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Standardizing the Collection and Measurement of Glucose in Exhaled Breath and Its Relationship to Blood Glucose Concentrations

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**PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance**

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STANDARDIZING THE COLLECTION AND MEASUREMENT IN EXHALED BREATH
GLUCOSE AND ITS RELATIONSHIP TO BLOOD GLUCOSE CONCENTRATIONS

For the degree of Master of Science in Biomedical Engineering



Is approved by the final examining committee:

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07/10/2014

Head of the Department Graduate Program

Date

**STANDARDIZING THE COLLECTION AND
MEASUREMENT OF GLUCOSE IN EXHALED BREATH AND ITS
RELATIONSHIP TO BLOOD GLUCOSE CONCENTRATIONS**

A Thesis

Submitted to the Faculty

of

Purdue University

by

Mark Hamilton II

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science in Biomedical Engineering

August, 2014

Purdue University

West Lafayette, Indiana

This Thesis is dedicated to my parents, Mark and Donna Hamilton, sister, Heather Hamilton, and girlfriend, Johanna Rennie Smith.

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ABSTRACT

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Blood glucose level control (glycemic control) is crucial in diabetes. Limitations in current commercially available monitoring devices include causing patient pain leading to poor blood glucose level management. The development of a non-invasive measurement system may lead to improved patient glycemic control, reducing unwanted side-effects and complications of poor blood glucose level maintenance.

This work explores the use of glucose within exhaled breath in attempt to establish an indirect method of blood glucose level measurement. Specifically, exhaled breath condensate (EBC) is examined. A breath condensing unit was designed to measure the temperature of the system, flow rate, volume of expired air, ambient humidity, and remove exhaled dead volume before condensing breath. A fluorometric assay was used to analyze and measure the glucose concentrations in the EBC samples. The results directly relate to the feasibility of developing a noninvasive EBC-based glucose measuring device.

A nebulizer study was performed to verify that the amount of glucose present in the condensate was predictable, given a known concentration of aerosolized glucose. The nebulizer study revealed that some glucose interferent is present in the ambient air.

Further exploration allowed for a humidity based model to be developed that can accurately and consistently predict the concentration of the condensate.

An IRB approved study, using a total of five human subjects, was employed to quantitatively evaluate the change in both blood and EBC glucose levels associated with the intake of either food or water. The human subject study results indicate that, with the use of the humidity based model derived from the nebulizer study, it is possible to predict blood glucose levels from EBC glucose levels. These results provide motivation for the further exploration of an EBC-based non-invasive blood monitoring device.

1 INTRODUCTION

1.1 Overview

Diabetes Mellitus, often referred to simply as diabetes, is a disease in which affected individuals are unable to autonomously control their blood glucose levels due to either a lack of insulin receptors or insufficient levels of insulin production [1]. Instead, diabetic patients require the aid of blood glucose monitoring systems and exogenous insulin to maintain normal physiological glucose levels. If blood glucose levels are left unchecked and allowed to remain too frequently outside healthy levels, the patient may become victim to more serious medical conditions including: lower-limb numbness, stroke, blisters, glaucoma, hypertension, neuropathy, and diabetic coma [2]. Diabetes is diagnosed as one of three categories, Type 1, Type 2, and Gestational Diabetes, which combined are estimated to affect 8.3% of the U.S. population or 25.8 million people [3].

Type 1 diabetes, previously known as juvenile diabetes, affects approximately 5% of the total diabetic population [4]. Patients with Type 1 diabetes cannot produce insulin because their bodies have attacked and destroyed their insulin producing pancreatic beta cells [4]. As such, the only form of treatment currently available to Type 1 diabetics is active blood glucose level monitoring coordinated with self-administered doses of insulin delivered via injection or pump.

Type 2 diabetes, commonly known as adult onset diabetes, is the most common form of the diabetes accounting from 90% to 95% of all diagnosed cases of diabetes [3].

This form of diabetes normally begins with the body's inability to properly use its insulin, or insulin resistance [5]. Initially the pancreas produces additional insulin to compensate, however, as the need for insulin continues to rise the pancreas gradually begins to lose its ability to make more [3]. The early stages of Type 2 diabetes can be controlled with a healthy meal plan and exercise, but as the disease progresses it will require Type 1 level treatment to maintain healthy blood glucose levels.

Gestational diabetes is a temporary form of glucose intolerance that may develop during pregnancy. It is often diagnosed around the 24th week of and does not indicate diabetes outside the pregnancy [6]. However, 5% to 10% of women diagnosed with gestational diabetes are diagnosed with Type 2 diabetes immediately after birth and 35% to 60% are diagnosed with Type 2 diabetes within 20 years [3].

Most complications of diabetes manifest themselves when blood glucose levels are allowed to shift drastically, or attain and sustain dangerous levels; thusly, strict blood glucose level control (glycemic control) can prevent or delay complications [7]. This typically involves keeping blood glucose levels between 70 and 130 mg/dL before meals and less than 180 mg/dL two hours after a meal. To put these values into perspective, the fasting levels of blood glucose used to diagnose diabetes are enumerated in Table 1.1 [8]. In addition to mitigating health complications from diabetes, strict glycemic control also reduces the financial burden of the disease long term [9-11]. However, patients struggle to maintain these preventative measures [12-14] citing the following as reasons to monitor blood glucose less frequently than recommended: testing my blood sugar as often as recommended would be expensive, painful, and unpleasant [15]. Patient discomfort and unease provides motivation to develop a painless and cheap blood monitoring system in hopes to promote better glycemic control.

Table 1.1: Fasting blood glucose levels and their corresponding diagnostic significance. No food or drink, with the exception of water, should be consumed eight hours prior to the test.

Diagnostic Significance	Fasting Plasma Glucose
Normal	< 100 mg/dL
Prediabetes	100-125 mg/dL
Diabetes	≥126 mg/dL

1.2 Minimally Invasive and Commercially Available Methods of Blood Glucose Monitoring

1.2.1 Single reading self-monitoring blood glucose devices

The most widely accepted and universally used self-monitoring blood glucose (SMBG) device is the glucose meter and it is the standard of SMBG for the American Diabetes Association (ADA) [16]. The glucose meter has undergone much improvement since its release in 1970 including miniaturization, disposable sensors, smaller sampling volume, and shorter measuring time [17, 18]. Despite how far these devices have come, device accuracy remains an issue [19] and there is a large disconnect between commercial product and academic research [20]. Glucose meters typically operate via blood sampling. Patients sample capillary blood by using a lancet to prick their fingers and then apply a drop of blood to the sensor of the device [16]. The two primary methods of glucose quantification in most SMBG devices use electrochemistry or photometry to measure the oxidation of glucose through either glucose dehydrogenase or glucose oxidase [21]. Typical patients may have to measure their blood glucose levels three or more times a day, though blood glucose sample frequency will vary between individuals [22].

1.2.2 Continuous blood glucose monitoring

Continuous glucose monitoring (CGM), where a device monitors fluctuations of blood glucose in near real time, may be an achievable method of alleviating high glucose measurement frequency of some patients. Unfortunately, CGM systems are unavoidably at least minimally invasive. They range from implantable devices, which are fully invasive, to trans-dermal, which can be minimally invasive [19]. Additionally, CGM devices cannot entirely rid the patient of SMBG finger pricks as most of these devices will intermittently require calibration with a SMBG device, such as a glucose meter [23]. There are a number of CGM devices commercially available, and there is significant research in the field of a closed loop CGM device [23]. A closed loop CGM device

involves combining the device with an insulin pump and a controller, allowing the device to dose insulin as needed if the blood glucose level becomes elevated. The system would effectively allow the patient strict glycemic control with little device interaction. Current limitations of CGM devices include accuracy, durability, reliability, and lag time [23]. The perfection of such a device would in essence be the creation of an artificial pancreas, and this is the focus of much diabetic research [24]. Much progress has been made on the path to an artificial pancreas on the CGM, insulin pump, and controller fronts, but it is still far from being commercially available [25]. However, when it does become commercially available, the closed loop system has the possibility to take out patient error and negligence out of glycemic control.

1.2.3 Limitations of commercially available glucose monitoring devices

A majority of the diabetic patient population utilizes SMBG systems to aid their glycemic control [19, 21], it thereby seems prudent to focus on SMBG future directions and limitations. Each diabetic patient is recommended to test their blood glucose levels according to the severity of their diabetes, however, more frequent measurements allow for a more complete understanding of the patient's glucose metabolism and better glycemic control [16]. Accompanying each additional measurement is another prick of the finger, another test strip and lancet used, and another period of time patients need to allot out of their day. It is a direct tradeoff between better glycemic control and more discomfort (physically, mentally, and financially) to the patient [15]. A non-invasive method of blood glucose monitoring would mitigate the negative aspects of frequent blood glucose level measurements, and as such is at the forefront of diabetic research.

1.3 Non-Invasive Measurement of Blood Glucose Levels

1.3.1 Imaging

Many different imaging modalities are currently being explored in attempts to non-invasively measure blood glucose levels, including: near-infrared (NIR) spectroscopy, mid-infrared (MIR) spectroscopy, ultrasound, photoacoustic spectroscopy, and Raman scattering [26, 27]. Within non-invasive diabetic research NIR spectroscopy has accrued amongst the most depth and breadth of research [28-30], but most of these methods share similar limitations [31]. Glucose does not produce a strong consistent signal in most of these modalities and there is considerable interference from undesired tissues and substances, therefore precision and consistency are inherently two challenges to non-invasive blood glucose level imaging [30-35]. There are some imaging sensors already produced by companies, but unless these problems can be overcome they are currently not of use to the diabetic patient population [31].

1.3.2 Indirect measurement through biological liquids

Significant amount of non-invasive blood glucose monitoring research has been focused on using other, more readily available, patient liquids. Urine, saliva, and tears have all been analyzed in attempts to find a relationship between their constituents and blood glucose levels [17, 36-45]. Urine analysis was part of the foundation of diabetes diagnosis, as glucose is excreted in urine when blood glucose concentration gets too high [17]. Unfortunately this primarily happens upon atypically high glucose concentrations for healthy individuals, rendering urine ineffective as an indirect blood glucose measurement. Saliva analysis superficially would appear to be very relevant in monitoring glucose metabolism; however, saliva glucose concentrations are more closely related to the sugar content and timing of the previous meal [46]. This allows saliva analysis to aid in short term glycemic control but limits its all-day monitoring uses [39, 42, 43]. Functional relationships between tear glucose levels and blood glucose levels have been thoroughly investigated [36-38, 44, 45]. This work is leading toward a

wearable sensor that would be placed over the eye, akin to a contact lens [40, 41]. Some limitations of a contact lens form of indirect blood glucose monitoring are patient appeal, discomfort, and cost.

1.3.3 Indirect measurement through exhaled breath and exhaled breath condensate (EBC)

The body of this thesis focuses on the use of exhaled breath condensate (EBC) for use as an indirect measurement of blood glucose level due in part to its non-invasive nature and minimal perceived inconvenience to the patient. EBC sampling and analysis has been performed since the 1980s [47] and it has since expanded into a thriving and promising non-invasive testing method [48-50]. Breath acetone, for example, has been used to in diabetes diagnostics and has been investigated as a biomarker for glucose metabolism [51, 52]. Specifically, this work investigates glucose in EBC. Glucose has been detected in EBC samples but is not yet well characterized [46]. The physiology and theory behind EBC collection and analysis will be elaborated on later in the thesis.

1.4 Significance of a Non-Invasive Glucose Measuring Device

The main purpose of developing a non-invasive glucose measuring device is to make strict glycemic control more achievable for all diabetic patients. Assisting patients to properly dose their insulin or otherwise maintain their blood glucose levels will significantly lower the medical complication rate associated with diabetes and reduce the overall healthcare costs rooted in diabetes. The top three reasons cited from a patient survey monitor blood glucose less frequently than recommended: testing my blood sugar as often as recommended would be expensive, painful, and unpleasant [15]. EBC analysis has the potential to address each of these issues [49].

1.5 Immediate Goals

The American Thoracic Society (ATS) and the European Respiratory Society (ERS) created a joint task force in 2005 to address the state of EBC research. A major finding of the task force was a lack of consistency in measurement techniques for each biomarker [48]. As glucose is not yet well categorized in EBC samples, following the recommendation of the ATS/ERS task force seems prudent. Provided that the ideal final product of this research would result in an affordable, handheld glucose breathalyzer that can accurately monitor blood glucose levels, the immediate goals of this work are listed below:

- Standardize a method of collecting and measuring glucose concentrations from aerosol
- Design a device to collect exhaled breath condensate
- Evaluate the relationship between exhaled breath condensate glucose levels and blood glucose levels

2 BACKGROUND

2.1 Lung Physiology and EBC

Exhaled breath contains water vapor and various solutes originating from epithelial lining fluid (ELF) that can be collected and analyzed as liquid EBC [53]. EBC consists mostly of condensed water vapor, with the other molecules representing a very small fraction (~0.01%) [54]. ELF is diluted up to 10,000 times in EBC by water vapor, making measurement of ELF components in breath challenging due to low signal to noise ratios [46]. It is presumed that the contents of the ELF come to equilibrium with the blood in the surrounding capillary, thusly the content of EBC have been heavily investigated [50, 54-58]. It still remains uncertain how endogenous non-volatile molecules, such as glucose, are aerosolized during respiration. Two hypotheses prevail: (1) turbulent flow in the lungs may force droplets of ELF into the air [58]; (2) ELF droplets may be released into the breath when a film of ELF, formed during the prior exhalation, bursts during inhalation [59].

2.2 EBC and Diabetes

A majority of breath related diabetes work has focused on volatile endogenous biomarkers, some of which are byproducts of the disease itself [60-63]. Some examples of volatile diabetes related biomarkers include, but are not limited to, acetone, carbon dioxide, ethanol, and methyl nitrate [60]. Acetone had been an unquantified indicator of diabetes long before its use in breath research as physicians would recognize its fruity smell; it was later discovered to be the result of diabetic ketoacidosis and related to insulin resistance [52, 64]. While acetone has been detected and measured in breath, it is yet to be correlated with blood glucose levels and varies between diabetic and non-diabetic patients [65]. Carbon dioxide is a known product of both glycolysis and the Krebs cycle, both of which involve the breakdown of simple sugars. Based on this knowledge, a patent has been filed to utilize glucose labeled with high molecular weight carbon (^{13}C) to indicate metabolism [66]. The ^{13}C test works well as a diagnostic tool, to assess glucose metabolism over a certain period of time but is limited as not all sugar consumed by the patient can be ^{13}C labeled [67, 68]. Breath ethanol has shown promise in its relation to blood glucose levels and it is typically used in conjunction with acetone as a biomarker [60, 69]. Methyl nitrate, produced primarily by hyperglycemic oxidative stress, has been used to track some diabetic blood glucose levels but does not translate for all patients [60, 70, 71].

Regardless of the biomarker measured, it must be noted and accounted for that the measurement is not a direct measurement of the ELF. Any volatile biomarker condensed for sampling will be heavily diluted, approximately 2,500 times, by the simultaneously condensed water vapor [53, 54]. This estimate was obtained using sodium and chlorine ion measurements in EBC and ELF to determine dilution. The possibility of glucose measurements being similarly diluted should be considered. However, glucose cannot be directly compared to the prior potential breath biomarker examples because glucose is not volatile nor is it a by-product of diabetes.

There has been a push for both sensitivity and real time measurement within breath research with such technologies as selected ion flow tube mass spectrometry

(SIFT-MS) and cavity ring-down spectrometry [65, 72-78]. These approaches have been shown effective for volatile molecules, but they are currently lab bench technologies unsuitable for individual patient use. Additionally, most of these technologies focus on volatile biomarkers with little interest in the non-volatile components of the breath. Gas chromatography and anion-exchange chromatography are also frequent measurement techniques for breath research, unfortunately these often require lyophilization to be most effective [54, 79].

2.3 Commercially Available and Patented EBC Collection Devices

A majority of the commercial EBC collection devices pass breath through a cold condensation chamber where the breath is condensed [80-84]. The samples are then typically stored in sterile containers until they can be analyzed. The marketed devices are effective for measuring a set list of biomarkers, but have little direction for analysis of biomarkers not present on their lists [85, 86]. Additionally, these collection devices are very expensive [87].

2.3.1 ECoScreen

ECoScreen is a multiple use, non-invasive EBC collection system. The ECoScreen cools the passing breath to -10°C to condense the water vapor and aerosolized particulate and suggests a collection time of 5-15 minutes. Its suggested uses are for: detection of lower airway inflammation, assessment of bronchial challenge testing, and identification of tumor markers [85]. All analysis must be done by the researcher with little suggestion on how to test for biomarkers (Jaeger Teonies, Hoechberg Germany, (http://spira.fi/data/attachments/020_ECoScreen_Brochure.pdf)).

2.3.2 RTube

RTube is a single use, non-invasive EBC collection system. Using an RTube, the patient breaths into a vertical tube encased in a cooling sleeve [86]. The breath is

condensed in the vertical tube, then to be removed via plunger and stored in the RTube disposable tube. There is no set collection time and again little aid is offered in the means of analysis (Respiratory Research Inc., Charlottesville Va, <http://respiratoryresearch.com/products-rtube-overview.htm>).

2.3.3 EBC related patents

Many patents detailing many aspects of EBC collection and many potential biomarkers have been filed, even some that suggest the collection and measurement of glucose [88-92]. While these patents are promising for the progress of EBC related diabetic research, as of the writing of this thesis there has been no research released correlating the glucose measurement of EBC to that of the blood. In order for any glucose based EBC device to succeed, a function relationship between the breath and blood glucose levels must be established.

2.4 Glucose Detection and Measurement in EBC

Table 2.1, based on a table from a prior thesis on this work [93], displays non-lyophilizing methods of glucose measurement in liquid samples. Given a desired lower limit of 0.1 mg/L, it was found that BioVision's glucose assay kit (BioVision catalog number: K606-100, <http://www.biovision.com>) had sufficient sensitivity while being simple and relatively inexpensive. It should be noted that blood glucose measurements are typically reported in units of mg/dL and that $10 \text{ mg/L} = 1 \text{ mg/dL}$.

Table 2.1: Glucose measurement methods, associated detection limits, and references. Based on a table from a thesis by Arun Mohan [93].

Method	Detection Limit	Reference
Anion exchange chromatography with pulsed amperometric detection	0.18 µg/L	Baker (2007) [46]
Reagentless glucose biosensor using molecular excitation luminescence	7.2 µg/L	Der (2008) [94]
Amperometric glucose biosensor based on carbon nanotubes and platinum nanoparticle modified gold electrode	18 µg/L	Duan (2006) [95]
Glucose oxidase in poly(o-aminophenol) film on polypyrrole-Pt nanocomposite modified glassy carbon electrode	81 µg/L	Li (2007) [96]
Biosensor based on poly(methylene blue) and Au colloid modified glassy carbon electrode	90 µg/L	Liu (2005) [97]
Osmium redox polymer- based biosensor on thin film gold electrode	0.18 mg/L	Liu (2008) [98]
MnO ₂ -GOx carbon paste electrode	0.55 mg/L	Wang (2008) [99]
Biosensor based on immobilization of glucose oxidase in electrochemically polymerized polytyramine film and overoxidised polypyrrole film on platinized carbon paste electrode	0.9 mg/L	Liu (2007) [100]

2.5 Prior Studies

In a prior study, by Arun Mohan under the direction of Ann Rundell, using a total of 13 human subjects, EBC and blood glucose levels were measured over the course of three hours with a provided meal [93]. Five subjects returned to participate in the same procedure two more times on different days.

