

**Variability and trial effect of tidal-breath dose-response
citric acid cough reflex tests**

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Preface

This research was carried out between July 2016 and Dec 2017 at the Rose Centre for Stroke Recovery and Research, St.George's Medical Centre. This research was supervised by Prof. Maggie-Lee Huckabee and Dr. Phoebe Macrae, the University of Canterbury.

Preliminary results from this research have been presented as a poster at the following international conference:

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Abstract

Introduction: A reproducible method is crucial in establishing cough sensitivity. Although reproducibility of the tidal-breath dose-response (TB-DR) method using capsaicin has been validated; the same method using citric acid is uninvestigated. This study determined the variability across multiple cough reflex tests (CRT) of suppressed cough thresholds in healthy individuals.

Materials & Methods: Sixteen healthy volunteers underwent five CRTs (inter-test interval of at least 30 mins) within a single day. The TB-DR method was used to administer progressively increasing 0.1 mol/L increments of citric acid, ranging from 0.1-1.6 mol/L. Up to three 15-second trials of each concentration (45-second inter-trials interval) were administered via facemask. Placebo trials of saline were randomly interspersed. The suppressed cough threshold was defined as the lowest concentration producing two consecutive coughs on 2/3 trials. Data were analysed using a linear mixed effects model. The 95% prediction interval for within-participant variability was derived from the model using a bootstrapping method.

Results: The fixed effect results revealed that the mean SCT at baseline test across participants (model intercept) was 0.29 mol/L, 95% bootstrapped CI [0.12, 0.45]. The effect of test on SCT was 0.003 mol/L, 95% bootstrapped CI [-0.02, 0.03]. For the random effects, the variability due to participants at baseline SCT was 0.33 mol/L, 95% bootstrapped CI [0.21, 0.45]. The variability of the CRT effect between participants was 0.04 mol/L, 95% bootstrapped CI [0.02, 0.06] while the variability for the residuals (within participant variability) was 0.08 mol/L, 95% bootstrapped CI [0.06, 0.09]. The 95% prediction interval for the residuals (variability within a participant) was [-0.14mol/L, 0.16 mol/L].

Conclusions: This study defined the variability of citric acid CRT using the TB-DR method. There was no systematic trend in responses observed over the course of five CRTs. However,

the high percentage of variability for baseline SCT between participants suggests caution in establishing normative thresholds. The fairly consistent within-participant measures suggest CRT may be useful for repeated measures in tracking change as treatment effect within an individual participant or patient.

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List of Abbreviations

| | |
|----------------------|--|
| ACE | Angiotensin-converting enzyme |
| C₂ | Two consecutive coughs |
| C₅ | Five consecutive coughs |
| CI | Confidence interval |
| CRT | Cough reflex test |
| CVA | Cerebrovascular accident |
| ERS | European Respiratory Society |
| FEES | Fiberoptic endoscopic evaluation of swallowing |
| GERD | Gastroesophageal reflux disease |
| HEC | Human Ethics Committee |
| NaCl | Sodium chloride |
| NCT | Natural cough threshold |
| Q-Q | Quantile-quantile |
| SA | Silent aspiration |
| SB-DR | Single-breath dose-response |
| SCT | Suppressed cough threshold |
| SD | Standard deviation |
| SE | Standard error |
| TB-DR | Tidal-breathing dose-response |
| TB-SD | Tidal-breathing single dose |
| VFSS | Videofluoroscopic swallowing study |

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Introduction

Cerebrovascular accident (CVA), or stroke, was ranked as the third most common cause of death in New Zealand in 2012, after cancer and ischaemic heart disease, and approximately 9,000 new stroke cases are reported annually (Ministry of Health [MOH], 2015). The prognosis of stroke depends largely on the severity and site of lesion. Swallowing impairment, also termed dysphagia, is a frequently documented consequence of stroke. The prevalence of post-stroke dysphagia (detected using clinical and/ or instrumental assessments) ranges from 40-81% worldwide (Martino et al., 2005). About 44% of these patients will present with persisting swallowing disorders and episodes of aspiration (food or fluid entering the lungs) after the acute phase of the illness (Marik & Kaplan, 2003). In a large-scale study (N = 14,293) investigating stroke outcomes, it was reported that pneumonia increases the risk of 1-month mortality by up to three times, further indicating that efforts are required to reduce and prevent pneumonia among stroke patients (Katzan, Cebul, Husak, Dawson, & Baker, 2003).

Silent Aspiration

“Silent penetration” was first used by Linden and Siebens (1983) to describe patients who presented with laryngeal penetration on videofluoroscopic swallowing study (VFSS) while clinical sign such as cough was not observed. As research in identifying aspiration in dysphagic patients continues to expand, contemporary terminology identifies “silent aspiration” is the entry of saliva, liquid or food below the glottis without triggering a cough reflex or other overt signs such as choking, gagging, “wet-hoarse” voice, throat clearing response (Garon, Engle, & Ormiston, 1996; Garon, Sierzant, & Ormiston, 2009; Horner, Massey, Riski, Lathrop, & Chase, 1988; Linden & Siebens, 1983; McCullough, Wertz, & Rosenbek, 2001; Ramsey, Smithard, & Kalra, 2005; Sakai et al., 2016).

2.1 Prevalence of SA

An early study conducted by Horner and colleagues (1988) evaluated the clinical correlations and outcomes of aspiration following stroke through oral motor-sensory examination and VFSS in 47 patients. Patients were evaluated at mean 2.9 months post-stroke (ranging from one to 29 months). Subjective clinical signs such as coughing, gagging, choking, swallowing reflex, and vocal quality were documented through clinical examination. On VFSS, oral preparation, presence or absence of swallowing and coughing reflexes, as well as pharyngeal motility, were observed. Their findings revealed that just over half of the stroke patients (51.1%) aspirated on VFSS. Of these, 45.8% aspirated with an overt cough reflex, whereas 54.2% presented with silent aspiration. These findings highlighted the need for sensitive bedside clinical examinations in detecting SA among those patients. Subsequently, more studies were done to evaluate SA among dysphagic stroke patients. Daniels and colleagues (1998) conducted a study to evaluate the occurrence and clinical predictors of aspiration within five days of acute stroke. Of the 38% of patients who

aspirated, only 33% of them demonstrated overt signs of aspiration and 67% of them aspirated silently.

The lack of standardised protocol in detecting SA (e.g: cough reflex test [CRT] methodology, VFSS protocol and reproducibility of CRT results) across settings, reveals a range of prevalence from 17.6% to 46.9% of stroke patients (Guillén-Solà et al., 2015; Maeshima et al., 2007; Sato et al., 2012; Vilardell et al., 2017; Wakasugi et al., 2008, 2014). These findings suggest that SA is a crucial problem in managing patients while highlighting the need for a standardised and reproducible protocol for detecting SA.

2.2 Correlation between SA and aspiration pneumonia

While the high prevalence of SA among dysphagic stroke patients has been documented, Sekizawa and colleagues (1990) attempted to evaluate the correlation between the presence of cough reflex and occurrence of aspiration pneumonia in five elderly patients with neurological disorders. They recruited 10 controls who had neurological disorders and five patients who presented with aspiration pneumonia. Using the tidal-breath dose-response (TB-DR) method, all participants were administered citric acid aerosols in incremental concentrations from 0.03% to 36% and the lowest dose which triggered five consecutive coughs was recorded as an individual's corresponding cough threshold. In terms of methodology, details regarding the number of trials for each concentration, nebulisation time and output rate were not reported. All participants in the control group coughed at some point with a mean cough threshold of 1.06 (SE 0.18) logmg/ml. In contrast, all patients with aspiration pneumonia presented with an absent cough reflex across all concentrations. They concluded that the absence or attenuation of cough reflex could be one of the contributing factors to aspiration pneumonia in these patients. The emergence of this small-scale study

provoked further investigations into the association between cough reflex and aspiration pneumonia.

A more recent cohort study using capsaicin cough test discovered that reduced cough reflex might weaken protection against aspiration pneumonia (Niimi et al., 2003). Seven patients with a history of recurrent pneumonia were recruited and compared to a healthy control group. Patients with recurrent pneumonia were reported to have consistently higher cough thresholds compared to controls. The absence of cough reflex has been shown in multiple studies to highly correlate with aspiration pneumonia in patients with swallowing disorders (Marik & Kaplan, 2003; Nakajoh et al., 2000; Nakazawa, Sekizawa, Ujiie, Sasaki, & Takishima, 1993; Pontoppidan & Beecher, 1960) as well as mortality risk in acute ischemic stroke patients (Aviv et al., 1997).

2.3 Diagnosis of SA

2.3.1 Instrumental assessment (VFSS & FEES)

To date, the ‘gold standard’ instrumental assessment methods for evaluating cough and swallowing are the fiberoptic endoscopic evaluation of swallowing (FEES) and VFSS, despite the lack of standardisation across practitioners and documentation of acceptable reliability (Lee, Kim, Seo, & Kang, 2014; O’Donoghue & Bagnall, 1999; Ramsey, Smithard, & Kalra, 2003). During the VFSS, a range of textures and volumes are presented to a patient to ensure the most representative study of patient’s swallowing skill. The lateral plane is known for best detecting aspiration and providing optimum visualisation of the oropharyngeal function of swallowing (Miles, Moore, et al., 2013). By providing high-quality fluoroscopic imaging, the VFSS allows clinicians to identify anatomical or physiological dysfunction. Additionally, evaluation of compensatory approaches and therapeutic strategies can be assessed.

The FEES procedure was first introduced in 1988 by Langmore and colleagues. This procedure involves insertion of a flexible scope transnasally to a patient's pharynx. The high-resolution technology allows real-time viewing of anatomical structures and recording of task-specific movements such as at rest, speaking, breath-holding, pre- and post-swallow aspiration. Similar to VFSS, FEES enables clinicians to assess an individual patient's swallowing skill and detect aspiration, as well as presence or absence of a cough.

