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ORIGINAL ARTICLE

Impact of Inactivated SARS-CoV-2 Vaccines on Serum Glycan Profiles and Protein N-Glycosylation

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SUMMARY

Background: **This study aimed to investigate the effect of inactivated SARS-CoV-2 vaccines and booster shots on serum glycan profiles and protein N-glycosylation, specifically how vaccination influences glycan synthesis over time, how booster shots differentially impact populations with varying antibody titers, and which specific glycoproteins exhibit altered glycosylation. The goal was to explore a novel mechanism by which COVID-19 vaccines might exert antiviral effects through indirect inhibition of host glycosylation.**
Methods: **Serum glycan profiles were analyzed in individuals receiving two primary doses and a booster shot of inactivated SARS-CoV-2 vaccine, categorized by symptomatic status and antibody titers. Serum proteins were immobilized on AminoLink plus coupling resin, followed by sequential derivatization of α2,6- and α2,3-linked sialic acids. Glycans were released using PNGase F and analyzed by MALDI-TOF-MS with maltoheptaose as an internal standard. Glycoproteomics via LC-MS/MS identified site-specific protein glycosylation changes.**
Results: **Significant alterations in serum glycan profiles were observed. Overall glycan synthesis showed substantial suppression one-month post-vaccination, followed by gradual recovery. The booster vaccine inhibited glycan synthesis, and high-titer individuals exhibited a more pronounced N-glycan profile and faster recovery. Symptomatic status had no significant impact on glycan abundance. Glycoproteomic analysis revealed substantial alterations in glycosylation of stress and immune response proteins, including CP, HPX, SERPINA1, FN1, IgG, AGP, and C3, after vaccination.**
Conclusions: **This study demonstrates a novel mechanism: inactivated COVID-19 vaccines indirectly inhibit host glycosylation pathways. Vaccination significantly suppresses N-glycosylation, primarily reducing N-glycan abundance, with recovery influenced by antibody titer. These results highlight the intricate relationship between immune response, host glycosylation, and viral infection, suggesting avenues for developing novel therapeutic strategies targeting both the virus and host response to enhance antiviral protection.**
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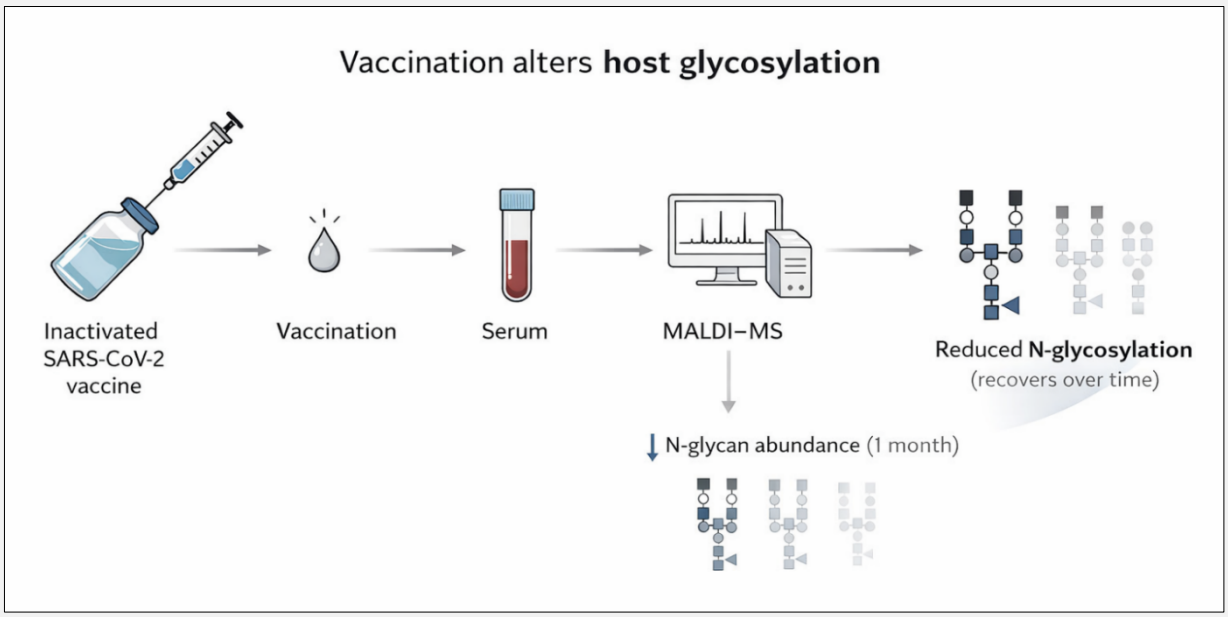
Graphic abstract



KEYWORDS

glycan, glycosylation, SARS-CoV-2, vaccine, antibody titer, mass spectrometry

LIST OF ABBREVIATIONS

ACE2 - Angiotensin converting enzyme-2
ACN - Acetonitrile
CDG - Congenital disorders of glycosylation
CFG - Consortium for functional glycomics
HIV - Human immunodeficiency virus
MALDI - Matrix-assisted laser desorption/ionization
MS - Mass spectrometry
NGI-1 - N-linked glycosylation inhibitor-1
NSP - Non-structural protein
ORF - Open reading frame
OST - Oligosaccharyltransferase
PNGase F - Peptide-N-glycosidase F
RNA - Ribonucleic acid
RNAi - Ribonucleic acid interference
SARS-CoV-2 - Severe Acute Respiratory Syndrome Coronavirus 2
TM - Tunicamycin
TOF - Time of flight

INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus responsible for the COVID-19 pandemic, is a positive-sense, single-stranded RNA virus encased in a protein shell. According to data from the Centers for Disease Control and Prevention (CDC) and Worldometers, over 700 million individuals have been infected with SARS-CoV-2 worldwide, resulting in more than 7 million deaths. COVID-19 is a highly contagious, infectious disease that can transmit from person to person [1]. The genetic information of the SARS-CoV-2 virus is carried by a single-stranded, positive-sense RNA genome of approximately 30 kilobases (kb) [2]. This genome encodes 16 non-structural proteins (NSP1-16), 4 structural proteins (Spike glycoprotein [S], Envelope [E], Membrane [M], Nucleocapsid [N]), and 6 accessory proteins (ORF3a, ORF6, ORF7a, ORF7b, ORF8 and ORF10, where ORF stands for open reading frame) [3]. While the NSP1-16 and accessory proteins facilitate the replication of the SARS-CoV-2 RNA within the cell through various mechanisms, the four structural proteins are essential components of the SARS-CoV-2 virion [4].

SARS-CoV-2 infects host cells through a multi-step process. The virus's spike glycoprotein first binds to the angiotensin converting enzyme-2 (ACE2) receptor on the host cell surface. Subsequently, a host protease cleaves the spike protein, allowing the virus to fuse with

the host cell membrane and enter the cell. This fusion creates a pore that enables the viral genome to reach the cytoplasm [4]. It is evident that the spike glycoprotein plays a crucial role in viral entry and transmission. The glycosylation pattern of the spike protein significantly influences its antigenicity and infectivity [5]. The spike protein contains a variety of N-glycans that not only initiate binding to ACE2 but also form a protective glycan shield to evade the immune system [6,7]. Additionally, the spike protein can possess complex O-glycans [8,9], further complicating the virus's interaction with host cell receptors.