2.5.1 Collection device

The experimental setup for the prior study is shown in Figure 2.5.1. The system was comprised of two chambers, the preliminary chamber and the condensation chamber. In order to prevent early condensates from forming, the preliminary chamber was kept at

37°C. It housed the capnometer (CO₂ analyzer), solenoid valve, and primary reservoir. As the subject exhaled, initially the airflow was directed out of the system. To remove air from dead space within the lungs and collect only air from the gas exchange region of the lungs, the capnometer triggered the solenoid and moved the air flow through the system when it read CO₂ values associated with end tidal air. That air was then collected in the primary 5L reservoir. Once the primary reservoir filled, the subject was finished with the breath sample.

Starting by emptying the filled primary reservoir, the collected breath was passed through the condensation chamber via a stainless steel tube, filling a secondary reservoir. The collected breath was passed back and forth between the two bags until enough EBC volume was produced. The air passing between the two bags was regulated with a flow regulator and that flow rate was monitored by a pneumotachometer.

2.5.2 Glucose assay kit and analysis

In order to detect and quantify glucose in the collected EBC samples, a BioVision glucose assay kit was used (#K606-100, <http://www.biovision.com>). The kit utilizes glucose oxidase to transform glucose into gluconic acid and hydrogen peroxide. Hydrogen peroxide then attaches to the kit probe. The mechanism is shown in Figure 2.5.2. The reaction is covered and kept at 37°C for 30 minutes. When the mixture is hit with excitatory 535nm light, the probe fluoresces at a wavelength of 590nm.

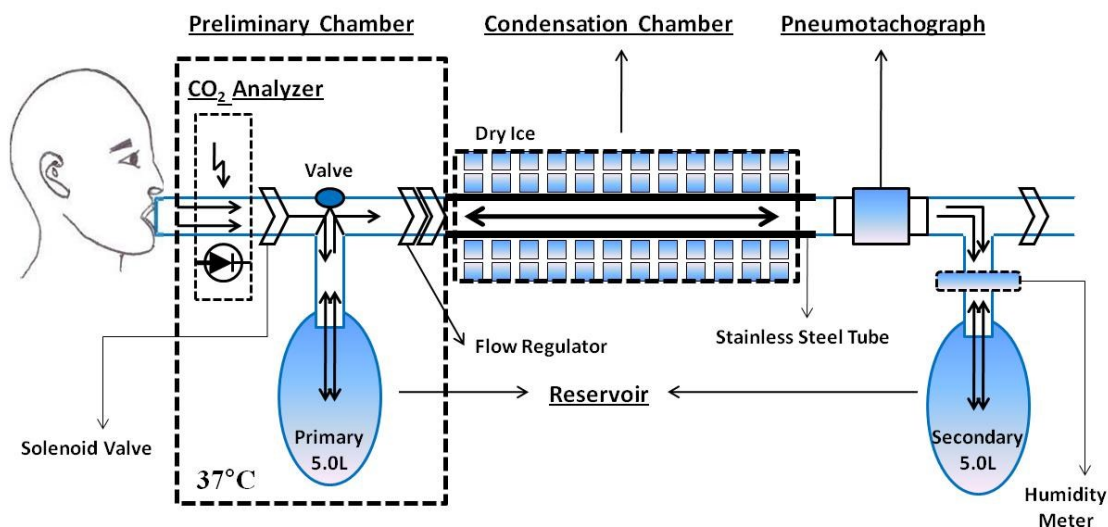


Figure 2.5.1: Experimental setup for Mohan thesis [93]. There are two main compartments, the preliminary chamber and the condensation chamber. The preliminary chamber is kept at 37°C to avoid premature condensation, while the condensation chamber is filled with dry ice.

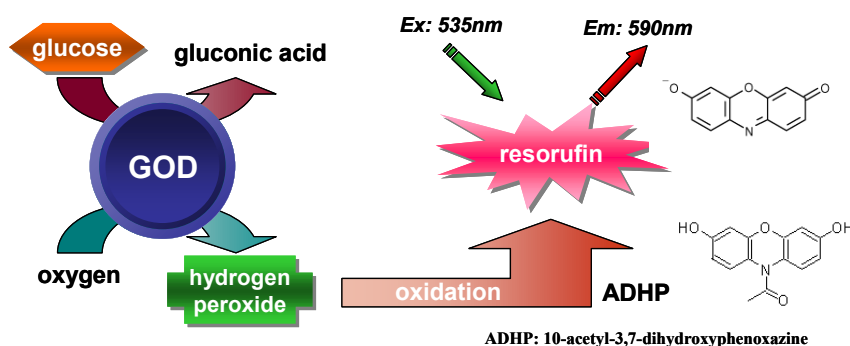


Figure 2.5.2: BioVision glucose assay kit mechanism [93]

The assay results were analyzed using a fluorescent spectrometer yielding relative fluorescence units (RFUs). The RFU are converted to glucose concentration units by the generation of a standard curve from known glucose concentrations.

2.5.3 Human subject EBC and blood collection procedure

All subjects were asked to fast three hours prior to participating in the study. Three breath samples were collected prior to the provided meal at approximately ten minute intervals. After the third collection, the subject was provided a meal, and then

breath samples were taken every ten minutes for two hours. With every breath sample, both before and after the meal, a blood glucose measurement was taken. In order to measure blood glucose levels, a drop of blood was obtained by pricking the subject fingertip with a lancet. The drop of blood was then analyzed for glucose concentration by an ACCU-CHEK Nano glucometer (Roche Diagnostics). Breath samples were collected using the device depicted in Figure 2.5.2 and analyzed using the BioVision glucose assay kit.

2.5.4 Results

One subjects test can be seen in Figure 2.5.3 with blood glucose levels denoted in red and EBC glucose levels in blue. Both blood and EBC show an increase in glucose level after the subject was provided a meal.

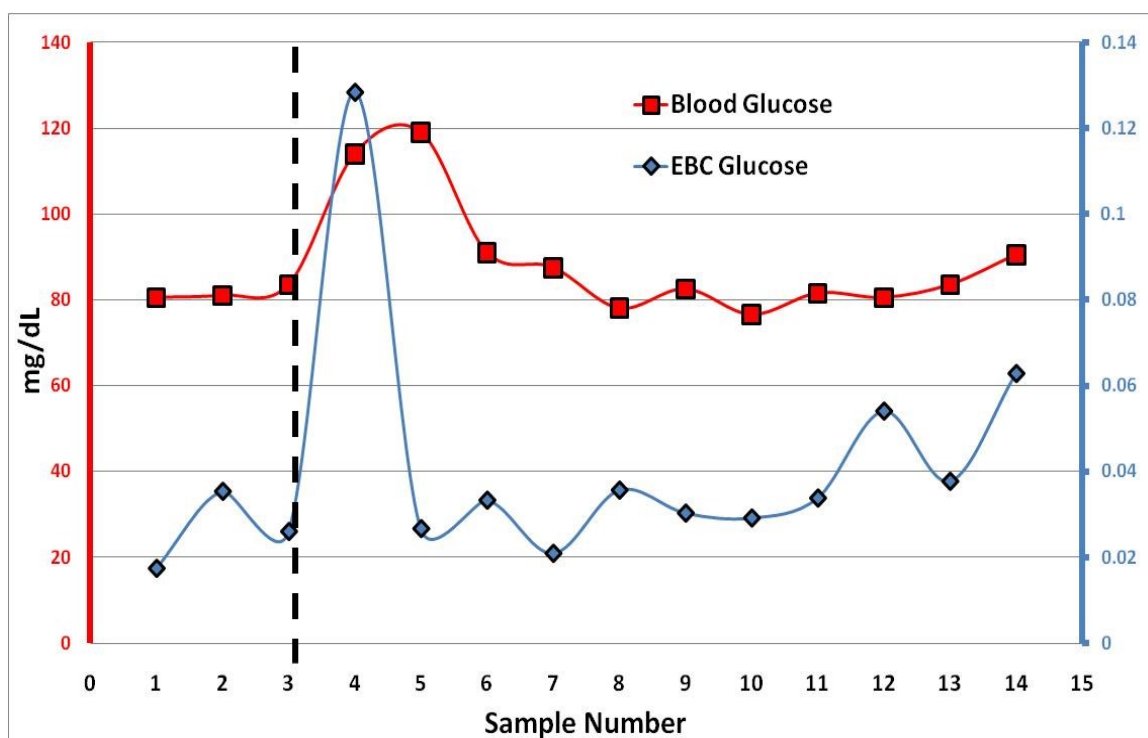


Figure 2.5.3: EBC (blue) and blood (red) glucose trend over 2.5 hours of sample collection. The dashed line represents when the subject was provided a meal [93].

The two major findings of this study were the dilution factor from blood glucose levels to EBC glucose levels and the effects of a meal on EBC glucose levels. The dilution factor was found to be ~ 1800 , but very variable with a standard deviation of a ~ 1400 . The EBC samples taken prior to the meal were significantly different than both the three samples taken directly after the meal and the last three samples taken from the patient.

2.5.5 Summary and conclusions

This study suggests the feasibility of an EBC based glucose monitoring device. EBC significantly changing after a meal provides a basis for future work in this area. However, the variability of the blood-to-breath glucose ratio suggests that more has to be done to understand the blood and EBC relationship. Following these promising results, we sought to learn more about the blood-to-breath relationship and the collection process overall.

3 PORCINE SUBJECT STUDY

From the prior study, it became clear that there is a lot yet to be understood about the blood-to-breath relationship. Given that the blood glucose levels within a human subject vary according to the patient's homeostatic control, it becomes difficult to assess the variability of EBC glucose due to its expected dependence on blood glucose levels. To thoroughly examine EBC variability and factors that may contribute to EBC variability, it would be beneficial to hold the subject blood glucose level steady. Using an animal model, porcine in this case, it is possible to control the subject blood glucose levels and use constant blood glucose levels to better understand its relationship to EBC glucose levels.

3.1 Breath Collection Setup

The collection setup used in the porcine study is very similar to the one described in the thesis by Arun Mohan (Figure 2.5.1) but there are a few key differences. For ease of collection the pigs used in this study were anesthetized, meaning that the anesthetic delivery circuit must be incorporated into the breath collection setup. During collection, the subject has a tracheal tube inserted allowing delivery of anesthetic gases in 100% O₂ while directing the exhalation toward the collection device. The collection and condensation setup can be seen in Figure 3.1.1.

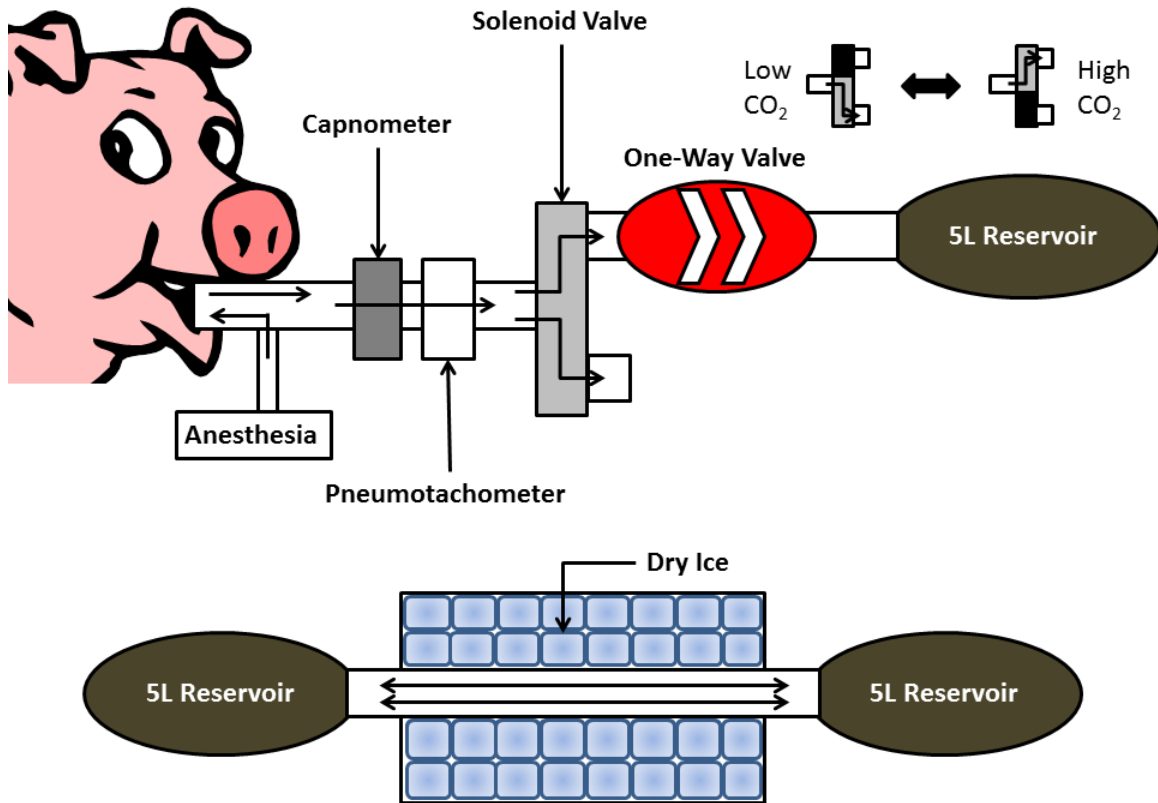


Figure 3.1.1: Porcine experiment setup for collecting and condensing breath.

The top portion of Figure 3.1.1 details the collection setup. Upon exhalation, the breath first passes through a capnometer followed immediately by a pneumotachometer to measure the partial pressure of CO₂ and air flow rate respectively. As the gas exchange responsible for the presence of glucose in the breath is suggested to occur at the alveolar level, the dead space that has no alveolar interaction does not contribute to the EBC glucose. Dead space air similarly does not acquire CO₂ from the gas exchange. By triggering the solenoid valve based on the capnometer output, the solenoid valve forces air with little CO₂ (dead space) to be dispelled out of the system while collecting the air with more CO₂. The dead space relationship to CO₂ content is detailed in Figure 3.1.2 (adapted from the Mohan thesis [93]) and the solenoid switching mechanism is represented in the top right corner of Figure 3.1.1. From the solenoid valve the air to be collected then passes through a one way valve into the collection reservoir.

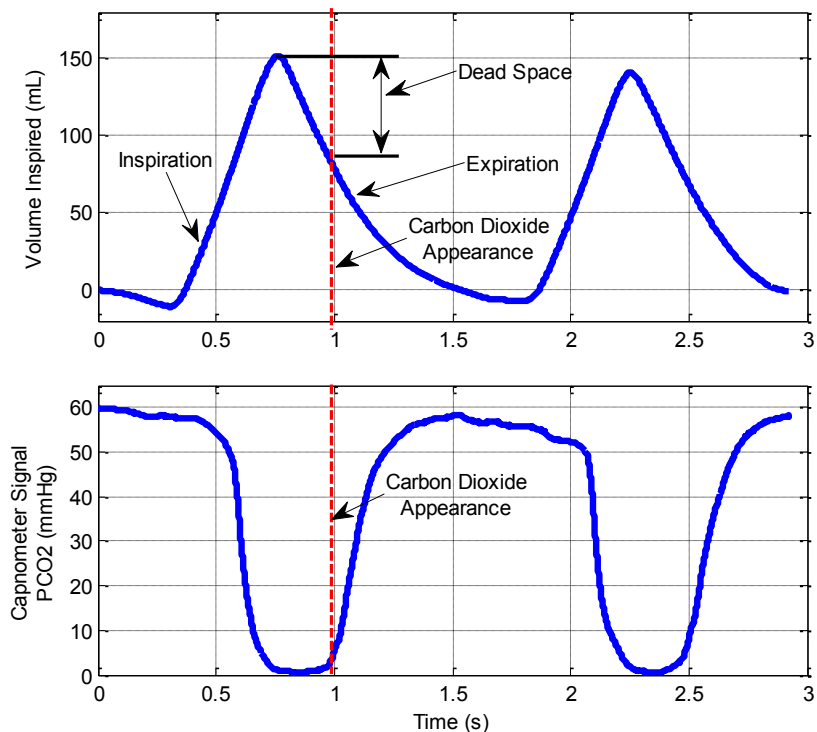


Figure 3.1.2: Illustration of dead space dependence on CO₂ content. The red dashed line represents a potential trigger level for the solenoid switch [93].

Once the collection reservoir is filled, its end is clamped shut until it can be attached the condensation setup (bottom of Figure 3.1.1). The collected breath is passed between reservoirs until each reservoir has been emptied ten times. Each time the breath travels from one reservoir to the other, it passes through a stainless steel tube surrounded by dry ice. After each reservoir has been emptied ten times in this setup, the reservoirs are removed and the sample is collected in a microcentrifuge tube via plunger.

3.2 Research Design

The Purdue Animal Care and Use Committee (PACUC) approved protocol 1203000611 included up to 30 porcine subjects for the following three aims:

- 1) Establish a quantitative relationship between the glucose levels in the EBC and blood using porcine subjects

- 2) Validation of the quantitative relationship from Aim 1 to accurately predict the blood glucose levels in the porcine subject
- 3) Optimize EBC instrumentation and collection parameters.

Up to ten pigs were allotted for each aim and each subject was to be anesthetized and ultimately sacrificed. Every subject had ECG and blood pressure monitoring in addition to a femoral vein catheter for the administration of fluids. The blood glucose levels were to be adjusted with intravenous (IV) administration of dextrose or insulin and sustained with somatostatin [101, 102]. Exhalation mode, respiration rate and inhale-exhale duty cycle were all to be altered to control the breathing style using phrenic nerve stimulation and abdominal compression [103].

None of the aims were completed and Aim 1 was the only one attempted. A total of seven subjects were run.

3.3 Establish a Quantitative Relationship between the Glucose Levels in the EBC and Blood Using a Porcine Subject

3.3.1 Methods

The experimental factors and the target levels at which they were evaluated were listed in Table 3.1 Table 1.1. The experiment design randomly ordered the three target blood glucose levels with six random combinations of ventilation related factors at each glucose level. Before any EBC sample was taken, the blood glucose had to be stable within 10% of the desired level for 10 minutes (as confirmed by 3 blood glucose samples collected at least 2-3 minute apart) and ventilation factors had to be stabilized for at least 5 minutes prior to EBC sample collection. Blood glucose levels were controlled with an IV dextrose or insulin. It took about 10 minutes to collect each breath sample using the setup described in 3.1 Breath Collection Setup. Prior to the experiment, the veterinary technician attached a cuff-electrode to the subject's phrenic nerve and an automated compression machine (thumper) was attached to its abdomen. The cuff electrode was

controlled by a grass amplifier. Together, the cuff electrode and thumper were used to control the subject's breathing patterns to match those listed in Table 3.1. Allowing for adjustments to the glucose levels and the stabilization of the ventilation factors, each study lasted between 6 to 8 hours for the anesthetized subject. At the conclusion of the day, the subject was euthanized.

