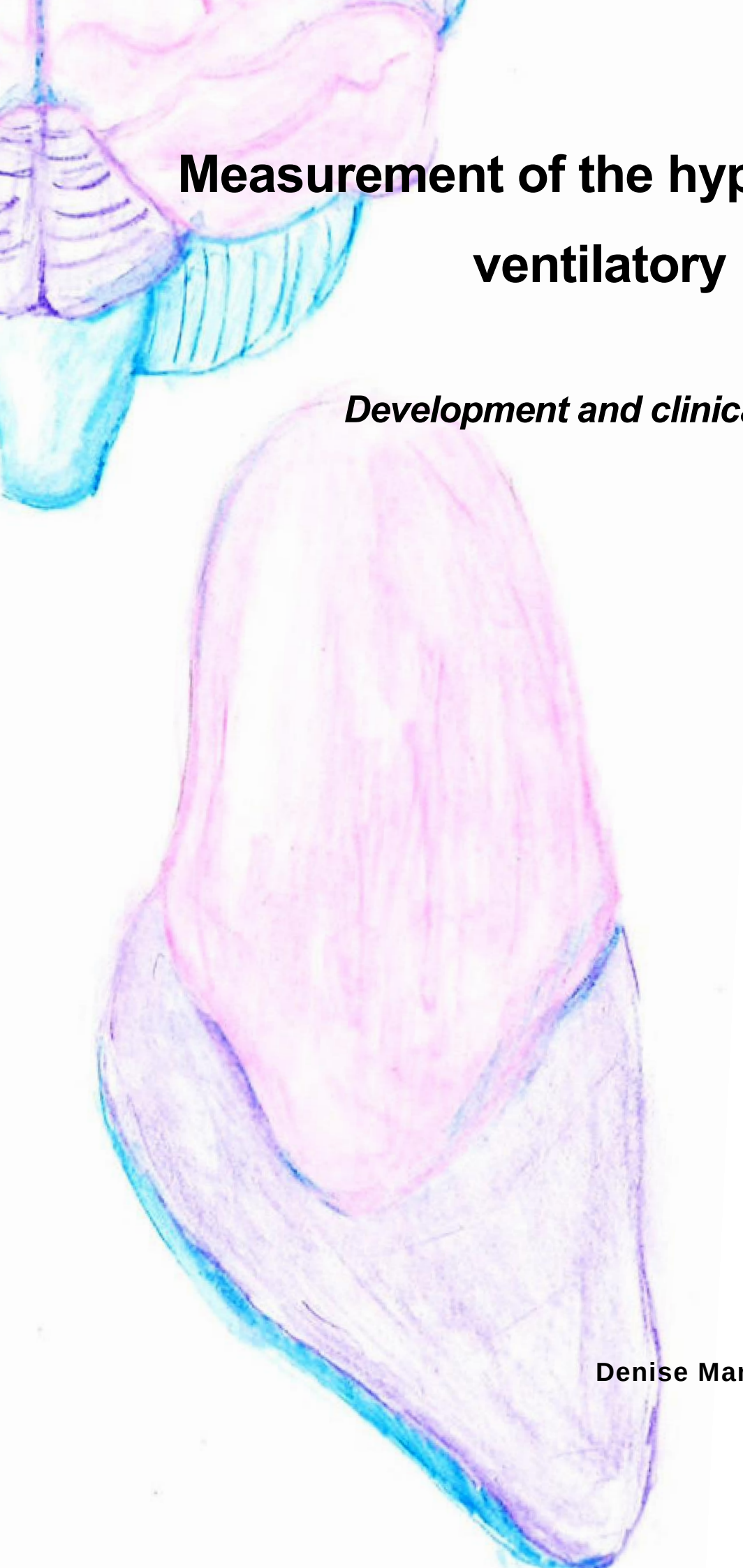

Measurement of the hypercapnic ventilatory response

Development and clinical application

Denise Mannée-Boele, BSc

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Technical Medicine, Medical Sensing and Stimulation

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Preface

Al van jongs af aan had ik een grote interesse in het menselijk lichaam en de werking daarvan. Op de basisschool wist ik al dat ik dokter wilde worden, over de jaren varieerde alleen het soort dokter nog van huisarts tot hartchirurg. Jaren later moest ik kiezen, koos ik voor het 'gewone' geneeskunde of het nieuwe technische geneeskunde? Uiteindelijk heb ik met oog op mijn interesses voor bètavakken, toch gekozen voor het laatste, en ben ik nog altijd gelukkig met die keuze. In mijn masterstages kwam ik via de urologie en de neurologie terecht bij de intensive care en de cardio-thoracale chirurgie. Hier vond ik mijn passie voor fysiologische problemen. Daarom leek mij de afstudeeropdracht die werd aangeboden op de Longgeneeskunde een passende uitdaging. Tien maanden later mag ik terugkijken op een goede en leerzame tijd. Ik mocht mij verdiepen in de regulatie van de ademhaling en de fysiologie daarachter. Daarnaast mocht ik mijn praktische skills tot uiting brengen bij het maken van een meetopstelling. Ik heb het hele proces van onderzoek kunnen doorlopen in deze afstudeerstage, van ontwerpen tot metingen uitvoeren en van METC-aanvraag tot data-analyse. Door de vele gesprekken met patiënten op de poli of spoedeisende hulp, heb ik mij meer kunnen bekwamen in de gespreksvoering en het diagnosticeren. Ik heb ervan genoten dat dit zo'n veelzijdige stage was. Maar dit alles was niet zo mooi geweest zonder de hulp van verschillende mensen.

Allereerst wil ik al mijn begeleiders hartelijk danken voor de tijd en energie die zij gestoken hebben in mijn begeleiding. Drs. Michiel Eijsvogel en dr. Michiel Wagenaar, bedankt voor jullie enthousiasme voor dit onderzoek. Ik heb veel van jullie mogen leren over gespreksvoering, patiëntzorg en het jullie zo geliefde vak van de longziekten. Dr. ir. Frans de Jongh, dank voor je kritische blik op mijn werk. Ik vond het fijn dat ik op elk willekeurig tijdsstip bij je binnen kon lopen voor vragen en discussie. Drs. Timon Fabius, ik heb genoten van onze discussies over uiteenlopende onderwerpen (die niet altijd wat te maken hadden met mijn onderzoek). Ik wil je bedanken voor je gezelligheid en de uitgebreide feedback die je me altijd kon geven over mijn functioneren en werk. Drs. Bregje Hessink-Sweep, als tutor heb ik veel aan je gehad. Op momenten dat ik wat onzeker was over mijn kunnen, liet je me terugkijken op mijn leerproces, waardoor ik kon zien hoeveel ik mij al ontwikkeld heb door alle stages heen. Dank je wel voor onze fijne en persoonlijke gesprekken, ik heb ze zeer gewaardeerd.

Naast mijn begeleiders, wil ik ook de andere leden van de afstudeercommissie bedanken. Prof. Bart Koopman, hartelijk dank voor het voorzitten van mijn afstuderen. Ook hartelijk dank aan dr. Erik ten Berge, een van de laatste keer dat ik je sprak, hebt je mij nog eens op het hart gedrukt dat ik écht moest gaan afstuderen op het gebied van longen. Dat was een goed advies! Verder wil ik ook alle longfunctie analisten bedanken, jullie hebben met mij meegedacht en me geholpen met praktische vragen. Ik wil alle gezonde vrijwilligers bedanken, zonder jullie had ik dit onderzoek niet kunnen uitvoeren.

Bedankt lieve pappa en mamma, voor jullie liefde en dat jullie het mogelijk voor me maakten om te gaan studeren. Als laatste, lieve Paul, wil ik jou bedanken, dat je me hielp relativeren als ik door de bomen het bos niet meer zag en me voor hield dat ik dingen wel kon, als ik er zelf niet zoveel vertrouwen in had.

Denise Mannée

Abstract

Introduction: Control of breathing is predominantly regulated by feedback of the central chemoreceptors and in lesser amount by the peripheral chemoreceptors. All chemoreceptors are sensitive to changes in partial pressure of carbon dioxide (PCO_2) and via acid-base reactions to hydrogen concentration ([H+]). However, peripheral chemoreceptors also respond to hypoxemia. The relationship between PCO_2 and the resulting minute ventilation is known as the hypercapnic ventilatory response (HCVR). Two types of methods are available to measure the HCVR: rebreathing (i.e. breathing in a closed circuit causing inspired CO_2 to gradually increase) and steady-state (i.e. breathing at two or three predetermined PCO_2 levels until stable ventilation is measured). The HCVR is assumed to be linear above the ventilatory recruitment threshold (VRT). The aim of this study is to investigate which method is best suitable for use in clinical practice. The key factor is the reproducibility of the test. Additionally, the experience of the subjects with the methods, the possibility to decrease the burdensome of the measurements, and the theoretical applicability in the clinic, were investigated.

Methods: Twenty healthy adults were enrolled in the study. At the first visit, the hypercapnic (hyperoxic) ventilatory response of all subjects was measured with a rebreathing and steady-state method, in random order. At the second visit, 5-9 days after the first, both measurements were repeated, in the same order. In both visits, two visual analog scale (VAS)-questionnaires about both measurements and a preference questionnaire were completed with questions about duration, breathing comfort and dyspnea sensation. The slope and projected apnea threshold were calculated. Furthermore, a short slope was calculated in rebreathing, which is the slope of the minute ventilation as function of the end-tidal CO_2 calculated over a smaller delta end-tidal CO_2 (VRT to VRT + 1.5 kPa). The intra-class coefficient (ICC) of the slopes was used to assess the reproducibility. A method with an ICC higher than 0.8 was considered reproducible.

Results: The ICC (95% confidence interval (CI)) of the rebreathing method was 0.89 (0.73-0.95), and 0.56 (0.14-0.81) for the steady-state method. The ICC of the short slope in the rebreathing method was 0.78 (0.51-0.91). In the preference questionnaire, the steady-state measurement was preferred over the rebreathing measurement by 16 out of 20 subjects, at both visits. Breathing was considered easier and dyspnea sensation was less in steady-state than in rebreathing ($p<0.04$). Breathing comfort was scored 5.0 (4.0-6.0) and 4.5 (4.0-5.8) for rebreathing and 6.0 (5.0-7.0) and 6.0 (4.0-7.0) for steady-state. Dyspnea sensation was scored 4.0 (4.0-5.8) in rebreathing and 3.5 (2.3-4.8) in steady-state.

Discussion: The rebreathing measurements are reproducible, with an ICC above 0.8. The steady-state measurements are not reproducible. The 95% CI is wide, suggesting that in some subjects the measurements are reproducible and in others not. The relative high variability of $PETCO_2$ may be a key factor in the poor reproducibility of the steady-state measurements. Other factors (e.g. non-linearities, variation in oxygen fraction, measurement duration, and gender) affecting the reproducibility are ruled out. Most subjects chose the steady-state as the method of preference. It is hypothesized that side effects (e.g. nausea, headache) are more present in rebreathing measurements. However, the absolute difference between VAS-scores was small. Rebreathing measurements were reasonably reproducible with the short slope. More variability is present in ventilation at lower levels of $PETCO_2$, and therefore

the slope can vary more between measurements. Variations in method can influence the measured slope, which should be considered while making clinical conclusions from the measurements.

Conclusion: Based on the reproducibility of the measurements, it is preferred to use the rebreathing method to measure the hypercapnic ventilatory response.

Recommendations: It is recommended to investigate the reproducibility in patients. Furthermore, the reproducibility of the rebreathing tests should be established under normoxic conditions with the peripheral chemoreceptors active. This could make the method more useful for clinical application. To assess other aspects of the ventilatory system, the measurement can be extended, including variability tests and time delay tests.

List of abbreviations

$[H^+]$	*Concentration of hydrogen molecules*
$[HCO_3^-]$	*Concentration of bicarbonate molecules*
95% CI	*95% Confidence interval*
AT	*Apnea threshold*
CBF	*Cerebral blood flow*
CO_2	*Carbon dioxide*
COPD	*Chronic obstructive pulmonary disease*
COV	*Coefficient of variation*
CSAS	*Central sleep apnea syndrome*
CVR	*Cerebrovascular reactivity*
e.g.	*Exempli gratia*
FiO_2	*Fraction of inspired oxygen*
HCVR	*Hypercapnic ventilatory response*
HF	*Heart Failure*
ICC	*Intraclass coefficient*
i.e.	*Id est*
IQR	*Interquartile range*
N. IX	*Glossopharyngeal nerve*
N. X	*Vagus nerve*
O_2	*Oxygen*
OHS	*Obesity hypoventilation syndrome*
OSAS	*Obstructive sleep apnea syndrome*
pAT	*Projected apnea threshold*
PCO_2	*Partial pressure of carbon dioxide*
$PETCO_2$	*End-tidal partial pressure of carbon dioxide*
$PETO_2$	*End-tidal partial pressure of oxygen*
PO_2	*Partial pressure of oxygen*
R^2	*Coefficient of determination*
RAR	*Rapid adapting receptor*
SAR	*Slowly adapting receptor*
Short slope-R	*The slope of minute ventilation as function of the end-tidal CO_2 calculated over a smaller delta end-tidal CO_2 (range: ventilatory recruitment threshold to ventilatory recruitment threshold + 1.5 kPa).*
Slope-R	*The slope of minute ventilation as function of the end-tidal CO_2 measured with rebreathing method*
Slope-SS	*The slope of minute ventilation as function of the end-tidal CO_2 measured with steady-state method*
VAS	*Visual analog scale*
VRT	*Ventilatory recruitment threshold*

Index

“Laat alles wat adem heeft de HEERE loven. Halleluja!”

Psalm 150:6 (HSV)

1. Introduction

To provide the human body of energy, nutrients are continuously converted to energy (catabolism). In this process oxygen (O_2) is consumed and carbon dioxide (CO_2) is produced as a waste product. The alveoli of the lungs are built to efficiently exchange gases between the air in the lungs and blood in the capillaries. O_2 diffuses into the body and CO_2 is eliminated from the body in the alveoli. The uptake of O_2 and elimination of CO_2 is decreased with decreased ventilation and vice versa. The body strives to maintain a constant level of O_2 and CO_2 molecules and hydrogen ions in the arterial blood. Balanced concentrations are maintained through control of breathing. The most essential sensors in control of breathing are the central and peripheral chemoreceptors. Central chemoreceptors react to changes in the partial pressure of carbon dioxide and concentration of hydrogen molecules (PCO_2 and $[H^+]$). Peripheral receptors also respond to PCO_2 and $[H^+]$. However, the response of the peripheral chemoreceptors can be modulated by the partial pressure of oxygen (PO_2). In hypoxia, the response of the peripheral chemoreceptors is increased. If the chemoreceptors are stimulated, ventilation is adjusted to restore PCO_2, $[H^+]$, and PO_2. In various diseases, control of breathing is attenuated or amplified. This leads to changed breathing patterns and dysregulation of PCO_2, PO_2 and $[H^+]$. [1]–[4]

1.1. Rationale

Control of breathing is dysregulated in individuals with diseases like central or obstructive sleep apnea syndrome (CSAS, OSAS), hypercapnic chronic obstructive pulmonary disease (COPD), obesity hypoventilation syndrome (OHS) or Phox2B gene mutation. Knowledge about the pathogenesis of an unstable control of breathing can help optimize disease and subject specific treatment or therapy.

As described, the most essential sensors in control of breathing are the chemoreceptors. The ventilatory response of the chemoreceptors can be described as the relation between the blood gas as input and ventilation as output. In this study, the main regulator of ventilation is studied: the central chemoreceptor, which respond to changes in PCO_2. Two types of methods are known to measure the ventilatory response: rebreathing methods and steady-state methods (e.g. Read's rebreathing, Duffin's rebreathing, dynamic end-tidal forcing, prospective targeting) [4]. In both methods inspired PCO_2 is regulated to manipulate PCO_2 at the central chemoreceptors. In rebreathing, the CO_2 in exhaled air of a subject is re-inhaled. The chemoreceptors are exposed to increasing CO_2 levels as CO_2 in the blood will increase gradually with inhaled CO_2. In steady-state, it is assumed that prolonged exposure to a certain PCO_2 is necessary to measure the corresponding ventilation, due to time delays. Therefore, in steady-state the PCO_2 is maintained at a constant level for 5-10 minutes. This can be realized through balancing the amount of exhaled air going back in the closed system, or using computerized systems with gas mixers.

Numerous systems are available to measure the ventilatory response via rebreathing or steady-state methods. The systems can be manually-controlled or computer-controlled systems (e.g. dynamic end-tidal forcing systems or prospective targeting with the RespirAct) [4]. None of these systems is known as the golden standard for measurement of the ventilatory response. Therefore, the aim of this study is to find the method most suitable for clinical application. In this study, a measurement setup was designed

and built to determine the ventilatory response with a manually-controlled system via both rebreathing and steady-state method. The applicability of both methods is determined by the reproducibility of the measurements, and the burden on the measured subject.

In various researches reproducibility of rebreathing or steady-state tests was tested. In 1973, Strachova et al. found the long-term reproducibility of Read's rebreathing method. The correlation coefficient between the mean of three measurements in the first session and the mean of three measurements in the last session was 0.939 [5]. A few years later, Berkenbosch et al. compared the mean of 2 to 6 hyperoxic rebreathing measurements of three subjects between two consecutive days. Two subjects had an almost identical mean response and one subject had an almost doubled mean response measured with the rebreathing test. The mean of the first and second steady-state response session showed good correspondence [6]. In the study of Cohen et al. 4 newborn subjects underwent steady-state measurements twice. The mean difference between measurement 1 and 2 was 150% [7]. In 1991, Nishimura et al. performed a study to find the reproducibility of rebreathing measurements with the coefficient of variation (COV), the ratio of standard deviation to mean. Six subjects underwent three consecutive measurements on 1 day, the session was repeated one and two week(s) after. The mean COV per subject ranged from 4.6 to 37.1% [8]. In 2010, Jensen et al. tested the variability of the ventilatory response in 20 human subjects as measured with Duffin's modified rebreathing technique under hyper- and hypoxic conditions. They used the intra-class coefficient (ICC) to describe the repeatability of the test. All subjects performed 4 hyper- and hypoxic rebreathing measurements on one day, and 1 hyper- and hypoxic measurement on 7, 14, 21 and 60 days thereafter. The ICC (95% confidence interval, 95% CI) within-day and between-days were 0.740 (0.478-0.889) and 0.782 (0.582-0.903), respectively [9]. The reproducibility of rebreathing and steady-state tests has not been compared in a large population. Therefore, the focus of this study is to compare the reproducibility of both methods.

It is known that (transient or mild) hypercapnia can cause dyspnea sensation, sluggishness, and headaches [10]. When the ventilatory response is measured, PCO_2 increases above 7 kPa [6], [11]. However, no studies are known to investigated the burden of the measurements on the subjects. Therefore, in this study, the experience of the subjects with the tests was monitored. In this study, another focus was to investigate if hypercapnia can be less extensive in measurements. Hypothetically, this could decrease the burden on the subject.

Both reproducibility and burden on the subject are investigated in this study to answer the main question:

"Which method, steady-state or rebreathing, should be implemented in clinical practice to measure the ventilatory response?"

The sub-questions deduced are:

- *To which extent are the measurements with the rebreathing and the steady-state method, reproducible?*
- *How do subjects experience measurements with both methods?*
- *Is it possible to decrease the burdensome of the tests on the subject?*
- *In what manner can both methods theoretically be applied?*

Chapter 2 provides a clinical background on the control of breathing. Chapter 3 mainly focuses on the technical aspects of control of breathing and its measurement. It gives a theoretical framework of how the rebreathing and steady-state methods can be applied. In chapter 4, the method to investigate the reproducibility, subject experience and the possibility to decrease the burdensome for subjects is described. The results are presented in chapter 5. Lastly, in chapter 6 and 7 the results are discussed and conclusions are drawn.

