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A Comparison of Exhaled Breath Nitric Oxide Between Old and Young Individuals

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by

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Dedication

I would like to dedicate this work to my wife, Kim, who has encouraged me to strive for excellence.

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I would like to thank Dr. Robert Haight for the countless hours of assistance he provided to me during this project. I would also like to thank Dr. Stuart Brooks for providing me encouragement and inspiration while I worked on this project.

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ABSTRACT

BACKGROUND: Older individuals suffer from higher rates of pulmonary infections than younger individuals. In addition, older individuals have increased morbidity and mortality due to pulmonary infections when compared to younger individuals. The physiological and immunological reasons for these aforementioned differences are not clear. Recently, non-invasive markers of the lung's physiologic and immunologic status have been recognized. This study employs one of these non-invasive markers, exhaled nitric oxide, in an attempt to determine how the airways may change with age, predisposing older individuals to pulmonary diseases and poorer outcomes as compared to younger individuals.

METHODS: Exhaled nitric oxide measurements were obtained from a group of 25 older subjects (61 to 79 years old, median 72 years old) and a group of 23 younger subjects (21 to 30 years old, median 24 years old) that were non-smokers with no history of pulmonary disease, no recent respiratory infections, and no history of environmental allergies. A focused history and physical exam along with spirometry were used to confirm the normal pulmonary status of each subject. Exhaled nitric oxide was measured following the American Thoracic

Society recommendations using the Sievers Nitric Oxide Analyzer 280i. The exhaled nitric oxide values for the old and young groups were compared using the Wilcoxon Rank Sum test.

RESULTS: For the older subjects, the median exhaled NO concentration was 36.9 ppb. For the younger subjects, the median exhaled NO concentration was 18.7 ppb. These exhaled NO concentrations are significantly different ($p = 0.0011$).

CONCLUSIONS: The exhaled NO concentrations are significantly higher in older individuals than in younger individuals. The reasons for this difference along with the significance are unclear and further studies will be necessary to further evaluate these issues.

Introduction

Background

Respiratory infections, particularly pneumonia, are known to cause significant morbidity and mortality in the elderly population.[1, 2] The incidence of pneumonia has been shown to increase with age with incidences of 91.6/100,000 for individuals younger than 45 years of age, 277.2/100,000 for individuals aged 45 to 64 years, and 1012.3/100,000 for individuals older than 65 years in one study.[3]

Factors that are known to predispose the elderly to pneumonia are vast and include: age greater than 65 years, underlying comorbid illnesses such as chronic obstructive pulmonary disease, diabetes mellitus, congestive heart failure, malignancy, poor nutrition, institutionalization, recent hospitalization, and smoking.[1, 4] It is evident, as individuals increase in age, they acquire more of the aforementioned risk factors bringing into question whether increasing age itself is an independent risk factor for developing pneumonia.

Aging in itself has been shown to lead to physiological and immunological changes that may predispose the aged population to pneumonia and poorer outcomes once contracted. The physiological changes include: impaired mucociliary transport, enhanced oropharyngeal colonization, increased aspiration potential, decreased ability to expectorate, decreased respiratory muscle

endurance, and decreased forced expiratory volume in one second (FEV_1).[1, 4] Although not fully elucidated, the immune system has been shown to undergo alterations as individuals age such that there is impaired immune surveillance, decreased ability to mount an appropriate immune response, and inappropriate immune responses.[5]

Exhaled respiratory markers have been recognized that correlate with infection and inflammation.[6] These include nitric oxide (NO), airway pH, eicosanoids, hydrogen peroxide, hydrocarbons, carbon monoxide, and NO-related products.[6]

NO has been studied extensively since Gustafsson recognized that it was present in exhaled breath.[7] As will be discussed later, NO has many immunological and physiological roles within the human. In particular, NO has been found to be positively correlated with inflammation and infection within the lower respiratory tract.[6, 8-10]

This study was conducted to determine if there is a difference in endogenous NO concentrations derived from the lower respiratory airways between younger and older individuals in an attempt to see how the airways may change with age, predisposing the older population to pulmonary infections and increased morbidity and mortality with pulmonary infections. Other studies have not shown a difference between these two groups, however, those studies have generally included less people, particularly older individuals.[11-13]

Airway Nitric Oxide

Biochemistry

NO is a free-radical which is formed enzymatically and non-enzymatically. NO itself is very reactive and undergoes a multitude of reactions with other molecules within the human respiratory tract to produce other compounds with varying functions.

NO may be formed when L-arginine is oxidized producing NO and L-citrulline. This reaction is catalyzed by a group of three enzymes known as nitric oxide synthases (NOSs) with the assistance of various co-factors. NOSs are divided into constitutive and inducible types. There are two types of constitutive NOS (cNOS) which are known as Type I or neuronal NOS (nNOS) and Type III or endothelial NOS (eNOS). These enzymes are dependent on calcium and calmodulin for activation. There is one type of inducible NOS known as Type II or inducible NOS (iNOS) which is not dependent on calcium and calmodulin for activation.

NO is also produced non-enzymatically within the airways. One method is via nitrite (NO_2^-) protonation to form nitrous acid (HNO_2) which then releases NO gas when an acidic environment is present such as during an asthma exacerbation.[14] Another source of NO is via the release of NO from S-nitrosothiols within the airway tissues.[6, 15] The nitrosothiols are originally produced by NO reacting with thiol groups (RSH) to produce the nitrosothiols

(RS-NO). The nitrosothiols are excellent carriers of NO and provide it stabilization to act at distant sites from production.[16]

Nitric oxide is highly reactive with other molecules besides the thiols. When NO combines with the superoxide anion, peroxynitrite ($OONO^-$) is formed.[16] This molecule is a pro-oxidant and is known to be toxic to many molecules such as nucleic acids, lipids, and proteins.[16, 17] Also, it is known to be very toxic to microbes.[6, 17] In addition, NO reacts with O_2 to form nitrite and nitrate in aqueous solutions by reactions catalyzed by transition metals such as iron.[9, 17] NO also reacts with the heme moiety of hemoglobin to form methemoglobin.[17]

Cellular and Anatomic Sources of the Nitric Oxide Synthases

All three of the nitric oxide synthases have been identified in airway mucosa.[18] Neuronal NOS has been found in neurons, bronchial epithelial cells, and skeletal muscles.[9, 19] Endothelial NOS has been localized to platelets, endothelial cells, endocardial cells, bronchial epithelial cells, and myocardial cells.[9, 20] Inducible NOS has been demonstrated in the epithelial linings of the airways, macrophages, neutrophils, eosinophils, and fibroblasts.[9, 17] As has been described above, the cNOSs are calcium/calmodulin dependent for activation whereas iNOS is not dependent on calcium/calmodulin for activation. Inducible NOS is induced indirectly by inflammatory cytokines such as interferon gamma (IFN-γ), interleukin (IL) – 1 β, tumor necrosis factor – alpha (TNF-α), and others. The inflammatory cytokines induce the production of transcription factors such

and nuclear factor- kappa B (NF-κB) and signal transducers and activators of transcription-1 (STAT-1) which in turn lead to the transcription of the iNOS gene sequence.[21-24] It is of note that iNOS produces up to 1,000 times more NO than cNOS and that cellular production continues for hours after the stimulus.[22] In addition, iNOs has now been shown to be expressed at low levels constitutively in airway epithelium.[25]

As mentioned earlier, all three of the enzymes are produced within the airways. However the upper airways need to be distinguished from the lower airways with regards to synthase activity and NO levels. The upper airways, which include the paranasal sinuses and nose, have been shown to produce several fold higher levels of NO than the lower airways.[22, 26, 27] The type of synthase in these areas is genotypically similar to iNOS of the lower airways.[26] However, it is expressed at relatively high levels constitutively.[26, 28] The lower airways are capable of producing NO via all three types of synthases. For years there was great debate over where the majority of the exhaled NO was derived from within the lower airways. It has now been determined that the majority of the exhaled NO is derived from the airways proximal to the alveoli.[29]

Function of Endogenous Nitric Oxide within the Lower Respiratory Tract

Nitric oxide is a mediator, either directly or via other NO containing reaction products for the nonadrenergic, noncholinergic (NANC) neural inhibitory impulses from nerves innervating the respiratory tract.[30] Neuronal NOS is the synthase which is involved in the production of NO for this neural function. The

NANC neural pathways are the only bronchodilatory pathways in the airways of humans.[31] It has been postulated that the bronchodilatory effect mediated by the NANC neural impulses is important in balancing cholinergic bronchoconstriction rather than causing significant independent bronchodilatation.[32] The bronchodilatory effect mediated by NO or NO containing products is via stimulation of the intracellular enzyme guanylate cyclase which catalyzes the conversion of guanylate triphosphate (GTP) to cyclic guanylate 3'5' monophosphate (cGMP) which ultimately leads to relaxation of the smooth muscles within the airways.[9]

NO and NO containing products are involved in vasodilatation in the pulmonary and bronchial circulations.[33, 34] Endothelial NOS is the synthase which is involved in the production of NO for this vasodilatory function at baseline. The vasodilatation is mediated in a similar fashion as the bronchodilatation.

When present in abnormally elevated amounts, NO is a mediator that is involved in the inflammatory process in multiple ways. Inducible NOS is the synthase responsible for production of NO in very large quantities which contribute to an inflammatory response. NO, due to its vasodilatory properties, causes exaggerated vasodilatation along with plasma exudation carrying inflammatory cytokines and cells to specific sites within the lung to cause or perpetuate an inflammatory response.[9]

NO, through an intermediary, peroxynitrite, may lead to significant inflammation within the airways through its oxidizing effects on tissues within the

lungs.[35] In addition, because of its toxic effect on microbes, it may serve as an innate defense mechanism within the lung.[17]

NO has also been shown to accentuate inflammation by modulating the immune system. It is believed that NO may selectively inhibit T-helper 1 (Th1) lymphocytes.[36] This in turn may lead to decreased levels of IFN-γ which is produced by the Th1 lymphocytes. As a result of decreased levels of IFN-γ, Th2 cells proliferate liberating substantial amounts IL-5 which drive eosinophilic inflammation in conditions such as asthma.[36-38] This effect is most prominent when elevated levels of NO are present after induction of iNOS.

NO has also been shown to mediate ciliary motion and stimulate airway secretions.[39] In individuals with primary ciliary dyskinesia (PCD), exhaled NO values have been shown to be very low. This is significant as only PCD has such a characteristically low exhaled NO value.[40]

As can be appreciated from the above discussion regarding NO production and function, NO's roles and the roles of the synthases are quite complex. Due to its many functions, NO may therefore have beneficial or deleterious effects depending on the underlying situation within the lung.

Clinical Significance of Nitric Oxide

Nitric oxide is present in the airways of healthy individuals and individuals with various airway diseases. In addition, all three of the synthases are produced in both healthy individuals and those with airway diseases.[6, 25] The expression of iNOS in the lower airways of healthy individuals had long been debated but there

is now evidence that it is indeed expressed at a basal rate, ramping up when it is specifically induced by inflammatory cytokines involved in various airway diseases resulting in elevated exhaled NO levels.[25] NO has been studied extensively in individuals with airway conditions as a potential marker for inflammation. Many believe that this marker will revolutionize the non-invasive monitoring of airway diseases.

Exhaled NO has been studied most extensively in asthmatics. Elevated levels of exhaled NO in asthmatics have been well documented in the literature.[41, 42] This rise in NO is believed to be related to the increased expression of iNOS due to an inflammatory state via inflammatory cytokines and to the non-enzymatic production of NO from nitrite in the acidic bronchial environment of asthmatics.[14, 43] This marker is not specific for asthma but may assist in differentiating healthy individuals from asthmatics when there is a question.[44] Another potential use of NO in asthmatics is for the monitoring of corticosteroid and/or anti-leukotriene therapies. Levels of NO have been shown to decline in asthmatics treated with corticosteroids (inhaled and oral) along with the leukotriene inhibitors.[45-48] The reason for the decline associated with corticosteroids has been postulated to be due to decreased iNOS levels secondary to inhibition of iNOS transcription factors such as NF-κB and a reduction of inflammatory cytokines.[45, 49] In addition, exhaled NO has been shown to increase before other markers of asthma exacerbation such as lung function, provocative concentration 20% (PC_{20}), sputum eosinophilia, or asthma

symptoms.[50] Due to this ability, it has been proposed that NO levels may be followed to determine the "loss of control" of asthma.[50]

Exhaled nitric oxide has also been shown to be elevated in other disease states. Adrie et al. described elevated exhaled nitric oxide levels in mechanically ventilated patients with pneumonia.[10] In addition, individuals with unstable chronic obstructive disease, bronchiectasis, and exposure to outdoor pollutants have also been shown to have elevated levels of exhaled NO.[51, 52] Elevated exhaled nitric oxide levels have also been demonstrated in individuals given L-arginine orally or via inhalation, and during exercise.[51-55] Table 1 summarizes the conditions in which exhaled NO may be increased.