Data analysis and model generation employed repeated measures ANOVA to analyze the EBC and blood glucose concentration, respiratory rate, peak inspiratory and expiratory air flow rates, tidal volume, exhale duration, and minute ventilation.

Table 3.1: Experimental Factors

Factors	Level 1	Level 2	Level 3
Blood glucose level	Hypoglycemic (50 mg/dL)	Normal (90 mg/dL)	Elevated (200 mg/dL)
Exhalation style	Passive	Forced with abdominal compression	N/A
Inspiration/Expiration duty cycle	Resting Rate	Held breath (inspiration 3 times longer than exhalation)	Shallow breath (inspiration 3 times shorter than exhalation)
Respiration rate	Resting Rate	Rapid (~40 breaths/min)	N/A

3.3.2 Results

With each subject, the blood glucose level was successfully controlled and maintained with dextrose, insulin, and somatostatin at all the desired levels of Table 3.1. However, as the chest compressions and phrenic nerve stimulation were applied they began to affect the health of the subject. While the breath rate and force were being controlled, the subject would become unstable before any pattern could be established for that subject. As such, it was decided to focus on the achievable different blood glucose levels. Regrettably, randomized glucose levels were unachievable as each blood glucose level change substantially increased collection time and frequent swings in blood glucose level also affected subject viability. The attempt at Aim 1 of the PACUC approved study was reduced to breath correlations of three constant blood glucose levels.

Figure 3.3.1 provides an example of the blood and EBC glucose levels collected from a porcine subject from Aim 1. The blood glucose levels were maintained at the

normal (~100 mg/dL) and elevated (~200 mg/dL) with repeatable consistency. The breath glucose levels seem to be relatively similar with the exception of two markedly higher readings. The two samples that read higher than the rest coincide with raised blood glucose values. Overall, there was no significant difference between EBC samples from elevated, normal, or hypoglycemic pig blood glucose levels ($p=0.382$; $n=7$). Refer to Appendix A

Whole Pig Study Data for data from the other six subjects.

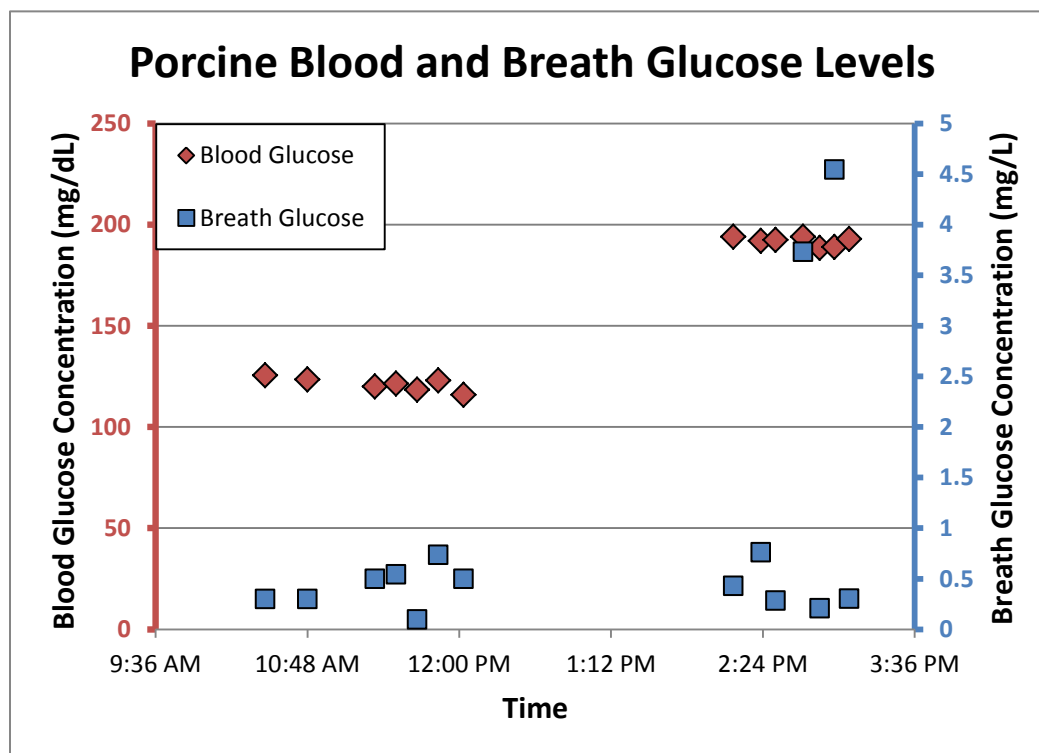


Figure 3.3.1: Blood (red) and EBC (blue) glucose trend of a porcine subject.

3.3.3 Discussion

The porcine studies did not yield the results expected. Constant elevated, normal, and hypoglycemic pig blood glucose levels did not have a significant effect on the EBC samples ($p=0.382$; $n=7$). Samples collected while the patient maintained normal blood glucose levels appear approximately as expected; they are low in the assay limits with an average value and standard deviation of 0.43 ± 0.23 mg/L. The EBC glucose levels

corresponding to elevated blood glucose levels are less logical. The average value and standard deviation for such samples are 1.47 ± 1.85 mg/L. In this case, the variance inherent in these measurements is greater than the average value. Looking at Figure 3.3.1, it is clear that primarily two samples are responsible for this variability. The relationship of the EBC sampled during normal glucose levels to the EBC values during elevated glucose levels is also perplexing, as the two highly concentrated values from the elevated glucose levels are more intuitive than the majority.

Provided the lack of understanding and progress achieved by the seven subjects in this study, it was decided that further in-vitro investigation of the collection and condensation process was needed.

4 COLLECTION AND MEASUREMENT OF GLUCOSE IN AEROSOL

The difficulty faced in the porcine study suggested that it is impractical to analyze biological variance when the collection mechanisms are not fully understood. Therefore, a method of assessing the collection and measurement protocols not involving biological subjects needed to be developed. One way to accomplish this would be through the use of simulated breath with known and controlled glucose levels. The following study used a nebulizer to simulate breath of known concentrations and investigate the process of collection and measurement of glucose in the resulting mixture. It should be noted that the data and some description of this following chapter was submitted in a different format to the Journal of Breath Research for review on April 22nd, 2014.

4.1 Initial Nebulizer Glucose Standard

In addition to removing the biological variable from the process to isolate the collection procedure itself, it seemed prudent to simplify the system further and focus primarily on the collection and measurement. Therefore, the extraneous elements of the pig collection setup were removed for the nebulizer study. Additionally, a majority of EBC research does not utilize any bag collection reservoir for smaller total volume [48].

Instead, the subject breathes through the device which ends with the condensation tube and the breath vacating into the room.

4.1.1 Nebulizer collection setup

The setup for the nebulizer experiment is depicted in Figure 4.1.1. The nebulizer used in this study is ultrasonic, meaning it pushes the liquid through a very fine vibrating mesh resulting in small (respirable) aerosolized droplets (Omron MicroAir, Omron, Kyoto, Japan). The dead space accrued by the connective tubing was 13 ml. Dry ice in the container surrounding the condensation tube lowered the temperature inside causing condensate to form and eventually freeze on the interior of the condensation tube. The condensation tubes were then warmed to room temperature in 3-5 minutes and the samples were poured into sterile microcentrifuge tubes and frozen at -80°C until assayed. Samples were thawed and assayed in a batch within three days of collection. Samples were generated by nebulizing five milliliters of solution into the collection device (Figure 4.1.1). Collection was performed for five minutes as the output was drawn through the condensation tube using a vacuum (Schuco Vac, Carle Place, NY) at a flow rate of six liters per minute.

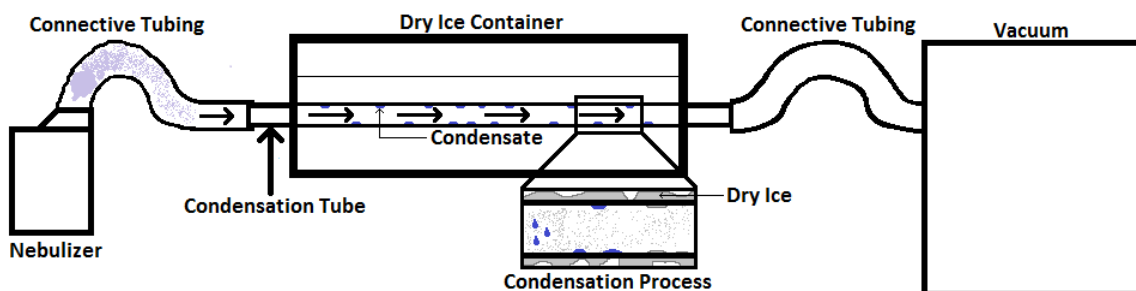


Figure 4.1.1: Aerosol condensation and collection device. The condensation tube runs through a container filled with dry ice, causing the breath and aerosol passing through the tube to condense. A section of the condensation tube is shown at a higher magnification to illustrate the condensation process. The vacuum draws the output of the nebulizer through the connective tubing and condensation tube.

4.1.2 Nebulizer collection methods

The concentrations to be used in the nebulizer were based on the BioVision glucose assay kit. The kit uses concentrations of 0, 0.72, 1.44, 2.16, 2.88, 3.6 mg/L to create its standard. Five milliliters of each listed concentration were added to the medicine cup of the nebulizer. The initial solution added to the nebulizer is referred to as 'stock' or 'nebulizer input' throughout this study. Once a collection was finished, liquid that was not aerosolized remains in the medicine cup of the nebulizer. That leftover liquid was referred to as 'remnant' in this study. Finally, the sample that condensed in the condensation tube from the nebulizer output is referred to as 'condensate' throughout this study. For this and all following studies, the condensation tube and connective tubing (Tygon® R-3606) were cleaned with ethanol and dried with dry, cleaned air (oil-free, 0.2 µm filtered pressurized air with a dew point of -40 °C) before initial use and between each sample collection. A generalized linear model was run to analyze the data with Tukey tests run for pairwise comparison.

4.1.3 Results

The expected study results of running the nebulizer standard were that all samples (stock, remnant, and condensate) have the same glucose concentrations. However, as indicated by Figure 4.1.2, all three samples do not produce the same glucose concentrations. The stock and the remnants samples were significantly different from the condensate collected ($p < 0.001$). Aerosolization of glucose solutions with concentrations of 1.44 mg/L and greater yielded condensate concentration lower than the stock, while solutions of 0.72 mg/L produce similar condensate concentration to the input and no glucose solutions generated condensate with glucose concentration higher than original solution.

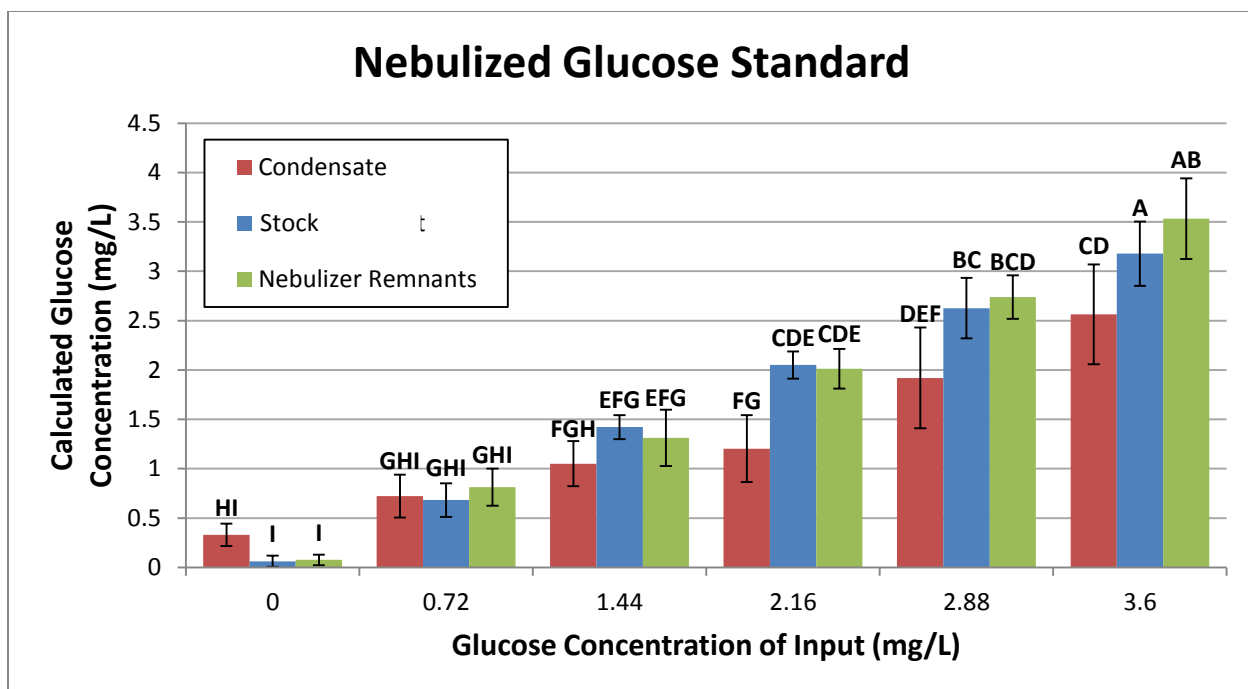


Figure 4.1.2: The glucose concentrations from condensate (n=9), remnants (n=9), and stock (n=9) samples from a nebulized glucose standard. Groups that do not share a letter are significantly different.

4.1.4 Discussion

The stock and the remnants had the same glucose concentrations for all stock values. However, the condensate differed in glucose concentration inconsistently throughout the range of stock concentrations. From this it is clear that the nebulizer itself has no effect on glucose concentration, as the stock and remnants have the same glucose concentration. However, there must be something unaddressed that is contributing to the condensate glucose concentration. This provides some explanation for why it was difficult to find a relationship between blood and breath glucose levels in the porcine subjects.

Standardization of EBC collection processes for individual biomarkers was recommended by the ATS/ERS task force in 2005 [48], and included in the findings are potential causes of variation when collecting breath samples. In order to troubleshoot the simulated breath setup, the task force suggestions were used as guidance.

4.2 Standardizing the Nebulizer Collection Procedure

The ATS/ERS task force identified problems that translate to the nebulizer setup and include: assay accuracy, material interaction, and background interference. A diagram detailing these three identified problems is depicted in Figure 4.2.1. Because the accuracy and variability associated with each problem is dependent on the steps that come later in the process, it seemed prudent to investigate these issues beginning with assay accuracy working backward through the collection and measurement process. For all statistics shown, a generalized linear model with pairwise Tukey tests were run for analysis.

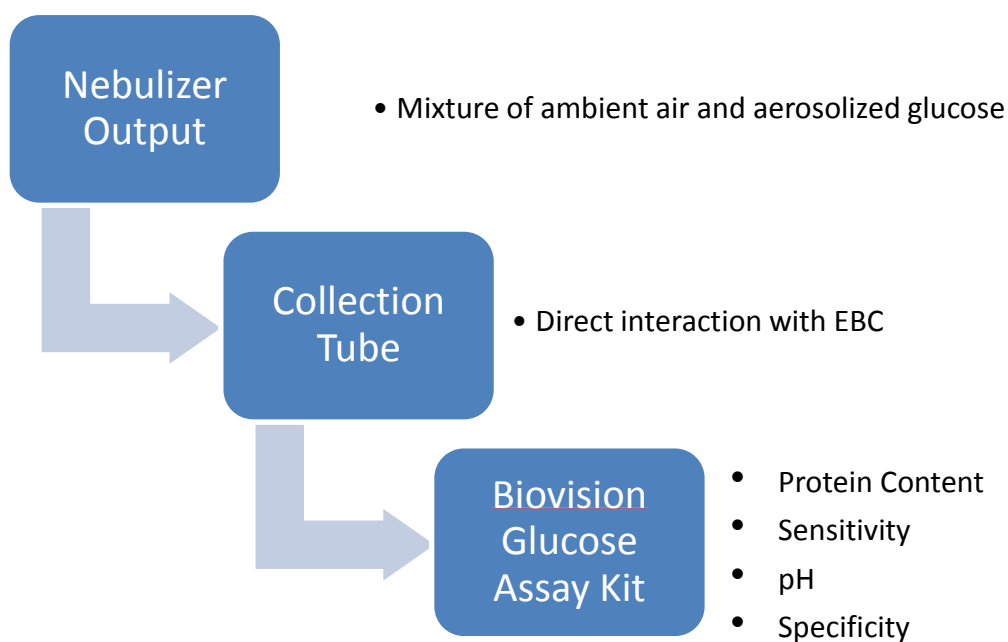


Figure 4.2.1: Diagram of ATS/ERS task force problems that apply to the nebulizer collection

4.3 Assay accuracy

The BioVision glucose assay kit is directly responsible for all the test results reported by this test. As such, it is imperative that the test is shown to be reliable for EBC samples and capable of providing consistent and accurate measurements.

4.3.1 Protein concentration

Differences between the compositions of the assay standard and EBC samples may affect accuracy. Most notably, the assay may require protein that is present in the provided standard but not collected during breath condensation [104].

4.3.1.1 Methods

The setup for the EBC collected to analyze protein concentration is depicted in Figure 4.3.1. For EBC samples from human subjects the user was asked to inhale through the nose and exhale normally into the mouthpiece for 5 minutes. The total protein concentration in the preliminary EBC samples, the glucose assay kit buffer, and the glucose assay kit standard at the highest (3.6 mg/L) concentration were determined using a BCA assay (Pierce Biotechnology Inc., Rockford IL) with a working range of 5-250 $\mu\text{g/ml}$.

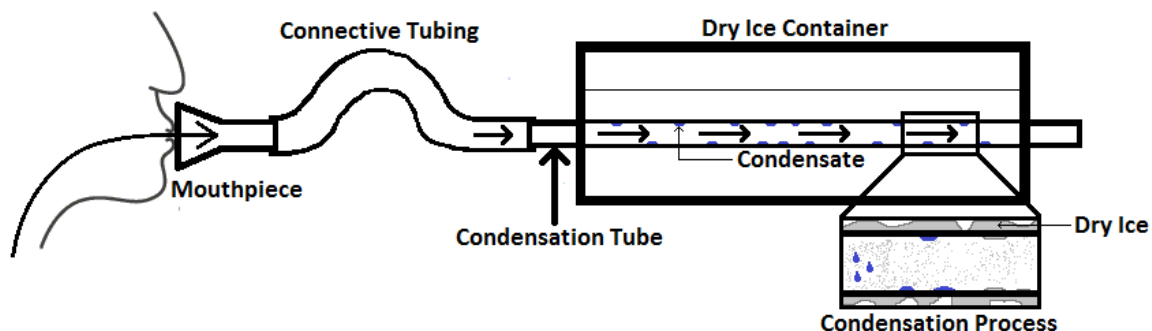


Figure 4.3.1: Exhaled breath condensation and collection device. The condensation tube runs through a container filled with dry ice, causing the breath and aerosol passing through the tube to condense. A section of the condensation tube is shown at a higher magnification to illustrate the condensation process. The user is shown breathing through a mouthpiece, connective tubing and condensation tube.

