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Original Research

Effects of sulphur thermal water inhalations in long-COVID syndrome: Spa-centred, double-blinded, randomised case–control pilot study

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ABSTRACT

Background: The long-COVID syndrome is characterised by a plethora of symptoms. Given its social and economic impact, many studies have stressed the urgency of proposing innovative strategies other than hospital settings. In this double-blinded, randomised, case–control trial, we investigate the effects of sulphur thermal water inhalations, rich in H_2S, compared to distilled water inhalations on symptoms, inflammatory markers and nasal microbiome in long-COVID patients.

Methods: About 30 outpatients aged 18–75 with positive diagnosis for long-COVID were randomised in two groups undergoing 12 consecutive days of inhalations. The active group (STW) received sulphur thermal water inhalations whereas the placebo group received inhalations of sterile distilled non-pyrogenic water (SDW). Each participant was tested prior treatment at day 1 (T0), after the inhalations at day 14 (T1) and at 3 months follow-up (T2). At each time point, blood tests, nasal swabs for microbiome sampling, pulmonary functionality tests (PFTs) and pro-inflammatory marker measure were performed.

Results: The scores obtained in the administered tests (6MWT, Borg score and SGRQ) at T0 showed a significant variation in the STW group, at T1 and T2. Serum cytokine levels and other inflammatory biomarkers reported a statistically significant decrease. Some specific parameters of PFTs showed ameliorations in the STW group only. Changes in the STW nasopharyngeal microbiota composition were noticed, especially from T0 to T2.

Conclusions: Inhalations of sulphur thermal water exerted objective and subjective improvements on participants affected by long-COVID. Significant reduction of inflammatory markers, dyspnoea scores and quantitative and qualitative changes in the nasopharyngeal microbiome were also assessed.

Introduction

Long-COVID, post-COVID-19 conditions (PCC), chronic-COVID, post-COVID syndrome (PCS) and post-acute sequelae of SARS-CoV-2 (PASC) are all names used to identify a postviral syndrome affecting up to 10% of people who have recovered from SARS-CoV-2 infection.[1] The actual number of people worldwide who have experienced symptoms attributable to long-term COVID has not been systematically measured yet. However, current estimates suggest that this postviral syndrome could affect at least 65 million people, since its incidence ranges from 10–30% of non-hospitalised individuals and raises up to 50–70% of hospitalised patients, in contrast to the 10–12% of the vaccinated pop-

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ulation experiencing prolonged symptoms.[2–4] Some long-term COVID symptoms are non-specific and similar to those detected in other viral respiratory diseases, whereas other persisting features are uniquely associated with SARS-CoV-2. Long-term COVID syndrome is characterised by a plethora of symptoms, ranging from respiratory to cognitive disorders, which adversely and strongly impair the quality of life of affected patients.[2] Misdiagnosis can easily rise after SARS-CoV-2 infection and accurate anamnesis is nowadays advocated to detect a clear linkage to post-viral onset.[5]

Early clinical studies, during the 2020 pandemic and the first post-pandemic months, described a few common and recurring symptoms, such as dyspnoea, chest tightness, mild to moderate fatigue, chills or sweats, myalgia, dry cough, fever, headaches, cognitive impairments, or difficulties in concentration generally described as 'brain fog'.[6-9] Since then, more than 200 symptoms affecting different organ systems have been attributed to long-term COVID syndrome, such as cardiopulmonary,[10] vascular[11] as well as the central and peripheral nervous systems.[12] The immune system, responsible for uncontrolled overproduction of pro-inflammatory cytokines and chemokines leading to systemic inflammation and autoimmune disease, plays a key role in the pathogenesis of both acute and long-term disease.[13,14] Although several factors have been identified for influencing the onset and magnitude of post-acute symptoms[15,16] very little is known about long-term effects and proper treatment to restore pre-infection health conditions.[17] In this context, given the social and economic burden linked to the health-deteriorating involvement of multiple organs, many studies have stressed the urgency of proposing innovative strategies, alternative treatments, and tailored interventions aimed at rehabilitating people with long-COVID syndrome.[18]

Water-based therapies are secular practices that have been recognised for their regenerative and therapeutic properties.[19] Crenotherapy is a generic term that refers to different approaches using mineral water for therapeutic purposes.[20-22] Among them, inhalations with thermal waters (TW) are firm established treatments used to prevent, treat, or avoid recrudescence of several respiratory diseases due to long-lasting outcomes and almost no side effects.[23] TWs used for inhalations are hypertonic solutions having different effects attributable to their specific mineral composition.[24,25] Among TWs, sulphur waters, defined as waters containing at least 1 mg/L hydrogen sulphide (H_2S) and/or its respective ions (sulphide ion, S_2^- and hydrosulphide ion, HS^-), have been considered for prevention and treatment of both SARS-CoV-2 infection and long-COVID syndrome.[20,26] The emerging data on the biological effects of H_2S, a gaseous signalling molecule involved in a multitude of physiological and pathological processes, are increasingly recognising it as a strong biological mediator with several properties, including antiviral and anti-inflammatory ones.[27,28] The same effects have been reported for H_2S donor compounds that can release H_2S under certain circumstances.[29,30] Recently, studies performed on airway epithelial cells infection highlighted a significant antiviral activity of H_2S and H_2S donors against a wide range of enveloped RNA viruses,[31] while other studies demonstrated the ability of H_2S to reduce the transmembrane protease serine-2 (TMPRSS2) expression, one of the two main proteins involved in the entry of SARS-CoV-2 into host cells.[32] Moreover, the therapeutic effect of H_2S has been investigated also from an anti-inflammatory point of view. It is effective in downregulating interleukin-6 (IL-6), one of the most expressed pro-inflammatory mediators in the serum of people affected both by COVID-19 and long-COVID and exerting its effect by inhibiting the NF-κB pathway, thus finally modulating the transcription of pro-inflammatory cytokines.[33] Since the first months of the pandemic, great interest has been given to the SARS-CoV-2 main gate of entrance: the nasal filter.[34] As previously shown, the nasal microbiota can play a determinant role in favouring[35] or discouraging[36] the engraftment and thus the diffusion of several pathogens.[37] Moreover, nasal microbiota can be influenced by several factors such as age, comorbidities[38] cleansing practices, smoking habits,[39] environmental factors as fumes and aerosol inhalations etc. In this view, we investigated if long-COVID resident nasal microbiome may have common features[40] or peculiar quantitative or qualitative changes following inhalations. In this pilot study, the effect of sulphur thermal water (STW) inhalations compared to sterile distilled non-pyrogenic water (SDW) inhalations in participants affected by long-COVID syndrome was investigated in a randomised case–control trial. Moreover, we also analysed the effects of active (STW) versus placebo (SDW) inhalations on blood test parameters, serum inflammatory cytokines, pulmonary function tests, as well as qualitative and quantitative changes in the nasal microbiome. To our knowledge, this pilot study represents the first randomised case–control trial on a rehab programme in a spa setting, aimed at evaluating the effect of sulphur water versus placebo inhalations on participants affected by long-COVID syndrome with pulmonary involvement. The study focused on demonstrating the possible effects elicited by endogenous administration of H_2S both on patients' symptomatology (questionnaires), and on changes of disease markers such as inflammatory cytokines, nasal microbiome, respiratory parameters and exertion tolerance.

Methods

Study design and participants

The study (www.clinicaltrials.gov identifier, NCT06294756) is a double-blind, interventional, randomised case–control pilot trial. The aim was to assess the efficacy of sulphur thermal water (STW) inhalations in patients diagnosed with long-COVID (Table S1). The study was performed from May to October 2023 at the Acque Albule, Terme di Roma spa facility (Tivoli Terme, Rome, Italy).