Exhaled nitric oxide has been shown to be decreased in various respiratory disease states and include: cystic fibrosis, PCD, and pulmonary hypertension.[51] Decreased exhaled nitric oxide levels have also been demonstrated in those who are smokers. In addition, various drugs have been shown to reduce exhaled NO levels such as inhaled NOS inhibitors, oral or inhaled corticosteroids, anti-leukotriene medications, and alcohol.[45-48, 51, 56, 57] Table 1 summarizes the conditions in which exhaled NO may be decreased.

Measurement of Nitric Oxide

The most commonly used method for detecting NO in exhaled breath is via chemiluminescence. With this method, exhaled NO reacts with an excess of ozone (O_3) within an exhaled breath NO analyzer to produce nitrogen dioxide (NO_2) with an electron in the excited state (NO_2*). NO_2* then transforms to the

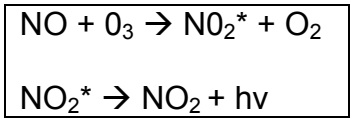
ground state (NO_2) while emitting electromagnetic radiation (chemiluminescence) ranging from 600-3,000 nm in wavelength. This chemiluminescence is detected by a photomultiplier tube and is converted into an equivalent electrical display of NO concentration. With this method, NO can be detected down to a concentration of less than 1 part per billion (ppb). Refer to Figure 1 for the chemical reactions involved with the chemiluminescence method of measuring exhaled NO concentration.

Table 1. Factors Which Influence Exhaled Nitric Oxide[6, 58]

Increased NO	Decreased NO
Drugs: L-Arginine ingestion or inhalation, nitroprusside, ACE inhibitors	Drugs: NOS inhibitors, oral or inhaled corticosteroids
Respiratory tract infections	Primary ciliary dyskinesis
Sarcoidosis	Pulmonary hypertension
Asthma exacerbation	Cystic Fibrosis
Unstable COPD	Smoking
Bronchiectasis	Alcohol ingestion
Allergic/atopic state	Forced exhalation
Exercise	Repeated spirometry
Exposure to outdoor pollutants	Sputum induction

ACE = angiotensin converting enzyme; COPD = chronic obstructive pulmonary disease; NOS=nitric oxide synthase

Figure 1. The Chemical Reactions Involved in the Chemiluminescence Method of Measuring Exhaled Nitric Oxide



There are two types of exhaled NO collection methods which may be used in order to analyze the breath within an analyzer. One method is called the "on-line" method. This method involves an individual exhaling a single breath into a mouthpiece which provides expiratory positive pressure against exhalation to prevent contamination of the sample by paranasal and nasal NO by closing the velopharyngeal aperture during the maneuver. The mouthpiece is connected to the breath analyzer by tubing which immediately analyzes the breath for NO and provides a real-time read-out of the NO concentration. The other method, known as the "off-line" measurement, involves an individual breathing continuously into a specialized bag via a mouthpiece for a defined amount of time. After collection, the sample is then analyzed by the chemiluminescence machine. With this method the sample is analyzed at a later time.

The American Thoracic Society (ATS) and the European Respiratory Society (ERS) have both published extensive guidelines on the measurement of exhaled NO in an attempt to standardize the procedure.[58, 59]

Materials and Methods

Study Design

Individuals for two groups, a younger group and an older group, were recruited to participate in this study which was performed in the Breath Lab at the University of South Florida in Tampa, Florida. Informed consent was obtained from each of the study participants prior to the conduction of the study after the research protocol along with the risks and benefits of the study were fully explained. All personal information and data gathered in this study were done per the guidelines of the Health Insurance Portability and Accountability Act (HIPAA). The Institutional Review Board at the University of South Florida approved the study protocol used for this particular study prior to its conduction.

The study subjects consisted of an older group of 25 individuals (61 to 79 years old, median 72 years old) and a younger group of 23 individuals (21 to 30 years old, median 24 years old) that were non-smokers with no history of lung disease, no recent respiratory infections, and no history of environmental allergies. A focused history using a questionnaire (Appendix A) and a physical exam were performed prior to beginning the testing to assess for any symptoms or signs of respiratory illness or impairment. None of the research participants had any symptoms or signs of respiratory illness or impairment that would have

excluded them from this study. In addition, the subjects abstained from eating or drinking except for water for 6 hours prior to the study.

Next, exhaled NO was assessed. After the exhaled NO measurement, spirometry was performed to further confirm normal respiratory status. Spirometry was performed after the NO measurement as it has been show to affect the exhaled NO concentration if done prior to the exhaled NO assessment.[58] After the spirometry, exhaled breath condensate (EBC) was obtained to assess the airway pH. The methods for obtaining exhaled NO, spirometric, and EBC measurements will be described in detail later in the materials and methods section.

Measurement of Exhaled Nitric Oxide

Exhaled NO measurements were performed in accordance with the protocol described by the ATS.[58] The on-line measurements of exhaled NO were made using a chemiluminescence analyzer (Sievers, NOA™ 280i; Boulder, CO) with a response time of 67 msec which is sensitive to NO over a range of approximately 0.5 ppb to 500,000 ppb. Standardization of the analyzer with the zero air filter (Sievers; Boulder, CO) containing $KMnO_4$ and activated carbon and the calibration NO gas of 45 parts per million (ppm) (Sievers; Boulder, CO) were performed each day prior to use per the manufacturer's recommendations. The chemiluminescence analyzer was connected to a personal computer that contained the NO analysis software (Sievers, NO Analysis™ Software Version

3.2; Boulder, CO) which provided a graphical display of exhaled flow rate and exhaled NO concentration during the analysis process.

Prior to the subject performing the test, the procedure was demonstrated by the examiner multiple times. Next, the subject was seated in a comfortable manner such that he could view the attached computer monitor which displayed the real-time measurements of NO and flow rate so that biofeedback would be provided to assist in the acquisition of acceptable measurements. The subject then inhaled through their mouth via the provided mouthpiece to total lung capacity (TLC). The inspired air was filtered of room NO by a cartridge attached to the distal end of the mouthpiece. Once reaching TLC, the subject exhaled in a continuous manner against resistance while maintaining the target ATS recommended exhalation flow rate of 50 ml/sec until a stable NO plateau of at least 3 seconds was obtained.[58] After each maneuver, the subject was given a 30 second break before performing the test again.[58] Repeated exhalations were performed until three or more exhaled NO values were obtained that met ATS criteria and were within 5% of the mean to assure reproducibility.[58] The resultant mean of these values was then taken to be the exhaled NO concentration for that subject. Figure 2 depicts the NO analyzer and mouthpiece. Figure 3 depicts the maneuver required for exhaled NO measurement.

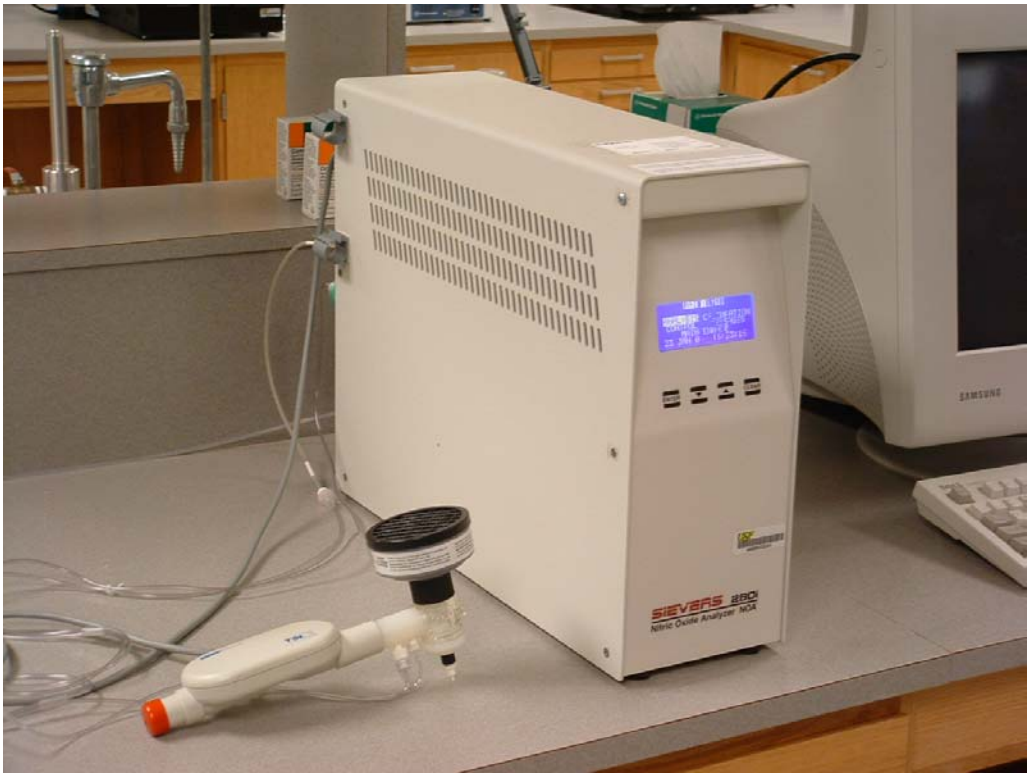


Figure 2: Sievers 280i Nitric Oxide Analyzer with Mouthpiece

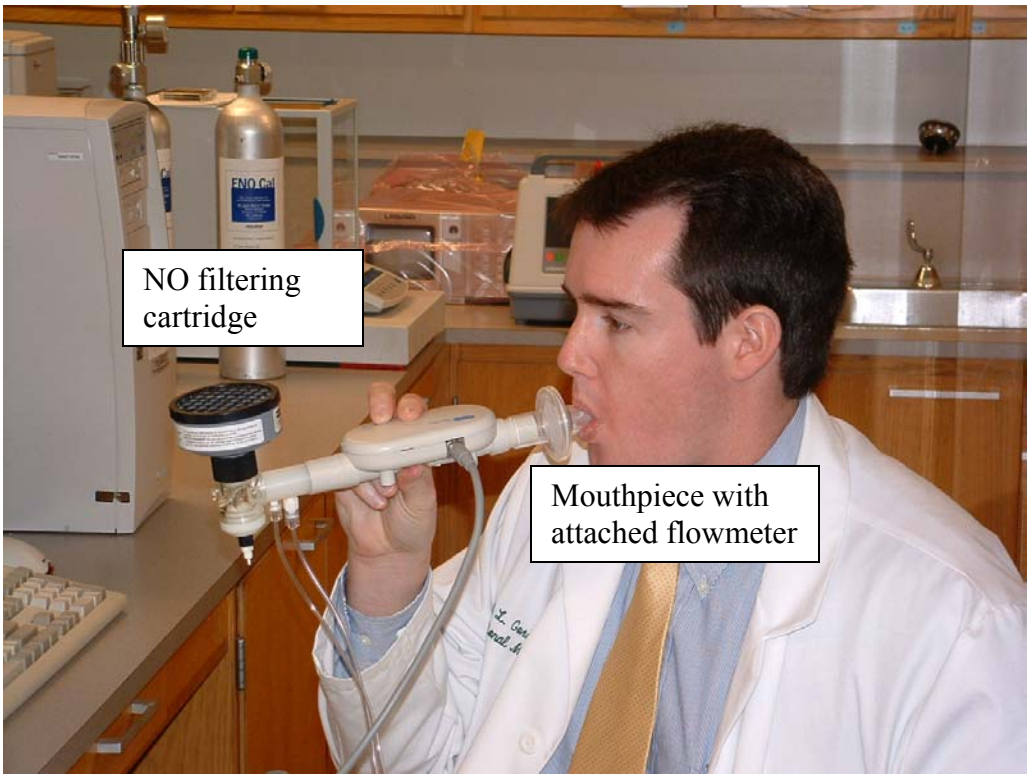


Figure 3: Performance of Exhaled Nitric Oxide Maneuver

Spirometry

Spirometry was performed on each of the subjects using the Puritan-Bennett, Renaissance II Spirometer (Carlsbad, CA) per ATS protocol.[60] Forced expiratory volume in one second (FEV_1) and forced expiratory volume in 6 seconds (FEV_6), a surrogate for forced vital capacity (FVC), were measured with the spirometer.[61] In addition, the spirometer calculated the FEV_1/FEV_6 ratio. Hankinson's spirometric reference values, which were incorporated into the spirometer, were used to determine if the subject's spirometric values were within normal limits.[62] In addition, the subjects' FEV_1/FEV_6 ratio had to be equal to or greater than 70% in the younger group and equal to or greater than 65% in the older group to be considered normal for this study.

Measurement of Exhaled Breath Condensate pH

Exhaled breath condensate collection and deaeration of the condensate were performed as has been described in the literature by using the RTube™ Exhaled Breath Condensate System (Respiratory Research, Inc; Charlottesville, VA) with the specialized pHTube™.[63] In this procedure, the subject breathed normally into the specialized plastic pHTube™ which was surrounded by a cooling sleeve for 15 minutes while wearing nose clips. Due to the surrounding cooling sleeve, the breath vapor was condensed into a breath condensate (liquid). After obtaining the condensate, the sample was deaerated with argon gas by utilizing the Exhaled Breath Condensate System "standard plunger" to remove the carbon dioxide from the sample while monitoring the pH to assess for

stabilization of the pH using a pH meter (Denver Instrument Company, Model 250 pH/Ion/Conductivity Meter; Arvada, CO). Once the pH stabilized, that value was taken to be the pH of the condensate for that subject. In addition, each of the condensate samples were tested spectrophotometrically (Thermo Spectronic, Genesys™ 2 Spectrophotometer; Rochester, NY) for amylase to assure that there was no salivary contamination of the exhaled breath condensate using a specialized kit which assayed for α-amylase quantitatively in a kinetic fashion (Pointe Scientific, Inc., Liquid Amylase Reagent Set; Lincoln Park, MI). Figure 4 depicts the specialized tube used for collection of condensate. Figure 5 depicts the process of measuring pH while the condensate sample is being deaerated with argon gas.

