Stress and Health

WILEY

RESEARCH ARTICLE OPEN ACCESS

Neurobiological and Anti-Inflammatory Effects of a Deep Diaphragmatic Breathing Technique Based on Neofunctional Psychotherapy: A Pilot RCT

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Received: 10 June 2024 | **Revised:** 26 September 2024 | **Accepted:** 24 October 2024

Funding: This research received a specific grant from the SIF—Società Italiana di Psicoterapia Funzionale and SEF—Scuola Europea di Psicoterapia Funzionale.

Keywords: allostatic load | cytokines | diaphragmatic breathing | mind-body therapies | stress | vagal tone

ABSTRACT

We examined the feasibility of using the neofunctional deep breathing (NDB) technique to reduce the allostatic load following the Trier Social Stress Test (TSST). Forty-four healthy subjects were randomised into experimental and control groups. Following the TSST procedure, participants underwent either a single session of NDB or an attention control intervention. The Procomp Infinity Biofeedback system measured breath per minute (BPM), respiratory amplitude, HRV linear domains, skin conductance, and trapezius muscle electromyographic activity. Cortisol and cytokine salivary concentrations, perceived stress, and anxiety levels were also assessed. These parameters were combined into an allostatic load index (ALI) to measure the intervention's effect. This pilot RCT demonstrated the feasibility of the study design and practicality of the intervention. The NDB group showed reduced ALI, increased respiratory abdominal amplitude, decreased BPM, increased HRV indicating parasympathetic activation, and decreased cortisol and inflammatory cytokines. This study highlighted the feasibility of testing the NDB technique in reducing allostatic load through a neurobiological and anti-inflammatory response after exposure to

Abbreviations: ALI, allostatic load index; ANS, autonomic nervous system; BMI, body mass index; BPM, breath per minute; BVP, blood volume pulse; DB, diaphragmatic breathing; EMG, electromyography; HF, high frequency; HPA, hypothalamic-pituitary-adrenal; HR, heart rate; HRV, heart rate variability; NDB, neofunctional deep breathing; NT, neofunctional therapy; RMSSD, root mean square of successive differences; RS, respiratory sensor; SAM, sympathetic-adrenal-medullary; SC, skin conductance; STAI, state-trait anxiety inventory; SUDS, subjective units of distress scale; TSST, Trier Social Stress Test.

Clinical or Methodological Significance of this Article: This is the first pilot RCT to explore the neurobiological and anti-inflammatory effects of a single session of the neofunctional deep breathing (NDB) technique following a structured stress protocol in healthy participants. The study's strength lies in using the allostatic load index (ALI) and a rigorous methodological framework. The NDB technique, combining breathing and physical touch, effectively increases the parasympathetic activity, reduces salivary cortisol and inflammation markers, indicating its potential as an adjunctive anti-inflammatory treatment.

psychosocial stress. This protocol can represent a non-invasive therapeutic adjutant in disorders related to a dysregulation of the HPA axis or to an inflammatory state.
Trial Registration: NCT04102813

1 | Introduction

The term 'stress' refers to the physical and psychological response to events, called stressors, that challenge a person's normal functioning (Selye 1998). Reduced perceived stress is associated with a decreased risk for several illnesses (Harriss et & al. 2009; He, Nowson, and MacGregor 2006; Sattelmair et al. 2011; Velten et al. 2014), greater wellness (Maniaci et al. 2018, 2020; Mintzer et al. 2019), and higher academic achievement (La Cascia et al. 2019; Maniaci et al. 2021).

It has been widely shown that stress has a deep impact on the functionality of both the immune and endocrine systems (Vitlic, Lord, and Phillips 2014). The effects of stress on the homoeostatic balance of these two systems are strictly related to the duration and intensity of the stimulus (Fali, Vallet, and Sauce 2018). An acute stressor triggers a brief upsurge in innate immune response cells, increasing pro-inflammatory cytokines such as IL-1β, IL-6 and, occasionally, TNF-α; and decreasing anti-inflammatory cytokines such as IL-10 and IL-4 (Goebel et al. 2000; Maes et al. 1998; Morey et al. 2015; Steptoe, Hamer, and Chida 2007). This immune response is mirrored by changes in both serum and salivary markers of inflammation (for a review see Slavish et al. 2015). Additionally, the activation of the sympathetic-adrenal-medullary and hypothalamic-pituitary-adrenal axes results in the release of catecholamines and glucocorticoids that modulate the immune response (Sapolsky, Romero, and Munck 2000; Kemeny and Schedlowski 2007), peaking approximately 15–20 min post-stress (Russell and Lightman 2019). Chronic stress leads to immune dysregulation, which may include immunosuppression and low grade chronic inflammation (Morey et al. 2015; Segerstrom and Miller 2004; Rohleder 2019). Dysregulation of the HPA axis, along with changes in pro- and anti-inflammatory cytokines profiles, contribute to the pathogenesis of anxiety, post-traumatic stress and obsessive-compulsive disorders (Furtado and Katzman 2015a).