2. Clinical background

Control of breathing is realized with a feedback system. A feedback system has three fundamental components, (1) a control system, (2) a sensor, and (3) an effector, see figure 1. Through negative feedback a disturbance to the system (change in PCO_2, PO_2 and $[H^+]$) can be restored. For example, a disturbance causes the PCO_2 in the controlled system to increase. The increase is sensed by the sensor (central and peripheral chemoreceptor), increasing the afferent signal. The afferent signal is 'interpreted' by the respiratory center and is compared to a reference value. The error (difference between actual and reference value) is converted to an efferent output to the effectors. The effectors (respiratory muscles) increase their work, increasing ventilation. More CO_2 is exhaled with an increase in ventilation and by this process PCO_2 levels are restored. The respiratory system maintains homeostasis by integrating the signals from all sensors (figure 2, left box) in the respiratory center including several parts of the brain and central nerve system (figure 2, middle box). Motor output to the effectors (figure 2, right box) is adjusted to meet ventilatory demands. [12]

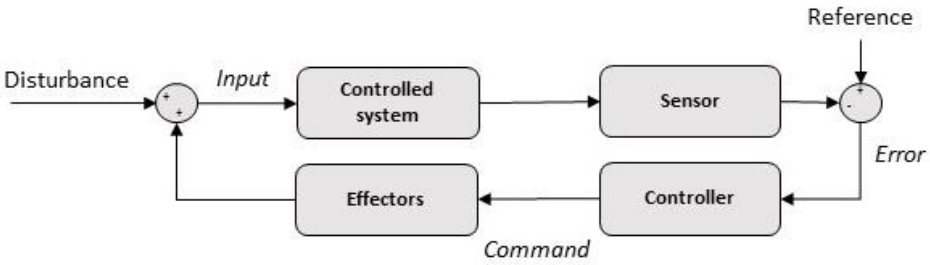


Figure 1: Schematic presentation of a feedback system. Sensor observes a disturbance of the measured signal; the control system compares the feedback with a reference value to estimate the error. The effectors will oppose the error to normalize the measured signal.

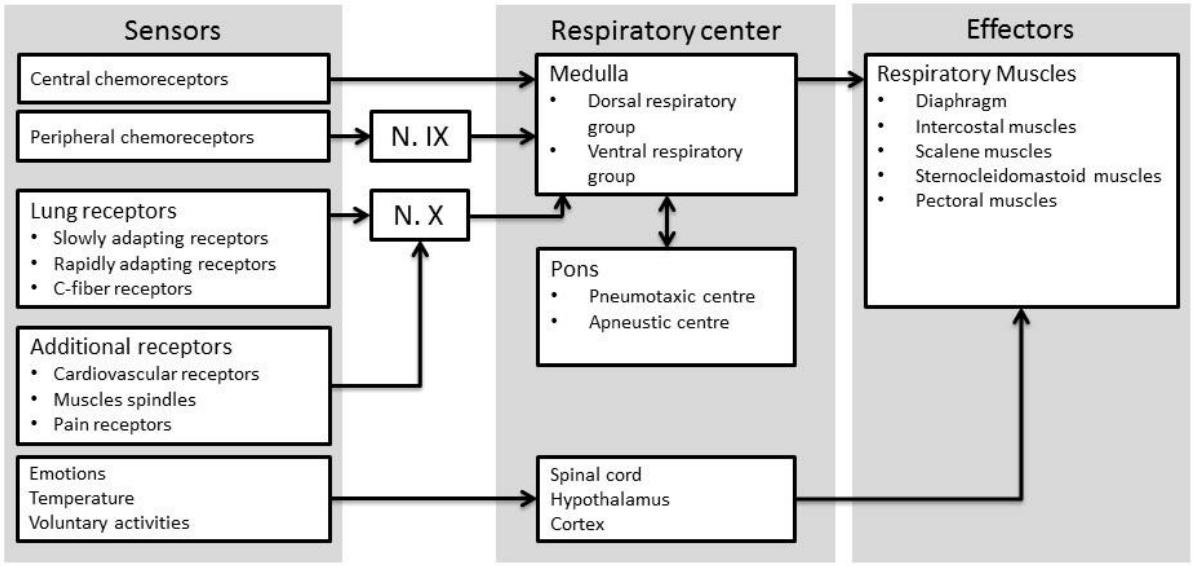


Figure 2: Respiratory control system. The respiratory system maintains homeostasis by integrating the signals from all sensors (left box) in the respiratory center including several parts of the brain and central nerve system (middle box). Motor output to the effector (right box) is adjusted to meet ventilatory demands.

2.1. Sensors

Several sensors are involved in the control of breathing. The main sensors are the central and peripheral chemoreceptors. The response of central chemoreceptors to elevating or decreasing levels of PCO_2 is the most important in the control of breathing. However, various lung receptors and additional receptors, such as muscular receptors and cardiovascular receptors, are also involved in the control of breathing.

2.1.1. Chemoreceptors

Chemoreceptors are sensitive to chemical changes in the blood and can be divided in two groups based on their location: (1) central and (2) peripheral chemoreceptors. Central chemoreceptors are found in the brain and peripheral chemoreceptors in the carotid / aortic bodies. The two types of chemoreceptors are linked anatomically and in various studies it is demonstrated that peripheral chemoreceptors influence central chemoreceptors. [12]

Central chemoreceptors can be found in the ventral part of the pons and the medulla oblongata. They lay in the extracellular brain fluid, and respond to changes in $[H^+]$. The blood brain barrier is highly impermeable to hydrogen ions; however, it is permeable to CO_2. CO_2 is buffered via the bicarbonate buffering system, it can be shifted through carbonic acid (H_2CO_3) to $[H^+]$ and bicarbonate ($[HCO_3^-]$), see eq. 1a. An increase in CO_2 causes a shift in eq. 1a to the right, increasing $[H^+]$, stimulating the central chemoreceptors. pH (logarithmic $[H^+]$) at the central chemoreceptors is dependent on PCO_2 in the cerebrospinal fluid, surrounding the central chemoreceptors (central PCO_2) and $[HCO_3^-]$, via the Henderson-Hasselbalch equation, see eq. 1b.

$$CO_2 + H_2O \leftrightharpoons H_2CO_3 \leftrightharpoons [H^+] + [HCO_3^-] \quad \text{(eq. 1a)}$$

$$pH = 6.1 + \log\left(\frac{[HCO_3^-]}{0.03 \cdot PCO_2}\right) \quad \text{(eq. 1b)}$$

Central PCO_2 is dependent on 3 main factors: (1) local metabolism, (2) arterial PCO_2 and (3) cerebral blood flow (CBF) [6], [11], [13], [14].

1. Local metabolism: an increase in metabolic activity at the central chemoreceptors causes an increase in central PCO_2, causing an increase in ventilation.
2. Arterial PCO_2: changes in arterial PCO_2 are not immediately 'measured' at the central chemoreceptors, due to the blood transit delay from systemic circulation to the cerebral blood circulation. Central PCO_2 changes with a time constant, which is dependent on the cerebral volume/ cerebral blood flow ratio. Furthermore, the diffusion of CO_2 over the blood brain barrier is dependent on the difference between arterial and central PCO_2. Cerebrospinal fluid contains less proteins than blood, making the buffer capacity of the cerebrospinal fluid smaller than the buffer capacity of blood. An increase in central PCO_2 will therefore cause a bigger fall in pH than the same change in arterial PCO_2. An increase in arterial PCO_2 causes an increase in central PCO_2, causing an increase in ventilation. If the cerebral pH is changed for a longer time, a compensatory mechanism will stabilize pH. For example, in patients with chronic hypercapnic COPD, $[HCO_3^-]$ in the cerebrospinal fluid is actively increased.

3. Cerebral blood flow: an increase in arterial PCO_2 (i.e. hypoventilation) or increase in central PCO_2 (e.g. caused by increased metabolism, increased arterial PCO_2) causes vasodilation at the local arterioles of the downstream vascular system, as a result CBF is increased. Cerebrovascular reactivity (CVR) refers to the vasomotor responsiveness of blood vessels in the brain to changes in blood gas stimuli. In a brain without CVR, central PCO_2 will increase with the same rate as arterial PCO_2 increases. In the healthy brain, an increased arterial PCO_2 will increase CBF and CO_2 is washed-out of the brain. The increase in arterial PCO_2 is more extensive than the increase in central PCO_2, the central chemo reflex is dampened. The same can be applied for a decrease in arterial PCO_2, CBF decreases, more CO_2 is 'washed-in' and the decrease in central PCO_2 is less extensive than the decrease in arterial PCO_2. The feedback loop of CVR and ventilatory regulation are integrated, figure 3 gives a representation of the feedback system.

Peripheral chemoreceptors can be found in the glomus caroticum and the glomus aorticum. In humans, the glomus caroticum, or carotid body, is the most important location for the peripheral chemoreceptors. Signals are sent from the carotid body to the central nerve system via the carotid nerve (which is a branch of glossopharyngeal nerve (N.IX)). The peripheral chemoreceptors are sensitive to arterial PCO_2, PO_2 and $[H^+]$. In hypoxia, the sensitivity of the chemoreceptors to changes in $[H^+]$ or PCO_2 is increased. At higher PO_2 levels, the peripheral chemoreceptors are silenced. [13]

2.1.2. Lung receptors

Lung receptors can be divided in three groups; slowly adapting receptors (SARs), rapidly adapting receptors (RARs), and C-fibers (J-receptors or juxta pulmonary capillary receptors). The first two are pulmonary volume receptors, both belong in the group of mechanical sensors [15]. All the fibers from SARs, RARs and C-fibers travel via the vagus nerve (N.X) to the regulation centers in the medulla. Differentiating between these three groups is often difficult, due to their similar compositions, and reflexes. Therefore, stimulation of one group of receptors, influences the other two groups.

SARs are neural afferent endings of N.X found in the tracheobronchial tree, mostly in the smaller airways. SARs can be found in the smooth muscle tissue of the airways. The place of the SARs in the lung influences the output of the SARs.

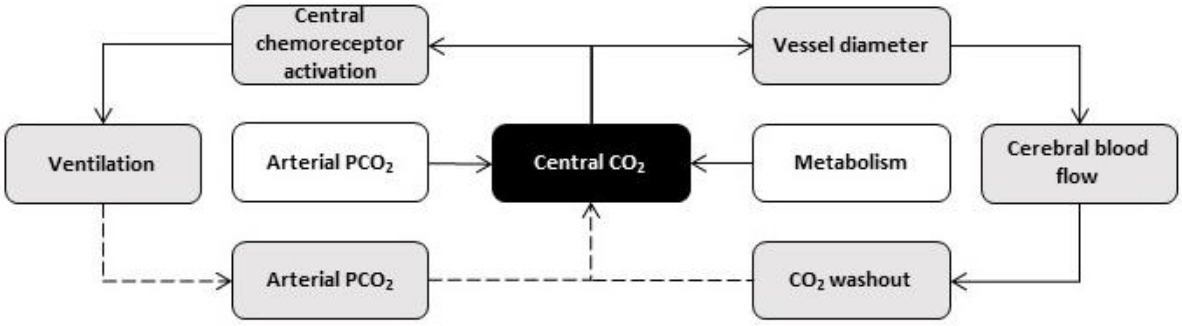


Figure 3: Feedback loop of cerebral reactivity (right) and feedback loop of ventilatory regulation (left). Dashed lines represent a negative effect and solid lines a positive effect. Modified from McKay et al. - Central respiratory chemo sensitivity and cerebrovascular CO2 reactivity: a rebreathing demonstration illustrating integrative human physiology [11].

Input to SARs could evoked the so-called Hering-Breuer reflex. It prevents the lungs from being overinflated, and is therefore also called the inflation reflex. Inspiration is early terminated, and the expiratory pause is prolonged. The counterpart of the inflation reflex is the Hering-Breuer deflation reflex; deflation of the lungs leads to an input to inspire. Furthermore, SARs affect airway tone. The Hering-Breuer reflex is suppressed by cortical activity. [16], [17]

RARs lay in between epithelium cells in the airways. They respond to mechanical and chemical irritant stimuli (e.g. damaging gases, cigarette smoke, inhaled dust and cold air) and to inflammatory and immunological mediators. Ventilatory reflexes are caused by stimulation of RARs, with augmented breathing (e.g. expiration reflex, aspiration reflex) and coughing as the main reflexes. The type of reflex is dependent on the location of the RARs. The receptors are probably nearly inactive in normal quiet breathing. Stimulation of RARs is accompanied with the stimulation of other airway receptors. [13], [16], [18]

C-fiber receptors are found in the alveolar wall, close to the capillaries. They can be grouped as nociceptors, which responded to damaging stimuli, they are not sensitive to lung volume changes, in contrast to the SARs and RARs. The stimuli of the C-fiber receptors are mechanical events (e.g. edema, congestion and pulmonary embolism). Stimulation of the C-fiber receptors, cause similar reflexes as in stimulation of the RARs (with exception of the coughing reflex). Severe stimulation of the C-fiber receptors can result in apnea. [13], [16], [19]

2.1.3. Additional receptors

It is believed that activation of the body, in case of exercise, causes muscles to send signals to increase ventilation. Furthermore, the muscles spindles of the intercostal muscles could be involved in the sensation of dyspnea, in case of abnormal attempt to move the chest wall (e.g. obstruction). Cardiovascular receptors can sense barometric changes in the blood. In case of increased blood pressure, the reflex can be hypoventilation or even apnea and vice versa. Pain can cause apnea, and afterwards a period of hyperventilation. [13]

2.2. Respiratory center

The respiratory center lays in the brain; in the pons and medulla, see figure 4. Ventilation can also be altered via signals from the cortex, hypothalamus or limbic system. Though the site of respiratory rhythm generator is controversial, it probably involves all the centers in the medulla and pons. Various rhythms can be generated by differential activation of the various centers. [20]

2.2.1. Medulla

Breathing rhythm is thought to originate in the medulla. Signals from various sensors are integrated in the medulla, via N. IX and N. X. The medulla contains a dorsal and ventral respiratory group (figure 4). The dorsal respiratory group is activated prior to inspiration and the ventral respiratory group regulates the switch between inspiration and expiration. It is thought that the pre-Bötzinger complex, which lays on the ventral medullary respiratory column, is the primary respiratory pattern generator of inspiratory rhythm. It is hypothesized that the complex exists of pacemaker neurons.[12], [13], [20]

2.2.2. Pons

In the pons two ‘centers’ can be found; the apneustic center and the pneumotaxic center (figure 4). The apneustic center has an excitatory effect on the parts of the medulla involved with inspiration (dorsal respiratory group), promoting inspiration. The pneumotaxic center stops or restrains the inspiration, and is considered the antagonist of the apneustic center. The pneumotaxic center regulates the inhaled volume, if absent, breathing pattern will exist of prolonged deep inspirations and brief expirations. [13], [20]

2.2.3. Other regulatory centers

Furthermore, ventilation is dependent on cortical signals and voluntary activities. For example, it is possible to hold your breath voluntarily and ventilation can be adapted when singing or talking. The ventilatory muscles can be directly controlled by the cortex and through the pyramidal tracts the centers in the medulla and pons are bypassed. When someone faints, or collapses by voluntarily breath holding or in extreme cases of hyperventilation, a ventilation reflex will occur, to restore PCO_2 and PO_2 levels. Emotions and temperature can cause changes in breathing pattern; the respiratory muscles are controlled via the limbic system and the hypothalamus. [13], [20]

2.3. Effectors

In control of breathing, the effectors are the respiratory muscles. The main muscle in inspiration is the diaphragm. The diaphragm contracts in inspiration and moves downwards, increasing the size of the thoracic cavity and creating a negative pressure in the lungs. The diaphragm is innervated by the phrenic nerve, which originates from the cervical spinal cord (C3-5). The diaphragm is assisted by the external intercostal muscles, which lift the ribs and expand the thoracic cavity even further. The external intercostal muscles are innervated by intercostal nerves (T1-12). With increased breathing work, the scalene muscles, the sternocleidomastoid muscles and the pectoral muscles are additionally activated. [13]

Normal expiration is a passive process. In forced expiration the abdominal muscles are active, pushing the diaphragm up and reducing the size of the thoracic cavity. [13]

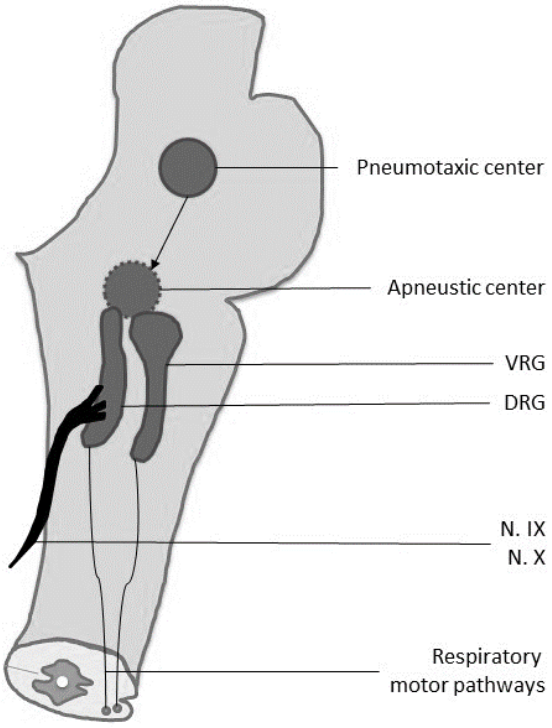


Figure 4: Regulation centers in the medulla and pons. The pneumotaxic and apneustic center lay in the pons, the ventral and dorsal respiratory group (in figure VRG and DRG resp.) can be found in the medulla. Both N. IX and N. X end in the medulla. Respiratory motor pathways are used to signal the effectors.

2.4. Diseases affecting control of breathing

Control of breathing is affected in various diseases, such as CSAS/OSAS, COPD, and hypoventilation syndromes. Below the causes and consequences of all three groups are described.

2.4.1. Sleep Apnea

Disturbance of breathing in sleep refers to cessations of breathing (apneas) or reductions in breath amplitude (hypopneas), resulting in hypoxemia and hypercapnia. The apnea or hypopnea can be either caused by a central event (CSAS) or an obstructive event (OSAS). A central event is a result of the reduction or stop of signals from the respiratory center to the effectors, while an obstructive event is caused by (near) closure of the extra thoracic upper airway [21]. OSAS and CSAS (with Cheyne-Stokes breathing pattern, which is a pattern of progressively deeper and sometimes faster breathing, followed by a gradual decrease that results in a temporary apnea) are common in patients with heart failure (HF) and reduced ejection fractions. Approximately 1–2% of the general population has HF, with the prevalence rising to approximately 10% in those older than 70 years, of whom about 50% will have a reduced ejection fraction [22]. It is estimated that 1/3th (20-40%) of the patients with HF have OSAS and approximately the same amount has CSAS with Cheyne-Stokes breathing pattern (30-50%) [22], [23]. The disturbed breathing patterns in sleep of patients with OSAS and CSAS can cause cardiovascular problems [21] and are associated with higher mortality [24]. The pathogenesis of OSAS and CSAS can vary per subject, and therefore patients may respond different to various treatments [25].

2.4.2. COPD

COPD is defined in the GOLD 2017 report as a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases [26]. COPD patients often complain about sleeping problems. Results of the study of McSharry et al. show that patients with severe COPD show poor sleep quality in comparison to normative cohorts of similar age, and that reduced sleep quality is associated with day-time hypoxemia [27]. Multiple causes can be appointed for the sleep disorders in COPD: hypoxemia, hypercapnia (as result of changed respiratory drive), inflammation, COPD medications, co-morbidities (e.g. OSAS, CSAS, restless leg syndrome) and/or nicotine use [27]. The effects of the sleep disorder can be worsened hypoxemia and hypercapnia, causing cardiac arrhythmias and pulmonary hypertension. Furthermore, nocturnal deaths may be induced in exacerbations [27]. Recognition of the pathogenesis of hypercapnia and sleep disorders in COPD patients can be used to appoint the correct treatment. Moreover, incorrect treatment can worsen the situation of the patient, leading to disastrous consequences, worsening the conditions of the patient [28], [29].

