB levels (expressed as mean γH2AX foci per nucleus) in PBMCs from young (n = 11) (blue) and aged (n = 9) (red) individuals before and after vaccination. P-values are derived from Wilcoxon signed-rank test and Mann-Whitney U test. **P < 0.01 (b) Confocal microscopy images showing γH2AX staining at each time-point after SARS-CoV-2 vaccination in a representative older and young donor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunity [25]. Regarding, recent studies have also shown a reduction in vaccination efficiency after SARS-CoV-2 vaccination in older adults [35–37]. Moreover, previous studies have shown that Influenza and *Salmonella typhi* vaccines lead to a systematic increase of oxidative stress [38–40]. Guided by these data, in this study, we investigated the mechanistic basis for the link between aging and the response to SARS-CoV-2 vaccination, by analyzing the oxidative stress and the accumulation of DNA damage in aged individuals before and after vaccination. We found that the baseline intracellular pro-oxidant imbalance was associated with reduced humoral responses following vaccination, independently of the vaccination-mediated oxidative stress. In support, the robust antibody responses after vaccination observed in few older individuals were coupled with their relatively lower intracellular oxidative burden. Reportedly, oxidative stress can impact on cellular macromolecules (DNA, proteins and lipids) functionality through the increased formation of ROS and RNS [25,40]. In fact, oxidative stress is a major contributor of DNA damage formation, thus activating the DDR network, as seen in patients with Systemic Lupus Erythematosus, Systemic Sclerosis and Rheumatoid Arthritis [28,32,41]. Along this line, patients with systemic autoimmune diseases may develop lower antibody responses after vaccination as compared to healthy controls, as observed after vaccination with either “traditional” vaccines (e.g., against influenza or pneumococcal) or the latest vaccine against SARS-CoV-2 [42–45], which could be related to the constantly increased oxidative stress burden and the subsequent chronic DDR activation.

Another functional link between oxidative stress and reduced humoral responses comes from obese patients. Obesity is associated with systematic low-grade inflammation, similar to what is observed in aged individuals [46]. Multiple vaccines show reduced antibody responses in obese patients, while obesity has been recognized as an independent risk factor for worse COVID-19 prognosis [47–51].

Persistent DDR activation and elevated oxidative stress can lead to stress-induced premature (cellular) senescence (SIPS), a state of permanent cell cycle arrest [52]. SIPS in human cells is accompanied by a specific secretory phenotype, mainly expressing pro-inflammatory chemokines (e.g., CXCL8) and cytokines (e.g., TNF-α, IL-6), along with increased resistance to cellular death [21,53]. Accordingly, vaccination efficacy in aged recipients is limited mainly due to senescence of the