The glycosylation sites and glycans of the spike protein can vary significantly depending on the expressing cell line. HEK293 cells produce diverse complex N-glycans on all but N-glycosylation site N616, which is predominantly expressed as high-mannose. In contrast, the baculovirus-insect cell line generates mostly high-mannose N-glycans at 14 N-glycosylation sites. Moreover, the spike protein contains nearly 60 O-glycosylation sites, four of which possess the most abundant and diverse O-glycans [8]. These glycoprotein and their glycans may affect the range of cells the virus can infect and could shield certain epitopes from antibody neutralization [10].

Vaccines have long been recognized as the most effective tool in preventing infectious diseases by strengthening the body's natural defenses and reducing morbidity and mortality from viral infections. Studies showed that COVID-19 vaccines prevented 14.4 million deaths from COVID-19 in 185 countries and territories between December 8, 2020, and December 8, 2021 [11]. Vaccine development often focuses on the structure of the spike protein, particularly its glycan moiety, to achieve better viral protection. Several types of COVID-19 vaccines have been developed, including RNA/DNA vaccines, adenovirus vector vaccines, protein subunit vaccines, and inactivated viral vaccines. Each of these vaccine types has been widely used worldwide, with mRNA and protein subunit vaccines predominating in the USA and inactivated vaccines being widely used in China. Despite their differences, all of these vaccines stimulate our bodies to recognize and protect us from the virus that causes COVID-19 by boosting the immune response to produce antibodies. While host glycosylation of the vaccine target can impede its antigenicity and immunogenicity, removing N-glycans has been shown to increase the effectiveness of human immunodeficiency virus (HIV) vaccine [12,13]. However, the potential impact of vaccines on host cell glycosylation has not been extensively studied.

Recent studies have demonstrated that inhibiting protein N-glycosylation can block SARS-CoV-2 infection, as N-glycosylation is essential for host cell invasion [14]. Ablation of host N-glycosylation using RNA interference (RNAi) or inhibitors significantly reduced the spread of SARS-CoV-2, including variants such as alpha, beta, gamma, or delta. Cells treated with these agents produced fewer virions or became completely non-infectious. Moreover, partial enzymatic deglycosylation of intact virions revealed that surface-exposed N-glycans are crucial for the virus to invade host cells [14]. These findings raise the question of whether vaccines can inhibit protein N-glycosylation in addition to producing antibodies against the virus. To investigate this, we collected sera from volunteers who had been vaccinated with two inactivated vaccines and one booster shot. Serum proteins were conjugated to an aldehyde resin for glycan derivatization and enrichment (Figure S2). The α2,6-linked sialic acids form an ethyl ester after ethyl esterification, while the α2,3-linked sialic acids remain underivatized. Further, the α2,3-linked sialic acids form a stable structure after p-Toluidine carbodiimide coupling [15,16]. Glycans were released using PNGase F and then comparatively analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). To further investigate changed glycoproteins, we performed glycoproteomics on serum to identify site-specific protein glycosylation. This involved liquid chromatography-tandem mass spectrometry (LC-MS/MS), and the resulting MS/MS spectra were analyzed using intact glycopeptide analysis software (MSFragger and Byonic).

MATERIALS AND METHODS

Reagents and materials

All chemicals or materials were ordered from Beyotime (Shanghai, China) unless otherwise noted. AminoLink plus coupling resin was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Snap-cap spin-columns (SCSC) and rubber caps were purchased from Beyotime. Sodium citrate ($Na_3C_6H_5O_7$), sodium acetate (Na_2CO_3), sodium cyanoborohydride ($NaCNBH_3$), and 1-hydroxybenzotriazole hydrate (HOBt) were purchased from Sigma-Aldrich (St Louis, MO, USA). *N*-(3-(Dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC) was ordered from Meryer (Shanghai, China). P-Toluidine (pT) was purchased from TDI (Osaka, Japan). Formic acid and sodium chloride (NaCl) were ordered from Sinopharm Chemical (Shanghai, China). Acetonitrile (ACN) was purchased from Tedia (Fairfield, OH, USA). PNGase F was ordered from New England Biolabs (Ipswich, MA, USA). Urine, ammonium bicarbonate, iodoacetamide (IAA), and *N,N′*-dimethylaniline (DMA) were purchased from Aladdin (Shanghai, China). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), trifluoroacetic acid (TFA) were ordered from Macklin (Shanghai, China). High-performance liquid chromatography (HPLC) grade water and 2,5-dihydroxybenzoic acid (DHB) were purchased from J&K Chemical (Zhejiang, China). Sequencing grade trypsin was purchased from Promega (Madison, WI, USA). Amide-80 gel slurry was ordered from Tosoh Bioscience (Tokyo, Japan). Silica C18 resin was purchased from Silicyle (Quebec, QC, Canada). Sinopharm inacti-

vated vaccine was purchased from Beijing Institute of Biological Products.

Sample collection

This study adhered to the ethical guidelines of the Institutional Review Board (IRB) at Shenzhen Hospital of Southern Medical University, with approval obtained prior to sample collection. All participants provided written informed consent before participating in the study. Serum samples were collected at specific time after participant receiving the second dose of the inactivated vaccine, with details as follows: sera were collected from all individuals at first month after the vaccination, sixteen of them were collected once at about three months and six months, respectively. Finally, the sixteen individuals were given a booster shot and their sera were collected after one month of the booster vaccination. Then, 200 µL of serum from each sample was used for glycosylation analysis. All samples were stored in -80°C prior to use.

Serum sample processing

Each serum sample was tested for protein concentration using a BCA assay kit (Beyotime) and a nanodrop spectrometer. Then serum samples were mixed into different testing samples according to Table S1. Twenty-seven individuals were not treated with the vaccine, and 62 individuals were treated with the vaccine. Samples were taken 1 - 2 months after their second shot. Of these 62 vaccinated individuals, 18 had follow-up measures recorded at 3 months, 6 months, and 1 month after their booster shot. A total of 35 samples were prepared, and each consists of more than 50 µL in volume for further processing (Table S1).