Despite their advantage in detecting overt and silent aspiration, VFSS and FEES often require special training and skilled staff to perform the procedure. Additionally, there are significant costs related to procurement and maintenance of the FEES and VFSS equipment apart from the radiation exposure during the VFSS procedure. As with other invasive procedures, during a FEES procedure, insertion of the flexible laryngoscope through an individual's nasal passage and into the pharynx often leads to discomfort and may interrupt swallowing in some patients (Adachi, Umezaki, & Kikuchi, 2017). Though, Leder et al. (1997) through a double-blind randomised clinical trial revealed that FEES procedure is well tolerated even without the used of topical anaesthetic, vasoconstrictor or lubricant to the nasal mucosa prior to the procedure. Having addressed all the pros and cons of the instrumental procedures, alternative screening assessments are required to help to increase the sensitivity of clinical outcome and minimise health care expenditures significantly.

Citric Acid Cough Challenge

Irritant-induced cough challenge was first explored over 60 years ago (Bickerman, Barach, & Drimmer, 1954; Bickerman, German, Cohen, & Itkin, 1957) to assess the cough-suppressing effect of pulmonary pharmaceutical drugs. Over the years, it has been used as an objective assessment tool of cough reflex sensitivity in humans (Wright, Jackson, Thompson, & Morice, 2010). In clinical practice, irritant-induced cough plays two major roles: (1) disease diagnosis and (2) progression tracking for disease or treatment (Fuller, 2002). Several cough stimulants – primarily capsaicin (Brandimore, Troche, Huber, & Hegland, 2015; Dicipinigaitis, Chang, Dicipinigaitis, & Negassa, 2016; Millqvist & Bende, 2001; Wise, Breslin, & Dalton, 2012, 2014), distilled water (Fontana et al., 1999; Lavorini et al., 2007; Lowry, 1994), tartaric acid (Stephens, Addington, & Widdicombe, 2003) and citric acid (Chou, Scarupa, Mori, & Canning, 2008; Ebihara et al., 2011; Gui et al., 2010; Kallesen, Psirides, & Huckabee, 2016; Kashiwazaki et al., 2013; Katsumata, Sekizawa, Ebihara, & Sasaki, 1995; Rostami-Hodjegan, Abdul-Manap, Wright, Tucker, & Morice, 2001) – have been used in these studies, with tartaric, citric acid and capsaicin most commonly documented (Chung, 1996; Morice et al., 2007).

Recently, irritant-induced inhalation cough challenge using tartaric or citric acid has been introduced as an adjunct to clinical assessment of swallowing in dysphagic patients and is commonly known as the CRT. Numerous studies have demonstrated that CRT can be a stand-alone or an adjunct screening tool for predicting risk of SA in patients with dysphagia or elderly people (Addington, Stephens, Gilliland, & Rodriguez, 1999; Fujiwara et al., 2017; Kallesen et al., 2016; Lee et al., 2014; Miles, Moore, et al., 2013; Nakajoh et al., 2000; Oba et al., 2017; Sakai et al., 2016; Sato et al., 2012; Wakasugi et al., 2014, 2008; Yamanda et al., 2008). However, most of these studies did not address the reproducibility of CRT. The test-retest reliability of CRT is not well-defined, especially in the patient cohort. Being cognizant

of the importance of the test, the standardisation of CRT methodology guidelines have been established by the European Respiratory Society (ERS) task force to improve the consistency of test administration across research laboratories.

3.1 Dosing methods

Selection of stimulant concentration has been addressed in several ways. Usually, the serial dilution method is used to produce a range of concentrations. The serial dilution method involves stepwise dilution of a stimulant in solution. Generally, the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion. Across cough literature, the most frequently used dilution factors are half-logarithmic and two-fold. The half-logarithmic dilution ($10^{0.5}$ -fold as the constant dilution factor) gives rise to a range of concentrations from 0.001 to 1 mol/L (e.g. 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 mol/L) regardless of the type cough stimulants used.

On the other hand, the two-fold dilution gives rise to a range of doubling concentrations of stimulant used. Nonetheless, the range of concentration varies across laboratories as do the type of cough stimulants involved. Examples of commonly used range of concentrations using two-fold dilution: 0.98-1000 $\mu\text{mol/L}$ (Dicpinigaitis, 2003a), 0.49-4000 $\mu\text{mol/L}$ (Dicpinigaitis et al., 2016; Nejla, Fujimura, & Kamio, 2000), 0.61-1250 $\mu\text{mol/L}$ ([Varechova et al., 2007] – a study done in 27 children), 0.016-50 $\mu\text{mol/L}$ (Midgren, Hansson, Karlsson, Simonsson, & Persson, 1992) for capsaicin; 7.8-1000 mmol/L (Wright et al., 2010), 0.028-1870 mmol/L (Watando et al., 2004), 0.01-3.3 mmol/L (Barros, Zammattio, & Rees, 1990) and 0.025-0.85 mol/L (Pounsford & Saunders, 1985) for citric acid. In addition, the 0.1 mol/L and 0.2 mol/L dilution factors are extensively used by one laboratory in New Zealand (Miles, Moore, et al., 2013; Monroe, Manco, Bennett, & Huckabee, 2014; Perry & Huckabee, 2017).

The lack of standardisation across laboratories, given that ERS guidelines also overlooked this issue, hinders the interpretation and translation of research findings into clinical works.

3.2 Administering methodologies

At present, the two widely used administration methodologies are single-dose (SD) and dose-response (DR) methods (Morice et al., 2007; Morice, Higgins, & Yeo, 1992; Morice, Kastelik, & Thompson, 2001; Pounsford & Saunders, 1985; Wong & Morice, 1999). The SD method involves the use of only one concentration of the cough stimulant. This method is time efficient, has a lower tendency for tachyphylaxis (a rapid improvement of tolerance in response to an active substance after a few initial doses; [Nichols, 2010]) and is commonly used in clinical practice. The DR method can be conducted either through single-breath (SB; also known as vital capacity method) or tidal-breath (TB).

The SB method usually requires participants to exhale to residual volume and then inhale the cough stimulant to their total lung capacity which usually takes place within 1-1.2 seconds (Pounsford & Saunders, 1985). In contrast, the TB method generally demands participants to breathe normally during the presentation of increasing concentrations of cough stimulant over a fixed duration, which is usually 15-60 seconds (Morice et al., 2007). The testing protocol would usually require the participant to breathe through their mouth either via a mouthpiece or facemask. Placebo trials of 0.9% sodium chloride (NaCl) are typically interspersed randomly between increasing concentrations throughout the testing procedure to increase participant blinding. Usually, the number of coughs in 15 seconds immediately after each trial will be recorded. The lowest concentration to elicit two (C_2) or five (C_5) consecutive coughs will be treated as an individual's cough threshold (Morice et al., 2007, 2001). Wright et al. (2010) reported that only about 43% of total participants achieved a C_5

response suggests a poor reproducibility of the C₅ response; C₂, therefore, is a preferred and extensively used outcome measure across laboratories (Morice et al., 2007).

3.3 Sensitivity and specificity of CRT

Sensitivity and specificity of CRT as a bedside screening tool in predicting aspiration pneumonia have been explored and are reported to range from 17-92% and 67-100%, respectively, across the literature (Addington et al., 1999; Guillén-Solà et al., 2015; Lee et al., 2014; Miles, Moore, et al., 2013; Oba et al., 2017; Sato et al., 2012; Wakasugi et al., 2008, 2014). The lack of standardised methodology used in conducting CRT and incomplete details reported across research laboratories have led to this wide range of sensitivity and specificity.

Miles and colleagues (2013b) using the TB-DR method, conducted a study to evaluate the sensitivity and specificity of citric acid CRT. They evaluated the validation of a simple, quick and portable citric acid CRT against instrumental tests in identifying aspiration in 181 patients. A total of 80 patients underwent VFSS and the remaining 101 patients received a FEES. All patients completed a CRT either before or after the instrumental test in a random manner. A portable nebuliser with an 8L prefixed free-flow output and restricted flow rate of 6.6 L/min was used to administer citric acid aerosol. The citric acid concentration of 0.4, 0.6 and 0.8 mol/L were administered in an ascending manner (1-minute interval). Each concentration was presented over 15 seconds and up to three doses per concentration. Placebo trials were interspersed between incremental concentrations to increase participant blinding. All patients were instructed to breathe comfortably and cough whenever they felt the need to cough. Production of cough over the 15 seconds delivery period was recorded. A positive result was considered if a C₂ response was generated on the presentation of citric acid. The lowest concentration with a positive result on 2/3 of the trials was treated as the patient's cough threshold. When compared against VFSS, the sensitivity and specificity for CRT in

detecting aspiration within patients who aspirated were maximised at 0.6 mol/L – 71%, 71% respectively. On the other hand, the optimal sensitivity and specificity for CRT were 85% and 71% at 0.4 mol/L, when compared against FEES.

The authors concluded that the low citric acid concentrations (0.4-0.6 mol/L) are more sensitive for predicting SA than high concentration (0.8 mol/L). They also suggested that in identifying aspiration, CRT is superior to other components of bedside examinations such as assessment of jaw strength, vocal quality and the structure of the soft palate. Their argument did not provide good support for CRT as the assessment of jaw strength and the structure of the soft palate have been known to be poorly correlated with aspiration (McCullough et al., 2001). The difference in terms of the optimal concentration between VFSS and FEES may be due to other limitations of the study: 1) the not equally experienced clinicians who conducted the FEES and VFSS and 2) the difference in patients' age in VFSS and FEES. In addition, the findings reported in their study did not account for between-participant variability, diurnal variation and test-retest reliability which could be a concern when the test is used to track change in clinical settings.

On the other hand, more recently, Guillén-Solà and colleagues (2015) conducted a prospective study to evaluate the sensitivity and specificity of citric acid CRT as a screening tool for SA in 134 stroke patients. The author reported the participants' demographic information in great detail. CRT was conducted using the TB-SD method. Without mentioning whether a facemask or mouthpiece was used, the authors used an ultrasonic nebuliser (3 ml/min flow rate) in administering the citric acid. All patients were given a VFSS immediately after CRT and Penetration-Aspiration Scale (Rosenbek, Robbins, Roecker, Coyle, & Wood, 1996) was used as the VFSS outcome while CRT result was blinded from the rater. In their study, sensitivity and specificity of CRT in identifying SA was 19% and 74%. They concluded that CRT results could not be used as an independent measure

of SA in subacute stroke patients. Critically, this study used a different method than that used by Miles and colleagues (2013). They exposed patients to citric acids for 60 seconds instead of 15 seconds. Tachyphylaxis could take place on continuous citric acid exposure over 60 seconds (Morice et al., 2007). A 1-minute exposure time has been shown to affect the reproducibility of the citric acid CRT (Morice et al., 1992). Another limitation of their study was the variability such as the test-retest reliability, between participants variability and diurnal variation were not taken into account. These variabilities might be the confounding factors that influence on the reproducibility of CRT results and may not reliably represent the true picture of patient's cough sensitivity.