2.4.3. Hypoventilation syndromes

More and more, obesity becomes a problem in the western society, leading to sleep-disorders, such as previously described OSAS and CSAS. OHS is another sleeping disorder, which is closely related to OSAS. The prevalence of OHS in patients with OSAS is estimated to be 10-38%. The difference between OHS and OSAS is that in the former, longer periods of nocturnal hypoventilation occur and day-time hypercapnia is present, while the pathophysiology of OHS is not fully understood. In obese

subjects, sensitivity to CO_2 is augmented to maintain normocapnic blood values. It is thought that patients with OHS have a reduced CO_2 sensitivity or an insufficient augmented CO_2 sensitivity, causing day-time hypercapnia [30]. In OHS patients, several factors can cause the respiratory failure and changes in CO_2 sensitivity. Examples of these factors are: resistance to leptin, increased mechanical load to the respiratory system and decreased muscle endurance [31]. A good diagnosis is desirable, as untreated OHS can lead to life-threatening cardiopulmonary problems.

Another group of patients suffering of hypoventilation syndrome is the group of patients with a heterozygous Phox-2B gene mutation. The mutation causes a patient to hypoventilate in sleep due to dysfunctional hypoxic and hypercapnic responses [32], [33]. People generally require tracheostomy and lifetime mechanical ventilation or, less invasive biphasic ventilatory support [34]. However, lifetime ventilation is associated with pneumonia and infections. Less invasive therapies are preferred if applicable. Mild cases of the Phox-2B gene result in central sleep apnea [35], which can still affect the overall health of a patient.

3. Technical background

In this part, the control of breathing will be further explained, by means of the terms 'plant', 'controller' and 'loop gain'. As described in the previous part, ventilation is regulated through various feedback loops. The most important feedback loop in the control of breathing is the feedback loop controlling $[H^+]$, PCO_2 and PO_2 [4], as it is thought that other sensor reflexes are suppressed in normal breathing. The feedback system can be divided into two parts: the plant and the controller. The plant is the 'passive' part of the system, and is also referred to as the controlled part of the system. The plant in case of the chemoreflex represents the relation between ventilation as input, and PCO_2, PO_2 and $[H^+]$ as output, see figure 5. The reaction to a disturbance is determined by the physical properties of the system. The controller describes the relation between PCO_2, PO_2 and $[H^+]$ and ventilation. Normally, respiration is determined by equilibrium between the two subsystems. To find the response of both the plant and the controller, an open loop situation can be created. Which means that ventilation or PCO_2 is used as an input, without an attempt to compensate for the changes in the system due to the input.

3.1. Controller

The controller can be described with PCO_2 or PO_2 as the dependent variable. This means the function of the controller can be described with changing PCO_2 values under a constant PO_2 level and with changing PO_2 values under a constant PCO_2 level. As described previously, this study focused on measurement of the reflex of the central chemoreceptors, therefore PCO_2 was the dependent variable. The reflex of the central chemoreceptors solely, can be measured under hyperoxic conditions, as the peripheral chemoreceptors are silenced. This response is called the hypercapnic (hyperoxic) ventilatory

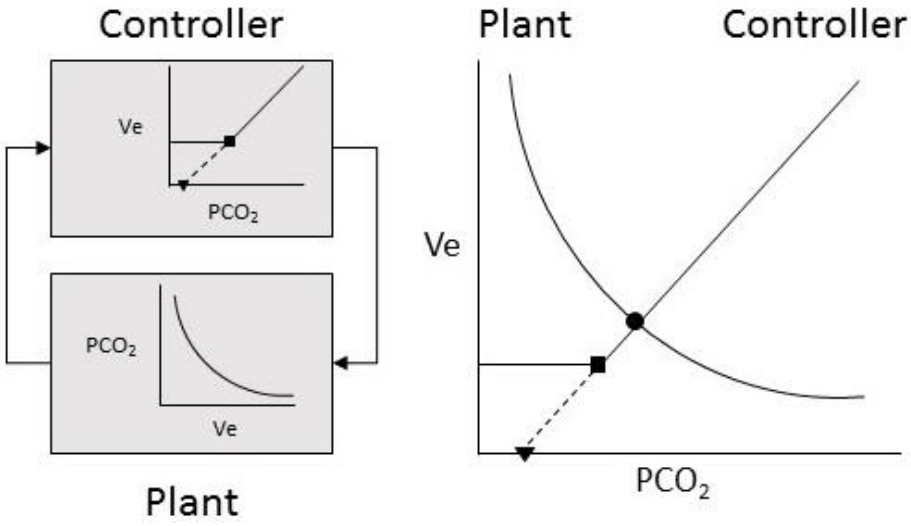


Figure 5: Function of the plant and controller of the ventilatory control system, adapted from Miyamoto et al. [36]. The function of the controller gives the relation between input partial carbon dioxide pressure (PCO_2) and output ventilation (Ve). Up to a certain PCO_2 level, there is no reaction from the sensors, ventilation is driven by the wakefulness drive. Above the point of inflection (square), called the ventilatory recruitment threshold, ventilation increases linearly with PCO_2. The apnea threshold (triangle) is the PCO_2 level were ventilation ceases if wakefulness drive is not present. The function of the plant gives the relation between input ventilation and output PCO_2, it is referred to as the isometabolic hyperbola. In the right part of the figure, plant and controller function are combined, the 'working point' of the system is indicated with a circle.

response (HCVR). The function of the controller with PCO_2 as dependent variable is linearly proportional to CO_2 above the ventilatory recruitment threshold (VRT). This linear relation is referred to as 'slope' (other terms are 'CO_2-sensitivity slope, sensitivity slope or sensitivity). At PCO_2 levels below VRT, chemoreceptors are not sensitive to PCO_2 changes, and basal ventilation can be found, which is driven by the so called 'wakefulness drive'. In sleep, the wakefulness drive disappears and it is hypothesized that the function of the controller becomes linear over the whole $PETCO_2$ range (figure 5). The apnea threshold (AT) is the intercept with the x-axis, it indicates $PETCO_2$ were ventilation ceases. The HCVR (in sleep) can be described with eq. 2 [37], in which Ve is ventilation, PCO_2 is the partial pressure of carbon dioxide, AT is the apnea threshold and S the slope.

$$Ve = S(PCO_2 - AT) \quad \text{(eq. 2)}$$

As early as 1892, research is performed on humans to find the effects of changes in PO_2 and PCO_2, on ventilation. A lot of methods to measure the ventilatory response are developed over time, nonetheless all methods can be traced back to two main groups; rebreathing and steady-state methods. [4]

3.1.1. Steady-state methods

The first steady-state measurements were performed in the late 50s and early 60s [4]. The main idea of a steady-state measurement, is that it takes time to measure the central chemoreceptor response to inspired CO_2 or O_2 due to a time delay and wash-in time in the cerebral circulation. Therefore, inspired PCO_2 and PO_2 levels were kept constant for 5-20 minutes to equilibrate with the PCO_2 in the reservoir of the measurement setup (usually a bag) and tissue PCO_2. One of the weaknesses of this method was that the researchers did not consider changes in the chemo-sensitivity from the prolonged exposure to hypercapnia/hyperoxia [4], [38]. Furthermore, the effects on the response due to CVR were not included [6]. Lastly, inspired PCO_2 could not be kept constant because it was increased by the exhaled CO_2. To address the latter problem, a new form of steady-state measurement was developed, called dynamic end-tidal forcing. Inspired CO_2 and O_2 levels can be controlled with use of a sophisticated system with high gas flows to keep end-tidal PCO_2 and PO_2 (resp. $PETCO_2$ and $PETO_2$) constant [39]. The problem with the dynamic end-tidal forcing system is that, since a lot of equipment is needed to perform the measurements, it is bulky and therefore not easily moved. Experiments have to take place in a lab environment [4]. Nowadays, sequential gas delivery and prospective targeting are used to perform steady-state measurements [4]. Prospective targeting is a smaller system. A controlled gas mixture and the gas in the lungs is constantly blended with use of two reservoirs and a cross-over valve [40]. In sequential gas delivery, a variable dead space is used to control $PETCO_2$ and $PETO_2$ [41]. Combination of the two systems results in a system which can control arterial PCO_2 and arterial PO_2 [4].

3.1.2. Rebreathing methods

The first rebreathing method used a large rebreathing bag with the possibility to keep hyperoxic conditions. A subject had to breathe in the bag, so CO_2 would accumulate, and inspired PCO_2 would increase central PCO_2. The size of the rebreathing bag ensured a slow increase of CO_2 in the bag, and equilibration of $PETCO_2$ and central PCO_2. The problem of slow rebreathing creates the same problem as in steady-state measurements, the effect of CVR on the response is not considered. To overcome this problem, Read [42] used a smaller rebreathing bag with an initial CO_2 concentration of 7% and 93%

oxygen. With the prefilled bag, he assured that $PETCO_2$ and arterial PCO_2 were quickly increased to venous PCO_2, decreasing the effect of cerebral blood flow on the ventilatory response. Oxygen levels decreased over the measurement (nonetheless hyperoxia was maintained). Another advantage obtained with Read's rebreathing method was that the time of rebreathing was shortened, preventing changes in the sensitivity of the chemoreceptors to occur over the measurement. The main disadvantage of Read's method is that measurements of the ventilatory response under isoxic conditions could not be performed. [4], [6], [42]

In 1988 Duffin et al. modified Read's rebreathing method by adding a hyperventilation maneuver before starting rebreathing. The second modification was the possibility to keep the same percentage of oxygen through a valve system. With the Duffin's rebreathing method, the ventilatory recruitment threshold and the ventilatory response under isoxic and hypoxic conditions can be measured. The latest form of rebreathing is dynamic rebreathing, in which a fast, computerized system is used to provide a flow of gas with fixed CO_2, O_2 and nitrogen levels, which makes it possible to maintain a stable oxygen level and repeat the measurement exactly. [4], [43]

3.2. Plant

The plant can be described by a hyperbola function (figure 5), with a positive asymptote [36]. The function is called the isometabolic hyperbola, as it changes with increased or decreased metabolic production [44]. At the steep part of the hyperbola, a minor change in ventilation can cause the PCO_2 to fall or rise quickly, while at the less steep part of the hyperbola, a fall or rise in PCO_2 is only accompanied by a major fall or rise in ventilation. The isometabolic hyperbola can be measured with a hyper- and hypoventilation test. $PETCO_2$ is measured as a function of the ventilation (contrariwise to HCVR measurement) [45].

3.3. Loop gain

Loop gain is a concept in electronics and control systems theory. Simplified, loop gain determines the behavior of the system in terms of the stability of the system. A stable system has a loop gain between 0 and 1, while an unstable has a loop gain above 1. If a disturbance is applied to an (almost) unstable system, oscillations in the system can be the result. Loop gain is often assessed in the open loop situation, both plant and controller have their own loop gain. In practice plant and controller are not easily separated, as they are inherent related to each other in the feedback system. Therefore, system loop gain should also be measured in the closed loop situation.

3.3.1. Open loop

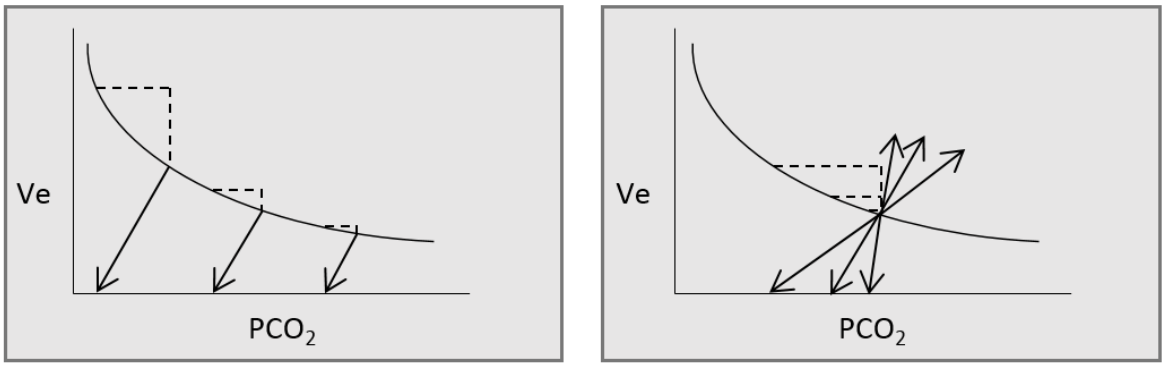
CO_2 reserve is the difference in PCO_2 in normal breathing (eupnea) and apnea. A disturbance to the system can cause apnea more easily as the CO_2 reserve is smaller. A small CO_2 reserve can therefore be interpreted as a high gain, promoting instability. In figure 6 changes in the CO_2 reserve by changed controller or plant gain can be found. Figure 6a shows the plant function, with a low and high gain. Hyperventilation raises ventilation and lowers PCO_2 along the isometabolic hyperbola. This means that a greater increase in ventilation and reduction in PCO_2 is required to reach the apneic threshold than it would be under normocapnic conditions; CO_2 reserve is increased. In hyperventilation, the system is

more stable and has a low gain. Figure 6b, shows the controller function, with a low and high gain in sleep (as the wakefulness drive is gone). With an decreased slope the system is more stable, the gain is low [21]. Central chemoreceptor activity and CVR define the controller gain [11].

System instability can be the result of increased controller sensitivity and/or increased plant gain [46]. A combination of hypoventilation and increased slope is the most susceptible for instability. Furthermore, increased lung wash-out times or delay from lung to chemoreceptors can promote instability. Due to an increased time delay, more fluctuations in PCO_2 and ventilation appear in the system. The chance that the CO_2 reserve is exceeded is higher than in a normal subject. [21]

Controller and plant gain can be a useful tool to describe diseases affecting the control of breathing. As described in Section '*2.4. Disease affecting control of breathing'*, the slope is decreased in subjects with OHS and hypercapnic COPD, controller gain is thus decreased. In patients with CSAS and heart failure, controller gain is increased and patients are therefore at risk for instability of ventilation (especially) in sleep [47]. Various studies suggest that CVR is reduced in subjects with OSAS [21], [48], therefore controller gain is increased. In older subjects CVR is reduced, it can also be reduced with use of various medications [47].

Plant gain can be altered in disorders causing hyper- or hypoventilation, or in metabolic alkalosis [47]. Furthermore, plant gain is increased with restricted lung volumes [49]. In healthy subjects, the plant and controller can react fast on changes induced by the other component [21], due to a small time delay. The time delay is caused by the time it takes for CO_2 to move from lungs to chemoreceptor side, in which cardiac output has a key role. A crucial factor in the pathogenesis of CSAS in heart failure patients is the increased time delay [21]. Due to heart failure, cardiac output decreases resulting in venous congestion which causes the time delay from lungs to chemoreceptor to increase. With an increased time-delay, the system is prone for instability.



a b

Figure 6: Results of changed controller or loop gain. A: change in plant gain due to hyper- or hypoventilation and the effect on the CO_2 reserve (dotted lines). In hyperventilation CO_2 reserve is increased, gain in decreased, in hypoventilation vice versa. B: change in sensitivity to CO_2 and the effect on the CO_2 reserve (dotted lines). With increased sensitivity to CO_2, CO_2 reserve is decreased, gain is increased. With decreased sensitivity, vice versa. Adapted from Dempsey et al. [21]

One should realize that the choice of method and protocol can influence the measured controller gain [6], [38], [50]. With steady-state measurement and with rebreathing measurement as proposed in this study, the response of the 'whole' controller, chemoreceptor sensitivity and CVR, is tested. As described in Section '*2.1.1. Chemoreceptors'*, CVR tends to dampen the effect on CO_2 by increase or decrease of CBF, stabilizing fluctuations in central PCO_2. In theory, CVR and chemoreceptor sensitivity can be measured apart from each other, with Read's and Duffin's rebreathing method, using the prefilled bag as described in Section '*3.1.2. Rebreathing methods*'. The arterial-venous difference in PCO_2 is diminished or completely removed, by taking three breaths from the bag at the start of the test [4], [6]. CO_2-induced changes in CBF do not washout CO_2. Both the cerebrovascular response and central chemoreflex occur, however the effects of both can be separately measured [11]. As the treatment of an unstable system due to a decreased CVR differs from the treatment of an increased sensitivity to CO_2, it is necessary to measure the effects of both separately.

Numerous variations can be made on the steady-state or rebreathing measurements. The protocol can have implications on the measured slope and consequently on the controller gain. At high levels of oxygen the peripheral chemoreflex is almost zero, while in normoxic and hypoxic conditions the sensitivity to CO_2 is affected by the peripheral chemoreceptors [3], [4], [51]. Controller gain is increased in normoxic hypercapnia, relative to hyperoxic hypercapnia. Subjects with instable breathing patterns, should be measured under normoxic conditions, as the controller gain could be underestimated if measured under hyperoxic conditions. Furthermore, left or right shifts can be caused by the protocol of choice. In human studies, $PETCO_2$ is used as a measure for central and arterial PCO_2, as it is too invasive to arterial or central PCO_2. In healthy subjects, $PETCO_2$ and $PETO_2$ can be used as reasonably well measures for arterial PCO_2 and PO_2, as it can be assumed that there is no diffusion limitation. However, a natural existing small difference in arterial and end-tidal pressures can be found, because of under perfusion of parts of the lung (ventilation/perfusion mismatch). Resulting in a $PETCO_2$ slightly smaller (~0.4kPa) than arterial PCO_2, and $PETO_2$ slightly higher than arterial PO_2. At any given work point, $PETCO_2$ and $PETO_2$ are unreliable measures for central CO_2 and O_2. Central PCO_2 is a mixture of arterial and venous PCO_2, while $PETCO_2$ is lower than arterial PCO_2. In the dynamic situation, as is the case in measurement of the controller, three other factors play a role in the relationship between end-tidal and arterial partial pressures; (1) blood transit delay, (2) cerebral blood flow, and (3) the blood-brain barrier [4], [6], [11], [13]. In steady-state measurements, $PETCO_2$ is kept constant for 5-10 minutes, CO_2 can equilibrate over all tissues. Therefore, $PETCO_2$ is a good measure of central PCO_2. In rebreathing, the delay of CO_2 from lungs to brain is not considered, causing $PETCO_2$ to be higher than central PCO_2. Resulting in a right shift of the measured response. In Read's and Duffin's rebreathing method, arterial and venous PCO_2 are equilibrated at the beginning of the test. Therefore, arterial and central PCO_2 are approximately equal, however a small difference is still present. As well as in normal rebreathing a right shift of the curve is expected, however less than in 'normal' rebreathing.

Lastly, the controller gain / slope in steady-state measurements can depend on the chosen levels of $PETCO_2$. It is common to measure only two increased levels of $PETCO_2$, as prolonged exposure can cause the sensitivity to change. It is important to choose the two measured levels $PETCO_2$ above VRT

as the response below VRT is not linear, see figure 5 [4]. If a point below VRT is chosen, the slope and the controller gain are underestimated.

3.3.2. Closed loop

Some subjects have a changed controller gain and/or plant gain, with stable open loop situations. However, closed loop gain (gain of the whole system, a combination of controller and plant gain) can become > 1. Knowledge of the closed loop system is of importance, to find what the controller and plant gain together do to the stability of the system.

Van den Aardweg et al. analyzed the variability of breathing to make it possible to derive information on the performance of the chemoreflexes, instead of finding the capability of the reflex to response. They concluded that variability in $PETCO_2$ and ventilation is not a random process, and that the variability is dependent on loop gain (time delay and time constants). The dependency on loop gain can be found with coherency analysis (power spectra and cross-spectral analysis) of $PETCO_2$ and ventilation data. Analysis of variability could be a new tool to study the chemoreflexes without application an external stimulus [20]. According to Sands et al. two distinct phenotypes of ventilatory instability can be described: instability by increased gain and instability with increased biological noise. In all subjects 'noise' to the system can be induced by e.g. neural variability, sighs, and behavioral effects [31].