Eligible participants were adult outpatients who went to the spa facility with an independent prescription of inhalation therapy with sulphur water for long-COVID respiratory issues. The participants had previously tested positive in certified polymerase chain reaction (PCR) screening for SARS-CoV-2 infection (data from the Regional Archive of Health Service for SARS-CoV-2 Infection) and, at the time of the study, had a positive diagnosis of long-COVID syndrome with pulmonary involvement. Other inclusion criteria were age 18–75 years, to avoid bias due to physiological pulmonary ageing issues, and a negative swab for SARS-CoV-2 infection at enrolment (Visit 1, T0). Exclusion criteria were the presence of pre-existing comorbidities affecting the airways (such as asthma, rhinitis, etc), prescriptions of inhalatory, intravenous or intramuscular steroid therapy, pre-existing diagnosis of depression, psychological or psychiatric disorders, and severe obesity (BMI>32 kg/m^2). The ability to walk was a mandatory requirement for performing the 6-minute walking test (6MWT). The willingness and ability to comply with scheduled visits, laboratory tests and other study procedures, together with the absence of major ECG alterations, were also mandatory prerequisites to be enrolled (Table 1). All patients' identification data were substituted with a three-digit cardinal number (001, 002 etc), which were assigned according to patients' order of arrival. The same identification method has been applied to all formats administered, as well as to all samples and specimens acquired. The only copy matching list (identification data-numbers) was kept by the study designer. Neither the participants nor any of the medical researchers or laboratory staff involved in the screening, enrolment, clinical evaluation, monitoring, and laboratory as well as statistics of the participants' analyses were aware of the study intervention received (STW vs SDW). A randomisation list (1:1 STW vs SDW) was created prior to recruiting through a randomisation sequence generator[41] set with ranges: minimum value 1; maximum value 30 and formatted in two columns. The only copy list, with randomisation sequence obtained by generator, was directly given to the inhalation assistant, who administered the intervention. Therefore, the inhalation assistant was unblinded to the treatment assigned but blinded to the medical condition of the participants. All the activities performed at visits 1, 2 and 3, respectively T0, T1 (at the end of inhalatory therapy) and T2 (90 ± 7 days from the end of inhalatory therapy) are reported in Table S2.

Table 1
Participants' eligibility criteria: Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
• Adults aged from 18–75. • Willing and capable of giving informed consent. • Participants with smoking habits or not. • Participants with COVID vaccination or not. • Negative to SARS-CoV-2 rapid swabs at screening visit. • Certified previous SARS-CoV-2 infection (Regional Public Health Service archives of SARS-CoV-2 infection). • Certified diagnosis of long-COVID (post-infective onset symptoms only, lasting more than 4 months since swab negativisation). • Any severity of COVID-19 symptoms during acute infection (home care, ICU admission, ventilation). • Participants treated with inhaled bronchodilators or not. • Willing and able to comply with scheduled visits, laboratory tests and other study procedures.	• Obesity (BMI>32 kg/m^2). • Walking impairment. • Pre-existing other comorbidities affecting the airways (eg asthma, rhinitis). • Major ECG alterations. • Therapy with inhaled, IV or IM steroids. • Pre-existing diagnosis of depression, psychological or psychiatric disorders. • Patients currently recruited to other clinical trials.

Inhalation therapy

All participants followed an inhalation protocol based on 12 consecutive sessions, 20 min each, once a day from T0 (visit 1) through a conventional thermal water aerosolisation Faset^{TM}system (Faset Spa, Trezzano sul Naviglio, Milan, Italy) delivering particles of TW with a diameter between 0.6 μm <MMAD < 5 μm (Fig. S1). Treatment consisted of 10 min warm steam and 10 min aerosol inhalation with nasal prongs. Inhalations with STW Acque Albule were administered to patients belonging to the active group, called STW, while patients belonging to the placebo group, called SDW, were addressed to modified inhaler modules, previously disconnected from the hydraulic circuit that supplied TW and connected to non-pyrogenic sterile water reservoirs (highly depurated water- pharmaceutical grade FU-for external and internal use, Makeitlab, Canosa di Puglia, BT, Italy). In order to preserve patients' blindness to randomised treatment, any possible difference in perceived odour (sulphur vs water) during inhalation was assessed before starting the study: due to the sulphur smell of the inhalation room, no difference among the active or placebo therapy was perceivable.

The chemical composition of Acque Albule sulphur thermal water is determined by the Department of Public Health and Infectious Diseases of the Sapienza University of Rome twice a year and reported on the webpage of the Acque Albule, Terme di Roma[42] (Table S3).

Questionnaires

The illness severity was assessed according to the Symptoms Burden Questionnaire- Long-COVID, version 1.0 (SBQ-LC).[43]

The Patient Health Questionnaire-9 (PHQ-9)[44] together with the seven-item Generalised Anxiety Disorder (GAD-7) scale[45] were used for screening/enrolment as an assessment to mood alterations or depression diagnosis for exclusion criteria.

St. George Respiratory Questionnaire (SGRQ),[46] designed to assess health impairment in patients affected by asthma or chronic obstructive pulmonary disease (COPD), was administered to both the active and placebo groups at T0 and T2. In this study, we used a validated English-to-Italian translated version of SGRQ. Results obtained were analysed using St. George's Respiratory Questionnaire Application.[47]

The 6MWT is a simple and easily reproducible test to assess the cardiopulmonary response involved during physical activity.[48] In this study, we used a validated version by previous studies focusing on long-COVID-related pulmonary issues.[49] Being set during summer, the test was performed indoor in a fully air-conditioned environment on a flat pathway. After each session of 6MWT, we assigned to each participant the relative Borg score describing the level of dyspnoea after physical exercise.

The MoCA test is a mini-mental state test providing score adaptation to each patient's scholar level, used to determine cognitive lack and in this case the presence and severity of cognitive involvement.[50]

Spirometry test

Whole pulmonary functional tests (PFT) were performed with a cabin spirometer (Quark PTF-Q box, Cosmed®, Rome, Italy), allowing resting, forced and diffusing capacity of the lungs for carbon monoxide (DLCO) evaluation both in active and placebo groups. Spirometry evaluation was performed by the same trained medical researcher at T0, T1 and T2 with patients in a sitting position.

Blood tests

Blood analyses and tests were assigned to an independent UNI EN ISO 9001:2015 accredited laboratory for complete blood count, glycaemia, total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, transaminases, creatinine, azotaemia, uric acids, high-sensibility- C-reactive protein (Hs-CRP). Blood group determination was performed at T0 only. Fasting blood tests were performed at T0, T1 and T2 on both groups, 25 mL blood were taken at each session using two 5 mL EDTA tubes and three 5 mL no-reagent tubes. Serum was obtained by in loco centrifugation of blood samples at 4,000 × *g* for 10 min (Thermo Scientific SL-16, Thermo Fisher Scientific, Inc.Waltham, MA, USA). Patients' serum samples for ELISA assay were immediately coded for double-check identification and separately stored at −20°C.

ELISA assay

The amount of interleukin-6 (IL-6), interleukin-1β (IL-1β), serum S-100B protein, angiotensin-converting enzyme (ACE) and glutathione synthetase (GSS) in the collected serum was determined using enzyme-linked immunosorbent assay kits (Fine Test ELISA, Fine Biotech Co., Ltd., Wuhan, China) according to the manufacturer's instructions. Optical density (OD) absorbance was measured at 450 nm by a microplate reader (NB-12-0035, NeBiotech, Holden, MA, USA).

Nasal swab samples

The nasopharyngeal secretions were collected on handkerchiefs with swabs (Copan^{TM} eNat 2 mL, fine tip, cod. C-606CS50P, Copan Italia, Brescia, Italy). Both nostrils were sampled. All specimens were immediately coded for double-check identification and further refrigerated at 4°C before being processed.

DNA extraction

Each swab was inserted into the semipermeable NAO Baskets and 20 Ll proteinase K and 400 µL buffer AL were added. Each sample was slightly vortexed, and centrifuged at 10,000 × *g* for 1 min, allowing the elution of the digestion solution. After incubation at 56°C for 10 min and addition of 400 µL ethanol, the washing step and DNA purification were performed in accordance with the manufacturer's instructions (QIAmp DNA Mini Kit and DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany). Finally, DNA elution was completed in 60 µL elution solution (10 mM tris(hydroxymethyl)aminomethane hydrochloride and 0.5 mM ethylenediaminetetraacetic acid at pH 9.0)] as previously described.[51,52] Moreover, A Nextrator-48S system (Genolution, Seoul, Korea) was used according to the manufacturer's recommendations for DNA extraction. Briefly, an aliquot of 200 µL of each sample was loaded to the well of the plate and extraction was performed using a program named VN in Nextractor-48S software system, following the manufacturer's instructions. At the end of the process, eluted DNA (50 µL) was collected and stored at −20°C until the analysis.