3.4 Reproducibility of CRT

Morice et al. (1992) have demonstrated short and long-term tachyphylaxis following prolonged exposure to citric acid over one minute or associated with repetitive CRTs. Their first experiment studied short-term tachyphylaxis using the TB-SD method (citric acid at 0.68%) where participants were required to tidally breathe through a spacer for one minute while cough frequency was recorded within the same period (Collier & Fuller, 1984). All participant received two CRTs at a 1-week interval. The authors reported a marked reduction in cough frequency following one-minute of continuous citric acid exposure (mean cough frequency dropped from 4.9 to 0.5 coughs at the end of one minute for first CRT and 3.1 to 0 for the second CRT). They reported a statistically significant difference within participant between tests, in which cough frequency for the first CRT was consistently higher than the second CRT when TB-SD method was used. In experiment two, the single breath dose-response (SB-DR) method was utilised to evaluate long-term tachyphylaxis (Pounsford & Saunders, 1985). A series of CRTs were repeated within three hours using half-logarithmic incremental concentration of citric acid (0.01, 0.03, 0.1, 0.3 and 1.0mol/L). During each

CRT, participants were administered four one-second inhalations of each concentration with 10-second inter-inhalations intervals. They demonstrated a decline in cough frequency following four consecutive CRTs in 40 minutes and did not return to baseline even after 180 minutes which was an indication of long-term tachyphylaxis. Tachyphylaxis was evident regardless of the methods of administrations used. Their findings suggest that short- and long-term tachyphylaxis might play an important role in affecting test-retest reliability. The reduction in cough frequency over time, either due to adaptation to the cough stimulant or familiarity of the testing procedure might lead to a nonreproducible finding; hence, a poor test-retest reliability.

Dicpinigaitis (2003) attempted to define short- and long-term reproducibility of CRT using capsaicin. In studying short-term reproducibility, all 40 participants received two CRTs with a 14-day interval. In terms of long-term reproducibility, a separate group of 40 participants underwent two CRTs at least six months between tests. CRTs were conducted using SB-DR method. Incremental doubling concentration of capsaicin aerosols ranging from 0.98 – 1000 $\mu\text{mol/L}$ (one inhalation each concentration) were administered via nebulisers with an output of 1.007 ml/min. Each inhalation was presented for 1.2 seconds at 1-minute inter-inhalation interval while cough frequency during 15 seconds immediately after each inhalation was recorded. The lowest concentration which triggered C_2 and C_5 would be treated as an individual's cough threshold. Interestingly, they reported a remarkably good reproducibility between CRTs across participants. In terms of short-term reproducibility, they reported that C_2 and C_5 responses were reproducible within one doubling concentration in 92% of total participants. For long-term reproducibility, they reported 100% and 79% reproducibility for C_2 and C_5 responses respectively, within one doubling concentration.

Nejla and colleagues (2000) compared the TB-DR and SB-DR methods using capsaicin CRT to validate the reproducibility of two different methodologies in 22 healthy adults and

four patients with a pulmonary illness. All participants received two CRTs with a three-week inter-test interval. The TB-DR method required participants to tidally breathe from a 300 mL spacer for 15 seconds every one minute. Details about the number of trials administered per concentration and the used of facemask or mouthpiece were not reported. On the other hand, the SB-DR method required participants to exhale to residual capacity then inhale to total lung capacity. The capsaicin aerosols were administered using breath-activated dosimeter at 1.6 seconds every one minute. For both methods, capsaicin aerosols ranging from 0.49-4000 $\mu\text{mol/L}$ were administered in incrementally doubling concentration manner. Cough frequency over the 15 seconds (TB-DR method) and one minute immediately after the aerosol was discharged (SB-DR method) were recorded and the lowest concentration which elicited C_5 response would be considered as an individual's cough threshold. Using Pearson correlation analysis, they reported a good correlation between test one and test two using TB-DR method ($r = 0.84$) and SB-DR method ($r = 0.76$).

Similar to that reported by Dicipinigaitis (2003), they concluded that capsaicin CRT has good reproducibility, regardless of the methodology used. However, their findings were conflicting with that reported by Morice et al. (1992). The main drawback from these studies is the lack of consistency in the type of irritants used, which could be a confounding factor influencing the test-retest reliability of CRT. Different studies used different irritants, thus comparison of the results across studies is impossible. Also, administration methodology such as the control of the inspiratory flow rate could be another factor that influences the reproducibility of CRT. For example, Dicipinigaitis (2003) limited the inspiratory flow rate to 0.5 L/s by adding an inspiratory flow regulator valve to the setup which therefore optimised the reproducibility of inspiratory effort with each breath; hence gives rise to a reproducible CRT results.

To further validate the reproducibility of CRT, Wright et al. (2010) validated ERS standard citric acid CRT using the SB-DR method. In their study, they compared the conventional SB-DR method with a novel SB-DR method. The conventional SB-DR method involves four presentations of each half-logarithmic concentration (1, 3, 10, 30, 100, 300, 1000 mmol/L). Each trial was presented for one second with a 30-second inter-trial interval. On the other hand, the novel method required participants to inhale only once of each doubling concentration (7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 mmol/L). All trials were presented for 1.2 seconds at 30 seconds interval. For both conventional and novel methods, production of cough during 15 seconds immediately after each inhalation was recorded and the lowest concentration which triggered C_2 and C_5 responses were treated as an individual's cough threshold. CRTs were conducted at baseline, one, two, and four hours, as well as two weeks later. Both methodologies were refined through the use of a dosimeter to administer discrete doses within and across tests. They eliminated C_5 from their analysis due to lack of reproducibility across participants. For novel CRT, the mean change from baseline was -1.57%, -3.14%, -2.08%, and 1.36% at 1, 2, 4 hours and 2 weeks later, respectively. Using the conventional method, the mean change from baseline at the same four time points were -9.79%, -10.70%, -11.69%, and -8.24%, respectively.

The authors demonstrated a systematic trial effect (the mean threshold increased over time) for the conventional method with the effect lasting for two weeks. For the novel method, they observed a gradual increase in cough threshold at one and two hours which began to recover when the test was repeated at four hours after baseline, although it was still higher than baseline threshold. Unlike the conventional method, they observed a lower mean cough threshold two weeks after baseline measurement. Their findings revealed that the novel method has a higher intra- and inter-day reproducibility compared to conventional method. The limitation of this study was it did not describe the variability due to participants

such as within- and between participants variability. Their findings further confirmed that administration protocol, dosing method and equipment used could impact the reproducibility of CRT. For example, administration method using one inhalation per concentration is more reproducible than four inhalations per concentration. The explanation for this could be that tachyphylaxis is more likely to take place when an individual receives four inhalations of each concentration more than once (Telaga et al., 1974). Their findings questioned the reproducibility of citric acid CRT using the TB-DR method, when the citric acid delivery time is as long as 15 seconds and there will be up to three exposures per concentration.

3.5 Confounding factors affecting test-retest reliability

3.5.1 Voluntary suppression of cough

It has been known that cough can be voluntarily initiated and suppressed. Hutching et al. (1993) conducted a study to evaluate voluntary cough suppression using capsaicin CRT in 28 healthy adults. For each CRT, five concentrations of capsaicin solutions (0, 10, 33.3, 100 and 333 $\mu\text{mol/L}$) were administered using SB-DR method with one 1-second inhalation each concentration (1-minute inter-inhalations interval). All participants were administered two CRTs (natural cough test vs suppressed cough test) at five-minute inter-tests intervals in a random order. During the natural cough test, participants were told to relax and cough if they needed to. The suppressed cough test required participants to suppress their cough. Cough frequency within 30 seconds immediately after each inhalation was recorded. The authors compared pre- and post-CRT mean cough frequency of all concentrations for both conditions. For natural cough test, the mean cough frequency for the highest concentration was 2.94 ± 0.34 (n=23) coughs; whilst for the suppressed cough test, it was 0.29 ± 0.18 (n=3) coughs. They proposed that there is some degree of voluntary suppression of cough in responding to irritant-induced cough test. However, they did not evaluate the effect of repetitive CRTs for

natural and suppressed cough tests. For example, each of their participants received only one natural and suppressed cough test. It remains unknown if the higher SCT was due to voluntary suppression or variability of the test?

Monroe et al. (2014), through a randomised control trial, established normative data of citric acid CRT using TB-DR method in 80 healthy individuals recruited from the community which being separated into two groups (group A and B). Group A participants (n=40) received citric acid ranging from 0.8 to 2.6 mol/L, with 0.2 mol/L increments; whereas, in Group B participants (n=40) received citric acid ranging from 0.1 to 1.2 mol/L with 0.1 mol/L increments. The study protocols for both groups were similar to that by Miles et al. (2013b); the only difference was Monroe et al. (2014) evaluated natural cough threshold (NCT) and suppressed cough threshold (SCT). They reported a consistently higher threshold across groups when participants were instructed to suppress their cough. Their findings were in line with that by Hutching et al. (1993). Meanwhile, Eccles (2009) reported voluntarily suppression of natural cough until the reflexive cough pathway overrides the voluntary system; which is when a true reflexive cough is elicited. Their findings suggest that SCT is a preferred outcome measure that more closely reflects a true reflexive cough as part of the airway protection. A study involves a series of CRTs using SCT as the outcome measure is ideal for evaluating the variability of CRT.

3.5.2 Diurnal variation

A study by Pounsford & Saunders (1985) attempted to describe diurnal variation of citric acid CRT in 10 healthy adults. CRTs were conducted using SB-DR method at four different time points (two between 9am-12pm and two between 2-5pm); each CRT was conducted on a separate day. Order of the tests was randomised across participants. Citric acid aerosols ranging from 0.025-0.91 mol/L were administered. No details about the number

of inhalations per concentration and inter-concentrations interval were reported. They revealed that the overall mean cough threshold for the afternoon session (0.23 [0.10] mol/L) was statistically higher than the morning session (0.15 [SD 0.02] mol/L). Using the SB-DR method, they confirmed the presence of diurnal variation on cough threshold among healthy adults.