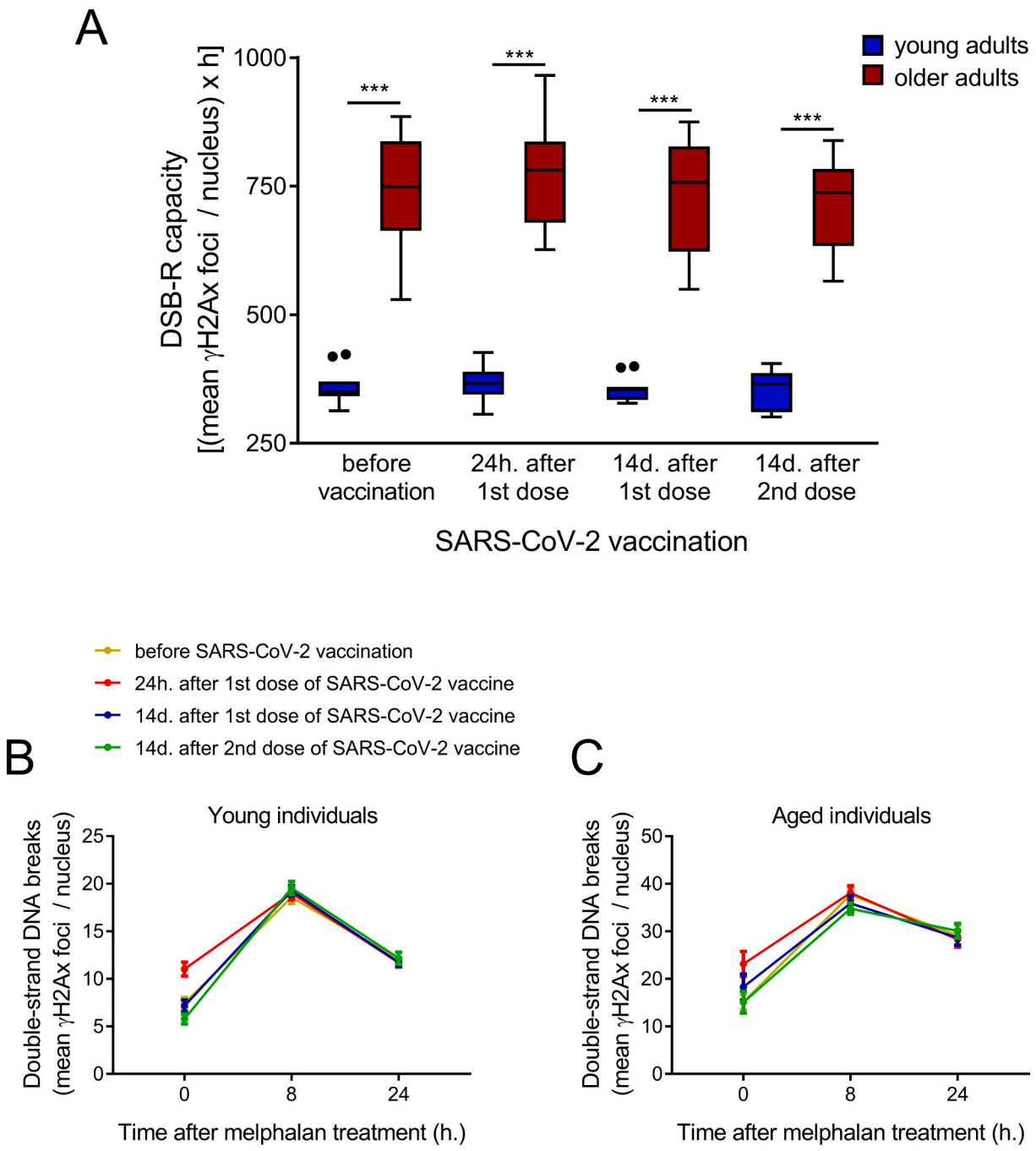

Fig. 3. Double-strand breaks repair (DSB-R) capacity in older and young individuals is unaffected after BNT162B2 vaccination. (a) Tukey boxplots representing the melphalan-induced DNA double-strand breaks repair (DSB-R) kinetics, as assessed by measuring γH2AX foci via confocal microscopy, and expressed as AUC (0-24 h after melphalan treatment) in PBMCs of young ($n = 11$, blue) and older individuals ($n = 9$, red). P-values are derived from Wilcoxon signed-rank test and Mann-Whitney U test. ***$P < 0.001$ (b-c) Line graphs representing DSB-R capacity, by showing the formation and removal of γH2AX foci (mean ± standard error) after ex vivo melphalan treatment in PBMCs of young (n = 11) and aged (n = 9) adults before and after SARS-CoV-2 vaccination. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immune system (immunosenescence) that is also accompanied by an increased baseline inflammatory status (inflammaging) [21,54]. This chronic, low-grade inflammation can inhibit antigen-specific immunity, as suggested by the negative correlation between baseline TNF-α levels and antibody production after Influenza vaccination in previous studies [55]. Moreover, PBMCs of frail individuals exhibit elevated gene expression associated with T-cell exhaustion and oxidative stress compared with non-frail subjects, which may partly account for reduced efficacy of Influenza vaccination [56]. In line with these observations, we observed herein that increased baseline oxidative stress and accumulation of toxic DNA DSBs, which could both induce the immunosenescence, were associated with less efficient antibody production following vaccination.

