NOVEL METHODS FOR BIOMARKER ASSESSMENT IN RED BLOOD CELL STORAGE FOR TRANSFUSION MEDICINE

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ABSTRACT

Blood banking, a meticulously regulated process endorsed by both the Food and Drug Administration (FDA) and the World Health Organization (WHO), plays a pivotal role in collecting and preserving red blood cells (RBCs) for transfusion medicine. Each day, the United States alone administers around 29,000 units of RBCs, addressing the diverse medical needs occurred from surgeries, diseases, traumas, and cancer treatments. However, conventional blood storage solutions employed, while serving as anticoagulants and preservatives, contain glucose levels that substantially exceed the typical "healthy" blood glucose range (4-6 mM). These solutions induce hyperglycemia, which is linked to significant negative alterations in RBCs. Vulnerable populations, such as people with diabetes, face severe consequences with chronic hyperglycemia. Transfusion medicine is undeniably lifesaving, but it is not without its drawbacks. It is theorized that adverse storage-related changes, referred to as storage lesions, are responsible for a multitude of post-transfusion complications. These storage lesion markers encompass metabolic and physical transformations that RBCs undergo during storage, with notable examples including advanced glycation end products (AGEs) and oxidative stress.

High glucose concentration storage solutions adversely affect stored RBCs used for transfusion medicine. The Spence group proposed a potential alternative to the hyperglycemia issue: develop a normoglycemic blood storage solution. This novel RBC additive storage solution has already demonstrated improvements in key RBC function, such as adenosine triphosphate (ATP) levels and deformability. This dissertation reveals additional advantages through the evaluation of other storage lesion markers. Key components of this work include novel methods to detect and quantify AGEs and explore connections with oxidative stress, involving measuring free reduced glutathione (GSH).

The core focus of this dissertation lies in investigating the ramifications of using a normoglycemic storage solution to reduce AGEs, particularly Nε-carboxymethyl-lysine (CML) and Nε-carboxyethyl-lysine (CEL), mitigate oxidative stress (as evidenced by GSH levels), and promote ongoing research involving normoglycemic storage conditions. This research is of paramount importance because it holds the potential to enhance the quality of RBCs for transfusion. Thus, directly impacting patient outcomes and quality of life, as well as offering insights into aging via *in vivo* patient samples. The methodologies employed in this study encompass the development of a pioneering approach utilizing ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) for the detection and quantification of CML, CEL, and lysine on the RBC membrane. A comparative analysis was performed between hyperglycemic (AS-1) and normoglycemic (AS-1N) storage solutions over 43 days. These findings played a critical role in determining AGE and GSH levels through weekly sample testing and involving innovative "feeding" techniques to ensure sterile normoglycemic glucose concentrations. In addition to comparing the two storage solutions, samples from type I diabetic (T1D) patients were utilized to explore the correlation between elevated blood glucose, glycated hemoglobin A1c (HbA1c%), and storage lesion markers. The methodologies and potential biomarkers presented in this study hold the promise of enhancing patient screenings and refining future C-peptide drug therapy clinical trials.

In summation, this dissertation strives to pioneer novel methodologies and quantify storage lesion markers to advance transfusion medicine interventions. Ultimately, this research has the potential to improve the lives of countless individuals who depend on life-saving transfusions and future clinical trial drug discoveries.

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“Think progress – success is in the numbers. The quality is in the quantity and achievement often comes to the person who makes the most attempts.” – Gary Keller

“Serenity now!” – Frank Costanza

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LIST OF ABBREVIATIONS

2,3-DPG: 2,3-diphosphoglycerate

AD: Alzheimer's disease

AGE: Advanced glycation end product

AIDS: Acquired immunodeficiency syndrome

ADP: Adenosine diphosphate

AMP: Adenosine monophosphate

ATP: Adenosine triphosphate

AS: Additive solution

AS-1: Additive solution 1

AS-1N: Additive solution 1 (normoglycemic)

BSA: Bovine serum albumin

cGMP: Cyclic guanosine monophosphate

CEL: Nε-carboxyethyl-lysine

CGM: Continuous glucose monitoring

CML: Nε-carboxymethyl-lysine

CPD: Citrate-phosphate-dextrose solution

CPD-N: Citrate-phosphate-dextrose (normoglycemic)

CVD: Cardiovascular disease

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbent assay

EMA: European Medicines Agency

eNOS: Endothelial nitric oxide synthase

FDA: Food and Drug Administration

GC: Guanylyl cyclase

GCS: γ-glutamylcysteine synthase

gHSA: Glycated human serum albumin

GO: Glyoxal

GPD: Glucose degradation products

GPx: Glutathione peroxidase

GS: Glutathione synthase

HB: Hemoglobin

HbSN: Hemoglobin count from the RBC sample supernatant

HbRBCs: Hemoglobin count from the packed RBCs

HIV: Human immunodeficiency virus

HSA: Human serum albumin

GSSG: Oxidized glutathione

GSH: Reduced glutathione

ICH: International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use

ICU: Intensive care unit

IS: Internal standards

LC-MS/MS: Liquid chromatography with tandem mass spectrometry

LOD: Limit of detection

LOQ: Limit of quantification

MGO: Methylglyoxal

MS: Multiple sclerosis

NaCl: Sodium chloride

NAD: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen

NO: Nitric oxide

PBS: Phosphate buffered salt solution

PC1: Type I proprotein convertase 1

PC2: Type II proprotein convertase 2

PC3: Type I proprotein convertase 3

pRBC: Packed red blood cells

PSS: Buffered physiological salt solution

PVC: Polyvinyl chloride

RAGE: Receptor for advanced glycation end products

RBC: Red blood cell

RBS-EC: RBC-endothelial cell

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RU: Response units

SADH: Streptavidin in dextran hydrogel

SD: Standard deviation

SEM: Standard error of the mean

SPR: Surface plasmon resonance

T1D: Type I diabetes

T2D: Type 2 diabetes

TRALI: Transfusion-related acute lung injury

TACO: Transfusion-associated circulatory overload

UPLC-MS/MS: Ultra-performance liquid chromatography with tandem mass spectrometry

WB: Whole blood

WBC: White blood cells

Zinc: Zn^{2+}

Chapter 1: Blood Banking, Glycolysis, and Advanced Glycation End Products

1.1 History of Blood, Blood Banking, and Transfusion

Approximately 13.6 million units of whole blood (WB) were transfused in the United States in 2019.[1] Recently, 5 million people in the U.S. require blood transfusions each year, equating to 29,000 units of red blood cells (RBCs) each day.[2] Blood transfusions are often only thought of as the treatment for traumatic blood loss and hemorrhage; however, blood banking and storage of RBCs are also used during various cardiovascular procedures, surgeries, extreme cases of anemia, cancer, and for patients with sickle cell disease to improve hemoglobin (Hb) levels.[3–5] Due to the traumatic nature in which a person requires a blood transfusion, it is important to ensure the viability, safety, and efficacy of the blood used in the transfer. Therefore, the Food and Drug Administration (FDA) regulates this transfusion process. Currently, a Hb count less than 7 g/dL is the threshold for a blood transfusion, since healthy Hb levels are approximately 12 g/dL for women and 14 g/dL for men.[6,7]

A blood transfusion is used to transfer blood donated from one person (donor) to another (patient) and is typically required due to extreme circumstances or known medical conditions.[8,9] The first known mention of a blood transfusion occurred in 1615 by chemist Andreas Libavius, but the first major advancements in "cross-circulation experiments" did not occur until the late eighteenth century.[10] In the late nineteenth century, knowledge of certain constituents, such as sodium bicarbonate, sodium phosphate, and oxalic acid, became available and could be used to prevent blood coagulation.[10] The interest in anticoagulants for blood draw prompted the discovery of sodium citrate in 1915, which became the first major transfusion development.[8,11] Other major blood related advancements included the first blood storage solution invented by Rous and Tuner in 1915, and the first blood bank during World War I setup by Robertson.[12,13] Prior to World War

I, transfusions were accomplished by having a donor "hooked-up" to a patient via a venipuncture tube connection system utilizing gravity. The advancements in transfusion medicine were prompted by the war, when the issue of viable direct transfusions forced scientists to develop new methodology.[14] The concept of storing blood and transporting it across battlefields or larger areas of Europe paved the way for anticoagulants and storage containers. In military settings, the donor is considered an emergency "walking blood bank" because blood transfusions are often needed in larger quantities due to traumatic blood loss and hemorrhages.[4,5] Glass vacuum bottles were used to transport WB until 1952 when polyvinyl chloride (PVC) bags were invented, which provided a more sanitary and safer mode of transportation.[15–17] The first recorded use of sodium citrate and glucose in blood storage was in 1914 when they were used as anticoagulation components added to blood for transfusions.[10]

By the start of the twentieth century, there was greater understanding of specific blood types and the adverse interactions that could cause acute hemolytic transfusion reaction, which is a reaction from the human body fighting incompatible RBCs, a reaction which can be lethal if left untreated.[18] The discovery of blood types and groups by Landsteiner, the 1930 Nobel Prize winner in Physiology or Medicine, trailblazed blood transfusion safety practices.[18] The four major blood types (A, B, AB, and O) known today were accepted internationally by 1927.[10]

It would take another 60 years before the first RBC additive solution, saline-adenine-glucose (SAG) was developed and used to create saline-adenine-glucose-mannitol (SAG-M) in 1981, which was the precursor to the FDA licensed solutions used today.[19] The FDA approved additive solutions (AS) today are AS-1 (Adsol, Baxter Healthcare Corp., Deerfield, IL), AS-3 (Nutricel, System Pall Corp., Port Washington, NY), and AS-5 (Optisol, Terumo Corp., Somerset, NJ).[20–22] AS-7 (SOLX, Haemonetics Corporation, Boston, MA) was not incorporated until 2013, and was

developed as a more effective solution in preventing the storage lesion; however, there is limited research utilizing AS-7, as opposed to the three previously mentioned solutions.[23] The current blood collection protocol and subsequent storage are governed by World Health Organization (WHO) and FDA protocol (**Figure 1.1**).[24–26]

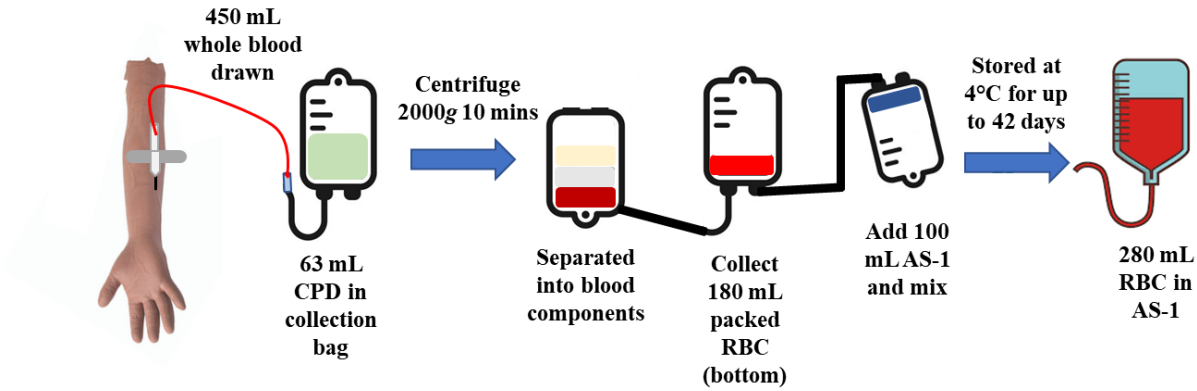

Figure 1.1. FDA approved blood storage protocol. Whole blood is drawn from a healthy, consenting donor via venipuncture and collected into a 500 mL sterile PVC collection bag that contains an anticoagulant solution. The blood bag is centrifuged for 10 minutes, and the blood components separate into three layers (plasma, buffy coat, and packed RBCs). The packed RBCs are drained into a secondary collection bag which contains an additive solution. The blood is then stored at 4°C for up to 6 weeks. [32]

1.2 Current Blood Storage Anticoagulants and Additive Solution Constituents

According to the 2019 National Blood Collection and Utilization Survey, there were almost 12 million units of RBCs collected that year, which was a significant decrease in comparison to 2017 at 13.6 million RBC units.[27–29] However, there was a 2.5% increase in RBC units transfused in 2019.[28] This survey suggests there is a significant decline in the blood supply, but the demand is increasing. During the start of COVID-19, there was a 10% decrease in blood donations, which was deemed the worst in 10 years by the American Red Cross.[30,31] Thus, it is more important than years prior to ensure the safety and longevity of collected and subsequently stored blood.

There is no specific timeframe before 42 days that stored blood must be transfused into a patient, but there are numerous reports that show there are increased adverse reactions after 35 days or more in storage, which will be discussed further.[30,31]As previously mentioned, the blood storage protocol is upheld by the FDA in the United States, and there are currently four anticoagulants (CPD, CPDA-1, CPD-2, ACD-A), and four additive solutions (AS-1, AS-3, AS-5, AS-7) approved (**Table 1.1** and **Table 1.2**) Although SAG-M was the first additive solution, and most widely used today in other countries, the FDA has not approved it for official use in transfusion medicine.[21] The solutions highlighted in this dissertation research are CPD and AS-1.[32–36]

Table 1.1. Anticoagulant Constituents

Constituent	CPD (mM)	CPDA-1 (mM)	CP2D (mM)	ACD-A (mM)
Sodium Citrate	89.4	89.4	89.4	85
Citric Acid	15.6	17	15.6	40
Glucose	129	177	258	136
Monobasic Sodium Phosphate (Monohydrate)	16.1	5.8	16.1	-
Adenine	-	2	-	-

Table 1.2. Additive Solution Constituents

Constituent	SAG-M (mM)	AS-1 (mM)	AS-3 (mM)	AS-5 (mM)	AS-7 (mM)
Dextrose (Glucose)	45.4	111	55.5	45.4	80.0
Adenine	1.26	2.0	2.22	2.22	2.0
Monobasic Sodium Phosphate (Monohydrate)	-	-	23.0	-	-
Dibasic Sodium Phosphate (Heptahydrate)	-	-	-	-	12.0
Mannitol	29.0	41.2	-	45.4	55.0
Sodium Chloride	150	154	70	150	-
Citric Acid (Monohydrate)	-	-	2.0	-	-
Sodium Citrate (Dihydrate)	-	-	23.0	-	-
Sodium Bicarbonate	-	-	-	-	26.0

Each constituent of the anticoagulant and additive solution serves a specific purpose to ensure the longevity and safety of blood storage.[32,36,37] An anticoagulant prevents the formation of blood clots with naturally occurring fibrinogen in plasma contained in the WB. This anticoagulant in blood collection tubes is to keep blood in a fluid-type state for *in vitro* diagnostics or clinical chemistry testing.[38,39] The FDA has approved sodium citrate based anticoagulants.[33] Sodium citrate inhibits calcium ions, which are naturally occurring in blood, from participating in the blood clotting process. Blood collection tubes contain either 3.2% or 3.8% citrate and it chelates ionized calcium, which creates a citrate-calcium complex.[40,41] Monobasic sodium phosphate is added to the CPD solution to reduce the phosphate concentration gradient difference between cytosol and WB supernatant.[19] Also, monobasic sodium phosphate inhibits phosphate leakage from stored RBCs.[19] Citric acid, a well-known constituent in CPD, is primary used for maintaining the pH balance at 5.6 due to the ability to be heat-stable at this lower pH.[42,43]

Another type of anticoagulant used in blood draw is potassium ethylenediaminetetraacetic acid (EDTA).[44] Similar to sodium citrate-based anticoagulants, there is a minimum volume and

concentration of the anticoagulant required to properly prevent clots without changing the structure of the RBCs. EDTA is a type of chelating agent that binds to metal ions in whole blood (calcium, magnesium, lead, and iron).[39,45] It is used to prevent blood clotting by binding to the calcium ions and which prevents the activation of coagulation, which inhibits the ability of the blood from clotting.[46,47] Heparin is the most used clinical anticoagulant, although it has been associated with contamination issues.[48] This type of anticoagulant is a sulfated polysaccharide and inhibits thrombin to prevent coagulation in blood draw tubes.[48,49] However, it is not used in blood storage due to the adverse effects to RBC morphology and osmotic fragility over time.[44]

An additive solution preserves sterilely stored packed RBCs (pRBCs) by adding a sterile isotonic solution that contains some of the necessary constituents to extend storage life of blood for transfusion.[37,50,51] The current additive solutions are adjusted to pH 5.6-5.8, which is well-below the physiological pH of 7.4.[21] It is frequently reported that a glucose-containing solution pH of 5.8 or above can be autoclaved and does not modify the sugars, although this theory will be evaluated in chapter 3.[52–55] Once the pRBCs at physiological pH are added to the additive solution, the pH will significantly increase to approximately 6.5 during 42 days of storage, one of the reasons being lactic acid formation.[56] Remaining in this acidic environment impacts the overall health and function of the RBCs, specifically intracellular biochemical pathways: adenosine 5'-triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG).[15] Maintaining significant levels of ATP and 2,3-DPG are crucial for the survival of RBCs, and a decrease in both molecules adversely affects stored blood, which will be critically evaluated in this work.[21] One of the major concerns of storing pRBCs is the loss of important cell functions. Adenine is used to extend the storage life of pRBCs by promoting ATP synthesis.[15,57]

To maintain an isotonic solution, sodium chloride (NaCl) must be included in the additive solutions. An isotonic solution is critical to maintain a low level (<1%) of hemolysis by minimizing RBC membrane damage.[15,23] Typically, the amount of intravenous NaCl is 0.9%, forming an isotonic solution to maintain proper fluid and electrolyte balance.[58] Thus, this dissertation research will utilize 0.9% NaCl as a basis for all blood banking protocols.

Hemolysis, the rupture or death of RBCs and subsequent release of Hb, usually occurs when pRBCs are added to artificial solutions, such as buffers and additive solutions, or exposed to high-stress environments (freezing and homogenizing).[59] For FDA protocols, hemolysis must be below 1% of total RBCs quantity for a sample to be considered acceptable for human subjects.[26] This rigid criteria is to limit complications of free Hb, which can include injury to the endothelium, tissues, and kidney proximal tubule due to free Hb redox effects.[59,60]

Mannitol prevents hemolysis by preventing osmotic swelling that leads to rupture.[61] Osmotic lysis has been reported to be further decreased (50%) by the addition of mannitol to additive solutions containing NaCl.[50] Together, NaCl and mannitol prevent pRBCs from immediate hemolysis, maintain pH, and provide the ability to easily adjust when transfused into humans with an isotonic solution.

Dextrose, or D-glucose, is considered one of the most important and controversial constituents of blood storage solutions. Glucose is a known energy supplier for RBCs, and approximately 90% of glucose is converted to pyruvate and lactate by being anaerobically catabolized *in vivo*.[62] CPD contains 129 mM glucose and AS-1 contains 111 mM glucose, and both were initially shown to extend the life of stored RBCs and increase levels of 2,3-DPG, even as a hyperglycemic solution.[15,63,64] After the pRBCs are added to additive solution AS-1, the final glucose concentration is 40 mM, which is 8-10x more concentrated than normal healthy glucose

levels (4-6 mM).[21] However, it has been reported that stored blood requires higher glucose levels to survive the 42 day storage period.[15,19] Recent studies have shown the adverse effects of storing blood in a hyperglycemic storage solution for 6 weeks and the importance of further investigation into blood transfusion health, which will be discussed in more detail throughout this dissertation.[65]

There are several studies highlighting the decline in RBC health throughout storage, and the FDA has approved a "rejuvenating" solution, *Rejuvesol*. This solution can be prescribed by a physician and added to a blood bag 24 hours before transfusion.[20] The purpose of *Rejuvesol* is to reduce the metabolic and physicochemical storage lesions that occur during typical blood storage and to eliminate possible transfusion related reactions.[20] Storage lesion is a term coined to describe the physical and chemical adverse effects RBCs experience during blood storage.[15,66] The storage lesion affects metabolism, physiology, and function of RBC energy production, which will be discussed further in this chapter.[66,67] Thus, this "rejuvenation" solution can be added to stored blood, incubated up to 24 hours, washed to remove cell debris, and then transfused to a patient.[68] *Rejuvesol* is not regularly added to stored blood in current blood banking practices. The existence of this solution does provide insight into the damaging effects of the current FDA blood storage protocols and studies that could improve transfusion health.

1.3 Normoglycemic Storage Solution Innovation

As previously mentioned, the FDA-approved storage solution is hyperglycemic, and is still above the average blood glucose level of a person with diabetes of ~7-9 mM.[70] The Spence group has compared ATP release from RBCs obtained from people with diabetes and healthy control donors and discovered that healthy control RBCs released twice the amount of ATP.[71,72] Also, RBCs obtained from people with type 2 diabetes (T2D) have shown similarities to the storage lesion formation in stored RBCs, including changes in reduced glutathione (GSH) production and

sorbitol accumulation.[71,73] The research does not suggest that transfusing a person with the hyperglycemic storage solution would result in the patient developing diabetes, but it does suggest that hyperglycemic environments, similar to the bloodstream of a person with diabetes, contribute to lesion formation. Thus, the Spence group has proposed a new anti-coagulant and additive solution that contains 5.5 mM glucose to mimic *in vivo* blood glucose levels (**Table 1.3**).[74]

Table 1.3. Proposed Normoglycemic Storage Solution Constituents Compared to FDA Approved Solutions

Constituent	**CPD (mM)**	**CPD-N (mM)**	**AS-1 (mM)**	**AS-1N (mM)**
Dextrose (Glucose)	129	5.5	111	5.5
Sodium Citrate (Dihydrate)	89.4	89.4	-	-
Monobasic Sodium Phosphate (Monohydrate)	16.1	16.1	-	-
Citric Acid (Monohydrate)	15.6	15.6	-	-
Sodium Chloride (Dihydrate)	-	-	154	154
Adenine	-	-	2.0	2.0
Mannitol	-	-	41	41
pH	5.6	5.6	5.8	5.8

The AS-1N solution (normoglycemic version of AS-1 solution) has been used in previous experiments, and the Spence group has reported lower glucose levels has reduced the storage lesion, specifically, lowering intracellular sorbitol changes, increased RBC deformability, increased RBC-derived ATP release, and increased endothelium-derived NO release.[71,74–76] These reports focused on understanding more about the 4–6-week storage time and the importance of

glucose control to reduce the total accumulation of storage lesions and other common transfusion related issues, which will be covered later on in chapter 1. Glucose control in AS-1N was achieved by various techniques to manually monitor and control glucose levels by adding a sterile glucose solution to maintain a 4-6 mM glucose concentration.[74] Studies to determine the effect of normoglycemic storage were completed *in vitro* utilizing both static systems and dynamic systems involving a 3D printed transfusion device to mimic RBC circulation.[74] The normoglycemic storage solution was reported to have a 25% increase in downstream nitric oxide (NO) production, and the hyperglycemic solution had 20% decrease in deformability with pRBCs and a 50% decrease in ATP release from the stored RBCs.[75,77]

Most studies involving the storage lesion, including advanced glycation end products (AGEs), focus primarily on fresh RBCs, day 1 of storage, or the last day of storage.[78,79] By examining the pRBCs in shorter time intervals during the entirety of the storage period, it provides greater insight into reversibility or recovery and the commonly cited two-week period of storage as a turning point for the viability of the pRBCs.[74,80,81] In 2013, the mean storage time for blood transfused to intensive care unit (ICU) patients was 16-21 days, which has been associated with adverse effects such as increased infections, organ and renal failure, and patient time spent in ICU.[82] It has also been observed at the end of 14 days in storage, there have been recorded increased levels of potassium and lactate, and decreased levels of sodium and glucose in RBC storage bags.[83] To further investigate the role of glucose and storage lesion marker accumulation, the normoglycemic and hyperglycemic storage solutions will be utilized and studied throughout the entirety of this dissertation.

