**SUPPLEMENTAL MATERIAL**

The goal of this study was to utilize functional lung imaging to assess regional lung volume displacement in healthy volunteers. The methodology for the functional lung imaging (X-ray Velocimetry) image acquisition is previously described in detail. The supplemental information extends the methodology to assist in describing factors of the image and data acquisition and/or analysis that provide insight into sources of methodologic variability and individual responses.

**METHODS AND STUDY DESIGN**

**Participants**

The study enrolled ten healthy adult participants recruited from the general community through a combination of research websites and printed fliers, designed to reach a broad audience interested in participating in health-related studies. Each potential participant was fully informed about the study’s objectives, procedures, and any potential risks involved before providing written consent to participate. Ethical approval for this study was granted by the University of Miami Institutional Review Board (IRB), ensuring adherence to established guidelines for conducting research involving human subjects, which included the assurance of participant confidentiality and safety throughout the study.

Upon enrollment, each participant completed a detailed demographics questionnaire to document age, sex, height, weight, and other relevant background information that could potentially influence pulmonary function outcomes. Following this initial data collection, participants underwent a battery of pulmonary function tests. These tests included spirometry, which measures airflow and lung volume to assess respiratory efficiency; body plethysmography, a more comprehensive test that evaluates lung volumes and airway resistance; and a measurement of the diffusing capacity of the lung for carbon monoxide (DLCO), an indicator of the lungs’ capacity to transfer gas from inhaled air to the bloodstream. These assessments were conducted to detect any potential obstructive or restrictive ventilatory impairments that could influence the study’s outcomes.

All participants reported being asymptomatic, with no history of specific respiratory issues or ongoing respiratory symptoms at the time of study enrollment. To further minimize any risk of confounding factors, each participant was screened to confirm the absence of an active COVID-19 infection prior to participation. This screening process included a negative COVID-19 test, as respiratory function could be temporarily or permanently altered by an active infection. However, no data was collected on previous COVID-19 infections, meaning that prior exposure to COVID-19 was not an exclusion criterion.

During the data collection phase, one participant was excluded from further analysis due to an observed decrease in tidal volume during bilevel ventilation. This reduction was attributed to the individual’s voluntary control of breathing, which suggested an inability to fully relax and adapt to pressure-assisted breathing. This exclusion was based on the need to maintain the integrity of the study’s findings, as voluntary control could introduce variability not representative of typical respiratory function under assisted ventilation.

To ensure that this exclusion did not unduly affect the study’s results, a sensitivity analysis was conducted. This analysis involved re-evaluating both the primary and secondary outcomes of the study with and without the data from the excluded participant. The results indicated that excluding this individual did not significantly alter the findings, thereby reinforcing the robustness and reliability of the study’s overall conclusions.

**Airway Airflow and Non-Invasive Positive Airway Pressure**

Participants were fitted with a leak-free ventilation full-face mask and an inline pneumotachograph for continuous airflow measurement.12 Participants were also assessed with a NIPPV device (Resmed S8 Autoset, San Diego, CA). The study protocol involved monitoring while breathing spontaneously and on NIPPV both in the supine position. For NIPPV, a spontaneous-time mode with an inspiratory pressure of 15 cm H2O, an expiratory pressure of 5 cm H2O, and a backup rate of eight breaths per minute was used. This configuration was chosen to simulate a typical therapeutic setting, optimizing airway patency and gas exchange while ensuring comfort and safety. We reasoned that in the current study it would provide a realistic representation of standard noninvasive positive pressure ventilation (NIPPV) conditions for use by individuals without respiratory disease, allowing for sufficient assessment in the cross-section of individuals studied. The settings used were aimed to simulate the supportive effects of NIPPV as commonly administered in clinical practice, particularly focusing on the balance between effective ventilation support and participant comfort. As the data collection procedure requires the capture of five fluoroscopic images during the complete breath cycle (from the start of inspiration to the end of expiration) we set the NIPPV back-up rate to limit breath duration to a maximum of 7.5 seconds per breath.