Adaptation through compensatory and anticipatory mechanisms is usually required to achieve or restore physiological stability, which is referred to as allostasis. Notably, the burden of chronic exposure to stressful events and the associated neuroendocrine responses can be conceptualised as an allostatic load (McEwen and Stellar 1993) and summarised into an individual measure, namely the Allostatic Load Index (ALI). Chronic stress triggers an imbalance in biomarkers, increasing the allostatic load and facilitating larger decrements in cognitive and physical functioning and cardiovascular diseases increased risk (Seeman et al. 1997). Primary mediators, including stress hormones and cytokines, disrupt cellular function (Picard, Juster, and McEwen 2014). Over time, dysregulation extends to metabolic, cardiovascular, and immune biomarkers (secondary outcomes), culminating in clinical endpoints (tertiary outcomes) (McEwen and Seeman 1999). Higher ALI values indicate higher physiological strain, while lower values indicate better adaptation to stress. To date, research in this field has employed numerous physiological variables to construct ALI indices, diverging from the variables originally used in the initial model (Mauss et al. 2015).

Diaphragmatic breathing (DB) is an evidence-based, low-cost technique that involves deep breathing, ensuring the expansion of the lungs into the diaphragm rather than relying solely on the abdomen or ribcage. This method has been shown to stimulate the vagus nerve, leading to a marked increase in parasympathetic and cardiac vagal activities (You et al. 2022). Such stimulation results in the normalisation of blood pressure and heart rate (Janet and Gowri 2017; Joshi et al. 2016) and modulation of HRV (heart rate variability) values (Kromenacker et al. 2018). Alongside these neurovegetative adjustments, reductions in blood (Kiecolt-Glaser et al. 2010) and salivary cortisol (Perciavalle et al. 2017) levels have been observed following DB. Interestingly, cortisol changes also correlate with alterations in cytokines patterns (Twal, Wahlquist, and Balasubramanian 2016).

DB is often used in Mind-Body Therapies, that are designed to bridge the gap between physical and mental well-being (Hopper et al. 2018), improving cognition and emotion regulation (Ma et al. 2017). Mind-body therapies encompass a range of treatments, including yoga, qigong, tai chi, and mindfulness (Bower and Irwin 2016). These approaches typically merge DB with meditation and moderate physical activity, aiming to foster relaxation and stress mitigation (Morgan et al. 2014). A unique method within Mind-body therapies, the neofunctional deep breathing (NDB) technique, emphasises enhanced DB in conjunction with a reduced respiratory rate, falling within the 5–6 BPM range. Distinguishing itself from other mind-body therapies, NDB amplifies the effect of DB by directly manipulating the thoracoabdominal compartment. This intervention seeks to harmonise the movement between the ribcage and abdomen during regular breathing (Di Nuovo, Rispoli, and Genta 2011; Di Nuovo and Rispoli 2011). The overarching objective of the NDB protocol is to diminish conscious control, facilitating the resurgence of spontaneous physiological functioning (Di Nuovo, Rispoli, and Genta 2011; Di Nuovo and Rispoli 2011; Rispoli and De Vita 2016). This technique (Rispoli 2004) is characterised by several steps and is applied while the patient is in a supine position (for the full description of the technique, see Supporting Information S1: Supplementary 1).

Although these techniques are often used in common practice, more methodologically robust studies are needed to examine their efficacy.

1.1 | Objective

To the best of our knowledge, this is the first single session RCT investigating the effect of an NDB technique on both physiological and psychological stress variables, including cytokine patterns. We hypothesised that compared to the control group, the experimental group would will display a significant decrease in ALI, highlighted by an increase of vagal tone, in association with a reduction of cortisol levels, pro-inflammatory cytokines, state anxiety and perceived stress.

2 | Method

2.1 | Participants

All the procedures were conducted in the outpatient clinic of the Psychiatry Section of the Department of Biomedicine, Neuroscience and Advanced Diagnostics at the University of Palermo. Subjects were recruited through university facilities and social media advertisements during a 5-month period from October 2019 to January 2020. This pilot study did not include a formal sample size calculation prior to data collection. Instead, a post-hoc sensitivity analysis using G*Power was conducted, which indicated that the study had 80% power to detect a moderate effect size (Cohen's $f = 0.36$). Healthy volunteers were screened through a structured telephone interview in order to assess their eligibility in terms of the following exclusion criteria: age < 18 or > 55 years old, contemporary participation to other clinical trials, regular physical activity practise (at least 1 h/day), BMI > 40, past or current drug abuse problem or drug addiction (including nicotine), chronic inflammatory diseases, serious infections (e.g., HIV, hepatitis B and C), cancer in the previous 6 months, therapies with immunoactive (e.g., antibiotics, antihistamines, corticosteroids), beta blockers, or psychotropic drugs during the 2 weeks prior to the trial procedure, and regular use of anti-inflammatory drugs for more than 15 days/month.