1.4 Background on Human Blood

Blood storage cannot be fully understood without a thorough comprehension of blood and blood components *in vivo*. Blood, a major fluid in the body, is essential for human and animal life.[84] Hematopoietic stem cells, located in the bone marrow, produce blood cells through the process of hematopoiesis.[85] This process generates RBCs, white blood cells (WBCs), and platelets, which account for approximately 46% of WB volume.[84] As shown in **Figure 1.2**, 55% of WB is plasma (water, ions, proteins, nutrients, and hormones), <1% are platelets and WBCs (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), and the remaining 45% are RBCs.[18,84]

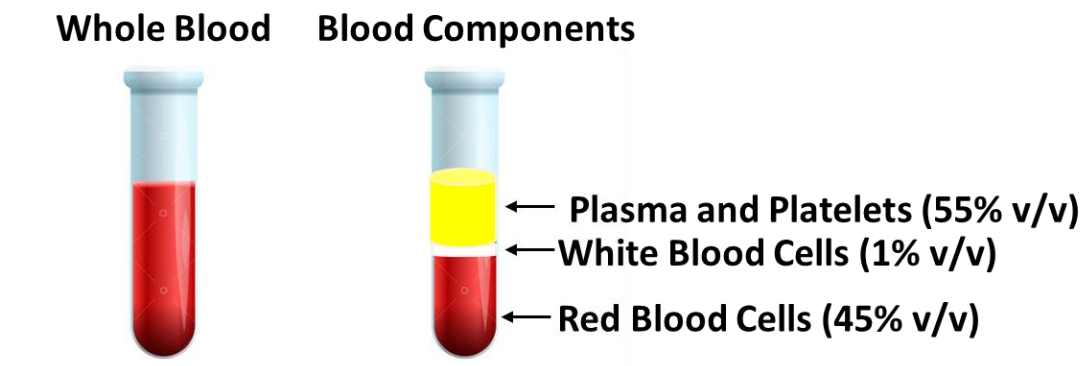

Figure 1.2. WB components after centrifugation. Blood consists of 3 main parts including plasma, white blood cells and platelets, and pRBCs. Most of the volume (55%) includes plasma and platelets and it is typically a yellow-ish color. White blood cells (<1%) are usually a white color and change depending on an influx of white blood cells produced due to illness. RBCs (45%) are a bright red color at the bottom of the tube and are usually viscous.[18,51,84]

There are five major functions of blood, including (1) the transportation of oxygen to lungs and tissues, (2) carrying other cells and antibodies, (3) preventing excess blood loss or thrombosis by clotting, (4) transporting waste products, and (5) regulating body temperature.[18,84,86] The average male has about 12 pints of WB and the average female has about 9 pints. Plasma contains

essential clotting factors (fibrinogen) for hemostasis and has a mixture of salts, water, fat, sugar, and proteins. Some hormones are transported by the plasma blood cells by binding to proteins in the plasma, along with essential antibodies.[18,87]

RBCs are a unique and abundant cell that carry oxygen to tissues following inhalation and carbon dioxide to the lungs for exhalation.[18] These types of cells do not contain a nucleus and account for about 45% of the volume of WB. RBCs transport 98% of total oxygen from the lungs to the tissues using Hb, a metalloprotein that carries oxygen.[18] Hb contains iron, which can bind up to four oxygen molecules, and oxygenation gives RBCs the well-known red coloring.[18,88] The iron heme group of Hb is attached to a globin (group of heme globular proteins) polypeptide chain, giving it the name hemoglobin. Therefore, each oxygen molecule binds to an iron atom of the heme group, so it can transport a maximum of four oxygen molecules.[18,88] The disc type shape of the RBC allows for increased oxygen saturation, deformability, and survival, allowing the cells to move through small blood vessels maintaining a flexible shape for the least amount of flow resistance.[89,90] Erythropoietin is a hormone in the kidneys that controls the production of RBCs, which have a lifespan of ~120 days in circulation.[84,91] The old or damaged RBCs are removed by macrophages located in the spleen and liver and are broken down into components that are later metabolized.[18]

WBCs, also called leukocytes, protect the body from illness and infections. The major types of WBCs are neutrophils and lymphocytes, which both fight infections and regulate other immune cells to attack infected cells, such as tumors.[92] WBCs account for <1% volume of WB and are produced in the bone marrow.[84,93]

Platelets, also called thrombocytes, account for <1% of cells suspended in WB. The platelets are fragments of cells that assist in blood clotting (or coagulation) when fibrin is present.[18]

Understanding blood coagulation is important, as too much fibrin can form clots, which can increase in size to form blood clots that cause strokes and heart attacks.[94,95] Alternatively, not enough fibrin produced can lead to "thin" blood, which does not clot easily and can increase blood loss.[94]

Serum, or a blood fluid with the clotting components and cells removed, is commonly used in research to study WB.[96] It contains less protein than plasma and has a higher amount of potassium.[87] Serum is separated after blood is drawn into a blood collection tube with no anticoagulants present.[87] There is a difference between serum and plasma, since serum is the fluid remaining post blood clotting, and plasma is the fluid remaining in a tube with anticoagulant and post centrifuging.[96]

1.5 Glycolysis and Blood Flow

RBCs do not have a nucleus, nor mitochondria[18,97,98] and there are three main pathways that are important to RBC metabolism: glycolysis, the hexose monophosphate shunt (Luebering-Rapoport shunt), and base salvage pathway for purine synthesis.[50] The anaerobic glycolysis pathway main function is to generate ATP, which is the source of energy for cells.[99] Glycolysis is the energy mechanism using glucose, and the starting point of the pathway converts glucose to glucose-6-phosphate by hexokinase phosphorylation, then utilizes phosphoglucomutase to regulate glucose-6-phosphate formation, and in later steps the Na^+, K^+ ATPase activation produces ATP.[100,101] The glycolysis pathway continues until producing protons and an end product, pyruvate, which by anaerobic glycolysis with lactate dehydrogenase is converted to lactic acid.[100] The first half of the mechanism process requires two ATP molecules per one glucose molecule.[99] The second half of the mechanism, the generation of pyruvate, results in two ATP molecules produced for each one glyceraldehyde phosphate molecule, making a total of four ATP

molecules.[99] Thus, resulting in RBCs that produce two molecules of ATP from one glucose molecule.[99] ATP is needed to phosphorylate RBC membrane proteins, and if ATP is not consistently produced, cells can experience hemolysis. A lack of ATP in stored blood has been reported and is believed to be due to storage solution decreases in pH and changes in glycolytic metabolism.[74,102,103] ATP is stable between 6.8-7.4 pH ranges, but otherwise it hydrolyzes to adenosine diphosphate (ADP) and phosphate, which can be measured in stored blood above 6.8 pH.[104,105]

The Luebering-Rapoport shunt is important to the production of 2,3-diphosphoglycerate (2,3-DPG).[106,107] This glycolytic intermediate is used to indirectly support Hb affinity for oxygen and RBC survival.[108] Thus, enough 2,3-DPG is critical to oxygen transport, as the oxygen would bind too tightly to Hb and would not release from the RBCs if 2,3-DPG is too low.[88,108] 2,3-DPG is highly dependent on pH, so a decrease in pH effects the amount of 2,3-DPG bound to RBCs.[109] Specifically, if the pH decreases to <7.0, there is a significant decline in production of 2,3-DPG mutase.[109] It is hypothesized that the cause of significant pH decrease (especially between days 14 to 35 in stored blood) can be attributed to the increased amount of protons in solution from an increased amount of lactate generated.[103] Studying pH for stored blood solutions is important as the storage environment becomes acidic (6.5-6.8) over the 6 weeks of storage, which indicates increased lactic acid formation.[50,103,110] The adenylate pool, or the total amount of adenosine monophosphate phosphate (AMP), ADP, and ATP, is an important component to RBCs.[111–113]

Another pathway that is important for stored blood is the well-studied polyol pathway, where glucose is reduced to sorbitol and then converted to fructose.[115] This pathway is an important aspect to blood banking because it is a mechanism often increasing in hyperglycemic environments, such as in people with diabetes or stored blood.[115] The cellular toxicity of a high

glucose environment for extended periods of time has shown to increase common diabetic complications, such as retinopathy, nephropathy, neuropathy, and cardiovascular disease.[116] Typically, glycolysis is the main pathway for converting glucose to ATP in normoglycemic environments.[100] However, in the presence of elevated glucose, the glycolysis pathway cannot regulate this concentration increase, so other pathways are utilized.[117] The two-step metabolic polyol pathway utilizes the enzyme aldose reductase to reduce glucose to sorbitol and sorbitol dehydrogenase to convert sorbitol to fructose (**Figure 1.3**).[117] Sorbitol dehydrogenase, another enzyme, utilizes nicotinamide adenine dinucleotide (NAD^+) to produce nicotinamide adenine dinucleotide hydrogen (NADH), and subsequently produces fructose.[118,119] This process highlights the importance of the redox reaction in conjunction with the metabolization of glucose.[119]

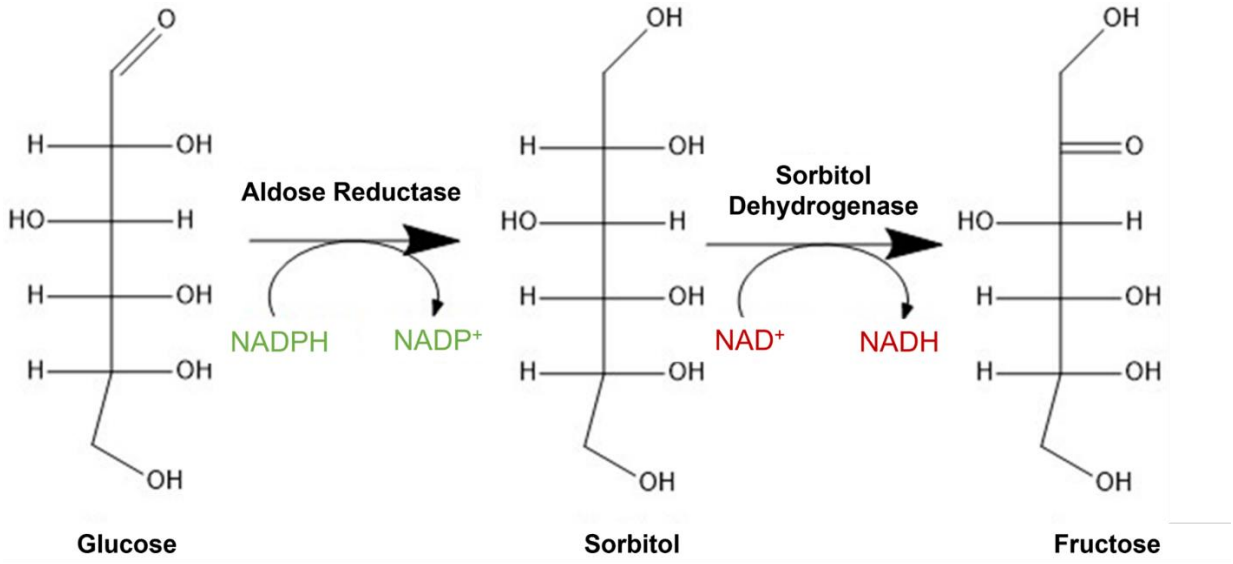

Figure 1.3. Two-step glucose to fructose mechanism. The aldolase reductase enzyme uses the donated hydrogen group from NADPH to $NADP^+$, and this mechanism produces sorbitol for the second step of this process. Sorbitol dehydrogenase, another enzyme, donates hydrogen to NAD^+ to create NADH, and subsequently produces fructose. This process highlights the importance of the redox reaction in conjunction with the metabolization of glucose.[118,119]

Blood storage lesions include increased cell membrane rigidity and lysis, pH changes and decreased levels of potassium, lactate, sorbitol, and ATP.[67] Clinical studies report decreased deformability and aggregation of RBCs after two-weeks of storage and significantly increased morbidity of the cells.[66,90,120] The Spence group has shown the adverse effects of hyperglycemic conditions and a decrease in flow-induced ATP release from RBCs stored in AS-1 when compared to AS-1N stored cells.[74–76] ATP release stimulates the production of NO in endothelial cells, which relaxes smooth muscle cells and, in turn, results in vessel dilation (**Figure 1.4)**.[67] Robert Furchgott, Louis Ignarro, and Ferid Murad were awarded the Nobel Prize in Physiology or Medicine for their discoveries identifying NO as the gaseous signaling molecule that was previously known as the endothelium-derived relaxing factor.[121] When stimulated by mechanical deformation or flow-induced shear stress, RBCs release ATP which stimulates production and subsequent release of NO from endothelial cells, known as endothelial NO synthesis.[122–126] NO is synthesized endogenously from L-arginine and oxygen, via an enzyme, NO synthase (eNOS).[127,128]

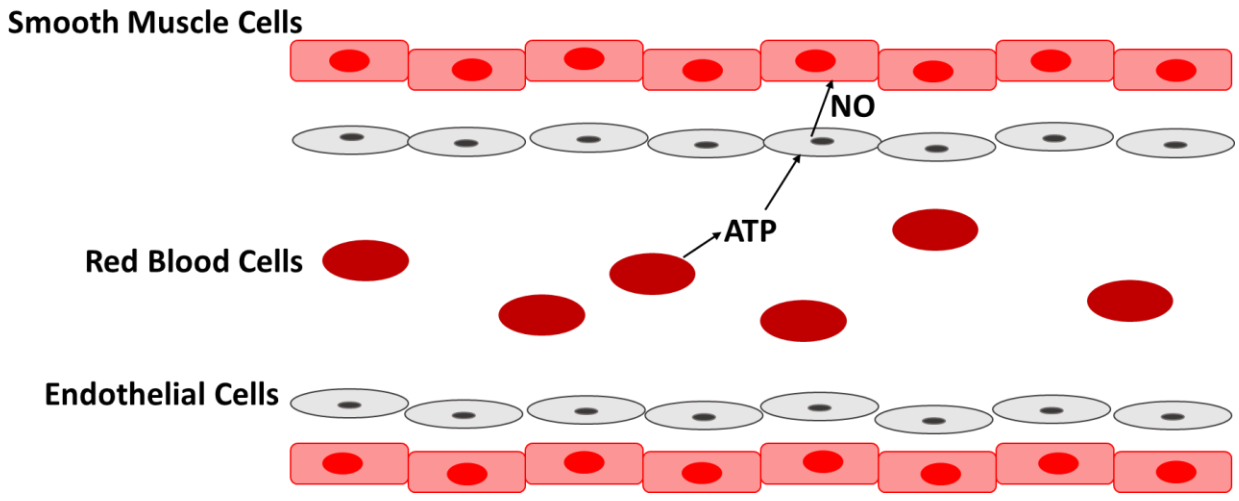

Figure 1.4. ATP and NO release in the bloodstream. Red blood cells release ATP that induces subsequent NO release from endothelial cells. The NO induces vasodilation in the smooth muscle cells and therefore increases blood flow.[104,122,127,132]

The endothelium derived relaxing factor, NO, diffuses into smooth muscle cells next to the endothelium and subsequently binds to a prosthetic heme group of guanylyl cyclase (GC).[129,130] When NO binds to GC, it increases cyclic guanosine monophosphate (cGMP) levels, which in turn initiates relaxation of smooth muscle cells.[129,130] This mechanism results in vasodilation and increased blood flow that are essential to stimulate vessel dilation and prevent high blood pressure (hypertension).[131–133]

In the process of oxidizing L-arginine to L-citrulline to produce NO, endogenously made NO can undergo different reactions in which the mechanism results in the formation of free radicals.[134,135] L-arginine contributes indirectly to the increased blood flow and necessary vasodilation. A vital component of this cellular messenger is the free radical signal-transducing agent effect.[133,134] These secondary free radicals increase oxidative stress (a common storage lesion) to the cells.[136] The signaling process associated with free radicals, or reactive oxygen species (ROS), known as redox signaling, is important in regulating homeostasis for cell functions.[137] However, large amount of ROS can result in various adverse effects to the cells, including protein damage, lipids, DNA, cell processes, and incurring various diseases associated with cancer. [137] Antioxidants are utilized to control the amount of free radicals and are used as important defense mechanisms against oxidative stress and subsequent damage.[138]

Glutathione or γ-l-glutamyl-l-cysteinyl-glycine is an important antioxidant and is ATP-dependent. Therefore, the production of ATP directly relates to glutathione, which is critical to cell signaling and overall homeostasis.[139] Also, glutathione stimulates total L-arginine, and therefore correlates to the nitric oxide synthase (NOS) activity and subsequent vasodilation process.[127,128,133] Cell metabolism and functionality can be altered if homeostasis is not maintained properly, as the role of an antioxidant is vital to decrease ROS and oxidative stress.[139–142] Typical

mammalian tissues contain 1-10 mM glutathione, and this non-protein thiol defends against oxidative stress, which will be discussed further in chapter 3.[143] Significantly altered levels of glutathione can contribute to various conditions such as neurogenerative diseases, cancers, increase inflammation, and other pathologies associated with premature aging.[139,144–146] Glutathione can exist in two different intracellular forms, GSH, which accounts for over 98% of thiol in cytosolic compartments (including intracellular fluid of RBCs), and oxidized glutathione (GSSH).[133,143] Therefore, an important biomarker for oxidative stress and impaired vasodilation via ATP and NO production is measuring the GSH/GSSH ratio and the effect of the glycation mechanism in hyperglycemic environments as shown in **Figure 1.5**.[147]

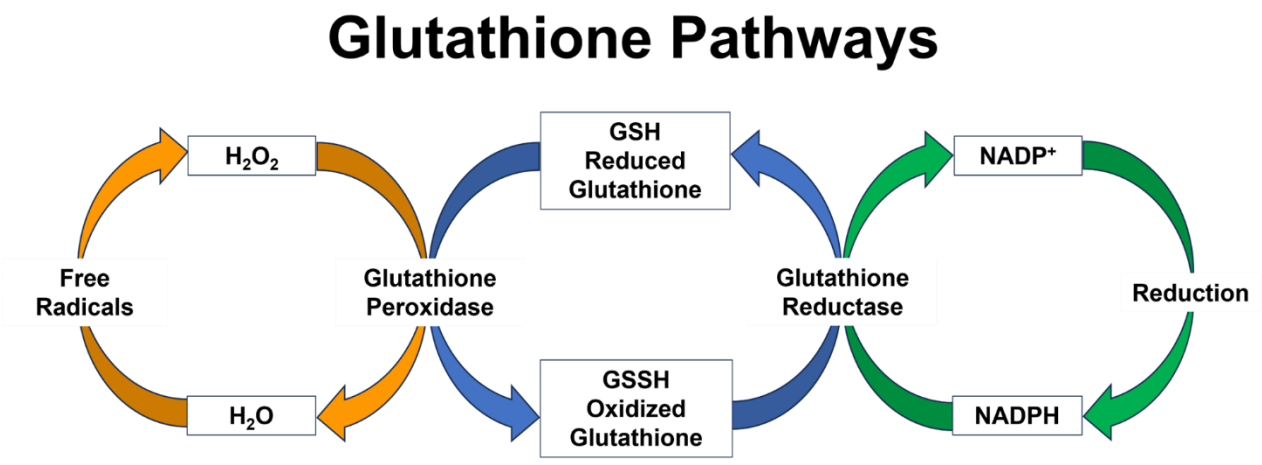

Figure 1.5. The glutathione pathway. Glutathione exists in the thiol-reduced (GSH) and disulfide-oxidized (GSSH) forms. Aerobic metabolism generates hydrogen peroxide (H_2O_2) and, therefore, free radicals. Glutathione peroxidase utilizes H_2O_2 to be metabolized and then is used by GSH/GSSH cycle. GSSH can be reduced to GSH via Glutathione Reductase utilizing NADPH.[143,147,148]

The GSH/GSSH ratio determines intracellular redox potential and is used to indicate extreme oxidative stress.[143,148] In increased oxidative stress, GSSH cannot be reduced to GSH as efficiently, which increases disulfides and depletes intracellular GSH.[143] Elevated GSH levels typically indicate an increase in resistance to oxidative stress and antioxidant capacity.[143,148] A decrease in the GSH/GSSG ratio is often linked to various diseases associated with oxidative stress, such as cancer, Parkinson's disease, and Alzheimer's disease.[148] There is a key relationship between NAD NADH formation and sorbitol, which highlights the indirect relationship between the glutathione ratio and sorbitol accumulation.[119,149]