**Non-Invasive Functional Lung Imaging**

During spontaneous breathing and while on NIPPV, functional imaging was performed using X-ray Velocimetry (XV LVAS, 4DMedical, Los Angeles, CA). This involved acquisition of five separate fluoroscopic images each over a complete and continuous tidal breath (81 kV, a 6.4 ms pulse width, and a radiation dose of 0.080 µGy per frame). Images all sharing the same center of rotation were captured sequentially at five angles: 0° PA (Posterior-Anterior axis), ± 36° from PA, and ± 72° from PA acquired at 15 frames per second and used for subsequent analysis as previously described.13 Each scan was performed at each of the five views for approximately 8 seconds, allowing sufficient time to capture at least one complete respiratory cycle (inspiration and expiration) both with and without NIPPV.

Fluoroscopy Imaging Mode: Automatic Exposure Control (AEC) on the X-ray system’s detector was enabled to ensure that the images maintained a high signal-to-noise ratio regardless of individual participant factors such as size and tissue density. Throughout the imaging sequences, participants remained in a supine position. To precisely synchronize the imaging data acquisition with airflow data, a Geiger counter with an analog output was connected to the auxiliary input of the digital recording system, recording at a frequency of 50 Hz for accurate post-hoc alignment.

**Computed Tomographic (CT) images for registration**

Participants were instructed to perform a sequence of breathing maneuvers (deep inspiration and expiration) to ensure rapid acquisition of images. In the supine position, a dual-source CT system (Siemens Healthcare, Germany) was used to perform chest scans during inspiration and expiration. The scanning parameters used were: collimation of ≤1, pitch of 1, peak voltage of 120 kVP, and exposure-time product of between 40-200 mAs for the CT scans. Image reconstruction was performed from the thoracic inlet to the lung base using B30f kernel with a slice thickness of 1mm.

In brief, Lung fissure analysis was performed semi-automatically using lung segmentation module. The module uses threshold-based, region-growing, and machine learning algorithms to differentiate lung tissue from surrounding anatomy. Trained biomedical engineers or graduates refine and adjust segmentations manually to define the lobar anatomic regions.

**Data Collection and Functional Lung Imaging Analysis**

Data from the pneumotachograph and corresponding roentgenographic indicator (Geiger counter) were collected and analyzed using the RemLogic 3.4.3 software (Embla, Buffalo, NY; [Figure](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3639000/figure/F2/) 1).(13) Custom algorithms were used for semi-automated peak detection and cyclic waveform algorithms used to extract breath-by-breath values for flow from respiratory signals acquired at 50 Hz.(14) In brief, analysis is targeted to select periods of the raw airflow trace coinciding with the fluoroscopic image acquisition (15 fps) for the entire breath (15 frames per breath).(14) (Figure 1)

Analysis of fluoroscopic data requires digital recombination with Computed Tomographic (CT) images for registration and quantification of the inspiratory tissue expansion over the duration of a tidal breath.(13, 15) Fluoroscopic image registration with the CT is designed to be an automatic during tidal breathing, although in this case whereby the NIPPV intervention had not previously been combined with the XV analysis all image registrations were manually assessed by an experienced engineer. The XV analysis provides a measure of lung tissue expansion derived from 8 mm x 8 mm x 8 mm voxel units from images of the whole lungs or from selected regions. Specific ventilation (SV) is derived by the change in lung volume of a given voxel from the start of inspiration (*Vinsp – Vexp*) normalized by the volume at the start of inspiration (Vexp). (Figure 2) During the study, data were collected to evaluate regional SV in participants both during tidal breathing and under NIPPV.