3.5.3 Order effect

Interestingly, Perry and Huckabee (2017), adopting a similar TB-DR methodology used by Miles et al. (2013b) and Monroe et al. (2014) but different from that of Pounsford and Saunders (1985), and found no diurnal variation. The authors applied the same study design as that by Pounsford and Saunders (1985), except they controlled oral hygiene to limit the potential effect of oral bacterial on SCT. All participants were required to brush their teeth for two minutes prior to CRT. Citric acid aerosols ranging from 0.1-1.2 mol/L were administered in 0.1mol/L increments via a facemask. Participants were told to suppress their cough while breathing through their mouth normally. Each concentration was administered up to three times, 15 seconds each dose with placebo trials being randomly interspersed between incremental concentrations. Production of C_2 over the 15 seconds period was considered as a positive result. The lowest concentration with at least two positive results out of three trials was considered as individual's SCT. No diurnal variation was observed in their study. However, they discovered an order effect between first and the second CRTs - a higher mean SCT for the second CRT. It remains unknown if the inconsistent findings were due to different methodologies used in conducting CRT or learning effect across participants over time led to an increased SCT. However, this finding is not in line with the results found using capsaicin CRT as previously described. It would be interesting to evaluate the reliability of CRT using the TB-DR method over repetitive tests in healthy adults.

The test-retest reliability of citric acid CRT using TB-DR method is unknown. When there are data showing diurnal variation, order effect and technical factors can influence responses to CRT (Barber et al., 2005; Barros et al., 1990; Dicipinigaitis, 2003b, 2007; Dolovich, 1985; Khalid, 2012; Morice et al., 2007, 2001; Ryan et al., 1981). It is important to validate the reproducibility of citric acid CRT using TB-DR method, especially when it is a commonly used method in research and clinical settings. A sensitive and reproducible objective measure is crucial in establishing the association between cough sensitivity and pathology for clinical and research applications. This thesis will focus on the reliability of CRT using TB-DR method over five separate tests.

3.6 Research aim

To determine the variability and trial effect of citric acid CRTs on SCT in healthy individuals.

3.7 Research questions

1. What is the within-participant, test-retest variability?
2. Is there a statistically significant trial effect of repetitive CRTs on SCT in healthy individuals?

Methodology

4.1 Screening/ exclusion criteria

A screening was carried out through a face-to-face interview prior to recruitment; only healthy participants who met certain criteria were recruited. Individuals with current respiratory illness, history of drug abuse, past or current issue of swallowing difficulty, and/or current gastroesophageal reflux disease (GERD) (Harding & Richter, 1997; Irwin, 2007) were excluded from the study. Smokers or people with prolonged exposure to second-hand tobacco smoke were also excluded (Bergren, 2001; Dicipinigaitis, 2003a; Dicipinigaitis et al., 2016; Lewis et al., 2007; Stravinskaite, Sitkauskiene, Dicipinigaitis, Babusyte, & Sakalauskas, 2009). All participants using opioid-based medication such as painkillers (codeine, hydrocodone, Vicodin, OxyContin, Demerol, etc.), narcotics, or angiotensin-converting enzyme (ACE) inhibitors were not eligible in participating the study (Bolser, 2007; Morice et al., 2007; Morice, Marshall, Higgins, & Grattan, 1994). To avoid a ceiling effect, individual who did not cough at 1.6 mol/L were excluded from the present study.

4.2 Participants and human ethics approval

This study was approved by the Human Ethics Committee (HEC) of University of Canterbury (HEC 2016/82). A total of 16 healthy participants (15 females; mean age 30.25, range 20-48 years) were randomly recruited for this study and provided informed consent. Seven participants had previous experience with citric acid CRT.

4.3 Oral hygiene

As oral hygiene may influence cough reflex sensitivity in healthy individuals and patients (Watando et al., 2004), all participants were given a toothbrush to clean their mouth

without dentifrice (to avoid any contamination of chemical effect) prior to each CRT as oral hygiene control.

4.4 CRT procedures

Citric acid CRTs were carried out using the TB-DR protocol described by Monroe et al., (2014). The baseline test was administered at the same time of the day (11 am) for all participants and repeated five times with an inter-test interval between 30 and 90 minutes (mean $42 \pm SD 12$). During each test, participants were instructed to sit upright on a chair. Citric acid solutions were prepared in the university chemistry department by diluting citric acid powder in 0.9% NaCl. A placebo dose (0.9% NaCl) was presented at the beginning of each test to acclimatise participants to the procedure. A portable nebuliser (PulmoMate Compressor, Model 46501, DeVilbiss Healthcare LLC, Pennsylvania, USA) with a predetermined free-flow output of 8 L/min and restricted flow output of 6.6 L/min was used to administer citric acid aerosol. Citric acid, ranging from 0.1-1.6 mol/L, was administered in an ascending order of 0.1 mol/L increments with a 45-second inter-trial interval. Each concentration was administered up to three times for 15 seconds through a facemask. Placebo trials were randomly interspersed between incremental concentrations through the test to increase participant blinding. Participants were instructed to relax and breathe normally and to try to suppress their cough. Production of coughs during the 15-second delivery period was recorded. A test response was considered positive if a C₂ response was generated on the administration of citric acid. The lowest concentration producing a positive result on 2/3 trials was deemed to be the participant's SCT. The cut-off point of the test was either when SCT had been determined or when a participant did not cough at 1.6 mol/L (Miles, Zeng, McLauchlan, & Huckabee, 2013; Monroe et al., 2014; Morice et al., 2007).

4.5 Data analysis and model stability assessment

The response variable in this study was participants' SCT. To account for correlation in the data, due to each subject having five measurements of SCT, linear mixed effects model was performed using the lme4 package in R (version 1.0.143; Bates, Machler, Bolker, & Walker, 2014; R Development Core Team, 2013). Test number was treated as a continuous variable (one to five) and was entered in the model as a fixed effect. Intercept for participant was entered in the model as a random effect, this allowed each participant to have their own intercept randomly deviating from the mean intercept. Similarly to the random intercept, the random slope for participants allowed for the possibility that the effect of test on SCT can vary from participant to participant. The following parameters were estimated and reported from the model:

- 1) Model fixed effects
 - a. Model intercept: average SCT at baseline test across participants
 - b. Model slope: average effect of test on SCT across participants
- 2) Model random effects (variance estimates were derived for)
 - a. Random intercept: Baseline SCT between participants
 - b. Random slope: CRT effect between participants
 - c. Residuals: SCT between tests within a participant
 - i. The 95% prediction interval for the residuals: the prediction interval provides a way to quantify the uncertainty of a single future observation from a population and can be used to derive a range where 95% of the SCT for a participant values will possibly fall in.

Linear mixed models are valid under the following assumptions: 1) normality of residuals, 2) normality of random effects - all random effects (random slopes and random

intercepts) follow a normal distribution, and 3) residuals have constant variance. In order to investigate whether all the assumptions were met, visual inspection of the residuals and random effects using histograms, quantile-quantile plots (QQ-plot) and residuals-box-plot (Figure 1-4) were evaluated to detect deviations from normality and homoscedasticity

The final model was as follow:

$$\text{lmer [SCT} \sim 1 + \text{CRT} + (1 + \text{CRT} | \text{Participant})]$$

4.6 Model assumptions

Visual inspection of the residuals (Figure 1) did not appear to be extremely skewed and no extreme outliers were found; however, a heavy-tailed Q-Q plot has been noted. Visual inspection of the random effects (Figure 2 & 3) appeared to be skewed and revealed notable deviations from normality.