1.6 Advanced Glycation End Products

Oxidative stress induces oxidative damage, which is associated with increased inflammation and the formation of AGEs. These AGEs are a common storage lesion and are used as a biomarker of the glycation mechanism and inflammatory conditions. AGEs are typically formed by two routes: exogenously by diet and endogenously by hyperglycemia.[150] There are three common ways that increased glycation results in heterogenous AGEs: fluorescent cross-linking, non-fluorescent cross-linking, and non-fluorescent non-crosslinking.[151–154]

Glycation **(Figure 1.6**) is the chemical reaction of a carbonyl group of a reducing sugar binding to an amino group of proteins, lipids, or peptides.[154,155] The glucose, fructose, or trioses bind to the amino groups of proteins without the presence of enzymatic control.[154,155] The glycolysis mechanism produces a Schiff base intermediate, and because these are unstable and reversible, the more stable and irreversible Amadori product formed via rearrangement is the precursor for the irreversible AGEs.[155,156]

There are several pathways to produce various types of AGEs, but the most common are the autoxidation of glucose and lipid peroxidation (discussed further in chapter 2).[150,155,156] The most

common AGEs are Nε-carboxymethyl-lysine (CML) and Nε-carboxyethyl-lysine (CEL), which are formed from the glyoxal or methylglyoxal reaction (respectively) with free lysine groups of proteins.[157,158] These AGEs are associated with common adverse effects, such as oxidative stress, protein cross-linking, increased free radical production, lower ATP release, and less cell deformability.[150,157,158]Also, AGEs in general are commonly associated with various conditions such as diabetes,[159–161] kidney disease,[162,163] cardiovascular disease,[164] neurodegenerative disorders,[165] and cancer.[150,166]

Hyperglycemic conditions, such as the FDA approved additive solutions, promote the glycation process and Maillard reaction. CML and CEL can be used as indicators of RBC membrane glycation and increased oxidative stress, which will be discussed more in the following chapters.[160]

Even though the *in vivo* formation of these two AGEs have been studied, the extent of CML and CEL formation during typical 42-day blood storage has not been reported. There have been previous reports of utilizing liquid chromatography with tandem mass spectrometry (LC-MS/MS) and enzyme-linked immunosorbent assay (ELISA) kits for fresh RBCs and 42-day RBCs but examining the rate in weekly increments over 42 days using several types of biomarkers to determine oxidative stress has yet to be investigated.[167]

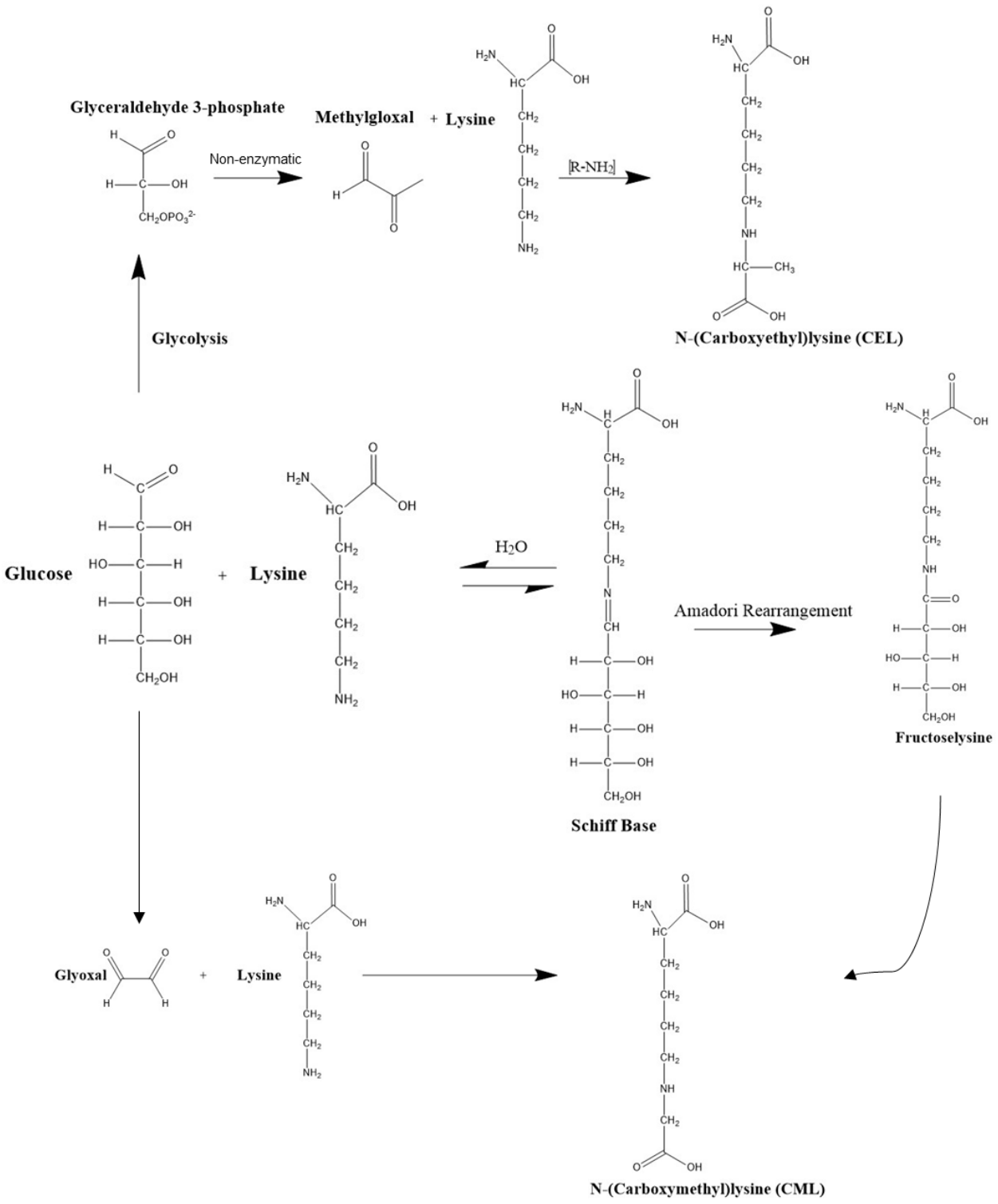

Figure 1.6. Proposed pathway for formation of CML and CEL. Major pathways of AGE formation are through autoxidation of glucose and lipid peroxidation. CML formation can occur from a glyoxal intermediate or fructoselysine, and CEL formation occurs from methylglyoxal intermediate and lysine.[150,154,221,222]

1.7 Previously Reported Techniques to Study AGEs

AGEs have been a point of research since 1985 when Dr. Mahtab Ahmed first reported CML in processed foods while studying the Maillard reaction.[168] He also discovered the presence of CML in various biological samples, such as human skin collagen, lens proteins, crystalline, and various intracellular proteins.[168,169] The pathophysiologic significance of CML was present in discovering the linkage between aging and increased concentrations of CML. Also, CML accumulation was present in people with diabetes, atherosclerosis, renal disease, and cardiovascular disease.[158,170]

Dr. Ahmed also discovered the second most abundant AGE years later, CEL.[168] There was evidence of increased CEL concentrations in tissues linked to increased signs of aging, and therefore CEL is also considered a biomarker for increased oxidative stress.[168,171] A major measurement obstacle with past and current AGE research is the limited number of modified proteins, as only 1% of lysine in proteins are involved in the glycation process to form CML and CEL.[168] Even though CML has been detected in several tissue sites in vivo (liver, muscles, RBCs, mitochondrial proteins, skin collagen, and fetal tissues), it has been complicated to identify these specific target proteins that exist in such low quantities.[168,171] There have been advances in research further linking plasma AGE accumulation and increase severity in diabetic complications, such as retinopathy, neuropathy, nephropathy, and cardiomyopathy.[150,158,172] Clinical studies have explored inhibiting AGE formation to lead to less severe diabetic complication with aging.[173] However, diabetic complication research has been mostly focused on limiting exogenous AGEs through food and diet rather than studying *in vivo* AGE prevention.[150,174,175]

There have been several types of analytical techniques to measure AGEs in food, tissues, plasma, and RBCs. Competitive ELISA kits for CML have been utilized for cells such as skin,[176]

adipocytes,[177] lipoproteins,[178] blood,[179] and various proteins.[180,181] Semba reported that ELISA kits did not show cross-reactivity with other compounds to study CML in blood samples by preparing plasma from patients 65 years or older.[162] This report correlated elevated plasma CML levels to increased chronic kidney disease patients, and thus, further suggesting diet intervention could assist in lowering serum or plasma CML.[162] The researchers also suggested pharmacological intervention with pyridoxamine, which is an AGE inhibitor, to help reduce circulating AGEs and improve renal function in people with diabetes and chronic kidney disease.[163] CML and CEL levels were also reported in multiple sclerosis (MS) patients using ELISA kits.[182] This report evaluated the AGE accumulation and linked it to increased serum CML levels in MS disease in comparison to healthy controls.[182] There have been reports that neurodegeneration, specifically in Alzheimer's and MS, is linked to increased AGEs, and these mechanisms are another clinical route that can be explored further.[182,183] Other techniques to study AGE formation and accumulation in clinical studies include immunocytochemical staining, Western Blot Analysis, and surface plasmon resonance (SPR).[184–186]

One of the routine clinical research methods that eliminates common sample matrix and specificity issues that are common in ELISAs is by utilizing LC-MS/MS.[167,187–189] Common areas of clinical research detecting and quantifying AGEs using LC-MS/MS include using human plasma from subjects with renal failure and T2D. Other research includes studying genetic polymorphisms and other pathophysiological related diseases in human plasma from healthy volunteers and subjects with renal failure and T2D.[79,167,190,191] However, using LC-MS/MS to detect and quantify CML and CEL in different stored blood solutions needs further method development and long-term experiments. In the Spence lab, the group has focused on comparing different storage solutions (normoglycemic and hyperglycemic), and there is good evidence to

move forward studying AGE formation and develop a method to compare CML and CEL over 42 days.[74] Often hemoglobin A1c (HbA1c) is associated with a diagnosis of diabetes, as well as long-term glucose management, but the relationship between other glycated proteins on the RBC membrane and HbA1c levels is unclear. Other commonly used biomarkers to detect the extent of hyperglycemic conditions include glutathione (GSH and GSSH) and sorbitol accumulation, but these are indirectly related to the AGE formation. Therefore, studying AGE formation with these two-storage solutions overtime using a well-developed analytical method such as LC-MS/MS is an important aspect of this dissertation.

1.8 Transfusions and Clinical Outcomes

While it is important to consider blood storage for these trauma events, there are also patients with pre-existing conditions that require massive transfusions.[193] Another area of research yet to be fully explored involves multiple blood transfusion to people with anemia. Blood transfusions are one of the most common medical applications for trauma patients and people with anemia.[3] A typical transfusion is 1.5 L of blood, or ¼ of total blood volume in a human.[192] Anemia, affecting almost a quarter of the population, is a condition with an extremely low level of Hb in the body (due to low levels of healthy RBCs).[194,195] This condition is also referred to as iron deficiency; with low levels of Hb (<8 g/dL), there are low levels of iron, since Hb carries the iron necessary for the body.[3,194,196] Also, there are reports both supporting and contradicting the premise of lower oxygen delivery to important organs associated with anemia.[197] Too low levels of oxygen can induce hypoxia and lead to systematic deterioration of vascular blood flow, the ability of peripheral tissues to adequately regulate oxygen, and lack of oxygen delivery to the heart that can cause coronary flow issues to the myocardium (myocardial ischemia).[3,198]

One of the clinical approaches assisting in increasing Hb count for a person with anemia is prescribed blood transfusions. In current practice, there is no set limit of how many blood transfusions a person can have. However, there are associated risks with each blood transfusion, which can increase morbidity and mortality. Some transfusion related risks include transmission of diseases, errors associated with administration of blood components, immune suppression, and acute respiratory distress syndrome.[3] Hendrickson reported that 15% of all hospitalized patients require blood transfusions.[199] This statistic does not include pre-existing conditions that require transfusions. The benefits-to-risk ratio for transfusing a patient is always considered prior to clinical application. Therefore, it is important to prioritize the health of the pRBCs and decrease the approximate 1% cell lysis associated with damaged blood due to transfusion-related-storage.[199]

There are studies that consider other drawbacks of current storage solutions, such as the known decrease in 2,3-DPG and ATP/NO production.[108,200,201] A patient who has known pre-existing conditions due to a trauma related event or anemia could have other associated risks of the treatment due to the declined metabolic, physical, and chemical properties of current pRBCs in storage. Some of the important physical and chemical properties are determinants of current reported studies that reappraise pRBC storage. The storage lesion markers are known to increase in the presence of compromised RBC membranes.[67] These damaged membranes and proteins are linked to adverse clinical outcomes. A study involving cardiac patients (over 6,000 subjects) compared incidence of adverse outcomes for transfusions with RBCs that were stored for 14 days or less to RBCs stored more than 14 days, and researchers found that RBCs stored for longer periods of time were associated with a significant increase in number of adverse outcomes to the patients.[202] Therefore, even though RBC transfusions can be a life-saving clinical treatment, it can be associated with severe post-transfusion complications.[203]

According to recent FDA claims, blood transfusions have improved dramatically with donor screening and automated data systems.[204] According to the FDA fiscal year 2014 and 2018 reports, in combined years, transfusion-associated circulatory overload (TACO) cases had the highest reported number of fatalities (32%), and second (26%) was associated with transfusion-related acute lung injury (TRALI).[204] It is imperative that studies report increased storage lesion markers and subsequent possible adverse post-transfusion related outcomes for future considerations in conducting a reappraisal of the current FDA blood storage protocols.

1.9 Storage Lesion

The storage lesion is often associated with the various transfusion-related complications as previously described.[67] There are different types of storage lesion markers, each affecting various aspects of the RBC (**Table 1.4**).

Table 1.4. Types of Adverse Effects Related to the Storage of RBCs

Type	Adverse Effect
Damage to Proteins	Oxidative Damage to RBC Structure and Band 3 Anion Transport Protein
Increased RBC Adhesion	Adhesion to Endothelial Cells Promotes Inflammation
Cell Rigidity/Deformability	Decreased Hemoglobin Flexibility, Shorter RBC Circulation Lifespan, Cause of Vaso-Occlusion
Oxidative Stress and Inflammation	Formation of AGEs
Slowed Metabolism	Less ATP Formed, Less NO Released
Acidosis	Less 2,3-Diphosphoglycerate (2,3-DGP)

Healthy RBCs have limited RBC-endothelial cell (RBC-EC) interactions.[205,206] In comparison, RBCs associated with various vascular disorders or adverse effects of storage solutions have increased RBC-EC adherence due to biochemical changes to the outer membrane and plasma protein concentration (**Figure 1.7**).[150,205,207,208] Increased adherence to endothelium is linked to various vascular disorders and closely associated with diabetes mellitus, polycythemia vera (thrombotic complications), and retinal vascular disorders.[208] Sickle cell disease, with RBC morphology changes and deformation of the membrane, is also associated with increased adhesiveness to the endothelial cells.[205,206,208] One of the newer avenues of adhesion research is focusing on the correlation between diabetes/aging and modified RBC membranes.[208] An example

of this modification that could be linked to increased adherence to endothelium is the addition of the receptor for advanced glycation end products (RAGE).[207]

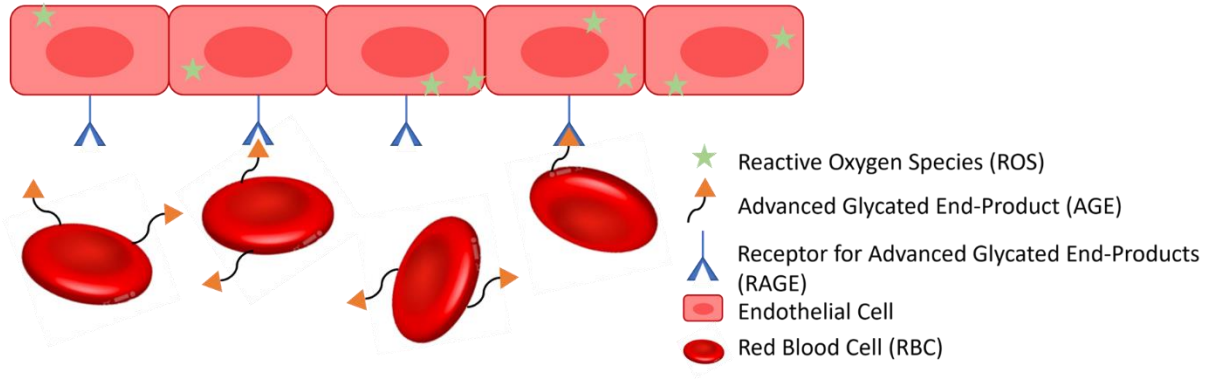

Figure 1.7. Red blood cell AGE and endothelial cell receptors. RBC membranes contain various proteins, some of them glycated by exposure to hyperglycemic environments and oxidative stress. The modified proteins can increase cross-linking and cellular dysfunction. These AGEs have a receptor (RAGE) located on the endothelial cells, and the adverse effects from AGEs can be transmitted via the RAGE. The generation of ROS production occurs mainly through the NADPH oxidases by RAGE stimulation. The increasing ROS has been linked to increased oxidative stress and diabetic complications.[223–226]

There are several biochemical and morphological changes to pRBCs reported in storage solutions. The shape of the cell changes from a biconcave disk to crenated, known as echinocytes, and the cells are less deformable, smaller in size, and have increased cell rigidity after 14 days of storage.[209–211] Additionally, the increased cell lysis and decreased asymmetry of the membrane phospholipids lead to an overall cell membrane protein loss and modifications.[56,212,213] The irreversible morphological changes could begin as early as day 12 in storage, and these changes have been reported to shorten the overall lifespan of the RBC *in vivo*.[214]

One of the possible explanations of these adverse physiochemical changes is due to the metabolism, acidosis, oxidation, and overall protein modification/arrangements.[214] It is well known that ATP and 2,3-DPG levels change during pRBC storage. ATP is essential for

intracellular activity, and studies have shown a significant decrease in ATP activity after 5 weeks of storage.[66] However, once stored blood is transfused, the ATP levels increase dramatically along with associated reversible morphological changes.[214] Low levels of ATP can lead to less efficient activity of Na^+, K^+ pump mechanism, thus leading to increased potassium leakage and higher chances of high potassium related transfusion complications.[215–217] Ideally, instead of introducing a rejuvenation solution or waiting until transfusion, the ATP concentration could be maintained at healthy levels to promote healthy pRBCs. Another concern for stored blood transfusions that is not well investigated *in vivo* is the altered metabolism from increased glycolysis in a hyperglycemic environment.[218] In hyperglycemic environments, the inflammatory response switches to accommodate glycolysis to meet the demands of more energy and effects ATP utilization.[218,219] Moreover, less stress on the cells in a normoglycemic environment that maintains ATP concentration would result in more viable RBCs which can help increase positive patient outcomes.[209,214]

Blood transfusion has been utilized for over 100 years, but the solutions to store blood are currently the topic of discussion for improving transfusion medicine.[213] The extensive research behind adverse effects to RBCs shows a need for storage improvement, and one avenue to explore is the amount of glucose concentration.[9,13,59,220] By examining known biomarkers of AGEs, oxidative stress, and protein glycation, more information can be obtained to fully evaluate the use of a normoglycemic blood storage solution alternative. The clinical applications for this solution include transforming transfusion medicine for people with diabetes, sickle cell, anemia, and other at-risk patients (neonates).

There are a few different hypotheses for this dissertation work. The first is there will be a difference in biomarkers between the two solution types (hyperglycemic and normoglycemic). The

second, there will be a need for method development to evaluate the sample preparation, bioanalytical guidelines, and LC-MS/MS usage for blood samples. Finally, the biomarkers evaluated for stored solutions can be used for additional testing utilizing screening T1D patients for a C-peptide related clinical drug trial.

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Chapter 2: Method Development to Detect and Quantify Advanced Glycation End Products (AGEs) Using UPLC/MS-MS

2.1 Introduction

2.1.1 Background

Transfusion medicine is a critical component of modern healthcare, evident by the millions of units of red blood cells (RBCs) transfused annually to patients.[1] The collection of whole blood and subsequent storage of various blood components (such as the RBCs or plasma) into units ready for transfusion is relatively simple. Briefly, the process involves collection of ~ 450 mL of whole blood from donors, centrifugation to separate the RBCs from the plasma and leukocytes, followed by storage in separate bags at 4°C for various lengths of time depending on the component (plasma or RBC) and country regulations.[2,3]

A key feature of current protocols for RBC storage is the collection solution into which the whole blood (WB) is drawn, and the solution into which the separated RBCs are stored (the latter also known as additive solution).[4] The most popular collection solution is citrate-phosphate-dextrose (CPD), which contains citrate, phosphate, and dextrose (glucose).[5,6] Following centrifugation, the RBCs are then stored in one of multiple available additive solutions (e.g., AS-1, AS-3, AS-5, or AS-7).[5,7,8] While the current blood storage procedure has been in place since the 1970s, there are many reports showing adverse effects of storage over time on the RBC's chemical and physical properties.[9–13] These adverse effects, collectively known as the RBC storage lesion, involve chemical, physical, and metabolic changes, as well as functional changes, to the RBC while in storage.[14,15] Unfortunately, the exact origins of the storage lesion are not known, nor is the mechanism leading to the various changes to the stored RBC well understood.

An interesting feature of the collection and additive solutions used in RBC storage is the high level of glucose in the CPD and the AS-1. Specifically, typical blood glucose concentration ranges from 4-6 mM in a healthy person, while a person with diabetes often has a fasting blood glucose level ranging from 7-9 mM.[16] Currently, approved versions of CPD and AS-1 have glucose concentrations that exceed 110 mM; even after the RBCs are added, and the AS-1 is diluted due to mixing of the RBCs with the AS-1, the concentration of the glucose in the RBC/AS-1 solution is still in excess of 40 mM, a value much higher than that of healthy humans and humans with diabetes.[10,17] It is noteworthy that after transfusion of the ~ 280 mL of pRBCs into a human, the glucose concentration in the storage bag will not affect the glucose levels in the human transfusion recipient (due to dilution of the 280 mL pRBCs into a human who typically has a total blood volume of ~ 5 L); rather, the concerning feature of the high glucose is the effect on the RBC properties during storage.