16S rDNA amplicon sequencing and bioinformatic analysis

Libraries for next-generation sequencing were prepared according to the 16S Metagenomic Sequencing Library Preparation Guide (part 15044223 rev A; Illumina, San Diego, CA, USA). The PCR amplicons were obtained using primers containing overhang adapters, as previously described.[52,53] Tagged PCR products were generated using primer pairs with unique barcodes through two-step PCR. In this strategy, target primers containing overhang adapters were used in the first PCR to amplify the target gene; that product was then used in the second PCR using primers containing barcodes. Each amplification reaction had a total volume of 25 µL, containing 12.5 µL KAPA HiFi Hot Start Ready Mix (Roche, Pleasanton, CA, USA), 5 µL each primer (1 mM) and 2 µL template DNA. Reactions were carried out on a Techne TC-PLUS thermocycler (VWR International, LLC, Radnor, PA, USA). Following amplification, 5 µL PCR product from each reaction was used for agarose gel (1%) electrophoresis to confirm amplification. The final concentration of cleaned DNA amplicon was determined using the Qubit PicoGreen dsDNA BR assay kit (Invitrogen, Grand Island, NY, USA) and validated on a Bioanalyser DNA 1,000 chip (Agilent, Santa Clara, CA, USA). Libraries were prepared using the ISeq100 reagent kit preparation guide (Illumina, San Diego, CA, USA). Raw sequence data were processed using an in-house pipeline that was built on the Galaxy platform and incorporated various software tools to evaluate the quality of the raw sequence data (FASTA/Q Information tools, Mothur). All data sets were rigorously screened to remove low-quality reads (short reads 200 nucleotides (nt), zero-ambiguous sequences). Demultiplexing was performed to remove PhiX sequences and sort sequences; moreover, to minimise sequencing errors and ensure sequence quality, the reads were trimmed based on the sequence quality score using Btrim (an average quality score of 30 from the ends and remove reads that are less than 200 bp after end trimming).[54] OTUs (operational taxonomic units) were clustered at a 97% similarity level, final OTUs were generated based on the clustering results, and taxonomic annotations of individual OTUs were based on representative sequences using RDP's 16S Classifier 2.5. Observed OTUs were defined as observed species. A level of 97% sequence identity is often chosen as representative of a species and 95% for a genus.[55] The sequence reads were analysed in the cloud environment also. BaseSpace through the 16S Metagenomics app (version 1.0.1; Illumina): the taxonomic database used was the Illumina-curated version. Relative abundances of community members determined with rarefied data and summarised at each taxonomic level. Availability of data and material at NCBI Sequence Read Archive project (PRJNA1123150).

Statistical analysis

Determination of sample size

The determination of sample size for this pilot study, considered a confidence of 95% and a gender/age independent prevalence of 13% (data of the UK Office for National Statistics[56] for long-COVID at 12 weeks) was calculated[57] using a calculator software.[58] The sample size used in this study (n=30) exceeds the 95% of confidence sample size (n=21.5) corresponding to 98.5% confidence. Moreover, our sample size respects the thumb rule of 12 components/group for pilot studies.[59] Based on the literature and considering the aim of the trial as a pilot study to investigate for the first time the effect of STW on participants affected by long-COVID syndrome, a target of 30 participants in total was chosen to be randomised in a 1:1 ratio, in a sample size of 15 per group.[60] All participants randomised who received all 12 consecutive sessions of study treatments (STW and SDW) were included in statistical analyses. Between-group differences in the primary outcome at 14 and 90 days were assessed for the whole pulmonary functionality and cardiopulmonary response involved during physical activity. The quality of life was assessed as a secondary outcome looking at the percentage of participants with health impairments and affected by residual dyspnoea and fatigue. Biomarkers of the inflammatory response, and other clinical variables were analysed by a non-parametric one-way ANOVA (Kruskal–Wallis test) for comparing within the same group at different time points, and between the two groups (STW vs SDW) using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered significant. Percentage variations were calculated using a calculator software.[61]

Microbiological statistical analysis

Relative abundances of community members were determined with rarefied data and summarised at each taxonomic level. The proportion of the microbiome at each taxonomic rank, such as phylum, order, class, family and genus, was determined using the RDP classifier and the Greengenes Database. Alpha and beta diversity were calculated using Primer software (version,[62]) at a level of 97% sequence similarity. Regarding alpha diversity, the Shannon index and equitability index at the species level were computed.[63,64] Principal-component analysis (PCA) was performed using the METAGEN assist platform and R (version 3.1.3, www.R-project.org) with packages 'ggplot2', 'ape', 'psych' and 'vegan'.[63,64] Multivariate analysis, the PCA, and partial least square-discriminant analysis (PLS-DA) were performed to investigate dissimilarity between groups. Feature selection was performed using PLS-DA and 10-fold cross validation to tune algorithm parameters and to check model validity. Dendrogram and clustering analysis were based on the Euclidean distance and Ward's method.

Clinical trials

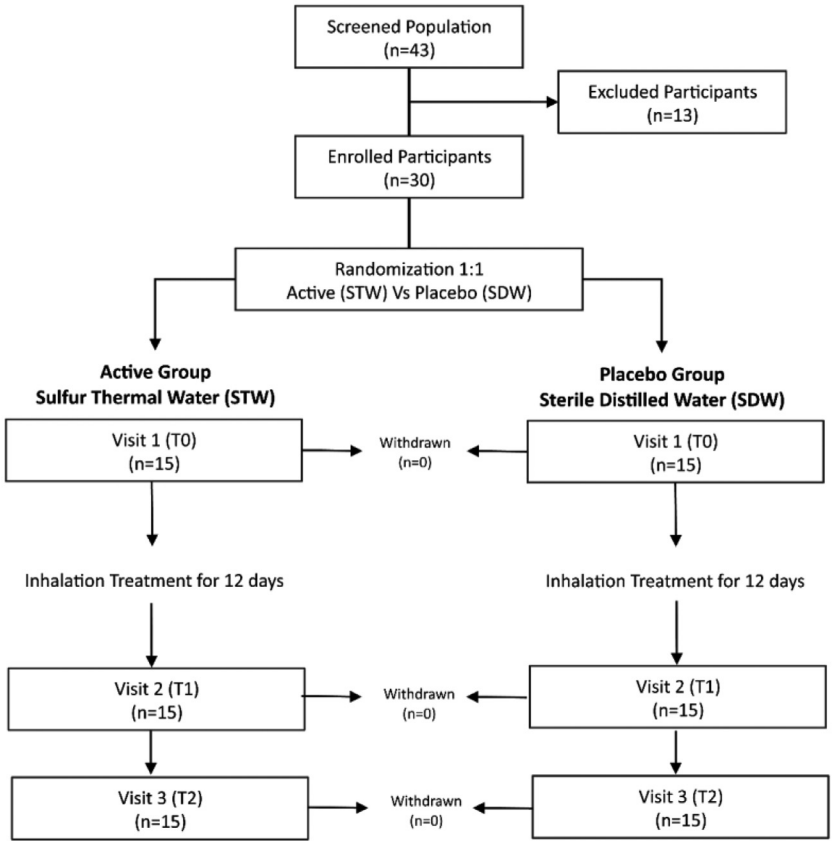
The study was registered on Clinicaltrial.com with ID NCT06294756.

Results

In total 30 individuals, 13 men and 17 women, all of Caucasian ethnicity, with a mean age of 52.7 ± 13.35 years, joined the study. All participants completed the study up to its natural term at 3 months' time. No drop out, loss in follow-up nor adverse events occurred, no changes to selection criteria, outcomes nor treatment protocol were made since their formulation (Scheme 1).

Demographic, physiological findings

The STW group was composed of 15 patients, seven men and eight women, one of whom was at the 20th week of gestation at enrolment. The average of STW participants was 57.06 ± 11.82 years, all participants had a SARS-CoV-2 infection with a mean duration of 22.75 ±



Scheme 1. Study flowchart.

10.03 days, two needed intensive care unit (ICU) admission, four had reinfection although all participants were vaccinated with a mean of 2.6 doses, SBQ-LC mean score was 15.25. The prevalence of symptoms affecting both groups of participants during the acute phase of SARS-CoV-2 infection and those characterising the long-COVID phase are reported in Fig. S2. The main frequent comorbidities of STW participants were hypertension (n=7), autoimmunity (n=5), allergies (n=5), minor cardiovascular diseases (n=3), hypercholesterolaemia (n=3). Two participants referred to previous diagnosis of thrombophilia: one female with heterozygous factor V Leiden mutation associated with heterozygous MTHFRII C677T mutation and one male with heterozygous MTHFRII C677T mutation. The main familiarities retrieved were in hypertension (n=9) diabetes (n=3), cardiovascular diseases (n=3), thrombophilia (n=3). Five participants were current smokers, three were previous smokers (quit >36 months), seven denied having ever smoked. Three participants reported domestic or working mould exposure, six reported to be exposed to fumes. Three participants used non-medicated nasal spray regularly, two confirmed previous thermal water inhalation treatments in the pre-COVID era (>36 months).