After this first screening, a consultation with a clinical psychologist or a psychiatrist was scheduled to administer the Structured Clinical Interview for DSM-5 (SCID-5) Personality Disorders (PD) and Clinician Version (CV) (First, Williams, Benjamin, et al. 2016; First, Williams, Karg, et al. 2016) to exclude any psychiatric disorders. All participants provided their written informed consent, and all measures were administered with respect for their privacy. Due to the outbreak of the COVID-19 pandemic, the study was interrupted. This phase marked the first data collection wave. Following the release of such measures, we restarted the recruitment in March 2021.

New eligibility criteria were established and added to the prior ones: not having contracted SARS-CoV-2 infection during the previous months and having completed the vaccination cycle against SARS-CoV-2. Therefore, a new group of participants was recruited in the period from March 2021 to May 2021. Seven participants (17.5%) were recruited pre-pandemic, and the remaining 33 participants (82.5%) were recruited during-pandemic starting in March 2021. Furthermore, all subjects were submitted to a nasopharyngeal swab in our laboratory before undergoing the intervention. Researchers involved in the procedure were vaccinated and used the appropriate PPE (FFP2-mask, visor and gloves).

2.2 | Randomisation Procedure

Participants were allocated to experimental group or control group through a randomisation procedure performed on IBM SPSS 24. A stratified random sampling for age and sex was performed to ensure a better representation of the volunteer population and equal sex distribution in both groups. A block design procedure of 11 blocks of size 4 was adopted to obtain equally sized groups.

2.3 | Procedure Overview

This study was approved by the Ethical Review Board of the AOUP 'P. Giaccone', Palermo, Italy (Verb. N° 06/2019), and registered in Clinical Trials.Gov (NCT04102813) and was carried out in accordance with the Declaration of Helsinki principles. Two participants per day were tested at 08.30–10.30 and 11.00–13.00 sessions respectively. The experimental procedure is overviewed in Figure 1 below.

1. Upon arrival, all participants were provided with an informative leaflet about the NDB and NT approach and signed the informed consent. Trained researchers proceeded to assess anthropometric parameters, including height and weight. At T0, subjects were invited to take off shoes, belts, necklaces and bracelets. To avoid excessive saliva dilution, 1–2 sips of water were consumed at specific time points (10 min before each saliva collection). A wearable respiratory sensor (RS) was fitted around the abdominal area at the level of the lowest rib. Patients were

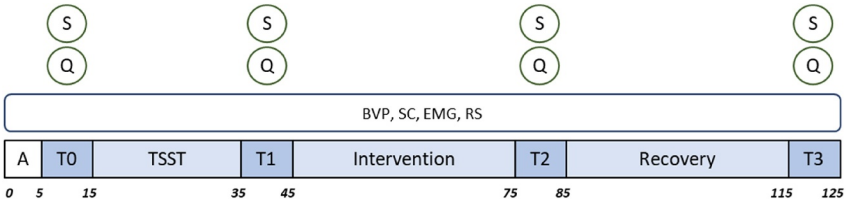

FIGURE 1 | Experimental procedure, with timeline reported in minutes. Following acclimation (A) and baseline physiological recording, participants were exposed to TSST, intervention (NDB or AC), and recovery procedures. Testing occurred at baseline (T0), at the end of the TSST (T1), at the end of the experimental procedure (T2), and at the end of recovery (T3). BVP, blood volume pulse; EMG, electromyography; Q, STAI and SUDS questionnaires administration; RS, respiratory sensor; S, saliva samples for cortisol and cytokines measurements; SC, skin conductance.

instructed to sit comfortably, ensuring their back was resting against the chair. Then, blood volume pulse (BVP), skin conductance (SC), and electromyography (EMG) biofeedback sensors were placed, and participants were instructed to still rest their hands, with palms facing upwards, on their legs. After 5-min calibration and acclimation interval, seated basal physiological data were recorded, and State-Trait Anxiety Inventory (STAI) and Subjective Units of Distress Scale (SUDS) questionnaires were filled out with researcher help in order to avoid hand movements; the STAI trait form was administered only at T0. Subsequently, participants were instructed to chew on a swab for 2.5 min to collect the first salivary sample for measuring cytokines and cortisol.

2. Birkett's (2011) Trier Social Stress Test (TSST) was administered as a standardised procedure for acute psychosocial stress induction. In this version, participants were not provided with paper and pencil for speech preparation and delivered their speech to a panel of two evaluators. Participants were given 10 min to mentally prepare a 5-min speech on why they were the ideal candidate for their desired job. The speech was delivered to two interviewers and videotaped. If a pause exceeded 20 s, participants were asked to continue. Following the speech, participants performed a mental arithmetic task by subtracting 13 from 1022, restarting from 1022 upon error. Continuous physiological measurements were taken throughout the task. At the end of TSST, the second psychological assessment was carried out and the second salivary specimen was collected (T1).