4. Methods

4.1. Subjects

Twenty healthy adults were enrolled in the study. On the subject of reproducibility the largest study was the study of Jensen et al. which performed HCVR measurements on 20 subjects [9], therefore we chose to include 20 subjects. The medical ethics committee of Twente (Enschede, the Netherlands) approved the study, as did the local board of directors. All patients provided written informed consent. To be eligible to participate, the subject was aged between 18 and 65 years. A potential subject was excluded if he/she had an existing cardio-pulmonary disease and/or neuro(muscular) disease and/or kyphoscoliosis, was unable to understand and read the English or Dutch language had a history of drug abuse, used respiratory stimulants or depressants (e.g. analeptics and opioids), or was pregnant.

4.2. Study design

At the day of the visit, subjects were instructed to abstain from caffeine, as caffeine acts as a respiratory stimulant, affecting the HCVR [52]. The HCVR was measured with a rebreathing method and steady-state method. Within 5 to 9 days a second visit took place, to repeat the rebreathing and steady-state measurements. During the measurements, the subject was seated and watched an episode of a nature documentary (‘Planet Earth’), to suppress cortical/voluntary activity of the ventilation. There was no follow-up of subjects. The order in which the measurements were performed was randomized (first rebreathing, second steady-state or vice versa). The randomization was performed with a pseudo random number generator. At the second visit, measurements were performed in the same order.

4.3. Methods of measurement

The HCVR was assessed by the rebreathing and steady-state method. The subjects were connected to the Oxycon Pro (Care Fusion, San Diego, USA), which measured inspiratory and expiratory flow with the use of the Triple-V flow sensor, placed in a Hans Rudolph mask (size XS, S, M, L). The airflow through the sensor was sampled to measure breath-by-breath O_2 and CO_2 levels. All measurements were performed under hyperoxic conditions, inspired fraction of oxygen (FiO_2) > 60%, to suppress peripheral chemoreceptor activity. Each day a gas calibration was performed. Before each measurement, a volume calibration procedure was performed on the closed system.

The parts of the rebreathing setup were: an anti-bacterial filter, rebreathing bag, y-piece with valves for in- and expiration, inlet for oxygen and connection tubes (figure 7a). The steady-state setup existed of the same components, with in addition a CO_2-absober canister with filters, two valves (Jaeger closing valve used in body plethysmography at Medisch Spectrum Twente), two Arduino controlled servomotors (SG90 9g micro servo, Towerpro), and an Arduino Mega board (figure 7b). The valves are used to control the flow through both limbs of the expiratory side of the system, with use of two Arduino controlled servomotors. Via an Arduino board, the angle of the valves could be set between 0 and 90 degrees, respectively resulting in a completely open and closed tube. The valves move inverse of each other, thus when the CO_2 bypass valve opens the bypass valve closes and vice versa.

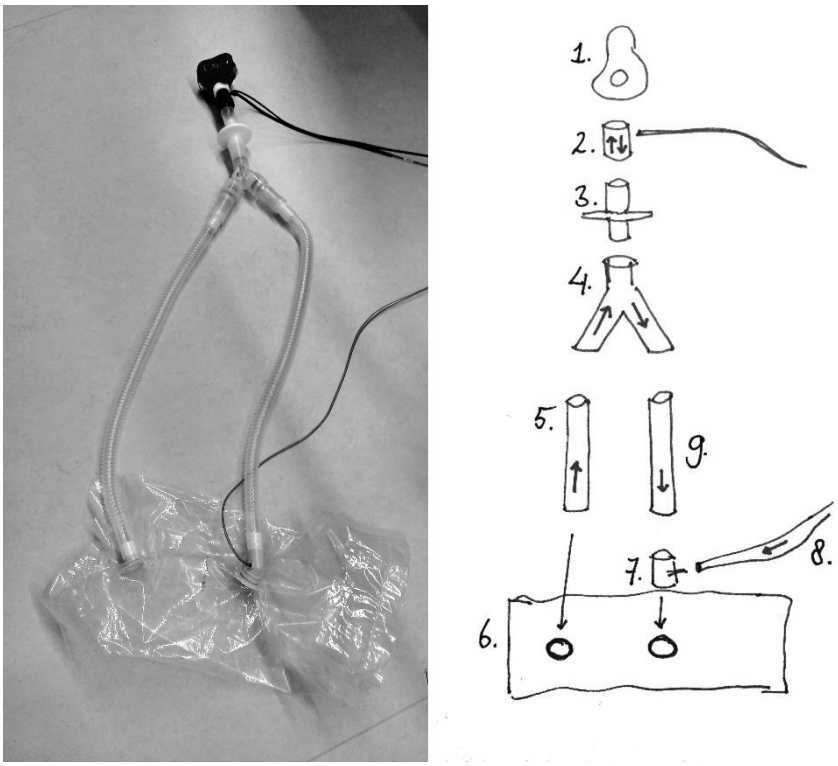


Figure 7a: Setup rebreathing method. 1. Hans Rudolf mask, 2. Triple V transducer with gas sample fiber, 3. Anti-bacterial filter, 4. Y-piece, 5. Connecting tube, 6. Rebreathing bag, 7. Oxygen inlet piece, 8. Oxygen tube, 9. Connecting tube

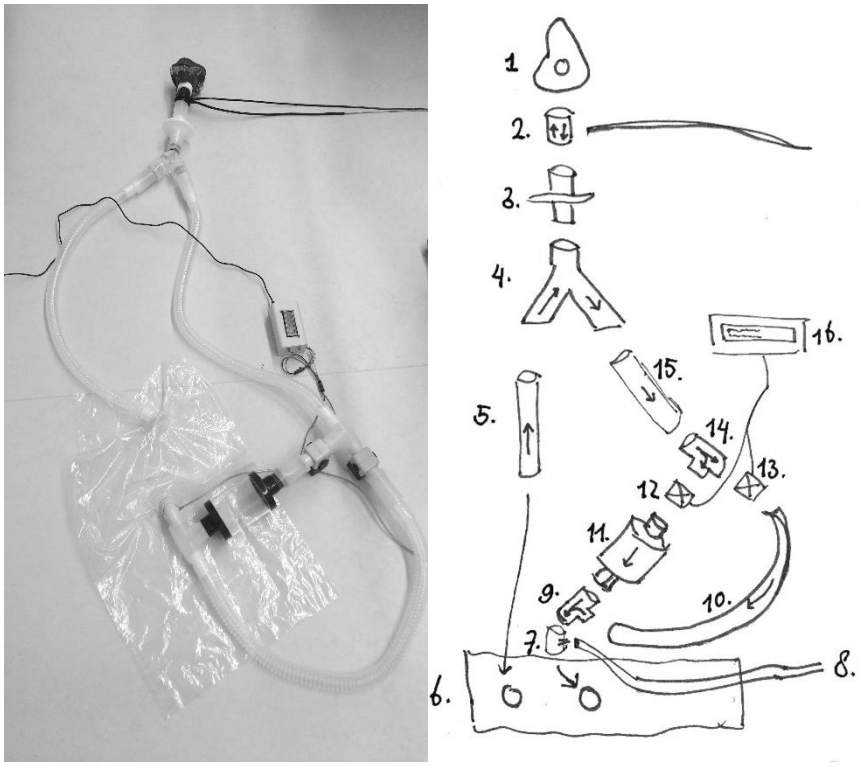


Figure 7b: Setup steady-state method. 1. Hans Rudolf mask, 2. Triple V transducer with gas sample fiber, 3. Anti-bacterial filter, 4. Y-piece, 5. Connecting tube, 6. Rebreathing bag, 7. Oxygen inlet piece, 8. Oxygen tube, 9. Silicone t-piece, 10. Connecting tube, 11. CO2-absorber canister, 12. CO2-absorber valve + servomotor, 13. Bypass valve + servomotor, 14. Silicone t-piece, 15. Connecting tube, 16. Arduino Mega board.

An appropriate sized mask was placed on the face of the subject and was checked for air leaks. A pulse oximetry sensor (Nonin Avant 9600 Pulsoximeter, PT Medical, Leek, The Netherlands) was placed on the finger of the subject. Oxygen was added to the system continuously, to keep FiO_2 above 60%. All measurements were stopped if $PETCO_2$ increased above 8.5 kPa, oxygen saturation became below 90% or the subject indicated he/she was not feeling well. After the stop, recovery of each subject was measured for 3 minutes. Protocols of both measurements can be found in Appendix '*A2. Protocol of measurements*'.

4.3.1. Rebreathing method

The subject started to breathe in an open system (mask, flow sensor and y-valve). After stabilization of the minute volume, rest ventilation was measured for approximately 3 minutes. Subsequently, the rebreathing bag was connected to the y-piece to close the system. Rebreathing increased PCO_2 in the bag and the lungs of the subject, until one of the stop criteria was reached. During the measurement, the start time of the measurement, the start of the rebreathing phase, the start of the recovery phase and the end time of the measurement were listed on a worksheet.

4.3.2. Steady-state method

The subject started to breathe in a closed system (mask, flow sensor, y-valve, bypass arms and rebreathing bag). At the start, the CO_2-absorber arm was open, the bypass valve was closed. Ventilation at 3 levels of $PETCO_2$ were measured with the steady-state method. After stabilization of the minute ventilation, rest ventilation was measured for approximately 3 minutes, this is the reference phase or level 1. Since it is uncertain if the level 1 is above VRT, two levels above level 1 were measured. The increase in $PETCO_2$ cannot be too substantial, because high ventilation levels are not sustained by a subject for 5-10 minutes. Therefore, the two levels above level 1 were $PETCO_2$ of level 1 + 0.5 kPa and + 1.5 kPa. To keep $PETCO_2$ stable at the predetermined levels, the valves were used to increase or decrease CO_2 elimination from the closed system. If ventilation was stable (for approximately 3 minutes), $PETCO_2$ was increased to the next level. After measurement of level 3, $PETCO_2$ was brought back to normal by opening the CO_2 absorber valve. During the measurement, start time of the measurement, start time of each level, start of the recovery phase and the end time of the measurement were listed on a worksheet.

4.3.3. Questionnaires

In total, 6 questionnaires were filled in by each subject over the course of the study. After each measurement, a visual analog scale (VAS)-questionnaire (see Appendix '*A1.1. VAS-questionnaire*') was filled in, with 3 questions to assess the experience of the subject with the method:

1. How did you feel about the duration of the test (1 = very unpleasant, 10 = very pleasant)?
2. How did you experience your breathing (1= very unpleasant, 10=very pleasant)?
3. To what extent did you experience dyspnea (1= no dyspnea, 10 = very dyspneic)?

At the end of a visit, the subject registered in a preference questionnaire (see Appendix '*A1.2. Preference questionnaire*') which method was preferred on basis of the 3 components as answered in the VAS-questionnaire, in addition the subject had to choose the overall preference.

4.4. Analysis

All data was analyzed offline with use of Mathworks MATLAB 2016a. Five parameters were used from the data; time, inspired fraction of oxygen (FiO_2), tidal volume, total breath time, and $PETCO_2$. Before analysis all parameters were filtered with a moving average filter of 5 breaths.

Rebreathing method: VRT had to be determined objectively from the $PETCO_2$-ventilation curve. A coefficient of determination (R^2) provides information on the accuracy of the fit of a linear regression through various data points. If R^2 was close to zero, the fit was poor, if it was close to 1 the fit was very good. It was assumed that VRT was reached within 10 breaths from the start of the rebreathing phase. Linear regression was performed on the rebreathing phase minus the first n samples, with a maximum of 10 samples. R^2 of all 10 regression lines were used to determine VRT. Theoretically, R^2 reaches a maximum at VRT. The data points above $PETCO_2$ values at VRT were used to calculate the sensitivity to CO_2 (slope-R), with linear regression (see figure 8). To answer if rebreathing could be less burdensome for the subject, the slope (short slope-R) was also calculated as the slope of the minute ventilation as function of $PETCO_2$ calculated over a smaller delta $PETCO_2$ (VRT to VRT + 1.5 kPa). If a subject did not increase 1.5 kPa above VRT, the short slope-R was not calculated.

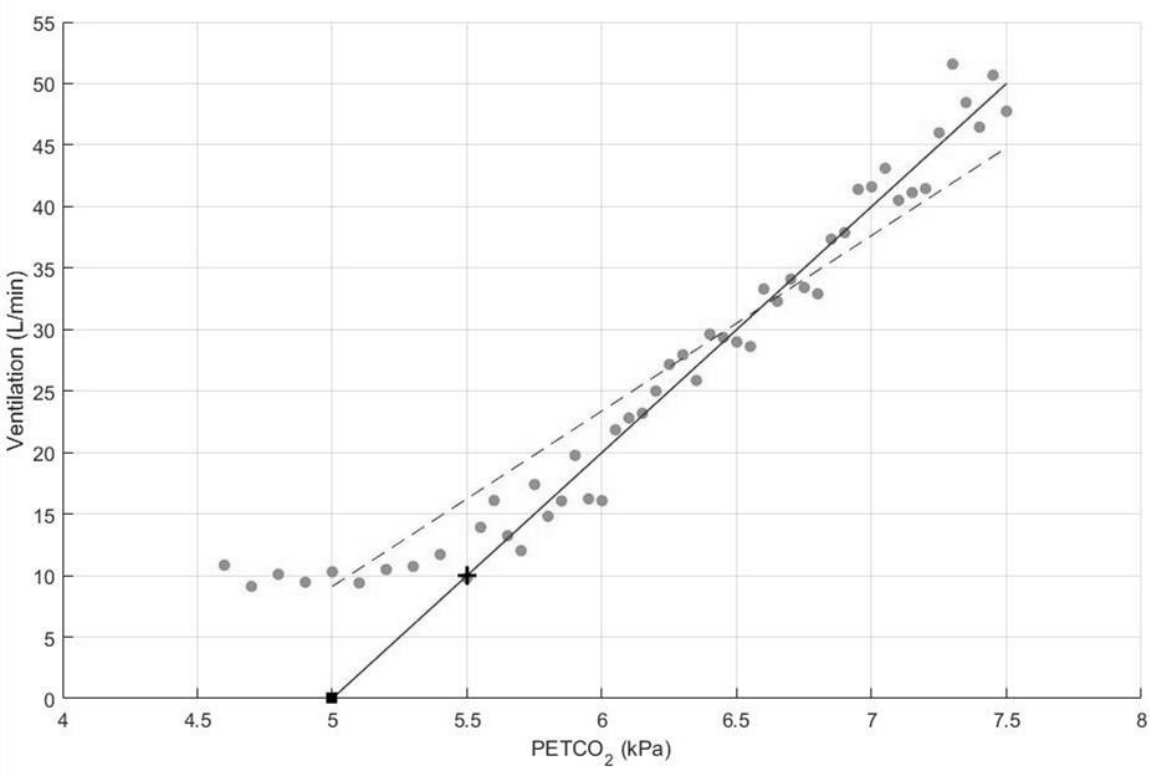


Figure 8: Analysis of rebreathing method, simulation of a measurement. Circles represent the ventilation response with $PETCO_2$ (kPa) on the x-axis and ventilation (L/min) on the y-axis. The plus sign indicates the ventilatory recruitment threshold, $PETCO_2$ above ventilatory recruitment threshold is used to calculated the linear relation between $PETCO_2$ and ventilation (slope-R), the continuous line. The dashed line is an underestimated slope, as a result of the use of points below VRT (as can be recalled from Section '*3.3.1. Open loop*'). The projected apnea threshold is indicated by a square.

Steady-state method: In the last minute of the reference phase (level 1), level 2 and level 3, mean $PETCO_2$ and mean ventilation were calculated. The sensitivity to CO_2 (slope-SS) was calculated by linear regression of the 3 mean points, see figure 9. The total duration of each level was calculated. To test if ventilation was indeed stable at the end of a level, the COV of the ventilation data in the last minute of all three levels was calculated. To tests if $PETCO_2$ could be kept adequately stable with the valves, the COV of $PETCO_2$ data was calculated in level 2 and 3, the rise to the predetermined $PETCO_2$ was not used in this calculation.

Projected apnea threshold (pAT): The 'projected' (as it not existing in awake subjects) apnea threshold is calculated, by projecting slope-R and slope-SS onto the x-axis, as can be seen in figure 8 and figure 9 (black squares).

FiO_2: All measurements were performed under hyperoxic conditions (FiO_2>60%), to silence the peripheral chemoresponse. To evaluate whether these conditions were met, the percentage of time FiO_2 is above 60% was calculated.

4.5. Statistics

Statistics were performed with IBM SPSS Statistics 24. All parameters were tested for normality. All continuous variables were expressed as the mean with standard deviation or as the median with interquartile range (IQR), as appropriate.

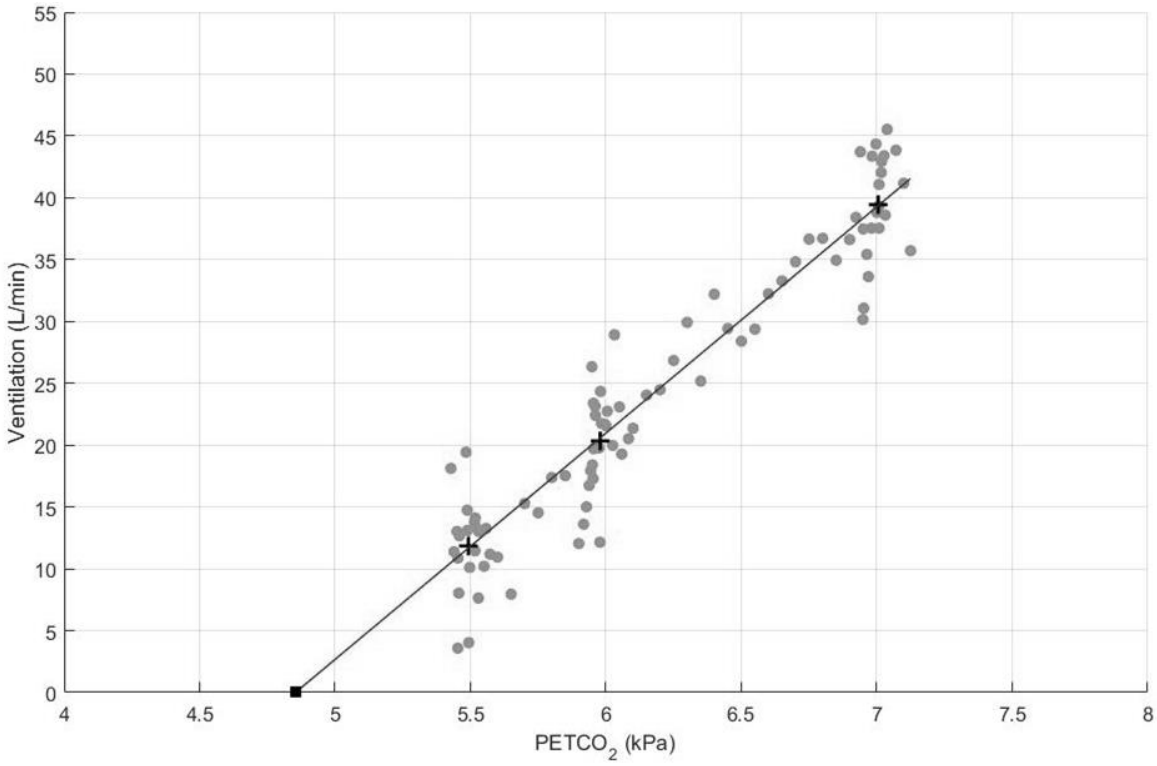


Figure 9: Analysis of steady-state method, simulation of a measurement. Circles represent the ventilation response with PETCO2 (kPa) on the x-axis and ventilation (L/min) on the y-axis. Three levels can be distinguished (~5.5, ~6 and ~7 kPa). The mean $PETCO_2$ and ventilation per level are shown as the plus signs. Linear regression of the three mean $PETCO_2$ and ventilation points gives the sensitivity to CO_2 (slope-SS), the continuous line. The projected apnea threshold is indicated by a square.

A Bland-Altman plot was made of the slopes between first and second measurement of the same method. Furthermore, Bland-Altman plots were made of the slopes and projected apnea threshold between the two methods. The limits of agreement were calculated with 1.96 times the standard deviation of the difference in slopes or projected apnea thresholds.