Figure 4: pHTube Used to Collect Breath Condensate (Cooling Sleeve Not Attached)

Figure 5: Measurement of pH of Breath Condensate While Undergoing Deaeration with Argon Gas

Statistical Analysis

The statistical software package JMP IN Version 5.1 (SAS Institute, Inc.; Cary, NC) was utilized to perform the statistical analysis. The exhaled NO values and the exhaled breath condensate pH values were compared for the younger and older groups using the Wilcoxon Rank Sum test. The Wilcoxon Rank Sum test was used to analyze the data because the frequency distributions of the data were found not to be normally distributed when plotted. The Holm method was then applied to limit the chances of Type I error that may have been introduced due to multiple testing.[64]

Results

Baseline Characteristics of the Study Populations

Twenty-three younger subjects were recruited with a median age of 24 years and a range of 21 to 30 years. Fourteen (61%) were females and 9 (39%) were males. One individual in the younger group had ulcerative colitis. The rest did not have any medical conditions. Twenty-five older subjects were recruited with a median age of 72 years and a range of 61 to 79 years. Eleven (44%) were females and 14 (56%) were males. Health problems included: hypertension, coronary artery disease, hyperlipidemia, hypothyroidism, gastroesophageal reflux disease, and cancer. Refer to Table 2 for a complete description of baseline characteristics of the study populations. In addition, none of the individuals in either group had smoked within the past ten years, had been diagnosed with or had symptoms of allergies (allergic rhinitis or sinusitis), had been diagnosed with any lung disease including asthma, had a respiratory tract infection recently, or used anti-histamines recently.

Spirometric Measurements

Spirometric measurements are summarized in Table 3.

Table 2. Baseline Characteristics of Study Populations

Characteristic	Age Category	
	Old (n =25)	Young (n = 23)
Gender	Female (11) 44% Male (14) 56%	Female (14) 61% Male (9) 39%
Median age	72 years old	24 years old
Health problems	Hypertension (12) 48% CAD (3) 12% Hyperlipidemia (6) 24% Hypothyroidism (2) 8% GERD (2) 8% Cancer (2) 8% None (5) 20%	None (22) 96% Ulcerative colitis (1) 4%
Medications	NSAIDs/ ASA (10) 40% Vitamins and minerals (12) 48% Statins (6) 24% ACE inhibitors (5) 20% PPIs (2) 8% Synthroid (2) 8% None (1) 4%	Birth control pill (6) 26% Imuran (1) 4% Antibiotic (1) 4% Vitamins and minerals (1) 4% None (15) 65%
History of tobacco use (>10 years ago)	Yes (16) 64% No (9) 36%	Yes (2) 9% No (21) 91%
Occupation	Retired (19) 76% Nurse (1) 4% Professor (3) 12% Restaurant manager (1) 4%	Adm. assistant (4) 17% Nurse (3) 13% Student (16) 70%
Exposure to secondary smoke	Yes (3) 12% No (22) 88%	Yes (1) 4% No (22) 96%
Past exposures	Firefighter (1) 4% Coal dust, coke oven (1) 4% None (23) 92%	Chlorine gas (1) 4% None (22) 96%

CAD = coronary artery disease; GERD = gastroesophageal reflux disease; NSAIDs/ASA = nonsteroidal anti-inflammatory drugs / aspirin; PPIs = proton pump inhibitors; Adm. assistant = administrative assistant.

Table 3. Spirometric Measurements of Study Populations

Measurement	Age Category	
	Old	Young
Median FEV_1 (percentage of normal)	94%	95%
Median FEV_6 (percentage of normal)	95%	92%
Median FEV_1/ FEV_6	78%	87%

FEV_1 = forced expiratory volume in 1 second; FEV_6 = forced expiratory volume in 6 seconds

Exhaled Breath Condensate pH Measurements

The median deaerated pHs for each of the groups are depicted in Table 4. The range of pH for the older group was 5.26 to 8.12. The range for the pH for the younger group was 6.44 to 8.12. The median pHs of the older age group and the younger age group were both 7.72.

Exhaled Nitric Oxide Measurements

The median exhaled nitric oxide concentrations for each of the groups are depicted in Table 4. The range for exhaled NO for the older group was 6.0 ppb to 103 ppb. The range for exhaled for NO for the younger group was 6.4 ppb to 214 ppb. The median exhaled NO concentrations of the older and younger age groups were 36.9 ppb and 18.7 ppb, respectively.

Table 4. Exhaled Breath Nitric Oxide and Exhaled Breath Condensate Deaerated pH

Measurement	Median for Old	Median for Young	P Value
Deaerated pH	7.72	7.72	0.959
Nitric Oxide (ppb)	36.9	18.7	0.0011

ppb=parts per billion

Discussion

Exhaled NO has been shown to be elevated in lower respiratory conditions in which inflammation is present such as asthma and pneumonia. Two mechanisms are thought to be responsible for the increase in NO. One is the up-regulation of iNOS due to inflammatory cytokines and the other is due to an increased release of NO from molecular intermediates due to an acidic airway.[14, 43] Despite the uncertainty with regards to the mechanism of NO elaboration from the lower airways, exhaled NO has the potential for assisting in the diagnosis and prognosis of airway diseases and monitoring their treatments.

The current study compared NO concentrations between a younger group of 23 individuals (median age of 24 years) and an older group of 25 individuals (median age of 72 years) that were non-smokers with no history of pulmonary disease, no recent respiratory infections, and no history of environmental allergies. This study was conducted to determine if there is a difference in endogenous NO concentrations derived from the lower respiratory airways between younger and older individuals in an attempt to see how the airways may change with age, predisposing the older population to pulmonary infections and increased morbidity and mortality with pulmonary infections.

This study showed that exhaled NO concentrations of the older and younger age groups were 36.9 ppb and 18.7 ppb, respectively. This represented a significant statistical difference between the two groups with a ρ value of

0.0011 per the Wilcoxon Rank Sum test. This statistical significance held true when adjusted for multiple testing with the Holm method. In addition, there was no significant difference between the exhaled breath condensate pHs between the two groups with each having a median of 7.72.

This is the first study to show a significant difference in exhaled NO between a younger and an older population. Previous studies have not shown a difference. Ekroos et al. found no correlation between age and NO.[11] However, the age range was 21-48 years and the majority of the subjects were in their twenties and thirties. Their study did not really address a possible difference between a young population and an aged population due to its limited age sampling. Tsang et al. studied a group of 121 healthy adults with an age range of 20-79 years and did not find a relationship between age and NO levels.[13] On evaluation of their data, there were noted to be several high NO outliers in the younger age ranges that may have significantly affected their data. In addition, Tsang et al. did not divide their sample into two distinct groups in order to analyze for a difference as was done in this study using the Wilcoxon Rank Sum test. Instead, they looked for a correlation using regression. With their method, a linear relationship would have been necessary to reveal a difference as opposed to the method used presently which would pick up any difference between the two distinct age groups. Therefore, the difference in the findings between the current study and the study of Tsang et al. may be due to the use of different statistical methods. It is also possible that the difference may be related to the different population samples as the current study was conducted in the

United States of America and the study of Tsang et al. was conducted in China. In an abstract by Duncan et al., no correlation between age and exhaled NO was found in a group of 59 healthy individuals.[12] However, this study had a limited age range with the oldest category reaching a peak of 60 years. Also, there were only two individuals in the oldest age category of "51-60." In addition, the "off-line" technique was used which differed from the "on-line" technique used in the present study.

The mechanisms responsible for the elevated NO concentrations in the older population are not clear at present. This study showed a difference in exhaled NO concentrations between the age groups despite similar pHs between the groups. Therefore, this is more compatible with a NOS-mediated increase rather than an airway pH-mediated increase in NO. If indeed the elevated NO concentration is due to NOS, what is the underlying reason for the increased NOS activity and what type of NOS is responsible?

It has been proposed that aged individuals have a baseline airway inflammation despite being clinically healthy.[12, 65, 66] Bronchoalveolar lavage examination has supported this hypothesis by showing that older individuals have elevated lymphocytes with an elevated CD4+/CD8+ T cell ratio, elevated IgM, IgA, and IgG levels, elevated IL-6 and IL-8, and elevated neutrophils.[5] It is therefore possible that a low-grade inflammation may provide the necessary constituents such as inflammatory cytokines to induce the expression of iNOS in bronchial epithelial cells, macrophages, and neutrophils leading to the elevated NO levels in the older population.

Another potential reason for the elevated NO concentrations in the older population may be due to an immune system dysregulation without inflammation leading to inappropriate NOS (iNOS or cNOS) expression.

It is also possible that NO concentrations are elevated at baseline in the aged in an attempt to compensate for an impaired immune system as NO has been shown to be toxic to microorganisms and therefore may be of assistance to the immune system. This baseline compensatory increase could potentially be mediated by elevated cNOS or iNOS expression.

Lastly, the increase in NO concentrations in older individuals may be secondary to an incompetent soft palate allowing for contamination of exhaled lower airway NO with upper airway NO despite performing the maneuver properly.

The significance of the elevated NO concentrations in the aged population is not clear. Whether the elevated levels further support the hypothesis of baseline airway inflammation in the aged remains to be determined. If the elevated NO levels are due to an immune system dysregulation or a compensatory mechanism to assists an impaired immune system then NO levels may be useful in identifying those with immune systems which are not completely competent to fight infection. If this is indeed the case, an elevated NO concentration may serve as a marker for a dysregulated or impaired immune system which would predispose older individuals to pulmonary infections such as pneumonia with poorer outcomes. Finally, it is possible that the difference in NO concentration between the older and younger groups is not clinically significant.

Additional investigations are necessary to elucidate the mechanisms and significances of elevated NO levels in the aged. A study which employed immunohistochemical methods to determine the differential expression of the NOSs between the age groups would elucidate which NOS is responsible for the elevated NO concentrations in the aged. Another study of value would be to create a prospective study in which baseline serial measurements of NO would be taken in an older population while they are free of respiratory disease. The group would then be followed for the incidence of pulmonary infections along with the morbidity and mortality secondary to pulmonary infections in order to determine if a relationship exists between baseline elevated NO levels and pulmonary infections in the aged. An additional study that would be interesting would be to determine the effect of baseline inflammation in the aged on NO levels. This could be accomplished by performing bronchoscopic biopsy evaluations for inflammation in younger and older individuals and correlating the levels of inflammation with the exhaled NO concentrations. Another potentially valuable study would be to perform bronchoalveolar lavages or induced sputum studies on older and younger individuals to assess the milieu of cells and mediators and their relationships with exhaled NO concentrations.

Conclusion

This study evaluated exhaled NO levels between younger and older populations to gain further insight into possible differences between the airways of these two groups. It was found that older individuals have significantly higher NO concentrations than younger individuals despite each group having similar pHs. The reasons for this difference along with the relationship to pulmonary infections are unclear and further studies will be necessary to further evaluate these issues.

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Appendices

Appendix A: Exhaled Breath Condensate and Exhaled Breath Nitric Oxide Study Questionnaire

Subject number:_____

Exhaled Breath Condensate and exhaled Breath Nitric Oxide Study Questionnaire

Today's Date _____

Gender: Male Female (circle one)

1) How old are you? _____years
2) Do you have any health problems that you see a physician for? Please list them below.

1._____

2. _____

3. _____

4. _____

5._____

3) Are you taking any medications? If so please list them below. (Including over the counter medications)

1._____

2. _____

3. _____

4. _____

5._____

4) If you have ever smoked, answer the following.

How many packs per day did you smoke? _____

For how many years did you smoke? _____

When did you stop smoking? _____

5) On what date were you last ill? _____

6) What illness did you have? _____

7) What is your occupation? _____

8) Are you exposed to second hand smoke at home or at work? Please circle "yes" or "no".

YES or NO

9) Were you or are you exposed to any gases, dusts, or fumes at your job?