3. Participants were helped to assume a supine position and submitted to a single session of 30 min of NDB (experimental group) or to an attention control intervention (control group), which consisted of listening to a 30 min audiobook. Consistent with past works (Bennett et al. 2018; Borchardt and Zoccola 2018; Ditto, Eclache, and Goldman 2006), an audiobook with a neutral content was chosen in order not to determine a psychophysical activation of the participants. Therefore, the audiobook selected, '*La dittatura delle abitudini*', was about the importance of following healthy and productive habits. The listening session was followed by a brief questionnaire to control attention levels. Five therapists with 4-year postgraduate training on functional approach delivered the NDB session in the case of experimental group, or listened to the audiobook together with the participant in the case of control group, in order to control Hawthorne and body position effects (McCambridge, Witton, and Elbourne 2014; Vrachatis et al. 2014). At the end of the session, the third psychological assessment was administered, and the third salivary sample was collected (T2).

4. Lastly, the participants underwent a recovery period, designed to assess the biofeedback and cytokines' slow responses to stress and relaxation procedures. The subjects of both groups were provided with a 30-min session of audiobook listening, followed by a brief questionnaire to control attention level through the session. At the end of the session, the fourth psychological assessment was carried out, and the last salivary sample was collected (T3).

2.4 | Measures

2.4.1 | Autonomic Nervous System (ANS) Response

All the ANS parameters were recorded and converted from analogue to digital data (A/D) using the Procomp Infiniti multimodality encoder and related sensors (Thought Technology, Montreal, Quebec).

2.4.1.1 | Heart Rate Variability (HRV). Blood volume pulse (BVP) was detected through a photoplethysmography sensor (BVP-Flex/PRO, SA9308M) and was sampled at 2048 Hz. According to a review by Schäfer and Vagedes (2013), photoplethysmography technology was shown to be a reliable technology for collecting inter-beat-intervals (IBIs) data for HRV analysis for healthy and resting subjects. The sensor was placed on the dominant hand's thumb pad to prevent artefacts caused by light interference (Combatalade 2010). A digital trigger detecting the point of maximum deviation was applied to the BVP raw signal to obtain heart rate (HR) and IBIs values. All the BVP data were visually examined, and artefacts were corrected. Kubios HRV Analysis Software 3.3 (Tarvainen et al. 2014) was used to compute short-term (~5 min) time and frequency domain HRV indices. As a time domain measure, the root mean square of successive differences (RMSSD, in ms) was estimated, as an index of vagal activity in the short-term measurements (Shaffer and Ginsberg 2017). As a frequency domain measure, fourier analysis was used to calculate the high-frequency power (HF: 0.15–0.40 Hz, in ms^2 and nu) as a proxy for parasympathetic tone (Gerritsen and Band 2018). However, according to previous research (Shaffer and Ginsberg 2017), frequency domains of HRV should be analysed in relation to respiratory rate measurements.

2.4.1.2 | Skin Conductance Response (SCR). Skin's conductance (in μS) was measured through Ag/AgCl electrodes (SC-Flex/Pro SA9309M), fixed with two finger bands and positioned at the second phalange of the index and little finger of the non-dominant hand, and sampled at 256 Hz. SC is a well-known measure of sympathetic activity in response to stress stimuli.

2.4.1.3 | Electromyogram (EMG). Muscle tension was measured by detecting surface voltages using the dry Triode Ag/AgCl electrode and Myoscan-Pro sensor (SA9401M-50). The sensor has an active range of 20–500 Hz and can record signals up to 1600 μS. Data were sampled at 256 Hz and preprocessed through a deep smoothing algorithm. The sensor was placed on the trapezius muscle with positive and negative electrodes parallel to the muscle fibres.

2.4.1.4 | Respiratory Amplitude and Breath Per Minute (BPM). Respiratory parameters were measured through the respiration sensor & cable (SA9311M), a woven elastic band approximately 52 inches (132 cm) in length with a stretch adjustable belt. Throughout the session, the band was positioned around the abdominal area, at the lowest ribs height. A stretch sensitive device is strapped to the abdomen in order to register the respiratory amplitude and the BPM rate. During inspiration, the ribcage expands and stretches the device. When exhaling, the stretch relaxes and the sensor returns to its neutral position. The resulting waveform is displayed on the screen.

2.4.2 | Salivary Samples Collection

Samples of saliva were collected by Salivette device (Sarstedt, Numbrecht, Germany). All subjects were instructed to abstain from drinking, eating, and brush their teeth 60 min prior to the collection. The cotton roll was directly introduced in the mouth's participant without handling and chewed for 2.5 min by doing circular movements. The cotton roll was not manually handled during insertion to maintain sample purity. Samples were collected and centrifuged at 1500*g* for 10 min, after which the retrieved saliva was transferred to 1.5 mL tubes and stored at −80°C until further analysis. All laboratory tests were conducted at the institute of Clinical Biochemistry, Clinical Molecular Medicine and Clinical Laboratory Medicine of the University of Palermo.