Glycan profile using glycoprotein immobilization

As described previously, glycoprotein immobilization and sialic acid modification are necessary for their stabilization during MALDI-MS analysis [17,18]. Briefly, 20 µL of each testing sample were mixed with 180 µL of DI water and denatured at 90 - 95°C for 10 minutes. After cooling, the samples were mixed with 1 × PBS buffer pre-conditioned AminoLink plus coupling resin (200 µL slurry). The sample-resin mixture was added with 50 µL of 10 × binding buffer (100 mM sodium citrate and 50 mM sodium carbonate, freshly prepared). Proteins were coupled to the resin in 1× binding buffer (4 hours at room temperature (RT)) followed by the addition of 50 mM $NaCNBH_3$. After washing the resin with 1 × PBS (500 µL, 3 ×), the samples were further incubated in 1 × PBS for 4 hours in the presence of 50 mM $NaCNBH_3$. Unreacted aldehydes remaining on the resin were blocked with 1 M Tris-HCl (pH 7.4) (30 minutes at RT). The 2,6-linked sialic acid was then derivatized with 0.25 M EDC (200 µL) and 0.25 M HBot (200 µL) in ethanol at 37°C for 1 hour (note: water should be avoided for complete esterification). After removing the reagents and washing the resin with deionized water, the 2,3-linked sialic acid was further modified with 1 M p-toluidine (pT) (500 µL). The reagents and chemicals were washed off using 10% ACN, 10% formic acid, 1 M NaCl, and DI water (500 µL each, 3 - 5 times wash per solution). Then, 0.5 µL PNGase F and 120 µL 25 mM NH_4HCO_3 (pH = 8.0) were added to the resin and incubated for 2 hours at 37°C to release N-glycans, which were then analyzed by Bruker ultrafleXtreme MALDI-MS. The MALDI matrix was prepared by mixing 4 µL of DMA with 200 µL of DHB (100 µg/µL in 50% ACN, 0.1 mM NaCl). Then, 2 µL of glycan and 1 µL of MALDI matrix were deposited on a MALDI plate and dried completely in vacuum. All spectra were acquired in a positive mode with a mass range of 900 - 4,000 and a laser power of 70%. One microliter of 1 mM DP7 was added to the sample as an internal standard for semi-quantification by MALDI-MS. A total of 1,000 shots were accumulated per spectrum. Each group received three technical replicates. Glycan peak lists were created with Bruker flexAnalysis software (signal-to-noise ratio ≥ 2). Glycan composition was determined by searching the database in GlycoWorkbench [19].

MALDI TOF MS data analysis

Glycan spectra were uploaded to Bruker flexAnalysis, where peaks with a signal-to-noise ratio greater than two were labelled for intensity and mass-to-charge ratio. The mass list was then output into Excel format, where the average and standard deviation were calculated (at least three technical replicates were tested for each sample). Glycan composition was assigned based on the CFG human glycan database and precursor mass. To compare changes in glycans among different groups, we classified them into high-mannose, fucose-only, sialic acid-only, and fucose-sialic acid N-glycans.

Glycan data were extracted from Bruker flexAnalysis software for standardization, which involved calculating ratios based on the total and individual intensities of N-glycans. Data analysis was performed using Python, including the third-party libraries pandas, numpy, matplotlib, and seaborn (exploratory data analysis) [20]. Preprocessing and reading of Excel files were carried out using pandas, which creates all diverse mapping relationships of data in a data type named DataFrame. After transposing the frame, the program converted the intensity values from their original format to a logarithmic scale to amplify the main effect. The combination of these third-party libraries directly produced the box plots for visualization.

Intact glycopeptide mass spectrometric analysis

Protein concentration of each serum was quantified using a NanoDrop spectrophotometer. Twenty-microliter serum samples underwent denaturation with 8 M urea prepared in 1 M ammonium bicarbonate. Subsequently, disulfide bonds within the proteins were reduced by adding dithiothreitol (DTT) to a final concentration of 12 mM, followed by incubation at 37°C and 600 rpm for one hour. Alkylation of the reduced cyste-

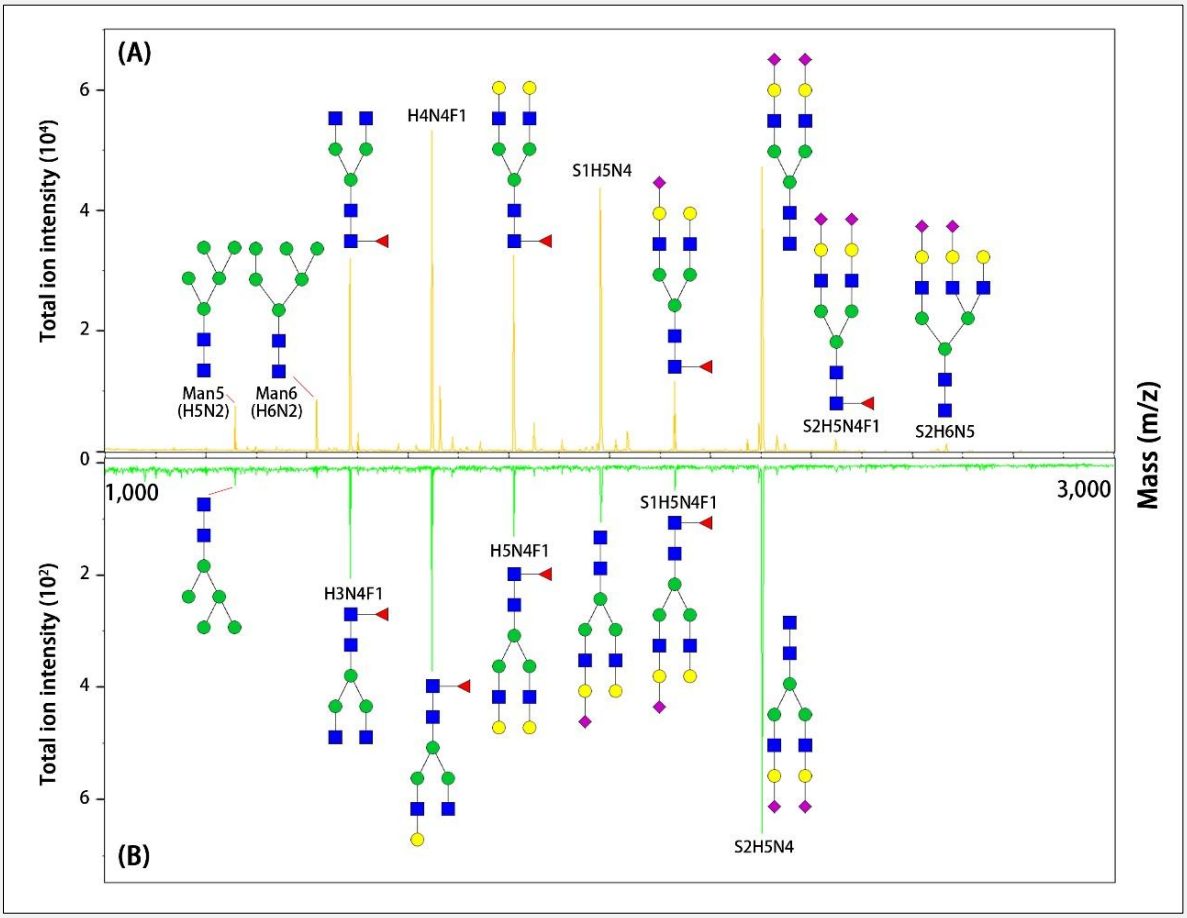


Figure 1. MALDI-ToF-MS serum N-glycans of healthy volunteers without vaccination compared to those who had two vaccination shots. Serum was collected after two months of the second vaccination.