An ICC was calculated to evaluate the correlation between (1) the first and second measurement of the same method and (2) between the two methods. The former answered the question on the reproducibility of the measurement, and was the primary outcome of this study. The ICC was calculated for long and short slopes. If the ICC was above 0.8, the measurements were assumed highly correlated. A p-value below 0.05 indicated a significant correlation. A two-way mixed model was used.

Paired t-tests were performed to find significant difference between the mean of various parameters. The compared parameters were: (1) slope-R and slope-SS, (2) $PETCO_2$ level 1 (steady-state) and $PETCO_2$ at VRT (rebreathing), (3) $PETCO_2$ level 2 (steady-state) and $PETCO_2$ at VRT (rebreathing) (4) total duration measurement 1 and 2 of the same method, (5) duration of levels in steady-state in measurement 1 and 2, (6) percentage of time FiO_2 was above 60% of measurement 1 and 2 of the same method and (7) outcome of the questionnaires between rebreathing and steady-state. The paired t-test had the null hypothesis that both samples are from the same population. A p-value < 0.05 indicated a significant difference in mean between the two methods. If data was not-normally distributed a Wilcoxon signed rank test was performed.

5. Results

5.1. Subjects

Twenty-six subjects were assessed for eligibility of whom two did not meet the inclusion criteria and two declined to participate. Twenty-two healthy volunteers participated in the study, one subject retreated from the study after the first visit and one subject was excluded, because they were uncomfortable during the rebreathing test. Of the remaining twenty subjects, eleven were men. The median (IQR) age was 39 (26-53) years, the median length was 179 (169-183) centimeters, and the median weight was 72 (65-87) kilos. All obtained parameters were not normally distributed.

5.2. Slopes

To visualize the results, a Graphical User Interface (the HCVR-GUI) was made, see Appendix '*A3. Graphical User Interface*'. Two subjects were not able to reach level 3 in the steady-state measurements, these two are excluded if calculations are made with slope-SS of measurement 2. Median slope-R over the population is 12.9 (9.2-16.3) L/min/kPa in measurement 1, and 11.6 (7-17.2) L/min/kPa in measurement 2. Median slope-SS over the population is 13.5 (8.3-17.3) L/min/kPa in measurement 1, and 13.4 (8.4-16.9) L/min/kPa in measurement 2. In figure 10, a scatterplot of slope-R of measurement 1 vs 2 (circles) and slopes-SS of measurement 1 vs 2 (squares) can be found.

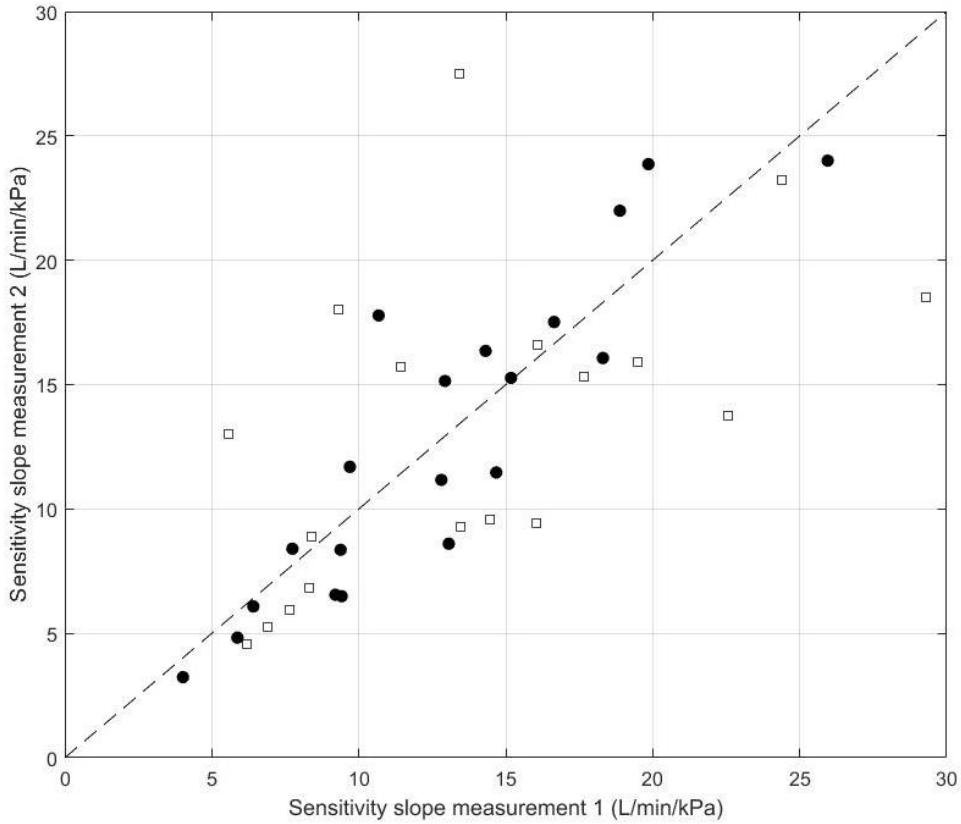


Figure 10: Scatterplot of measured slopes (L/min/kPa) of first vs second measurement. In the scatterplot, the slopes of the first and second measurement are plotted against each other, rebreathing slopes are circles (20 subjects), steady-state slopes are squares (18 subjects). Each circle or square indicates one subject. Measurement 1 is on the x-axis, measurement 2 on the y-axis. The dashed line indicates y=x.

Short slope-R was calculated with $PETCO_2$ at VRT to VRT + 1.5 kPa. Two subjects did not reach $PETCO_2$ at VRT + 1.5 kPa, these two subjects were excluded for calculation of ICC. Median short slope-R is 10.0 (6.7-15.6) L/min/kPa and 8.9 (6.4-15.0) L/min/kPa, for measurement 1 and 2 respectively.

The ICC of all combinations made can be found in table 1. The ICC of slope-R measurement 1 vs measurement 2 is 0.89, the ICC of slope-SS measurement 1 vs measurement 2 is 0.56. An ICC > 0.8 is considered a good agreement. The ICC was calculated for slope-R vs slope-SS for both measurements. The ICC between methods of the first measurement is 0.50, and 0.87 for the second measurement. With a Wilcoxon Signed Rank test it was demonstrated that slope-R and slope-SS showed no significant difference in median (p-values: 0.15 and 0.35, respectively first and second measurement). The ICC of short slope-R between measurement 1 and 2 was 0.78 (p-value < 0.05).

Two Bland-Altman plots were made of slope-R of measurement 1 and 2, and of slope-SS of measurement 1 and 2, see figure 11. The mean slope difference in measurement 1 and 2 is for both methods close to zero. The limits of agreement of slope-R are ~12 L/min/kPa and of slope-SS ~26 L/min/kPa. There is no trend in the mean slope.

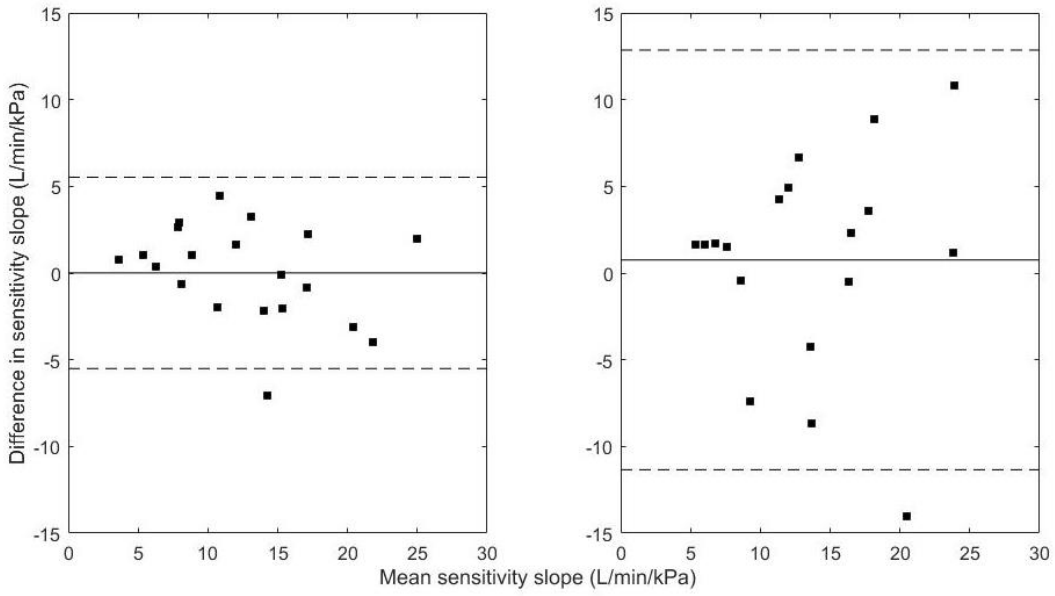


Figure 11: Bland-Altman of slopes of measurement 1 and 2. Mean of slopes of on the x-axis in L/min/kPa and the difference between slopes on the y-axis in L/min/kPa. Left: rebreathing method (20 subjects), right: steady-state method (18 subjects).

Table 1: ICC of slopes (first 4 are within method, last 2 are between methods)

Measurement	ICC	95% CI	p-value
slope-R measurement 1 vs 2	0.89	0.73-0.95	<0.01
slope-SS measurement 1 vs 2	0.56	0.14-0.81	0.01
short slope-R measurement 1 vs measurement 2	0.78	0.51-0.91	<0.01
slope-R vs slope-SS measurement 1	0.50	0.08-0.76	0.01
slope-R vs slope-SS measurement 2	0.87	0.69-0.95	<0.01

Furthermore, two Bland-Altman plots were made of slope-R versus slope-SS for both measurements, see figure 12. The mean difference between slope-R and slope-SS over the population is close to zero. The limits of agreement are ~24 L/min/kPa for measurement 1 and 13 L/min/kPa for measurement 2. There is no trend in the mean of slope-R and slope-SS.

5.3. Projected apnea threshold

Median pAT in rebreathing is 4.11 (3.76-4.78) kPa in the first measurement, and 4.14 (3.61-4.51) kPa in the second measurement. Median pAT in steady-state is 3.92 (3.46-4.31) kPa in the first

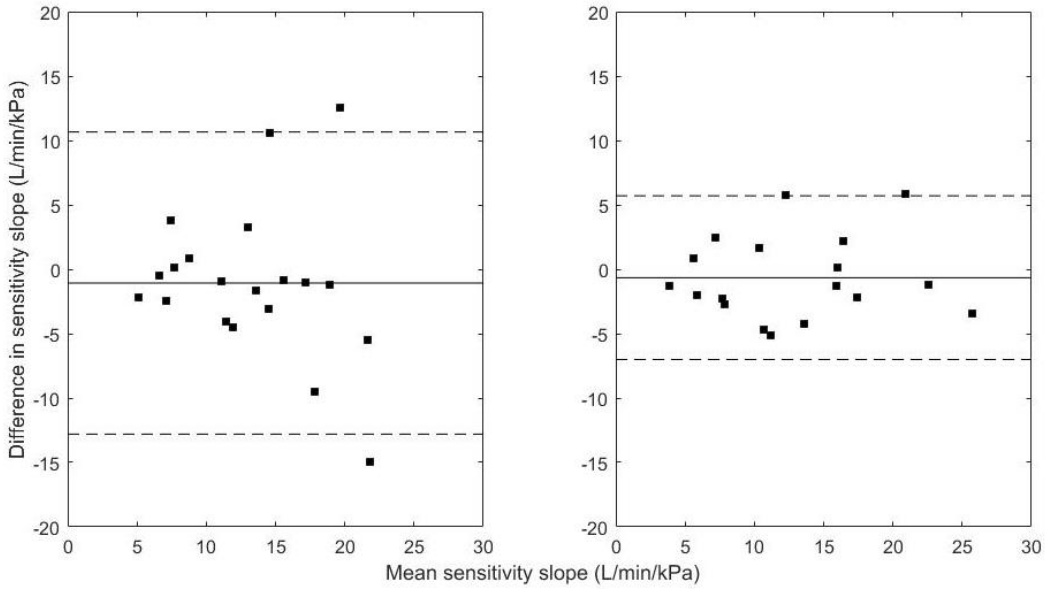


Figure 12: Bland-Altman of slope of the rebreathing and steady-state method. Mean of the slopes on the x-axis in L/min/kPa and the difference between slopes on the y-axis in L/min/kPa. Left: measurement 1 (20 subjects), right: measurement 2 (18 subjects)

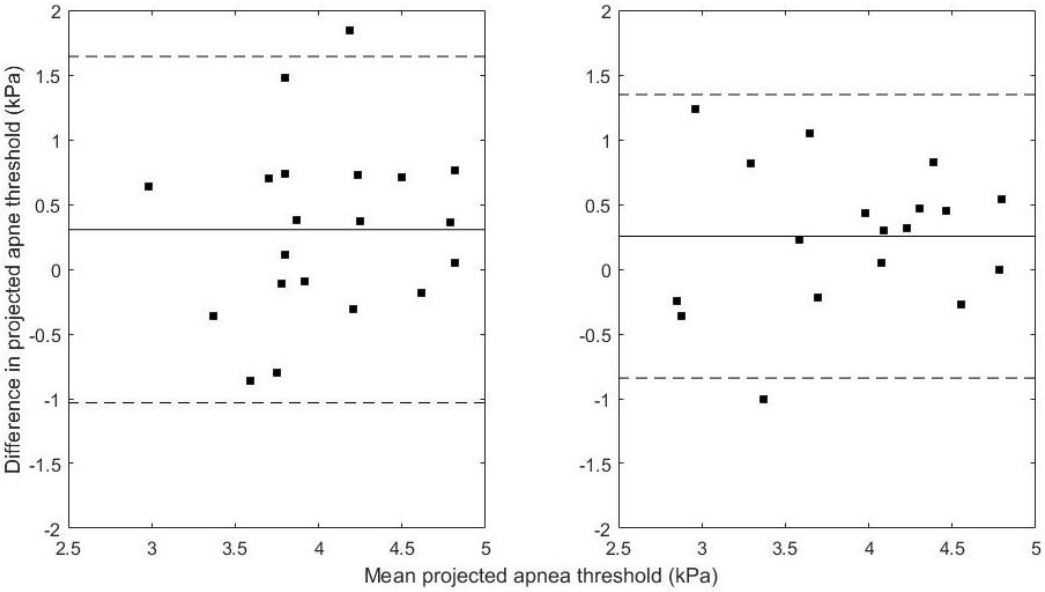


Figure 13: Bland-Altman of projected apnea threshold of the rebreathing and steady-state method. Mean of the projected apnea threshold on the x-axis in kPa and the difference between projected apnea threshold on the y-axis in kPa. Left: measurement 1 (20 subjects), right: measurement 2 (18 subjects)

measurement, and 3.84 (3.00-4.07) kPa in the second measurement. Furthermore, two Bland-Altman plots were made, in figure 13, the Bland-Altman of pAT of measurement 1 and measurement 2 can be found. A mean systematic error between the projected apnea thresholds between methods can be found in the plots of 0.2 kPa. The limits of agreement are ~2.7 kPa for measurement 1 and ~2.1 kPa for measurement 2. There is no trend in the mean of slope-R and slope-SS.

5.4. Variability

The variability of the steady-state data was expressed with the COV of the ventilation and $PETCO_2$ data. Table 2 shows the median ventilation COV and the median $PETCO_2$ ventilation.

Table 2: Median (IQR) coefficient of variation of ventilation and PETCO2 per level per measurement

Measurement	Level	Median Ventilation COV	Median $PETCO_2$ COV
1	1	3.0% (2.6-5.6%)	-
	2	2.8% (2.1-4.5%)	19% (16-28%)
	3	4.0% (2.1-5.9%)	38% (28-43%)
2	1	5.4% (3.3-6.8%)	-
	2	2.9% (2.2-5.1%)	20% (16-27%)
	3	3.4% (2.8-4.8%)	39% (24-42%)

5.5. End tidal carbon dioxide levels (steady-state)

Over the population, median $PETCO_2$ at level 1 was 5.0 (4.6-5.2) kPa, and 4.8 (4.5-5) kPa, respectively measurement 1 and 2. Median $PETCO_2$ at level 2 was 5.5 (5.1-5.8) kPa, and 5.3 (5-5.5) kPa, respectively measurement 1 and 2. Median $PETCO_2$ at VRT in rebreathing was 5.2 (4.8-5.5) kPa, and 5.1 (4.8-5.3) kPa, respectively measurement 1 and 2.

To see if $PETCO_2$ at level 1 and 2 exceeded $PETCO_2$ at VRT (derived from the rebreathing test), $PETCO_2$ level 1 and 2 are compared with $PETCO_2$ at VRT using a Wilcoxon signed rank test. In 16 out 20 cases $PETCO_2$ level 1 was lower than $PETCO_2$ VRT for measurement 1. In 18 out of 20 cases $PETCO_2$ level 1 was lower than $PETCO_2$ VRT for measurement 2. In both measurements, there was a significant difference in median between $PETCO_2$ at level 1 and at VRT (p-value: 0.01 and <0.01). In 17 out 20 cases measured $PETCO_2$ level 2 was higher than $PETCO_2$ at VRT for measurement 1. In 19 out of 20 cases measured $PETCO_2$ level 2 was higher than $PETCO_2$ at VRT. In both measurements 1 and 2, there was a significant difference in median between $PETCO_2$ at level 2 and at VRT (p-value: <0.01 and <0.01).

5.6. Duration

The median duration of the steady-state measurements was 23.5 (21-25.8) minutes and 22.5 (20-25) minutes respectively for the first and second measurement. The median of the duration of the rebreathing measurements was 18.5 (17-21.5) minutes and 17 (15-21) minutes respectively for the first and second measurement. A Wilcoxon Signed Rank test was performed on the duration of the first measurements, and on the duration of the second measurements. The duration of the rebreathing tests was significantly shorter than the duration of the steady-state tests, with a p-value of <0.01 (measurement 1 and measurement 2). The mean difference in duration for measurement 1 was 4.5 (2-

6.8) minutes, for measurement 2 it was 5.5 (3.3-6.8) minutes. With a Wilcoxon signed rank test it was tested if median duration of steady-state level 1, 2 and 3 differed significantly between measurement 1 and 2. The duration of level 1 and level 2 did not significantly differ in median, p-value was 0.66 and 0.84, respectively. The duration of level 3 did not significantly differ in the two measurements, with a p-value of 0.08. In 14 out of 18 subjects level 3 duration was longer in measurement 1 than in measurement 2.

5.7. Inspired oxygen fraction

FiO_2 was above 60% for 93% (66– 97%) of the time and 95% (78– 97%) of the time for the first and second rebreathing test, respectively. FiO_2 was above 60% for 100% (93– 100%) of the time and 100 (78 – 100%) of the time for the first and second steady-state test, respectively. A Wilcoxon Signed Rank test was used to find if there was a difference between percentage of time FiO_2 was above 60% in measurement 1 and 2. It was not-significantly different, with a p-value of 0.60 (rebreathing method) and 0.77 (steady-state method).

5.8. Questionnaires

The six questionnaires were completed by all subjects. In table 3, the VAS-scores of measurements 1 and 2 can be found. VAS-scores were compared between steady-state and rebreathing, with a Wilcoxon Signed Rank test. Time duration of the rebreathing and steady-state test was not scored significantly different (p-values: 0.62 and 0.94 for measurement 1 and 2, respectively). The breathing comfort was scored significantly different (p-values: 0.01 and <0.01 for measurement 1 and 2, respectively). The dyspnea sensation was significantly different in measurement 1 (p-value: 0.04) and not in measurement 2 (p-value: 0.06). Breathing was experienced to be easier in steady-state. The dyspnea sensation was less in steady-state.

In both measurements, 16 out of 20 subjects chose the steady-state as method of preference, while 4 chose the rebreathing method. On all three areas (duration, breathing comfort and dyspnea sensation), the steady-state method was preferred by most subjects, see table 4.