4.3.1.2 Results

To determine the most accurate assay parameters for quantifying glucose in aqueous samples (similar to EBC), we quantified the amount of protein in the standard glucose assay kit and compared that to the protein concentration in EBC and water samples. Protein concentration in deionized water, EBC samples and components of the glucose assay kit are provided in Table 4.1.

Table 4.1: Protein concentration in deionized water (n=3), EBC sample (n=3), the glucose assay kit glucose buffer solution (n=3), and the glucose assay kit glucose standard at concentrations of 3.6 mg/L (n=3) and 0.36 mg/L (n=3). Groups that do not share a letter are significantly different.

	DI Water	EBC	Buffer	3.6 mg/L	0.36 mg/L
Protein Concentration ($\mu\text{g/mL}$)	0.42 \pm 1.11	6.26 \pm 5.00	18.45 \pm 0.73	26.13 \pm 5.79	22.92 \pm 5.25
Groups	B	B	A	A	A

4.3.1.3 Discussion

Since the protein content of the EBC sample is significantly less than the protein concentrations of the glucose assay kit standards, the provided standard may not provide an accurate representation of EBC glucose concentration. A standard more similar to EBC constitution, created of deionized water and commercial glucose, was created for use with EBC samples.

4.3.2 Sensitivity

To provide a more direct comparison with EBC samples using the glucose assay kit, a customized standard solution with no protein was created and compared to the original kit standard.

4.3.2.1 Methods

The custom standard was created using deionized water and D-(+)-Glucose (Sigma-Aldrich, St. Louis, MO) to obtain final concentrations of 0, 0.72, 1.44, 2.16, 2.88, and 3.6 mg/L, which are the same concentrations used in the glucose assay kit. Due to the low glucose concentrations of the samples being tested, the no-protein standard was also

run at even lower concentrations of 0.003, 0.0075, 0.015, 0.03, 0.06, 0.12, and 0.36 mg/L to extend the working range of the assay. The glucose assay kit was applied to both its provided standard and the custom standard to verify that the glucose assay kit could be used with EBC samples.

4.3.2.2 Results

Glucose measurements in standards created with the kit standard and the customized no-protein standard are shown in Figure 4.3.2. Both standards show low standard deviations, but the custom standard shows higher dynamic range of RFU values from the same glucose concentrations. Additionally, the no-protein standard remains linear at low glucose concentrations (Figure 4.3.3) below the stated accuracy range of the glucose kit. For the remainder of the results in this study, we used the customized no-protein standard since it was linear and had a large dynamic range.

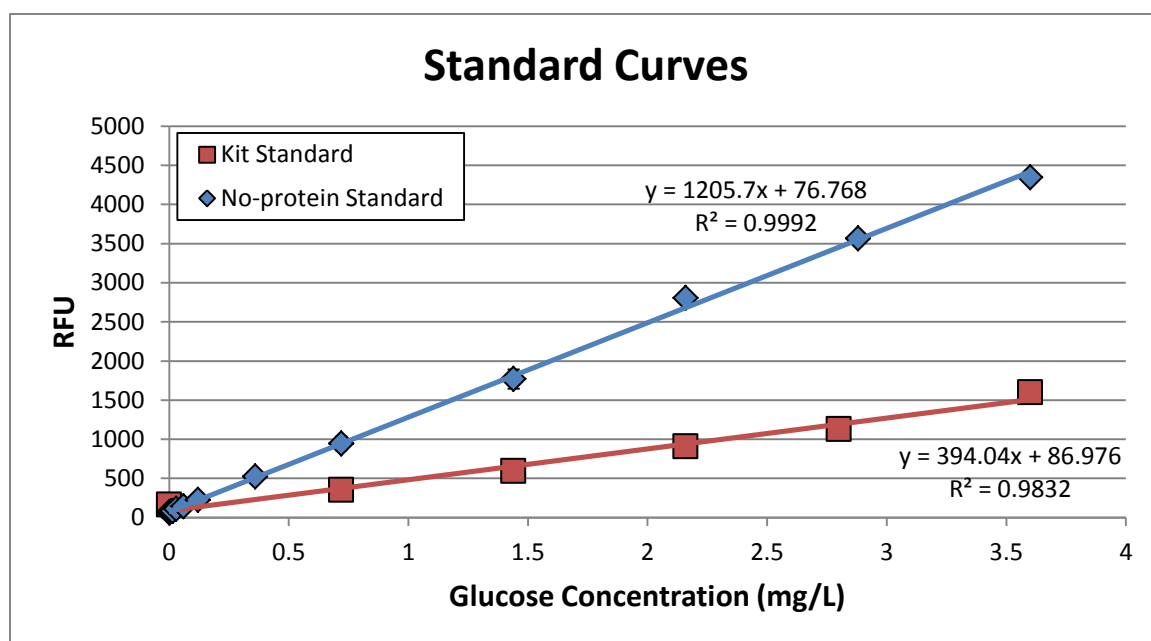


Figure 4.3.2: The standard curve generated by the kit standard (n=4) and the customized no-protein standard (n=3). Error bars represent the standard deviation of the samples.

4.3.2.3 Discussion

As the no-protein glucose standard showed improved performance over the kit standard (Figure 4.3.2 and Figure 4.3.3) while maintaining a more physiologically relevant protein level, it was used for measurements of glucose concentrations.

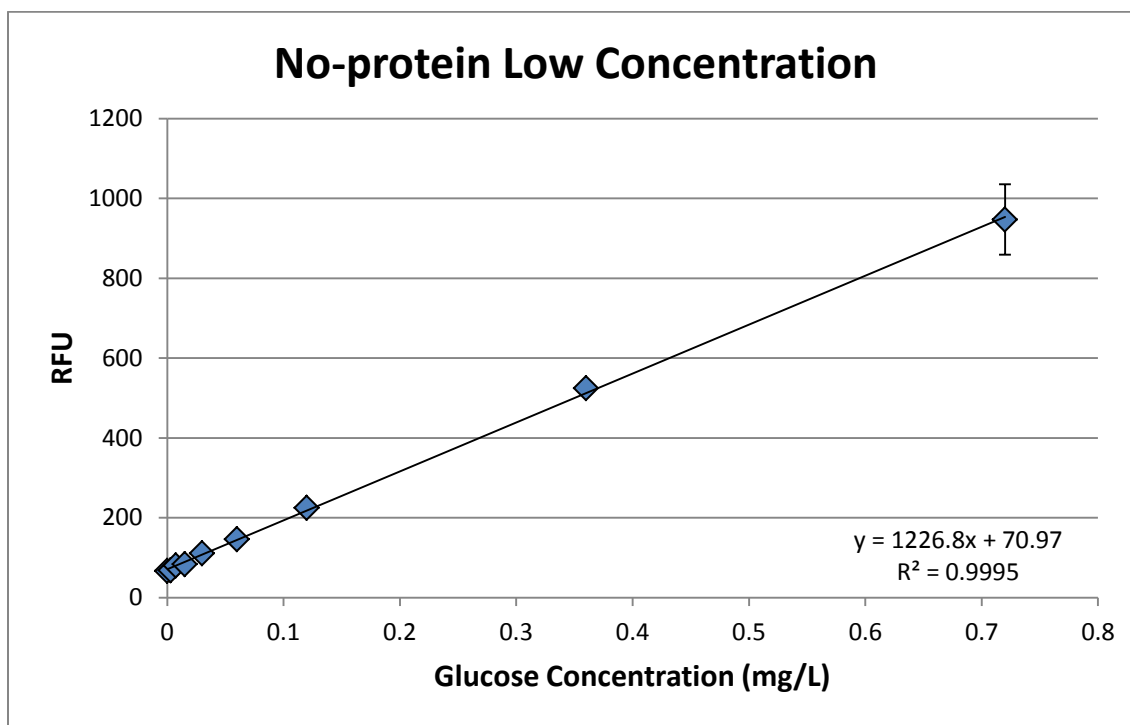


Figure 4.3.3: The standard curve generated by the customized no-protein standard (n=3) with an emphasis on the low glucose concentrations. Error bars represent the standard deviation of the samples.

4.3.3 pH

Previous work examining EBC acidification in acute lung injury found EBC pH to range between 5.5-6.5 [105]. As pH may influence the assay outcomes [106], a pH meter was used to measure pH before and after assaying samples.

4.3.3.1 Methods

The effect of EBC pH on glucose assay performance was evaluated using nebulized glucose solutions with pH in the range of prior work. Solution pH was measured using an electrode (MI-410, Microelectrodes, Inc., Bedford, NH) before ('Initial Samples') and after ('With Reactive Mix') the addition of the working reagents

of the glucose assay. To evaluate interaction between glucose concentration and pH, collected glucose solutions within and above the range expected in EBC were tested (0, 3.6, 7, and 36 mg/L).

4.3.3.2 Results

We measured the pH values before and after adding the glucose reaction mix to confirm that the glucose assay results will not be affected by the pH of the glucose samples. The pH values of the different glucose solutions were not different from each other after assay buffer application (Fig. 4; ‘With Reactive Mix’). The samples, reported in Figure 4 along with pH values, are all buffered to approximately the same pH by the glucose reaction mix (‘With Reactive Mix’; $p < 0.001$).

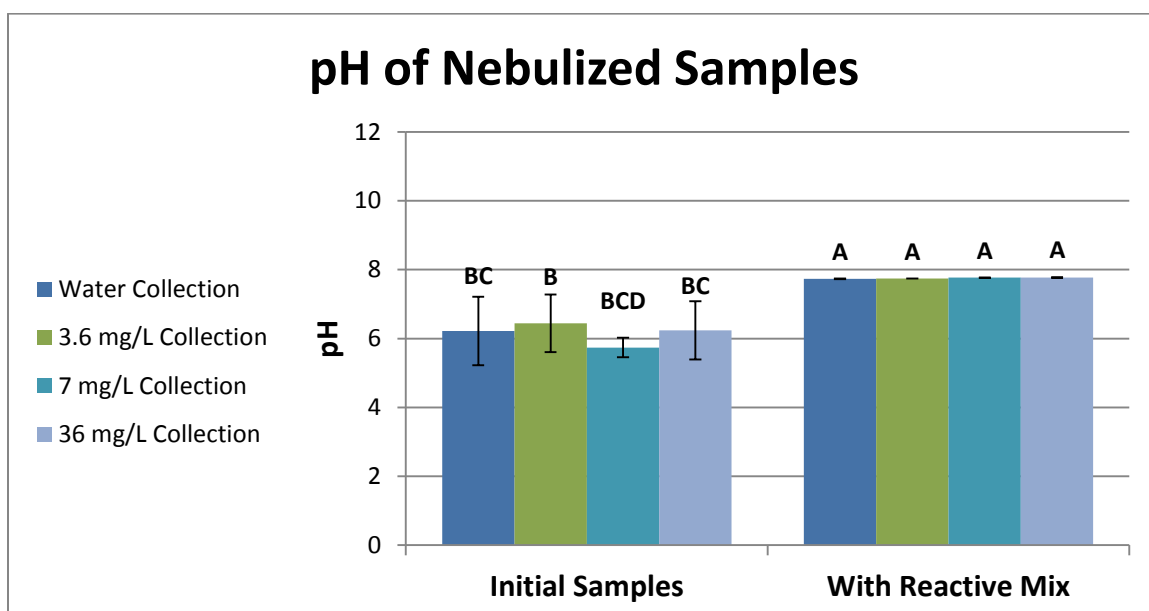


Figure 4.3.4: pH measurements of different samples before ($n=3$) and after ($n=3$) the addition of assay reaction mix. Groups that do not share a letter are significantly different.

4.3.3.3 Discussion

Varying pH levels can also lead to inconsistent measurements in some chemifluorescent assays [106]. Exploring this possibility, we found that a physiologically relevant span of pH values are all buffered as the kit reaction mix is added to the solutions.

4.3.4 Specificity

To ensure that the BioVision glucose assay kit does not react with other similar molecules, the other simple monosaccharides were tested with the kit.

4.3.4.1 Methods

To confirm assay specificity for glucose, standards of deionized water and both D-(-)-Fructose (Avantor, Center Valley, PA) and D-(+)-Galactose (Sigma-Aldrich, St. Louis, MO) were made with concentrations of 0, 0.72, 1.44, 2.16, 2.88, and 3.6 mg/L and compared.

4.3.4.2 Results

To ensure that the glucose assay kit is quantifying the concentration of glucose and not some other monosaccharides, we test the assay output for fructose and galactose. The glucose kit showed no cross-reaction with other monosaccharides, such as fructose or galactose, suggesting that the assay is highly specific for glucose as reported by the manufacturer (Figure 4.3.5).

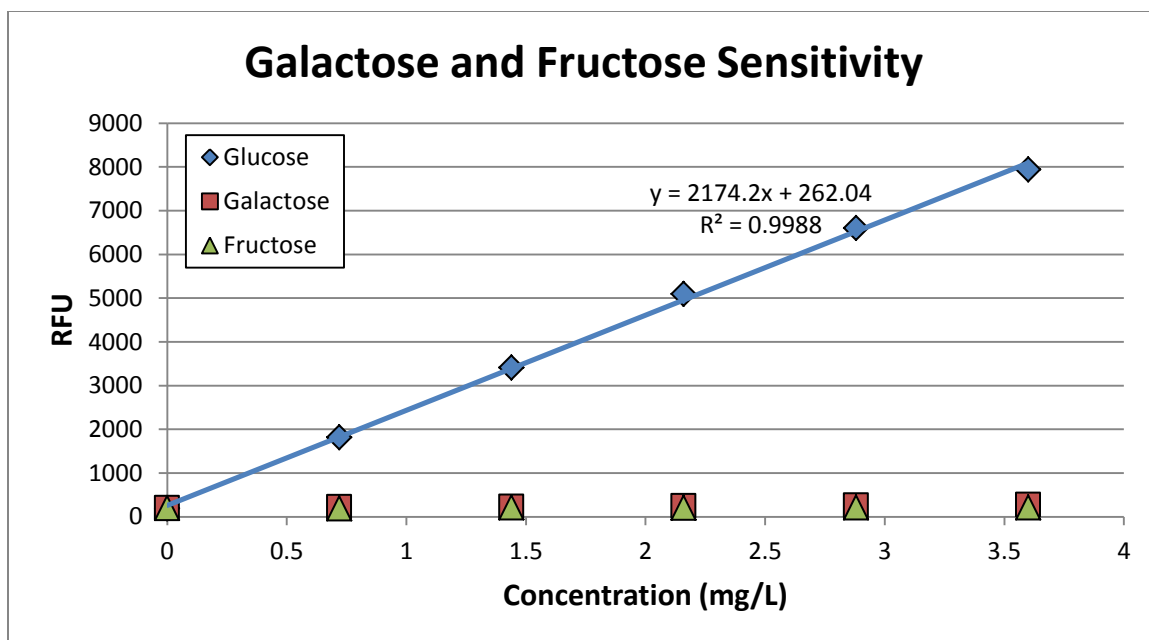


Figure 4.3.5: Equivalent concentrations of glucose, galactose, and fructose in solution as assayed by the glucose assay kit. (n=3)

4.3.4.3 Discussion

The kit is specific for glucose and does not cross react with other monosaccharides.

4.3.5 Conclusion

With the simple standard of deionized water and glucose replacing the kit standard, the BioVision Glucose Assay Kit could act as a viable EBC glucose quantification assay. Confidence in the assay kit readings lends to analyzing the next step in the nebulizer set up diagram, material interaction.

4.4 Material Interaction

The ATS/ERS task force notes that each desired biomarker should be individually analyzed for ideal collection materials [48]. Potential materials to be evaluated were selected from commercialized EBC collection devices and materials appearing in current EBC research [55].

4.4.1 Methods

Condensation tubes of Teflon, stainless steel, and glass were used with outer diameters of 9/32" (with the exception of Teflon with an OD of 1/4") and wall thickness ranging from 0.0625"-0.14". A pipette was used to insert one milliliter of glucose solution of various concentration (0, 1.8 or 3.6 mg/l) into a tube of each material. The tubes were rolled for five minutes before pouring the sample into a microcentrifuge tube and assaying the sample with the glucose assay kit.

To assess the material effects on glucose measurement as the samples change physical states, a second test was run to assess material interactions as glucose solutions were frozen and thawed within the tubes. In this test, 1 ml of the glucose solution was placed in the tube with a pipette, and the entire tube was placed in a container filled with dry ice, as depicted in Figure 4.1.1, for 5 minutes. The tube was then thawed and the resulting solution was removed and assayed for glucose.

4.4.2 Results

Interaction effects for the four potential collection materials tested are provided in Figure 4.4.1. While all of the materials with the exception of glass have no statistical effect on the glucose measurement, Teflon showed the highest correlation with glucose concentration in the original solution. Also noteworthy is how much variance a glass collection system introduces to the samples. As this variability is undesired in the system, the glass collection device was left out of the freeze/thaw experiment.

The effect of freezing and thawing on the glucose solution measurements are shown in Figure 4.4.2. None of the materials showed a significant effect on the glucose measurement.

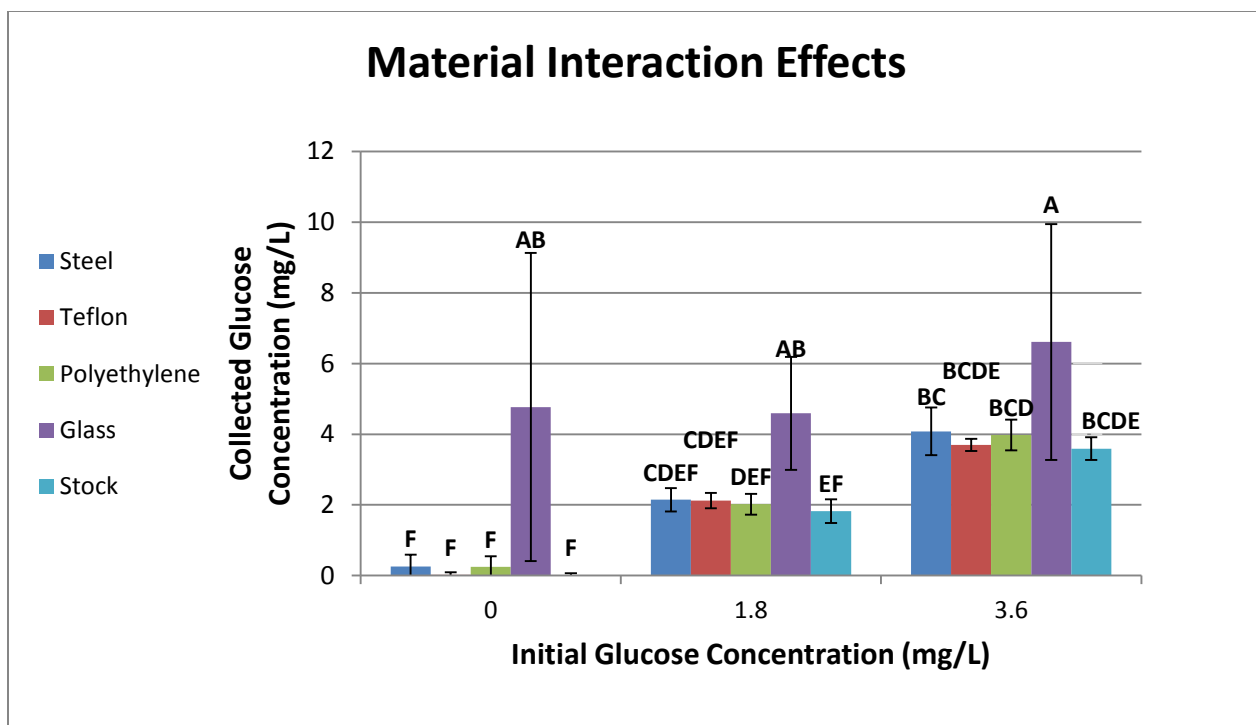


Figure 4.4.1: Results of material contact interaction test for Stainless Steel (n=6), Teflon (n=6), Polyethylene (n=6), and Glass (n=6). Stock solution (n=6) is shown for comparison. Groups that do not share a letter are significantly different.