The SDW group consisted of 15 participants, six men and nine women; average age 47.92 ± 13.31 years; all participants had SARS-CoV-2 infections with a mean duration of 14.92 ± 8.04 days, one needed ICU admission, seven had reinfection although all participants were vaccinated with a mean of 2.57 doses, SBQ-LC mean score was 13.21. The main frequent comorbidities of SDW participants were allergies (n=12), diabetes (n=3), hypercholesterolaemia (n=3), hypertension (n=2), autoimmunity (n=2), minor cardiovascular diseases (n=1). The main familiarities assessed were in cardiovascular diseases (n=8), hypercholesterolaemia (n=5), hypertension (n=4), thrombophilia (n=4), diabetes (n=3). Four participants referred diagnosis of thrombophilia: one male with heterozygous factor V Leiden mutation associated to heterozygous MTHFRII C677T mutation, one female with heterozygous MTHFRII C677T mutation only and two participants, a male and a female, referred homozygous MTHFR II C677T mutation. Five participants were current smokers, two were previous smokers (quit >36 months), eight denied having ever smoked. Four participants reported domestic or working mould exposure, eight reported to be exposed to fumes, four used non-medicated nasal spray regularly, four confirmed previous thermal water inhalation treatments in the pre-COVID era. Other demographic, physiological and medical outcomes are reported in Table 2.

Haematological results

Both STW and SDW patients were subjected to blood samplings at T0, T1 and T2.

The blood test results are reported in Table S4 and Table S5. No major alterations were encountered at baseline in both groups. All percentage variations are expressed as delta among T0/T1 and T0/T2. Although no significant variations have been detected among T0/T1, T1/T2 nor T0/T2.

Changes in test results and questionnaires in STW participants

The results of tests and questionnaires administered once or during all the three phases of the study are reported in Table 3. The SBQ-LC questionnaire administered at T0 only showed a higher score in STW (15.25) than in SDW (13.21), thus meaning a more remarkable impact of post-COVID sequelae affecting the former group. The PHQ-9 test administered at T0 showed a higher score in STW (4.06) than in SDW (2.85) but with an equal maximum score of 7 for both groups. A similar result has been assessed for the GAD-7 test, which showed a higher score in STW (7.31) than in SDW (5.64), but in this case STW presented also the highest maximum score (15 vs 12). Both tests are coherent with the findings assessed for the impact of sequelae, the STW group had higher scores both for depression and anxiety tests; however, none of the participants individually reached values close to the cut-off for depressive

Table 2
Participant demographics and medical history.

	Total	Active group (STW)	Placebo group (SDW)
Number of participants	30	15	15
Male	13	7	6
Average age in years (min–max)	52.70 (26–75)	57.06 (45–69)	47.92 ± 13.31 (26–75)
Male	52.84 (31–75)	60.85 (46–75)	43.5 (31–67)
BMI mean in kg/m^2	26.34	27.72	24.76
Male	28.08	31.06	24.62
Retired	6	4	2
Educational level			
Middle school	7	5	2
High school	10	4	6
University	13	6	7
Working environment			
Rural	2	1	1
Urban	28	14	14
Domestic environment			
Rural	2	0	2
Urban	28	15	13
SARS-CoV-2 infection history			
SARS-CoV-2 duration (days)	19.1 ± 9.95	22.75 ± 10.03	14.92 ± 8.04
Days since SARS-CoV-2 negativization			
Min–max	60–1,140	60–1,140	150–930
Mean	480	450	522
n participants ICU hospitalisation	3	2	1
n participants reinfection	11	4	7
SARS-CoV-2 vaccinated participants	100%	100%	100%
Doses of vaccine			
4	4	2	2
3	15	7	8
2	7	4	3
1	4	2	2
Nasal swabs			
Negative	88	44	44
Positive to SARS-CoV-2	1	0	1 (T0)
Positive to GABHS	1	1 (T0)	0
Medical history			
Autoimmunity	7	5	2
Allergies	17	5	12
Diabetes	4	1	3
Familiarity in diabetes	6	3	3
Cardiovascular diseases (CVD)	4	3	1
Familiarity in CVD	16	8	8
Hypertension	9	7	2
Familiarity in hypertension	13	9	4
Hypercholesterolaemia	6	3	3
Familiarity in hypercholesterolaemia	8	3	5
Thrombophilia	6	2	4
Familiarity in thrombophilia	7	3	4
Smoker			
Previous	5	3	2
Current	10	5	5
Never	15	7	8
Mould exposure	7	3	4
Fumes exposure	14	6	8
Use of nasal sprays	7	3	4
Previous inhalation treatments	6	2	4

or anxiety issues likely to interfere with the subjective tests (SBQ-LC and SGRQ) administered. The MoCa test administered at T0 showed, likewise, a lower mean score in the STW group (25.80) than in the SDW one (27.70), thus probably assessing a higher cognitive involvement of the former group. Two of the four parts of SGRQ, the total score and the activity score, showed an evident decrease, −36.43% and -43.60%, respectively, in the STW group from T0 to T2 (Fig. 1). In STW subjects also the impact score together with the symptoms score showed a marked decrease (−39.78% and −15.24%, respectively), whereas no significant decrease was assessed in the SDW where SGRQ total score had a lower decrease (−7.23%), as well as the activity score (−11.16%) and impact scores (−11.02%); symptoms score showed an increase (+12.81%), thus meaning that SGRQ in almost all its components showed no significant ameliorations in the SDW group (data not shown).

The 6MWT performed on both groups has shown that although STW participants covered a mean lower distance at baseline (402.81 metres), they presented a significative ($p<0.005$) increase at T2 (+15.9%), whereas SDW participants traversed a longer distance at baseline (459.28 metres) but then they showed a minimum improvement at T1 (+ 1.71%) and at T2 (+2.33%) (Fig. 2, **left plot**). Saturation measured prior and after the performance of 6MWT in the STW group showed a lower mean value at T0 (97.62% SpO_2) than the SDW group (97.85% SpO_2), but a higher increase was determined after physical exertion both at T1 (95.06% SpO_2) and T2 (96.18% SpO_2). The Borg score, a dyspnoea index performed during the 6MWT whose value is directly proportional to symptomatology (lower is better), resulted higher at baseline in the STW group, showing then a decreasing trend both at T1 (–18.86%) and T2 (–23.58%), whereas in SWD, despite the lower score at baseline, it

Table 3
6-minute walk test, Borg score, SpO_2 and questionnaire outcomes.

	Total	Active group (STW)	Placebo group (SDW)
6-minute walk test (6MWT)			
Min and max T0 (metres)	300–60	300–520	340–540
T0 mean (metres)	429.16	402.81	459.28
Min–max T1 (metres)	370–560	370–520	380–560
T1 mean (Δ%)	457.33 (+6.56%)	448.75 (+11.40%)	467.14 (+1.71%)
Mean Δ T1–T0 (metres)	+30.16	+46	+12.14
Min–max T2 (metres)	360–520	370–550	360–520
T2 mean (Δ%)	468.33 (+9.12%)	466.87 (+15.9%)	470 (+2.33%)
Mean Δ T2–T1 (metres)	+10.33	+18.00	+1.42
Borg score			
Min–max	19–9	19–10	17–9
T0 mean	13.36	14.63	11.92
Min–max	17–8	16–8	17–9
T1 mean (Δ%)	12.53 (−6.21%)	11.87 (−18.86%)	13.28 (+11.41%)
Min–max	15–7	14–7	15–9
T2 mean (*Δ%)	11.73 (−12.20%)	11.18 (−23.58%)	12.15 (+1.93%)
Oxygen saturation (SpO_2)			
Min–max	96–99	96–99	97–99
Initial mean			
T0	97.73	97.62	97.85
T1	97.80	97.75	97.85
T2	97.90	98.00	97.78
Final mean			
T0	92.83	91.87	93.92
T1	95.33	95.06	95.64
T2	96.46	96.18	96.78
Symptoms Burden Questionnaire- Long-COVID (SBQ-LC)			
Min–max	1–37	3–37	1–34
Mean score	13.86	15.25	13.21
PHQ-9 test			
Min–max	0–7	1–7	0–7
Mean score	3.46	4.06	2.85
GAD-7 test			
Min–max	2–15	4–15	2–12
Mean score	6.53	7.31	5.64
Montreal Cognitive Assessment (MoCA)			
Min–max	18–30	18–30	24–30
Mean score	26.70	25.80	27.70
St George Respiratory Questionnaire (SGRQ)			
Total score			
T0	21.83	24.84	18.38
T2 (Δ%)*	16.45 (−24.64%)	15.79 (−36.43%)	17.05 (−7.23%)
Symptoms			
T0	25.14	28.21	21.62
T2 (Δ%)*	24.13 (−4.08%)	23.91 (−15.24%)	24.39 (+12.81%)
Activity			
T0	33.47	38.99	27.15
T2 (Δ%)*	22.99 (−31.31%)	21.99 (-43.60%)	24.12 (-11.16%)
Impacts			
T0	13.35	15.03	11.43
T2 (Δ%)*	9.58 (−28.24%)	9.05 (−39.78%)	10.17 (−11.02%)

Δ% (Δ T0/ T1).
[a] Δ% (Δ T0/ T2).

was increased at T1 and T2 (+11.41% and 1.93%, respectively) (Fig. 2, right plot).