Table 3: Median VAS-scores on for both methods for the first (M1) and second measurement (M2)

	Rebreathing		Steady-state	
	M1	M2	M1	M2
Question 1: Duration	6.0 (5.0-7.0)	5.5 (5.0-6.8)	5.5 (4.0-7.8)	6.0 (5.0-7.0)
Question 2: Breathing comfort	5.0 (4.0-6.0)	4.5 (4.0-5.8)	6.0 (5.0-7.0)	6.0 (4.0-7.0)
Question 3: Dyspnea	4.0 (4.0-5.8)	4.0 (4.0-6.0)	3.5 (2.3-4.8)	4.0 (2.3-5.0)

Table 4: Number of subjects per preference (rebreathing, steady-state, no preference) per measurement (M1, and M2)

	Rebreathing		Steady-state		No preference	
	M1	M2	M1	M2	M1	M2
Question 1: Least problems with duration	n=5	n=5	n=10	n=11	n=5	n=4
Question 2: Easiest breathing	n=4	n=4	n=16	n=15	n=0	n=1
Question 3: Least dyspneic	n=3	n=2	n=14	n=17	n=3	n=1
Question 4: Method of preference	n=4	n=4	n=16	n=16	-	-

6. Discussion

The aim of this study was to answer: *"Which method, steady-state or rebreathing, should be implemented in clinical practice to measure the ventilatory response?"*. To answer this, the reproducibility of both methods was investigated. Secondly, the experience with the methods of all subjects was obtained and it was determined if the methods could be less burdensome for the subjects. Furthermore, a theoretical framework provided insights in the clinical application of both methods. In this chapter, the outcomes will be discussed. It can be concluded that the rebreathing method is the method of preference for implementation in clinical practice.

6.1. Reproducibility

The ICC (95% CI) of the rebreathing measurements was 0.89 (0.73-0.95). The ICC of the steady-state measurements was smaller, i.e. 0.56 (0.14-0.81). The rebreathing measurements are considered reproducible with an ICC > 0.8, whilst the steady-state measurements are not. The 95% CI of the steady-state measurements is wide, suggesting that the measurements are reproducible in some subjects and in others not.

As can be recalled from Section '*1.1. Rationale*', two studies calculated a correlation coefficient to determine reproducibility. In the study of Strachova et al. the long-term reproducibility of Read's rebreathing method was tested in 13 subjects [5]. They found a correlation coefficient of 0.939 between the mean slopes of the first and second session, which is higher than in our study. In the study of Strachova et al., the correlation was determined with a Spearman's ranked correlation coefficient, which implicates that a systematical error between measurements is not detected. Furthermore, the correlation was calculated between averaged values of three measurements. This accounts for the small disagreement between the study of Strachova et al. and our study. In 2010, a larger study was performed by Jensen et al., they calculated the ICC of Duffin's rebreathing method under hyper- and hypoxic conditions between-days and within-days. An ICC of 0.78 (0.58-0.90) was found between-days. All tests were performed in the same manner, first hypoxic test, thereafter hyperoxic test. The addition of hypoxic tests may have resulted in a higher variation in measured slope, as hypoxia can change the sensitivity of the chemoreceptors [4]. This could explain the lower ICC and the broader 95% CI, than found in this study.

Berkenbosch et al. concluded that the slopes measured with Read's rebreathing and a dynamic end-tidal forcing steady-state method showed good correspondence over consecutive days [6]. This contradicts the results of the steady-state measurements obtained in this study. In the study of Cohen et al. 4 newborn subjects underwent steady-state measurements twice, the mean difference in the slopes between measurements was 150% [7]. This is in correspondence with the poor reproducibility of this study. Nevertheless, crucial differences in study design exist between our study and the study of Berkenbosch et al. [6] and Cohen et al. [7]. In the study of Cohen et al. newborns were measured, whereas in this study adult subjects were measured. Therefore, the comparison between our study and Cohen's study may not be in place, as control of breathing in newborns shows more variation [53], which may have caused the measurements to not be reproducible. Berkenbosch et al. used the mean of 3 to 6 slopes of only 3 subjects to determine the correspondence in slope over several days. In our study,

steady-state measurements are performed manually, whereas Berkenbosch performed the steady-state measurements with the dynamical forcing technique. With the use of this technique $PETCO_2$ and $PETO_2$ can be regulated very well. Secondly, they accounted for non-linearities in the steady-state measurement. Thirdly, Berkenbosch et al. accounted for the length of the measurement as longer exposure to CO_2 can cause the sensitivity to change while measuring. Lastly, the subjects measured by Berkenbosch et al. were all men. If the small population size in the study of Berkenbosch et al. is ignored, several hypotheses can be made for the overall poor reproducibility of steady-state measurements in this study based on comparison of the study designs; (1) non-linear part of the ventilation response leads to underestimation of slope, (2) difference in steady-state method, (3) duration of the measurement can change the sensitivity of the chemoreceptors intra-measurement and (4) gender differences. These hypotheses will be discussed further.

6.1.1. Non-linearities

In most subjects (16/20 and 18/20), $PETCO_2$ of level 1 in the steady-state measurement was below $PETCO_2$ at VRT in the rebreathing measurement. $PETCO_2$ of level 2 was in 18 out of 20 in the first measurement and 19 out of 20 in the second measurement above VRT. To account for the non-linearity of the slope at level 1, the slopes were calculated with mean $PETCO_2$ and ventilation of level 2 and 3, instead of all three levels. Tests were performed retrospectively, see Appendix '*A4. Reproducibility of short steady-state slopes'*. The ICC of the slope with the levels above VRT were 0.62 (0.23-0.84).

In the study of Berkenbosch et al., $PETCO_2$ levels inducing a severe ventilatory response (50 L/min) were avoided. Subjects may be unable to sustain these high minute volumes for 5 to 6 minutes, due to fatigued respiratory muscles, causing non-linearities. Non-linearities at high $PETCO_2$ levels were not observed in this study. Likewise, it is important to choose $PETCO_2$ levels above the VRT, as can be recalled from Section '*3.1. Controller'*. In our study, level 1 was in almost all subjects below VRT. Therefore, slope-SS could have been underestimated [4], [6], [9], [38], [50]. The underestimation could differ between measurements, causing the steady-state slopes to show more variation, causing ICC to be low. However, even when accounting for the non-linearities at level 1, reproducibility increased only slightly, and was poor in most subjects (ICC: 0.62), which is in contradiction to the results of Berkenbosch et al. [6].

6.1.2. Difference in steady-state method

$PETCO_2$ level 2 and 3 showed a mean COV of 19% and 39% for measurement 1 and 20% and 39% for measurement 2. The median percentage of time FiO_2 was above 60% did not differ significantly between measurement 1 and 2. In the protocol of the steady-state method, two main actions must be performed by the observer: keeping FiO_2 above 60%, and regulation of $PETCO_2$ by changing the valves manually. The position of the valves was subjectively changed on basis of $PETCO_2$ levels on the screen. Opening or closing the valves has a delayed effect on $PETCO_2$. The delay is caused by the volume in the rebreathing bag and minute ventilation. Due to poor prediction of the time delay by the observer, $PETCO_2$ is prone to fluctuations. In contrast with our study, $PETCO_2$ and $PETO_2$ levels of the study of Berkenbosch et al. were constant, as they used dynamic end-tidal forcing. As can be recalled from Section ', with steady-state measurements it is intended to keep $PETCO_2$ constant, to establish an

equilibrium in all tissues. In the study of Berkenbosch et al. it is certain that an equilibrium between $PETCO_2$ and central PCO_2 is reached. In this study $PETCO_2$ shows a high variation. The high variability may be a key factor in the poor reproducibility of the steady-state measurements, as it is not certain that $PETCO_2$ reflected the true central PCO_2. Which can introduce shifting of the curve. If this has occurred at one of the levels, the calculation of the slope was influenced. Resulting in higher variability and a poor ICC. It is assumed that there is no oxygen-caused variation in sensitivity, as there was no difference in FiO_2 between the measurements.

6.1.3. Duration of the measurement

In this study, the exposure times to CO_2 on each level were not different between both measurements. The duration of level 1, level 2 and level 3 did not significantly differ in median between measurement 1 and 2. Furthermore, the coefficients of variation of ventilation were low. In measurement 1, 3.0%, 2.8%, and 4.0% for levels 1,2 and 3 and in measurement 2, 5.4%, 2.9% and 3.4% for levels 1, 2 and 3.

Poor equilibration between $PETCO_2$ and central PCO_2 is not solely caused by variation in $PETCO_2$, it can also be caused by insufficient exposure time to CO_2. Resulting in right shifts of the measured response, as the CO_2 measured it higher than the actual CO_2 at the central chemoreceptors. If this has occurred at one of the levels, the calculation of the slope was influenced. However, the coefficient of variation is low in ventilation, suggesting that in both measurements stable ventilation levels were obtained, concluding that it is likely that the subjects were in an equilibrium state.

In the study of Berkenbosch et al. two levels were measured to prevent a change in the sensitivity to occur within a measurement. In our study, we measured three levels, however only in two levels the subject was exposed to hypercapnia. It is therefore not likely that the subjects were exposed to CO_2 for too long, causing the sensitivity to change within the measurements.

6.1.4. Gender differences

Additional tests were performed to find the reproducibility of the data, based on gender, see Appendix '*A5. Reproducibility in men and women*'. Both men and women show a significant ICC with a wide 95% CI for steady-state measurements. ICC for men is 0.51 and for women 0.66, suggesting that there is no difference between how men and women perform in the steady-state measurement. These results suggest that the steady-state reproducibility was not affected by the inclusion of women. The rebreathing measurements were good reproducible, despite the inclusion of women. There is no consensus about the effects of female sex hormones on the sensitivity to CO_2. Beidleman et al. performed HCVR measurements in healthy subjects with Read's rebreathing method in 8 women at two different days in their menstrual cycle, they found no significant mean difference in the slope between the two days (follicular phase and luteal phase) [54]. Likewise, MacNutt et al. performed a study to find differences between sensitivity of the chemoreceptors on two days in the menstrual cycle. They found that the sensitivity to chemical stimuli was unaffected by menstrual-cycle phase [55]. In contrast, Jensen et al. concluded that the ventilatory response in pregnant women is augmented due to the effects of increased progesterone [56]. In our study, the women who participated were not pregnant, therefore the augmentation of the slope found by Jensen et al. was not likely to occur in this study. Furthermore, most

women in this study used anti-conceptive medicine, stabilizing hormone levels. Reducing the chance that female hormones would change the ventilatory response between measurements.

6.1.5. Agreement between rebreathing and steady-state slope

Based on the previously stated causes of poor reproducibility of the steady-state measurements, it can be concluded that there exists no substantial difference in protocol between measurement 1 and 2. However, when the agreement between rebreathing and steady-state slope is investigated, the agreement between the method increases between measurement 1 and 2. The ICC between the methods, reflecting the agreement of rebreathing and steady-state method, is 0.50 (0.08-0.76) and 0.87 (0.69-0.95) respectively for measurement 1 and 2. It is hypothesized that a certain learning curve for the steady-state method exists. Resulting in more agreement between rebreathing and steady-state measurement 2, and poor reproducibility between steady-state measurement 1 and 2.

6.2. Subject's preference

One subject was excluded after the first rebreathing measurement, due to persistent complaints of headache and nausea. Another subject quitted after the first visit, being uncomfortable to performed the measurements again. 16 out of 20 subjects chose the steady-state as the method of preference at both visits. The preference for the steady-state measurement can be explained with the results of the VAS-questionnaire. Breathing experience was more pleasant in both steady-state measurements relative to the rebreathing measurements and in the first measurement, the dyspnea sensation was lower in the steady-state measurement than in the rebreathing measurement. No other studies that we are aware of have studied the experience of the subjects with both rebreathing and steady-state tests. However, the absolute difference between VAS-scores was small. We concluded that the difference in burden of both methods on subjects is not clinically relevant.

It is hypothesized that the rebreathing test is experienced less pleasant, due to the higher levels of $PETCO_2$ reached in the measurement. With higher $PETCO_2$ levels, ventilation is increased more in rebreathing than in steady-state, which could have caused the sense of dyspnea to be higher in rebreathing. Furthermore, the fall in $PETCO_2$ (from maximal $PETCO_2$ to $PETCO_2$ in recovery) is more substantial in the rebreathing method. The quick fall in $PETCO_2$, causes vasoconstriction in the brain, due to reactivity of the vascular bed to CO_2 [11]. It is hypothesized that side effects (e.g. nausea, headache) are more present in rebreathing measurements, due to the more substantial vasoconstriction and rise in $PETCO_2$. However, this is merely speculation as the side effects were not listed apart for each test.

6.3. Decrease of burdensome of measurement

In steady-state it is not possible to decrease the burdensome of the measurement. It is necessary to expose subjects to at least two levels of $PETCO_2$ above rest $PETCO_2$. Furthermore, the exposure times cannot be decreased.

Based on the results of the questionnaires, it was hypothesized that the high $PETCO_2$ levels in rebreathing were associated with increased burden on the patient. The data of 18 subjects was used to calculated short slope-R. The ICC of short slope-R between measurement 1 and 2 was 0.78 (0.51-0.91)

and was significant, indicating that the rebreathing measurement were reasonably reproducible with less increase in $PETCO_2$. However, not as reproducible as the normal slope-R.

No studies were found in which it was investigated what the necessary increase in $PETCO_2$ was to find a reliable slope. In all studies including rebreathing measurements, subjects were exposed to $PETCO_2$ levels more than 7 kPa at the central chemoreceptors (e.g. Mackay et al. 55 Torr ~ 7.3 kPa central PCO_2 [11], Berkenbosch et al. ~ 8.3 kPa central PCO_2 [6]). In various studies it has been postulated that CO_2 stabilizes the respiratory controller, and that therefore a more stable breathing pattern is expected on hypercapnia [57], [58]. More variability exists in ventilation at lower levels of $PETCO_2$, and therefore the slope can vary more between measurements. This could explain the difference in ICC between slope-R and short slope-R (0.89 and 0.78, respectively). The same datasets were used to obtain slope-R and short slope-R, which could cause the results to be less reliable.

6.4. Clinical Application

The steady-state method as proposed in this study, is not applicable in clinical practice, as the reproducibility is poor. In contrast, the rebreathing method was reproducible and could be a good method to assess the ventilatory response of patients. However, it should be realized that the choice of method can influence the measured CO_2 sensitivity or controller gain and it can have consequences for the application of treatment. As described in Section '*3.3.1. Open loop*', variations in method can influence the measured controller gain through (1) effects of CVR, (2) oxygen levels, (3) shifting of curves, and (4) exposure time. The latter can be disregarded for future experiments with the methods used in this study. As the exposure times to hyperoxia and hypercapnia, in this study, are not long enough to change the sensitivity during the measurement [59].

Furthermore, it is possible that the cause of instable control of breathing, is not found with measurement of the controller only. A combination of plant and controller gain can be the problem. Furthermore, a time delay and noise can cause problems, while the controller is functioning normal.

6.4.1. Cerebrovascular reactivity

One subject performed measurements with the rebreathing and steady-state method as proposed in this study and with Read's rebreathing method. The results can be found in Appendix '*A6. Read's method*'. However, as only one subject was measured no conclusions can be drawn. Both the rebreathing and steady-state method as used in this study do not account for effects on CVR. Read's and Duffin's rebreathing method can account for the effect of CVR, as breathing from bag with 5-7% CO_2 causes $PETCO_2$ to equilibrate with central PCO_2 quickly.

Numerous studies suggest that CVR should be measured detached from the chemo sensitivity, disregarding the fact that together they determine the stability of the controller. If it is uncertain what the cause of an instability is, a method which can separate the two effects is preferable to optimize treatment.

6.4.2. Oxygen levels

In this study, the central chemoreceptors solely were measured, by maintaining hyperoxic conditions. However, in reality, the peripheral chemoreceptors do affect the stability of the controller as oxygen level can change the sensitivity to CO_2. The use of hyperoxic conditions can decrease the sensitivity to CO_2 relative to normoxic conditions. This could give the wrongful suggestion of a stable system, as the gain is lower than it would be in normoxia. Furthermore, it is more realistic to measure patients under normoxia conditions, as this is the normal situation for patients.

6.4.3. Shifts

If knowledge about the apnea threshold is needed, it should be remembered that certain methods can shift the slope, with consequently a shifted apnea threshold. The shift of the slope is visible in the data of the one subject performing rebreathing, steady-state and Read's rebreathing method, see Appendix '*A6. Read's method*'. Moreover, the apnea threshold changes consequently to changes in the slope.

6.5. Recommendations

Directly based on the results of this study it is recommended to investigated the possibility to develop a computer-controlled steady-state method to remove or reduce the effect of variations in $PETCO_2$. In addition, the possibility to make the rebreathing measurements more comfortable (and keep high reproducibility) could be investigated.

In this study, healthy subjects were measured under hyperoxic conditions to establish the reproducibility of the measurement. To use the method in clinical practice it would be necessary to reassess the reproducibility of the rebreathing measurements in patients and under normoxic conditions. Instead of filling the rebreathing bag with 100% oxygen prior to the tests, the rebreathing bag should be filled with room air. Over the course of the rebreathing measurement, FiO_2 should be kept stable at ~20%.

When reproducibility is established in normoxic conditions and with patients, more research should be performed to find normal values of the slopes in sick and healthy subjects. However, most patients with affected control of breathing develop problems in sleep. Therefore, besides day-time normal values, normal values in sleep should be investigated, as the sensitivity to CO_2 changes with the onset of sleep and in different phases of the sleep cycle [21]. The rebreathing method as suggested in this study would be a good method to obtain the sensitivity to CO_2 in sleep. Other methods (Read's and Duffin's) tend to provoke an arousal as the initial increase in CO_2 is not gradually [60].

As explained before, it is possible that the cause of instable control of breathing, is not found in the controller gain. Plant gain, time delay and noise can cause problems with control of breathing (e.g. increased time delays in CSAS with HF and increased noise in rapid eye movement sleep). Therefore, the possibilities to expand the measurement of control of breathing should be investigated. The first proposal is to use the recovery phase to investigate time delays in the system. In this study, it is hypothesized that the step change in PCO_2 at the end of rebreathing to the recovery phase could give insights on time delay of the system. Time constants can be calculated, oscillations and under-/overshoots can be studied, and the time to recover to eupneic level. A suggestion on the method to obtain these parameters can be found in Appendix '*A7. Recovery phase of rebreathing measurement*'.

Additionally, this method has been applied to the rebreathing data of this study. The second proposal is to extend the reference phase (level 1) to 5-10 minutes to measure variability in ventilation and to determine coherency between ventilatory and PCO_2 data, as proposed by Van den Aardweg et al. [58].

7. Conclusion

The main goal of this study was to determine which method, rebreathing or steady-state, should be used in clinic to measure the ventilatory response. The primary outcome was reproducibility, by means of an ICC between the slopes of both methods in measurement 1 and 2. Based on the reproducibility of the measurements, it is preferred to use the rebreathing method to measure the hypercapnic ventilatory response. The steady-state method is proven to be not reproducibility, due variations in $PETCO_2$. To make the rebreathing method more useful for clinical application, the reproducibility should be established in patient population and under normoxic conditions. To assess other aspects of the ventilatory system, an extended test is needed, including variability tests and time delay tests.

8. References

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Appendices

A1. Questionnaires

A1.1. VAS-questionnaire

Vragenlijst naar tevredenheid van de metingen met verschillende opstellingen (Versie 2, 21-02-2017)

Deelnemer nummer (in te vullen door onderzoeker): .....

Bezoeknummer (in te vullen door onderzoeker): 1 2

Metingnummer (in te vullen door onderzoeker): 1 2

Omcirkel het cijfer naar keuze

1. **Hoe vond u de tijdsduur van de meting?**

1	2	3	4	5	6	7	8	9	10

Heel onprettig Redelijk Heel comfortabel

2. **Hoe vond u het ademhalen gaan?**

1	2	3	4	5	6	7	8	9	10

Heel onprettig Redelijk Heel comfortabel

3. **Hoe benauwd was u tijdens de test?**

1	2	3	4	5	6	7	8	9	10

Niet benauwd Redelijk benauwd Heel benauwd

Als u nog opmerkingen heeft, dan kunt u die hieronder opschrijven.