In continuance, people with high blood glucose levels, such as people with diabetes, have RBCs with increased levels of advanced glycation end products (AGEs), which are thought to be a negative determinant in overall cell health.[18,19] Past work involving AGEs on the RBCs in the hyperglycemic bloodstream of people with diabetes provided the rationale to investigate the possible formation of AGEs on the RBCs in AS-1. A previous report[20] suggests the formation of AGEs later in storage and provides motivation to (1) quantitatively determine the concentrations of the AGEs being formed and (2) evaluate these concentrations of RBC-bound AGEs from the beginning (day 1) to the end of storage duration (42 days).[15,20,21] Such time-based studies of AGE formation on stored RBCs are without precedent. Here, we describe novel mass spectrometric determination of two AGEs, Nε-carboxymethyl-lysine (CML) and Nε-carboxyethyl-lysine (CEL).[22] The glyoxal mechanism produces CML, the first AGE discovered and the most widely

studied AGE.[23] Another important AGE, CEL, is associated with diabetes-related complications and derived from the methylglyoxal pathway.[24] Measurement of the formation of these two AGEs (CML and CEL) on the stored RBC is the focus of this study.

Current interest in studying the physiochemical effects on long term blood storage involves measuring CML and CEL simultaneously, while examining protein-bound lysine (not glycated) changes. The aims of the present study were to develop a method to quantify and compare CML, CEL, and lysine from blood samples stored in hyperglycemic and normoglycemic storage conditions, expand upon previous Spence lab storage lesion studies, and provide a well-characterized validation method for future blood sample studies. Future studies can use CML and CEL as biomarkers for clinical use in transfusion medicine and to understand more about the link between AGEs and diabetes.

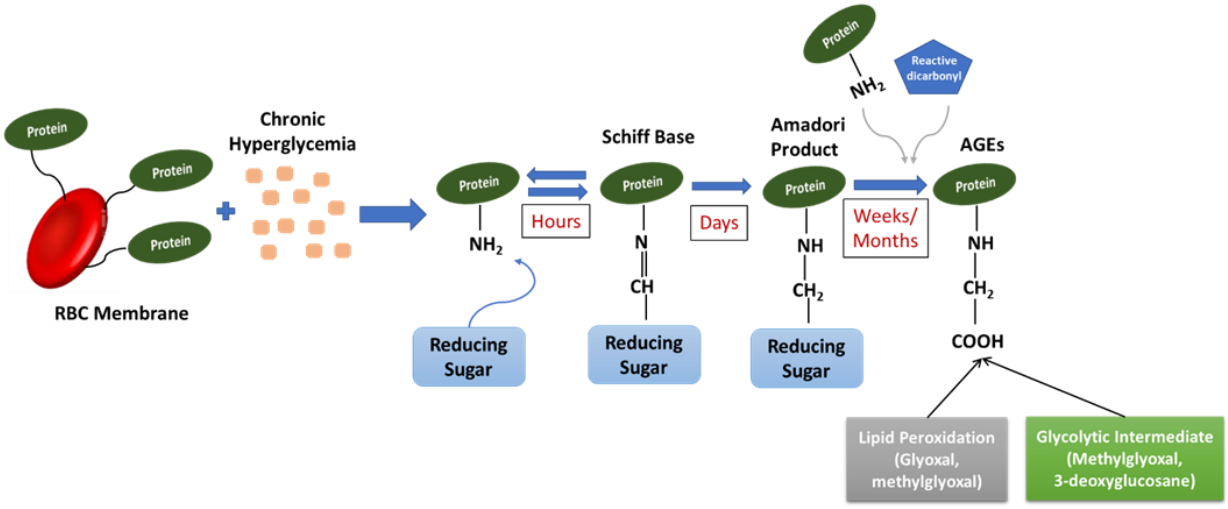

Figure 2.1. RBC glycation mechanism to produce AGEs through the Maillard reaction and chronic hyperglycemia exposure. The RBC membrane proteins become glycated with increased glucose concentration in the blood stream due to chronic hyperglycemia. The reducing sugar interaction with the protein produces a Schiff base a few hours after exposure, which is a reversible reaction. The irreversible reaction occurs days after chronic hyperglycemia and results in the Amadori product of the Maillard reaction of glycation. Weeks/months of chronic hyperglycemia results in the formation of AGEs due to increased reactive dicarbonyls from oxidative stress. Through the lipid peroxidation or glycolytic intermediate, various AGEs are formed, including CML and CEL, respectively. [24]

2.1.2 CML and CEL Mechanism

These two AGEs are the focus of this study as a biomarker for increased oxidative stress and other effects of storage lesions due to hyperglycemic conditions during a 6-week storage period, as previously described in chapter 1. Current methods of detecting and quantifying AGEs include non-selective enzyme linked immunosorbent assay (ELISAs), which use antibodies and are often limited to reliable types of antibodies and decreased sensitivity.[22] Specifically, commercial kits often cannot reliably distinguish between similar byproducts in the CML and CEL mechanism. Other types of analytical methods include time-consuming immunohistochemical detection, fluorescence spectroscopy and size exclusion chromatography with fluorescence detection.[25–27] None of the current research includes comparing AGEs in hyperglycemic and normoglycemic blood storage solutions on a weekly basis for 6-weeks using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Due to the major differences in sample conditions when RBCs are stored in blood bags as compared to freshly drawn whole blood in blood collection tubes, there is not a cohesive methodology for studying both CML and CEL for pre-clinical applications.

As previously discussed, glycation is considered a non-enzymatic reaction between the reducing sugar (glucose) and proteins.[29] This reversible reaction produces a Schiff base, and the Amadori Rearrangement produces irreversible products such as the oxidation of fructosyl-lysine and the subsequent product CML.[30] This product is directly from the reaction of glyoxal and lysine.[30] CML is a specific type of AGE formed through the non-enzymatic reaction between reducing sugars, particularly glucose, lysine, glyoxal, or methylglyoxal.[31] This reaction, known as glycation or the Maillard reaction, leads to the modification of lysine residues in proteins, resulting in the formation of CML.[32] CML is one of the most well-studied and commonly studied AGEs due

to its abundance in various tissues and its potential role in age-related and chronic diseases.[33] CML is known to accumulate with age, particularly in tissues with slow turnover, such as the extracellular matrix of blood vessels, skin, and cartilage.[23,34,35] The presence of CML in tissues and organs is associated with several adverse effects including cross-linking, inflammation and oxidative stress, tissue damage, and elevated CML levels have been associated with various chronic diseases and may be used as a potential marker of disease risk or progression.[34,36] CEL is another specific type of AGE formed through the non-enzymatic reaction between reducing sugars, particularly glucose, and lysine residues in proteins.[24,37] Like other AGEs, the formation of CEL occurs through glycation or the Maillard reaction.[37,38] CEL is structurally similar to another AGE, CML, but they differ in the side chain structure attached to the lysine residue.[30,39] Its abundance in tissues has been associated with age-related changes and chronic diseases.[36]

Overall, CML and CEL are important markers of glycation and AGEs formation, providing insight into the impact of glycation reactions on protein modifications and their potential role in age-related and chronic diseases.[31,38] Research on CML and other AGEs continues to shed light on their involvement in disease pathogenesis and potential therapeutic interventions to mitigate their adverse effects[31,40,41].

There are current hypotheses for measuring CML, CEL, and protein-bound lysine on the RBC membrane for the two types of storage solutions. Due to the different mechanisms of AGE formation, most likely there will be overall differences in concentrations of CML and CEL. Protein-bound lysine, even though some will become glycated into AGEs, is not expected tp change significantly. AS-1 is a hyperglycemic storage solution, it is anticipated that more CML and CEL will form over the course of 6-weeks, and more than in the AS-1N solution.

2.1.3 Method Development Using FDA, EMA, ICH Guidelines

The bioanalytical method validation by the European Medicines Agency (EMA) provides guidance and recommendations for bioanalytical assays.[43] By using the FDA approved guideline M10 by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), this study will use sample analysis recommended chemical, biological, and metabolite drug guidelines to explore various analytical parameters, such as limit of detection (LOD), limit of quantification (LOQ), matrix effects, intra- and inter-assay accuracy and precision, and interferences.[43]

2.1.4 Limitations For Using Biological Samples and LC-MS/MS

There are several challenges when developing a robust method for detection and quantification of AGEs, including the ten-thousand-fold difference between the concentrations of protein-lysine on RBC membranes versus modified lysine adducts (CML and CEL); only 1% of RBC proteins that contain lysine are glycated and form AGEs.[33,44] Acid hydrolysis is a well-known technique that can be used to extract proteins on the RBC membrane to adequately prepare various biological samples (urine, pRBCs, plasma) for high throughput analysis such as ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS).[45,46] There are many drawbacks with the previous methodology, such as biological interferences and matrix effects enhancing or suppressing chromatographic signal.[47–49]

A custom-made glass pipettor was created out of a need to extract the samples from the acid hydrolysis tubing (**Figure 2.2**). The samples were at the bottom of a 6 mL vacuum tube and the shape of the tubing did not allow for commonly purchased laboratory equipment, such as 9” Pasteur pipettes, syringes, and serological pipettes. After further investigation, there was no straightforward way to remove the sample without contamination or loss. There are no approved

or suggested materials to extract the samples without “free pouring” out into sample collection tubes. Using the design of smaller pipettes and rubber pipette bulbs, the chemistry glass shop used the measurements of the vacuum tubing and collaborated to design simple, effective, and reusable glass pipettes that could easily reach the bottom of the tubing and extract the sample. It was important to include glass to limit corrosion over time with weekly 6 M HCl exposure.

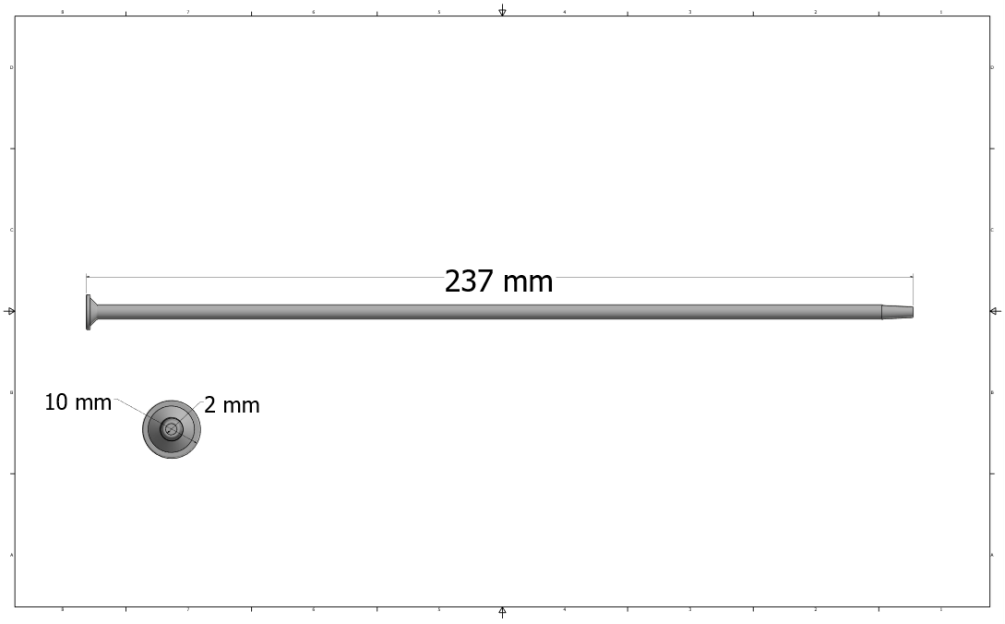

Figure 2.2. Engineering sketch of glass pipettor. The device was customized to fit inside a 6 mL Thermo Fisher Scientific vacuum tube to reach the hydrolyzed sample at the bottom. It is made from glass and is 237 mm in length, the interior diameter is 2 mm and the top diameter to attach a rubber pipette bulb is 10 mm.

The HCl needed to be dried off before the samples could be separated and analyzed via UPLC-MS/MS (**Figure 2.3**). It is important to note that the samples could not contain HCl because the acidic vapors can damage the UPLC separation column. The samples needed to be dried using a SpeedVac with an acid trap, which is specific to removing acid solvents to maintain sufficient instrument integrity. Initially, the samples were immediately taken to the SpeedVac and dried at 75°C and 0.1 bar, for 8 hours. The samples were frozen overnight at -80°C and dried the following

day. The introduction of freezing the samples overnight before drying and using a more efficient instrument assisted in lowering the dry down time from 8 hours to 4 hours. The remaining sample consisted of dried down pRBCs and was stored at -80°C until ready for separation and analysis.

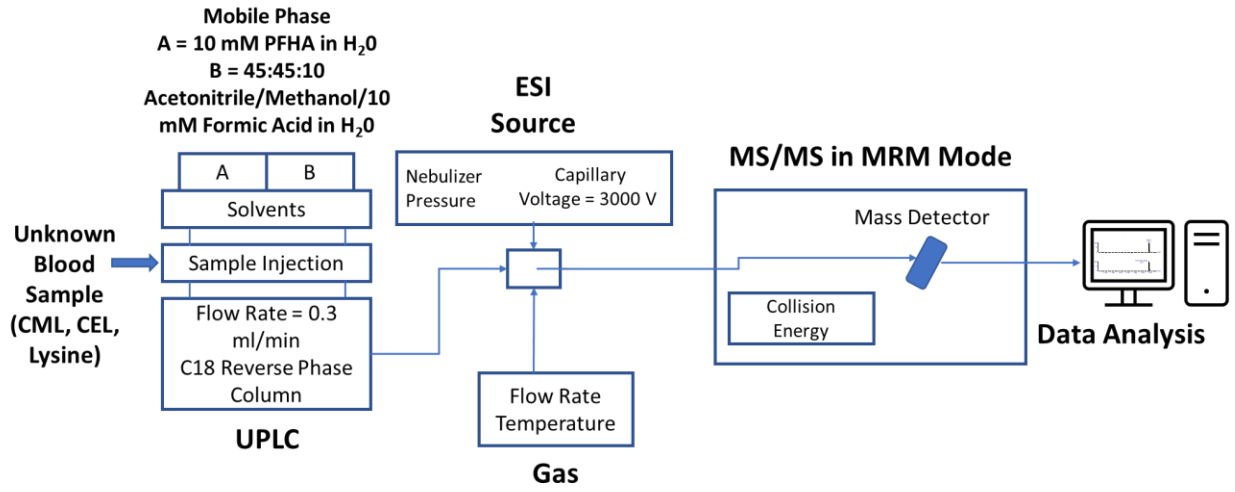

Figure 2.3. Method development parameters and instrument setup for the Waters Agilent UPLC-MS/MS instrument workflow. The unknown sample, mixed with internal standard, is injected via automated sample injector. The initial ramp begins with the mobile phases A and B, and continues until the sample is ready to be flowed through the C18 reverse phase column. The initial solvent conditions were 90 % mobile phase A and 10% mobile phase B for 5 minutes and then changed to 35% mobile phase A and 65% mobile phase B before switching to 1% mobile phase A and 99% mobile phase B between 5.01 and 6 minutes. Finally, at 6.01 minutes, mobile phase A was 90% and mobile phase B was 10%, the sample was injected, and these conditions were held until the 8-minute run was complete. The column was maintained at 40°C and the flow rate was 0.3 mL/min. Prior to sample injection, a needle wash was utilized containing 80/20 isopropyl alcohol (IPA)/water with 10 mM ammonium formate to maximize the sample delivery from the needle to the column. The sample was ionized by electrospray operating in positive ion mode (ES+), with capillary voltage of 1.00 kV. The source temperature was 150°C, desolvation temperature was 350°C, desolvation gas flow was 800 L/hr, and cone gas flow was 40 L/hr. Once the collision energy was applied to create fragment ions, the analyte masses was detected for mass spectrometry analysis.

2.2 Methods

2.2.1 RBC Collection and Purification

The blood draw followed a protocol approved by the Institutional Review Board of Michigan State University. Blood was obtained from healthy humans and informed consent was obtained from all donors. All record keeping complied with Health Insurance Portability and Accountability Act regulations. Whole blood was collected via venipuncture (140 mL total whole blood) into multiple 10 mL uncoated collection tubes (Thermo Fisher Scientific, Waltham, MA) that were previously prepared to contain either 1 mL of CPD or 1 mL of a normoglycemic version (CPD-N). Each blood draw was divided into CPD or CPD-N solution tubes to maintain consistency between storage solution results; that is, each donor had 5 tubes of blood collected in CPD and 5 tubes collected in CPD-N. All whole blood tubes were centrifuged at 2000*g* for 10 minutes, the plasma and buffy coat (containing the white cells or leukocytes) were removed by aspiration, and packed RBCs (pRBCs) were kept in the tubes.

2.2.2 Storage Solution Preparation

Each storage solution was prepared in total volumes of 50 mL with the concentrations in **Table 2.1**. Each constituent (except for glucose) was added to a beaker containing 40 mL of H_20 and dissolved. The pH was checked and adjusted with either 1 M HCl or 1 M NaOH. The beakers were covered with aluminum foil and placed in the autoclave for 30 minutes at 120°C and pressure of 20 bar. The solutions were allowed to cool in an ice bath before transferring to the biosafety cabinet to ensure continual sterile procedures. The glucose solutions were prepared separately with filter sterilization to limit glucose degradation (more detail is provided in chapter 3). The glucose solutions were prepared in 10x concentrated glucose in 20 mL H_2O, and the glucose was added slowly, to ensure it dissolved, to a stirring solution of H_2O as to not saturate the solution too

quickly. These solutions were added to a 250 mL, 0.22 µm PVDF filter membrane stericup filter system (Fisher Scientific, Hampton, NH) and moved to the biosafety cabinet before capping the bottles to limit contamination. To each additive solution, 5 mL of the 10x glucose solutions were added and diluted up to 50 mL to achieve the correct concentrations. Once the solutions were diluted correctly and mixed thoroughly, they were added to their respective containers until ready for use.

2.2.3 Glucose Concentration and Hematocrit Percentage

The pRBCs were added to either AS-1 or AS-1N in a 2:1 volume ratio and mixed and stored at 4°C for one hour before initial glucose concentration and percent hematocrit (the percentage of volume occupied by the RBCs) readings. The glucose concentration was determined with an Aimstrip Plus Blood Glucose Meter (VWR, Radnor, PA) using a 22-gauge needle and 1 mL syringe to collect and transfer a drop of RBC sample onto an Aimstrip Plus Blood Glucose Test Strip (VWR). The glucose reading was repeated, and the average glucose concentration (mg/dL) was converted to a mM value. The glucose was adjusted to 5.5 mM after one hour for the AS-1N sample by adding an appropriate volume (typically between 50-400 µL) of a 100 mM glucose solution in 0.9% saline. The same volume of 0.9% saline was added to the AS-1 sample to maintain similarity in handling between the AS-1 and AS-1N. The RBC sample hematocrit was determined using a StatSpin MP microhematocrit centrifuge (Beckman Coulter, Brea, CA) and a hematocrit reader (StatSpin CritSpin). The remaining pRBCs were used for analysis either the same day or stored at 4°C. Periodic feeding of the RBCs stored in AS-1N was achieved using a closed and automated feeding system. Stored samples were removed from bags on day 1, 8, 15, 22, 29, 36, and 43 for LC-MS/MS analysis following sample preparation techniques described below.

2.2.4 Protein Precipitation for Free Lysine Quantification

To promote cell lysis, packed RBCs (100 µL) were frozen at -20°C for one hour and then thawed for 10 minutes at room temperature before mixing with 300 µL of HPLC grade acetonitrile. The sample was centrifuged at 12,000*g* for 10 minutes at room temperature, and the supernatant was removed and stored at -80°C until dried using a SpeedVac (Savant SpeedVac Concentrator, Thermo Fisher Scientific) with an acid vapor trap (Savant Refrigerated Vapor Trap, Thermo Fisher Scientific). The dried sample pellet was stored at -80°C until prepared for measurement.

2.2.5 Acid Hydrolysis Sample Preparation

RBC samples (100 µL) for acid hydrolysis were frozen for one hour at -20°C and thawed for 10 minutes at room temperature before centrifugation at 10,000*g* for 10 minutes at 4°C. The supernatant was removed, and pRBCs were collected for acid hydrolysis. The pRBCs were diluted to make a 1% RBC solution in constant boiling sequencing grade 6 M HCl (Thermo Fisher Scientific) in glass tubes and transferred to 10 mm, 6 mL vacuum hydrolysis tubes (Thermo Fisher Scientific). The samples were hydrolyzed at 110°C for 16 hours using a 120 V Digital Dry Bath/Block Heater and Dry Bath Block Insert (Thermo Fisher Scientific). The samples were then removed using customized glassware (MSU Chemistry glass shop) designed to attach a 2 mL Pasteur rubber pipette bulb (Sigma Aldrich, St. Louis, MO) and extract the sample (**Figure 2.1**). Using the glassware, the sample was placed into 1.7 mL tubes and frozen overnight at -80°C. The following day, the samples were dried to completion using a SpeedVac at 75°C for 4-5 hours and then kept at -80°C until ready for measurement.