2.4.2.1 | Endocrine Response. Salivary free cortisol levels were determined on the automated immunoassay analyser eCobas by using the Elecsys Cortisol II assay (Roche Diagnostics, Mannheim, Germany), a competitive polyclonal antibody immunoassay that employs a magnetic separation step followed by electrochemiluminescence quantitation. Samples were measured using 20 μL of saliva. The measurement range was 0.054–63.4 μg/dL (1.5–1750 nmol/L). Salivary cortisol is highly related to serum free cortisol (Kirschbaum and Hellhammer 1994; Teruhisa et al. 1981).

2.4.2.2 | Inflammatory Response. Human TNF-α, human IL-6 IL-1β high sensitivity (hs) as well as human IL-10 hs were measured from saliva samples by Diaclone ELISA kits (Medix Biochemica, Besançon, France). These assays employ the quantitative sandwich enzyme immunoassay technique. Absorbance was measured on a spectrophotometer using 450 nm. The sensitivity or minimum detectable dose of hsTNF-α was < 8 pg/mL, for IL-1β was 6.5 pg/mL, for hsIL-6 was 0.81 pg/mL, and for IL-10 was 0.98 pg/mL.

2.4.3 | Psychological Response

State-Trait Anxiety Inventory (STAI-Y) was used as a self-report questionnaire to investigate anxiety levels (Spielberger et al. 1983). Subjective Units of Distress Scale (SUDS) was employed to rapidly assess stress levels during the different phases of the clinical trial (Tanner 2012).

2.4.4 | Allostatic Load Index

ANS (RMSSD, HF, SC, EMG, respiratory amplitude, and BPM), endocrine (salivary cortisol, IL-6, IL-1B, TNF-a and IL-10), and psychological variables were aggregated into a continuous ALI. The primary advantage of this approach is that it utilises the full information available for each variable, allowing a detailed measurement of variations in allostatic load (Hickson et al. 2012; Widom, Horan, and Brzustowicz 2015). To build the ALI, *z*-scores were calculated for each variable at every time point; the *z*-scores for the variables respiratory amplitude, HF, RMSSD and IL-10 were inverted, as higher values in these parameters indicate a reduced allostatic load. Finally, the *z*-scores were summed for each participant and at each time point to derive the ALI (Langelaan et al. 2007).

2.5 | Statistical Analysis

Group-based differences in demographic features and pre-treatment measures were analysed using independent samples *t* tests and χ^2 tests. A repeated measures ANOVA with 'time' as within-subjects factor, 'group' as between-subjects, and 'timeslot' as covariate factor for all collected measures was performed to evaluate pre-post treatment effects. Greenhouse–Geisser corrected *p*-values were used when appropriate. All analyses assumed an alpha risk of 5%. All statistical analyses were performed using IBM SPSS 24 software for Windows.

3 | Results

3.1 | Study Sample

A sample of 44 subjects, 29 females (67.5%) and 15 males (32.5%), voluntarily participated in the study. Age ranged between 18 and 48 years ($M = 26$; SD = 7.64). The subjects were randomised into an experimental group ($N = 22$) and a control group ($N = 22$). Four subjects were excluded from the analysis due to major artefacts in heart rate data, thus 40 subjects (experimental group, $N = 20$; control group, $N = 20$) were included in the analysis. The CONSORT diagram is available as Supporting Information S2: Supplementary File 2. The mean BMI was 22.92 (SD = 3.15) for experimental group and 23.07 (SD = 3.79) for control group. We did not find sociodemographic differences between groups at baseline.

3.2 | Intervention Effect on ALI

A two (Group: Experimental, Control) × four (Time: baseline, post TSST, post intervention, post follow-up) repeated measures ANOVA was conducted to assess variation in ALI based on *z*-scores. We did not find main effects of time, irrespective of the assumption of sphericity or corrections applied ($p > 0.34$). However, an interaction effect between time and group was observed $F(3, 2.651) = 8.036$, $p = 0.001$, $\eta^2 = 0.178$. The observed power for this interaction was high at 0.981, suggesting a moderate-to-large effect size (Table 1).

A two (Group: Experimental, Control) × two (Time: baseline-post TSST) repeated measures ANOVA was conducted to assess variation in ALI based on *z*-scores and explore differences between the two groups regarding the magnitude of change in the ALI across the specified time points. This analysis was

TABLE 1 | Repeated measures ANOVA for ALI.

Factor	*F*	df	MSE	*p*	ηp^2
ALI: Time	0.949	2	0.031	0.411	0.025
ALI: Time × timeslot	1.041	2	0.034	0.372	0.027
ALI: Time × group	8.036	2	0.266	****	0.178

*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$.

subsequently repeated for the paired time points of post-TSST-intervention and intervention-follow-up. Results showed the following Group × Time interaction for the post-TSST-intervention pair: $F(1, 1) = 13.915$, $p = 0.001$, $\eta^2 = 0.273$. Notably, significant results emerged only for the post-TSST-intervention pair. The observed power for this interaction was high at 0.953, and the effect size, as indicated by Cohen's d, was −1.23, suggesting a very large effect.