A1.2. Preference questionnaire

Vragenlijst keuze tussen twee methodes (Versie 1, 21-02-2017)

Deelnemer nummer (in te vullen door onderzoeker): .....

Bezoeknummer (in te vullen door onderzoeker): 1 2

Omcirkel het juiste antwoord

Van welke meting vond u de tijdsduur het prettigst?

1	Maakt niet uit	2

Welke methode vond u het makkelijkst ademen?

1	Maakt niet uit	2

Van welke methode werd u het **minst** benauwd?

1	Maakt niet uit	2

Welke methode zou u aanbevelen om te gebruiken in het ziekenhuis

1	2

A2. Protocols of measurements

A2.1. Rebreathing protocol

Doel

Het meten van de hypercapnische ventilatoire respons.

Handelingsbevoegdheid

-

Contra-indicaties

Tijdens de meting vinden er verschuiving plaats in de zuurgraad van de patiënt. Dit kan als gevolg hebben dat de patiënt hoofdpijn ervaart, kortademig wordt en/of duizelig. Vooral patiënten in een instabiele conditie, hebben een verhoogd risico op complicaties. Onder instabiele conditie verstaan wij respiratoire acidose, alkalose zonder compensatie.

Stop criteria

Ernstige kortademigheid
Duizeligheid of flauwvallen
$PETCO_2$ > 8.5 kPa
SpO_2 < 90%

Duur

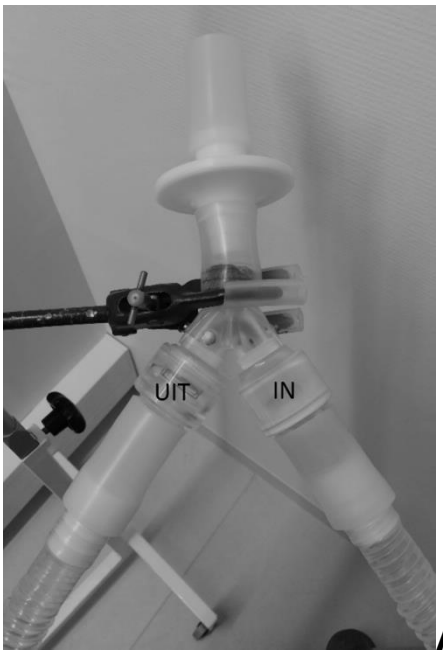
20 minuten

Voorbereiding van de handeling

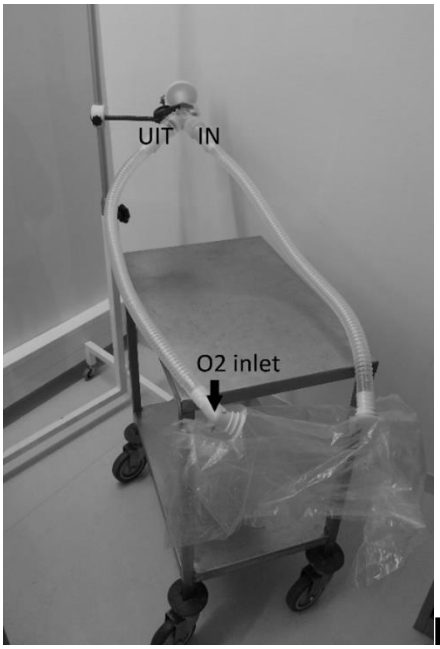
Benodigdheden			
Hans Rudolf masker met bevestigingsbanden	Triple V volume transducer	Y-klep	Kniestuk met zuurstofaansluiting
Ademanalyseapparaat (Oxycon Alpha)	Antibacterieel filter	2 x Spirometerslangen	Rebreathing bag
Siliconen tussenstukken	Statief	3 liter ijkspuit	Pulsoximetrie sensor
Steriel water	Alcohol 70%	Laptop	Standaard

Opstelling klaarzetten

Zet op het antibacterieel filter twee siliconen stukken, een kant verbonden met triple V sensor, een kant met de y-kleppen. Let erop dat de uitademingskant van de y-klep aan dezelfde kant zit als de zuurstofaansluiting (figuur 1B). Plaats dit als aangegeven is in figuur 1A, in de standaard. Sluit de spirometer slangen aan op de y-kleppen met siliconen stukken en op de slangen de rebreathing bag, met aan de uitademingskant het kniestuk met zuurstofaansluiting. Sluit de zuurstof slang aan op de zuurstofaansluiting.

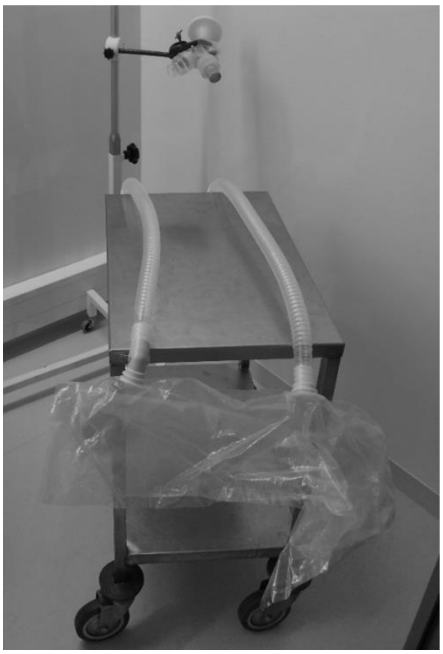


A



B

Figuren 1A, 1B: (A) y-kleppen gekoppeld aan spirometerslangen en antibacterieel filter. Waarbij alle drie de onderdelen verbonden zijn met siliconen stukken. (B) gesloten systeem in rebreathing fase.



C

Figuur 1C: (C) open systeem in rust fase en herstelfase.

Bediening apparatuur

IJking Oxycon Pro	- Voorafgaand aan de 1ste meting 's ochtends en de 1ste meting 's middags moet een gaskalibratie worden uitgevoerd. - Voorafgaand aan elke meting moeten de omgevingsfactoren gekalibreerd worden.
IJking triple V flow transducer	- Voorafgaande aan elke meting moet de triple V flow sensor worden gekalibreerd. - Er kan geen automatische ijking plaatsvinden voor de triple V flow sensor. - Sluit de triple V flow sensor aan op de **gesloten** opstelling (figuur 1B), zorg ervoor dat er voldoende lucht in de rebreathing bag zit, zodat deze niet vacuümtrekt als de ijkspuit gebruikt wordt. - Selecteer 'Volume calibration', start kalibratie. - Zet laptop met film klaar, ter afleiding van de patiënt.
Patiëntgegevens	- Vul patiëntgegevens in. - Noteer leeftijd, gewicht, lengte en geslacht.
Open systeem	- Eerst wordt er basis ventilatie van de patiënt gemeten en daarvoor moet het systeem **open** zijn (figuur 1C). Ontkoppel de rebreathing bag door de slangen te verwijderen van de y-klep.
Patiënt voorbereiding	- Zet de patiënt neer in de stoel vóór de opstelling. - Leg de procedure uit. **N.B.** vertel dat de patiënt ten alle tijden zelf de meting mag stoppen.
Sluit masker aan	- Terwijl patiënt zit, wordt het masker vastgemaakt op het gezicht. - Er wordt gecontroleerd op evt. lekkage d.m.v. kaartje voor het masker-gat houden. Patiënt wordt geïnstrueerd in te ademen, dit zal niet lukken. Indien er lucht langs het masker lekt dan is dit meestal hoorbaar of door de patiënt waarneembaar als een luchtstroom langs het masker.
Start test	- Klik op het icoon Breath-by-Breath. Hierbij zal een startup scherm verschijnen. - Selecteer '**protocol'** HCVR_1, en lay-out '**Denise_HCVR**'. - Klik op 'OK'.
Start background zeroing	- Klik op 'F1'. Rechtsonder in beeld staat een oranje rondje op het moment dat de background zeroing bezig is.

	- Wanneer het rondje groen is mag het sample slangetje terug worden geplaatst.
Start meting	- Plaats de triple V flow sensor in het masker. - Sluit de patiënt nu aan op het open systeem.
Start referentie fase	- Als de proefpersoon op een stabiele ademhaling ademt, wordt de referentie fase gestart. Deze duurt 3 minuten. - Noteer de starttijd van de referentie fase (deze is nog niet automatisch uit het systeem te verkrijgen).
Start rebreathing fase	- Voor het starten van de rebreathing fase moet de zuurstof flow naar de rebreathing zak zijn gestart. - Nu wordt het systeem gesloten (figuur 1B) door de spirometerslangen aan te sluiten op de y-kleppen. De CO_2 in de zak zal nu beginnen te stijgen. - Laat de zuurstof kraan maximaal (15L) 30 seconden aanstaan in het gesloten systeem. Zorg ervoor dat de FIO_2 > 60% is. Als de FiO_2 < 60%, dan de zuurstofkraan langer aan laten staan. - Draai de zuurstof kraan terug naar 2L en check op de FiO_2 hierbij stabiel blijft, pas anders de zuurstof flow aan. - Noteer de starttijd van de rebreathing fase.
Stop meting	- Als de patiënt aangeeft zich niet goed te voelen of niet meer verder te willen. - Als $PETCO_2$ stijgt boven 8.5 kPa. - Als SpO_2 daalt onder 90%. - Ga terug naar het open systeem en meet het herstel van de patiënt.
Start herstelfase	- Klik op ‘F1’. - Noteer de starttijd van de herstelfase. - Patiënt zal nu op basis van kamerlucht herstellen.
Afsluiten test	- Einde meting - Ga naar file report - Klik op opslaan data - Kies output ‘cycleplotter2016’ - Ga naar de C-schijf, mapje ‘cycleplotter’, kopieer het juiste bestand.

Nazorg

t.a.v. patiënt

- Controleer of de proefpersoon zich goed voelt, geeft aan dat de proefpersoon moet herstellen van de ademhalingstest en rustig moet blijven zitten.
- Mocht de proefpersoon onwel worden, bel assistentie.
- Mocht de proefpersoon na de meting benauwd blijven dan kan extra zuurstof worden toegediend, bel assistentie.

t.a.v. materiaal

- Het masker in een sopje afwassen, afdrogen en met een gaasje met alcohol 70% afnemen.
- Maskerriempjes worden uitgespoeld in een sopje en worden opgehangen om te drogen.
- Triple V flow sensor wordt met alcohol 70% afgespoeld.
- Antibacterieel filter wordt weggegooid.
- Siliconen stukken worden met alcohol 70% afgenomen.
- Spirometerslangen doorspoelen met alcohol 70% en daarna te drogen ophangen.
- Rebreathing bag te drogen neerleggen.

A2.2. Steady-State protocol

Doel

Het meten van de hypercapnische ventilatoire respons.

Handelingsbevoegdheid

-

Contra-indicaties

Tijdens de meting vinden er verschuiving plaats in de zuurgraad van de patiënt. Dit kan als gevolg hebben dat de patiënt hoofdpijn ervaart, kortademig wordt en/of duizelig. Vooral patiënten in een instabiele conditie, hebben een verhoogd risico op complicaties. Onder instabiele conditie verstaan wij respiratoire acidose, alkalose zonder compensatie.

Stop criteria

Ernstige kortademigheid
Duizeligheid of flauwvallen
$PETCO_2$ > 8.5 kPa
SpO_2 < 90%

Duur

25 minuten

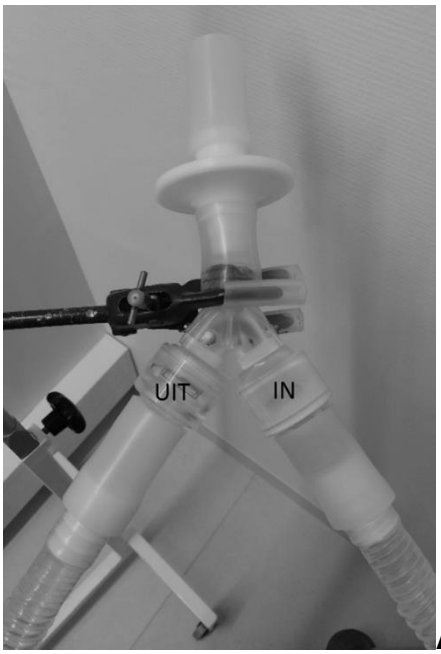
Voorbereiding van de handeling

Benodigdheden			
Hans Rudolf masker met bevestigingsbanden	Triple V volume transducer	Y-klep	Kniestuk met zuurstofaansluiting
Soda lime	Cannister + filters	Kleppen + Arduino board	2 x t-stukken
Ademanalyseapparaat (Oxycon Alpha)	Antibacterieel filter	3 x Spirometerslangen	Rebreathing bag
Siliconen tussenstukken	Statief	3 liter ijkspuit	Pulsoximetrie sensor
Steriel water	Alcohol 70%	Laptop	Standaard

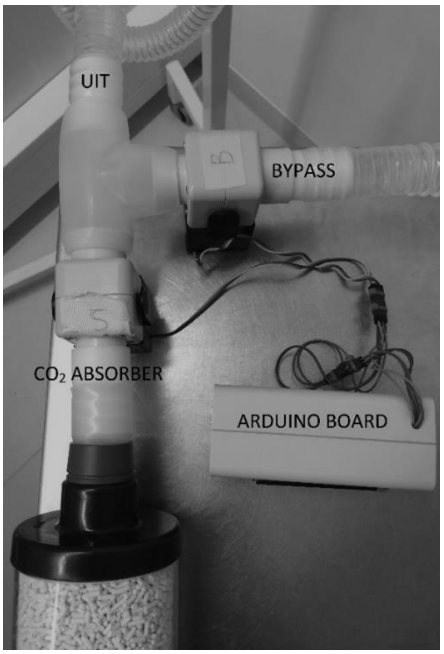
Opstelling klaarzetten

Plaats onderin de cannister een filter. Vul de cannister met soda lime, en plaats er bovenop nog een filter. Zet op het antibacterieel filter twee siliconen stukken, een kant verbonden met triple V sensor, een kant met de y-kleppen (figuur 1A). Let erop dat de uitademingskant van de y-klep aan dezelfde kant zit als de zuurstofaansluiting (figuur 1C). Plaats dit als aangegeven is in figuur 1A, in de standaard. Aan de uitademingskant wordt vervolgens een t-stuk geplaatst, aan het t-stuk worden de kleppen geplaatst. **NB** Koppel de juiste klep aan de juiste arm (S=soda lime, B=bypass). De cannister met soda lime en de bypass worden volgens figuur 1B verbonden met het t-stuk en de kleppen. **NB** op de cannister past geen ‘normaal’ silicone tussenstuk. Hiervoor is gebruik gemaakt van een dun-wandig silicone stuk samen met het losgemaakt uiteinde van een spirometrie slang, deze wordt in de cannister geschoven.

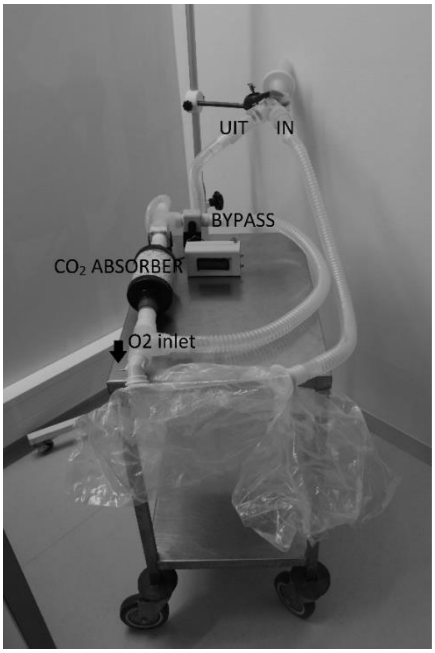
De beide armen worden vervolgens vlak voor de zuurstofaansluiting weer verbonden met een t-stuk. De derde uitgang van dit t-stuk wordt via het kniestuk met zuurstofaansluiting verbonden met de rebreathing bag (figuur 1B).



A



B



C

Figuren 1A, 1B, 1C: (A) y-kleppen gekoppeld aan spirometerslangen en antibacterieel filter. Waarbij alle drie de onderdelen verbonden zijn met siliconen stukken. (B) aansluiting t-stuk + absorber en bypass arm (C) gesloten systeem

Bediening apparatuur

IJking Oxycon Pro	- Voorafgaand aan de 1ste meting 's ochtends en de 1ste meting 's middags moet een gaskalibratie worden uitgevoerd. - Voorafgaand aan elke meting moeten de omgevingsfactoren gekalibreerd worden.
IJking triple V flow transducer	- Voorafgaande aan elke meting moet de triple V flow sensor worden gekalibreerd. - Er kan geen automatische ijking plaatsvinden voor de triple V flow sensor. - Sluit de triple V flow sensor aan op de **gesloten** opstelling (figuur 1B), zorg ervoor dat er voldoende lucht in de rebreathing bag zit, zodat deze niet vacuümtrekt als de ijkspuit gebruikt wordt.

	- Selecteer 'Volume calibration', start kalibratie. - Zet laptop met film klaar, ter afleiding van de patiënt.
Patiëntgegevens	- Vul patiëntgegevens in. - Noteer leeftijd, gewicht, lengte en geslacht.
Open systeem	- Eerst wordt er basis ventilatie van de patiënt gemeten en daarvoor moet het systeem **gesloten** zijn en staat de absorber arm **volledig open** (figuur 1C).
Patiënt voorbereiding	- Zet de patiënt neer in de stoel vóór de opstelling. - Leg de procedure uit. **N.B.** vertel dat de patiënt ten alle tijden zelf de meting mag stoppen.
Sluit masker aan	- Terwijl patiënt zit, wordt het masker vastgemaakt op het gezicht. - Er wordt gecontroleerd op evt. lekkage d.m.v. kaartje voor het masker-gat houden. Patiënt wordt geïnstrueerd in te ademen, dit zal niet lukken. Indien er lucht langs het masker lekt dan is dit meestal hoorbaar of door de patiënt waarneembaar als een luchtstroom langs het masker.
Start test	- Klik op het icoon Breath-by-Breath. Hierbij zal een startup scherm verschijnen. - Selecteer '**protocol'** HCVR_1, en lay-out '**Denise_HCVR**'. - Klik op 'OK'.
Start background zeroing	- Klik op 'F1'. Rechtsonder in beeld staat een oranje rondje op het moment dat de background zeroing bezig is. - Wanneer het rondje groen is mag het sample slangetje terug worden geplaatst.
Start meting	- Laat de zuurstof kraan maximaal (15L) 30 seconden aanstaan in het gesloten systeem. Zorg ervoor dat de FIO_2 > 60% is. Als de FiO_2 < 60%, dan de zuurstofkraan langer aan laten staan. - Plaats de triple V flow sensor in het masker. - Sluit de patiënt nu aan op het gesloten systeem. - Draai de zuurstof kraan terug naar 2L en check op de FiO_2 hierbij stabiel blijft, pas anders de zuurstof flow aan.
Start referentie fase	- Als de proefpersoon op een stabiele ademhaling ademt, wordt de referentie fase gestart. Deze duurt 3 minuten. - Noteer de starttijd van de referentie fase (deze is nog niet automatisch uit het systeem te verkrijgen).
Start level fases	- Voor het starten van de steady-state fase moeten de $PETCO_2$ levels bepaald worden. Level 1 is de gemiddelde $PETCO_2$ in de referentie fase, level 2 is $PETCO_2$ van de referentie fase + 0.5 kPa, level 3 is $PETCO_2$ van de referentie fase + 1.5 kPa. - Noteer de starttijd van level 2. - Nu wordt er naar het tweede level geswitcht door de kleppen om te schakelen naar volledig via de bypass. - Als het juiste $PETCO_2$ level is behaald, wordt met behulp van de kleppen de $PETCO_2$ stabiel gehouden. Als de $PETCO_2$ te ver stijgt, zet dan de absorber klep meer open en vice versa. - Als een stabiele ventilatie is behaald mag er worden doorgegaan naar het volgende level (dit zal ongeveer 5-10 minuten na de start van level 2 zijn). - **Herhaal deze stappen voor level 3.**
Stop meting	- Als de patiënt aangeeft zich niet goed te voelen of niet meer verder te willen. - Als $PETCO_2$ stijgt boven 8.5 kPa. - Als SpO_2 daalt onder 90%. - Als een stabiele ventilatie in level 3 behaald is. - Zet de klep van de absorber arm volledig open en meet het herstel van de patiënt.
Start herstelfase	- Klik op 'F1'.