2.2.6 Reagent Materials and Preparation

Immediately prior to measurement, the dried RBC sample used to detect free lysine sample pellet was reconstituted in 300 µL of 10 mM perfluorheptanoic acid (PFHA) in water, and

centrifuged at 13,000*g* for 10 minutes. The dried, hydrolyzed additive solution RBC samples were reconstituted in 500 µL of 10 mM PFHA in water, and centrifuged at 13,000*g* for 10 minutes. All supernatant samples were removed and used for measurement or stored at -80°C. The free lysine sample was diluted 1:100 using 10 mM PFHA in water. Each additive sample supernatant was divided into two categories: CML/CEL detection and lysine detection. The lysine detection samples were diluted 1:40,000 in 10 mM PFHA, and the CML/CEL detection samples were diluted 1:40 in 10 mM PFHA. The sample supernatants were mixed 1:1 (v/v) with an internal standard (IS) mixture containing: 0.1 µM Nε-(1-carboxymethyl)-L-lysine-d_3 (Cayman Chemical, Ann Arbor, MI), 0.1 µM carboxyethyl-L-lysine-d_4 (Toronto Research Chemicals, Toronto, ON), and 0.1 µM $^{13}C_6$, $^{15}N_2$ labeled L-Lysine (Sigma Aldrich). The standards include Nε-(1-carboxymethyl)-L-lysine (Cayman Chemical), Nε-(1-carboxyethyl)-L-lysine (Cayman Chemical), and $^{13}C_6$, $^{15}N_2$-lysine (Sigma Aldrich) and these constituents were used to prepare calibrator samples by dissolving the lyophilized samples in water, diluting with 10 mM PFHA for a ten-point calibration curve, and mixing with the same internal standard mixture as above. The ten-point calibration curve (0, 4, 8, 16, 64, 128, 512, 1500, 2048, 5000 nM) was used to quantify CML, CEL, and lysine by adjusting analyte peak area relative to associated internal standards to illicit an overall response. During initial sample preparation, AS-1 and AS-1N solutions were also hydrolyzed to determine if diluting the samples in reconstituted sample solution would decrease matrix effects. Quality control (QC) samples were prepared using a stock of RBCs in AS-1 or AS-1N following the procedure above, preparing aliquots of the hydrolyzed sample stock into 1.7 mL vials, drying, and freezing at -80°C until they were reconstituted for measurement.

2.2.7 Chromatography and MRM Mass Spectrometry

To determine if CML, CEL, and lysine were present, the reconstituted hydrolyzed RBC samples were analyzed by LC-MS/MS using a Waters TQ-S Micro UPLC system interfaced with a Waters Acquity UPLC. A 10 µL aliquot was injected onto a reverse phase column (High Strength Silica (HSS) T3 2.1 x 100 mm) and the compounds were separated using ion-pair chromatography. For multiple reaction monitoring mass spectrometry (MRM MS) of 9 channels (**Table 2.1**), the sample was ionized by electrospray operating in positive ion mode (ES+). CML, CEL, and lysine concentrations were calculated based on the integrated areas relative to the internal standard peak areas. The total protein-bound lysine helps evaluate total protein concentration over time, and thus the number of lysine that can be glycated.

Table 2.1. Mass Spectrometry for Analyte and Internal Standard Compounds

Analyte Compound	Precursor Ion Mass	Product Ion Mass	Cone Voltage (V)	Collision Energy (eV)
$^{13}C_6$, $^{15}N_2$ Lysine	147.1	84.0	19.0	14.0
N-CML	205.0	84.0	15.0	22.0
N-CEL	219.0	84.0	15.0	28.0
$^{13}C_6$, $^{15}N_2$ labeled L-Lysine	155.1	90.1	19.0	14.0
CML-d_3	208.0	87.0	15.0	28.0
CEL-d_4	223.0	134.0	15.0	15.0

2.2.8 Linearity, Detection Limit, and Quantification Limit

To assess linearity of the developed method, nine calibration standards and a zero standard were measured for each analyte in three experiments; specifically, each standard was diluted using either 10 mM PFHA in water, AS-1, or AS-1N reconstituted supernatant. To test the effect of acid hydrolysis on the reconstitution solution, AS-1 and AS-1N solution containing no sample were hydrolyzed and dried under vacuum. After initial analysis, it was determined that

the hydrolyzed sample solutions did not statistically alter the peak shape, retention time, or matrix effects. Therefore, to simplify sample processing, all standards were simply diluted in 10 mM PFHA in water. The correlation coefficient for standard curves using 10 mM PFHA in water (R^2) are reported in **Table 2.2.** The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the signal-to-noise ratio of the blank signal and the sample signal as reported in **Table 2.2** for each analyte.

2.2.9 Intra- and Inter- Assay Variation

Intra- and inter-assay variations were assessed utilizing quality control (QC) reconstituted hydrolyzed RBC samples spiked with known concentrations of CML, CEL, or lysine for each batch analysis. The assay variation was determined by reading the plate samples (n=6) for over 20 hours kept at 4°C in the sample manager of the autoinjector. Inter-assay variation was determined by measuring the analytes on 4 different samples (n=4) over a 2-day period at 4°C in the sample manager. Both intra- and inter- assay variations were prepared for the analytes and the results are reported in **Table 2.4**. As seen in equation (**1**), the stock QC sample expected concentration (spike concentration) was determined and either diluted 1:40 for CML/CEL or 1:40,000 for lysine. The two spiked concentrations (concentration of spiking solution) relate to a low (10 times the exogenous concentration), and high (40 times the exogenous concentration) concentration of the analytes. Percent recovery (% of target) in equation (**2**) was used to determine the accuracy and subsequent percent relative standard deviation (RSD %) in equation (**3**) for precision.

$$Volume\ of\ Spiking\ Solution = \left(\frac{Spike\ Concentration \times Volume\ of\ Sample}{Concentration\ of\ Spiking\ Solution}\right) \quad \textbf{(1)}$$

$$Percent\ Recovery = \left(\frac{C_{spiked}[measured] - C_{nonspiked}}{C_{spiked}}\right) \times 100 \quad \textbf{(2)}$$

$$Percent\ Relative\ Standard\ Deviation = \left(\frac{Std\ Dev}{Average}\right) \times 100 \quad \textbf{(3)}$$

2.2.10 Matrix Effects

There are no commercially available RBC samples in AS-1/AS-1N that are free of the three analytes, so it is impossible to obtain analyte-free biological samples for method validation. However, to adhere to the bioanalytical method guidelines, the hydrolyzed RBC samples were prepared and analyzed to determine the overall extent of suppression or enhancement of signal. The matrix effects were determined using only the IS mix in either sample or blank (water). The matrix effects were calculated using equation (**4**).

$$Matrix\ Effects\ \% = \left(\frac{IS\ Peak\ Area\,[Analyte]}{IS\ Peak\ Area\,[Water]} - 1 \right) \times 100 \qquad \textbf{(4)}$$

The matrix effects were calculated and reported in **Table 2.3.** According to bioanalytical method validation, less than 13% matrix effects are acceptable criteria.

2.2.11 Carry-over

For each analyte, there was significant carry-over of analyte signal (>20%), but less than 5% of IS signal. Therefore, after each standard curve, at least 5 blanks (water only) were run before continuing sample analysis. After analysis, 5 blanks were sufficient to remove any remaining analyte eluting. Also, a minimum of two blank samples were run in between each analyte sample.

2.2.12 Data Analysis

Calibration plots of analyte/IS peak area ratio versus CML, CEL, and lysine concentrations were constructed, and a linear regression was used for all analytes. The peak area ratio of sample versus associated IS was used to produce a response to determine the concentrations from the calibration line. SigmaPlot (Systat Software Inc.) was used to plot results and R software (Rstudio version 4.2.2) was used to perform statistical testing in the “rstatix” package (0v.7.2; Kassambara, A. 2023). One-way ANOVA tests for each analyte were conducted to examine differences between

storage solution types. Prior to conducting the ANOVA test, the assumptions of normality and homogeneity of variances were assessed using Shapiro-Wilk and Levene's tests, respectively. If these assumptions were met, the ANOVA test was performed. In the case of a significant ANOVA result, post hoc Bonferroni tests were conducted to determine which specific solution types exhibited statistically significant differences on the 4 analytes.

2.3 Results

2.3.1 Free Lysine Interferences

The free lysine samples were used to identify the amount of lysine not bound to the RBC membrane to ensure the lysine quantified in the samples were RBC membrane protein-bound lysine groups. Data analysis revealed that <1% of lysine was considered “free,” and therefore, the data collected for quantifying lysine can be considered lysine that was attached to the RBC membrane prior to acid hydrolysis.

2.3.2 Matrix Effects

According to bioanalytical method validation, less than 13% matrix effects are acceptable criteria. Due to presence of endogenous concentrations of the three analytes tested, the matrix effects were evaluated using IS peak areas. Matrix effects were found to be in all analytes tested (**Table 2.2.**). All matrix effects were below 13%, which shows there is no significant enhancement or suppression of chromatogram signal due to the blood component matrix. Therefore, the chromatograms can be used to quantify analytes during further analysis.

2.3.3 Linearity, LOD, LOQ

The calibration curve of all three analytes using stable IS was measured and indicated good linearity within the concentration range selected (see **Table 2.2**). The relative peak area versus injected relative concentration to IS was found to be linear with a regression coefficient R^2=0.999 for all three analytes. The LOD and LOQ were determined using standard deviation of the blank response divided by the slope of the calibration curve multiplied by 3 or 10, respectively.

2.3.4 Accuracy and Precision

Table 2.2. Linearity, LOD, LOQ, and Matrix Effects Percentage[a]

Analyte	Linearity (R^2)	LOD (nM)	LOQ (nM)	Matrix Effects (%)
CML	0.999	1.07	3.58	7.48
CEL	0.999	0.494	1.65	8.99
Lysine	0.999	0.345	1.15	6.33

[a] Validated method utilized (*n=6*).
CML: Nε-carboxymethyl-lysine (CML); CEL: Nε-carboxyethyl-lysine (CEL).
LOQ: Limit of quantification (10*Standard Deviation $_{\text{blank response}}$/Slope); LOD: Limit of detection (3*Standard Deviation $_{\text{blank response}}$/Slope).
Matrix effects calculated from ratio of ((IS area in matrix/IS area in blank sample)-1)*100.
IS: Internal standard.

The sample accuracy and precision were evaluated at two different concentrations of spiked analyte (low and high) as reported in **Table 2.3**. Intra-assay accuracy ranged between 113% and 116% for CML, 93.7% and 97.4% for CEL, and 94.2% and 102% for lysine. All analyte intra-assay accuracy results are in the 80-120% range, which is acceptable for EMA and US FDA bioanalytical guidelines. Each analyte precision was below the EMA and US FDA threshold of 15%. Inter-assay accuracy ranged between 92.8% and 102% for CML, 93.5% and 94.4% for CEL, and 93.4% and 98.8% for lysine. These intra-assay accuracy results are also in the acceptable range for EMA and US FDA bioanalytical guidelines, as is the inter-assay precision.

Table 2.3. Intra- and Inter-assay Method Validation

Analyte	Intra-Assay (n=6)[a]				Inter-Assay (n=4)[b]			
	Accuracy (% of target)		Precision (RSD %)		Accuracy (% of target)		Precision (RSD %)	
	Low	High	Low	High	Low	High	Low	High
CML	113	116	4.26	4.47	92.8	102	12.69	12.2
CEL	97.4	93.7	14.3	1.87	93.5	94.4	5.25	3.58
Lysine	94.2	102	3.36	1.27	93.4	98.8	8.14	7.06

[a] Intra-assay (six repeated analyses within one experiment during 20 h with samples stored in sample manager).

[b] Inter-assay (three independent experiments within 2 days) validation results. Concentration Low: ten-times endogenous levels, High: forty-times endogenous levels for all analytes.

2.3.5 Selectivity

All three analytes were successfully detectable in all blood samples from five healthy controls assayed in biological duplicate, (**Figure 2.4).** Lysine retention time eluted at 8.83 minutes (**Figure 2.4A**), CML eluted at 6.79 minutes (**Figure 2.4B**) and CEL eluted at 8.82 minutes (**Figure 2.4C**), all with their respective IS.

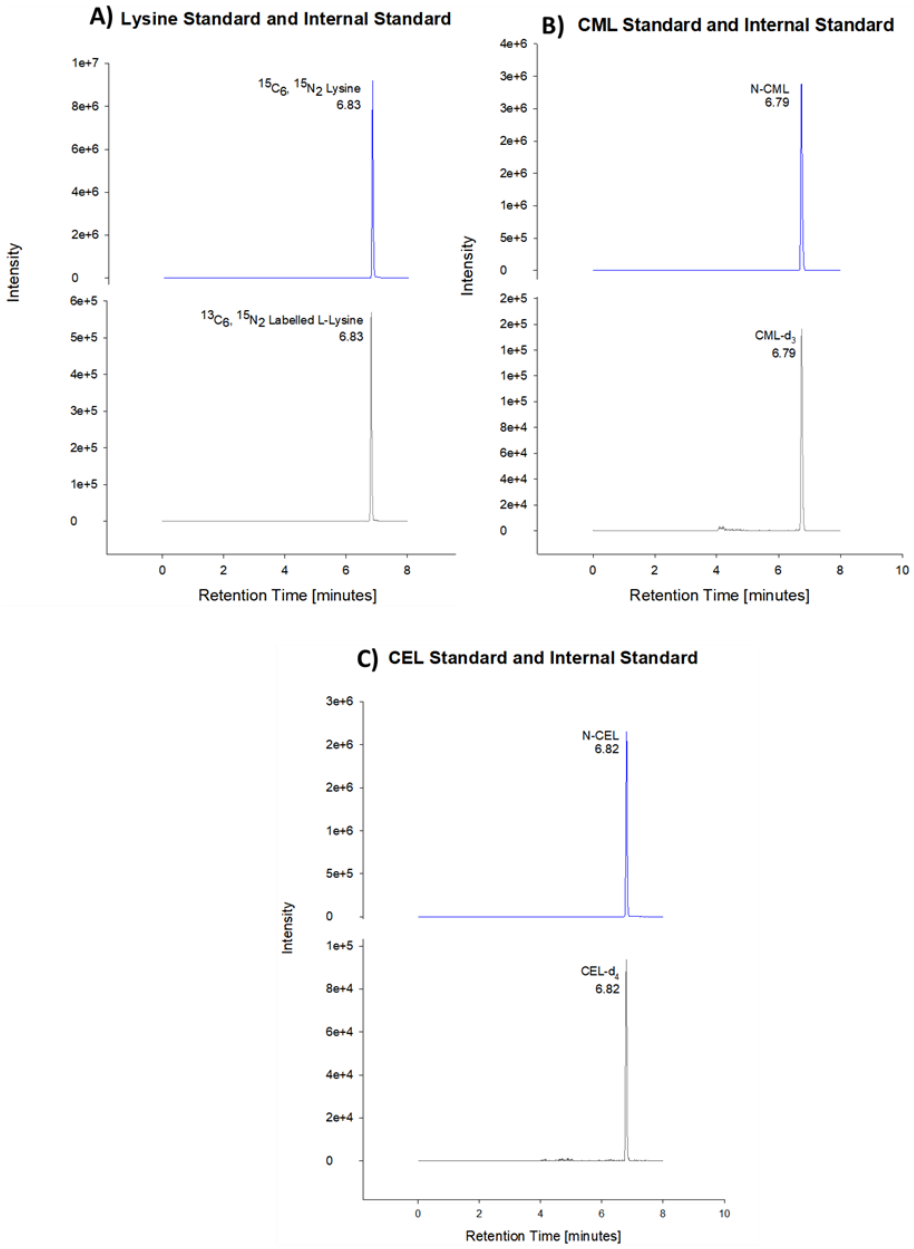

Figure 2.4. Chromatograms for analyte standards and internal standards showing excellent selectivity of LC-MS/MS method. A) Lysine chromatogram for 515 nM calibration curve. Above standard lysine eluting at 6.83 minutes with good peak shape and limited tailing. Below labeled lysine internal standard eluting at the same time of 6.83 minutes with good peak shape and limited tailing. B) CML chromatogram for 515 nM calibration curve. Above standard CML eluting at 6.79 minutes with good peak shape and limited tailing. Below labeled CML eluting at the same time of 6.79 minutes with good peak shape and limited tailing. C) CEL chromatogram for 515 nM calibration curve. Above standard CEL eluting at 6.82 minutes with good peak shape and limited tailing. Below labeled CEL eluting at the same time of 6.82 minutes with good peak shape and limited tailing.

2.3.6 Absolute Quantification of CML, CEL, and Lysine

CML, CEL, and lysine levels were identified and quantified in each unknown blood sample (**Figures 2.5-2.7**). Each figure represents two solutions, AS-1 (blue) on the left and AS-1N (gray) on the right.

For CML, there were no significant differences found between AS-1 and AS-1N, p=0.466 (**Figure 2.5).**

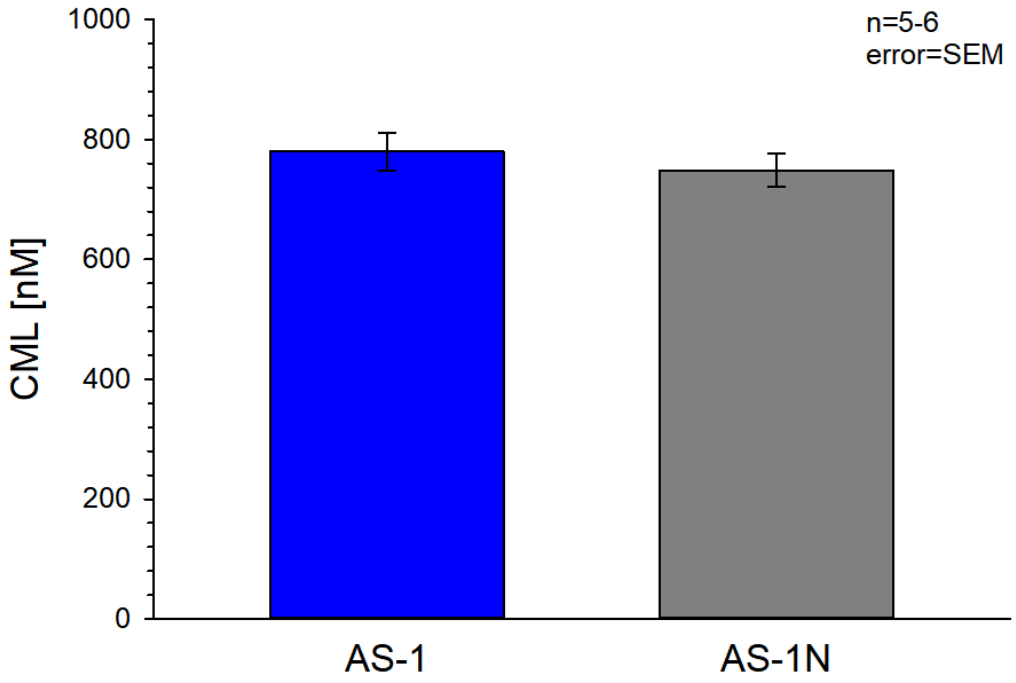

Figure 2.5. CML (nM) quantification for both AS-1 and AS-1N storage solutions for day 1 of storage. There was no statistically significant difference between AS-1 (779.9 nM ± 31.2) and AS-1N (748.8 nM ± 27.6) p= 0.466. n=5-6, error=SEM.

For CEL, there were no significant differences found between AS-1 and AS-1N, p=0.369 (**Figure 2.6**).

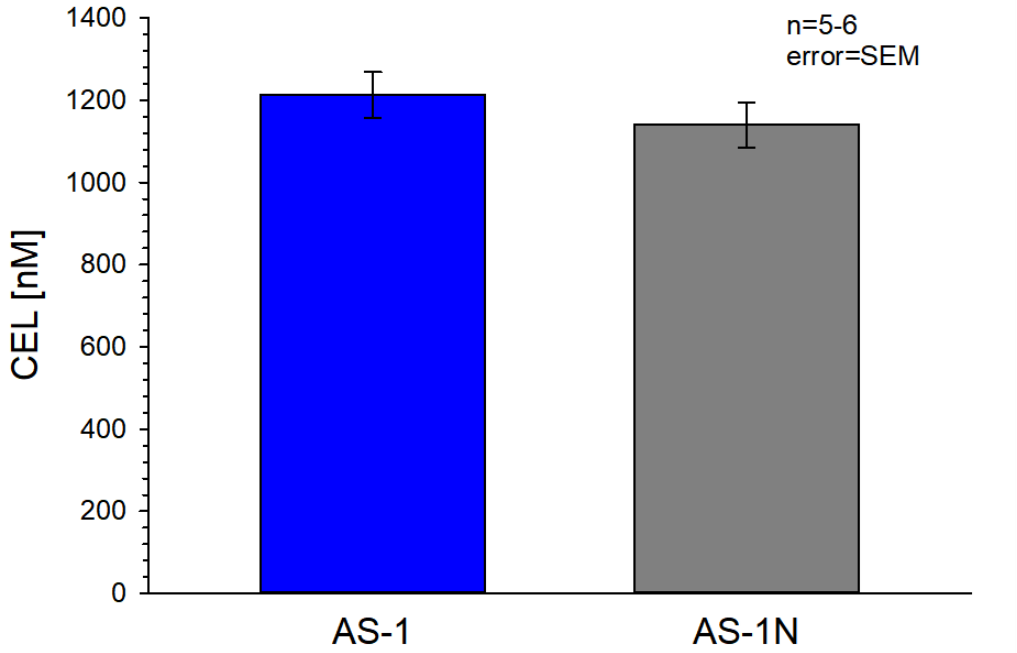

Figure 2.6. CEL (nM) quantification for both AS-1 and AS-1N storage solutions for day 1 of storage. There was no statistically significant difference between AS-1 (1212.6 nM ± 56.1) and AS-1N (1139.7 nM ± 54.3) p= 0.369. n=5-6, error=SEM.