A) Healthy volunteers without vaccination (Table S1 Tab #9). B) Volunteers with two vaccinations (Serum was collected after one-month of vaccination, Table S1 Tab #10). H Hexose, N HexNAc, S Neu5Ac, F Fucose, square GlcNAc, circle Mannose, circle Galactose, Diamond purple Neu5Ac, triangle Fucose.

ine residues was achieved by adding iodoacetamide (IAA) to a final concentration of 16 mM, and the samples were incubated in the dark at room temperature and 600 rpm for 30 minutes. To prepare for enzymatic digestion, the samples were diluted five-fold with HPLC-grade water, and trypsin was added at a protease-to-protein ratio of 1:50. This digestion step proceeded overnight at 37°C and 600 rpm. The resulting peptide mixture was then purified using a C18 solid-phase extraction (SPE) column, followed by enrichment of intact glycopeptides using a self-packed hydrophilic interaction chromatography (HILIC) column containing Amide-80. The eluted glycopeptide fractions from the HILIC column were pooled and concentrated to dryness using a SpeedVac. Finally, these dried samples were analyzed by LC-MS/MS.

The enriched intact glycopeptides were separated using an Easy-nLC 1200 system and directly coupled to a Q-Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) for analysis. Data acquisition was performed in data-dependent acquisition (DDA) mode, employing a static spray voltage, static gas mode, and EASY-IC internal mass calibration. MS1 analysis was conducted at a resolution of 120,000 across a mass-to-charge ratio (m/z) range of 375 - 2,000, with an automatic gain control (AGC) target of 10^6. For tandem mass spectrometry (MS2), the standardized AGC target was set to 250%, and positive ion polarity was used, selecting precursor ions with charge states ranging from 2 to 7. MS2 analysis was performed at a resolution of 60,000 with an isolation window of 1.6 m/z, and the scan range was set with a first mass of

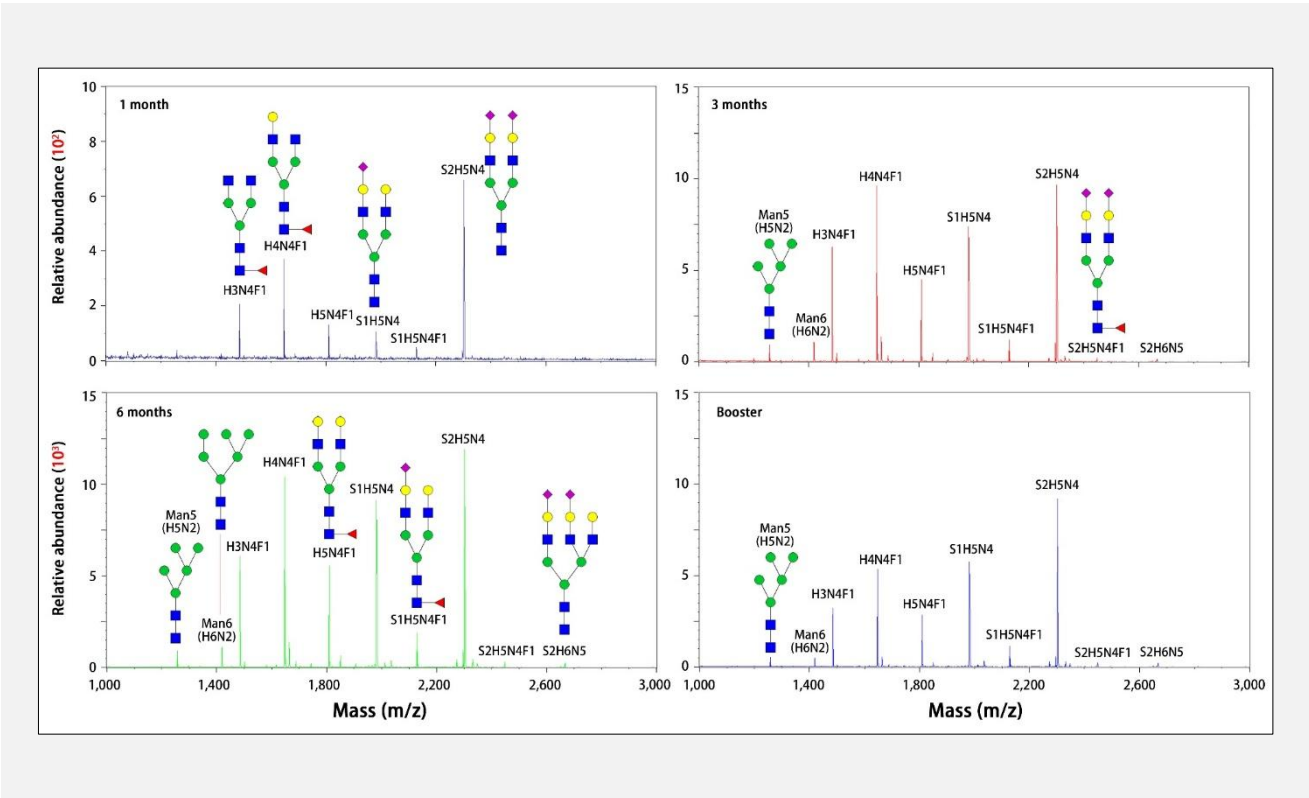


Figure 2. MALDI-ToF-MS profiles of serum N-glycans at different periods of times after vaccination and booster.

A) One month after two vaccinations. B) Three months after two vaccinations. C) Six months after two vaccinations. D) One month after booster shot. Glycan relative abundance continuously increases after 3 months. The data is average of serum mixture from 16 people. (Table S1 Tab #10, #11, #12 and #13).

110 m/z. Fragmentation was induced using higher-energy collisional dissociation (HCD) with stepped collision energy (20, 30, 40%). Further details regarding the specific procedures and parameters used for the mass spectrometric analysis of glycopeptides can be found in our previously published studies [21].

RESULTS AND DISCUSSION

Vaccination significantly suppresses N-glycosylation

To verify whether vaccination can inhibit glycosylation synthesis, we first compared the glycan profiles of individuals in the vaccinated (1 month) and non-vaccinated populations. Figure 1 shows that although the overall glycan profile is similar between non-vaccinated (A) and vaccinated populations (B), the total ion intensity is much lower in people after vaccination. Bi-sialoglycan (S2H5N4, where S = Neu5Ac, H = Hexose, N = HexNAc) is generally the most abundant N-glycan in serum, with an α2,6-linked sialic acid, and fucosylated N-glycans are another characteristic of the serum glycan profile. Following two vaccinations, the abundance of these N-glycans significantly decreased from forty thousand intensity (area under curve) to approximately 600, representing nearly 100-fold reduction. This substantial change may be advantageous in minimizing viral infection, potentially by altering the glycan pattern on host receptors like ACE2 and consequently hindering the binding of viral spike proteins [22]. This is due to the fact that glycans participate in mutual recognition between viruses and host receptors. For example, the binding of HIV-1 gp120 to CD4 was substantially decreased by removing the glycan chains from gp120 using endoglycosidase treatment in HIV-1 [23].