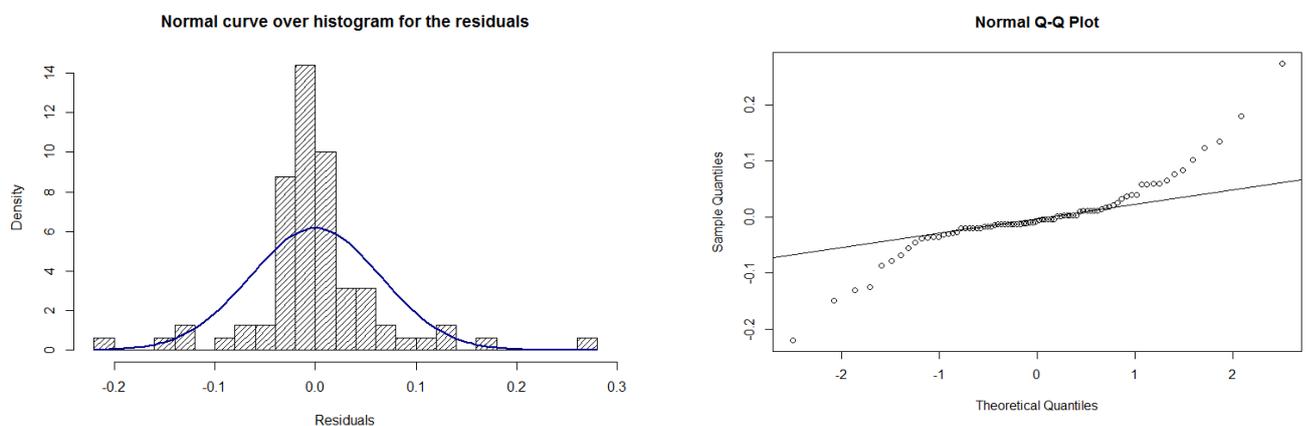


Figure 1. Histogram (left) and QQ-plot (right) of the residuals

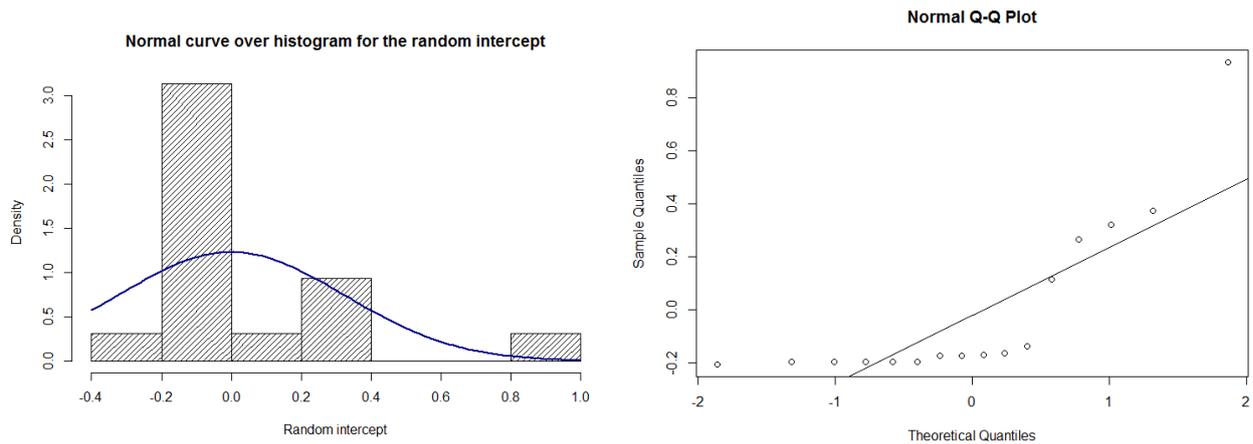


Figure 2. Histogram (left) and QQ-plot (right) of the random intercept

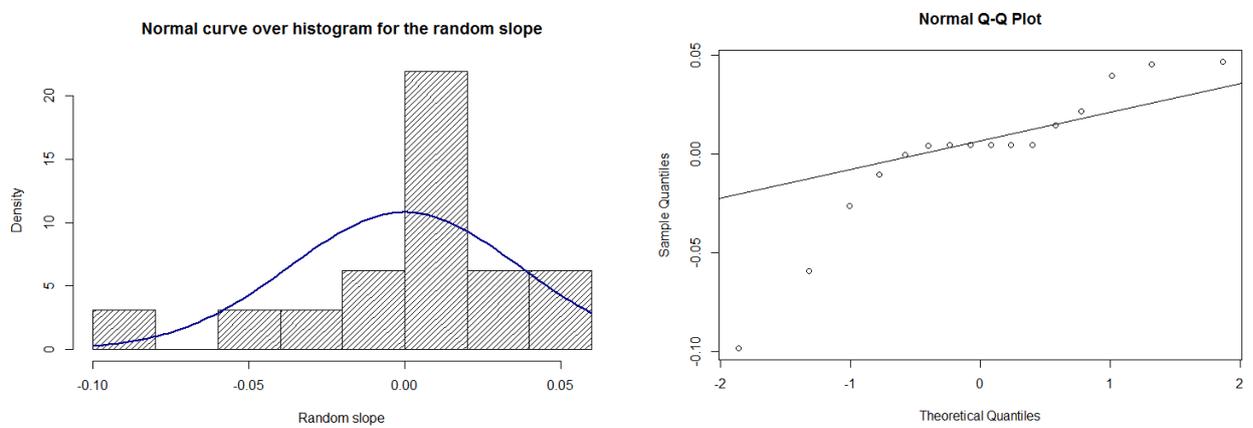


Figure 3. Histogram (left) and QQ-plot (right) of the random slope

A lack of homogeneity was observed when evaluating the variance of the residuals across participants. As it could be seen in Figure 4, some participants showed larger variability when compared to other participants (e.g: participant 3, 6, and 9). In addition to the visual inspections, a Levene's test was employed to evaluate the equality of variance of the residuals across participants. Results from the Levene's test revealed a statistically significant in the variance of the residuals [$F(15, 64) = 2.53, p < 0.005$].

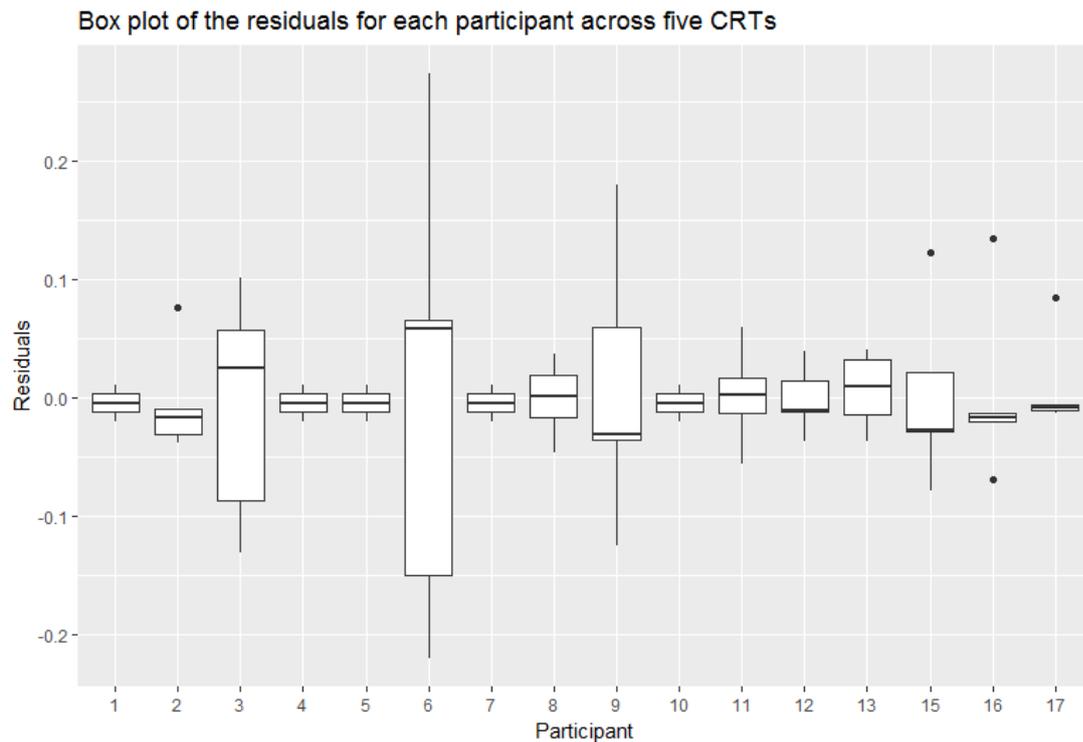


Figure 4. Box plot of the residuals for each participant across five CRTs

4.7 Bootstrapping method

To account for the non-normality of the residuals and random effects, a bootstrapping approach was preferred over data transformation as the transformation of the data would make the findings of the study difficult to be translated into clinical application. Bootstrapping confidence intervals (CIs) were calculated using the `confint.merMod` function in R. The `Confint` computes CIs on the parameters of a mixed model fit object. A thousand simulations were used to calculate the CI.

4.7.1 Bootstrapping approach for residual prediction interval

The bootstrapping approach to calculating the prediction interval of the residuals was carried out as the following:

1. The parametric model (e.g. the linear mixed effects model) was implemented and the 80 residuals were resampled with replacement using 1,000 iterations.

2. Empirical prediction intervals with a lower margin 2.5th percentile and upper margin 97.5th percentiles for the residuals were calculated on every iteration.
3. Finally, an average of the lower and upper margin prediction interval was generated by averaging the prediction intervals obtained through each iteration.

Results

The fixed effect results revealed that the mean SCT at baseline across participants (model intercept) was 0.29 mol/L, 95% bootstrapped CI [0.13, 0.45]. The effect of test on SCT was 0.003 mol/L, 95% bootstrapped CI [-0.02, 0.03]. For the random effects, the variability due to participants at baseline SCT was 0.33 mol/L, 95% bootstrapped CI [0.21, 0.46]. The variability of the CRT effect between participants was 0.04 mol/L, 95% bootstrapped CI [0.02, 0.06] while the variability for the residuals (SCT between tests within a participant) was 0.08 mol/L, 95% bootstrapped CI [0.06, 0.09]. The 95% prediction interval for the residuals (variability within a participant) was [-0.14 mol/L, 0.16 mol/L].

From a ratio of variance estimates of the random effects, the percentage of variability was derived for (Figure 5): 1) Baseline SCT between participants, 2) CRT effect between participants, and 3) SCT between tests within a participant. Figure 5 illustrates that between participants variability at baseline contributed the most to overall variance, followed by the variability between tests within a participant and the variability of the CRT effect between participants. Figure 6 displays the SCT for all participants across five CRTs.

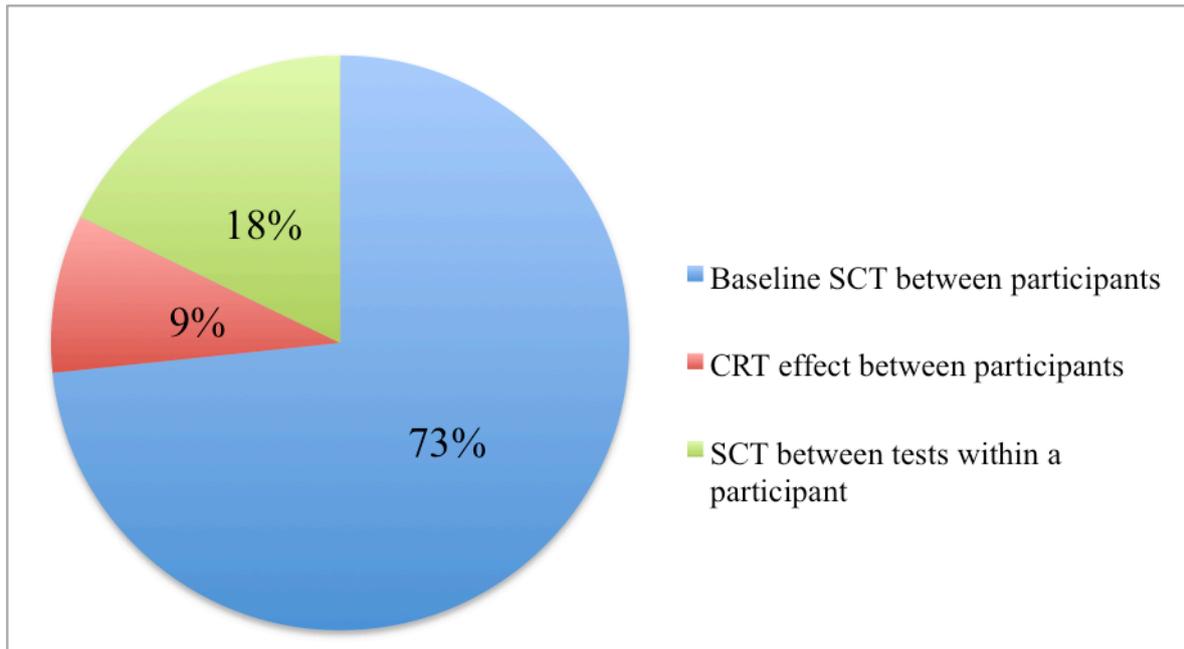


Figure 5. Pie chart showing the distribution of the percentage of variability of CRT