For lysine, there was no significant differences found between AS-1 and AS-1N, p=0.451 (**Figure 2.7).**

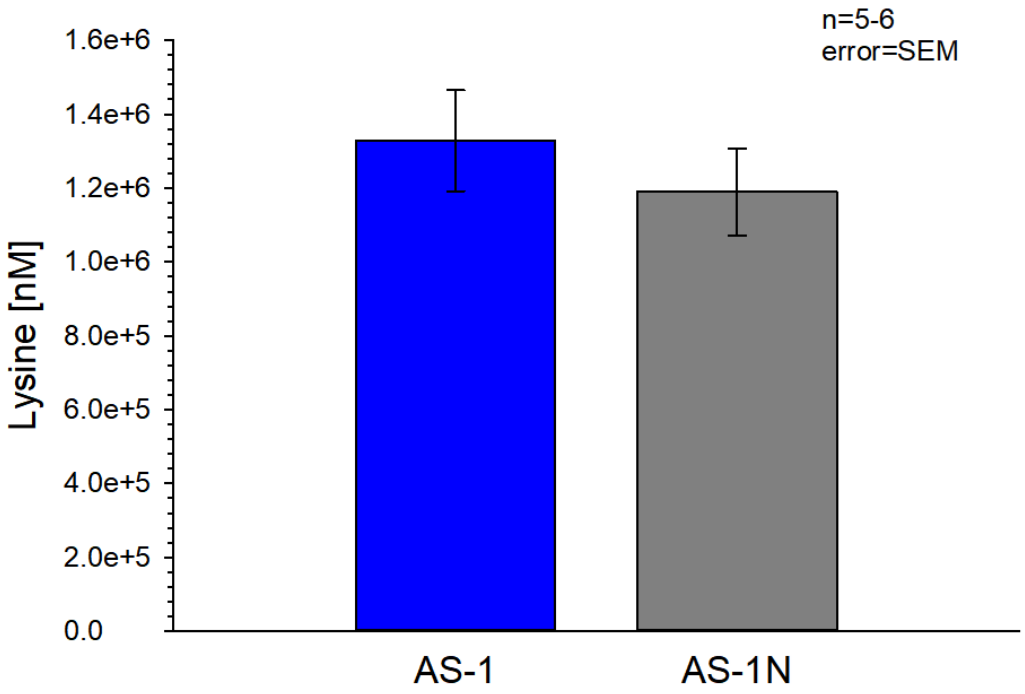

Figure 2.7. Lysine (nM) quantification for both AS-1 and AS-1N storage solutions for day 1 of storage. There was no statistically significant difference between AS-1 (1328734.8 nM ± 136593.5) and AS-1N (1189683.9 ± 118062.7) p= 0.598. n=5-6, error=SEM.

2.4 Discussion

This study includes a new protocol with high accuracy and precision for the quantification of two types of AGEs using UPLC-MS/MS. In the present study, UPLC-MS/MS with MRM was highly useful for the measurement of AGEs in blood samples. Specifically, this method was used to report the concentrations of CML, CEL, and lysine in two different blood samples on day 1 of storage. This study focused on CML and CEL without the addition of common byproducts because these are the most abundant and widely studied AGEs related to complications associated with diabetes. Due to the hyperglycemic storage conditions used in RBC storage for transfusion medicine, it was anticipated that the stored RBCs would also exhibit AGE formation. There is

limited research for detecting and quantifying both CML and CEL, and almost no present research involving stored blood. Current methods of detecting and quantifying AGEs include ELISAs, which use antibodies and are often limited to reliable types of antibodies and decreased sensitivity.[22] Current ELISA kit assays are not selective enough for only CML or CEL without other glycation mechanism byproducts. Other types of analytical methods include time-consuming immunohistochemical detection, fluorescence spectroscopy and size exclusion chromatography with fluorescence detection.[25–27] Thus, this work showed a reliable, sensitive, specific, and reproducible method to detect CML, CEL and lysine in human blood samples.

The bioanalytical method validation by the EMA provides guidance and recommendations for bioanalytical assays, which can be seen as the "gold standard" in other types of method development.[52] By using the FDA approved guideline M10 by the ICH, this study used sample analysis recommended chemical, biological, and metabolite drug guidelines to explore various analytical parameters, such as LOD, LOQ, matrix effects, intra- and inter-assay accuracy and precision, and matrix interferences. The EMA bioanalytical method validation guidance provides clear acceptance and reliability for biological assays and analytical results.

To date, most reports reporting AGE formation have primarily focused on AGEs in food, biological plasma or serum, or human tissues (retina, kidney, endothelial and smooth muscle cells).[24,27,29,31,53–58] Increased protein glycation has been reported in clinical studies involving people with diabetes, and it is linked to various complications associated with increased AGEs and oxidative stress.[19,59–61] Although glycation occurs on most cell types, AGE detection of bloodstream components may provide insight into increased pathologic conditions.[44,62] For example, glycated albumin (gHSA) has been shown to influence delivery of biologically active

peptides to healthy RBCs, while that same delivery was reduced in RBCs from people with diabetes.[63,64]

Through this process, our results showed acceptable linearity, LOD, and LOQ. The matrix effects did not contribute to chromatographic signals, which is a major concern for biological samples. There was clear specificity, peak separation, and distinguishable IS correlation to the analyte detected. The intra- and inter-assay results showed EMA and US FDA bioanalytical guideline acceptable accuracy and precision. Overall, the results showed this methodology to detect and quantify CML, CEL, and lysine in stored blood solutions can be used in other analyses involving suspected AGE formation on blood components.

Here, there were no statistically significant differences in the two blood storage solutions on day 1 of storage. Even though there were no statistically significant differences in the two blood storage solutions at day 1 (freshly drawn blood), the novel method confirms the potential for more studies involving longer storage times and diabetic patients to closely study AGE formation. AGEs are typically formed over longer periods of time than 4 hours of storage, but a comprehensive analysis can be a future project using this novel methodology. Also, it is unclear if other AGEs, such as CML/CEL byproducts, could increase oxidative stress and associated adverse effects to RBCs as seen in storage lesions. Storage lesions are a known issue in blood storage, the extent of AGEs causing adverse post-transfusion related complications and how longer storage time can change the amount of CML and CEL on RBCs is without precedent. A future direction utilizing this novel methodology can explore the 6-week blood storage time and the overall change in AGEs.

AGEs are known to be related to the pathomechanism of diabetes and other degenerative disorders.[39,42,61] This study involved utilizing blood from healthy donors to explore methodology that can reliably and accurately detect and measure AGEs, while maintaining sensitivity and

bioanalytical merits to other reports using different instruments or assays. In this chapter, the detection and quantification of CML, CEL, and lysine present on stored RBCs, without significant issues of matrix effects and with analyte recovery, was successfully confirmed. The purpose of this small initial study was to validate the robustness of the biomarkers and provide a pilot study for future blood banking AGE research. The data encourages further investigation of the accumulation of AGEs. Specifically, using this method to expand freshly drawn blood to stored blood over 42 days and evaluate the change of AGEs on a weekly basis (chapter 3). This method will be key in future blood banking studies in this dissertation to evaluate the difference in hyperglycemic and normoglycemic blood storage conditions and the overall glycation process of RBCs.

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Chapter 3: Weekly Blood Storage Evaluation for 43 Days

3.1 Introduction

3.1.1 Background

In times of war, natural disasters, and worldwide pandemics, there is typically a blood shortage because donation rates drop as much as 40%, yet the demand for transfusions remains constant or even increases.[1,2] It is imperative that donors are screened to prevent transfusion complications such as disease transmissions, alloimmunization, and other possible fatal occurrences.[3,4] However, screening donor blood for such issues was not always a priority before the early twentieth century, when little was known about blood typing and blood borne pathogens.[5,6] The lack of knowledge concerning blood types, Rh factor (a protein on red blood cells to determine positive or negative blood type), and blood diseases lead to mismatched transfusions and transfusion-related reactions and transferring of significant diseases.[5,6] Advancements in blood component separation techniques allowed for the preparation of specific blood products, such as red blood cells (RBCs), platelets, and plasma, allowing for safer and more personalized patient care.[7] The human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) epidemic in the 1980s led to a heightened focus on blood safety and the implementation of strict screening and testing protocols for blood donors.[8–10]

The development of blood substitutes and artificial blood has been an ongoing area of research to address blood shortages and increase blood availability.[11–13] Today, blood donation and storage are crucial focal points of modern healthcare systems worldwide. Blood banks and donation centers play a pivotal role in ensuring a safe and sufficient blood supply for medical treatments, surgeries, and emergency situations.[11,14,15] Research and advancements in blood collection,

storage, and transfusion practices continue to improve patient outcomes and reduce mortality rates.[16–18]

The Food and Drug Administration (FDA) has approved four additive storage solutions to extend the shelf-life and improve stored RBCs.[19,20] However, all current FDA approved additive solutions exhibit glucose concentrations that are at least 5 times greater than concentrations found *in vivo* (5-6 mM).[21,22] Reports in the literature suggest that storage of RBCs for more than 14 days could be linked to incidents of adverse outcomes after cardiac related surgery, and it is common practice to only transfuse blood before 5-8 days of storage for neonate patients to limit the onset of adverse reactions.[23–25]

There is also evidence that supports blood storage causes irreversible metabolic and physiological damages to the RBCs known as the red cell storage lesion.[26,27] As previously discussed in chapter 1, these physical and metabolic changes that occur to blood components during the typical 42 day storage period compromise the health of the RBC, and can lead to adverse effects when transfused.[14,27–31] Storage lesion markers call into question the overall safety and efficacy of stored RBCs transfused into a patient.[32] Blood banks and healthcare facilities follow strict guidelines for blood storage and use blood components with optimal storage conditions to minimize storage lesions and preserve RBC quality.[7,33,34] Monitoring the quality and functionality of stored RBCs, including hemolysis, and adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (DPG) concentrations, is crucial to ensure safe and effective transfusions for patient recipients.[17,34,35]

Previously, the Spence group reported that normoglycemic storage increases stored RBC ATP release, reduces oxidative stress, osmotic fragility, while increasing deformability, as discussed in chapter 1.[36,37] Normoglycemic storage has been shown to improve these adverse

effects, but the storage conditions were performed via manual "feeding" techniques while RBCs were stored in Eppendorf tubes. As shown in **Figure 3.1**, RBCs stored in normoglycemic conditions maintained initial ATP release levels throughout the storage duration, while RBCs stored in hyperglycemic conditions exhibited significantly decreased ATP release, typical of the storage lesion.[37] Perhaps not surprisingly, deformability measurements displayed a similar trend, with normoglycemic stored RBCs maintaining their initial relative deformability throughout storage, while hyperglycemic stored RBCs became more rigid overtime, a known inhibition of ATP release.[36–38] Overall, these results highlighted a key relationship between time of storage and damage permanence, specifically the importance of days 8, 12, and 15 for ATP release and deformability, indicating irreversible damages occurring to stored RBCs in hyperglycemic conditions during this time frame. It is expected that both time and type of storage will affect the results, so it will be important to examine the statistical relationship to determine the total interaction effects as well as individual variable effects.

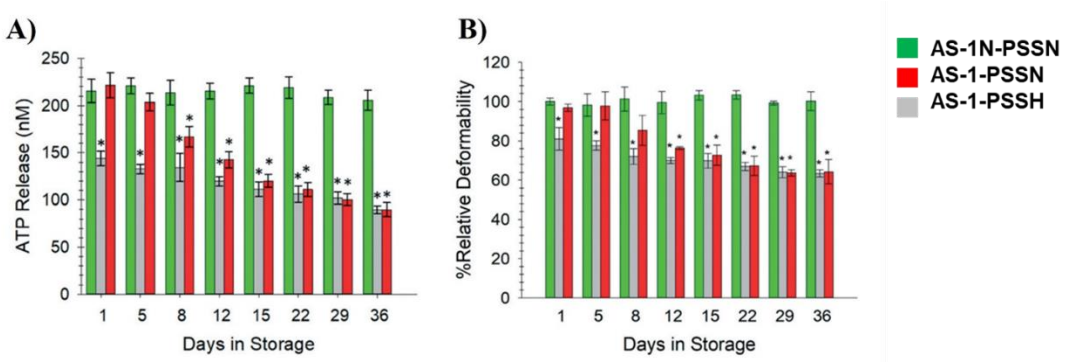

Figure 3.1. RBC release of ATP and relative deformability. A. Flow-induced release of ATP from ERYs stored in various additive solutions and transferred to various buffer systems prior to introduction to the fluidic device. This data suggests that the FDA-approved AS-1 storage solution may be damaging to the stored RBCs and that the damage is non-reversible after two weeks of storage. Error bars are ±SEM, n = 6 for all, *p <0.05 to AS-1N–PSSN day 1. B. AS-1N–PSSN samples possessed the highest deformability and maintained a stable level throughout 36 days of storage. Deformability of RBCs in AS-1–PSSN samples were able to completely recover (day 8) to a normal level of deformability or were no longer able to recover (beyond day 12) upon transfer to PSSN. Error bars are ± SEM, n ≥ 3, *p < 0.05 to AS-1N–PSSN day 1. Borrowed from Liu, Y. et. al.[37]

A major limitation of previous normoglycemic storage methods resides in the glucose utilization by the RBCs throughout storage. Without the addition of glucose throughout storage, normoglycemic stored RBCs become hypoglycemic after only 24 hours. This required a manual "feeding" of glucose to normoglycemic stored RBCs to maintain physiologically relevant glucose levels throughout storage. The stored RBC samples made up in 2 mL Eppendorf tubes were transferred to cut polyvinyl chloride (PVC) bags (ULINE, Pleasant Prairie WI) and sealed for storage with a heat sealer. The AS-1N samples were "fed" using 20 μL of 400 mM glucose in saline every 3 days. The AS-1 samples were not "fed" and were only opened to remove sample volumes. These conditions, however, were not equivalent and thus introduced a potentially confounding variable that could cause differences in the results.

3.1.2 Innovations

Although this initial normoglycemic storage approach was successful in alleviating storage lesion markers, it was not ideal for future application. The manual feeding involved opening the stored RBC bag, removing a small volume to measure the glucose, determining how much volume of a concentrated glucose solution was required to maintain physiological levels, and then injecting the stored RBC bag with said amount of glucose solution (**Equation 5**).

$$(G \times V_i) + (GS \times V_1) = (DC \times (V_1 + V_i)) \qquad \textbf{(5)}$$

G = Initial Glucose Concentration [mM]
V_i = Initial Volume [μL]
GS = Concentration of Glucose Solution [mM]
V_1 = Initial Volume [μL]
DC = Desired Glucose Concentration [mM]

This breach of sterility did not pose an issue for the initial studies because no transfusion was being performed; rather, the studies were to generate preliminary data providing evidence that normoglycemic storage with feeding reduced storage lesion markers. However, it cannot be reasonably translated to current practices, as it breaches sterility and requires unreasonable labor efforts. Additionally, the small-scale storage (less than 5 mL) with in-house fabricated PVC bags may not illicit similar results in comparison to commercially available blood storage bags (380-400 mL of RBCs and additive solution). Thus, for the importance of translational clinical practices, it was critical to utilize a feeding system that not only maintained sterility but one that could be applied to more realistic blood banking laboratory practices, which would include full autonomy in its feeding method. Therefore, an autonomous feeding device was designed and utilized to maintain normoglycemic levels in an autonomous system (**Figure 3.2**).

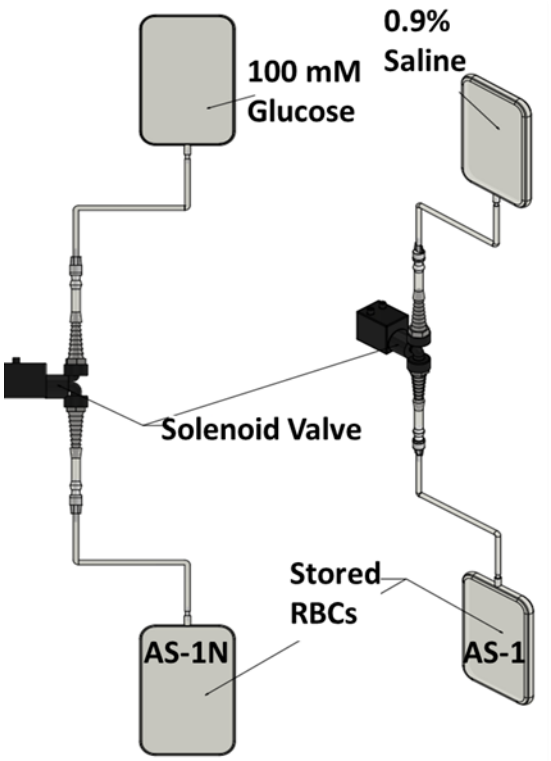

Figure 3.2. CAD drawing of automated glucose feeding system for hyperglycemic and normoglycemic stored RBCs. The pressure difference created between the saline/100 mM glucose bag and the stored RBC bag is driven by gravity, due to the 90 cm difference in height between the two. When the valve is closed, there is no fluid flow since the valve's inability to expand exerts an equal pressure on the fluid. However, when it is opened, the pressure differential is realized since the stored RBC bag is expandable. This drives fluid flow into the stored RBCs, thus allowing feeding volume and frequency to be controlled by the opening and closing of the solenoid valve. Borrowed from Soule, L. et. al., (*in review*).

Previous Spence lab members had developed the AS-1N additive solution by modification of the FDA approved solution AS-1, changing only the glucose concentration to 5.5 mM for normoglycemic conditions. However, the method used to sterilize the additive solutions was problematic. Previously, the d-glucose was added to the CPD/CPD-N and AS-1/AS-1N before autoclaving, which would turn the solutions a slight yellow color after sterilization was complete.

The heat to sterilize the solutions is important for practical applications regarding FDA protocols for *in vivo* infusion solutions limit bacteria and cell lysis. When the previous solutions were heated, the color change, even slight, indicates degradation, which led to a burnt sugar or caramel smell often associated with the Maillard reaction. This process is hypothesized to increase the likelihood of the formation of early and late AGEs, which would be problematic for the study of AGEs during storage.[39] In one report, the authors tested different autoclaving temperatures and varying glucose concentrations from different manufacturers and used liquid chromatography with tandem mass spectrometry (LC-MS/MS) to detect and quantify different glucose degradation products (GPDs).[40] Leitzen, S. et. al found there are significant differences associated with increased glucose concentration and autoclave temperature and increased GPDs, including glyoxal (GO) and methylglyoxal (MGO).[40]

Both GO and MGO are intermediates that form AGEs.[40] Based on the standard European Pharmacopoeia steam-sterilization method solutions, must be heated to at least 121°C for 15 minutes.[40] It is possible that increased GDP products in a glucose solution administered through intravenous application can increase the formation of AGEs on the blood vessel walls.[41] The extent of increased parenteral GPD solution administration research needs to be further investigated, but it is important to understand the risks of autoclaving glucose solutions for transfusion practices. So, in the interest of studying the AGEs formed from only the ERYs stored in high and low glucose storage solutions overtime, it was necessary to develop a new solution preparation protocol. That method change was discussed in chapter 2 section **2.2.2**, with the addition of glucose after the solutions were autoclaved. The glucose was instead filtered via stericups and added in a sterile environment to the solutions.

3.1.3 Glutathione

Once the feeding system was developed and validated, it could be used to store normoglycemic RBCs for additional *in vitro* testing of storage lesion markers. Specifically, ones related to oxidation and AGE formation, which was measuring glutathione. Glutathione is a crucial antioxidant and plays an essential role in cellular defense against oxidative stress, as previously discussed in chapter 1.[42] Glutathione is a tripeptide composed of three amino acids: cysteine, glutamic acid, and glycine (**Figure 3.3**).[43] The cysteine amino acid contains a thiol (-SH) group, which is crucial for the biological activity of glutathione.[44] This thiol compound is present in almost all cells and is involved in various physiological processes, including detoxification, immune function, and cellular protection against reactive oxygen species (ROS) accumulation.[43,45] Its thiol group is particularly important because it can donate electrons to neutralize harmful free radicals and help maintain the cell's redox balance.[44] The thiol group in cysteine is crucial for its ability to participate in these biochemical reactions and maintain cellular homeostasis.[44] The most prominent thiol in blood is glutathione, with the majority of glutathione residing in the cytoplasm of RBCs (0.4-3 mM).[46,47] Glutathione is present in relatively high concentrations in RBCs, where it serves as an important antioxidant and plays a vital role in protecting the cells from oxidative damage.[47] Due to its prominent role in antioxidant defense, glutathione level is often used as a key indicator of oxidative stress within cell types and was chosen for this study as an indirect measurement of oxidation in stored RBCs in various storage conditions.