Gradual recovery of glycan synthesis after vaccination

Since the vaccine can inhibit glycan synthesis, we wanted to know whether the glycosylation level can recover over time. To test this, we used sera collected at different time points: 1, 3, and 6 months, as well as 1 month after the booster shot (Table S1). A few changes were observed (Figure 2). The relative abundance of glycans gradually increased over time following vaccination, showing a more than 10-fold increase at six months compared to one month. This indicates that the vaccine's impact on the glycan profile is not sustained, and this recovery timeframe likely contributes to the observed decrease in vaccine effectiveness over time, ne-

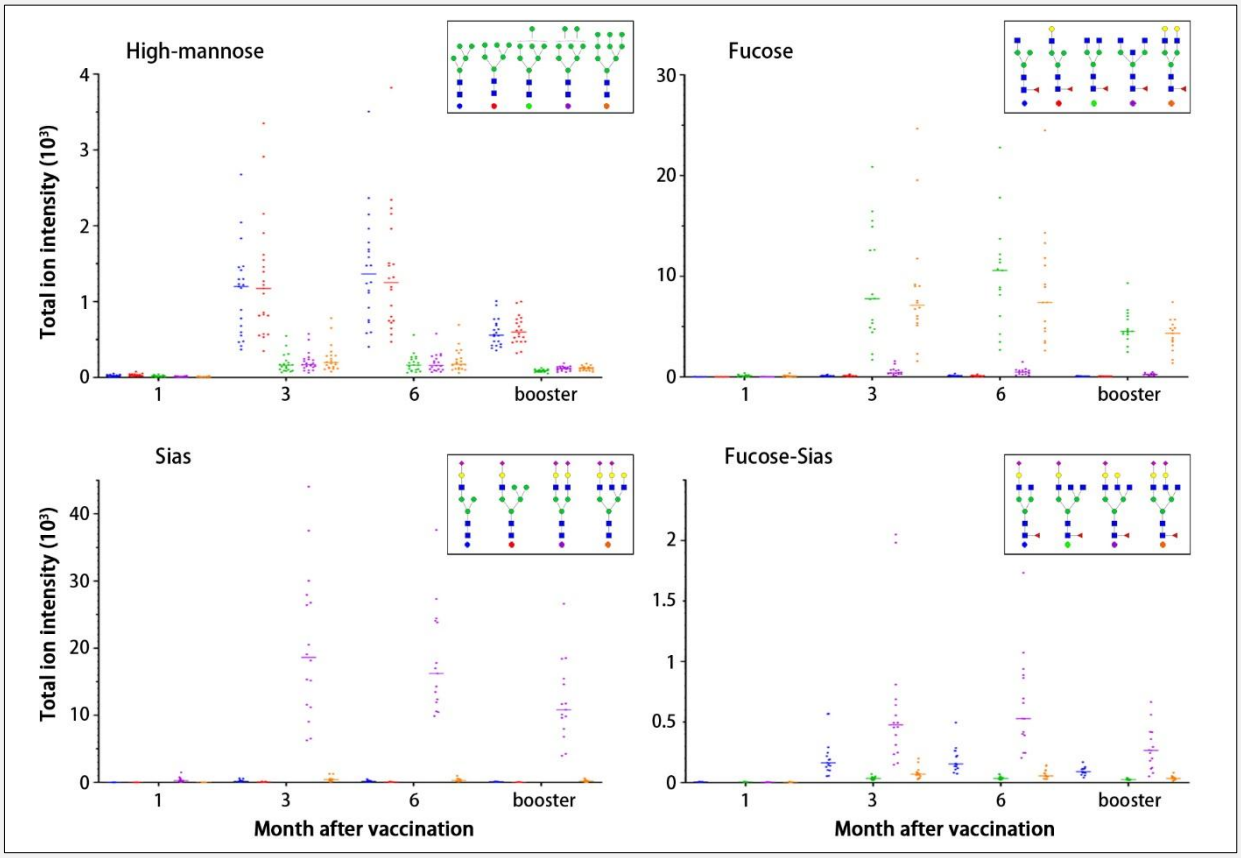


Figure 3. The relative abundance of different N-glycan types showed dynamic changes following vaccination and a booster shot. Initially, high-mannose glycans like Man5 and Man6 increased, peaking within six months, while Man7, Man8, and Man9 exhibited a smaller but gradual recovery, with all high-mannose glycans declining to a lesser extent after the booster. Among fucosylated N-glycans, H3N4F1 and H5N4F1 became most abundant, with limited recovery observed for others within six months. The sialoglycan S2(6)H5N4 displayed the highest abundance and a rapid six-month recovery. Notably, the sialylation and fucosylation of H5N4 to form S2(6)H5N4 and H5N4F1 persisted during the post-vaccination recovery phase, with S1(6)H5N5F1 becoming the most prevalent glycan, synthesized from H5N5 through the addition of Neu5Ac and fucose (analysis excluded sample #9 from Table S1).

cessitating booster shots. While the booster slightly reduced glycan relative abundance, the overall glycan profile remained consistent across the different time points examined. This result indicates that the vaccine inhibits N-glycan synthesis at the initial stage in the endoplasmic reticulum (ER). It may function like N-glycosylation inhibitor (NGI-1), engaging and blocking the activity of the oligosaccharyltransferase (OST) catalytic subunits (STT3B/STT3A) [24]. As it has been demonstrated that inhibiting STT3A using NGI-1 impaired SARS-CoV-2 infectivity and its variants alpha or beta [25], this suggests that the vaccine's suppression of N-glycosylation machinery synthesis is beneficial for enhanced protection.

Changes of specific types of glycans are summarized in Figure 3. Four different types of N-glycans were listed, including high-mannose, fucose, Sias (sialic acid), fucose-Sias. Overall, the relative abundance of N-glycans is gradually increased from 1 to 6 months, with most-abundant glycans belonging to Sias and fucose. Each subtype consists of several different glycans, all of which are reduced once the booster shot is given. However, the relative abundance after one-month booster shot is significantly higher than that after one-month second-shot. These results reveal that the booster vaccine has less inhibition on glycan synthesis.

The impact of vaccination on glycan synthesis in high antibody titer individuals

A titer is a blood test that measures the concentration of antibodies in the blood after vaccination to determine immunity to COVID-19 [26]. While higher antibody