	- Noteer de starttijd van de herstelfase.
Afsluiten test	- Einde meting - Ga naar file report - Klik op opslaan data - Kies output 'cycleplotter2016' - Ga naar de C-schijf, mapje 'cycleplotter', kopieer het juiste bestand.

Nazorg

t.a.v. patiënt

- Controleer of de proefpersoon zich goed voelt, geeft aan dat de proefpersoon moet herstellen van de ademhalingstest en rustig moet blijven zitten.
- Mocht de proefpersoon onwel worden, bel assistentie.
- Mocht de proefpersoon na de meting benauwd blijven dan kan extra zuurstof worden toegediend, bel assistentie.

t.a.v. materiaal

- Het masker in een sopje afwassen, afdrogen en met een gaasje met alcohol 70% afnemen.
- Maskerriempjes worden uitgespoeld in een sopje en worden opgehangen om te drogen.
- Triple V flow sensor wordt met alcohol 70% afgespoeld.
- Antibacterieel filter wordt weggegooid.
- Siliconen stukken en t-stukken worden met alcohol 70% afgenomen.
- Spirometerslangen doorspoelen met alcohol 70% en daarna te drogen ophangen.
- Ledig de cannister en spoel schoon.
- Kleppen worden met alcohol 70% afgenomen
- Rebreathing bag te drogen neerleggen.

A3. Graphical user interface

In the 'HCVR-GUI', the measurements can be visualized per measurement per subject, see figure A1.

A4. Reproducibility of short steady-state slopes

The reproducibility of slope-SS calculated with level 2 and 3 was tested with a ICC between measurement 1 and 2. The ICC was 0.62. The slope was also calculated with the first and second level and with the first and third level. The ICCs were 0.02 for slope-SS with level 1 and 2, and 0.56 for slope-SS with level 1 and 3.

Table A1: ICC of slope-SS calculated with 3 combinations of levels

Measurement	Levels	ICC	95% CI	p-value
Slope-SS measurement 1 vs 2	2-3	0.62	0.23-0.84	<0.01
Slope-SS measurement 1 vs 2	1-2	0.02	-0.41-0.45	0.46
Slope-SS measurement 1 vs 2	1-3	0.56	0.14-0.81	0.01

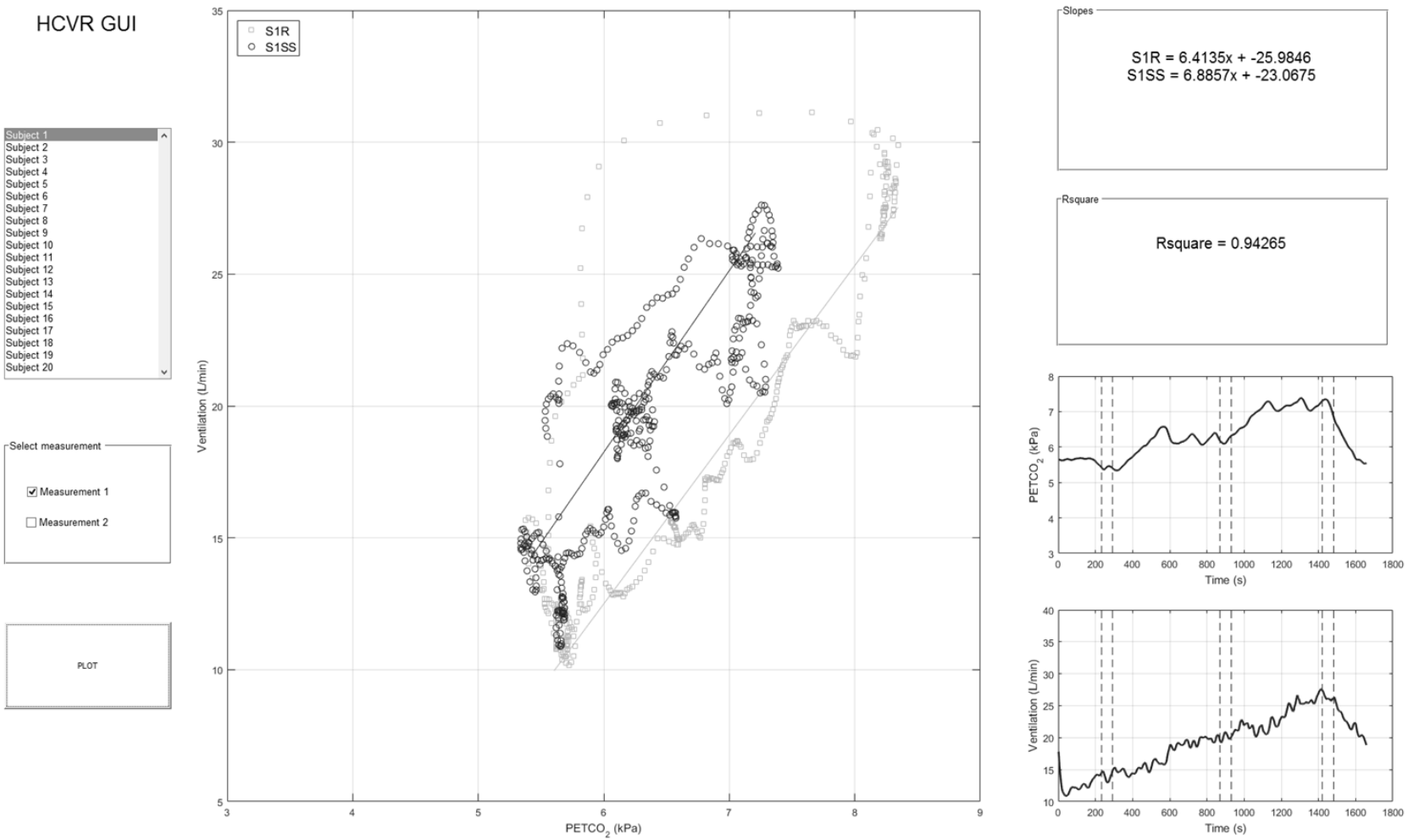


Figure A1: Graphical User Interface. HCVR-GUI. On the left side, a subject and measurement can be selected. In the middle, the responses are plotted (rebreathing = square and steady-state = circle). On the right side of the GUI, the function of all measurements is given, the fit of the rebreathing regression line is presented and $PETCO_2$ and ventilation of the steady-state over time are presented in a graph.

A5. Reproducibility in men and women

Reproducibility was also tested in groups, based on gender, see Section '*6.1.4. Gender differences*'. Both men and women show an ICC with a wide 95% CI for steady-state measurements. ICC for men is 0.51 and for women 0.66, suggesting that there is no difference between how men and women perform in the steady-state measurement.

Table A2: ICC between first and second measurement, differentiated between men and women

Measurement	Gender	ICC	95% CI	p-value
Slope-R measurement 1 vs 2	Men	0.92	0.73-0.98	<0.01
Slope-SS measurement 1 vs 2	Men	0.51	-0.10-0.84	0.05
Slope-R measurement 1 vs 2	Women	0.76	0.25-0.94	0.01
Slope-SS measurement 1 vs 2	Women	0.66	-0.10-0.93	0.05

A6. Read's method

One subject performed the rebreathing and steady-state measurement as described in Section '*6.4.1. Cerebrovascular reactivity*' and Section '*6.4.3. Shifts*' in Medisch Spectrum, additionally the rebreathing measurement was performed with a prefilled bag (93% O_2 and 7% CO_2) in Leiden University Medical Centre, according to Read's method [1]. The slope of all three measurement is calculated, and HCVRs are plotted. Fractional end-tidal, mixed expiratory and inspiratory CO_2 are compared.

Figure A2 shows the results of one subject, performing the rebreathing test and steady-state test, and in addition the rebreathing test according to Read. The slope of the rebreathing method as performed in this study is 28.4 ml/min/kPa, with a pAT of 4.2 kPa. slope with steady-state measurement is 15.5 ml/min/kPa, with a pAT of 2.8 kPa. The slope measured with Read's rebreathing method is 24.6 ml/min/kPa, with a pAT of 4.9 kPa.

In figure A3, the inspired, mixed expiratory and end-tidal fractions over time can be found. In the upper panel rebreathing with the rebreathing method of this study can be found, in the lower panel rebreathing with Read's method. In Read's method, rebreathing starts at t =0, in the rebreathing method, rebreathing starts at t=180. The upper panel shows that inspired CO_2 increases linearly over the course of rebreathing, while in the lower panel the inspired CO_2 increase to the same level as mixed expiratory almost instantly. There is only a small, constant gap between all fractions, while in the rebreathing method the gap decreases over time.

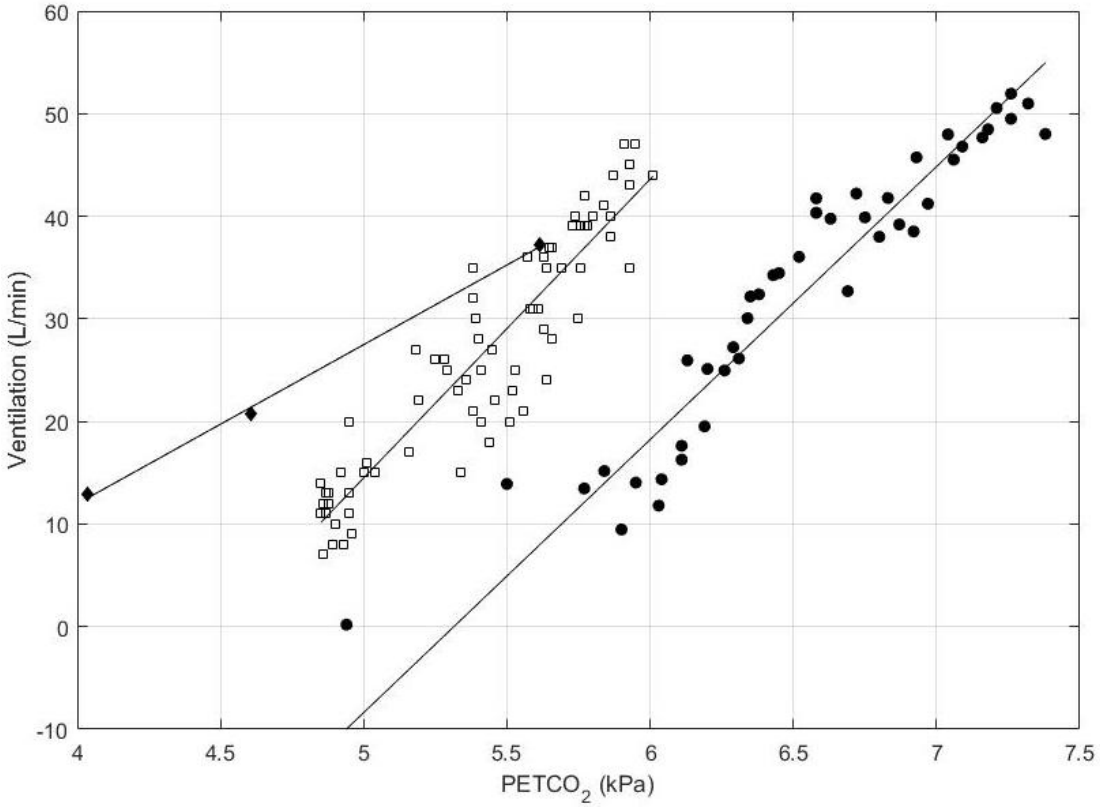


Figure A2: HCVR with three methods. The response measured with the rebreathing method (squares), with Read's rebreathing (circles) and with the steady-state method (diamonds)

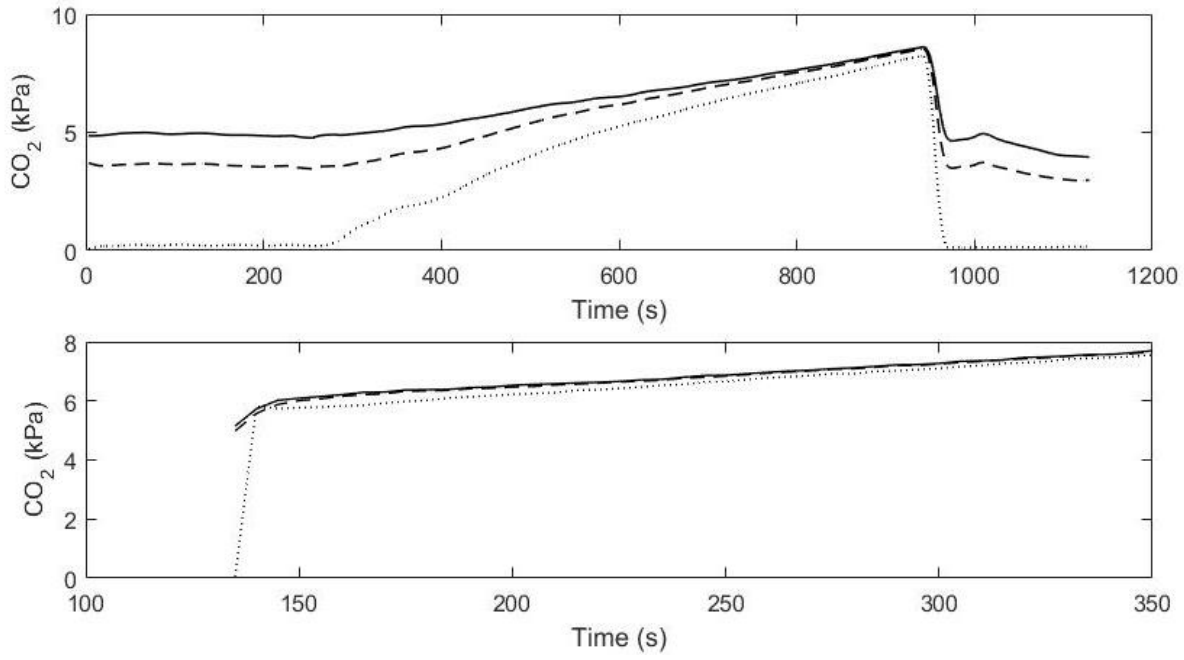


Figure A3: Course of fractions of PCO_2. $FETCO_2$ (solid line), $FECO_2$ (dashed line) and $FICO_2$ (dotted line), above rebreathing, below Read's rebreathing. In Read's rebreathing, almost instantaneously an equilibrium exists between inspired and expired fraction of CO2

A7. Recovery phase of rebreathing measurement

The recovery phase of the rebreathing measurement is studied, normalized to reference $PETCO_2$. Reference $PETCO_2$ is calculated as mean $PETCO_2$ in the last minute of the reference phase. Three $PETCO_2$ values are of interest: (1) maximum $PETCO_2$ in rebreathing, (2) initial decrease in $PETCO_2$, and (3) end-value $PETCO_2$ after three minutes recovery, see figure A4.

From these values, several parameters are derived: (1) Decrease time, the time between maximum $PETCO_2$ and the initial decrease $PETCO_2$, (2) Decrease $PETCO_2$, difference between maximum $PETCO_2$ and initial decrease $PETCO_2$, (3) Decrease slope (decrease $PETCO_2$/ decrease time), (4) Overshoot, initial decrease $PETCO_2$, (5) Maximum slope in the last minute, and (6) End-value of $PETCO_2$ after three minutes recovery.

For all subjects three minutes of recovery was recorded. The recovery phase of the rebreathing measurement was analyzed, and various parameters were calculated. Data of the parameters was not normally distributed. See table A3.

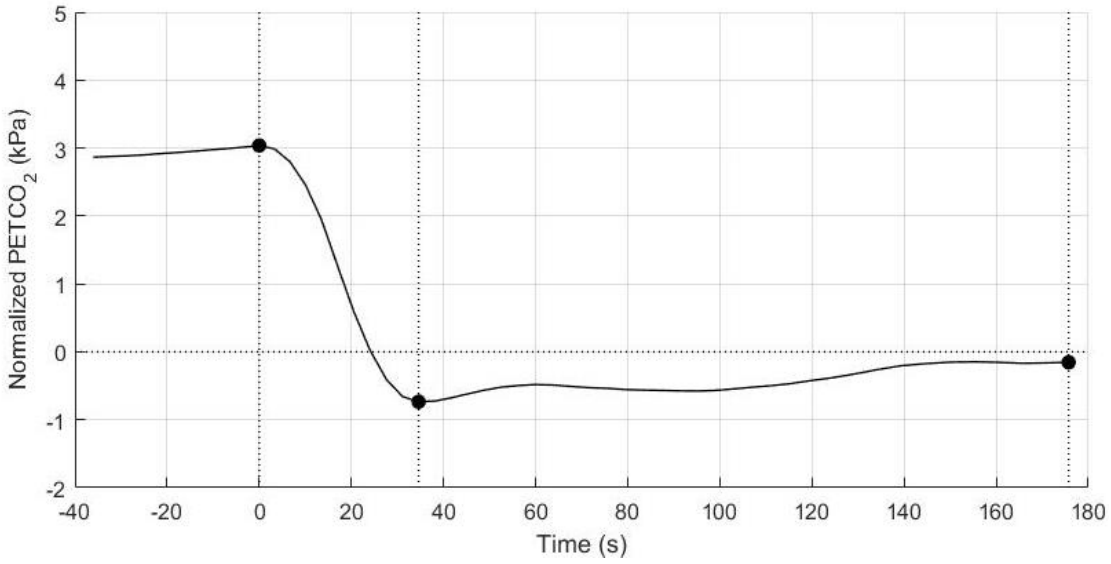


Figure A4: Time course of PETCO2 normalized in respect to reference PETCO2. Circles: (from left to right) maximum PETCO2 in rebreathing, initial PETCO2 decrease from start recovery, end-value PETCO2 after three minutes recovery. Time = 0 indicates the start of the recovery phase

Table A3: Median (IQR) of recovery parameters per measurement

	Measurement 1		Measurement 2	
	Median	IQR	Median	IQR
Decrease slope (kPa/second)	0.07	(0.04-0.11)	0.07	(0.05-0.10)
Decrease $PETCO_2$ (kPa)	2.64	(2.15-3.68)	2.56	(2.11-3.47)
Decrease time (seconds)	35.0	(28.0-61.0)	33.0	(28.0-49.0)
Reference - End $PETCO_2$ (kPa)	-0.27	(-0.48- -0.07)	-0.37	(-0.45- -0.17)
Maximum slope (kPa/second)	0.15	(0.12-0.20)	0.15	(0.11-0.20)
Overshoot (kPa)	0.23	(-0.33-0.73)	0.29	(-0.18-0.55)

A8. Resistance of the measurement setup

Calibration was performed in various conditions, to check if the change from open to closed (in rebreathing) and changes in the valve angles (in steady-state) resulted in other calibration settings. The resistance of the y-valve was also tested. The subject was placed in a body-box and performed a normal resistance maneuver. Hereafter, the y-valve (as used in the measurement setup) was placed in series with the pneumotach, and the maneuver was performed again. Lastly, a resistance maneuver was performed with a new y-valve. The difference between the two y-valves is that the latter has elastic silicone valves, while the first has hard silicone valves with springs.

The most notable change in correction factor for in- and expiration was found, between two conditions; (1) triple V flow sensor, and (2) y-valve with triple V flow sensor. For the steady-state setup the calibration was performed on all the different valve angles, which showed that no-significant difference between correction factors was found between the angles.

The subject was seated in the body-box and performed 2x3 maneuvers, first, the resistance of the persons lungs was measured, secondly the resistance of the persons lungs + y-valve, and lastly the resistance of the persons lungs + new y-valve. All measurements were found reproducible by the lung function analyst, based on the measured vital capacities. The mean resistance of lungs of the subject was 0.31 kPa/L/s, with y-valve 0.33 kPa/L/s and with the new y-valve 0.37 kPa/L/s.