3.3 | Intervention Effects on Biomarkers

A two Group (Experimental, Control) × two Time (post TSST-post intervention) separated repeated measures ANOVA were employed to investigate each variable. The Group × Time interaction showed an increase of abdominal amplitude $F(1, 33) = 35.871$, $p < 0.001$, and a decrease in BPM $F(1, 34) = 290.972$, $p < 0.001$. Additionally, we observed increase of HRV (RMSSD) $F(1, 37) = 8.588$, $p = 0.006$; a decrease of salivary cortisol $F(1, 36) = 12.056$, $p < 0.001$; and a decrease of IL-6 $F(1, 35) = 7.158$, $p = 0.011$, and TNF-α $F(1, 36) = 8.135$, $p = 0.007$. The control group showed a modification on frequency domains HRV, specifically increasing the HF $F(1, 37) = 9.132$, $p = 0.005$, and a decrease of SC $F(1, 37) = 5.299$, $p = 0.027$. Both groups showed a significant main effect of Time through a reduction of HR $F(1, 37) = 110.048$, $p < 0.001$, IL-1β $F(1, 31) = 4.527$, $p = 0.041$, STAI-Y1 $F(1, 36) = 63.822$, $p < 0.001$, and SUDS $F(1, 36) = 66.711$, $p < 0.001$, without significant differences between groups. No effects were found for IL-10 and EMG.

4 | Discussion

The aim of this RCT was to assess the feasibility of the NDB technique in mitigating the experienced allostatic load within a sample of healthy subjects after the administration of the TSST. As highlighted in a recent editorial by Juster and Misiak (2023), the significance of the allostatic load construct lies in its capacity to delineate the organism's responses to stressful events by amalgamating various ongoing physiological changes. When these initially adaptive short-term responses become chronic, the organism enters a state of allostatic overload, thereby elevating the risk of the emergence of various pathological conditions (Beckie 2012). In our study, we confirmed that the allostatic load is a valid outcome measure for this study. This load includes a comprehensive set of physiological, behavioural, and psychological indicators, as validated in the literature by Mauss et al. (2015), specifically including respiratory parameters, vagal tone, cortisol levels, pro-inflammatory and anti-inflammatory cytokines, perceived stress, and state anxiety. Notably, we observed variation in the allostatic load in response to the intervention, with the experimental group exhibiting a reduction in ALI, accompanied by an increase in parasympathetic activity. This was anticipated to coincide with decreased cortisol levels, reduced pro-inflammatory cytokines, and a more pronounced reduction in perceived stress and state anxiety compared to the control group.

This first pilot RCT introduces novel elements. First, while the efficacy of mind-body therapies in modulating physiological parameters has been established (Tung and Hsieh 2019), as far as we know, no study has yet analysed the effects of the administration of a single session deep DB technique based, which incorporates physical touch and manual intervention on the participant, such as the NDB protocol. Furthermore, the use of ALI as an indicator of the clinical intervention's significance is very relevant to inform future RCTs, given the scarcity of RCTs grounded in ALI literature.

4.1 | ALI Response

The results of the study suggested a clinically significant decrease in ALI within the experimental group following the application of the NDB technique. The effect size was indicative of a robust effect in decreasing allostatic load induced by an acute social stressor. All the improvements were not maintained in the recovery period (T3), when the two groups were involved in an attention control task, such as listening to an audiobook. This finding is consistent with the literature investigating variations in biomarkers comprising ALI influenced by mind-body therapies (D'Alessio et al. 2020). Furthermore, this study represents a preliminary contribution regarding the potential of Mind-body therapies in general, and the NDB intervention in particular, to moderate individuals' allostatic load after the administration of an acute stress such as the TSST. The analysis of variations in individual biomarkers comprising the ALI may provide insight into how the NDB technique exerts its clinical efficacy.

4.2 | ANS Response

At the end of the intervention (T2), subjects randomised in the experimental group showed higher levels of RMSSD over time and in comparison to the control group. As reported in the literature, this parameter is a time domain of HRV and may serve as a reliable index of parasympathetic activity (Nunan, Sandercock, and Brodie 2010). Indeed, HRV is defined as a non-invasive index of the autonomic nervous system's activity (Buccelletti et al. 2012) and is used as an indicator of physical health. Higher levels of HRV are associated with lower stress levels and better health outcomes (Gerritsen and Band 2018; Kaushik et al. 2006). The NDB technique has been demonstrated to effectively elicit a parasympathetic response at the cardiac level, consistent with literature pertaining to other Mind-body therapies (Amihai and Kozhevnikov 2015; Shearer et al. 2016; Tavares et al. 2017; You et al. 2022; Wells et al. 2012). Consistent with other studies, therapies based on physical touch can lead to a strong increase in vagal activity via stimulation of dermal and subdermal pressure receptors, which are innervated by vagal afferent fibres, and consequently to a decrease of HR (Field 2010). Therefore, the development of an intervention combining deep DB and physical touch, such as the NDB protocol, could have some clinical relevance.