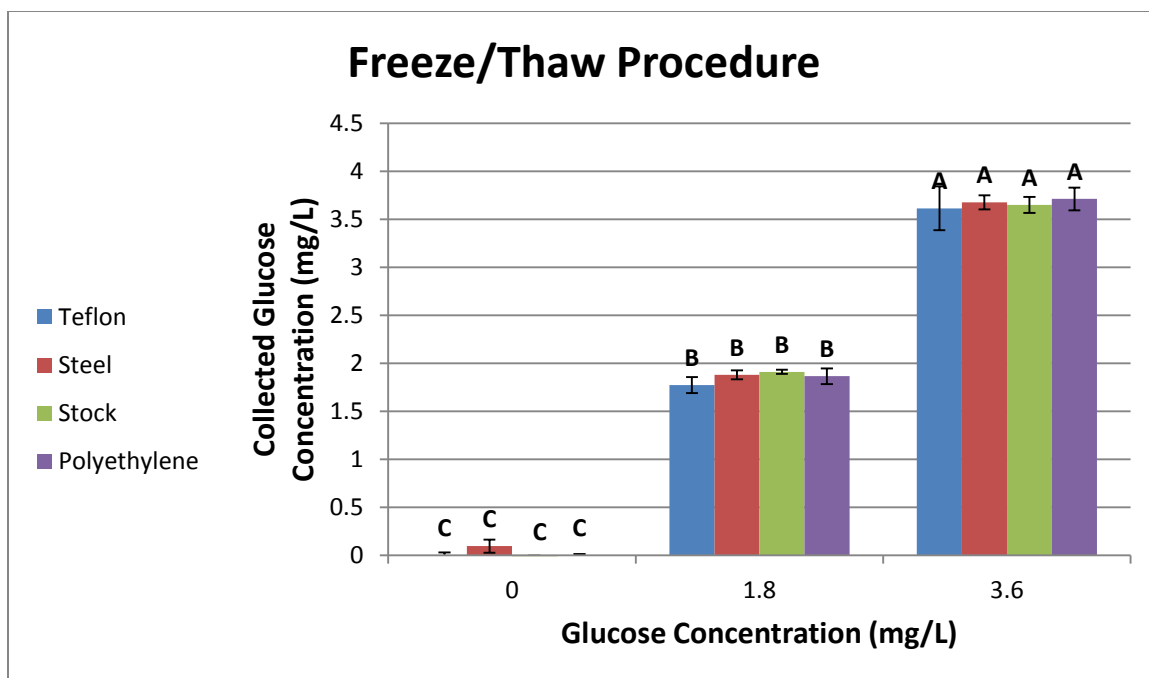


Figure 4.4.2: Results of material freeze/thaw test for Stainless Steel (n=3), Teflon (n=3), Polyethylene (n=3), and Stock solution (n=3) is shown for comparison. Groups that do not share a letter are significantly different.

4.4.3 Discussion

Teflon performed the most consistently of all the materials and provided no distinguishable change in solution concentration as can be seen in Figure 4.4.1 and Figure 4.4.2. Its reliability and inert nature toward glucose suggest that Teflon is an appropriate material for glucose collection. As both stainless steel and polyethylene also showed no statistical alteration in glucose concentration, either material could be potential EBC glucose collection device materials allowing some adaptability to any glucose measurement setup. However, as directed by the ARS/ETS task force, consistency amongst experiments is the goal of this study and as such Teflon is recommended for the quantification of glucose in EBC. The erratic nature of the glass tube measurements may be explained by the glucose in the tube gaining charge and preferentially adhering to the sides of the tube [107].

4.4.4 Conclusion

Teflon will be used for glucose EBC collection purposes as it had no statistical effect on glucose solutions and had the highest correlation coefficient with the stock solution. With an identified condensation material, it is possible to assess the final portion of the collection diagram, the background interference.

4.5 Background Interference

Exhaled breath is largely comprised of inhaled air [56], which may contain interfering compounds. The composition of exhaled air has been shown to have some dependence on the composition of the air inhaled [108]. In order to accurately measure components of EBC, the starting composition of the air must be determined.

4.5.1 Ambient measurements

Before background interference can be corrected for, it must be detected and measured.

4.5.1.1 Methods

Background air collection was performed using a setup similar to that seen in Figure 1b without a nebulizer. Total collection time was 5 minutes. The Teflon tube was then removed from the dry ice and thawed to room temperature, and the resulting solution was poured into a microcentrifuge tube and assayed for glucose. To examine different background air samples, this same test was performed in the laboratory, in a nearby park, and using dry, cleaned air (oil-free, 0.2 μm filtered pressurized air with a dew point of $-40\text{ }^{\circ}\text{C}$). As the dry, cleaned air does not contain enough moisture to condense with the use of dry ice alone, it was bubbled through deionized water before collection.

4.5.1.2 Results

The glucose concentration reported from condensed samples from laboratory air, outside air, dry and clean air (bubbled through deionized water), and water nebulized in the laboratory were compared to deionized water in Figure 4.5.1. Air collected from outside contained a higher glucose concentration than all the other samples ($p < 0.001$), while laboratory air and the condensate collected from the nebulizer run with deionized water output were statistically not different and significantly higher than the water, remnants of the nebulizer and clean air collections ($p < 0.001$).

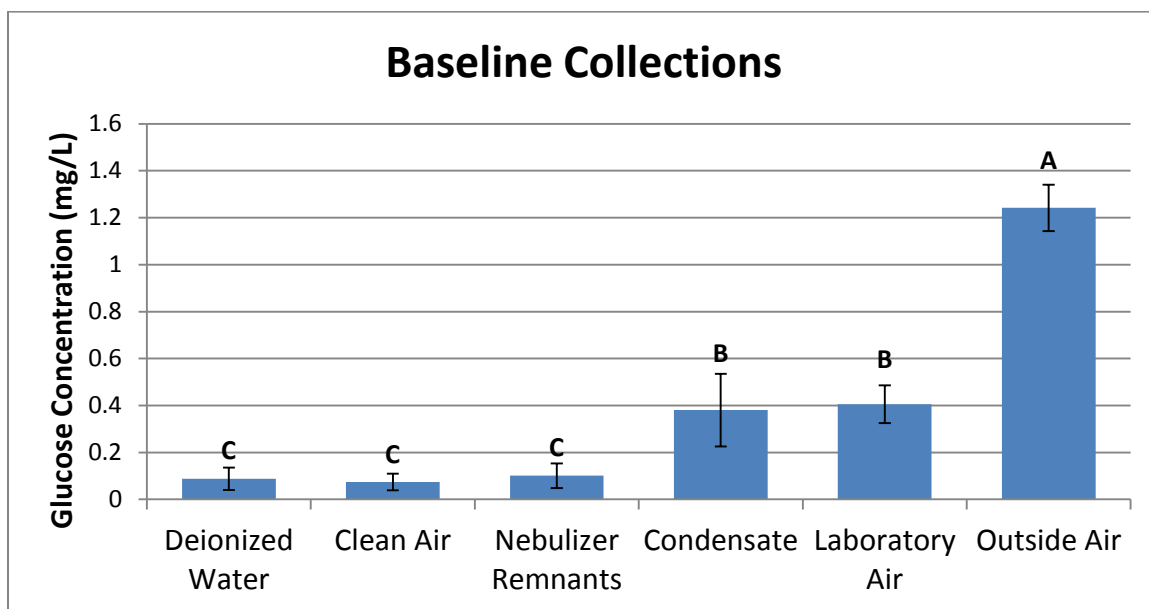


Figure 4.5.1: Glucose concentration from stock deionized water (n=18), dry and cleaned air bubbled through deionized water (n=3), nebulizer remnants of deionized water (n=18), condensate collected from the nebulizer run with deionized water (n=18), condensed lab air (n=12), and condensed outside air (n=3). Groups that do not share a letter are significantly different.

4.5.1.3 Discussion

As can be seen in Figure 4.5.1, condensed ambient air contains detectable atmospheric glucose contamination that would affect EBC measurements. Additionally, the amount of interferent present appears to depend on location. We hypothesize that differences in laboratory and park flora, fauna, and potential industrial sources (e.g. Tate & Lyle) may contribute to the stark differences seen in the condensation collections.

Collections from aerosolized deionized water, the corollary to EBC with no glucose, yielded concentrations indistinguishable from condensing background air. It is therefore imperative that a measurement of the environmental glucose be taken prior to any breath analysis.

4.5.2 Nebulizer mixture model

Nebulized glucose collection may be modeled as the result of a mixture of atmospheric interferent and input glucose solution. The contributions from the input glucose and the atmospheric interferent are dependent upon the fraction of the air sample that can be condensed in our device: this is directly related to the humidity of the air samples. In this case it is possible to relate collected glucose measurements to the input glucose solution concentration by measuring the humidity of the atmosphere and the air to be condensed. The relation between the glucose concentrations and humidity is defined below:

$$[Condensate] = [Input] * Fraction_{Input} + [Atmosphere] * Fraction_{Atmosphere}$$

Equation 4.1: Nebulizer mixture model

where brackets represent glucose or atmospheric interferent concentrations. The fractions of condensed sample may be estimated with humidity:

$$Fraction_{Atmosphere} = \frac{Humidity_{Atmosphere}}{Humidity_{Air\ to\ be\ Condensed}}$$

$$Fraction_{Input} = 1 - Fraction_{Atmosphere}$$

4.5.2.1 Methods

A repeat of the experiment detailed in section 4.1.2 was performed with the addition of ambient humidity measurements. Humidity measurements were performed with a VWR humidity sensor (VWR, Arlington Heights, IL). The stock glucose concentration, ambient glucose concentration, and ambient humidity are used in conjunction with the nebulizer mixture model to predict the condensate glucose concentrations.

4.5.2.2 Results

Since the relationship between the measured glucose concentrations of the condensed samples differed from the stock solution concentrations that were nebulized, we applied the mixture model to correct for the effects of the background interference. Using the mixture model in Equation 4.1, we show that it is possible to estimate the glucose concentrations of the collections from the stock solutions in Figure 4.5.2. The generalized linear model found no significant difference between the model estimation and measured output.

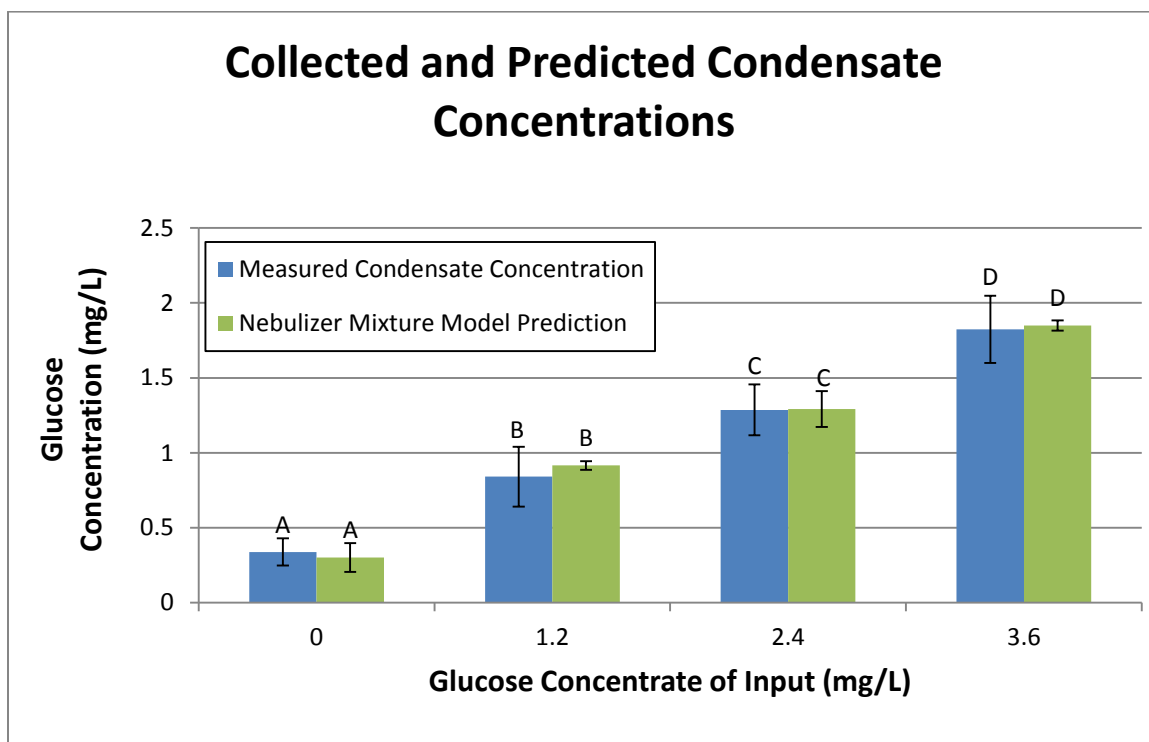


Figure 4.5.2: Mixture model estimated glucose concentration of the condensate concentration from the known stock sample compared to the measured collection glucose concentrations. Groups that do not share a letter are significantly different.

4.5.2.3 Discussion

Attempting to account for the mixture of the background air with the sample air, the concentration of the glucose in the condensate is estimated from the known stock solutions by the nebulizer mixture model (Figure 4.5.2). The nebulizer mixture model can be adjusted for anticipated use with EBC collections. Assuming that EBC collections are

the result of a mixture of atmospheric interferent and ELF glucose we can relate EBC glucose measurements to ELF concentrations once again by measuring the humidity of the atmosphere and the condensed air collected. The glucose concentration of the EBC as parallel to the nebulizer model:

$$[EBC] = [ELF] * Fraction_{ELF} + [Atmosphere] * Fraction_{Atmosphere}$$

Resulting from this model the glucose concentration in the ELF can be estimated:

$$[ELF] = \frac{[EBC] - [Atmosphere] * Fraction_{Atmosphere}}{Fraction_{ELF}}$$

A relationship as demonstrated above may provide insight connecting EBC samples to blood glucose levels; as such, humidity measurements and ambient glucose measurements are recommended to complement glucose EBC work. These measurements elucidate the environmental contribution to an EBC measurement, minimizing the uncertainty of changing environments and the variables therein.

4.5.3 Conclusion

Following the indications from these findings yields a viable EBC collection protocol. With the ability to confidently monitor the glucose concentration in exhaled breath, glucose can be more readily used as a biomarker in EBC. In particular, blood glucose may then be inferable from the EBC measurements, as ELF comes to equilibrium with the blood in the capillaries surrounding the alveoli [53]. Although no simple equilibrium explains how all of the concentrations of molecules in ELF relate to blood, some such relationships have been explored [109]. If there exists such a relationship for glucose between ELF and blood, breath glucose may be used to monitor metabolism non-invasively [49].

4.6 Standardized Collection and Measurement of Glucose in Aerosol

An accurate and reliable measurement technique for glucose from aerosol is detailed in this study. The procedure includes a customized standard using the BioVision Glucose Assay Kit to quantify the glucose, Teflon to collect the sample, and a nebulized glucose standard curve to relate the collection results to the glucose concentration in the aerosol. It has been found that a glucose signal is measured in the ambient air, and this contributes to a variation in the glucose level in nebulized glucose solutions, especially when the glucose concentration is low. Thus, it is critically important to compensate for the background glucose signal originating from ambient air in accurate estimation of the glucose present in EBC. The tested protocol for aerosolized glucose collection provided insight that should enable the reliable measurement and should prove to be a reliable method to quantify glucose in exhaled breath condensates. Future research will apply these techniques to investigate the relationship between glucose concentrations exhaled in the breath and that found in the blood.

5 HUMAN SUBJECT STUDY

The IRB approved study number 1205012274 R002, entitled ‘Glucose Detection on Exhaled Breath Samples’, set out to utilize the standardization achieved in the previous chapter to establish a functional relationship between blood and breath glucose levels in human subjects.

5.1 Methods

5.1.1 EBC collection procedure

The EBC collection device is based on the findings of previous chapter and is depicted in Figure 5.1.1. The breath collection setup device is similar to that of the porcine study seen in Figure 3.1.1, with a few major differences. The human setup has no solenoid valve, one-way valve, nor collection reservoir and instead has a Teflon condensation tube. Removing the solenoid valve removed a significant amount of non-functional device space; however, it was still necessary find another way to remove the lung dead space. The dead space in human adults ranges from 150-200 mL [110]. To remove this from the collection, the subjects were asked to inflate a 200 mL balloon prior to breathing into the collection device. The one-way valve and collection reservoir were

removed based on the findings of the ARS/ETS task force, which suggest that a majority of EBC research is being performed with direct collection as seen in Figure 5.1.1 [48].

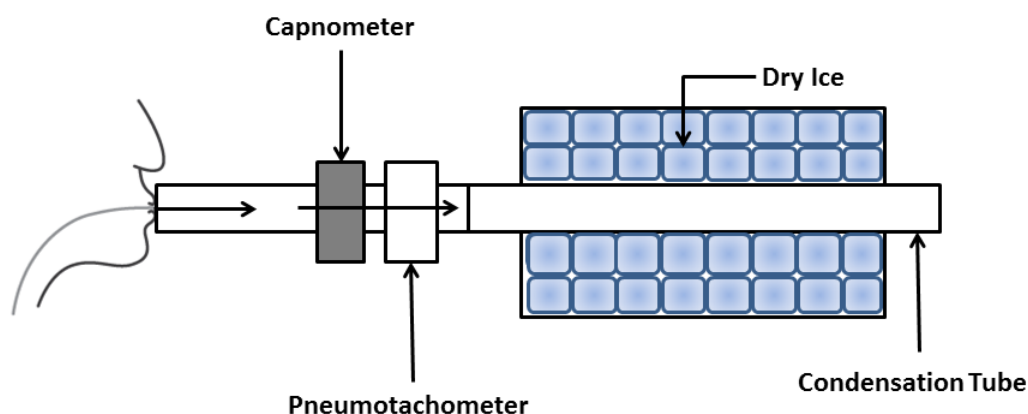


Figure 5.1.1: Human breath collection setup

The subject wore a nose-clip during collection. A typical breath cycle for a patient during EBC collection is as follows: inhale through mouth, exhale into 200 mL balloon, and then exhale into device. When breathing into the collection setup, the subject's mouth was in contact with an individual mouthpiece containing a saliva trap. The breath is then passed through a capnometer, pneumotachometer, and temperature sensor (not shown in Figure 5.1.1) before condensing in the condensation tube. Each EBC sample consisted of 50 L of condensed breath as measured by the pneumotachometer, taking ~10-15 minutes per sample and providing enough condensate for three assay readings. Additionally, ambient humidity and background glucose measurements, as described in Chapter 4: COLLECTION AND MEASUREMENT OF GLUCOSE IN AEROSOL and seen in Figure 5.1.2, were collected simultaneously with EBC collection. Once the patient exhaled 50 L, the condensation tube was removed and allowed to thaw to room temperature. The tube contents were poured into a 2 mL microcentrifuge and stored at -80°C.