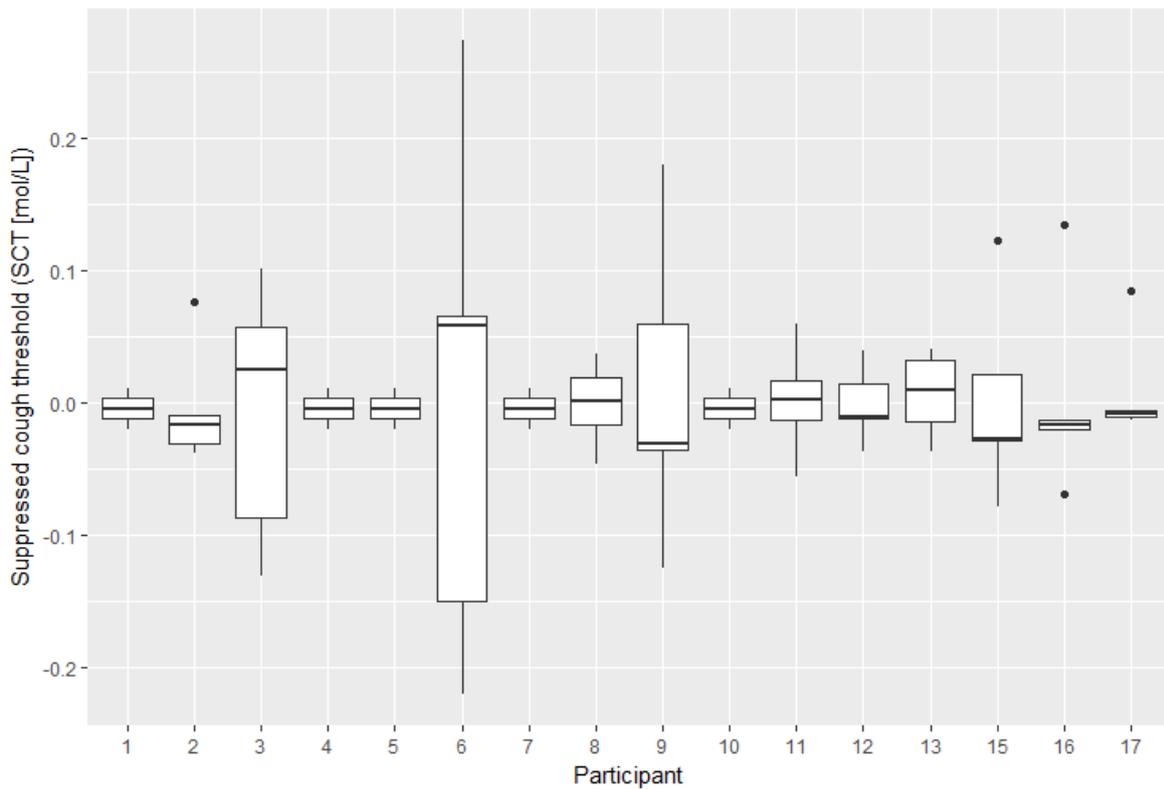


Figure 6. Box plot showing the SCT for all participants across five CRTs

Discussion

6.1 Trial effect of repetitive CRTs

This study is the first to evaluate the variability and trial effect of citric acid CRT using TB-DR method. This study reveals no trial effect of repetitive CRTs with variability close to zero (0.003 mol/L). The 95% CI includes zero effect reflects that there is no statistical significance trial effect of CRTs. Previous studies (Morice et al., 1992; Wright et al., 2010) have demonstrated a significant trial effect following repetitive exposures to citric acid; however, there is no distinct trial effect evident in this study. It may be due to different methodologies used in previous studies (e.g. TB-SD and SB-DR methods in Morice et al. [1992]; SB-DR method in Wright et al. [2010]). This finding suggests that citric acid CRT using TB-DR method may be a stable and effective tool for pre- and post-treatment measures.

6.2 Variability of CRT

This study discovered that variability due to baseline SCT between participants is the greatest which is about three dose levels (0.3 mol/L), followed by the within-participant variability which is about one dose level (0.1 mol/L) and the variability due to CRT effect between participants (almost zero). The baseline SCT appears to be quite variable across participants as shown by the participant random intercept (variability due to baseline SCT between participants), suggesting that different people behave differently towards CRT. For example, participant 1, 2, 4, 5, 7 and 10 had a much lower baseline SCT compared to that of participant 6 and 9 who had a much higher SCT (Figure 6, Appendix III). The reasonably high variability due to baseline SCT between participants suggests caution in establishing a normative threshold. It raises a concern when CRT using only single concentration is implemented clinically in detecting SA. The high variability due to baseline SCT between participants might affect the sensitivity of the CRT, for example, patients with a naturally

high SCT might be rated as failing the CRT when they did not cough at 0.6 mol/L (the concentration commonly used clinically). This might explain the poor sensitivity reported by Guillén-Solà and colleagues (2015) as participants in their study were tested using only single concentration without addressing the high variability in baseline SCT between participants.

The box plot (Figure 4) and Levene's test revealed that the variability for each participant was different with some participants showing a greater variability (e.g. participants 6 and 9) when compared to others with respect to repeated measures of CRT. This variability does not appear to be associated in a systematic way with the participant specific average SCT (e.g. a greater variability is present when the SCT is higher). However, the margins of error for the prediction intervals calculated in the bootstrapping technique may be too conservative for some participants while overestimates the others.

Moreover, this study reveals fairly consistent (variability was within 0.1 mol/L) within-participant measures. The 95% prediction interval for the residuals (variability within a participant) includes the value of zero effect which can be inferred that there is no statistical significance of within-participant variability. These findings reflect that citric acid CRT using TB-DR method may be useful for assessing change within a patient. When being assessed individually, an individual's SCT is stable across tests using the TB-DR method. This study observes no learning effect for repeated CRTs within-participant. In other words, when a larger variability is observable in dysphagic patient population or research population, it may reflect a pathological condition or may be due to a therapeutic effect (treatment outcome measure).

6.3 Possible contributing factors

It is almost impossible to identify the contributing factors of these variabilities separately when the physiological and psychological factors are confounding the technical

factors in contributing to the variability of CRT. The potential contributing factors to the between participants variation reported in this study might be the physiological and psychological contributions such as diurnal variation, individual personality, emotional state, level of attention, fatigues and etc. For example, participants with a competitive personality may use considerable effort to suppress their coughs whilst the others might give up and cough much earlier. As a result, participants with a competitive personality might have a higher SCT than the rest. This might be one of the contributing factors to the high variability at baseline SCT between participants evident in this study.

The present study used the TB-DR protocol similar to previous studies (Monroe et al., 2014; Perry & Huckabee, 2017). Without using an inspiratory flow regulatory valve, this method allows variability in individual's breathing pattern, especially at high concentration (Khalid, 2012; Morice et al., 2001; Newman, Pitcairn, Hooper, & Knoch, 1994). It has been validated that inspiratory flow is in association with the inconsistent inhalation rate among participants through tidal breathing which could lead to various doses being inhaled (Dolovich, 1985; Morice et al., 2007; Ryan et al., 1981). The instruction to participants was to breathe normally; however, as the concentration increased, the researcher observed that some participants learned to manipulate their breathing pattern by using shallow tidal breathing, more frequent swallows or prolonged exhalation to minimise the amount of inhaled citric acids. As a result, participants who changed their breathing pattern tended to have a higher SCT than those who didn't. The uncontrolled inspiratory flow may further explicit the impact of breathing method on the variation revealed in this study. It is highly recommended for future study to use an inspiratory flow regulatory valve to maintain a consistent inspiratory flow and a spirometry to monitor participants' breathing pattern throughout the test.

In general, this study adopts a simple, affordable and portable method which uses disposable jet nebulisers across tests and participants. There is evidence showing the high variability in nebuliser output across nebulisers (Dolovich, 1985; Hollie, Malone, Skufca, & Nelson, 1991; Ryan et al., 1981). The variability across nebulisers might contribute to the variability seen in this study; therefore, it is important for the future study to be consistent in using only one sterilisable nebuliser across tests and participants. The nebuliser needs to be calibrated regularly after multiple usages to ensure optimum performance and repeatable output.

It has been shown that the nebulization method and reservoir volume, on the other hand, play a role in influencing the aerosol output. An increase in operating time changes the solution temperature that will affect the rate of evaporation. The greater the nebulization time, the lesser the overall output per minute, when there is no constant refilling of the reservoir (Dolovich, 1985). However, the nebulization time used in this study was short enough and consistent (15 seconds) across tests and participants; therefore, it is less likely to be a contributing factor to the variability observed in this study.

6.4 Limitations

In terms of the data analysis method, the SCT was introduced as a continuous variable. However, in reality, the SCT is not a continuous measure but an interval variable. This may be problematic as for some participants; their SCT might not fall in the interval value which leads to a less precise result. On the other hand, the box plot (Figure 4) and Levene's test revealed a lack of homogeneity in the variance of the residuals across participants. The margin of error for the prediction interval calculated using the bootstrapping approach may overestimate or underestimate an individual's SCT.

Moreover, this study did not have enough data to generalise for gender and age. The findings reported in this study might not reflect the true population data but a specific group of the population. In addition, it was difficult to monitor an individual's breathing pattern without having some measurement such as a spirometry. Participants were told to breathe normally through their mouth while suppressing their cough. However, they were not told to suppress swallowing. Some participants tended to swallow during the 15 seconds delivery period which could affect the amount of citric acid being inhaled. It has been known that respiration is ceased during swallowing (Matsuo & Palmer, 2009).