4.3 | Respiratory Parameters

The results regarding heart rate variability can be explained by observing variations in the subjects' respiratory patterns. A

reduction of BPM was registered in experimental group during the application of the NDB procedure, in association with a significant increase in abdominal excursions in comparison to the control group. The average frequency of roughly 6 BPM achieved by the participants aligns with studies concerning resonance frequency between respiratory rate and baroreflex loop (Lehrer and Gevirtz 2014; Vaschillo, Vaschillo, and Lehrer 2006). Consistent with our results, diaphragmatic breathing to resonance frequency increases baroreflex and pulmonary stretch stimulation, inducing an augmented cardiac vagal activity (Shaffer and Meehan 2020; Tatschl and Schwerdtfeger 2022). The application of the NDB protocol aims to modulate the respiratory rate and facilitate the diaphragmatic excursions with the assistance of the therapist's manual support. As expected, during the recovery period (T3), we registered a reduction in the abdominal excursions' amplitude in experimental group, accompanied by an increase of BPM. However, the respiratory rate values recorded in experimental group subjects was lower than in control group, suggesting a potential long-term ability of NDB to regulate breathing rhythm.

4.4 | Cortisol

Following the application of the NDB technique, we found a reduction of salivary cortisol in experimental group participants compared to control group participants. During the recovery period (T3), we further observed a reduction in cortisol in both groups. High levels of cortisol are deeply involved in the pathogenesis of multiple diseases associated with a dysregulation of the HPA axis (Furtado and Katzman 2015b; Sapolsky 2000). Mind-body therapies, through an increase of the vagal tone, may contribute to modulating the neuroendocrine system, for example reducing cortisol release (Kiran et al. 2017), cortisol awakening response (Cahn et al. 2017), and cortisol reactivity to an acute stressor (Creswell 2017). Furthermore, there is some evidence suggesting that the administration of a long-term slow-paced breathing protocol may lead to a decrease in salivary cortisol concentration (Ma et al. 2017; Perciavalle et al. 2017). The use of physical touch in the NDB technique to induce specific changes in respiratory patterns may contribute to cortisol reduction by eliciting a vagotonic response (Field 2010). Overall, the components of the NDB technique make it a useful non-invasive method for effectively lowering cortisol levels.

4.5 | Cytokines

Following the administration of the NDB technique, we observed a decrease in the salivary concentration of IL-6 and TNF-α in experimental group compared to control group. Furthermore, we observed a greater, but not statistically significant, reduction of IL-1β levels in experimental group compared to control group. However, these improvements were not maintained in the recovery period (T3). Finally, no difference was found either between groups or over time in IL-10 levels. Our results are in line with previous evidence highlighting the beneficial effects of mind-body therapies on inflammatory patterns (Morgan et al. 2014). Concerning the non-linear trend of IL-10, Twal, Wahlquist, and Balasubramanian (2016) found similar results, suggesting the activation of a complex immunity response which modulates both pro- and anti-inflammatory signalling pathways (Cahn et al. 2017). Furthermore, the physical touch component of the NDB technique could have contributed to lowering levels of inflammation (Thomas and Kim 2021). Thus, the NDB protocol has been shown to produce an anti-inflammatory effect through potentially multiple mechanisms: by altering neural responses with a positive impact on the cross-talk between the brain and the immune system (Dutcher et al. 2021); and by reducing pro-inflammatory gene expression profiles, such as the nuclear factor kappa B (NF-κB) pathway (Buric et al. 2017), resulting in decreased pro-inflammatory signalling (Bower and Irwin 2016).

4.6 | Skin Conductance and Electromyogram Response

We observed a tendence to decreasing of SCR, during the intervention in both groups. In this regard, the literature is not univocal. Several studies have underlined no difference in SCR after the application of Mind-body therapies, in comparison to controls (Rauschel et al. 2015) or have highlighted increases in SC response during some typologies of meditation practices (Busch et al. 2012; Amihai and Kozhevnikov 2015), especially those requiring a discrete amount of active attention. However, a lower reduction of SC response in experimental group could be interpreted as a reaction to the physical touch, which has been found to be able to increase SC (Etzi, Carta, and Gallace 2018). Further research should be conducted to evaluate the effects of NDB on SC response, verifying how this variable could change after several applications of the NDB protocol.

No difference was recorded in the electromyographic activity of the trapezius muscle between the two groups and over time. Psychosocial stressors could produce muscle activation of small motor units, determining a constant low-level muscle tension while reducing the opportunity for muscles to take periodic rest moments (Melin and Lundberg 1997), with detrimental effects on the osteo-muscular system homoeostasis (Schleifer et al. 2008). While Mind-body therapies can be used to induce muscular relaxation (Kaushik et al. 2006), we could hypothesise that the small variation in the state of muscular tension may be due to the change of position (sit to supine) during the intervention phase.