All samples were analyzed using the BioVision glucose assay kit with a deionized water and glucose standard within five days (#K606-100, <http://www.biovision.com>). At this point, the samples were again thawed to room temperature.

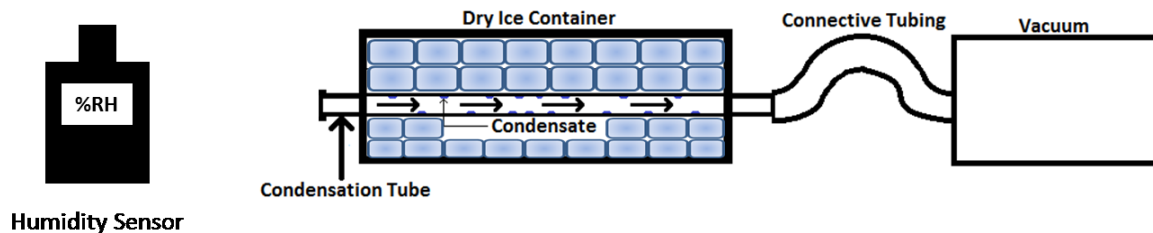


Figure 5.1.2: Background air collection setup

5.1.2 Correction for background

The nebulizer mixture model was adjusted for anticipated use with EBC collections. Assuming that EBC collections are the result of a mixture of atmospheric interferent and ELF glucose we can relate EBC glucose measurements to ELF concentrations once again by measuring the humidity of the atmosphere and the condensed air collected. The glucose concentration of the EBC as parallel to the nebulizer model:

$$[EBC] = [ELF] * Fraction_{ELF} + [Atmosphere] * Fraction_{Atmosphere}$$

Resulting from this model the glucose concentration in the ELF can be estimated:

$$[ELF] = \frac{[EBC] - [Atmosphere] * Fraction_{Atmosphere}}{Fraction_{ELF}}$$

The exhaled breath is assumed to be at 100% relative humidity, as in the case of the nebulizer [110]. Therefore, similar humidity fractions were used in EBC case as the original nebulizer mixture model. This model was used to estimate the glucose concentration in the ELF to provide ELF glucose data to compare to blood glucose levels.

5.1.3 IRB Procedure

‘Glucose Detection on Exhaled Breath Samples’ included the approval of two separate studies, Study A and Study B. A subject participating in one study was not disqualified from participating in the other.

5.1.3.1 *Study A*

For this study, subjects were asked to fast for four hours prior to the study. Three EBC collections, preceded and succeeded by blood glucose level measurements, were taken in succession prior to the provision of a meal. Upon collection of these initial three EBC collections, subjects were provided a meal in order to increase their blood glucose level. Immediately following the meal, six EBC samples along with their corresponding blood glucose level measurements were taken in succession. Each EBC sample was considered complete when the subject had exhaled a total of 50 L of air, taking ~10-15 minutes. Before and after each EBC collection a droplet of blood was acquired from the subject's fingertip using a lancet and applied to an ACCU-CHECK Nano glucometer (Roche Diagnostics) to quantify subject blood glucose level. The overall procedure took place over the course of ~2.5-3 hours.

5.1.3.2 *Study B*

For this study, subjects were asked to fast for four hours prior to the study. Three EBC collections, preceded and succeeded by blood glucose level measurements, were taken in succession prior to the provision of a glass of water. Upon collection of these initial three EBC collections, subjects were provided a glass of water to serve as a control to Study A. Immediately following the water consumption, three EBC samples along with its corresponding blood glucose level measurements were taken in succession. Each EBC sample was considered complete when the subject had exhaled a total of 50 L of air, taking ~10-15 minutes. Before and after each EBC collection a droplet of blood was acquired from the subject's fingertip using a lancet and applied to an ACCU-CHECK Nano glucometer (Roche Diagnostics) to quantify subject blood glucose level. The overall procedure took place over the course of ~1.5-2 hours.

It should be noted that the final three EBC collections of Study B can be used as the first three EBC samples of Study A. Refer to Appendix B
IRB Approval for the approved ARB protocols for Study A and Study B.

5.1.3.3 Subject demographics

A total of 3 normal healthy human subjects, all male ages 20-23, volunteered to participate in both studies.

5.2 Results

The raw EBC and blood glucose level collections of a representative human subject are shown in Figure 5.2.1. This data is a bit erratic and the EBC glucose values have no apparent relationship to the blood glucose values. Additionally, the EBC values are statistically not different before the water, after the water, nor after the meal ($p=0.884$; $n=3$).

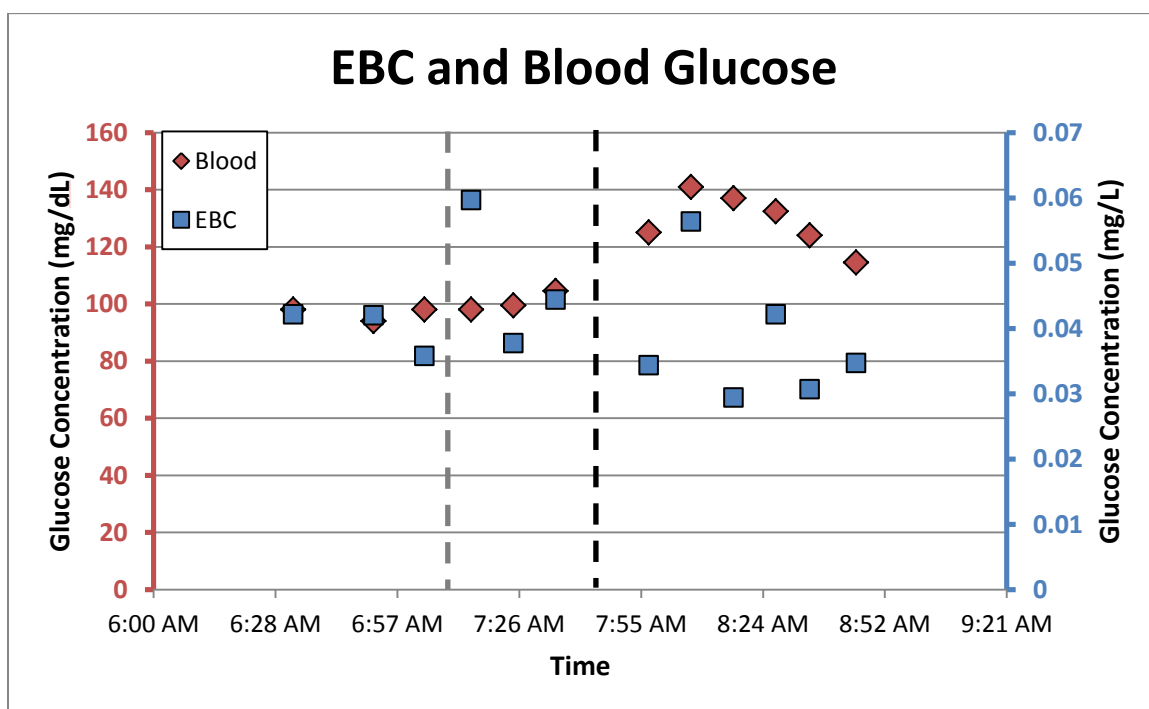


Figure 5.2.1: Whole blood (red) and EBC (blue) glucose level trend of a human subject. Provided water is indicated by the dashed gray line and provided meal is indicated by the dashed black line.

Figure 5.2.2 shows the EBC glucose values next to their corresponding background values and the glucose concentration the background is predicted to contribute the EBC concentration. Both the background and the predicted background contribution to EBC concentration are higher than the measured EBC glucose concentration ($p < 0.001$; $n=3$). Refer to Appendix C Whole Human Study Data for the remainder of the human data.

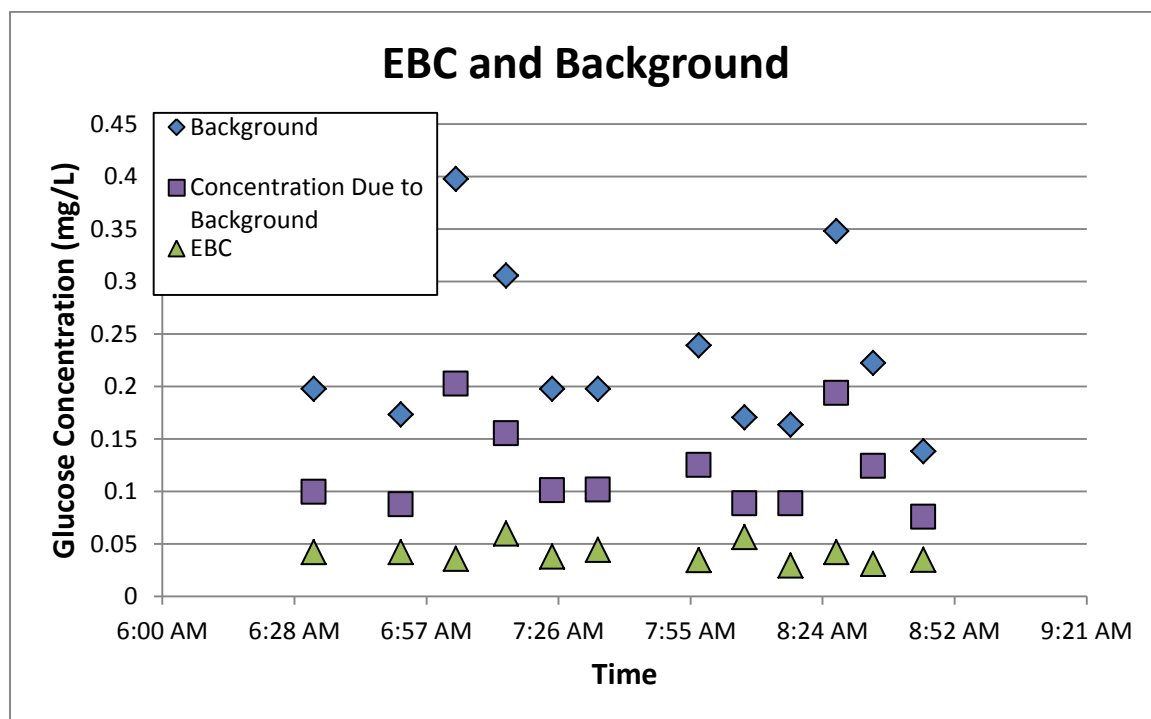


Figure 5.2.2: Comparing EBC glucose levels to background glucose levels

5.3 Discussion

The erratic EBC glucose values seen in Figure 5.2.1 were expected, as this is data before applying the nebulizer mixture theory. However, the expectation was that using the nebulizer mixture model to predict ELF concentration from EBC concentrations would allow a more straight-forward relationship between ELF and blood glucose concentrations. This is not the case, as seen in Figure 5.2.2. The measured EBC concentration is not only lower than its corresponding background glucose interference,

but less than that interference is supposed to contribute to the EBC concentration. The human subject inhaled a higher glucose concentration than was exhaled. This suggests that somewhere in the subject airways collection and retention of aerosolized glucose is occurring.

6 CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

In Section 1.5 the goals for this master's thesis were listed. Each goal and a summary of the conclusions from each goal follow.

- Standardize a method of collecting and measuring glucose concentrations from aerosol

This was initially intended to be accomplished through the PACUC approved porcine study. However, as that study was deemed too unethical to continue based on the procured results, another method of standardizing the collection process was developed. Using a nebulizer to remove the biological uncertainty, it was determined that utilizing the BioVision glucose assay kit to measure glucose concentration, a Teflon condensation tube surrounded by dry ice for collection, and the nebulizer mixture model to account for background interference allows for accurate measurement of glucose in aerosol.

- Design a device to collect exhaled breath condensate

A device that measures CO₂ content, air flow, temperature, and humidity was created while accounting for the standardization achieved in the first goal. The device has the subject breathe through a mouthpiece, capnometer, pneumotachometer, temperature sensor, and then the Teflon collection tube. Meanwhile, ambient humidity and background glucose concentration is recorded throughout the breath collection. Once the EBC is collected, the BioVision glucose assay kit is used to analyze the data.

- Evaluate the relationship between exhaled breath condensate glucose levels and blood glucose levels

An IRB approved study was employed to quantitatively evaluate the change in both blood and EBC glucose. The human subject study results indicate that humans are exhaling less aerosolized glucose than they are inhaling.

6.2 Future Work

6.2.1 Investigate origin of glucose presence in ambient air

This study would measure background glucose interference levels from many locations. It would be ideal to have measurements from high fauna population areas, high flora population areas, and different industrial sites. Comparing the glucose levels of these different background environments should allow for the identification of factors that contribute to glucose interference in background air. Additionally, it may be useful to simulate different methods of glucose aerosolization that fauna, flora, and factories may be performing.

6.2.2 Explore methods of glucose retention in respiratory system

Subjects could be asked to inhale different background air glucose concentrations for a regulated period of time, allowing the respiratory system to acclimate to the new background. Then EBC and background glucose measurements can be collected and compared to confirm that the subject removes glucose from the air inhaled. The different backgrounds will allow for a relationship between background glucose level and airway glucose retention to be identified; the clean air background would provide a control allowing for analysis of EBC glucose produced solely by the respiratory system. Additionally, taking EBC measurements of the dead space and deep lung air separately may indicate where in the airway glucose retention may be occurring.

6.2.3 Revisit breath-to-blood glucose level functional relationship

6.2.3.1 Multi-day fasting EBC study

This study would measure blood and EBC glucose levels while fasting several times a day for five days. Having fasting blood and EBC glucose levels over the course of the day and over the course of the week provides a thorough sample of fasting glucose levels. These data would provide means to properly assess the variability of the blood and EBC glucose level relationship while fasting.

6.2.3.2 EBC glucose study with extreme blood glucose levels

The human subject study run for this work analyzed the blood-EBC glucose level relationship through a normal range of blood glucose values. The proposed study would collect EBC samples from both hyperglycemic and hypoglycemic subjects. The abnormal blood glucose states could be induced with liquid dextrose and insulin and the study may have to be done in an animal model for ethical reasons. The data this study would collect would provide a more accurate understanding of the working range of the blood-EBC glucose level relationship.

6.2.3.3 Variable exhalation method study

Each subject participating in this study would be provided very detailed instructions of how to exhale while EBC collection is occurring. Referring to Table 3.1 provides a range of breathing styles that could be tested in this manner. However, with human subjects, the test would not be limited to how breath can be controlled via phrenic nerve stimulation and chest compression. Human subjects can be told to inhale until their lungs cannot take in anymore air and they could be asked to exhale their full lung volume. Additionally, given a metronome, humans will be able to breath in very controllable rhythms. This data would provide more information on the blood-EBC glucose level relationship, specifically if the relationship is breath style dependent. It may also give some insight to the mechanism of small, non-volatile particle aerosolization in the breath.

6.2.3.4 Subject diversity study

All the subjects that participated in the human subject study for this work were college age non-diabetic adults. Given that EBC glucose monitoring is a design to aid in the life of diabetic patients, it seems logical to confirm this relationship in diabetic subjects. While expanding the subject list, adding age groups and subjects with diabetes related complications will more appropriately represent the patient base this technology is geared toward.

6.2.3.5 Other directions

Once the studies proposed above have established the capabilities of an EBC based blood glucose monitoring system, the next steps would be to apply the findings of this work to a handheld device. The proposed devices thus far, for example, do not account for a background and do not contain humidity measurements. These will need to be added to any design in addition to the findings of future studies.

Furthermore, the method of EBC collection and measurement used in these protocols require at least ten minutes of collection time. For hand-held device purposes, a small sensor would likely serve better than an assay requiring a spectrophotometer.

Changing measurement methods may allow for a more convenient sampling time and simpler miniaturization of the device.

All future directions should continue toward getting a non-invasive blood glucose monitoring device market ready and available to patients.