Effects of inhalations on pulmonary functionality test (PFT)

The main respiratory pattern observed was the restrictive one, assessed in six STW participants (40%) and three SDW participants (20%). One obstructive pattern was only observed at T2 in an STW participant (6.66%), a pregnant woman at the 33rd week of gestation. All PFT parameters had lower mean values in STW sample at baseline and more participants belonging to this group presented values <80% of predicted respect to SDW subjects. Despite lower mean values and a higher number of participants presenting altered PFT in the STW group, parameters as FEV1/VC MAX %, ERV, DLCO (Fig. 3, left panel) and VA showed a continuous increase at T1 and T2, whereas the SDW group presented a lower or no increase at all at T1 and decrease or had a minimum increase at T2.

Some parameters such as FVC, FEV1, VC, IC decreased at T1 in both samples but then increased at T2 in SDW (FVC) or in SDW only (FEV1, IC), others as FEV1/FVC% increased at T1 in STW only (Fig. 3, right panel), and at T2 in STW only. All PFT are reported in Table 4.

Effects of STW inhalations on long-COVID serum markers

To assess the effects of STW inhalations on blood markers of long-COVID syndrome, serum from both STW and SDW participants enrolled in this study was collected and analysed by ELISA test. Considering the role played by inflammation in long-COVID symptoms, the expression of IL-6 and IL-1β proinflammatory cytokines, highly involved in COVID-

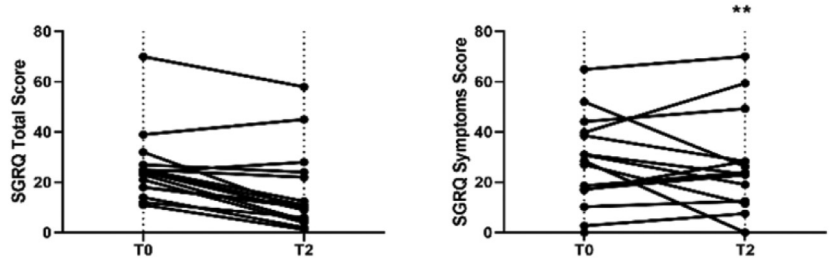


Fig. 1. SGRQ scores in STW participants from T0 to T2. Total SGRQ score (top left panel), SQRQ symptoms score (T0/T2 $p<0.005$) (top right panel), SGRQ impact score (T0/T2 $p<0.05$) (bottom left panel), SGRQ activity score (bottom right panel).

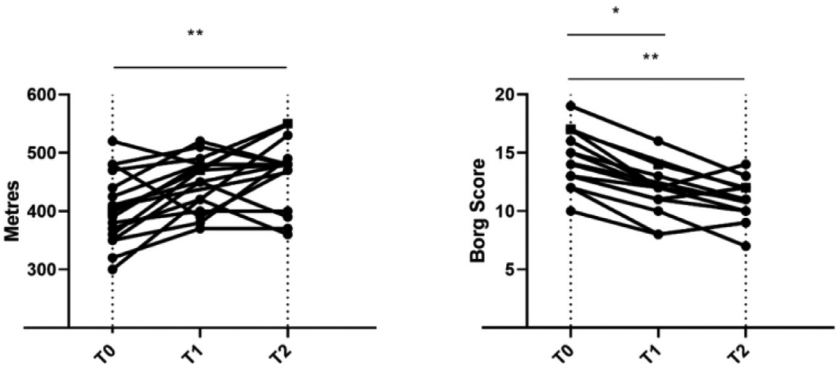


Fig. 2. 6MWT and Borg score in the STW group. 6MWT measured distance (metres) travelled by STW participants at T0, T1 and T2 (T0/T2, $p<0.005$) (left plot). Borg score index performed during 6MWT STW participants at T0, T1 and T2 (right plot) (T0/T1 $p<0.05$; T0/T2 $p<0.005$).

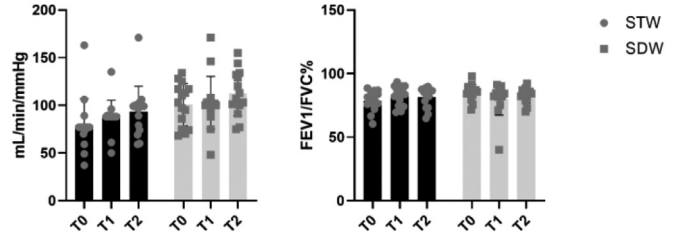


Fig. 3. PFT from T0 to T2 in STW and SDW. DLCO evaluation is expressed in mL/min/mmHg and reported in the left panel and FEV1/FVC% evaluation is reported in the right panel.

19 cytokine storm, was measured in both groups of participants at the three experimental time points, T0, T1 and T2 (Table 5).

Serum cytokine levels at T0 were comparable between the STW and SDW groups, measuring approximately 15 pg/mL for IL-6 and 80 pg/mL for IL-1β, thus indicating an effective patient randomisation among the two groups. Participants undergoing inhalations with STW showed a growing decrease in cytokine expression over time, which resulted statistically significant at T2. More specifically, a 48.49% percentage decrease of IL-1β and 44.57% of IL-6 expression was observed in STW participants. Regarding IL-6, a statistically significant ($p<0.05$) decrease of 40.24% was already observed after 12 days of inhalation (T1) in STW participants. This decrease was also observed in IL-1β cytokine expression but was not statistically significant ($p=0.12$). In contrast, in SDW participants no modulation of expression of the two analysed proinflammatory cytokines, compared to the basal levels at T0, was observed at T1 nor at T2 (Fig. 4A).

In addition to the levels of cytokines, responsible for cytokine storm and hyperinflammation typical of long-COVID syndrome, other characteristic proteins, such as S100B, serum ACE and GSS, used as a biomarker for severity in COVID-19, were analysed. As previously observed for the cytokine expression, those participants undergoing STW inhalations also showed reduced serum levels of S100B protein and ACE enzyme. The decrease of these biomarkers expression at the follow-up visit (T2) compared to T0 were respectively 59.50% for S100B and 93.46% for ACE. The decreased expression of the ACE enzyme has a strong statistical significance ($p<0.0001$), indicating a long-term protective effect of STW inhalations, which was already detected at T1 when a decrease (66.97%) in serum levels of these proteins was observed. No modulation was observed in the SDW participant group, either at T1 or at T2, compared to basal time (T0) (Fig. 4B and Table 5). GSS serum concentrations were comparable among the two groups at baseline (T0), ranging from 5.94 ng/mL in STW to 5.37 ng/mL in SDW. STW participants presented at T1 and T2 a marked decrease of −45.45% and −51.51% respectively compared to T0, whereas SDW participants showed a non-statistically significant reduction (Fig. 4B and Table 5). Hs-CRP was not significantly modulated in either group (STW and SDW) (Table 5).

Effect of STW on nasal microbiome

The structure of the nasopharyngeal microbial population was no different between STW and SDW participants at T0 (ANOSIM based on Bra−Curtis similarity, R=0.156; $p=0.001$). Both nasopharyngeal micro-

Table 4
PFTs mean values at T0, T1 and T2, Δ variations among T0/T1 and T1/T2, values < 80% attended in STW, SDW and whole population studied.