4.7 | Psychological Response

Both experimental group and control group experienced a decrease in perceived anxiety and subjective stress levels at T2, without significant differences between groups. Consistent with previous studies, the NDB technique has been shown to be effective in reducing anxiety (Chang et al. 2004; Ma et al. 2017) and stress levels (Goldstein, Lewin, and Allen 2020), but we should consider that the subjective perception of a condition of

well-being could be not consistent with the associated neurobiological parameters.

5 | Conclusions

To the best of our knowledge, this is the first RCT aimed at investigating the feasibility of a study examining the neurobiological and neuroinflammatory effects of a single session of NDB technique after the application of a structured stress protocol. A strength of the study lies in the use of ALI and the robust methodological framework. The NDB procedure, combining DB and physical touch has determined a robust vagotonia, as highlighted by the analysis of HRV linear and non-linear domains. Furthermore, our results suggest that the application of NDB technique may have an effect in reducing salivary cortisol levels and inflammation markers, such as IL-6 and TNF-α, after a stress induction. This result highlights a potential clinical impact of the NDB protocol as a coadjuvant anti-inflammatory treatment. The limitation we encountered to take into account regarding the feasibility of this study concerned some difficulties in recruitment. These were in part due to the SARS-CoV-2 pandemic, and in part to the strictness of the inclusion criteria. Moreover, we acknowledge that the lack of physical touch in the control group may be a limitation to this study design. Physical touch is known to alter physiological responses, including stress responses, which may confound our findings. Given that the NDB intervention involves multiple components, including physical touch, synchronised breathing, and relation with the therapist, it is impossible to determine which of these components contributed the most to the observed effects. Therefore, it is important to acknowledge that the effects we observed may be a result of any one or a combination of these components. Future studies should aim to differentiate the specific effects of deep breathing from those of physical touch.We also provide some initial evidence for the potential efficacy of the NDB intervention in reducing stress responses, which is important to examine in larger and more comprehensive RCTs. These should include a sham control group or other methods to control for the potential confounding effect of physical touch and to systematically investigate the individual effects of each NDB component. This will provide a more comprehensive understanding of the potential mechanisms underlying the observed effects and will increase confidence in the efficacy of NDB as an intervention for reducing stress. Moreover, to mitigate circadian effects, we ensured that participants from both the experimental and control groups were evenly distributed across the morning (8:30–10:30) and midday (11:00–13:00) sessions. While we focused on relative cortisol changes rather than absolute values, we acknowledge that future studies could benefit from using a narrower testing window.

Future studies should evaluate the effects of the NDB protocol as a non-invasive technique in different clinical conditions, such as psychiatric, cardiorespiratory diseases, and disorders related to a dysregulation of the HPA axis or to a LGCI condition. In a wider perspective, it could be applied as a non-invasive procedure to enhance and maintain psychophysical well-being.

In conclusion, this RCT provided evidence for the feasibility of a study examining the efficacy of a single session of NDB technique in reducing allostatic load of participants measured through ALI.

Author Contributions

Giuseppe Maniaci: conceptualization, investigation, methodology, data curation, formal analysis, writing–original draft, writing–review & editing. **Marco Daino:** investigation, data curation, formal analysis, writing–original draft, writing–review & editing. **Maria Iapichino:** investigation, data curation, resources. **Alessandra Giammanco:** investigation, resources. **Calogero Taormina:** investigation, resources. **Giuseppina Bonura:** investigation, resources. **Zaira Sardella:** investigation, resources. **Giuseppe Carolla:** investigation, resources. **Patrizia Cammareri:** investigation, resources. **Emanuele Sberna:** investigation, resources. **Maria Francesca Clesi:** investigation, resources. **Laura Ferraro:** resources, writing–review & editing. **Caterina Maria Gambino:** investigation, resources. **Marcello Ciaccio:** investigation, resources. **Luciano Rispoli:** conceptualization, methodology, writing–review & editing. **Caterina La Cascia:** writing–original draft, writing–review & editing. **Daniele La Barbera:** supervision, writing–review & editing. **Diego Quattrone:** writing–review & editing, project administration, supervision.

Acknowledgements

This research was conducted at the University of Palermo. We thank the therapists of the 'Centro Studi di Psicoterapia Neo-Funzionale' in Palermo, specifically Elisa Taormina, for her outstanding work delivering the psychotherapy protocol, and Federica Catalfamo and Elena Maria Fusco, MSc in psychology and MD respectively, for the passionate contributions given to the study during their graduation thesis.

Open access publishing facilitated by Universita degli Studi di Palermo, as part of the Wiley - CRUI-CARE agreement.

Ethics Statement

Ethics approval to conduct the main study was approved by the Ethical Review Board of the AOUP 'P. Giaccone', Palermo, Italy (Verb. N° 06/2019), and was carried out in accordance with the Declaration of Helsinki principles.

Consent

All participants provided written informed consent.

Conflicts of Interest

Luciano Rispoli is currently Founder and Director of Neo-functionalism and of the European School in Functional Psychotherapy (SEF), Naples, Italy. All other Authors declare that they have no conflict of interest.

Data Availability Statement

Research data are available upon request from the first author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.