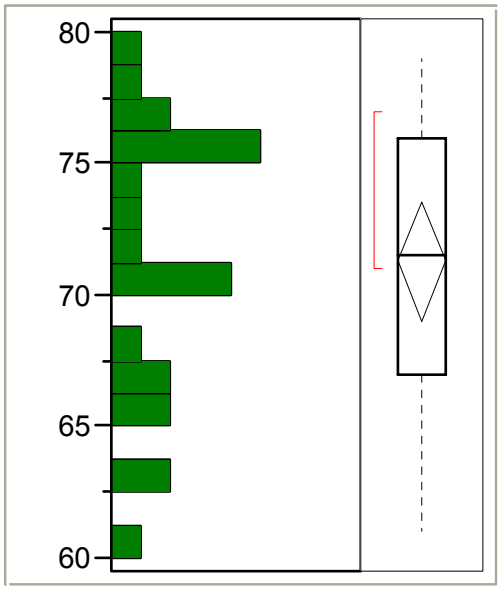
YES or NO

If so, please explain: _____

Appendix B: Age Distributions within Each Age Category

Category: Old

Distribution: Age



Quantiles

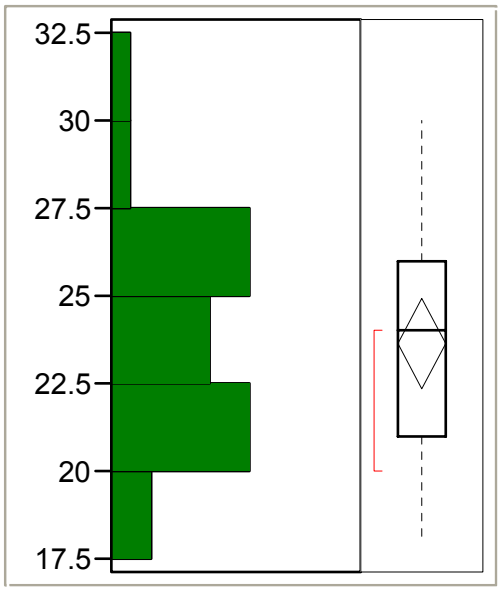
100.0%	maximum	79.000
99.5%		79.000
97.5%		79.000
90.0%		77.500
75.0%	quartile	76.000
50.0%	median	71.500
25.0%	quartile	67.000
10.0%		63.000
2.5%		61.000
0.5%		61.000
0.0%	minimum	61.000

Moments

Mean	71.291667
Std Dev	5.3363329
Std Err Mean	1.0892744
upper 95% Mean	73.545002
lower 95% Mean	69.038331
N	24

Category: Young

Distribution: Age



Quantiles

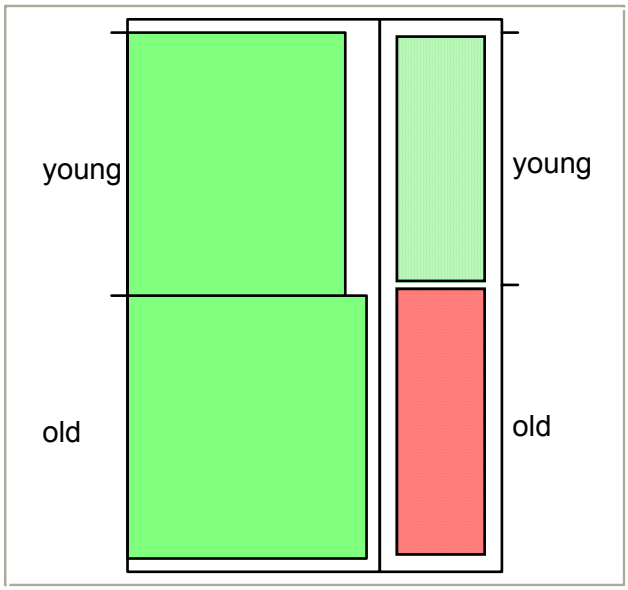
100.0%	maximum	30.000
99.5%		30.000
97.5%		30.000
90.0%		27.600
75.0%	quartile	26.000
50.0%	median	24.000
25.0%	quartile	21.000
10.0%		19.400
2.5%		18.000
0.5%		18.000
0.0%	minimum	18.000

Moments

Mean	23.608696
Std Dev	2.9808347
Std Err Mean	0.621547
upper 95% Mean	24.897705
lower 95% Mean	22.319686
N	23

Appendix C: Age Category and Gender Distributions for Entire Study Population

Age Category Distribution for Entire Study Population

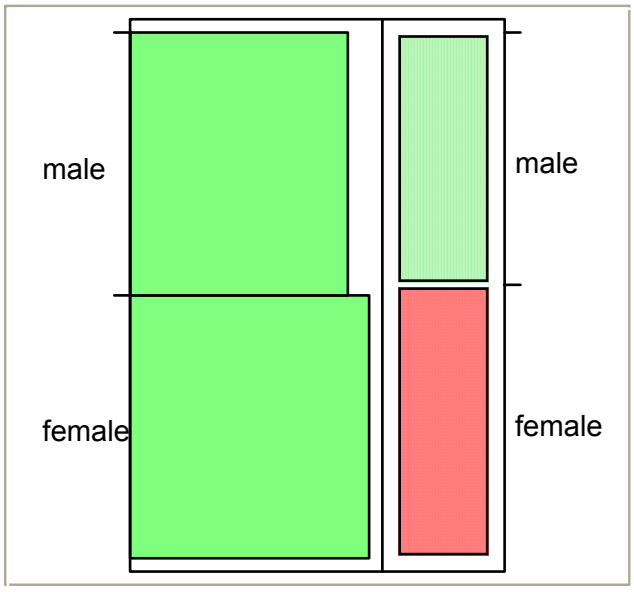


Frequencies

Level	Count	Prob
old	25	0.52083
young	23	0.47917
Total	48	1.00000

2 Levels

Gender Distribution for Entire Study Population



Frequencies

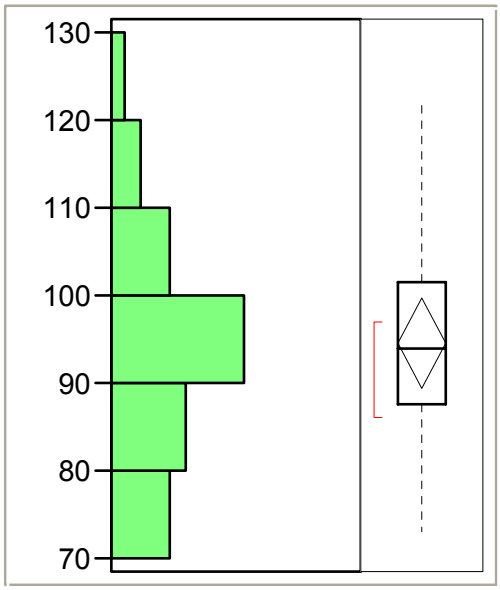
Level	Count	Prob
female	25	0.52083
male	23	0.47917
Total	48	1.00000

2 Levels

Appendix D: Distributions of Characteristics and Measurements by Age Category

Age Category: Old

FEV1



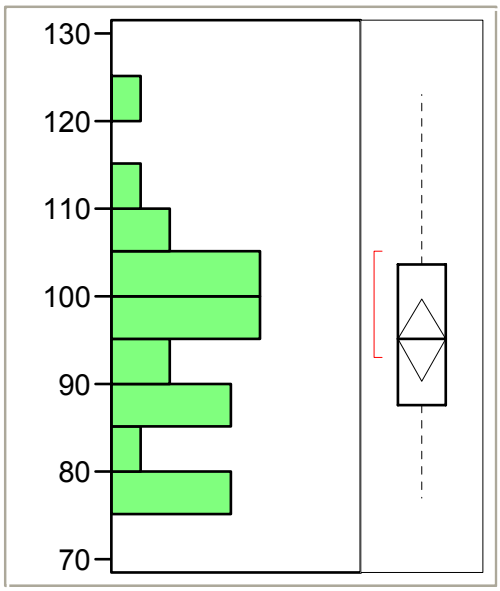
Quantiles

100.0%	maximum	122.00
99.5%		122.00
97.5%		122.00
90.0%		116.00
75.0%	quartile	101.50
50.0%	median	94.00
25.0%	quartile	87.50
10.0%		76.60
2.5%		73.00
0.5%		73.00
0.0%	minimum	73.00

Moments

Mean	94.48
Std Dev	12.467023
Std Err Mean	2.4934046
upper 95% Mean	99.626134
lower 95% Mean	89.333866
N	25

FEV6



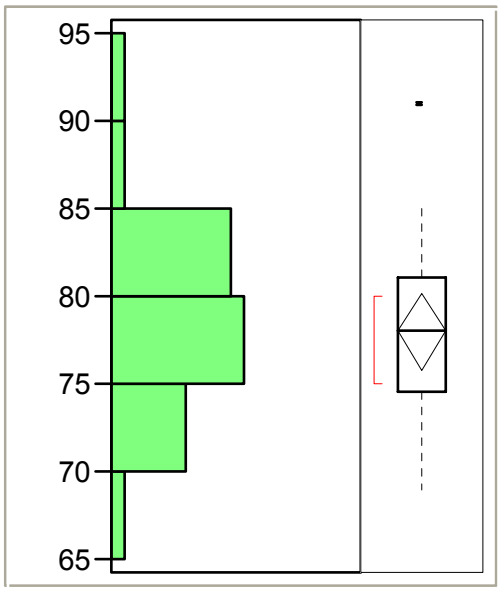
Quantiles

100.0%	maximum	123.00
99.5%		123.00
97.5%		123.00
90.0%		109.40
75.0%	quartile	103.50
50.0%	median	95.00
25.0%	quartile	87.50
10.0%		77.60
2.5%		77.00
0.5%		77.00
0.0%	minimum	77.00

Moments

Mean	95.04
Std Dev	11.570364
Std Err Mean	2.3140729
upper 95% Mean	99.816012
lower 95% Mean	90.263988
N	25

FEV1/FEV6



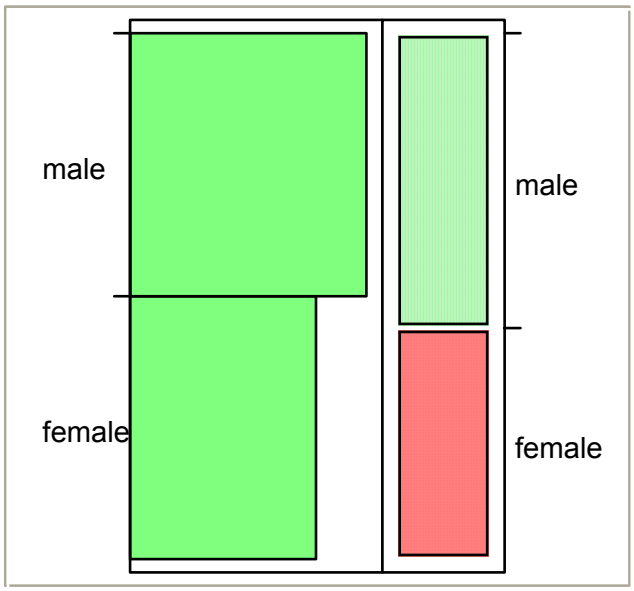
Quantiles

100.0%	maximum	91.000
99.5%		91.000
97.5%		91.000
90.0%		84.400
75.0%	quartile	81.000
50.0%	median	78.000
25.0%	quartile	74.500
10.0%		70.000
2.5%		69.000
0.5%		69.000
0.0%	minimum	69.000

Moments

Mean	77.96
Std Dev	5.2399109
Std Err Mean	1.0479822
upper 95% Mean	80.122929
lower 95% Mean	75.797071
N	25