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Appendices

Appendix A Whole Pig Study Data

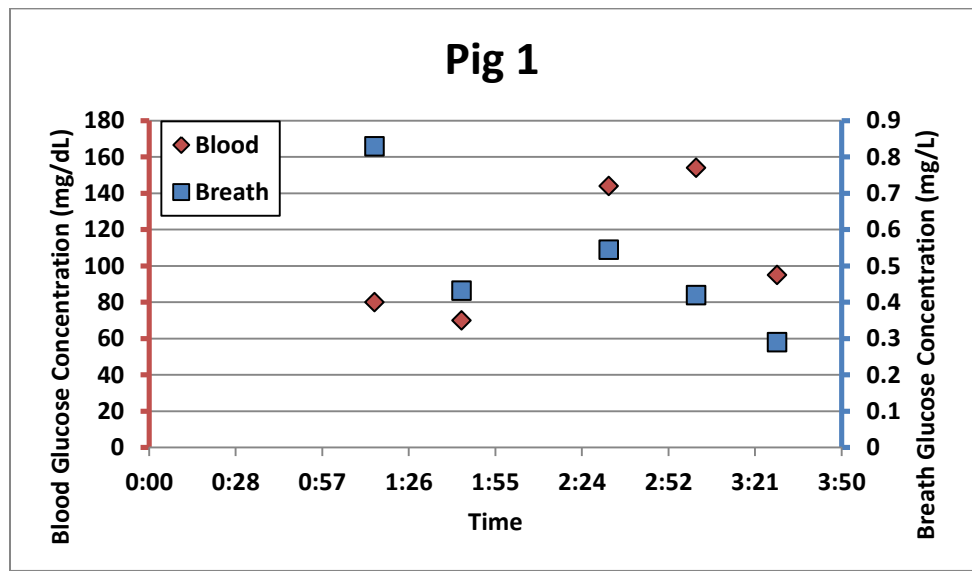


Figure A.1: Blood and breath data from Pig 1

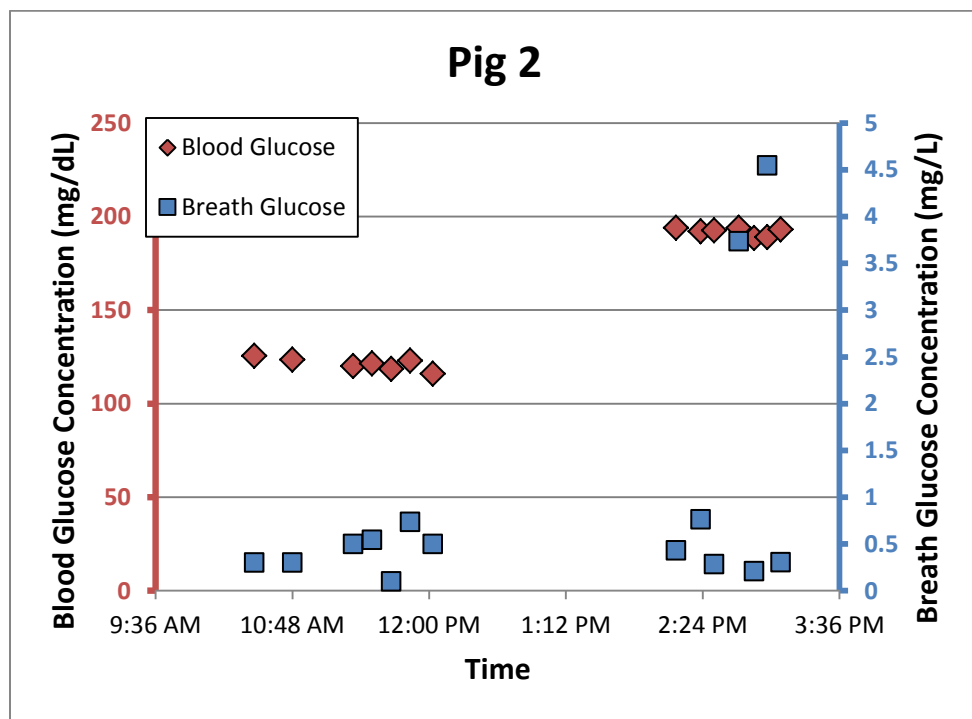


Figure A.2: Blood and breath data from Pig 2

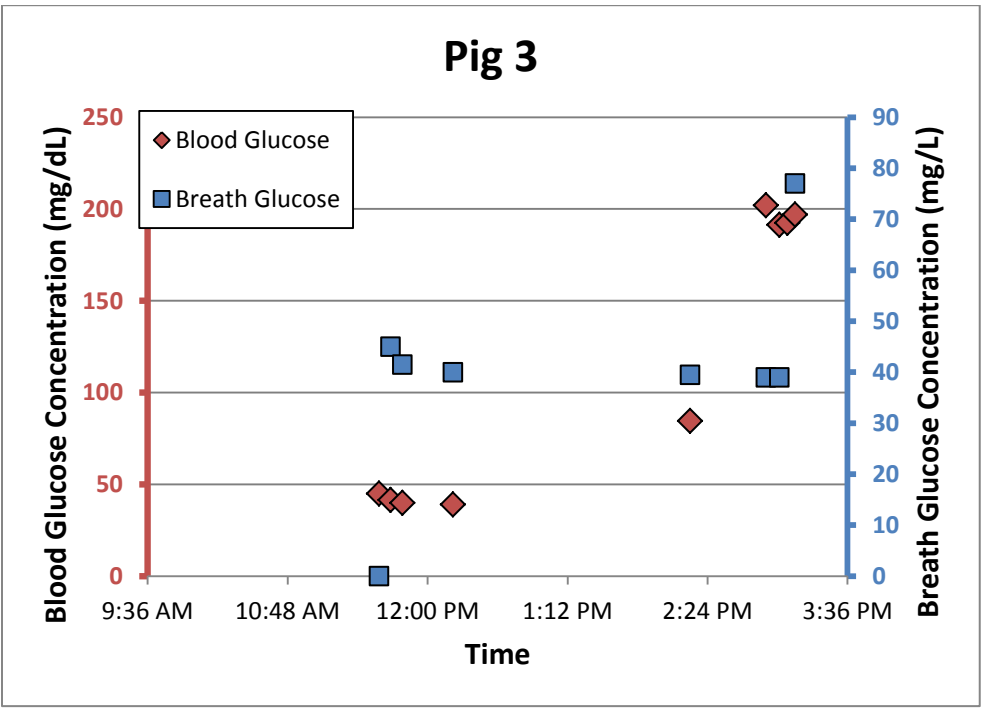


Figure A.3: Blood and breath data from Fig 3

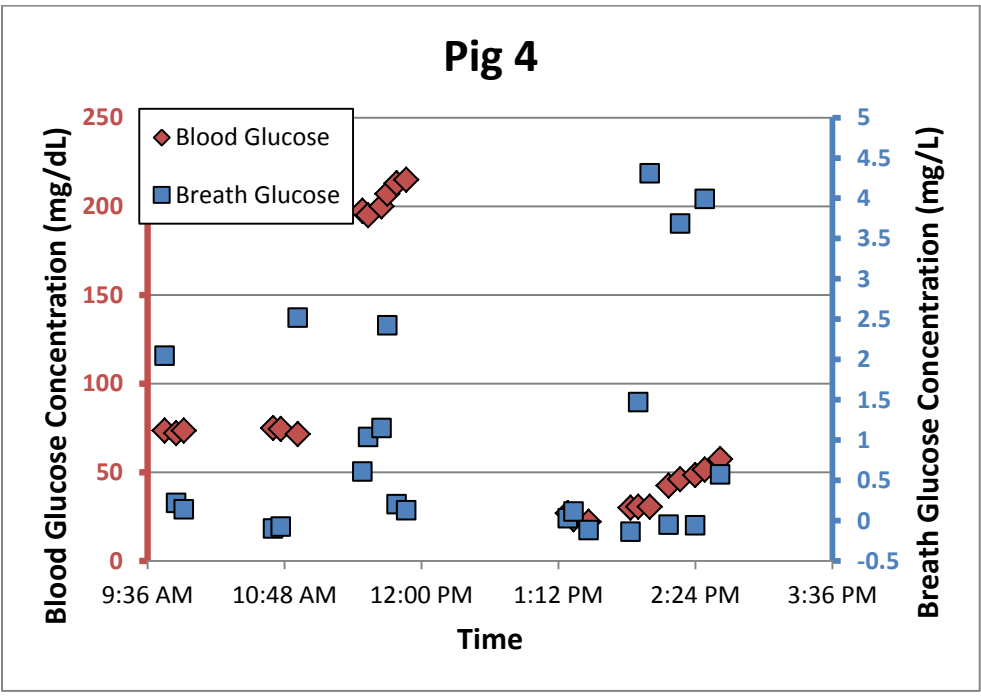


Figure A.4: Blood and breath data from Fig 4

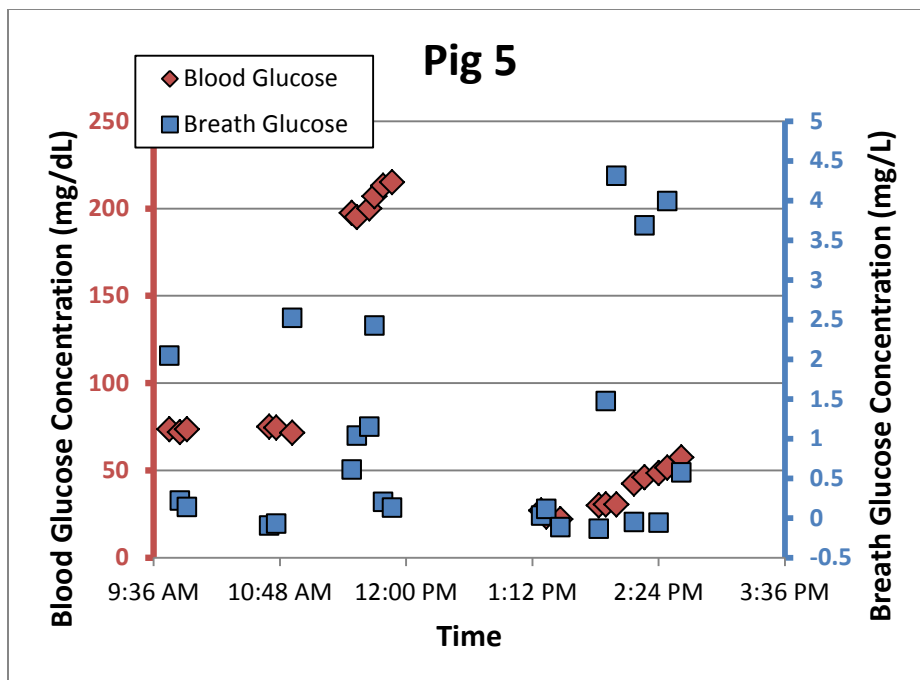


Figure A.5: Blood and breath data from Pig 5

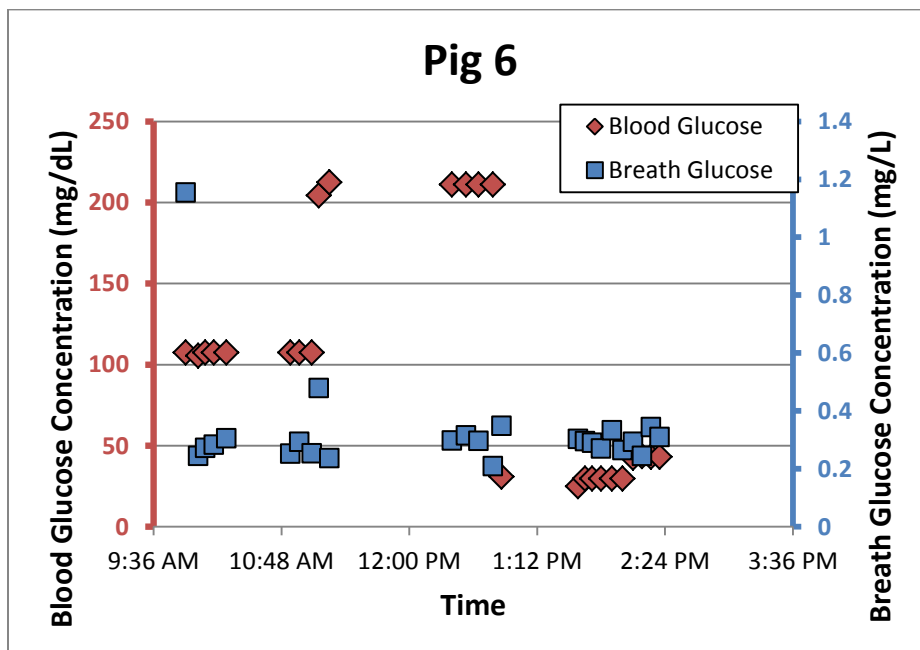


Figure A.6: Blood and breath data from Pig 6

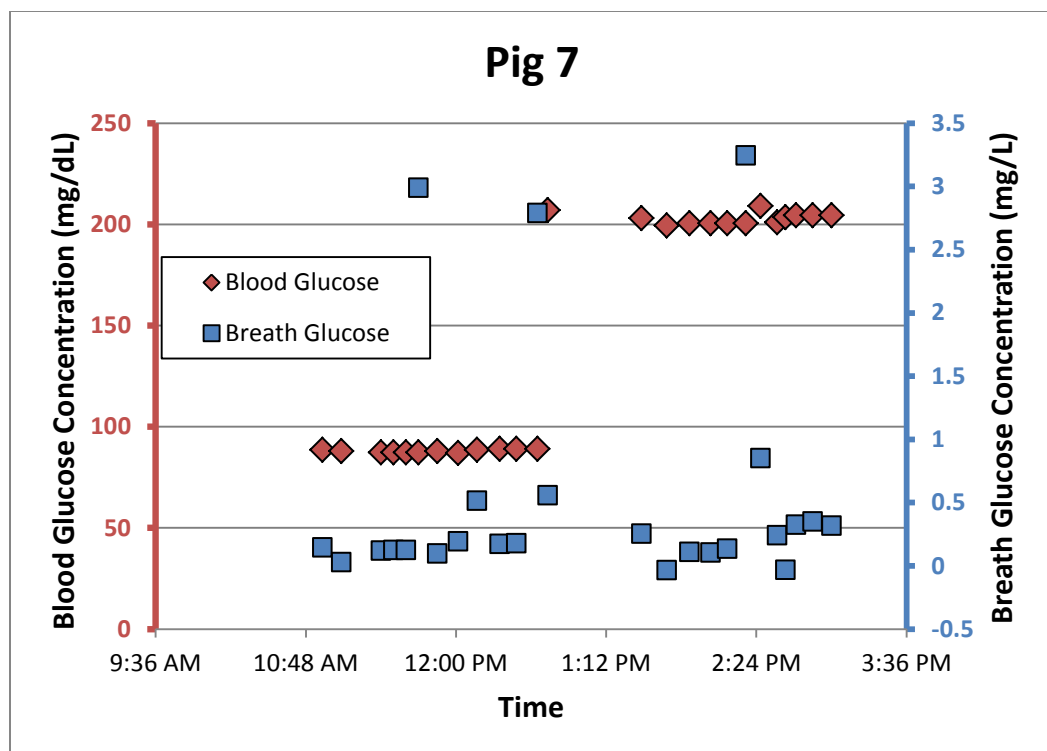
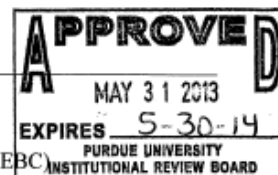


Figure A.7: Blood and breath data from Pig 7

Appendix B

IRB Approval

Research Project Number _____



RESEARCH PARTICIPANT CONSENT FORM

Measurement of glucose in Exhaled Breath Condensates (EBC)
 Professor Ann Rundell
 Purdue University
 Weldon School of Biomedical Engineering
 Study B

Purpose of Research

The purpose of this research is to evaluate the feasibility of a new, noninvasive glucose measuring device that uses exhaled air to replace the traditional use of blood samples. This research sets out to determine if stable blood glucose levels are also observed in the exhaled air samples.

The glucose concentration in the exhaled air sample will be measured as will the glucose concentration in the blood before and after consumption of water. Samples of the exhaled air and blood will be collected every ~15 minutes, starting 30 minutes before the consumption of bottled water and will continue until 2 hours after the drink. The glucose concentrations in the two sample types will be quantified and compared to determine if the glucose levels remain relatively unchanged.

All blood samples are fingerprick blood samples (<20uL/sample).

The breathing style during the collection of the exhaled breath sample will be measured. In particular the air flow rate in and out, the frequency of the inhalations and exhalations, and the duration of the inhalation and exhalation periods as well as the exhaled carbon dioxide levels will be recorded using a computer.

The glucose concentrations in the exhaled air and blood samples and measured breathing characteristics will be analyzed statistically and to establish a mathematical relationship. The results of this research will assess the feasibility of a non-invasive glucose monitor that uses exhaled breath samples. If a mathematical relationship cannot be found that accurately predicts the blood glucose levels from the glucose measured in the exhaled breath samples and the breathing style, then detection of the blood glucose in the exhaled air may not be feasible for clinical use.

Specific Procedures to be Used

You will be provided with the information to participate in this study in both a written and oral format. Upon completion of the inclusion/exclusion form and acceptance into the study, you will be given a subject code and a sample of exhaled air and a finger prick blood sample will be gathered approximately every 10 minutes for 30 minutes. A sealed bottle of water will be provided and samples of exhaled air and a finger prick blood sample will be gathered approximately every 10-15 minutes for the next two hours.

The collection of the exhaled air sample will be conducted by having you inhale and exhale through the collection device through a sterilized plastic tube. You will wear a nose clip to ensure all air goes in and out of your mouth. You will breathe in and out normally. It is anticipated that you will need to repeat breath like this for about 10 minutes until the desired volume of exhaled air (5L) is collected.

Participant's Initials

Date

Research Project Number _____

Immediately before and after the exhaled air collection, a finger prick blood sample will be taken from you by trained professional.

Duration of Participation

Your participation in this research involves an informational session about the research, a demonstration by the trained personnel, and then a 2.5 hour data collection period which would include taking multiple exhaled air and blood samples along with a drink of water. The total amount time is approximated at 3 hours. Please note that you can only participate in this study at most three times on three separate dates (provided you save your anonymous subject code). If you have misplaced or lost your subject code you will not be allowed to repeat the study.

Risks to the Individual

The risk is minimal and is no more than any other risk that you would encounter during everyday life. You probably will experience slight discomfort during the finger-prick blood drawing procedure and this procedure may possibly result in a tenderness and slight bruising of the fingers at the areas where the pricking was performed. Furthermore, there is a possibility of an infection at the site of blood collection. To minimize this risk of infection, we will provide you with a commercially available bottle of hand sanitizer to use as you see fit. There is a risk of breach of confidentiality. However, every safeguard will be used to minimize the risk as outlined in the confidentiality section.

Benefits to the Individual

You may not get direct benefits resulting from this research in the very near future, but the results obtained from this research may be used in support of the development of a non-invasive blood glucose monitor.

Compensation

You will be compensated for your time and participation a free bottle of water.

Confidentiality

Your identity will always be kept in strict confidentiality and the data will be used specifically for research purposes. Your signed consent form will be scanned and electronically stored in a password protected directory accessible only by the trained personnel involved in this study. When you complete the consent form, you will be given a unique random numerical prefix (your specific ID number) that will tag and link the collected breath, exhaled air and blood glucose results. This will ensure the data will be collected and handled in an anonymous format. The blood and exhaled air specimens will be destroyed as soon as the glucose concentrations have been measured. The computer recorded data on your breathing style, the results from the exhaled air and blood specimens and the feedback on the questionnaire will be linked using the unique random numerical prefix as an index. This data will be stored for five years electronically in the password protected directory. The results obtained with the donated blood and exhaled air will be published in scientific journals without any information that can be traceable to you.

Data may be used for future research purposes as appropriate.

In a group research environment even though we expect everyone to keep participants identity confidential, we cannot guarantee confidentiality because we cannot control what others might say outside of the research environment.

Participant's Initials

Date

Research Project Number _____

The project's research records may be reviewed by the National Institute of Health, Showalter Foundation, American Diabetes Association, Food and Drug Administration, Office for Human Research Protection, and by departments at Purdue University responsible for regulatory and research oversight.

Voluntary Nature of Participation

You do not have to participate in this research project, and you can withdraw your participation at any time without penalty.

Human Subject Statement

If you have any questions about this research project, you can contact you can contact Professor Ann Rundell whose office is Room 3029 in the Martin Jischke Hall of Biomedical Engineering (MJIS) Building with the telephone number of 496-7953. If you have concerns about the treatment of research participants, you can contact the Institutional Review Board at Purdue University, Ernest C. Young Hall, Room 1032, 155 S. Grant St., West Lafayette, IN 47907-2114. The phone number for the Board is (765) 494-5942. The email address is irb@purdue.edu.

Documentation of Informed Consent

I have had the opportunity to read this consent form and have the research study explained. I have had the opportunity to ask questions about the research project and my questions have been answered. I am prepared to participate in the research project described above. I will receive a copy of this consent form after I sign it.

Participant's Signature

Date

Participant's Name

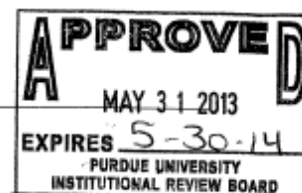
Researcher's Signature

Date

Participant's Initials

Date

Research Project Number _____



RESEARCH PARTICIPANT CONSENT FORM

Measurement of glucose in Exhaled Breath Condensates (EBC)

Professor Ann Rundell

Purdue University

Weldon School of Biomedical Engineering

Study A

Purpose of Research

The purpose of this research is to evaluate the feasibility of a new, noninvasive glucose measuring device that uses exhaled air to replace the traditional use of blood samples. This research sets out to determine if significant changes observed in the blood glucose level are observed in the exhaled air sample in a predictable manner.