	STW			SDW			Total population		
	T0	**T1**	**T2**	**T0**	**T1**	**T2**	**T0**	**T1**	**T2**
FVC	98.43	94.37	99.18	106.5	104.64	104.85	102.2	99.16	101.24
Δ		−4.06%	+0.75%		−1.86%	−1.55%		−3.04%	−0.96%
FVC < 80%	3	2	1	0	0	0	3	2	1
% sample	(20%)	(13.3%)	(6.6%)				(10%)	(6.6%)	(3.3%)
FEV1	100	99.43	99.56	110.28	104.57	111.07	104.8	101.83	104.82
Δ		−0.57%	−0.44%		−5.71%	+0.79%		−2.97%	+0.02%
FEV1<80%	2	1	1	0	1	0	2	2	1
% sample	(13%)	(6.6%)	(6.6%)		(6.6%)		(6.6%)	(6.6%)	(3.3%)
FEV1/FVC%	101.06	104.75	99.43	103.14	99.07	105.78	102.03	102.1	102.79
Δ		+3.69%	−1.63%		−4.07%	+2.64%		−0.07%	−0.76%
FEV1/VC MAX%	96.56	103.06	98.43	102.57	99	104.71	99.36	101.16	101.72
Δ		+6.05%	+1.87%		+3.57%	+2.14%		+1.80%	+2.36%
VC	99.5	88.06	89.37	99.85	97.21	96.14	99.66	92.33	92.27
Δ		−11.44%	−10.13%		−2.64%	−3.71%		−7.33%	−7.39%
IC	102.43	91.93	98.32	101.35	97.42	103.64	101.93	94.5	101.20
Δ		−10.50%	−4.11%		−3.93%	+2.29%		−2.43%	−0.73%
ERV	57.42	62.06	60.07	87.92	84	74.16	72.11	72.3	64.83
Δ		+4.64%	+2.65%		−3.92%	−13.76%		−0.19%	−7.28%
DLCO	77.88	88	93.33	103	103.87	114	92.72	96.46	103.8
Δ		+10.12%	+15.45%		+0.87%	+11.00%		+3.74%	+11.08%
DLCO <80%	6	4	2	2	3	1	8	7	3
% sample	(40%)	(26.6%)	(13.3%)	(13.3%)	(20%)	(6.6%)	(26.6%)	(23.3%)	(10%)
VA	83.44	93.42	97.77	103.35	101.66	107.69	95.56	98.06	102.66
Δ		+9.98%	+14.33%		-1.69%	+4.34%		+2.50%	+7.10%
KCO	90.77	93.14	93.88	95.30	97.87	100.83	93.45	95.66	97.45
Δ		+2.37%	+3.11%		+2.57%	+5.53%		+2.21%	+4%
IV	75.92	75.10	75.71	88.07	81.66	87.57	82.22	78.68	81.03
Δ		−0.82%	−0.21%		−6.41%	−0.5%		−3.54%	−1.19%
TLC	78.77	88.14	92.55	98.07	97.12	101.76	86.75	92.93	97.09
Δ		+9.37%	+13.78%		-0.95%	+3.59%		+6.18%	+10.34%
TLC <80%	5	3	2	2	2	1	7	5	3
% sample	(33.3%)	(20%)	(13.3%)	(13.3%)	(13.3%)	(6.6%)	(23.3%)	(16.6%)	(10%)

Table 5
Serum inflammatory markers at T0, T1 and T2.

	Total	Active group (STW)	Placebo group (SDW)
IL-1β (interleukin-1β) pg/mL			
T0	73.38	73.66	73.10
T1(Δ%)	66.55 (−9.30%)	58.41 (−24.78%)	74.69 (+2.17%)
T2 (Δ%)[a]	55.99 (−23.7%)	37.94 (−51.14%)	74.04 (+1.28%)
IL-6 (interleukin-6) pg/mL			
T0	13.58	16.13	11.03
T1 (Δ%)	10.07 (−25.84%)	9.64 (−40.24%)	10.51 (−4.71%)
T2 (Δ%)[a]	9.18 (−32.40%)	8.94 (−44.57%)	9.43 (−14.50%)
ACE (angiotensin-converting enzyme) ng/mL			
T0	45.56	61.13	30.00
T1(Δ%)	20.56 (−54.87%)	20.19 (−66.97%)	20.99 (−30.03%)
T2 (Δ%)[a]	14.50 (−68.17%)	4.00 (−93.46%)	26.51 (−11.63%)
GSS (glutathione synthetase) ng/mL			
T0	5.67 ng/mL	5.94 ng/mL	5.37 ng/mL
T1(Δ%)	4.16 ng/mL (−26.63%)	3.24 ng/mL (−45.45%)	5.20 ng/mL (−3.16%)
T2 (Δ%)[a]	3.63 ng/mL (−35.98%)	2.88 ng/mL (−51.51%)	4.48 ng/mL (-16.57%)
Serum S100B protein pg/mL			
T0			
T1(Δ%)	171.98	169.35	174.61
T2 (Δ%)[a]	125.00 (−27.31%)	90.75 (−46.41%)	159.26 (−8.79%)
	104.94 (−38.98%)	68.58 (−59.50%)	141.30 (−19.07%)
Hs-CRP (high-sensitivity C-reactive protein) mg/L			
T0	2.06	2.61	1.41
T1(Δ%)	1.82 (−11.65%)	2.19 (−16.09%)	1.39 (−1.41%)
T2 (Δ%)[a]	2.11 (+2.42%)	2.44 (−6.51%)	1.71 (−6.51%)

Δ% (Δ T0/ T1).
[a] Δ% (Δ T0/ T2)

bial communities were dominated by the Firmicutes phylum (34.7% ± 8.4%), followed by Proteobacteria (26.1% ± 11.7%) and Actinobacteria (20.2% ± 10.5%). At the genus level, *Corynebacterium* (45% ± 11.4%) and *Staphylococcus* (14% ± 8.2%) were the most abundant genera, following *Propionibacterium* (12.4% ± 2.3%), *Dolosigranulum* (6.1% ± 1.2%) and *Streptococcus* (5.6% ± 1.0%).

The structure of the nasopharyngeal microbial STW population is different between T0, T1 and T2 (ANOSIM based on Bray–Curtis similarity, R=0.566; p=0.001; Fig. 5). Specifically, ANOSIM analysis of the distance metrics revealed a significant difference in the metagenome among the SWT groups between T0 versus T2 (ANOSIM based on Bray–Curtis similarity as R=0.540; p=0.001) as represented in Fig. 5. To identify bac-

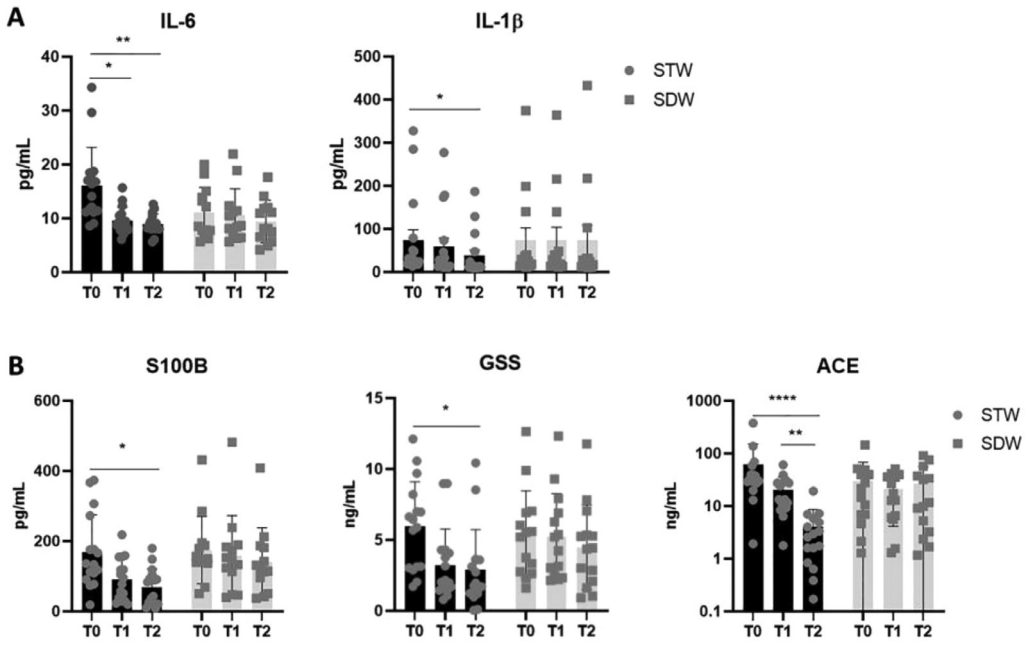


Fig. 4. (A) IL-6 and IL-1β and (B) S100B, GSS and ACE expression in serum of STW and SDW participants at T0, T1 and T2 was evaluated performing an ELISA test. Grey circles and grey squares represent individual values of STW and SDW participants, respectively. Results are expressed as mean ± standard deviation of data obtained by duplicate analyses.