Gender

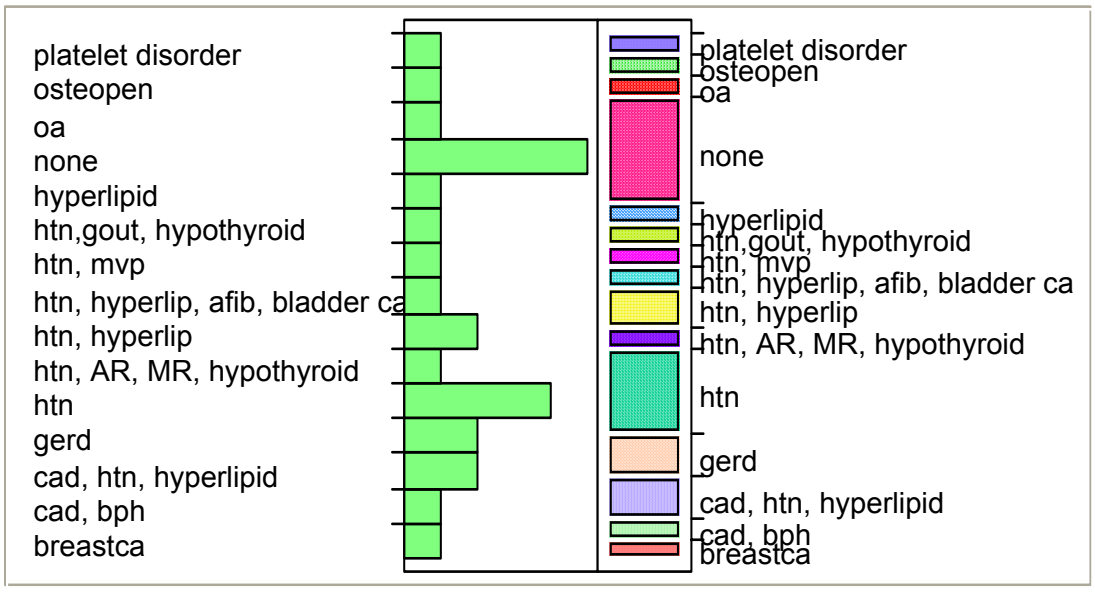


Frequencies

Level	Count	Prob
female	11	0.44000
male	14	0.56000
Total	25	1.00000

2 Levels

Health Problems

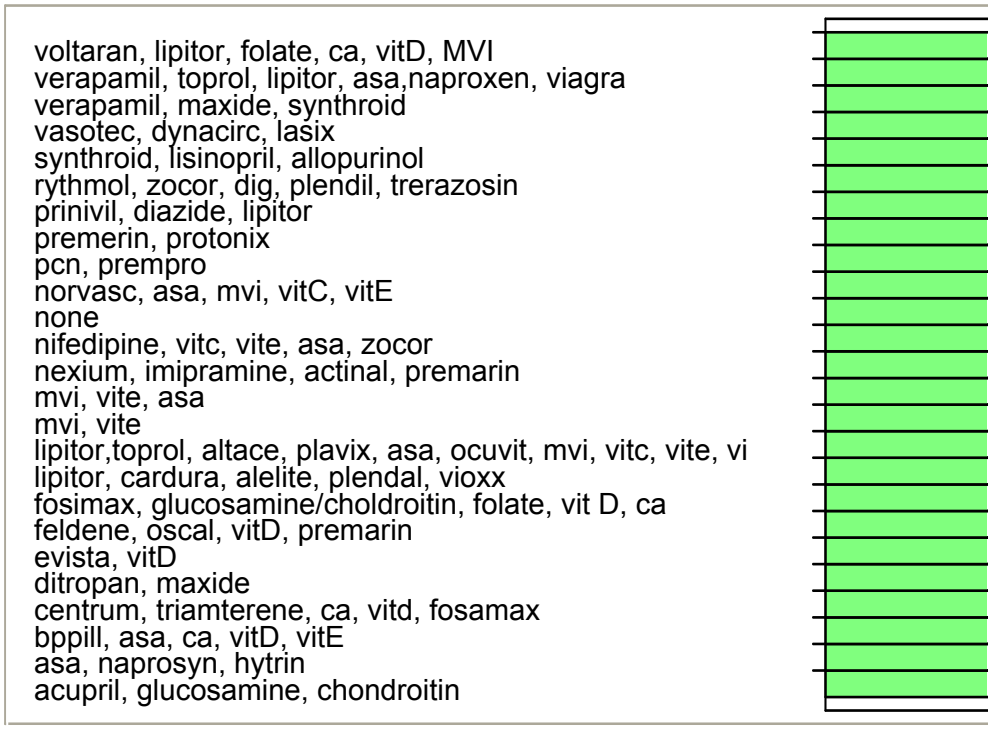


Frequencies

Level	Count	Prob
breastca	1	0.04000
cad, bph	1	0.04000
cad, htn, hyperlipid	2	0.08000
gerd	2	0.08000
htn	4	0.16000
htn, AR, MR, hypothyroid	1	0.04000
htn, hyperlip	2	0.08000
htn, hyperlip, afib, bladder ca	1	0.04000
htn, mvp	1	0.04000
htn,gout, hypothyroid	1	0.04000
hyperlipid	1	0.04000
none	5	0.20000
oa	1	0.04000
osteopen	1	0.04000
platelet disorder	1	0.04000
Total	25	1.00000

15 Levels

Medications

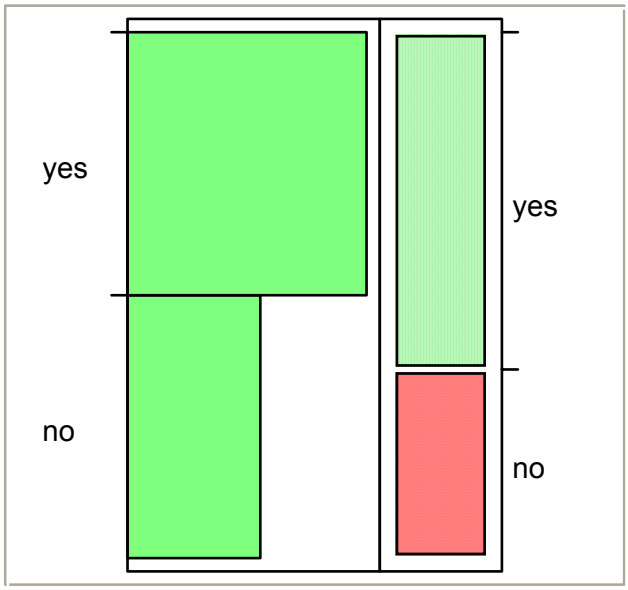


Frequencies

Level	Count	Prob
acupril, glucosamine, chondroitin	1	0.04000
asa, naprosyn, hytrin	1	0.04000
bppill, asa, ca, vitD, vitE	1	0.04000
centrum, triamterene, ca, vitd, fosamax	1	0.04000
ditropan, maxide	1	0.04000
evista, vitD	1	0.04000
feldene, oscal, vitD, premarin	1	0.04000
fosimax, glucosamine/choldroitin, folate, vit D, ca	1	0.04000
Lipitor, cardura, alelite, plendal, vioxx	1	0.04000
lipitor,toprol, altace, plavix, asa, ocuvit, mvi, vitc, vite, vi	1	0.04000
mvi, vite	1	0.04000
mvi, vite, asa	1	0.04000
nexium, imipramine, actinal, premarin	1	0.04000
nifedipine, vitc, vite, asa, zocor	1	0.04000
none	1	0.04000
norvasc, asa, mvi, vitC, vitE	1	0.04000
pcn, prempro	1	0.04000
premerin, protonix	1	0.04000
prinivil, diazide, lipitor	1	0.04000
rythmol, zocor, dig, plendil, trerazosin	1	0.04000
synthroid, lisinopril, allopurinol	1	0.04000
vasotec, dynacirc, lasix	1	0.04000
verapamil, maxide, synthroid	1	0.04000
verapamil, toprol, lipitor, asa,naproxen, viagra	1	0.04000
voltaran, lipitor, folate, ca, vitD, MVI	1	0.04000
Total	25	1.00000

25 Levels

Smoking History

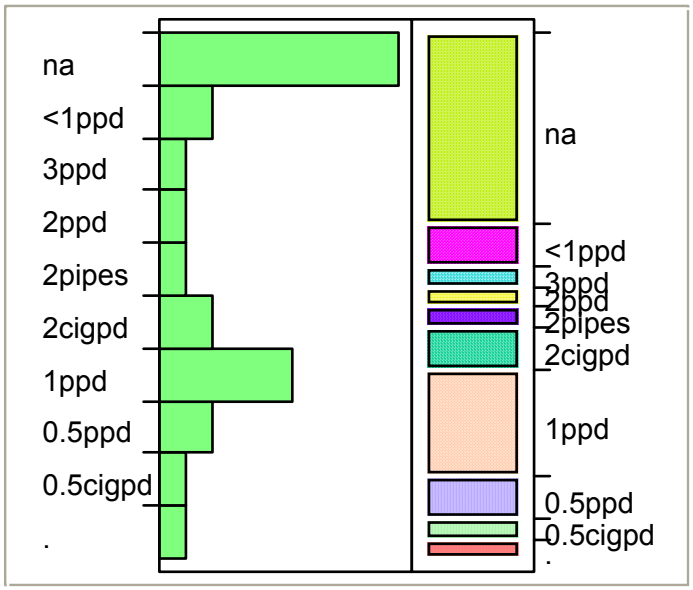


Frequencies

Level	Count	Prob
no	9	0.36000
yes	16	0.64000
Total	25	1.00000

2 Levels

Packs Smoked When Smoker

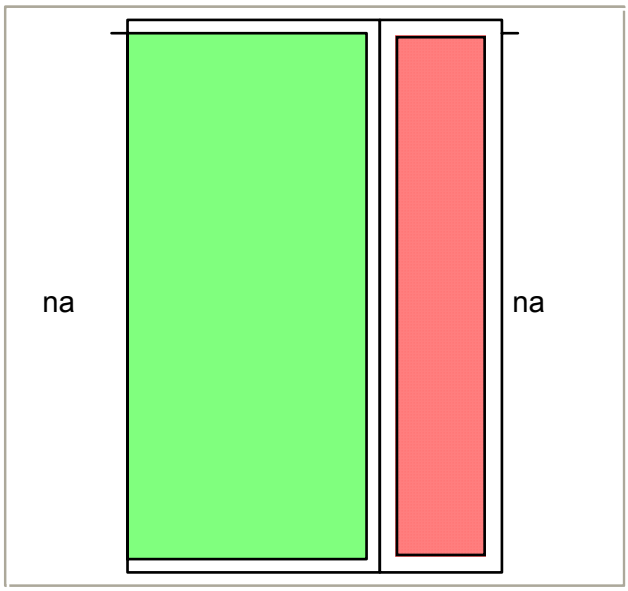


Frequencies

Level	Count	Prob
.	1	0.04000
0.5cigpd	1	0.04000
0.5ppd	2	0.08000
1ppd	5	0.20000
2cigpd	2	0.08000
2pipes	1	0.04000
2ppd	1	0.04000
3ppd	1	0.04000
<1ppd	2	0.08000
na	9	0.36000
Total	25	1.00000

10 Levels

Years Smoked

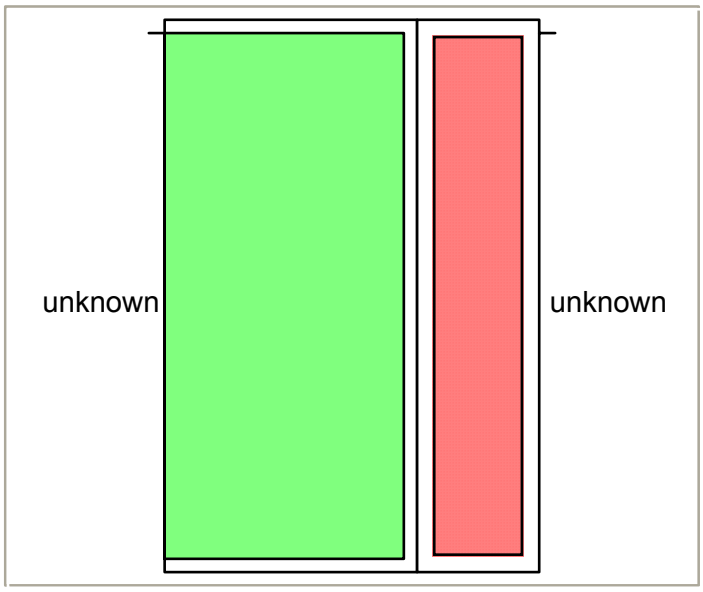


Frequencies

Level	Count	Prob
na	9	1.00000
Total	9	1.00000

1 Levels

Last III

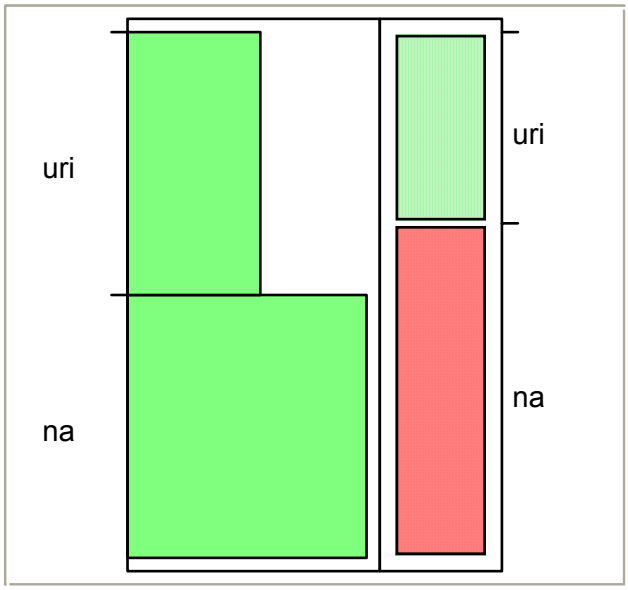


Frequencies

Level	Count	Prob
unknown	16	1.00000
Total	16	1.00000

1 Levels

Illness When Last Ill

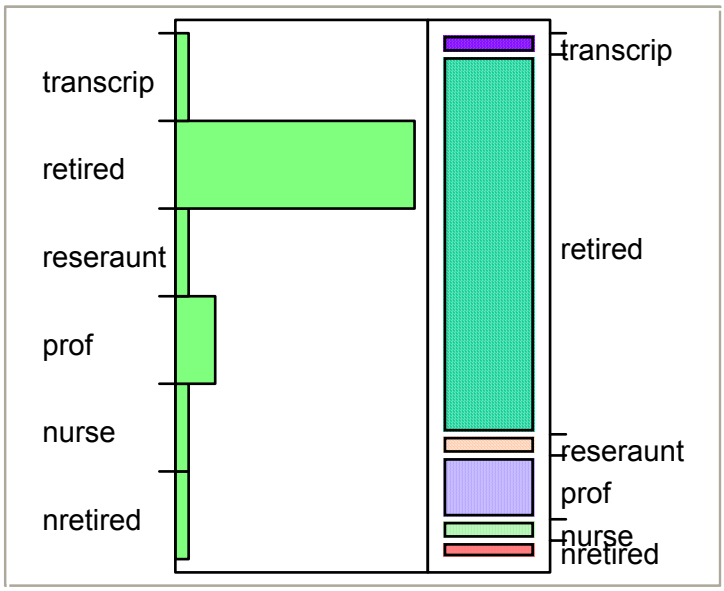


Frequencies

Level	Count	Prob
na	16	0.64000
uri	9	0.36000
Total	25	1.00000

2 Levels

Occupation

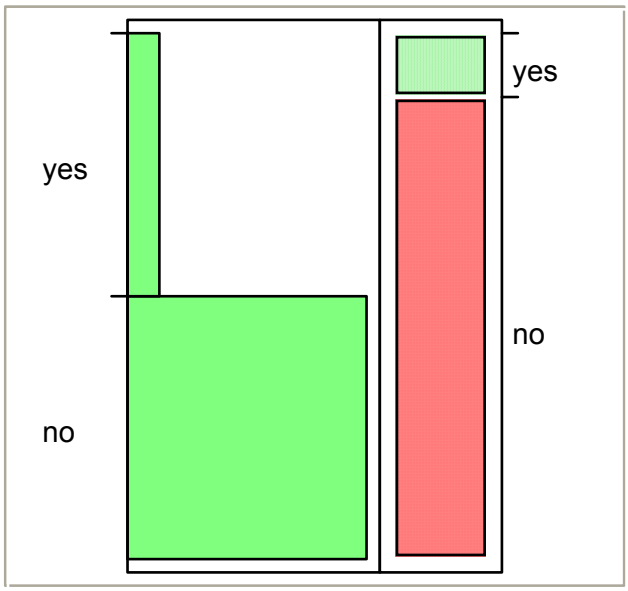


Frequencies

Level	Count	Prob
nretired	1	0.04000
nurse	1	0.04000
prof	3	0.12000
reseraunt	1	0.04000
retired	18	0.72000
transcrip	1	0.04000
Total	25	1.00000