The glucose concentration in the exhaled air sample will be measured as will the glucose concentration in the blood before and after a meal. Samples of the exhaled air and blood will be collected every ~15 minutes, starting 30 minutes before the consumption of a meal and will continue until 2 hours after the meal. The glucose concentrations in the two sample types will be quantified and compared to determine if the same trends are present in both sample types in response to a change in glucose levels.

All blood samples are fingerprick blood samples (<20uL/sample).

The breathing style during the collection of the exhaled breath sample will be measured. In particular the air flow rate in and out, the frequency of the inhalations and exhalations, and the duration of the inhalation and exhalation periods as well as the exhaled carbon dioxide levels will be recorded using a computer.

The glucose concentrations in the exhaled air and blood samples and measured breathing characteristics will be analyzed statistically and to establish a mathematical relationship. The results of this research will assess the feasibility of a non-invasive glucose monitor that uses exhaled breath samples. If a mathematical relationship cannot be found that accurately predicts the blood glucose levels from the glucose measured in the exhaled breath samples and the breathing style, then detection of the blood glucose in the exhaled air may not be feasible for clinical use.

Specific Procedures to be Used

You will be provided with the information to participate in this study in both a written and oral format. Upon completion of the inclusion/exclusion form and acceptance into the study, you will be given a subject code and a sample of exhaled air and a finger prick blood sample will be gathered approximately every 10 minutes for 30 minutes. Lunch will be provided and samples of exhaled air and a finger prick blood sample will be gathered approximately every 10-15 minutes for the next two hours.

The collection of the exhaled air sample will be conducted by having you inhale and exhale through the collection device through a sterilized plastic tube. You will wear a nose clip to ensure all air goes in and out of your mouth. You will breathe in and out normally. It is anticipated that you will need to repeat breath like this for about 10 minutes until the desired volume of exhaled air (5L) is collected.

Participant's Initials_____
Date

Research Project Number _____

Immediately before and after the exhaled air collection, a finger prick blood sample will be taken from you by trained professional.

Duration of Participation

Your participation in this research involves an informational session about the research, a demonstration by the trained personnel, and then a 2.5 hour data collection period which would include taking multiple exhaled air and blood samples along with a lunch. The total amount time is approximated at 3 hours. Please note that you can only participate in this study at most three times on three separate dates (provided you save your anonymous subject code). If you have misplaced or lost your subject code you will not be allowed to repeat the study.

Risks to the Individual

The risk is minimal and is no more than any other risk that you would encounter during everyday life. You probably will experience slight discomfort during the finger-prick blood drawing procedure and this procedure may possibly result in a tenderness and slight bruising of the fingers at the areas where the pricking was performed. Furthermore, there is a possibility of an infection at the site of blood collection. To minimize this risk of infection, we will provide you with a commercially available bottle of hand sanitizer to use as you see fit. There is a risk of breach of confidentiality. However, every safeguard will be used to minimize the risk as outlined in the confidentiality section.

Benefits to the Individual

You may not get direct benefits resulting from this research in the very near future, but the results obtained from this research may be used in support of the development of a non-invasive blood glucose monitor.

Compensation

You will be compensated for your time and participation a free lunch and free drink.

Confidentiality

Your identity will always be kept in strict confidentiality and the data will be used specifically for research purposes. Your signed consent form will be scanned and electronically stored in a password protected directory accessible only by the trained personnel involved in this study. When you complete the consent form, you will be given a unique random numerical prefix (your specific ID number) that will tag and link the collected breath, exhaled air and blood glucose results. This will ensure the data will be collected and handled in an anonymous format. The blood and exhaled air specimens will be destroyed as soon as the glucose concentrations have been measured. The computer recorded data on your breathing style, the results from the exhaled air and blood specimens and the feedback on the questionnaire will be linked using the unique random numerical prefix as an index. This data will be stored for five years electronically in the password protected directory. The results obtained with the donated blood and exhaled air will be published in scientific journals without any information that can be traceable to you.

Data may be used for future research purposes as appropriate.

Participant's Initials

Date

Research Project Number _____

In a group research environment even though we expect everyone to keep participants identity confidential, we cannot guarantee confidentiality because we cannot control what others might say outside of the research environment.

The project's research records may be reviewed by the National Institute of Health, Showalter Foundation, American Diabetes Association, Food and Drug Administration, Office for Human Research Protection, and by departments at Purdue University responsible for regulatory and research oversight.

Voluntary Nature of Participation

You do not have to participate in this research project, and you can withdraw your participation at any time without penalty.

Human Subject Statement

If you have any questions about this research project, you can contact you can contact Professor Ann Rundell whose office is Room 3029 in the Martin Jischke Hall of Biomedical Engineering (MJIS) Building with the telephone number of 496-7953. If you have concerns about the treatment of research participants, you can contact the Institutional Review Board at Purdue University, Ernest C. Young Hall, Room 1032, 155 S. Grant St., West Lafayette, IN 47907-2114. The phone number for the Board is (765) 494-5942. The email address is irb@purdue.edu.

Documentation of Informed Consent

I have had the opportunity to read this consent form and have the research study explained. I have had the opportunity to ask questions about the research project and my questions have been answered. I am prepared to participate in the research project described above. I will receive a copy of this consent form after I sign it.

Participant's Signature

Date

Participant's Name

Researcher's Signature

Date

Participant's Initials

Date

Appendix C

Whole Human Study Data

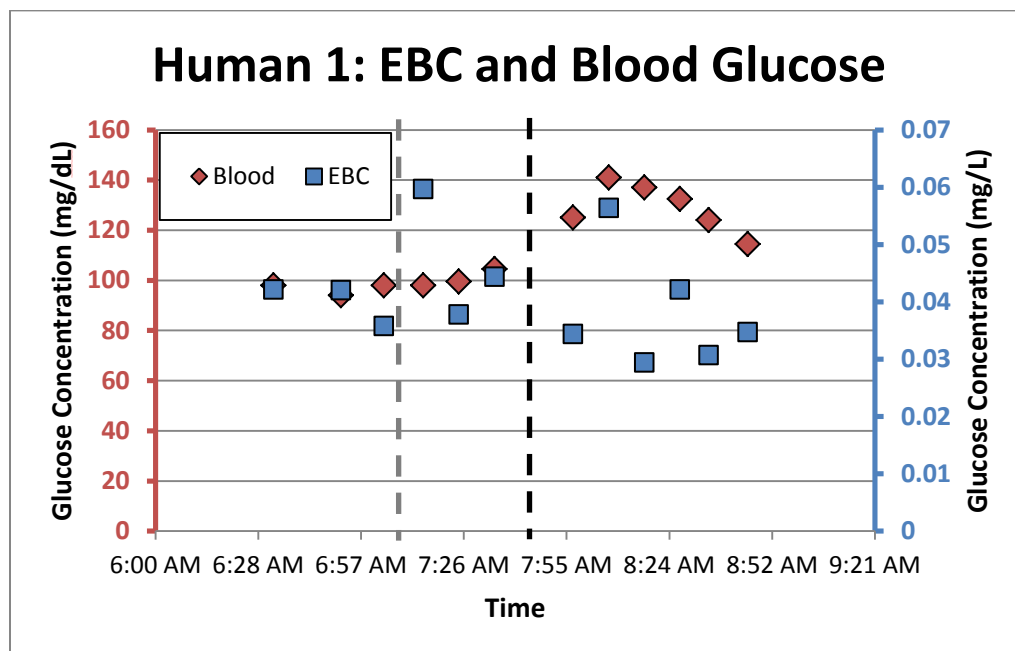


Figure C.1: Blood and breath data from Human 1

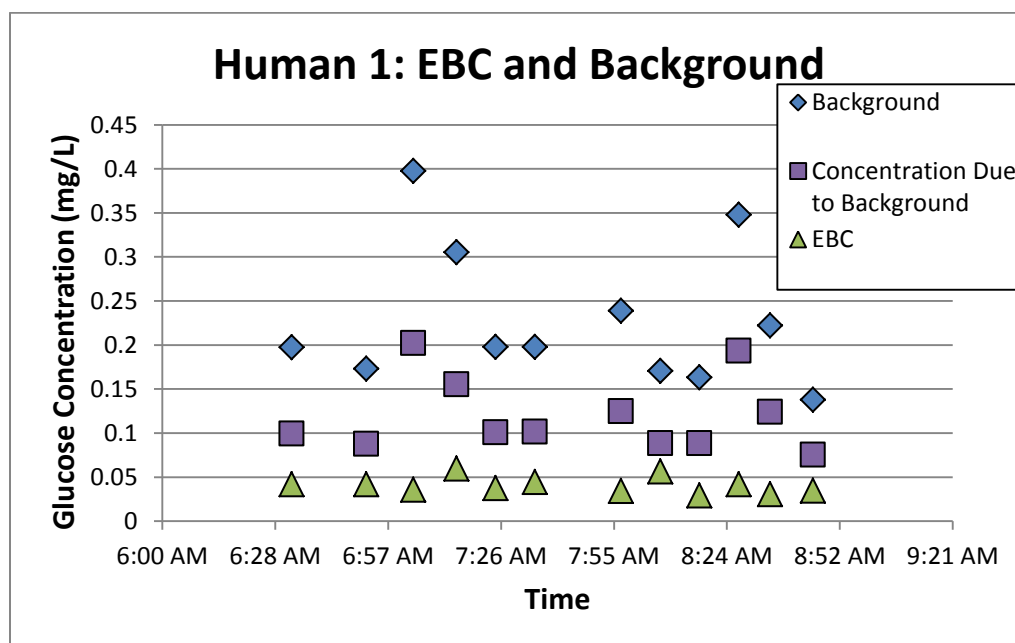


Figure C.2: Breath and background data from Human 1

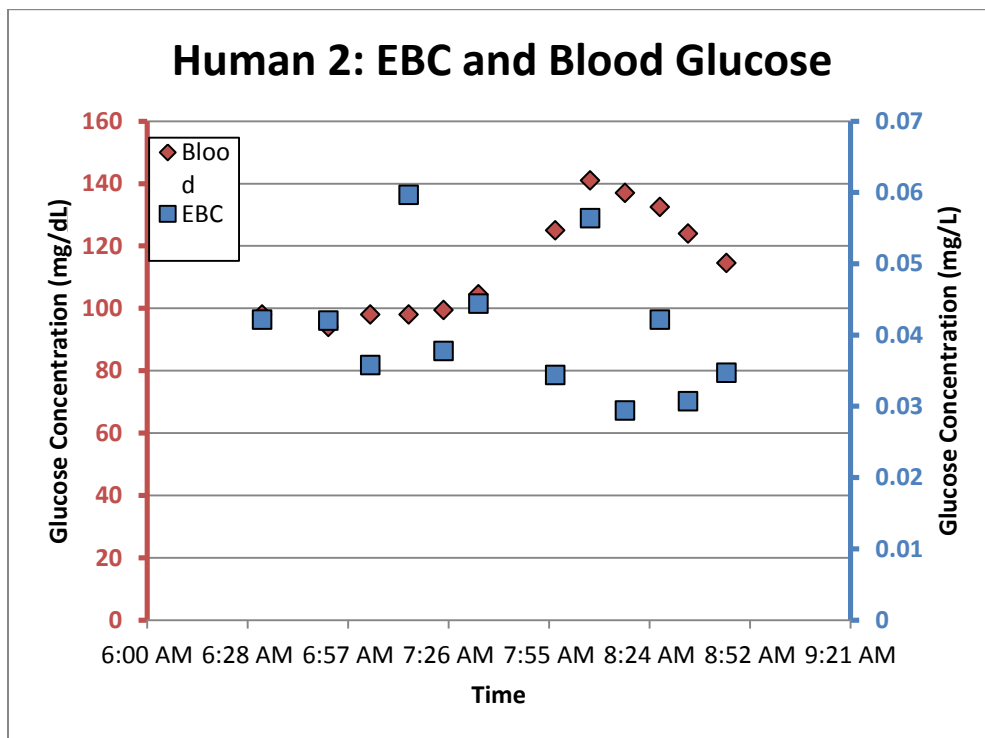


Figure C.3: Blood and breath data from Human 2

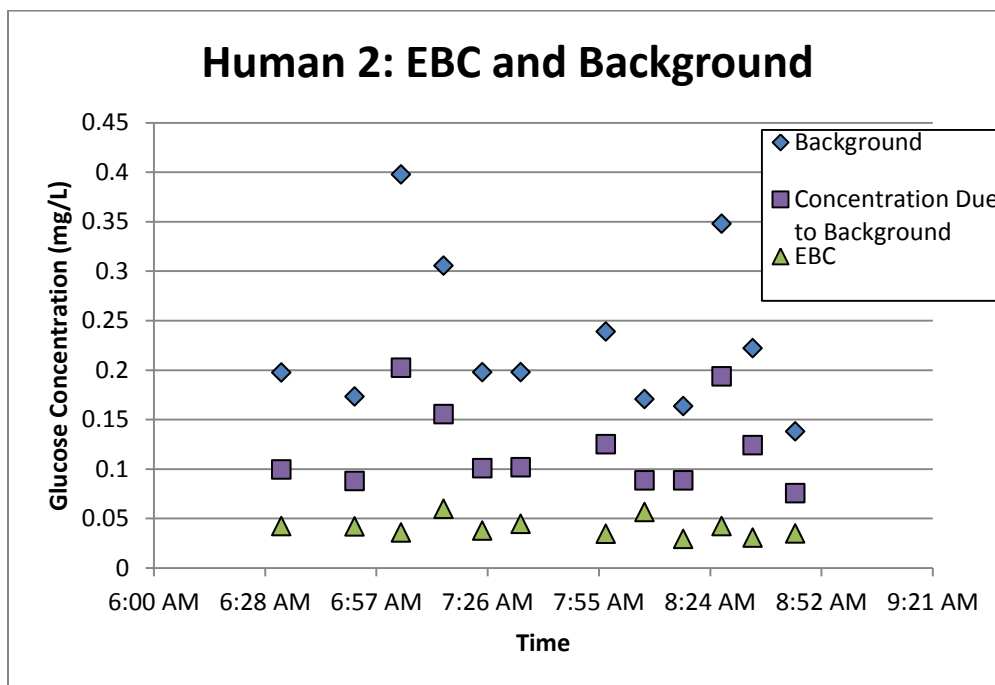


Figure C.4: Breath and background data from Human 2

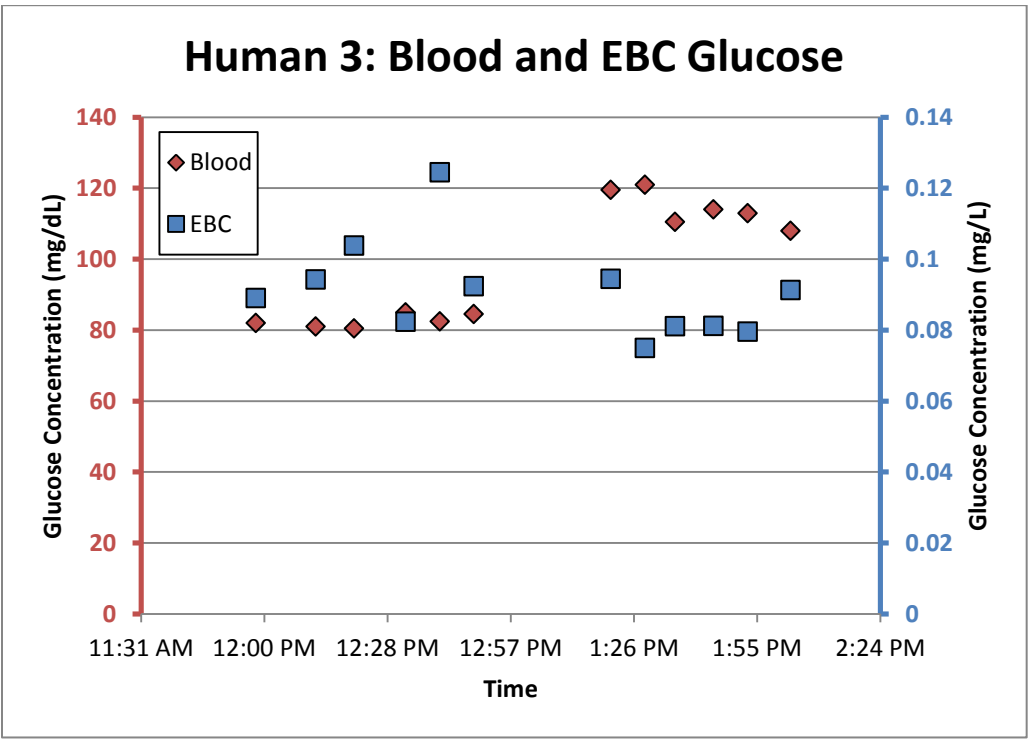


Figure C.5: Blood and breath data from Human 3

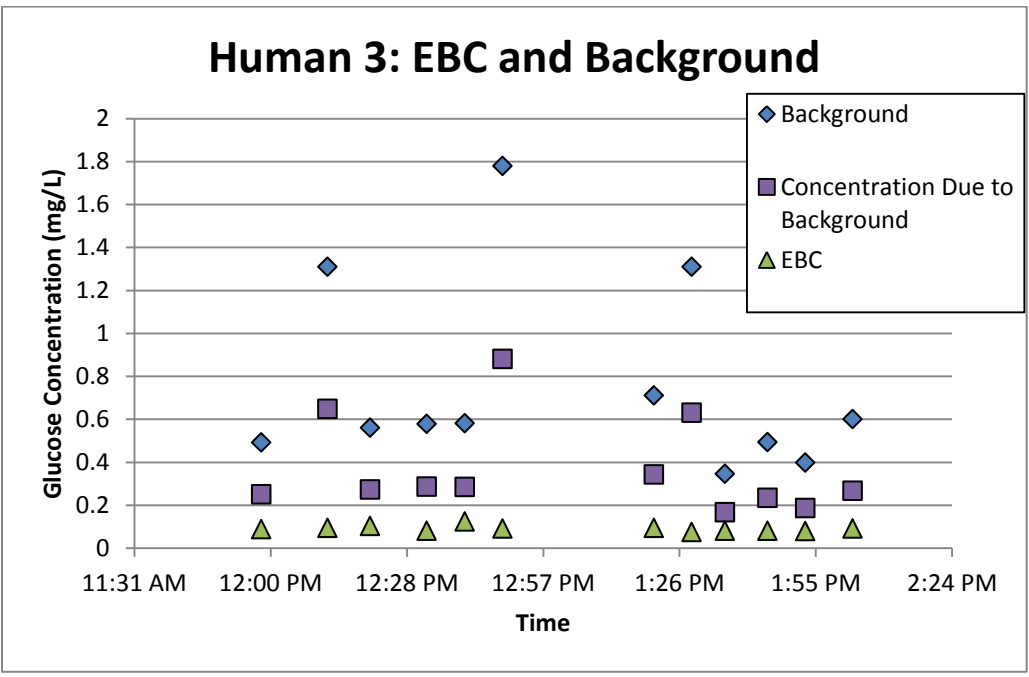


Figure C.6: Breath and background data from Human 3