Targeting senescence and inflammaging may prove beneficial for vaccine responses in older individuals or patients with chronic inflammatory diseases, where exhaustion of the immune system affects the response to vaccination. In support of this notion, a short treatment of co-cultured $CD4^+$ T cells and B cells with anti-TNF agents greatly enhanced immunoglobulin secretion [55]. Moreover, a phase II clinical trial showed treatment with senolytic drugs (e.g., mTOR inhibitors)

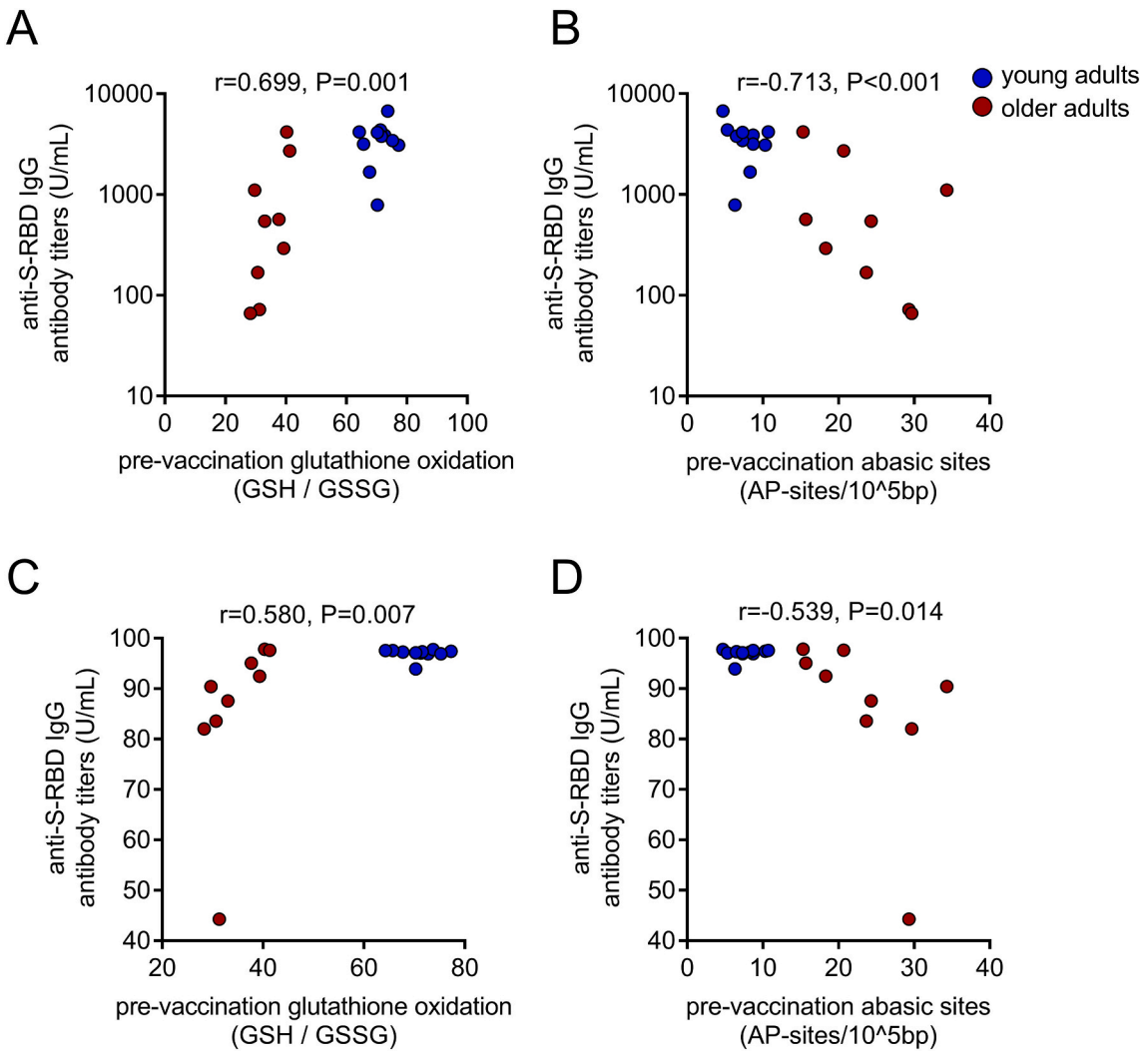

Fig. 4. Anti-S-RBD IgG antibody titers and neutralizing capacity of circulating antibodies following SARS-CoV-2 vaccination strongly correlate with endogenous oxidative stress markers. Scatterplots showing the relationship between endogenous oxidative stress, expressed as GSH to GSSG ratio and (a) anti-S-RBD IgG antibody titers or (b) neutralizing capacity of circulating antibodies (%) in PBMCs of young and older individuals. Scatterplots showing the relationship between endogenous AP-sites and (c) anti-S-RBD IgG antibody titers or (d) neutralizing capacity of circulating antibodies (%) in PBMCs of young and aged individuals. Correlation coefficients are derived from Spearman's test.

enhanced antiviral gene expression and antibody responses to Influenza vaccination [57]. In support, pre-treatment of older individuals with an oral small-molecule p38 MAPK inhibitor, which reduced baseline systemic inflammation, led to increased immune responses to cutaneous VZV antigen challenge [58]. Nonetheless, future randomized controlled trials are warranted to examine the safety and benefit of using short-term senolytic or anti-inflammatory treatment to boost vaccine responses in this population.

The observed aberrations in the PBMCs of older individuals (i.e., increased oxidative stress and DNA damage accumulation) may also provide insights into the clinical course of SARS-CoV-2 infection. Age is among the strongest prognostic factors of adverse outcomes among patients with COVID-19 [47] while biological age (frailty) has additive prognostic value over well-established algorithms such as the Sequential Organ Failure Assessment (SOFA) score [59].