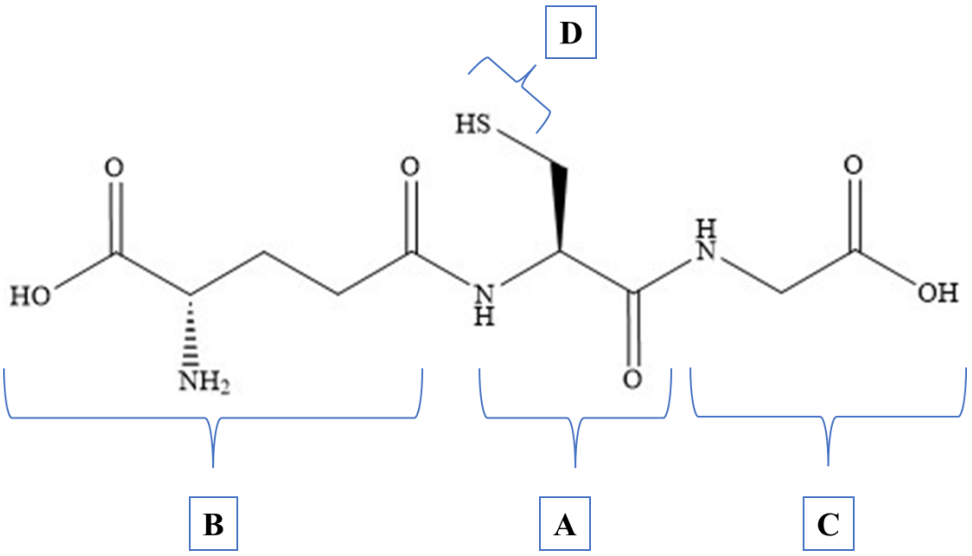

Figure 3.3. Glutathione molecule. Glutathione consists of a (A) cysteine, (B) glutamic acid, and (C) glycine. Within the cysteine, there is a thiol group, which is crucial for biological activity (D). A thiol is a type of organic functional group that consists of a sulfur atom bonded to a hydrogen atom. The sulfur atom in a thiol is also bonded to a carbon atom, making it part of an organic molecule. The general chemical structure of a thiol is R-SH, where R represents an organic group, which can vary and affect the specific functional properties of the thiol. Non-protein thiols are thiol-containing compounds that are not part of a protein's structure but are found in various biological and chemical contexts. These compounds contain a sulfur atom bonded to a hydrogen atom and are often involved in redox reactions, detoxification, and other cellular processes.[44,47,52]

The glycolytic pathway, also known as glycolysis, is a central metabolic pathway that occurs in the cytoplasm of cells.[48] It is a series of biochemical reactions that break down one molecule of glucose (a six-carbon sugar) into two molecules of pyruvate (a three-carbon compound).[49,50] These reactions are anaerobic, meaning they do not require oxygen.[48] Glycolysis is a universal pathway found in nearly all living organisms, from bacteria to humans.[48] It serves as a fundamental mechanism for glucose utilization and energy production.[48] Depending on the availability of oxygen, the fate of the pyruvate produced in glycolysis can vary.[49] In the absence

of oxygen, pyruvate can be converted into lactate or ethanol (fermentation) to regenerate nicotinamide adenine dinucleotide (NAD^+) for continued glycolytic activity.[49]

The polyol pathway, also known as the sorbitol-aldose reductase pathway or polyol-oxidative pathway, is a metabolic pathway in which glucose is converted into sorbitol and then into fructose through a series of enzymatic reactions.[49,51–53] The polyol pathway is of interest in medical research and clinical medicine because it is implicated in the complications of diabetes mellitus.[53] Elevated levels of glucose in the blood, as reported in diabetes, can lead to increased flux through the polyol pathway.[53] Specifically, hyperglycemia causes the increase activity for the sorbitol production.[54] The most important aspect of the polyol pathway is its association with reactive oxygen species (ROS) and oxidative stress.[49] Increased activity through the polyol pathway increases production of ROS that must be removed through antioxidant pathways such as the glutathione pathway.[49,54]

In the glutathione pathway, the conversion of glucose to sorbitol within cells can result in osmotic stress and damage due to the accumulation of sorbitol.[52,53] The osmotic stress and cellular damage associated with the polyol pathway are thought to contribute to various diabetic complications, such as cataracts (in the lens of the eye), neuropathy (damage to peripheral nerves), and nephropathy (kidney damage).[42,52,55–58] As a result, research efforts have focused on developing drugs that inhibit enzymes in the polyol pathway as a potential therapeutic approach to mitigate these complications in diabetic patients.[51]

By means of a selenium-dependent enzyme catalyzed process, glutathione peroxidase (GPx) facilitates the transfer of hydrogen from GSH to extremely reactive ROS, leading to their transformation into less reactive substances.[58,59] During these reactions, two GSH molecules come together through a disulfide linkage to produce oxidized glutathione (GSSG).[58,59] GSH is the active

and antioxidant form of glutathione, while GSSG is produced as GSH neutralizes ROS.[52,60] GSH is synthesized from cysteine and glutamate, by γ-glutamylcysteine synthase (GCS), which forms γ-glutamylcysteine.[52] This reaction is catalyzed by glutathione synthase (GS) and the addition of glycine.[52] This conversion of GSH to GSSG is an essential part of the antioxidant process.[61,62] To maintain an effective antioxidant defense, GSSG must be recycled back to GSH.[61] This regeneration process is catalyzed by glutathione reductase, which uses nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor.[52,53] The balance between GSH and GSSG plays a crucial role in maintaining cellular redox balance and protecting cells from oxidative damage.[42] Measuring the GSH/GSSG ratio in cells and tissues is of clinical importance in assessing oxidative stress and certain diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases.[42,55,57] The ratio of GSH to GSSG is an important indicator of the cell's redox state.[55,63] An elevated GSH/GSSG ratio is a sign of a reducing, healthy cellular environment, while a decreased ratio may indicate oxidative stress.[58,63,64] However, each of these components can be measured and evaluated separately, as seen in this chapter. There are no studies directly connecting normoglycemic blood storage, AGE formation, and changes in GSH levels, so these will be the subject of this chapter.

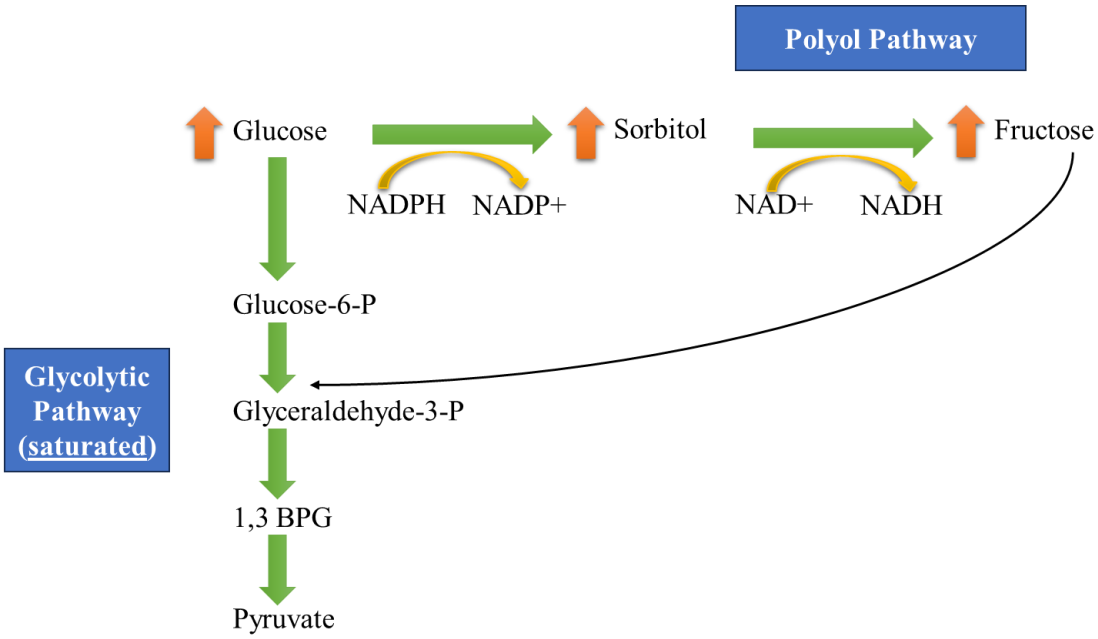

Figure 3.4. Glycolysis pathways. The favorable glycolysis pathway converts glucose to glucose-6-phosphate (Glucose-6-P), then to glyceraldehyde-3-phosphate (Glyceraldehyde-3-P), then 1, 3-bisphosphoglycerate (1,3 BPG), and finally pyruvate. In hyperglycemic environments, the glycolytic pathway will become saturated with excess glucose and the polyol pathway will increase to supplement the glycolysis. The initial step of the polyol pathway involves the reduction of glucose to sorbitol. This reaction is catalyzed by the enzyme aldose reductase. Sorbitol is then oxidized and converted into fructose by the enzyme sorbitol dehydrogenase. The conversion to fructose increases the NADP+/NADPH and thus is used for the conversion of Glyceraldehyde-3-P to 1,3 BPG and finally pyruvate. [48,49,52,53]

There are current methods for measuring free GSH, which can be used on the RBC membrane for the two types of storage solutions, including using commercially available fluorometric detection kits (Cayman Chemical, Ann Arbor, MI). AS-1 is a hyperglycemic storage solution, therefore it is anticipated that RBCs stored in AS-1 will exhibit low levels of free GSH over the course of 6-weeks and remain lower than RBCs stored in AS-1N. It is expected that both time and storage condition will affect the results, so it will be important to examine both variables to determine the interaction between the two.

3.1.4 Troubleshooting: Tubes Versus Bags

Initial blood storage methods involved storing the RBCs in 50 mL conical tubes with a septum cap (**Figure 3.5**). This sample vessel was chosen due to the limited supply of commercial blood storage bags due to a COVID-19 supply shortage and because the septum cap allowed sample removal and glucose addition without opening the entire system. For experimental purposes, it was important to keep the samples in a closed environment to mimic clinical blood bag storage sterility requirements, so the idea of utilizing septa caps was implemented. The septa caps were appealing because a small needle and syringe could be used to both "feed" and extract the sample without completely opening the tube caps. After further investigation and analysis, it was determined that the tube caps were faulty and therefore led to many leaks, increased cell lysis and possible bacterial contamination. Although this design was unsuccessful, it led to necessary optimization and experimental knowledge that was useful for the implementation of commercial animal veterinary blood bags with an automated "feeding" regime. Both methods and results will be described and differences in methodology will be highlighted.

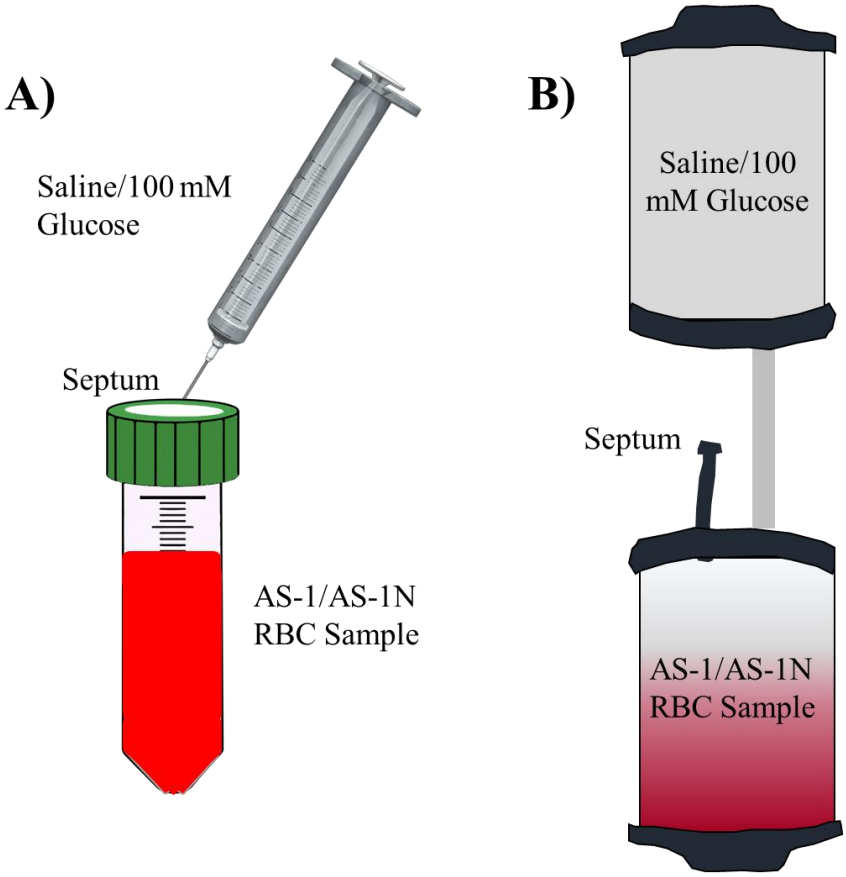

Figure 3.5. Blood banking troubleshooting using tubes and animal blood bags. **A**. 50 mL conical tubes with septa inserted screw top caps. Saline or 100 mM glucose solution could be "fed" using a syringe and needle by piercing the septum and depositing the solution. Samples were removed using the same system. **B**. Automated closed "feeding" system using 150 mL animal blood bags and tubing to distribute saline or 100 mM glucose solution directly into the bag when the valves were open. The bag system was kept sterile and completely closed and samples were removed via the septum at the bottom of the external tubing sampling port using a syringe and needle.

3.2 Methods

3.2.1 Sample Preparation

All storage solutions and RBC collection were prepared in a manner identical to those reported in

2.2.1. and **2.2.2.**

3.2.2 RBC Storage for Blood Bags

The plasma and buffy coat were removed via aspiration, and the pRBCs were placed into a 15 mL tube. The total volume of pRBCs was added to a 50 mL conical tube containing additive solution in a 1:2 ratio (e.g., 7 mL AS-1 and 14 mL RBCs). The 50 mL conical tubes were inverted and mixed thoroughly before being placed in either the prepared and sterilized blood storage tubes or bags. The samples were placed in the refrigerator at 2-6℃ for 1-2 hours before analysis of the blood glucose concentration to ensure it was 5.5 mM concentration. If the blood glucose concentration was below 5.5 mM, a 1 mL syringe and 22-gauge needle were used to deliver the appropriate amount of 100- or 400-mM glucose (**Equation 5**).

3.2.3 Glucose Concentration and Hematocrit Percentage

All glucose concentrations and hematocrit % were collected in a manner identical to those reported in **2.2.3.**

3.2.4 Preparation of Stored Blood Using 50 mL Conical Tubes with Septa Caps

The 50 mL conical tubes with septum screw caps (Syringa Lab Supplies, Boise, ID) were UV sterilized in the biosafety cabinet for 24 hours before use. The stored blood with additive solution was placed into the tube by removing the cap and pouring in the blood solution (12-23 mL). The tubes were kept with the caps on and stored at 4°C until use. The glucose was checked every 3 days using a 1 mL syringe and 22-gauge needle and removing approximately 10 µL of sample by piercing the septum. The sample glucose was analyzed by using a glucometer and strips as per manufacturer instructions. The sample was placed on the glucometer strips using the 1 mL syringe and 22-gauge needle, instead of using the conventional lancet approach by sticking a finger and using the blood drop to apply to the glucometer strip. The AS-1N sample was “fed” manually by calculating the necessary addition of 400 mM glucose solution to bring the sample glucose

concentration to 5.5 mM (**Equation 5**). The AS-1N sample was placed into the biosafety cabinet and the calculated volume was dispensed using the sterilized 400 mM glucose in 0.9% saline solution. The same amount of 0.9% saline solution was "fed" to the AS-1 sample for volume and sample preparation continuity. The hematocrit % was checked each week and utilized for acid hydrolysis using HematoSpin. The caps of the tubes were parafilmed to help with the leaking and contamination issue, although it was unsuccessful. The sample was removed from the tubes on day 1, 5, 8, 12, 15, 18, 22, 29, and 36 for the subsequent assays and sample preparation.

3.2.5 Acid Hydrolysis and SpeedVac Preparation

All acid hydrolysis and speedvac sample preparation were prepared in a manner identical to those reported in **2.2.5.**

3.2.6 Glutathione Assay Preparation and Optimization

The Thiol Detection Assay kit (Cayman Chemical, Ann Arbor, MI) was used as per the directions of the kit for cell lysate sample type. On the day of the scheduled blood experiments (as described above), 350 µL was allocated for the thiol assay. Aliquoted samples were stored at 4°C for one hour and then centrifuged at 2000*g* for 10 minutes. The supernatant was removed and 1 mL of cold diluted buffer (from thiol kit) was added. To ensure pRBCs were fully lysed, the RBCs were homogenized using a pestle (Fisher Scientific, Hampton, NH) and disposable mortar (VWR, Radnor, PA) for approximately 30 seconds, and the remaining samples were centrifuged at 10,000*g* for 10 minutes at 4°C. To promote maximum thiol retention from the samples, the supernatant was removed and placed into a 10 kDa MWCO Spin Filters device (Sigma Aldrich, St. Louis, MO) and centrifuged at 14,000*g* for 30 minutes at room temperature. The filtrate was collected and stored at -80°C until used. For the thiol assay kit, both types of standards (Glutathione and Cysteine) were assessed, and sample concentration was optimized to determine

the best dilutions. The Glutathione standards were used, and all results were analyzed as free reduced glutathione (GSH) concentration.

3.2.7 LC-MS/MS Sample Preparation and Usage

All LC-MS/MS preparation and instrument usage were prepared in a manner identical to those reported in **2.2.6.** and **2.2.7.**

3.2.8 Cell Lysis

A cell lysis assay was utilized to determine the level of hemolysis in both storage conditions, AS-1 and AS-1N, and in both vessels of storage (tubes and blood bags). A 400 µL volume of sample was removed from the tubes/bags. The sample was divided into 300 µL for cell lysis separation and 100 µL for hematocrit measurement and glucose determination. Hematocrit and glucose were determined using methods described (**methods 3.2.3**). The 300 µL aliquot was centrifuged at 500*g* for 10 minutes at room temperature and the supernatant was removed and stored at -20°C until analysis. The remaining pRBC sample was spun down at 2000*g* for 15 minutes at room temperature and 80 µL of pRBCs was removed and stored at -20 °C until analysis. On the day of the experiment, 72 mg of human hemoglobin (Sigma Aldrich) were added to a 15 mL tube. A 10 mL volume of Drabkin's reagent solution (Sigma Aldrich) was added to the hemoglobin tube and thoroughly mixed. This solution was used to make hemoglobin standards in the range of 0-0.8 g/L. The sample supernatant was diluted 1:10 in Drabkin's solution and the pRBCs were diluted 1:1000 in Drabkin's solution to fall within the range of the standards. Then, 200 µL of each standard or sample was added to a clear 96-well plate, gently mixed and then covered for 15 minutes. The absorbance was measured at 540 nm. Total cell lysis could then be determined using total hemoglobin levels in the supernatant and cell pellets. The cell lysis was determined using **Equation 6.**

$$Cell\ Lysis\ \% = \frac{[HbSN] \times [100 - Hematocrit]}{[HbSN] + [HbRBCs]} \qquad \textbf{(6)}$$

HbSN = Hemoglobin in Supernatant [g/L]
HbRBCs = Hemoglobin in packed red blood cells [g/L]

3.2.9 Blood Agar Plate

Blood agar plate steaking was used to determine the amount of bacterial contamination, specifically hemolytic bacterial contamination. The 5% sheep blood in tryptic soy agar plates (VWR) were removed from cold storage (4°C) and placed into an incubator (Troemner, Thorofare, New Jersey) to warm up. The work area was prepared by spraying with 70% ethanol/water and obtaining a Bunsen burner and match. Once the agar plate had equilibrated to approximately 37°C, it was removed from the incubator and placed in the prepared work area. The 22-gauge needle and 1 mL syringe were sterilized by holding near the flame of the burner for a few seconds, and then used to extract approximately 10 µL of blood sample through the septum of the conical tube cap. The agar plate was uncovered near the flame and the blood was injected onto the edge of the petri dish. The inoculating loop was sterilized using the flame and it was cooled before carefully streaking the sample in a zig-zag pattern, changing the directions for 4 zones on the plate. The agar plate was covered and placed upside down in a 37°C incubator and monitored culture growth each day for a week. By visual inspection, the bacterial growth was monitored (**Figure 3.7**).

3.2.10 Optimization of Valve System and Preparation of Sterilized "Feeding" Solutions

The valves were set up (**Figure 3.2**) and the 100-150 mL blood collection bags were filled with water and attached to the "feeding" system. An Arduino Uno microcontroller was used to control the valves and set up for automatic dispensing by Spence group member Logan Soule.

Empty Eppendorf tubes were weighed, and then water was dispensed from the valve system using various valve opening times (25-2000 milliseconds), and then weighed again. The mass difference was used to determine the volume dispensed at each valve opening period and then used to create a standard curve (**Figure 3.8**).

For proper function, the automated feeding system needed calibration to determine the appropriate amount of time to open the valve to deliver the necessary volumes of concentrated glucose to the stored RBCs. To do this, a simple mass subtraction method was utilized, dispensing the feeding solution into pre-massed sample tubes, and subtracting the mass before dispensing from the mass after dispensing to determine the volume of solution dispensed. Water was utilized as a surrogate to the 100 mM – 400 mM glucose solution since its density is 1 g/mL and easier to for calculation purposes. This calibration procedure also allowed for the determination of the lower limit of dispensing for the valve system, which dictated the glucose concentration. For example, the lower limit of dispensing for this system was determined to be 150 µL but using a 400 mM glucose feeding solution with a 150 µL dispensing volume would lead to significant glucose increases and hyperglycemia. To combat this, a lower glucose concentration feeding solution was required with greater dispensing volumes. However, dispensing greater volumes periodically throughout storage decreases the hematocrit, which would hinder the benefit of the transfusion.

So, based on maintaining a hematocrit range between 50-60% throughout the 43 days in storage while remaining above the lower limit of dispensing, the 100 mM glucose "feeding" solution was the best choice to implement into the system. Based on predicted hematocrit decreases over 42 days of feeding, the 100 mM glucose solution in 0.9% saline solution was chosen to be implemented into the automated feeding system (**Figure 3.9**). To make the 100 mM glucose "feeding" solution", 250 mL of a 0.9% NaCl solution (9g of NaCl diluted up to 1000 mL) was

added to a beaker with 4.504 g of dextrose and fully dissolved. The solution was then filter sterilized and placed into the biosafety cabinet. The remaining 0.9% NaCl solution was filter sterilized and placed into the biosafety cabinet. The solutions were added to previously prepared (**methods 3.2.1**), empty, and sterile (by UV light overnight) blood bags and closed and kept in sterile conditions until ready for "feeding" day. On the day of the experiment, a 10% bleach solution was attached to the valve system. Approximately 25 mL of 10% bleach was flowed through, allowed to sit in the system for 5 minutes, and another 25 mL of bleach was flowed. Immediately afterwards, 75 mL of sterilized water (0.22 µm stericup filtration system) was flowed through to ensure no bleach remained.

Finally, either the 100 mM glucose in 0.9% saline or 0.9% saline was attached and approximately 10 mL was delivered by opening the valve and utilizing the height and subsequent pressure difference to ensure all water was purged. This setup was then closed by attaching a luer lock cap to the end of the sterilized valve system and kept in the cold storage room until the RBC bags were ready.

3.2.11 Preparation of the Feeding Solution Regimen

Based on the total volume of the blood storage sample, the amount of glucose/saline to be periodically dispensed into the stored RBCs was determined. By utilizing the calibration curve generated previously and average glucose utilization rates calculated in preliminary experiments, the Arduino Uno was programmed to dispense an average of 300 µL every 3 days (**Figure 3.8**). The Arduino and breadboard were set up in the cold storage room and plugged in for continuous automated feeding. The blood bags were opened via the capped luer lock adapter and quickly attached to the luer lock adapter at the end of the valve system. This blood sample was considered

"Day 1" and kept in the cold storage room (4°C) attached to the automated feeding valve for 43 days.