6.5 Future directions

6.5.1 Patient cohort

Clinically, a simple and affordable protocol is preferred. It would be beneficial for future research to evaluate the reproducibility of citric acid CRT using the TB-SD method which is extensively used clinically in detecting SA among dysphagic patients. When the variability of CRT in the inpatient cohort is well-defined, clinicians will be more confident in interpreting the CRT result as it is more likely to reflect a true pathological condition or therapeutic effect (treatment outcome measure).

6.5.2 A refined methodology as a research tool

A well-controlled methodology using a minimum effective number of inhalations is vital to minimise trial effect while maintaining its sensitivity and reliability is vital when CRT is being used as a research tool. A more objective methodology would be ideal to improve the sensitivity of CRT and minimise rater error. It is strongly recommended for future studies to use an inspiratory flow regulatory valve to maintain a consistent inspiratory flow and measurement from spirometry to monitor participants' breathing pattern throughout the test.

Concluding Remarks

This study is the first to define the variability of citric acid CRT using TB-DR method in healthy individuals. Outcome measures have to be sensitive and reliable in providing empirical evidence for dysphagia assessment and treatment methods. These data suggest caution in establishing normative thresholds given the high percentage of variability for baseline SCT between participants. The fairly consistent within-participant measures suggest CRT may be useful for repeated measures in tracking change as treatment effect within an individual participant or patient. The variances reported in the present study suggest caution when interpreting the CRT results in both research and clinical purposes. If origins of variability can be identified and considered, the application of results from CRT can be improved. Despite these results, cannot be generalised to the pathologic population, perhaps, these findings can be used to compare the variances reported in patient groups while serving as a foundation for analysis of future research of healthy population.

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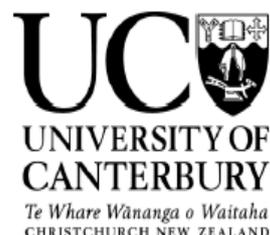
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Appendix I: Human ethics approval letter



HUMAN ETHICS COMMITTEE

Secretary, Rebecca Robinson
Telephone: +64 03 364 2987, Extn 45588
Email: human-ethics@canterbury.ac.nz

Ref: HEC 2016/82

29 August 2016

Su Hui Lim
Communication Disorders
UNIVERSITY OF CANTERBURY

Dear Su Hui

The Human Ethics Committee advises that your research proposal "Trial Effect of Cough Reflex Test on Cough Reflex Sensitivity in Healthy Individuals" has been considered and approved.

Please note that this approval is subject to the incorporation of the amendments you have provided in your email of 17th August 2016.

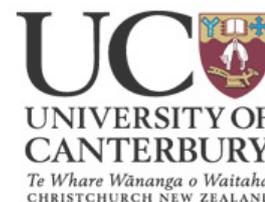
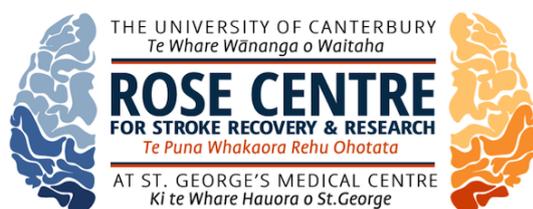
Best wishes for your project.

Yours sincerely

R. Robinson
pp.

Kelly Dombroski
Deputy Chair
University of Canterbury Human Ethics Committee

Appendix II: Information sheet



PARTICIPANT INFORMATION SHEET

Department of Communication Disorders

Telephone: +64 3 364 2307

Email: suhui.lim@pg.canterbury.ac.nz

13th June 2016

Trial Effect of Cough Reflex Test on Cough Reflex Sensitivity in Healthy

Individuals

Principal Investigator:

Su Hui Lim
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Communication Disorders
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I am an MSc student in Communication Disorders Department, performing a research project under the supervision of Prof Maggie-Lee Huckabee. You are invited to take part in a study determining the effect of repeatedly presented inhaled citric acid on the cough reflex sensitivity. The study will recruit 10 healthy adults who have no current issue of respiratory

and swallowing difficulty. Results from this study will aid us in developing rehabilitation approaches for people with absent cough reflex, like stroke patients.

You can take part in this study if you are 18 years or older, and have no medical problems that may affect your respiratory and swallowing. If you choose to take part in this study, your involvement in this study will be to inhale citric acid which smells like an orange via a facemask on each test. It is a single visit which lasts for three hours and consists of five cough reflex tests with 30 minutes inter-test interval. Each test will take 15-20 minutes, you will be administered different dose of citric acids and instructed to breathe through your mouth comfortably while suppressing your cough. Each test will stop when the researcher has gotten your cough reflex threshold.

Information about your cough reflex sensitivity will be stored on the computer and analysed at another time. The only data recorded will be the citric acid doses that represent your cough reflex sensitivity.

The only benefit to you is that your participation contributes important information about cough reflex sensitivity shifting in healthy adults. There are no known risks in the performance of the tasks and application of the procedures. The only after test-effect is citric acid CRT might induce short-term tickling sensation in the throat in some participants but will resolve within 15-30 seconds.

Participation is voluntary and you have the right to withdraw at any stage without penalty. You may ask for your raw data to be returned to you or destroyed at any point. If you withdraw, I will remove information relating to you. However, once analysis of raw data

starts on 3rd July 2017, it will become increasingly difficult to remove the influence of your data on the results. The results of the project may be published, but you may be assured of the complete confidentiality of data gathered in this investigation: your identity will not be made public without your prior consent. To ensure anonymity and confidentiality, no material which could personally identify you will be used in any reports on this study. Consent forms will be kept in a locked filing cabinet in the locked swallowing research laboratory or will be stored on password-protected laboratory computers. Research data will be stored for a period of 10 years after data collection is completed, at which time they will be destroyed.

A thesis is a public document and will be available through the UC Library. You will be offered copies of the final manuscript of this study or a basic summary. Please indicate to the principal investigator on the consent form if you would like to receive a copy of the summary of results of the study. However, you should be aware that a significant delay may occur between completion of data collection and the final report. Alternatively, or in addition, you can choose to have the results of the study discussed with you personally by the principal investigator.

You can contact the principal investigator if you require any further information about the study during work hours at (03) 364 2307 or through email: suhui.lim@pg.canterbury.ac.nz. She will be pleased to discuss any concerns you may have about participation in the study.

This project has been reviewed and approved by the University of Canterbury Human Ethics Committee, and participants should address any complaints to The Chair, Human Ethics Committee, University of Canterbury, Private Bag 4800, Christchurch (human-ethics@canterbury.ac.nz).

If you agree to participate in the study, you are asked to complete the consent form and return to the principal investigator.

Su Hui, Lim

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PARTICIPANT QUESTIONNAIRE

Department of Communication Disorders

Given code: 000__

Telephone: +64 3 364 2307

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3rd May 2016

Trial Effect of Cough Reflex Test on Cough Reflex Sensitivity in

Healthy Individuals

Screening questions:

- Do you have current respiratory infection or illness e.g. pneumonia, sore throat, chronic cough, running nose and etc.?

If YES, please list out _____

- Do you have history of drug abuse or current drug use (e.g. opioid-based medications – painkillers, codeine, hydrocodone, Vicodin, OxyContin, Demerol; narcotics; ACE inhibitors – hypertension medication; cough medication)?

If YES, please list out _____

- Do you have past or current issue of swallowing difficulty?

If YES, please detail _____

- Do you have current issue of gastroesophageal reflux disorder (GERD)?

If YES, please detail _____

- Do you have recent history of smoking or a current smoker?

If YES, please detail _____



PARTICIPANT CONSENT FORM

Department of Communication Disorders

Telephone: +64 3 364 2307

Email: suhui.lim@pg.canterbury.ac.nz

3rd May 2016

Trial Effect of Cough Reflex Test on Cough Reflex Sensitivity in Healthy Individuals

Declaration by participant:

I have read and I understand the Participant Information Sheet.

- I have been given a full explanation of this project and have had the opportunity to ask questions.
- I understand what is required of me if I agree to take part in the research.
- I understand that participation is voluntary and I may withdraw at any time without penalty. Withdrawal of participation will also include the withdrawal of any information I have provided should this remain practically achievable.
- I understand that any information or opinions I provide will be kept confidential to the researchers [the principal investigator, supervisor and co-supervisor] and that any published or

reported results will not identify the participants. I understand that a thesis is a public document and will be available through the UC Library.

- I understand that all data collected for the study will be kept in locked and secure facilities and/or in password-protected electronic form and will be destroyed after 10 years.
- I understand the risks associated with taking part and how they will be managed.
- I understand that I am able to receive a report on the findings of the study by contacting the researcher at the conclusion of the project.
- I understand that I can contact the researcher [suhui.lim@pg.canterbury.ac.nz] or supervisor [maggie-lee.huckabee@canterbury.ac.nz] for further information. If I have any complaints, I can contact the Chair of the University of Canterbury Human Ethics Committee, Private Bag 4800, Christchurch (human-ethics@canterbury.ac.nz).
- I would like a summary of the results of the project.
- I have been given a copy of the Participant Information Sheet and Consent Form to keep.
- By signing below, I agree to participate in this research project.

Name: _____ Signed: _____ Date: _____

Email address (*for report of findings, if applicable*): _____

| |
|-------------------|
| Given code: 000__ |
|-------------------|

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PARTICIPANTS WANTED FOR STROKE RESEARCH STUDY

Pilot Study 1: Trial Effect of Cough Reflex Test on Cough Reflex Sensitivity in Healthy Individuals

We are looking for healthy men and women, 18 years and above, to participate in a study determining the trial effect of citric acid cough reflex test on the cough reflex sensitivity. Results from this study will aid us in developing rehabilitation approaches for people with absent cough reflex, like stroke patients. You may be eligible to participate if you **do not have**:

1. Current issue of respiratory
2. Current and past history of swallowing problem
3. Current issue of gastroesophageal reflux disorder
4. History of drugs abuse
5. Prolonged exposure to tobacco smoke

If you agree to participate in the study, you will have to inhale citric acid which smells like an orange via a facemask on a three hours single visit. You will be instructed to breathe through your mouth comfortably while suppressing your cough. This study consists of five cough

reflex tests with 30 minutes rest in between tests; each test will take 15-20 minutes. This study will be carried out at the Rose Centre in St George's Hospital.