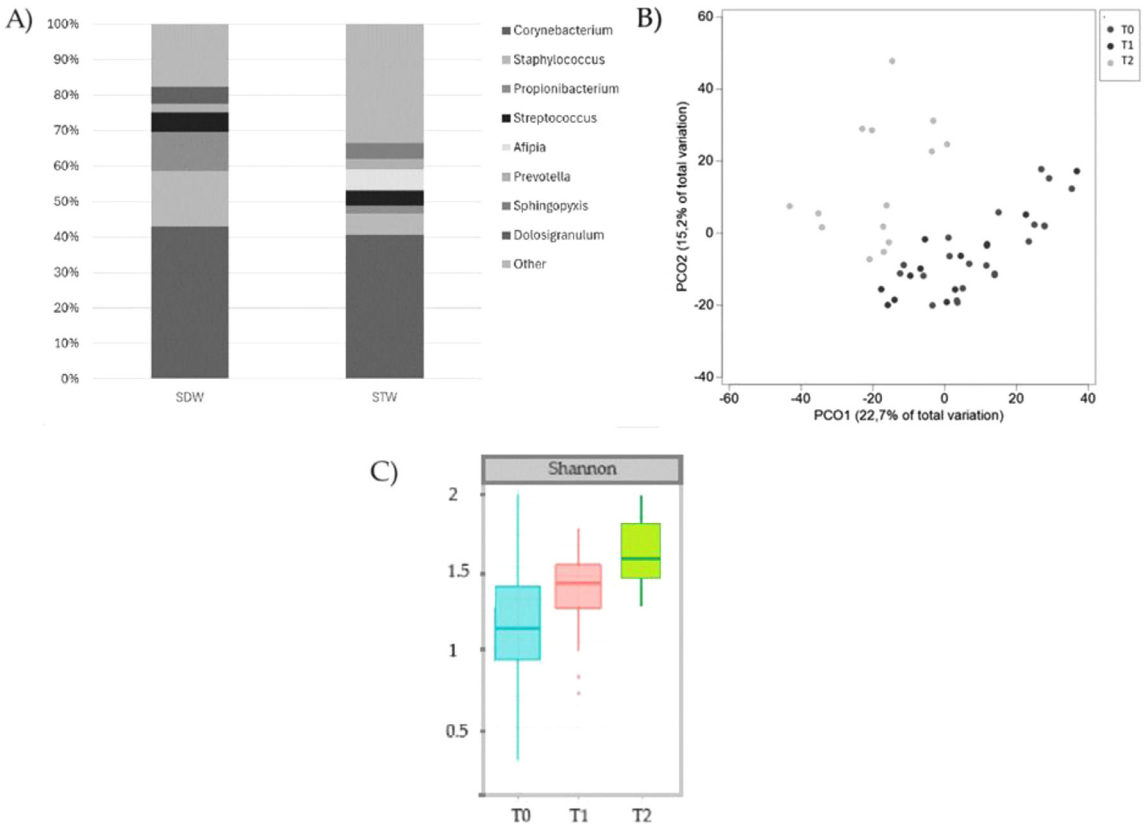


Fig. 5. The nasopharyngeal microbiota of STW participants is modified at T0, T1 and T2 during the treatment. (A) Bar-plots showing the composition of the nasopharyngeal microbiota at genera levels of SDW and STW participants at time T0. Genera with a relative abundance lower than 0.5% were grouped as 'Others' for plotting. (B) Principal coordinates analysis (PCoA) ordination plot based on unweighted UniFrac distances according to treatment. Each point corresponds to a sample. (C) Boxplots showing the biodiversity of the nasopharyngeal microbiota of STW participants at the three points (T0, T1 and T2). Statistical analysis of the differences between groups was calculated using the Kruskal–Wallis test with FDR correction for multiple comparisons.

Table 6
Some bacteria genera are correlated to the modification of the microbiota structure in STW.

Genus	STW_T2 (similarity respect T0=15.0%)		SDW_T2 (similarity respect T0=33.0%)		
	Average abundance (%)	Contribution of change respect T0 (%)	Average abundance (%)	Contribution of change respect T0 (%)	Dissimilarity STW_T2 vs SDW_T2 (85.7%)
Corynebacterium	40.7	24.2	20.7	2.0	10.0
Staphylococcus	25.1	18.5	5.9	9.0	12.0
Propionibacterium	7.7	7.7	2.2	1.0	4.0
Streptococcus	3.3	4.0	4.0	0.6	3.0
Prevotella	3.2	3.3	2.9	0.6	0.5
Sphingomonas	6.0	5.0	4.6	0.6	3.0
Dolosigranulum	4.5	0.7	0	3.7	0.5

Results of SIMPER analysis display the percentage of contribution in similarity and dissimilarity of each main genera, for both STW versus SDW at time T2 and for each condition at T2 vs T0.

terial taxa that contributed to the difference between times, we performed SIMPER analysis at the levels of genus. The results are shown in Table 6. At the genus level, microbial shifts were related to an increase in the relative abundance of several genera: *Corynebacterium, Propionibacterium, Staphylococcus* and *Streptococcus* at T2 compared to T0. Alpha diversity analysis showed that in STW participants at T2 harboured a higher Shannon diversity index (p=0.001) in their nasopharyngeal microbial populations than SDW. During the T2 time, the nasopharyngeal microbiota showed a decrease in the Bacteroidetes and Tenericutes phyla. These phyla were positively associated with higher alpha diversity indexes, as indicated by the Spearman correlation (p=0.05). On the other hand, a higher abundance of the Actinobacteria phylum was correlated with a higher Shannon index (rho=+0.58, p=0.001, q=0.001) and a higher number of OTU indexes (rho=+0.34, p=0.003, q=0.004). This was mainly due to the negative relationship between the *Corynebacterium* genus and both diversity indexes.

Discussion

The data reported in this pilot study represent the first spa-centred interventional trial focusing on long-COVID with pulmonary sequelae. In this study, a 12-day SWT inhalation therapy significantly improved exercise capacity and inflammatory biomarkers of patients with long-COVID. Notably, significant ameliorations were also observed in quality-of-life tests. Furthermore, nasopharyngeal microbiota findings showed qualitative and quantitative changes in participants undergoing SWT inhalation therapy only.

In Italy, the national health care system recognises spa therapies, as crenotherapy and balneotherapy, prescribed for rehabilitation of individuals affected by chronic and working pathologies, affecting almost the same systems involved by post-COVID condition, as consolidated and reimbursed interventions. In this context, several authors,[20,60,65,66] supported spa-centred rehab programmes since the very early phase of the pandemic for rehabilitation of people with long-COVID. Moreover, at least two studies reported that steam inhalations of tape water[67] and warm inhalations of sodium bicarbonate water[68] could both prevent and mitigate SARS-CoV-2 infection by creating a hostile environment to virus engraftment/replication through nasal mucosa and upper airways modifications in pH, humidity and temperature. The frailty of SARS-CoV-2 to steam inhalations was further confirmed by Chowdhury *et al.*[69] In our experience, in 3 month-time study, despite at least two subsequent flares of new viral strains, just one patient, undergoing placebo inhalations, presented a reinfection. Previous studies[19,60] focused more on the hydrotherapic and balneotherapic approach using other thermal water than sulphur one. According to recent studies, focusing on connections among serum levels of H_2S and SARS-CoV-2 infection,[70,71] we do really support that hypothermal sulphur waters, such as the Acque Albule tested in our trial, due to their H_2S content and availability, are the most suitable thermal waters to treat pulmonary sequelae.

In our subset population, most participants had mild to moderate COVID-19, but had substantial limitations in the form of persistent symptoms including reduced exercise capacity, dyspnoea, fatigue and functional impairment. In the baseline evaluation, SBQ-LC with its constitutive 17 independent symptom scales, thought to quantify the number of long-COVID-related symptoms and identify the most affected organ systems, resulted higher in STW population than in the SDW group, thus showing higher number of symptoms and involved systems among the active group subjects. Collectively, the STW group was composed of older subjects with higher scores of SBQ-LC. Moreover, higher BMI and lower platelet count were observed in the STW group. Several studies reported age and increased BMI, as well as low platelet count among the several significative risk factors predisposing long-COVID syndrome development.[72-74] Anyway, the affected individuals are not just older, but most of them represented the workforce of society, as demonstrated in our study; for this reason their recovery could not be ignored nor delayed.

The tests we performed, the SGRQ, the 6MWT, the Borg score, the PHQ-9 and GAD-7, were successfully used and validated by previous studies enrolling larger long-COVID population samples.[75,76] We excluded bias due to mood disorders, depression or anxiety. The 6MWT together with the Borg score were probably more sensitive than PFTs to detect improvements to physical exertion. In our study, 28 participants out of 30, when performing the 6MWT, walked at baseline lower distance in metres than expected according to the reference standards for Caucasian healthy population[77] calculated according to gender-based equation. The distances at T1 and T2 were much closer to expected values in STW participants only.

Some authors[15,78] suggested a hierarchy of feasibility among imaging and PFTs, in relation to the severity of post-COVID pulmonary involvement during the acute phase or later on. Experts retain PFTs, especially DLCO, as more sensitive than imaging to detect alterations to evaluate the long-term follow-up and the efficacy of respiratory rehab interventions.[79] In our sample, we assessed alterations in DLCO in 40% of STW participants and in 13.3% of SDW participants at T0. Although persisting for more than 12 months, these alterations showed a decrease just in STW participants after treatment, whereas the number of SDW participants presenting DLCO alterations showed no reduction. According to Mendez *et al*, DLCO reduction in COVID-19 survivors, three main factors were significatively associated with it: male sex, former or current smoking, log peak D-dimer.[80] In our study, probably due to the small sample size, no significant associated factors were detected; also, no significant differences in PFTs among smokers and non-smokers of STW and SDW were assessed.