Further, in the present study, we assessed the relationship of vaccination against SARS-CoV-2 with humoral adaptive immunity, by evaluating the neutralizing antibody formation effectiveness. Previous studies have revealed that cell-mediated adaptive immune responses are altered in the older individuals due to immunosenescence [54,60]. An age-associated decrease in naive ($CD45RO^{null}$) T cells with a simultaneous increase in memory ($CD45RO^{+}$) T cells is observed, partly due to the thymic involution and the persistent antigenic stimulation [60–62]. In addition, there are distinct deficiencies in the B-cell compartment with significant decreases in peripheral mature B-cells, influencing the antibody production [63,64]. Previous studies have shown that SARS-CoV-2 vaccination can successfully elicit a robust cell-mediated immune response [65,66]. Therefore, it would be particularly useful to examine whether cell-mediated adaptive immune response after SARS-CoV-2 vaccination is influenced by aging.

In summary, herein we show that all examined individuals showed an adequate response to the mRNA BNT162B2 vaccine, although antibody levels were higher in young compared to old subjects. The effectiveness of the antibody response strongly correlated with intracellular oxidative status. A question generated by our results is whether endogenous oxidative stress levels could act as a useful predictor for post-vaccination antibody production efficiency. A significant limitation of this study is the small sample size of vaccinated participants; increasing the number of participants will thus greatly increase the confidence of the generated data. However, our main finding that pre-existing oxidative stress and/or DSB levels correlate with neutralizing antibody response effectiveness further corroborates previous studies