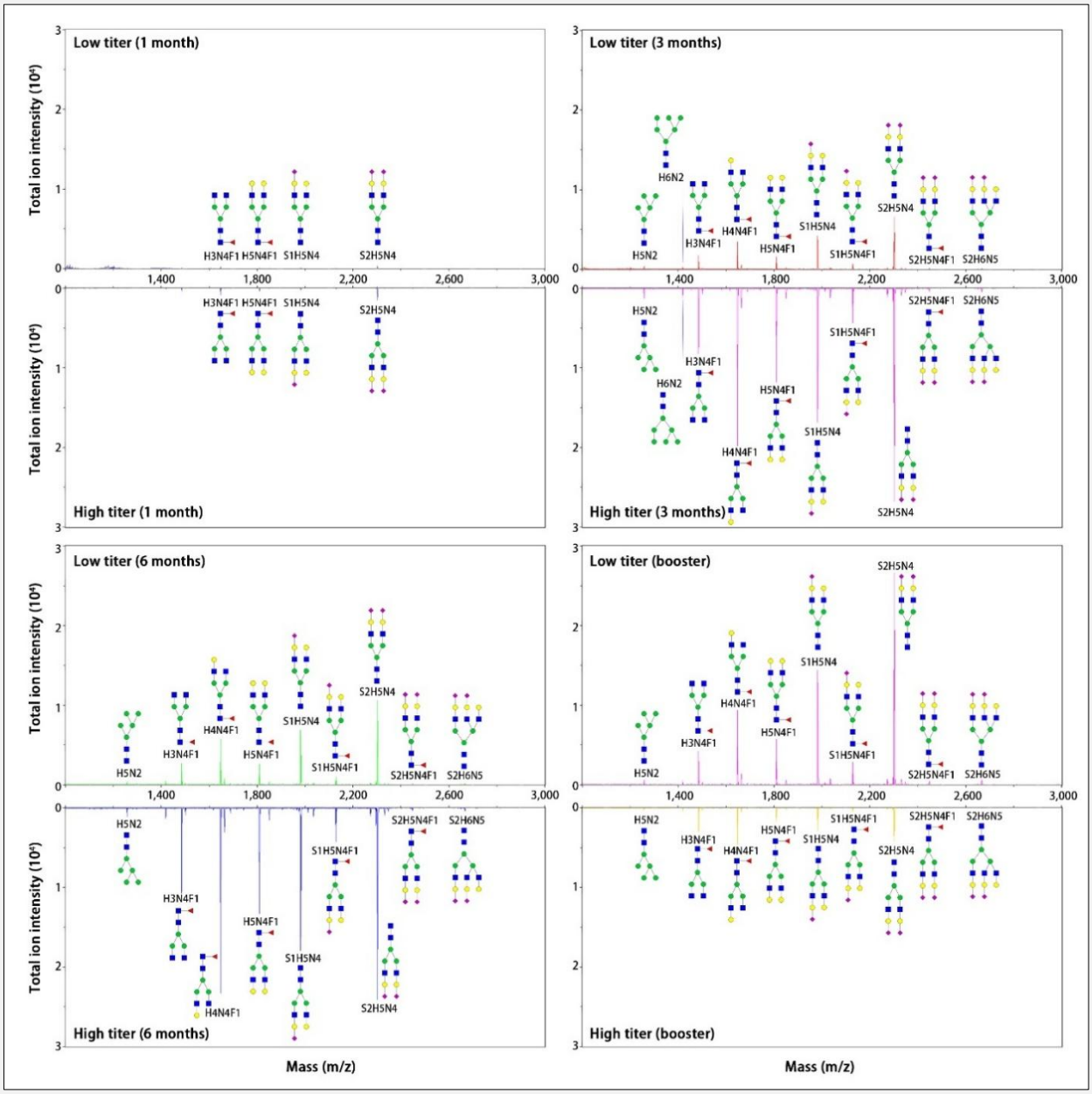


Figure 4. MALDI-MS profiles of serum N-glycans in a high-titer response individual. This figure depicts the MALDI-MS profiles of serum N-glycans from a volunteer who exhibited a high-titer response to a vaccine. The profiles were collected at one, three, six months after the second dose and one month after a booster shot. The total ion intensity of each N-glycan is represented on the y-axis, while the mass-to-charge ratio (m/z) is shown on the x-axis. The different monosaccharide colors represent different types of carbohydrate structures (see Figure 1 for the nomenclature). For high-titer response: The individual who demonstrated a high-titer response to the vaccine exhibited a more pronounced N-glycan profile, particularly in the early stages of recovery; low-titer response: individuals with low-titer responses showed a less intense N-glycan profile, suggesting a potentially weaker immune response; booster effect: The booster shot appeared to increase N-glycan abundance in low-titer individuals, indicating a potential enhancement of the immune response (Table S1 Tab #18, #19, #20, #21, #22, #23, #24 and #33).

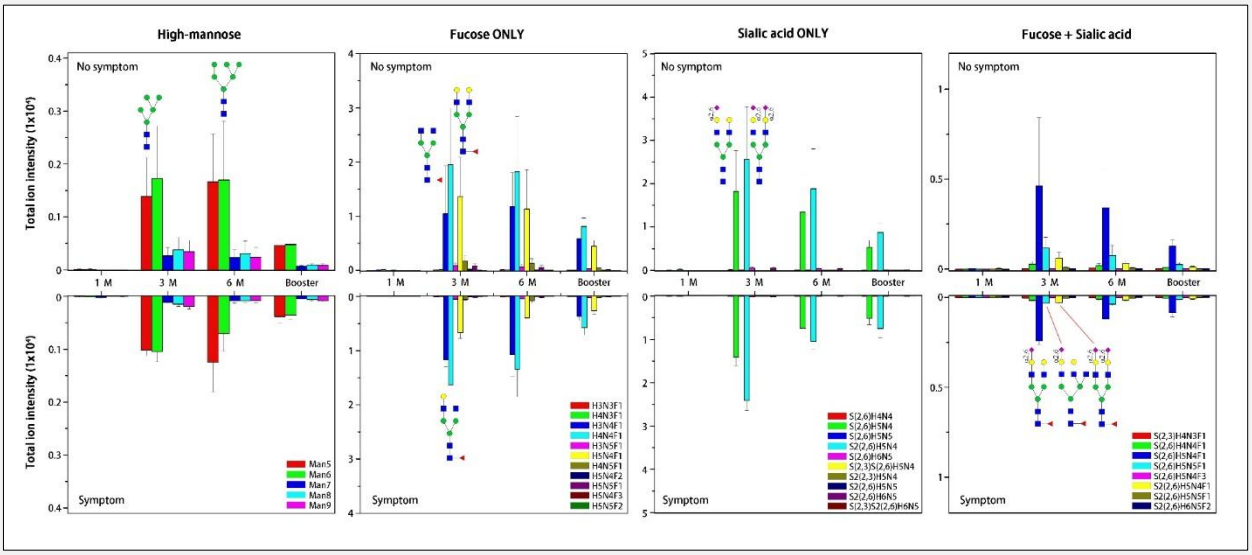


Figure 5. MALDI-MS profiles of serum N-glycans in Individuals with and without symptoms. This figure compares the MALDI-MS profiles of serum N-glycans between individuals with and without symptoms. Individuals without symptoms exhibited slightly higher overall N-glycan abundance, although the recovery rate of N-glycan synthesis appeared similar in both groups. Man5 and Man6 were the most abundant high-mannose N-glycans, while fucosylated and sialylated N-glycans were significantly more prevalent (approximately 10-fold) than high-mannose structures. Most sialic acid residues were linked in the α2,6 configuration, suggesting potential differences in N-glycan structure and immune response between individuals with and without symptoms. S Neu5Ac, H Hexose, N HexNAc, F Fucose (Table S1 Tab #1, #3, #5, #8 and #9).

titers are generally expected after vaccination, studies have shown that they do not correlate with the severity of vaccine-associated symptoms [27]. Neutralizing antibodies, or higher titer levels, are a strong indicator of immune protection against symptomatic SARS-CoV-2 infection. Research suggests a positive correlation between higher antibody levels and increased neutralizing antibody levels, which have been linked to greater protection from infection, reinfection, and severe disease [28]. In this study, we analyzed how these populations responded to glycan synthesis after vaccination, even though neutralizing antibody levels may gradually decline and disappear over time [29].