6 Levels

Second Hand Smoke

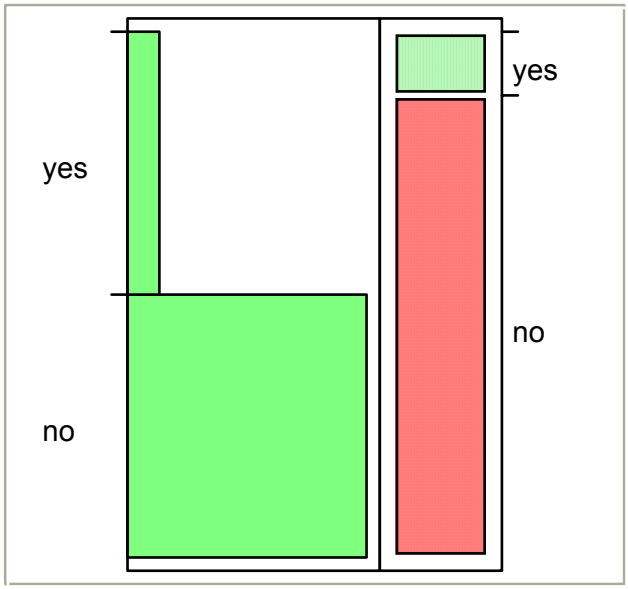


Frequencies

Level	Count	Prob
no	22	0.88000
yes	3	0.12000
Total	25	1.00000

2 Levels

Exposures

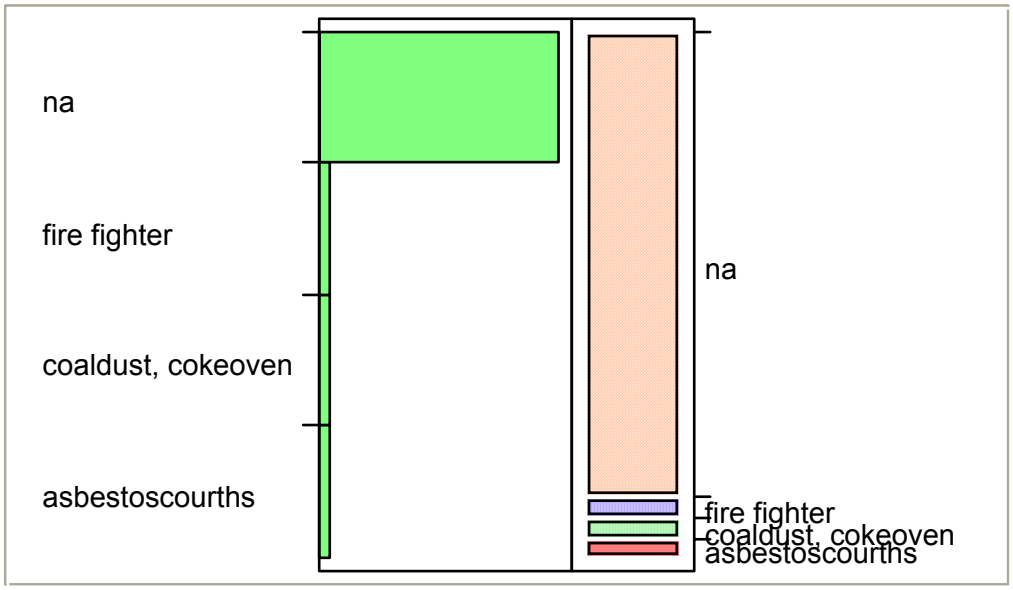


Frequencies

Level	Count	Prob
no	22	0.88000
yes	3	0.12000
Total	25	1.00000

2 Levels

Explanation of Exposures

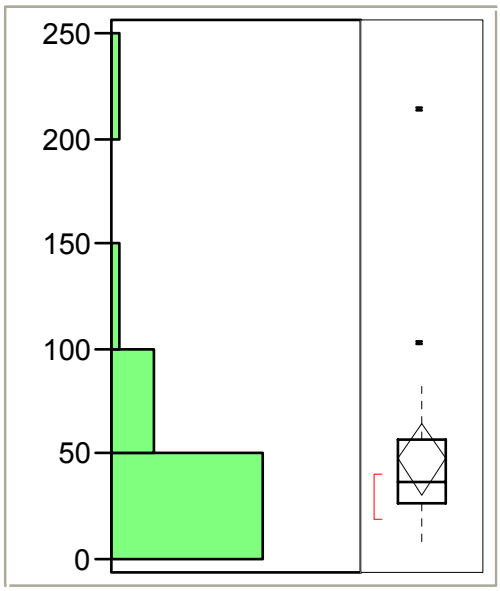


Frequencies

Level	Count	Prob
asbestoscourths	1	0.04000
coaldust, cokeoven	1	0.04000
fire fighter	1	0.04000
na	22	0.88000
Total	25	1.00000

4 Levels

NO Concentrations



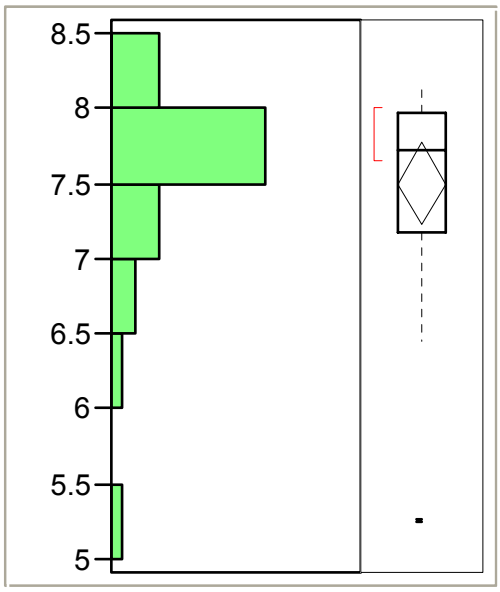
Quantiles

100.0%	maximum	214.00
99.5%		214.00
97.5%		214.00
90.0%		90.70
75.0%	quartile	56.60
50.0%	median	36.90
25.0%	quartile	26.30
10.0%		14.44
2.5%		6.00
0.5%		6.00
0.0%	minimum	6.00

Moments

Mean	47.772
Std Dev	41.525038
Std Err Mean	8.3050076
upper 95% Mean	64.912693
lower 95% Mean	30.631307
N	25

pH Deaerated



Quantiles

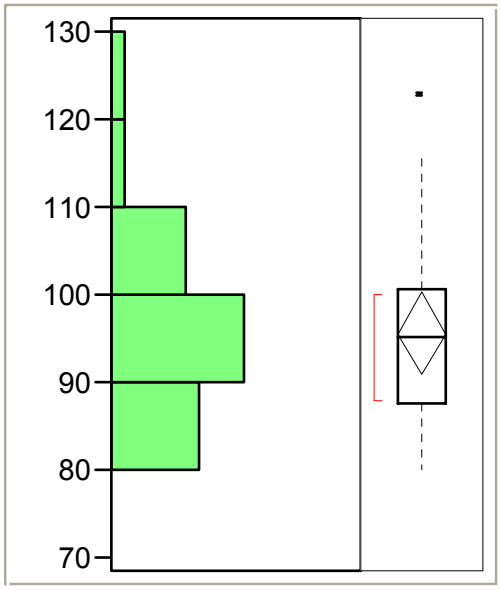
100.0%	maximum	8.1200
99.5%		8.1200
97.5%		8.1200
90.0%		8.0400
75.0%	quartile	7.9700
50.0%	median	7.7200
25.0%	quartile	7.1750
10.0%		6.5280
2.5%		5.2600
0.5%		5.2600
0.0%	minimum	5.2600

Moments

Mean	7.4996
Std Dev	0.6650343
Std Err Mean	0.1330069
upper 95% Mean	7.7741127
lower 95% Mean	7.2250873
N	25

Age Category: Young

FEV1



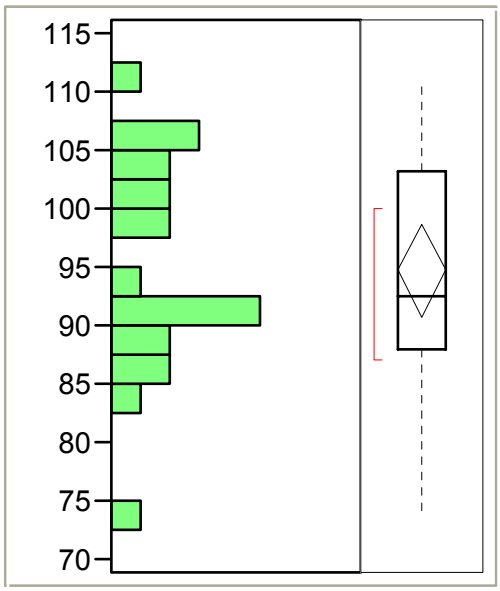
Quantiles

100.0%	maximum	123.00
99.5%		123.00
97.5%		123.00
90.0%		112.40
75.0%	quartile	100.75
50.0%	median	95.00
25.0%	quartile	87.50
10.0%		81.00
2.5%		80.00
0.5%		80.00
0.0%	minimum	80.00

Moments

Mean	95.590909
Std Dev	10.795321
Std Err Mean	2.3015702
upper 95% Mean	100.37729
lower 95% Mean	90.804532
N	22

FEV6



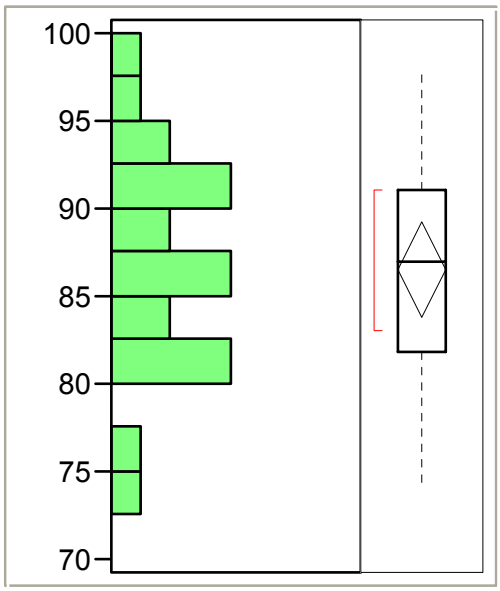
Quantiles

100.0%	maximum	111.00
99.5%		111.00
97.5%		111.00
90.0%		105.00
75.0%	quartile	103.25
50.0%	median	92.50
25.0%	quartile	88.00
10.0%		83.60
2.5%		74.00
0.5%		74.00
0.0%	minimum	74.00

Moments

Mean	94.681818
Std Dev	9.0679255
Std Err Mean	1.9332882
upper 95% Mean	98.702311
lower 95% Mean	90.661325
N	22