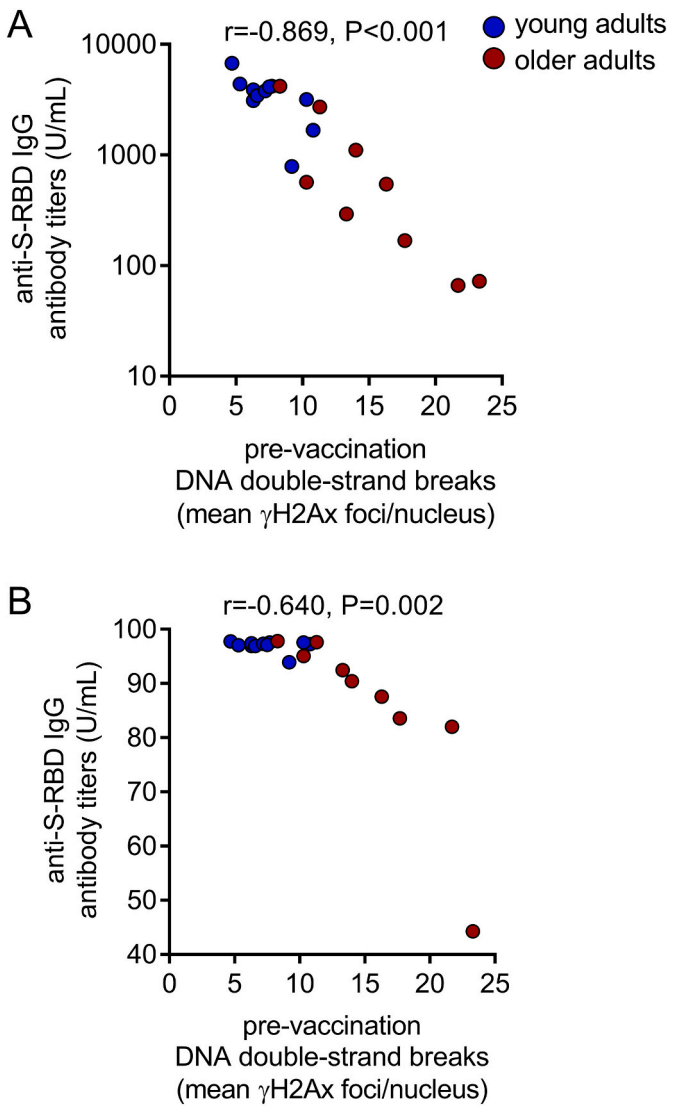

Fig. 5. Anti-S-RBD IgG antibody titers and neutralizing capacity of circulating antibodies following BNT162B2 vaccination strongly correlate with endogenous DNA damage levels at the time of vaccination. Scatterplots showing the relationship between endogenous DSBs (expressed as mean γH2Ax foci per nucleus) in PBMCs of young and aged individuals at the time of vaccination and (a) anti-S-RBD IgG antibody titers or (b) neutralizing capacity of circulating antibodies (%) 14 days after 2nd dose of the vaccine. Correlation coefficients are derived from Spearman's test.

showing that aging is associated with lowered antibody responses after vaccination [67].

5. Conclusions

To conclude, our study demonstrates that SARS-CoV-2 vaccination effectiveness may be influenced by the pre-existing intracellular oxidative stress levels. Older individuals demonstrate an increased pre-vaccination oxidative burden, accompanied by augmented DNA damage accumulation as compared to younger individuals. Interestingly, SARS-CoV-2 vaccine acted as an acute immune stimulant, inducing a transient oxidative stress and DNA damage accumulation in all participants. Of note, DNA repair mechanisms were not influenced by this acute stimulation, although a pre-existing repair deficiency in older individuals is observed. Whether such measurements may serve as biomarkers of vaccine efficacy warrants further studies in larger samples.

CRediT authorship contribution statement

Study conception: PAN, EK, VLS, PPS. Patient recruitment: PAN, EK. Experiments: PAN, VLS, IT, ET, MP. Drafting of manuscript: PAN, EK, NIV, VLS, PPS. Critical review of manuscript: all authors. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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