Figure 4 shows the individuals without symptoms (NS) but with different antibody titer responses to the inactivated vaccine. After one month following the second vaccine, all individuals exhibited lower glycan abundance. Three months post-vaccination, glycan levels steadily increased, but at notably different rates between patient groups. While glycans in low titer patients reached 4,000 to 6,000 intensity units, high titer patients exhibited a much more substantial increase, reaching approximately 25,000 intensity units, suggesting a potentially faster recovery of glycan synthesis in those with higher initial antibody responses. This trend continued at six months, with the most abundant N-glycan, S2H5N4, increasing 17-fold and 15-fold in low titer individuals at three and six months, respectively, whereas S2H5N4 increased 20-fold and 37-fold in high titer individuals during the same period. Interestingly, a booster shot appeared to inhibit glycan synthesis only in high titer individuals, resulting in a 16% relative abundance compared to the six-month level. Conversely, low titer individuals showed a marked recovery one month after the booster, with a 250% relative abundance compared to their six-month levels. These observations indicate a potential correlation between varying antibody titer responses and the dynamics of glycan synthesis recovery post-vaccination. Overall, the initial downregulation of glycan synthesis, particularly high-mannose glycans, following vaccination and the subsequent differential glycan abundance observed among responders may be linked to the complement pathway [30].

Symptom-dependent glycan synthesis post-vaccination

Concern about side effects, such as various post-vaccination associated symptoms, is a common reason for SARS-CoV-2 vaccine hesitancy. A study found that short-term systemic side effects of SARS-CoV-2 mRNA vaccination are correlated with greater long-lasting neutralizing antibody responses. In other words, experiencing symptoms after vaccination may be an indicator of a stronger immune response [31]. These side ef-

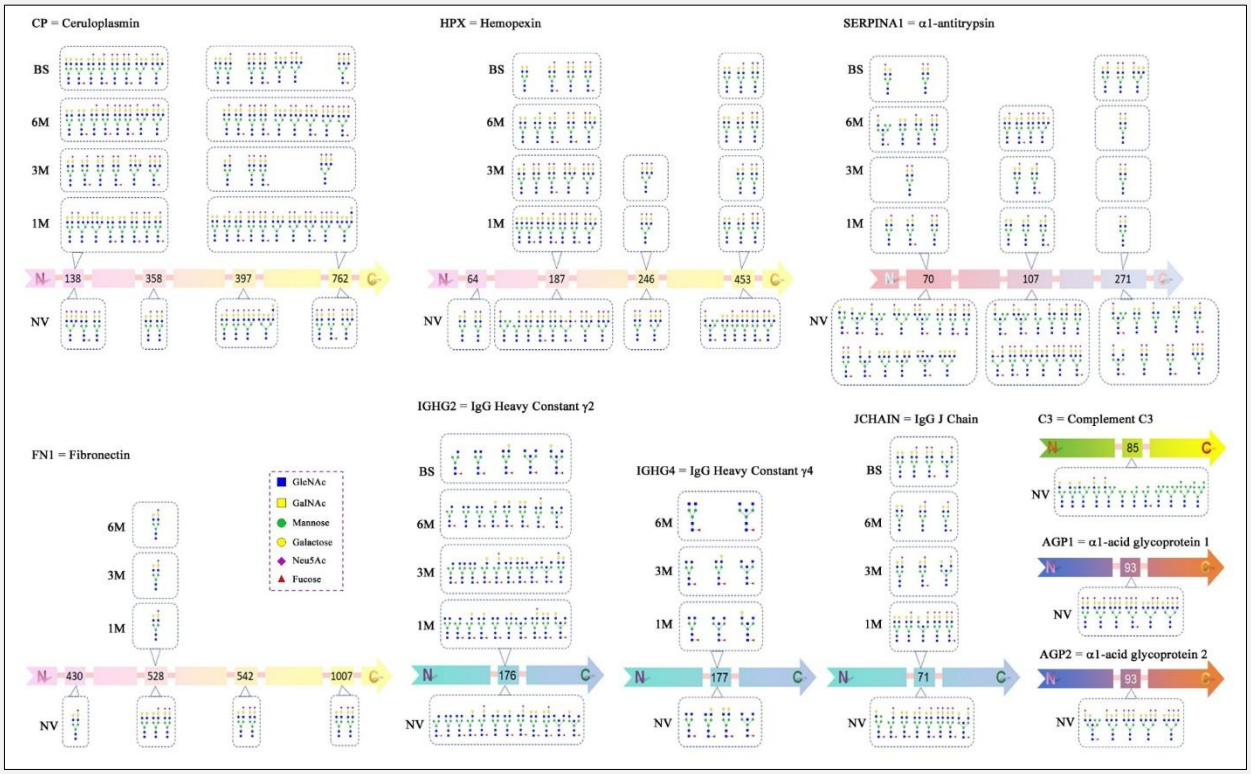


Figure 6. Comparative analysis of N-glycan profiles of ten abundant serum glycoproteins (CP, HPX, SERPINA1, FN1, IGHG2, IGHG4, JCHAIN, C3, AGP1, and AGP2) across non-vaccinated individuals (NV) and at 1M, 3M, and 6M post-vaccination, as well as 1M post-booster (BS). Generally, these glycoproteins displayed diverse and higher glycan abundance in the NV state, with a substantial decrease observed after the initial vaccination, even leading to glycan absence at some glycosides, suggesting vaccine-induced inhibition of serum protein glycosylation. Notably, individuals with a high-titer response exhibited a more pronounced N-glycan profile, especially during early recovery, while low-titer responders showed a less intense profile, potentially indicating a weaker immune response; however, the booster shot appeared to increase N-glycan abundance in these low-titer individuals, suggesting a potential enhancement of their immune response.

fects can attenuate over time after COVID-19 vaccination, and sustained improvement was observed after a second shot over a median follow-up of 67 days. Symptoms are commonly experienced by many people after vaccination, including muscle pain, fatigue, fever, headache, and sleepiness. The symptoms and severity of COVID-19 are possibly associated with changes in serum IgG glycosylation [32]. Therefore, it is interesting to investigate how glycosylation is altered among individuals who exhibit different symptoms and antibody titers.

The sera were compared from individuals who experienced symptoms and those who did not. For clarity, we plotted the relative abundance of glycans based on subtypes of N-glycans (high-mannose, fucose, sialic acid, and fucose-sialic acid) (Figure 5). All five high-mannoses were detected, with Man5 and Man6 being the most abundant. After 3 and 6 months, Man5 and Man6 levels increased substantially compared to those after 1 month. However, the booster shot re-suppressed high-mannose synthesis in both symptomatic and non-symptomatic individuals. Similar observations were found in other types of N-glycans, with biantennary N-glycans containing fucose and/or sialic acids being the most abundant and recovering faster than other species. As expected, most sialic acids had an α2,6 linkage, with fewer α2,3-linked sialic acids [33,34].

We then tested individuals with different symptoms one month after COVID-19 vaccination (Table S2). Five groups were tested: NS, fatigue, multiple symptoms (MS), muscle pain, and sleepiness (Figure S3A). A semi-quantitative analysis is provided in Table S3, comparing high-mannose, fucose, sialic acid, and fucose-sialic acid. Individuals with NS exhibited higher overall N-glycan abundance, while those with the lowest relative abundance. Figure S3A also demonstrates that gly-

can abundance gradually increases from MS, fatigue, and pain to sleepiness, suggesting that inhibition of N-glycosylation is less prominent in individuals experiencing sleepiness or without symptom. In another experiment, individuals with high and low titers were compared in the NS, sleepiness, and fatigue groups (Figure S3B and Table S4). Similar to the previous observation, individuals with high titers had slightly higher glycan abundance across different types of N-glycans. Results also indicated that individuals with NS experienced a quicker recovery in glycan synthesis. However, the initial recovery of N-glycans within the first month was minimal, as a substantial increase in glycan synthesis was observed within 3 to 6 months (Figure 2-5). These results suggest that single or multiple symptoms do influence glycan synthesis after vaccination.