FEV1/FEV6



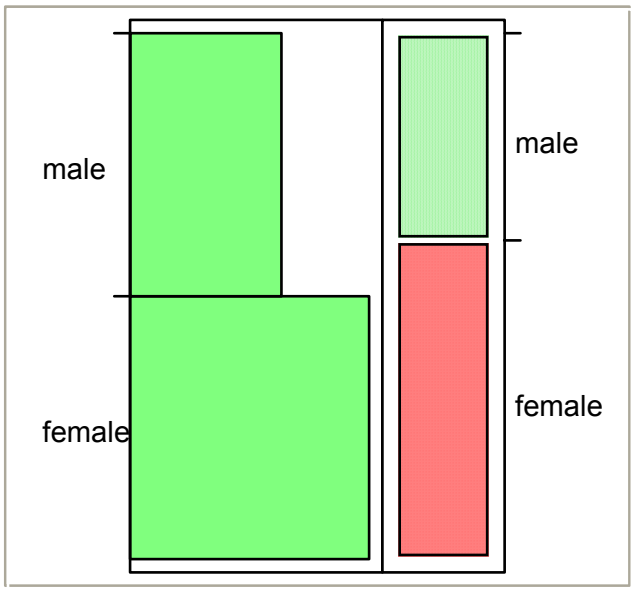
Quantiles

100.0%	maximum	98.000
99.5%		98.000
97.5%		98.000
90.0%		95.400
75.0%	quartile	91.000
50.0%	median	87.000
25.0%	quartile	81.750
10.0%		77.200
2.5%		74.000
0.5%		74.000
0.0%	minimum	74.000

Moments

Mean	86.545455
Std Dev	6.2467307
Std Err Mean	1.3318075
upper 95% Mean	89.3151
lower 95% Mean	83.775809
N	22

Gender

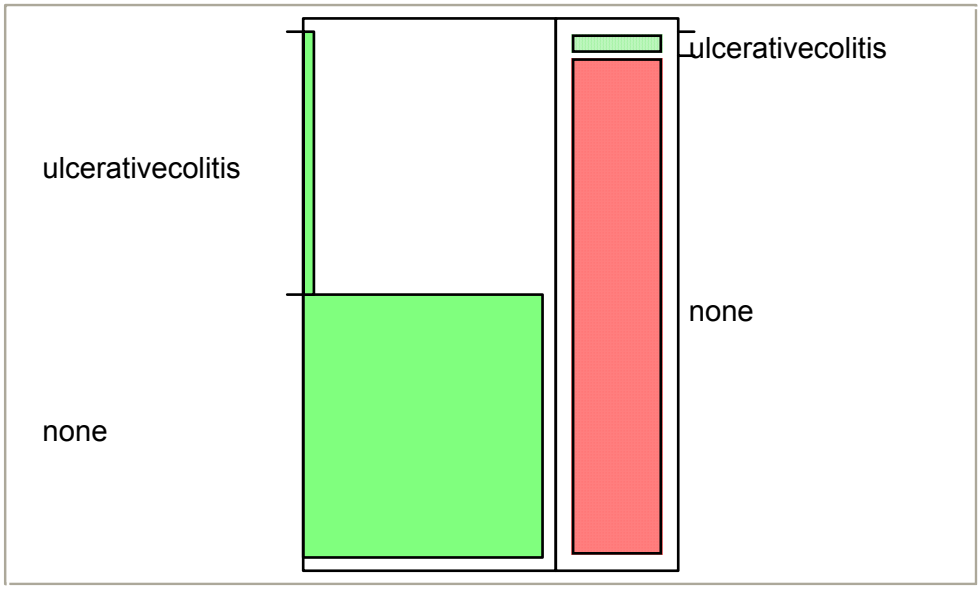


Frequencies

Level	Count	Prob
female	14	0.60870
male	9	0.39130
Total	23	1.00000

2 Levels

Health Problems

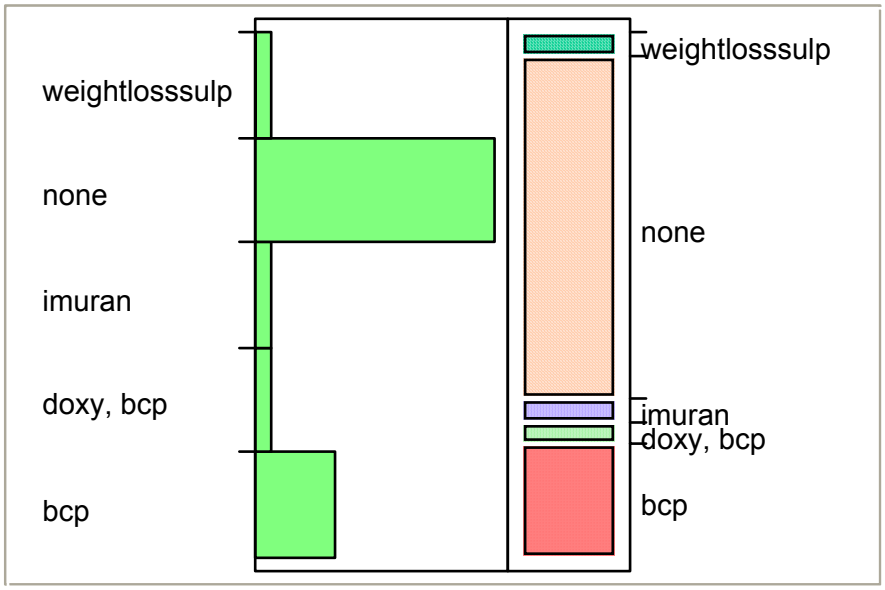


Frequencies

Level	Count	Prob
none	22	0.95652
ulcerativecolitis	1	0.04348
Total	23	1.00000

2 Levels

Medications

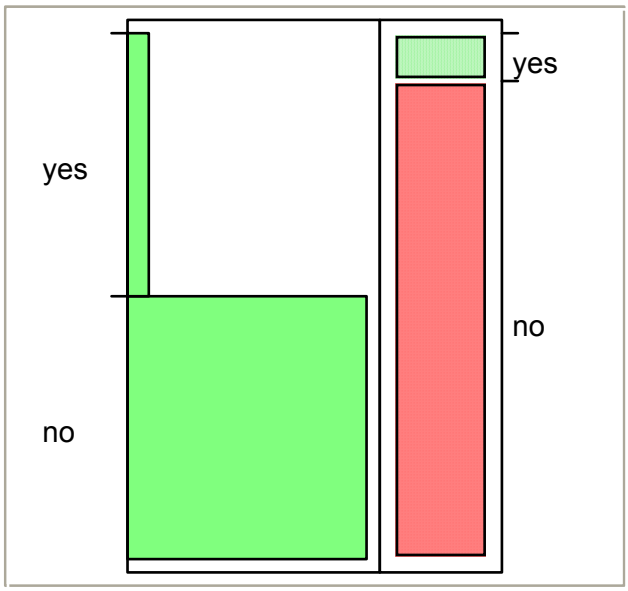


Frequencies

Level	Count	Prob
bcp	5	0.21739
doxy, bcp	1	0.04348
imuran	1	0.04348
none	15	0.65217
weightlosssulp	1	0.04348
Total	23	1.00000

5 Levels

Smoking History

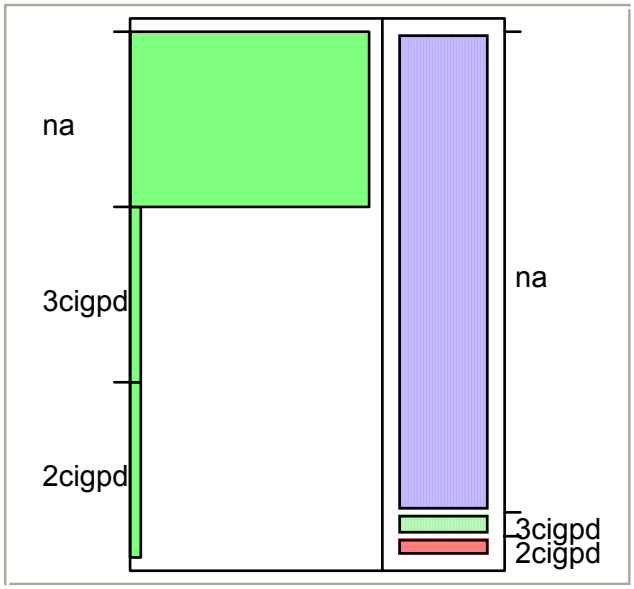


Frequencies

Level	Count	Prob
no	21	0.91304
yes	2	0.08696
Total	23	1.00000

2 Levels

Packs Smoked When Smoker

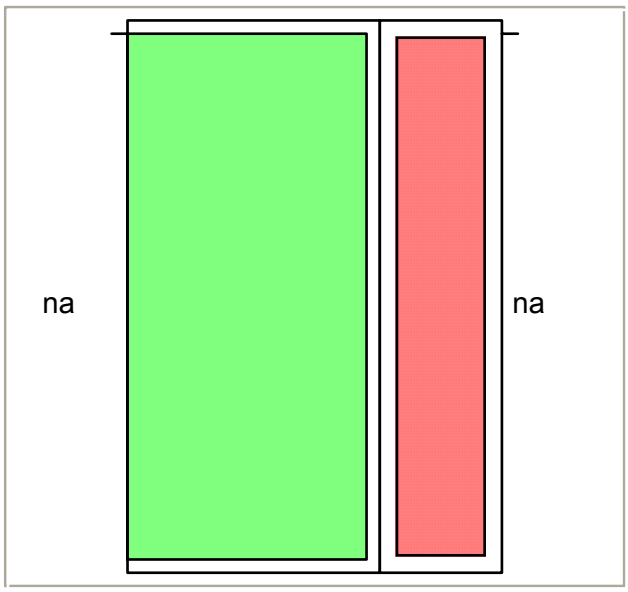


Frequencies

Level	Count	Prob
2cigpd	1	0.04348
3cigpd	1	0.04348
na	21	0.91304
Total	23	1.00000

3 Levels

Years Smoked

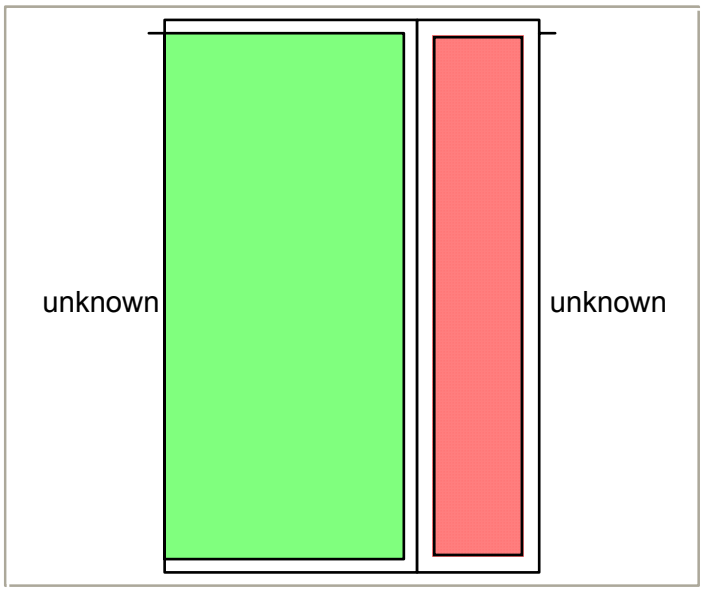


Frequencies

Level	Count	Prob
na	21	1.00000
Total	21	1.00000

1 Levels

Last Ill

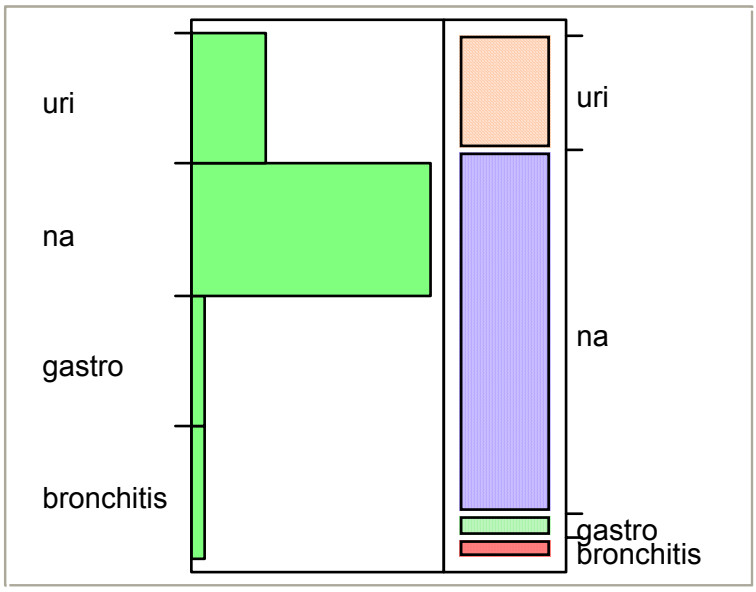


Frequencies

Level	Count	Prob
unknown	16	1.00000
Total	16	1.00000

1 Levels

Illness When Last Ill

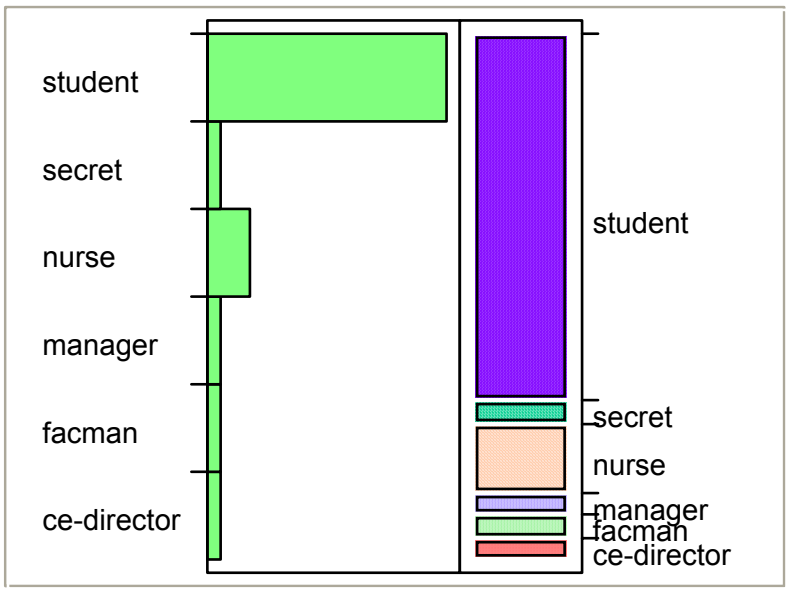


Frequencies

Level	Count	Prob
bronchitis	1	0.04348
gastro	1	0.04348
na	16	0.69565
uri	5	0.21739
Total	23	1.00000