**Lung Regions**

Post-hoc analysis of SV in the segmented regions of interest was then performed to identify values stratified into central and peripheral areas. Each individual lobe was segmented into a central zone, which is confined to the inner central zone (center of lobe to ½ outer edge), and a peripheral zone, with the designated outer regions (1/3 distance from outer edge to center). Central and peripheral regions from the lung were formed by combining all lobe central and peripheral regions. This stratification allowed for a focused comparison of ventilation patterns in different anatomical regions of the lung. By comparing the SV in central versus peripheral regions, the study aimed to uncover differences in response to NIPPV. (Figure 3)

**Derived Variables and Statistical Analysis**

The derived variables included the mean specific ventilation (MSV), low-volume region (LVR) percentage, and high-volume region (HVR) percentage. MSV is the average SV for a selected region and represented the average relative volume displacement for that region. Percent change of MSV was calculated as *[(MSV by NIPPV) – (MSV by Tidal Breathing)] / (MSV by Tidal Breathing) x 100.* LVR was defined as the percentage of voxels with SV less than 10%, while HVR was defined as the percentage of voxels with SV greater than 30%. These metrics were utilized to assess the contributions of changes in the total low or high-volume regions. Statistical analysis involved two-way analysis of variance (ANOVA) to determine differences between tidal breathing and breathing during NIPPV administration in different lung regions (central vs peripheral zones and lobar regions). Additionally, paired t-tests were conducted to determine the significance of these differences.

**Methodological Sources of Variability**

In brief, one of the biggest limitations in this field of investigation is that there have been no large-scale studies that include a spectrum from healthy participants to disease. Population studies using radiological imaging may prove ethically challenging, specifically those investigations that utilize multiple imaging steps due or repeat scans. It is therefore difficult to examine and control all the possible sources of variability in this proof-of-concept study. In the current study we were limited to scanning under two study conditions (A. Without and B. With NIPPV). We reasoned that in the current protocol that keeping the NIPPV constant allows us to observe varied responses as mentioned in the reviewer’s comment.

The most intuitive data to refer to in additional discussion of the individual variability in specific ventilation is to examine the changes in MSV from tidal to NIPPV in various lung regions illustrated in figure S1. While all for individual’s whole lung MSV was greater with NIPPV there was a range in the level of the change from ~1% to ~8%. Comparing MSV of the right and left lung the individual difference varies between +/- 4%, whereby in some the right lung MSV is greater than the left lung MSV, and vice versa. In examining the individual regional differences in MSV with NIPPV, examine the individual that demonstrates the greatest change in whole lung MSV. Left lung MSV was ~3% greater than in the right lung during tidal, however, during NIPPV both left and right lung MSV were increased and the was negligible difference between them. Now take for example the subject where right lung MSV is 0.2 and left lung MSV is 0.17 (bright green) during tidal breathing. Following NIPPV there was a decrease of 2% in the right lung MSV and an increase of 5% in the left lung MSV.

While overall, in all individuals, there was an increase in MSV for the entire lung, the specific amounts and the lung regions that contribute to the overall increase vary. We observed that there is individual variation in magnitude and regional volumetric changes to the set NIPPV levels in this healthy control population. It remains to be determined if there are physiological factors such as baseline minute ventilation, regional difference in lung compliance or chest wall, lung size, or regional airway dimensions that contribute to the observations.

An illustrative figure (Figure S1) provides additional detail which may further elucidate possible sources of error. The figure shows sequence and timing of the acquisition of fluoroscopic images relative to inspiration and expiration phases of the breath. The fluoroscopic image analysis parses images from the start of inspiration (or nearest frame) to the end of inspiration (or nearest frame), then based on the number of frames acquired during that period selects 7 approximately equal quantiles. Similarly, image frames for 7 approximately equal quantiles are selected from expiration. The accuracy of any specific frame may vary by half of the sampling frequency of the fluoroscopy ~0.035 s (or ~2.5% if inspiratory time is 1.5s).

Further, there is natural variation in inspiratory time in the schematic there is variation in the inspiratory time and volume between images acquired in the protocol. In analysis of the sensitivity to the between breath changes in the outcomes of VDP and VH we demonstrated that an +/-8% change in metrics between breaths (this includes the ~2.5% due to sampling frequency). These sources have been discussed in a previous manuscript. It is unclear what is the magnitude of the variability in regional MSV due to the image acquisition sampling limitation or the variance in breath magnitude or duration. Quiet tidal respiration was chosen to limit the variance and effort dependance. It is unclear if altered lung mechanics alter the relative proportion of the variance in region MSV due to image sampling frequency or breath dynamics.