Protein glycosylation alterations after vaccination

Which glycoproteins exhibit altered glycosylation following vaccination? Figure 6 presents the N-glycosylation profiles of ten abundant serum glycoproteins - ceruloplasmin (CP), hemopexin (HPX), α1-antitrypsin (SERPINA1), fibronectin (FN1), and immunoglobulins/complement components - at various time points post-primary vaccination (1M, 3M, 6M) and after a booster (BS), in comparison to non-vaccinated individuals (NV). The data reveal a consistent initial trend across most glycoproteins: a substantial reduction in glycan abundance one month after the primary vaccination, characterized by significantly decreased sialic acid levels in proteins such as CP, HPX, and SERPINA1, and the disappearance of high-mannose glycans in C3. This suggests a broad, early impact of the vaccine on the host's glycosylation processes; however, a gradual recovery of glycan abundance is observed for the majority of these proteins over the subsequent three to six months, indicating that the initial suppressive effect is not sustained long-term. Gene ontology (GO) analysis of these altered glycoproteins correlates these glycosylation changes to the acute-phase response, particularly acute inflammatory response and regulation of immune system processes. The booster shot elicited more variable, protein-specific responses in both glycan abundance and complexity, with some proteins showing a further decrease compared to the six-month mark, while others exhibited stabilization or a slight increase. Furthermore, the analysis indicates alterations in glycan structures throughout the vaccination course, implying changes not only in quantity but also in the composition and branching of these crucial protein modifications, which may have potential implications for protein function, stability, and interactions within the immune system, warranting further research to fully understand their significance in vaccine response and efficacy.

The effect of vaccine on glycosylation inhibition

Glycans on host receptors can influence viral entry by regulating the attachment of the virus to these receptors. Similarly, the glycans on the virus can not only affect initial binding by mutating the receptor-binding domain glycosylation of the spike protein but also play a crucial role in regulating viral entry [35]. Consequently, altering glycosylation on host receptors is desirable to enhance protection against infection. Studies have shown that vaccinated individuals exhibit lower levels of fucosylated N-glycans and higher levels of sialylation on mono-, bi-, and tri-sialoglycans in response to SARS-CoV-2 mRNA vaccination [36]. Moreover, viruses can undergo rapid mutations to alter their protein glycosylation, enabling them to evade recognition by the immune system and therapeutic antibodies, a process known as glycan masking [37].

While limited research exists on vaccine-induced inhibition of host glycosylation, reducing spike S glycosylation is known to enhance broad immune responses [6,35]. Our study corroborates this by demonstrating that an inactivated vaccine significantly suppresses host glycosylation within the first month post-vaccination (Figure 1) without altering the baseline N-glycan profile. Subsequently, N-glycan abundance rapidly rebounds between three and six months, a process partially reversed by the booster shot (Figure 2) and leading to a predominance of fucosylated and sialoglycans (Figure 3). Notably, the magnitude of the antibody titer response significantly impacts the recovery rate of glycan synthesis, with higher titers correlating with faster recovery (Figure 4). However, the booster shot's inability to inhibit glycan synthesis in individuals with low antibody titers suggests the involvement of a distinct synthesis pathway in this group. Although the booster effectively restores viral protection, it has a comparatively minor effect on the patient's N-glycan profile. Furthermore, asymptomatic individuals' post-vaccination exhibits higher glycan abundance and a slightly accelerated recovery (Figure 5). Interestingly, initial glycan abundance one month after vaccination varies among individuals, correlating with symptoms such as MS, fatigue, pain, and sleepiness, which rank from low to high glycan abundance (Figure S3). The most significant alterations in glycosylation were observed in glycoproteins involved in stress and immune responses (Figure 6). Overall, our findings indicate a substantial, though transient, suppression of glycan synthesis following the primary vaccination, with a relatively rapid recovery in the subsequent months, whereas the booster shot elicited a less pronounced suppressive effect on glycoforms.

The inhibition of host glycosylation pathways has been shown to interfere with the viral lifecycle, impairing secretion, fusion, and evasion of host immunity [38,39]. A deficiency or inhibition of ER glucosidases can alter N-linked glycan structures and modulate the host response to viral infections in congenital disorders of glycosylation (CDG). This can suppress viral replication by altering glycoforms from the normal [40]. Researchers have identified cases where glycosylation disorders with immunodeficiency have protected people from specific vi-

ral infections. Primarily, the defective glycosylation on their cells hinders the virus's ability to properly attach and enter the host cell, thereby limiting viral replication [41]. Consequently, partial inhibition of the N-glycosylation machinery can be effective in altering the course of viral infection. For example, tunicamycin (TM), an inhibitor of N-glycan synthesis, can significantly enhance the antiviral activity of interferon against viruses of L cells *in vitro*, demonstrating TM's antiviral activity [42]. Another inhibitor, NGI-1 (N-linked glycosylation inhibitor-1), is also found to have strong and broad antiviral activity against pan-Flaviviruses, Zika virus, and West Nile virus [43]. These observations suggest that an effective vaccine should have an inhibitory effect on N-glycosylation to enhance vaccine antiviral activity

CONCLUSION

Our findings demonstrate a novel mechanism by which COVID-19 vaccines can exert antiviral effects. Beyond their direct role in neutralizing the virus, these vaccines can indirectly inhibit the host's glycosylation pathways. This inhibition has significant implications for viral replication, as it can impair viral attachment, entry, and evasion of the immune system. We observed that the inactivated COVID-19 vaccine can dramatically suppress host glycosylation. While the overall profile of N-glycans remained similar, the abundance of these glycans was significantly reduced following vaccination. Interestingly, the recovery of glycan synthesis was influenced by the antibody titer elicited by the vaccine. Individuals with higher antibody titers exhibited faster recovery of glycan synthesis. However, individuals with lower antibody titers did not experience the same level of glycan recovery, suggesting alternative pathways may be involved. These findings highlight the complex interplay between the immune response, glycosylation pathways, and viral infection. By understanding these mechanisms, we can explore novel therapeutic strategies that target both the virus and the host's response to enhance protection against viral infections.

Supporting Information:
Additional experiments and experimental results (MALDI-MS raw data) are provided in the supporting information. Table S1 List of volunteer information, Table S2 Clinical data of samples, Table S3 Low titer versus high titer, Table S4 Multiple symptoms, Figure S1 Coronavirus structure, Figure S2 Workflow, Figure S3 Glycan profiles.

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Data Availability:
LC-MS/MS data can be accessed via PRIDE website (Accession: PXD064069, reviewer_pxd064069@ebi.ac.uk, Password: VE8SsSuvwo2V). MALDI-MS data can be accessed via GlycoPost (https://glycopost.glycosmos.org/preview/1004799118682b84291f8c8PIN, PIN CODE: 8762).

Declaration of Interest:
The authors declare no competing financial interests.

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