4 Levels

Occupation

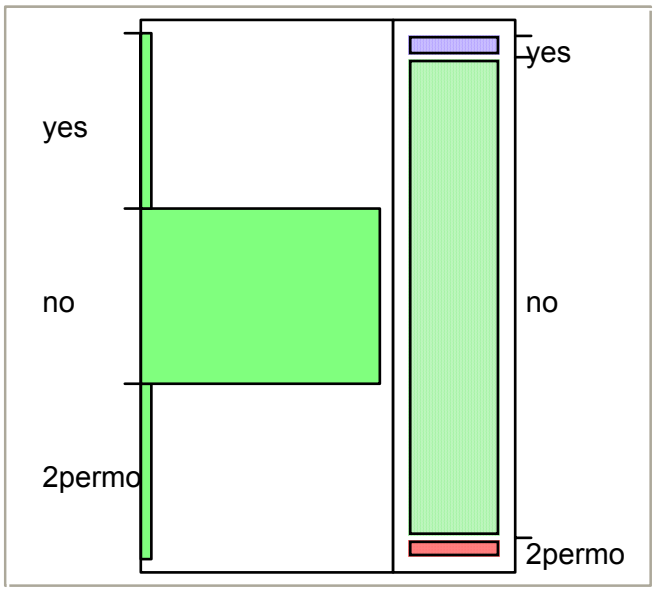


Frequencies

Level	Count	Prob
ce-director	1	0.04348
facman	1	0.04348
manager	1	0.04348
nurse	3	0.13043
secret	1	0.04348
student	16	0.69565
Total	23	1.00000

6 Levels

Second Hand Smoke

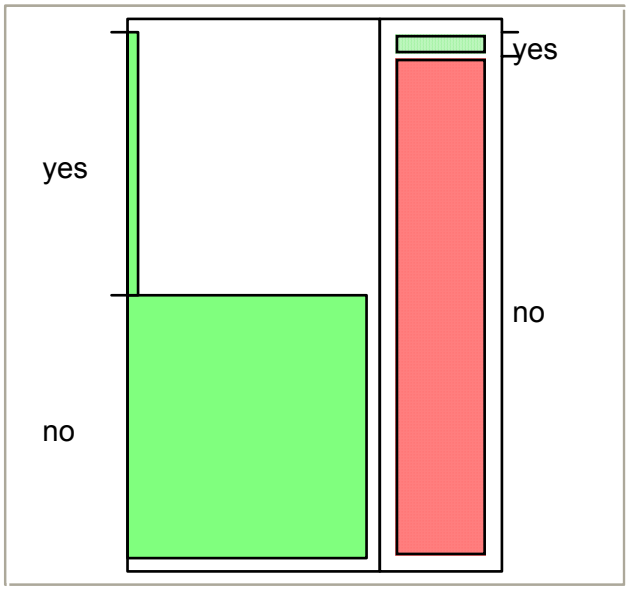


Frequencies

Level	Count	Prob
2permo	1	0.04348
no	21	0.91304
yes	1	0.04348
Total	23	1.00000

3 Levels

Exposures

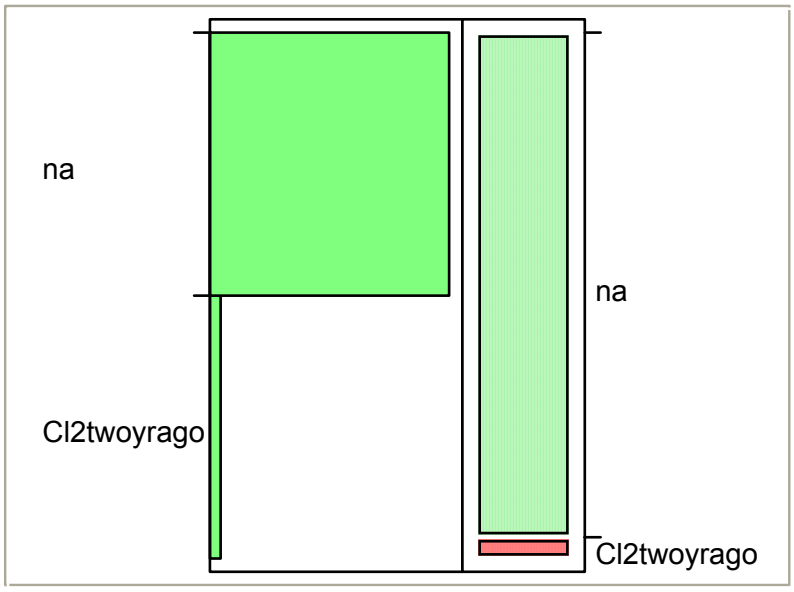


Frequencies

Level	Count	Prob
no	22	0.95652
yes	1	0.04348
Total	23	1.00000

2 Levels

Explanation of Exposures

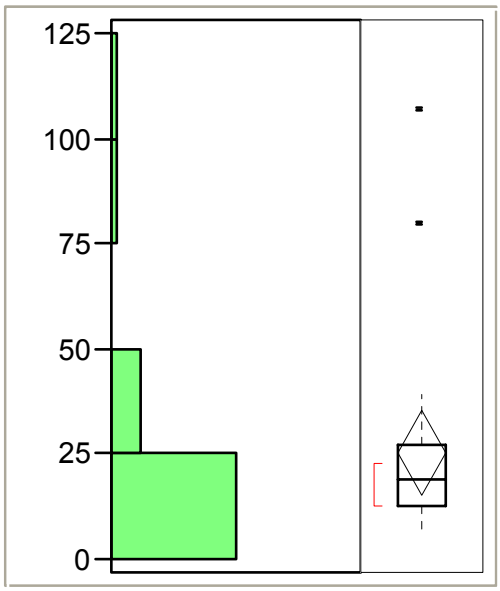


Frequencies

Level	Count	Prob
Cl2twoyrago	1	0.04348
na	22	0.95652
Total	23	1.00000

2 Levels

NO Concentations



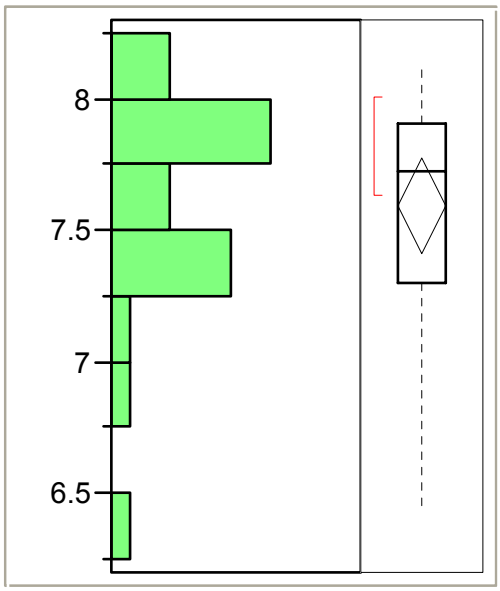
Quantiles

100.0%	maximum	107.00
99.5%		107.00
97.5%		107.00
90.0%		63.60
75.0%	quartile	27.00
50.0%	median	18.70
25.0%	quartile	12.70
10.0%		9.82
2.5%		6.40
0.5%		6.40
0.0%	minimum	6.40

Moments

Mean	25.221739
Std Dev	23.23606
Std Err Mean	4.8450535
upper 95% Mean	35.269765
lower 95% Mean	15.173713
N	23

pH Deaerated



Quantiles

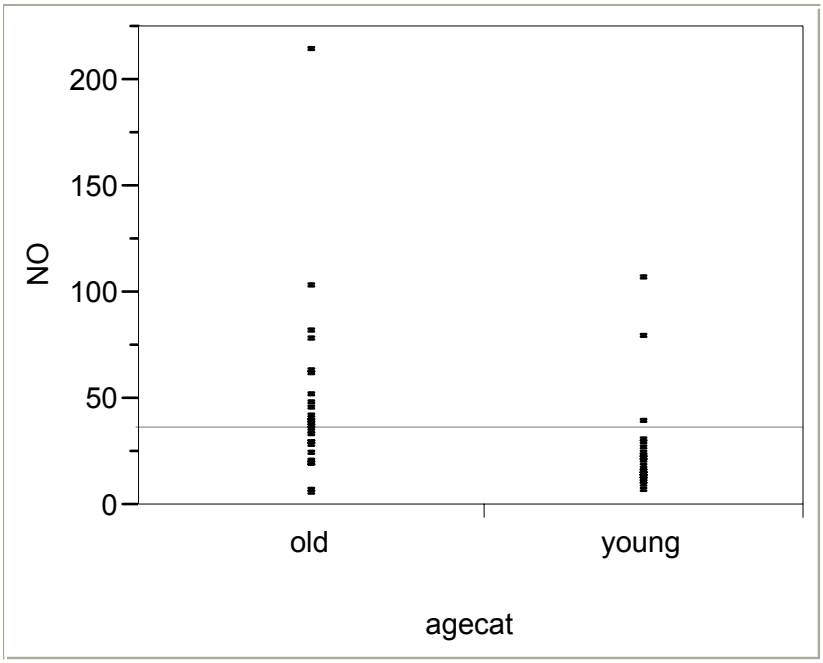
100.0%	maximum	8.1200
99.5%		8.1200
97.5%		8.1200
90.0%		8.0640
75.0%	quartile	7.9100
50.0%	median	7.7200
25.0%	quartile	7.3000
10.0%		6.9540
2.5%		6.4400
0.5%		6.4400
0.0%	minimum	6.4400

Moments

Mean	7.5917391
Std Dev	0.4261845
Std Err Mean	0.0888656
upper 95% Mean	7.7760351
lower 95% Mean	7.4074431
N	23

Appendix E: Analysis of NO and pH by Age Category

Oneway Analysis of NO By Age Category



Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Score Mean	(Mean-Mean0)/Std0
old	25	771	30.8400	3.261
young	23	405	17.6087	-3.261

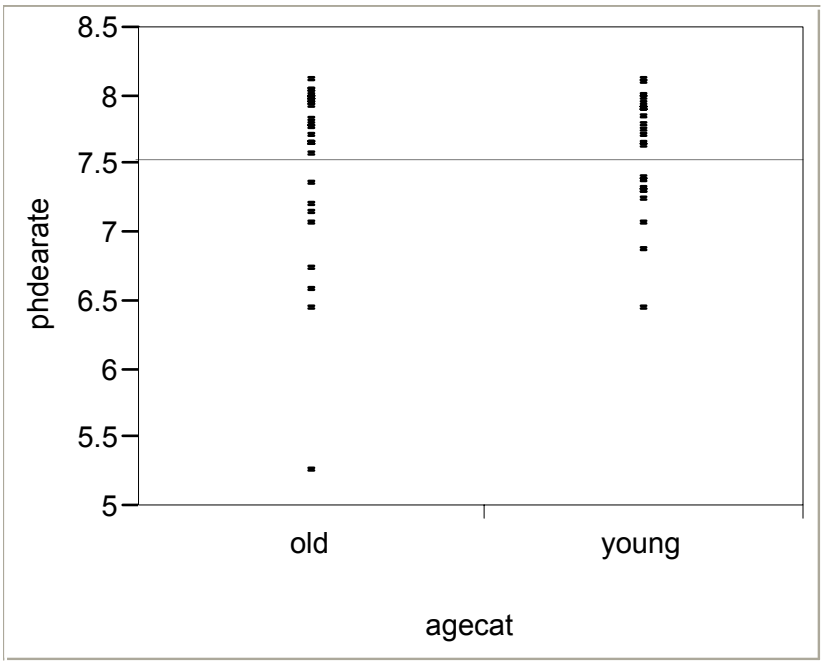
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
405	-3.26083	0.0011

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
10.7004	1	0.0011

Oneway Analysis of pH Dearate By Age Category



Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Score Mean	(Mean-Mean0)/Std0
old	25	615.5	24.6200	0.052
young	23	560.5	24.3696	-0.052

2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
560.5	-0.05161	0.9588

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
0.0038	1	0.9506