In a systematic review, aimed at assessing the most common findings at PFTs in participants presenting pulmonary manifestations following SARS-CoV-2 infection, a prevalence of mild restrictive patterns (15%) followed by obstructive patterns (7%) were reported.[81,82] In our sample, the main pattern detected was the restrictive one with mean DLCO value at T0 of 77.8% of predicted for Caucasians in STW participants

increasing of +10.12% at T1 and +15.45% at T2, whereas in SDW participants the mean DLCO value at T0 was 103% of predicted increasing of just +0.87% at T1 and of +11% at T2. Comparable behaviour was recorded for FEV1 and TLC, both showing lower values at baseline but higher increase in STW than in SDW participants. To determine the longitudinal outcome of patients with post-COVID respiratory syndrome, it is worth focusing on those having PFT <80% of predicted.[83,84] In our case, analysing both STW and SDW participants lower than 80% of predicted at T0, T1 and T2, the longitudinal outcomes can be summarised as follows: STW participants having DLCO, FEV1, TLC presenting lower than 80% of predicted at T0 decrease at T1 and T2, whereas SDW participants did not change. Moreover, in accordance with data reported by Bongiovanni *et al* describing the association between symptoms and DLCO, even in our sample persisting DLCO anomalies were coupled to more than three self-reported symptoms,[79] therefore those having a lower % of DLCO had higher score of SBQ-LC. DLCO ameliorations are well-known to be coupled to significant improvement in 6MWT, related symptoms and quality of life.[85] If conducting the same analysis of the 6MWT on our population and therefore, according to ERS guidelines considering 30.5 metres as the minimal clinically important difference (MCID), we obtain that in STW participants the mean increased distance was 46 metres at T0/T1 and 18 metres at T1/T2, with 12 participants reaching the MCID at T1 and six participants at T2; whereas in SDW participants the mean increased distance was 12.14 metres at T0/T1 and 1.42 metres at T1/T2, with six participants reaching the MCID at T1 and three participants at T2.

STW inhalations were significantly effective in reducing the expression of proinflammatory cytokines IL-6 and IL-1β, highly involved in the COVID-19 cytokine storm and in long-COVID syndrome. The cytokine storm is the main factor responsible both for COVID-19 symptoms and complications and for long-COVID syndrome.[86] Since the beginning of the pandemic, several clinical and experimental studies have been addressed to find strategies to counteract the cytokine release responsible for the hyperimmune activation.[87,88] In the present study, the effectiveness of sulphur thermal water inhalation has been observed in the STW group compared to the SDW group. In the STW group, the serum IL-6 and IL-1β cytokine level was statistically decreased already at T1, demonstrating an immediate effect of the inhalations, and it was further decreased at T2, in the follow-up analysis with respect to the T0 levels. This long-lasting effect confirms that the STW inhalation could represent a good strategy to treat long-COVID syndrome. S100B protein is an alarmin involved in the activation of the immune response and inflammation through the activation of the NF-κB pathway responsible for the transcription of the proinflammatory cytokines.[89] In people with COVID-19 and long-COVID, S100B has been identified as a marker of severity and onset of pneumonia.[90,91] The STW group showed a decrease overtime of S100B serum amount compared to the SDW group, further confirming the anti-inflammatory effects of sulphur thermal water inhalation in people with long-COVID. STW inhalations resulted effective in reducing serum ACE level. ACE receptor is the entry point for SARS-CoV-2 and it has been demonstrated that SARS-CoV-2 infection leads to increased serum ACE level.[92] Elevated circulating ACE amount predicts mortality and disease severity in people with COVID-19.[93] Moreover, persisting elevated levels were found in 22.6% of people with long-COVID still 3 months after the acute infection.[94] In this study, a strong decrease has been observed in the STW group at T1 and T2 compared to T0, suggesting the effectiveness of sulphur thermal water inhalations on this long-COVID marker also.

In all the inflammatory disease, as well as in COVID-19, inflammation and oxidative stress are strongly correlated. In our study, we took in consideration the modulation of GSS, the enzyme responsible for the synthesis of glutathione (GSH), which is controlled through negative feedback by reaction products.[95] We found a decrease over time of serum GSS amount in STW participants, while no modulation has been found in the SDW group. This result could be explained considering the negative feedback control mechanism of GSH, suggesting a probable increase of this antioxidant molecule in the serum of STW participants.

Since the first months of the COVID-19 pandemic, great interest has been given to the study of upper airways microbiota intended as the set of ecological communities of microorganisms including bacteria, archaea, fungi, viruses and protists playing a pivotal role both in health and disease.[96] Previous studies assessed that the nasopharyngeal microbiota plays a determinant role in influencing a patient's susceptibility to viral infections[97] by favouring[35] or discouraging[36] the engraftment of several pathogens such as SARS-CoV-2. The ACE2 receptor is modulated by the respiratory microbiota.[98] The nasal microbiome can be influenced by several factors such as age, ethnicity, systemic and respiratory comorbidities,[38,99] cleansing practices, intranasal medications[100] or systemic pharmacological therapies, ventilatory support,[101] hospitalisation, smoking habits.[39] A healthy nasal mucosa hosting a healthy microbiome constitutes the nasal barrier, which protects from viral entry and contributes to the regulation of the immune response to infections.[37] In participants involved in our study, the aforementioned factors together with at least 1-month abstention from antibiotics, pre-/probiotics and corticosteroid medications have been taken into consideration during the microbiota analysis. The composition of the nasal microbiome may undergo changes making the environment suitable for pathogenic species.[102] In this view, we investigated if long-COVID resident nasal microbiota may have common features[40] or peculiar quantitative or qualitative changes following inhalations.[34] In our study, we observed notable disparities in the abundance of nasal microbiomes between STW and SDW at T2. While there were no significant alterations in the overall microbial diversity at T0, discernible differences emerged in specific microbial abundances, particularly within the bacterial genera *Corynebacterium, Staphylococcus* and the family *Enterobacteriaceae* at T2. In our study, the increase of *Corynebacterium* at T2 in STW participants only is in accordance with the literature, which previously demonstrated that non-diphtheriae *Corynebacterium* spp. has important microbial interactions within the human nasopharynx[103] and appear to influence innate immune responses to viral infection.[104] The increase in biodiversity along with the increase in *Corynebacterium* and *Staphylococcus* has been linked to an improvement in eubiosis, which may in turn lead to an improvement in fatigue. Indeed, some authors posit that long-COVID persistent fatigue may have one of its causes in dysbiosis, whereby pathogens are prevalent, and the release of bacterial toxins could result in mitochondrial malfunctioning.[105,106]

Conclusion

Taken together, spa setting, with its long tradition in treating respiratory issues, and the effect exerted by H_2S-rich thermal water inhalation, could provide the multidisciplinary expertise and the innovative programme to approach long-COVID syndrome. H_2S-rich STW showed positive effects on different inflammatory markers that, alongside the aforementioned effects, demonstrated its clinical efficacy and suggests its application in long-COVID treatment. Sulphur thermal water inhalation in people affected by respiratory sequelae following SARS-CoV-2 infection is a safe procedure, even during pregnancy, leading to *in vivo* anti-inflammatory effects likely accelerating and maximising recovery and thus mitigating long-term effects.

A preventive use of STW in terms of onset, transmission, progression and exacerbations of infective or chronic and degenerative diseases may be possible, although these options have not been completely investigated yet. The complete spectrum of potentially beneficial effects and clinical applications of sulphurous mineral waters is probably still far from being completely sussed out. To date, spa therapies represent low-cost treatments, which are executable in the long term with almost no side effects. STW inhalations, not least, are performed out of hospital setting and could represent a bridge treatment from hospitalisation to rehoming.

CRediT authorship contribution statement

Serena Crucianelli: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Alessia Mariano:** Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Federica Valeriani:** Software, Investigation. **Nicholas Cocomello:** Investigation, Formal analysis. **Gianluca Gianfranceschi:** Investigation. **Alessia Baseggio Conrado:** Software, Formal analysis, Data curation. **Ferdinando Moretti:** Methodology. **Anna Scotto d'Abusco:** Writing – review & editing, Supervision. **Gioacchino Mennuni:** Methodology. **Antonio Fraioli:** Supervision, Resources. **Maria Del Ben:** Supervision. **Vincenzo Romano Spica:** Supervision, Methodology. **Mario Fontana:** Writing – review & editing, Validation, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Consent for publication

Written informed consent was obtained.

Ethics approval and consent to participate

The study was approved by the University Committee for Research (CAR), University of Rome Foro Italico (CAR 181/2024). Patients were not compensated.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author (MF). The data are not publicly available due to the privacy of research participants.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.clinme.2024.100251.

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