If you are interested in participating, please contact:

Su Hui, Lim

Master Student

University of Canterbury Rose Centre for Stroke Recovery and Research

Private Bag 4737, 249 Papanui Rd, Christchurch 8140

Telephone: (03) 364 2307

Email: suhui.lim@pg.canterbury.ac.nz

Appendix III: Raw data

Raw data from all 16 participants across five CRTs

| Participants (age) | Test number | | | | | | | | | |
|-----------------------|----------------|---------|----------------|---------|----------------|---------|----------------|---------|----------------|--------|
| | Baseline | | 1 | | 2 | | 3 | | 4 | |
| | SCT (mol/L) | Time | SCT (mol/L) | Time | SCT (mol/L) | Time | SCT (mol/L) | Time | SCT (mol/L) | Time |
| 001 (25) | 0.1 | 11am | 0.1 | 11.42am | 0.1 | 12.21pm | 0.1 | 12.56pm | 0.1 | 1.30pm |
| 002 (25) | 0.1 | 11am | 0.1 | 11.31am | 0.2 | 12.05pm | 0.1 | 12.43pm | 0.1 | 1.18pm |
| 003 (31) | 0.5 | 11.01am | 0.4 | 11.57am | 0.2 | 12.46pm | 0.1 | 1.27pm | 0.2 | 2.02pm |
| 004 (31) | 0.1 | 11am | 0.1 | 11.36am | 0.1 | 12.07pm | 0.1 | 12.46pm | 0.1 | 1.26pm |
| 005 (32) | 0.1 | 11.03am | 0.1 | 11.41am | 0.1 | 12.17pm | 0.1 | 12.57pm | 0.1 | 1.31pm |
| 006 (33) | 0.4 | 11am | 0.6 | 11.47am | 0.6 | 12.46pm | 0.8 | 1.40pm | 0.3 | 2.40pm |
| 007 (20) | 0.1 | 11.04am | 0.1 | 11.39am | 0.1 | 12.10pm | 0.1 | 12.40pm | 0.1 | 1.24pm |
| 008 (20) | 0.1 | 10.58am | 0.2 | 11.33am | 0.2 | 12.13pm | 0.2 | 12.51pm | 0.2 | 1.35pm |
| 009 (40) | 1.4 | 11.01am | 1.0 | 12.30am | 1.0 | 1.27pm | 0.9 | 2.09pm | 0.1 | 2.48pm |
| 010 (29) | 0.1 | 11.05am | 0.1 | 11.37am | 0.1 | 12.11pm | 0.1 | 12.45pm | 0.1 | 1.15pm |

| | | | | | | | | | | |
|----------|-----|---------|-----|---------|-----|---------|-----|---------|-----|--------|
| 011 (48) | 0.1 | 11.01am | 0.1 | 11.36am | 0.2 | 12.13pm | 0.3 | 1.02pm | 0.3 | 1.40pm |
| 012 (29) | 0.1 | 11.06am | 0.1 | 11.42am | 0.2 | 12.20pm | 0.2 | 12.55pm | 0.2 | 1.43pm |
| 013 (45) | 0.7 | 10.58am | 0.6 | 12.01pm | 0.6 | 12.50pm | 0.6 | 1.45pm | 0.6 | 2.38pm |
| 014 * | - | - | - | - | - | - | - | - | - | - |
| 015 (21) | 0.1 | 11.04am | 0.1 | 11.40am | 0.1 | 12.20pm | 0.2 | 1.00pm | 0.4 | 1.57pm |
| 016 (21) | 0.1 | 11.05am | 0.1 | 11.43am | 0.2 | 12.27pm | 0.4 | 1.10pm | 0.3 | 2.10pm |
| 017 (34) | 0.6 | 11am | 0.6 | 11.47am | 0.6 | 1.10pm | 0.6 | 2.30pm | 0.7 | 4.00pm |

*Participant 014 was excluded as he did not meet inclusion criteria, where he did not cough at 1.6mol/L.

Appendix IV: R Code

```
library(lme4)

trial_effects_study <- read.csv("//file/UsersSS/shl68/Home/My Documents/Trial effect
study.csv", head=TRUE, sep=",")

str(trial_effects_study)

# changing continuous variable to ordinal variable, adding 'factor' means adding another
column

trial_effects_study$Participants <- as.factor(trial_effects_study$Participant)

trial_effects_study$Test <- as.numeric(trial_effects_study$Test)

str(trial_effects_study)

library(lme4)

mod1 <- lmer(CRT~ 1 + Test + (1+Test|Participants), data = trial_effects_study)

library(lme4)

smod2 <- lmer(CRT~1+Test+(1+Test|Participants), data = trial_effects_study)

library(lattice)

#checking normality and variance of residuals

r_int<- ranef(mod1)$Participants$`(Intercept)`

r_slope<- ranef(mod1)$Participants$Test
```

```

plot(mod1)

hist(residuals(mod1), density=20, breaks=20, prob=TRUE,
     xlab="Residuals",
     main="Normal curve over histogram for the residuals")
curve(dnorm(x, mean=mean(residuals(mod1)), sd=sd(residuals(mod1))),
     col="darkblue", lwd=2, add=TRUE, yaxt="n")
qqnorm(residuals(mod1))
qqline(residuals(mod1))

hist(trial_effects_study$SCT, density=20, breaks=20, prob=TRUE,
     xlab="SCT",
     main="histogram for the outcome variable SCT")

#checking normality of intercepts
qqnorm(r_int)
qqline(r_int)
hist(r_int, density=20, breaks=6, prob=TRUE,
     xlab="Random intercept",
     main="Normal curve over histogram for the random intercept")
curve(dnorm(x, mean=mean(r_int), sd=sd(r_int)),
     col="darkblue", lwd=2, add=TRUE, yaxt="n")

#checking normality of slopes

```

```
qqnorm(r_slope)
```

```
qqline(r_slope)
```

```
hist(r_slope, density=20, breaks=6, prob=TRUE,
```

```
  xlab="Random slope",
```

```
  main="Normal curve over histogram for the random slope")
```

```
curve(dnorm(x, mean=mean(r_slope), sd=sd(r_slope)),
```

```
  col="darkblue", lwd=2, add=TRUE, yaxt="n")
```

```
#boxplot (residuals against participant)
```

```
ggplot(trial_effects_study, aes(Participants, residuals(mod1))) + geom_boxplot() +
```

```
  ggtitle("Box plot of the residuals for each participant accross five CRTs")+
```

```
  labs(x="Participant", y="Residuals")
```

```
#boxplot (SCT against participant)
```

```
ggplot(trial_effects_study, aes(Participants, residuals(mod1))) + geom_boxplot() +
```

```
  ggtitle("Box plot of participants' SCT across five CRTs")+
```

```
  labs(x="Participant", y="Suppressed cough threshold (SCT [mol/L])")
```

```
# estimated model coefficients and variance components
```

```
summary(mod1)
```

```
coef(mod1)
```

```
ranef(mod1)
```

```
# confidence intervals for test & participant effects to make inference
```

```

# about the variability of CRT within participant in a population of participants
confint(mod1, method="boot", nsim=1000, oldNames=FALSE)

library(boot)

# confidence interval for % explained variance by fixed and random effects
for (i in 1:1000) {

  ###Create the model

  mod1 <- lmer(CRT~ 1 + Test + (1+Test|Participants), data = trial_effects_study)

  ###Sample 80 points with replacement from the residuals (since there were 80 obs.)

  # we need replace to be true since this allows for reordering the residuals but also to use
the values randomly,

  # so that we can repeat some values. see example with sample(1:10, size = 10);
sample(1:10, size = 10, replace=T)

  Sample <- residuals(mod1)[sample(1:length(residuals(mod1)), 80, replace=T)]

  ###Sort the sample from smallest to largest, then find the 2.5th and 97.5th percentiles

  sample_sorted<-sort(Sample)

  lowerMOE<-

(sample_sorted[(0.025*(length(sample_sorted)))+sample_sorted[(0.025*(length(sample_s
orted))+1])/2

  upperMOE<-

(sample_sorted[(0.975*(length(sample_sorted)))+sample_sorted[(0.975*(length(sample_s
orted))+1])/2

```

```

###Put the results in the data frame, adding the intercept so they are on CRT scale

MOE$LowerMOE[i]<-lowerMOE

MOE$UpperMOE[i]<-upperMOE

}

c(mean(MOE$LowerMOE),mean(MOE$UpperMOE))

#ci for the fixed effects

foo_f <- function(m){

  i = j # 1 for intercept, 2 for slope

  return (fixef(m)[i])

}

# intercept

j =1

bsm<- bootMer(mod1, foo_f, 1000)

intercept_ci <- boot.ci(bsm, type= "perc")

intercept_value <- fixef(mod1)[j]

#slope

j = 2

bsm<- bootMer(mod1, foo_f, 1000)

slope_ci <- boot.ci(bsm, type= "perc")

```

```

slope_value <- fixef(mod1)[j]

# ci for random effects
foo_r <- function(m){
  i = j # 1 for intercept, 2 for slope, 4 for residual
  raneffects <- as.data.frame(VarCorr(mod1))[5]
  return (raneffects[j,1])
}

confint(mod1, level= 0.95,
        method = c("boot"),
        nsim = 1000,
        boot.type = "perc", oldNames = FALSE)

# intercept
j =1
bsm<- bootMer(mod1, foo_r, 1000, use.u=TRUE, type="semiparametric")
intercept_random_ci <- boot.ci(bsm, type= "perc")
intercept_random_value <- as.data.frame(VarCorr(mod1))[j,5]

# slope
j =2
bsm<- bootMer(mod1, foo_r, 1000)
slope_random_ci <- boot.ci(bsm, type= "perc")

```

```
slope_random_value <- as.data.frame(VarCorr(mod1))[j,5]
```

```
# residuals
```

```
j=4
```

```
bsm<- bootMer(mod1, foo_r, 1000)
```

```
residuals_random_ci <- boot.ci(bsm, type= "perc")
```

```
residuals_random_value <- as.data.frame(VarCorr(mod1))[j,5]
```