3.2.12 Preparation of stored blood using 50-150 mL veterinarian small animal blood bags

Prior to the day of the experiment, the butterfly needle and line were removed from the PVC 50-150 mL small animal blood storage bags (Animal Blood Resources International, Stockbridge, MI) and the "feeding" line was cut and a luer lock was added with vacuum grease. The prepared empty storage bags were sterilized by UV light for 24 hours prior to adding the RBC sample. The cut "feeding" line was attached to the end of the valve system and a luer lock adapter was added to the cut end using vacuum grease, as in the sterilized valve system in the cold storage room. Blood samples from consenting donors were collected and prepared as described in **methods 3.2.2**. After the AS-1/AS-1N blood samples were prepared in the 50 mL conical tube, the blood was poured into the appropriate empty blood storage bag using the luer lock opening created and then capped with a luer lock adapter. The blood was mixed using gentle movements of the hands and placed into 4°C storage for 1 hour. The sample was then mixed again and an aliquot was removed via a 22-gauge needle and 1 mL syringe to assess the blood glucose amount. If the blood was not between 5-5.5 mM, then the appropriate amount of 100 mM glucose in 0.9% saline was added via 1 mL syringe and 22-gauge needle in the biosafety cabinet and thoroughly mixed. An 800 µL RBC solution sample was removed from the blood bags on day 1, 8, 15, 22, 29, 36, 43.

3.2.13 Data Analysis

Calibration plots of analyte/IS peak area ratio versus CML, CEL, and lysine concentrations were constructed, and a linear regression was used for all analytes. The peak area ratio of sample versus associated IS was used to produce a response to determine the concentrations from the calibration line. Free GSH results were collected from the Thiol Assay kit results and a linear

regression was used for standards and analyte quantification. SigmaPlot (Systat Software Inc) was used to plot all results and R software (version 4.2.2) was used to perform statistical testing in the "rstatix" package (v0.7.2; Kassambara, A. 2023).

A repeated measures two-way ANOVA test for each analyte (CML, CEL, Total AGEs, Lysine, Total AGEs/Lysine, Free GSH) were conducted to examine for differences between storage solution types over 43 days. The storage solutions were treated as a between-subjects dependent variable. Prior to conducting the ANOVA test, the assumptions of normality and homogeneity of variances were assessed using Shapiro-Wilk and Levene's tests, respectively. If these assumptions were met, the ANOVA test was performed to determine which specific solution types in storage exhibited statistically significant differences on the analytes studied. In the case of a significant ANOVA result, post hoc Bonferroni tests were conducted to determine which specific solution types and/or days in storage exhibited statistically significant differences on the analytes.

3.3 Results

3.3.1 RBC Solution in Tubes: Cell Lysis

According to FDA regulations, appropriate cell lysis should be below 1% to be used for transfusion applications. In two tube samples (AS-1 and AS-1N), the starting cell lysis was 0.56% and 1.14% on day 14, respectively. By day 17, the cell lysis increased to 4.53% and 5.65% for AS-1 and AS-1N, respectively. By days 33 and 36, AS-1 had 8.48% and then 21.66%, and AS-1N had 7.20% and then 16.8%, respectively. Overall, there was a clear increase in cell lysis in the tubes, well beyond the 1% threshold (**Figure 3.6**). Thus, the tubes were considered an unusable blood storage vessel for future experiments.

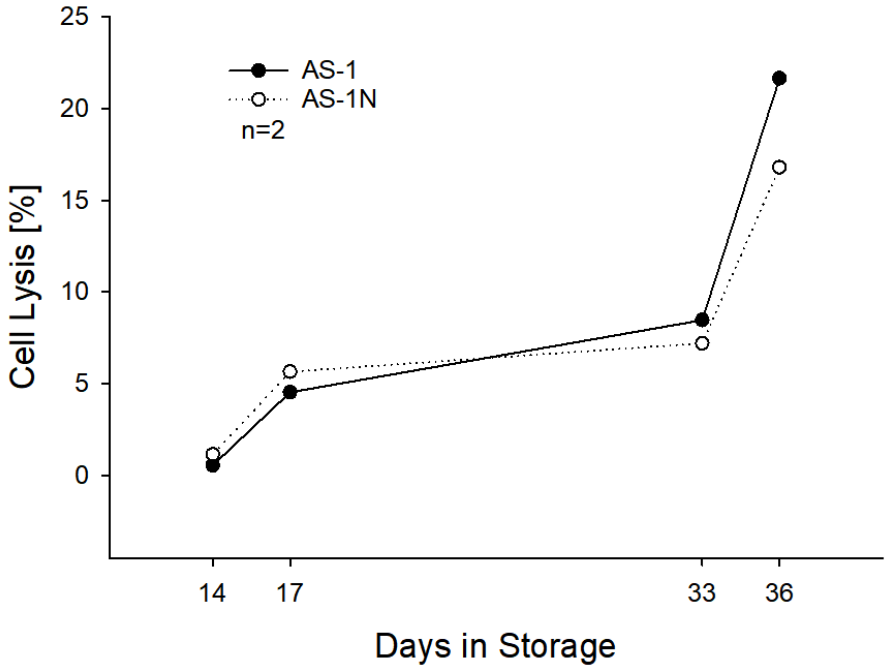

Figure 3.6. Cell Lysis from RBCs in two storage solutions, AS-1 and AS-1N, in tubes stored over 14-36 days. There was significant cell lysis present for both solutions and the lysis increased over time, resulting in extreme cell lysis (2-24%), n=2.

3.3.2 RBC Solution in Tubes: Blood Agar Streaking

The blood agar streaking test was used to determine if there was bacterial contamination in the tubes that could explain the increase in cell lysis. The stored blood samples were removed and streaked on the agar plates and stored in an incubator for at least a week. The plates were monitored, and bacterial contamination was not present in any of the samples. There was no visible color change, small areas of bacteria in media, or any cultures growing in the petri dish lids (**Figure 3.7**). This result does not indicate there was no bacteria at any point, but it does not lead to the assumption that the cell lysis was caused by the excess growth in bacteria due to the septa or caps leaking and an unsterile environment.

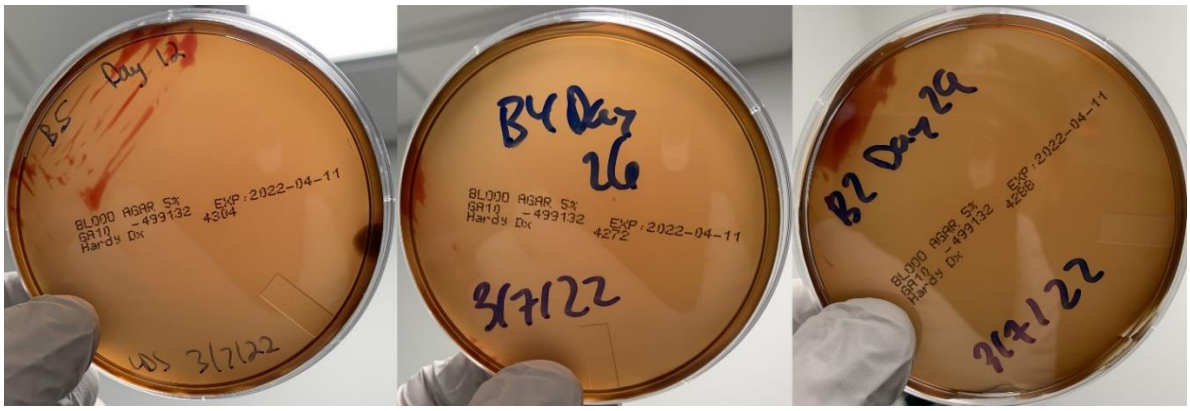

Figure 3.7. Agar streaking plates for bacterial growth in RBC solutions from tube storage. Several samples were examined for bacterial growth over various time points. None of the samples showed distinguishable bacterial growth, n=4.

3.3.3 IV Drip Calibration Curve for Automated Feeding

Since the septa tubes were no longer a viable option for storing blood, the next experiment was to utilize blood storage bags and set up a sterile, automatic feeding device. The valve IV drip system first needed to be calibrated to deliver the appropriate volume of solution to "feed" the samples, without compromising the necessary glucose range or hematocrit. The calibration curve for 0-2500 milliseconds was linear and showed good reproducibility, as seen in **Figure 3.8**. Thus, the calibration curve can be used to determine the amount of time needed to "feed" the RBC solution to achieve 4-6 mM glucose levels every 3 days without opening or invading the sterile environment.

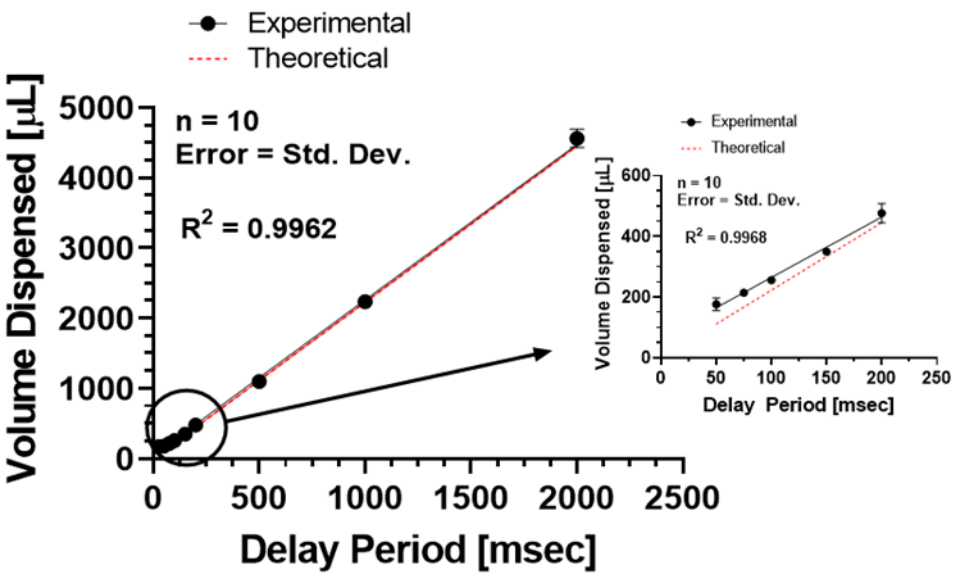

Figure 3.8. Calibration curve for dispensing volume via the opening of the solenoid valve. The volume dispensed from the automated feeding system with various valve opening intervals (50- 2000 msec) was determined by utilizing water (density = 1 g/mL) and a mass subtraction technique. The calibration curve is linear and can be used to determine the appropriate valve opening time that corresponds to the desired volume to dispense when feeding the concentrated glucose solution (n = 10, error = standard deviation). Borrowed from Soule, L. et. al., (*in review*).

3.3.4 Glucose and Hematocrit Changes over 43 Days

Samples were removed on a weekly basis, which accounts for lower hematocrit % than in the original experiment design (**Figure 3.9**). The glucose concentration was manually tested each week during sample removal from the bags to ensure the "feeding" solution kept the AS-1 and AS-1N samples in appropriate ranges (**Figure 3.10**). The glucometer saturates above 33 mM; therefore, it was assumed that AS-1 was at least 33 mM for the first 7 days. After day 8, glucose concentrations continually lowered until day 43 with the minimum glucose concentration of 23.3 mM. Although the AS-1 glucose concentration decreased significantly, it was consistently above AS-1N samples on both day 36 (6.85 mM) and 43 (8.30 mM), which for the purposes of this experiment, was considered sufficient and therefore not "fed" with a solution containing glucose.

The AS-1N samples maintained a glucose concentration between 4-6 mM, until day 36. This increase above 6 mM on day 36 and 8 mM on day 43 could be due to the decrease in hematocrit % with the same amount of solution "fed" every 3 days, and the RBCs possibly using less glucose to maintain metabolic function. Even though the AS-1N samples did exceed the desired glucose concentration range, the samples were still significantly lower than the AS-1 samples on both days 36 and 43.

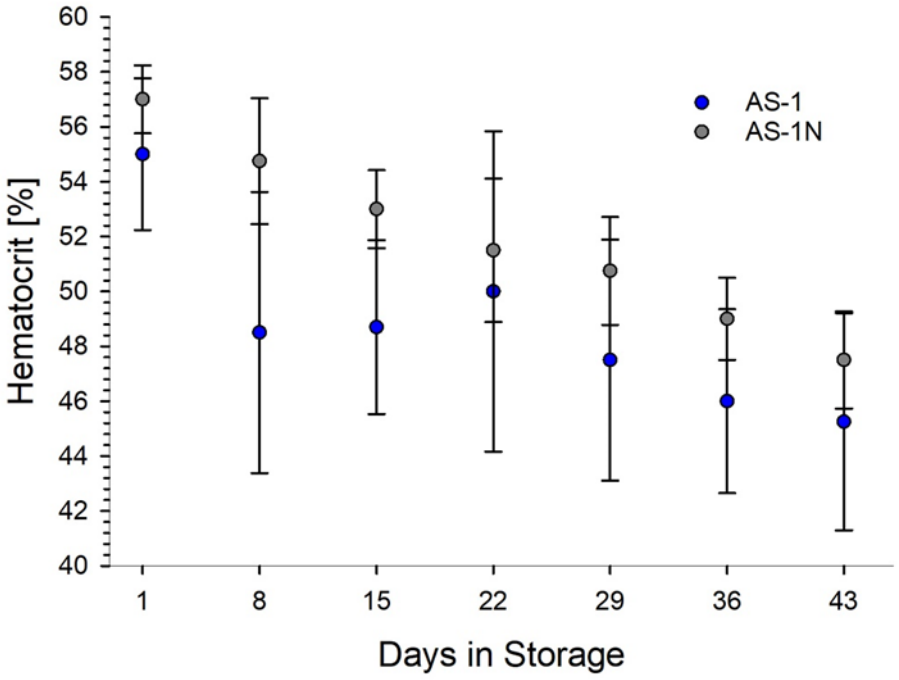

Figure 3.9. Hematocrit % of RBC solution in blood storage bags. There is no statistically significant difference between AS-1 and AS-1N storage solutions over 43 days. There is a distinguishable decline in the hematocrit due to removing samples and adding "feeding" solution. n=4-5, error=SEM.

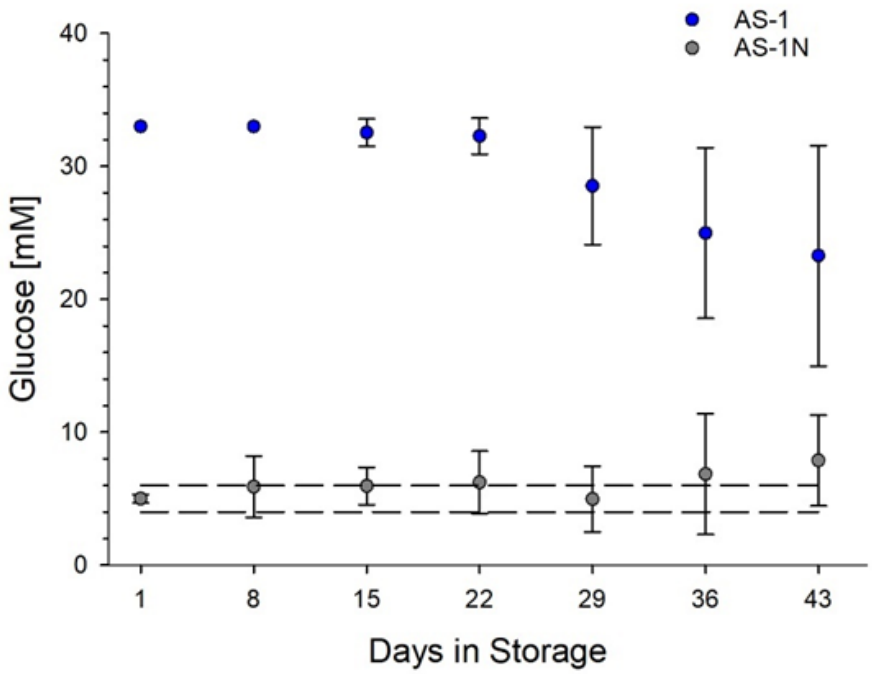

Figure 3.10. Glucose concentration [mM] of RBC solution in blood storage bags via glucometer. There is a statistically significant difference between AS-1 and AS-1N glucose concentration in storage solutions over 43 days. There is a distinguishable decline in the concentration of glucose in AS-1 due to removing samples and adding "feeding" solution (0.9% saline). The dashed lines indicate the desired range for the normoglycemic storage solution (4-6 mM) and AS-1N glucose concentration is within these bounds until days 36 and 43. n=4-5, error=SEM.

3.3.5 CML Over 43 Days

For CML, there were no significant differences found between samples stored in AS-1 and AS-1N ($p=0.399$). There were also no significant interaction effects between solutions and days in storage as seen in **Figure 3.11.** These results indicate that CML formation on RBC membrane was not affected by the lower glucose additive solution.

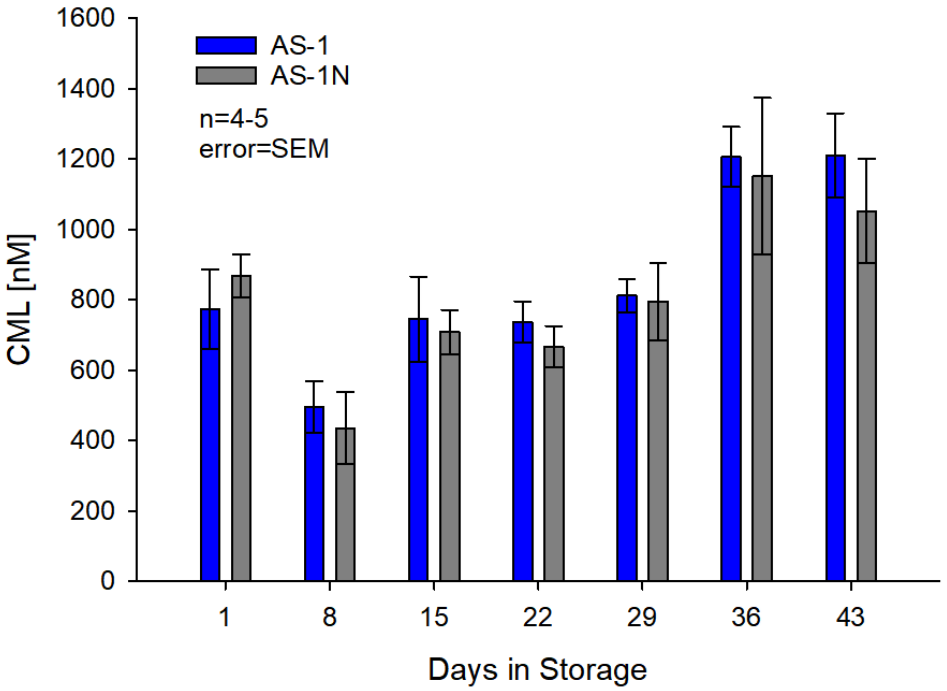

Figure 3.11. Carboxymethyl lysine (CML) concentration [nM] on RBC membranes after storage in AS-1 or AS-1N over 43 days. There is no statistically significant difference between AS-1 and AS-1N CML concentration in storage solutions over 43 days. p=0.399, n=4=5, error=SEM.

There was a significant effect of time on CML for AS-1, $F(6,18)=9.161$, $p<0.001$. For AS-1, Bonferroni tests revealed significant differences between days, visualized results are displayed below in **Figure 3.12**. There was a significant effect of time on CML level for AS-1N stored RBCs, $F(6,18)=2.882$, $p<0.05$. For AS-1N, Bonferroni tests revealed significant differences between days, visualized results are displayed below in **Figure 3.12**. These results indicate that CML formation was directly proportional with the progression of time for both AS-1 and AS-1N stored RBCs.

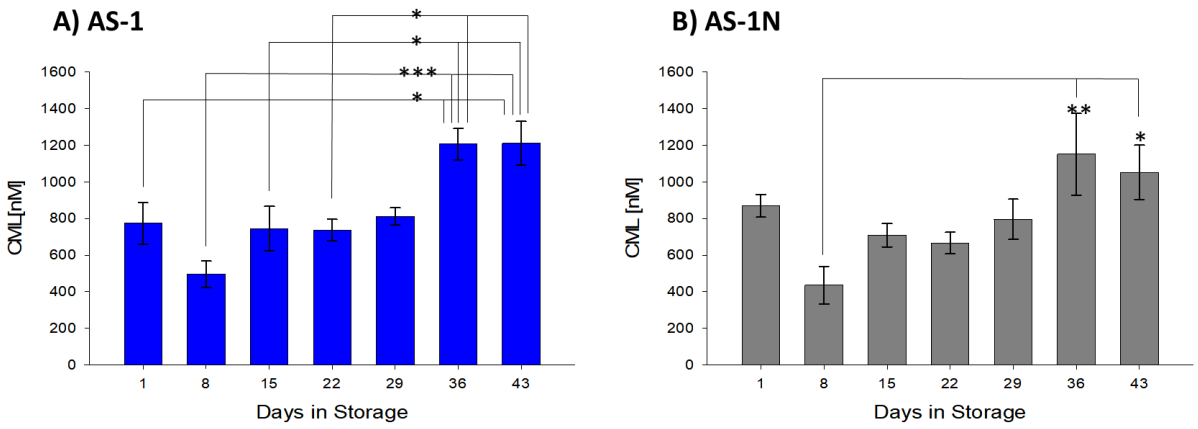

Figure 3.12. CML [nM] for both storage solutions over 43 days showed statically significant differences. **A.** The AS-1 solution had differences between day 1 and day 36, day 1 and day 43, day 8 and day 36, day 8 and day 43, day 15 and day 36, day 15 and day 43, day 22 and day 36, and day 22 and day 43. **B.** AS-1N solution had differences between day 8 and day 36, and day 8 and day 43. *p<0.05, **p<0.01, ***p<0.001, n=4-5, error=SEM.

3.3.6 CEL Over 43 Days

CEL was successfully detectable, and there was marginal significant differences found between samples in AS-1 and AS-1N (p=0.052). These differences, although not below p<0.05, showed the possibility of significance, if more samples were tested. There were also no significant interaction effects between solutions and days in storage as seen in **Figure 3.13.** These results indicate that CEL formation on RBC membrane was marginally altered with variance in solution.

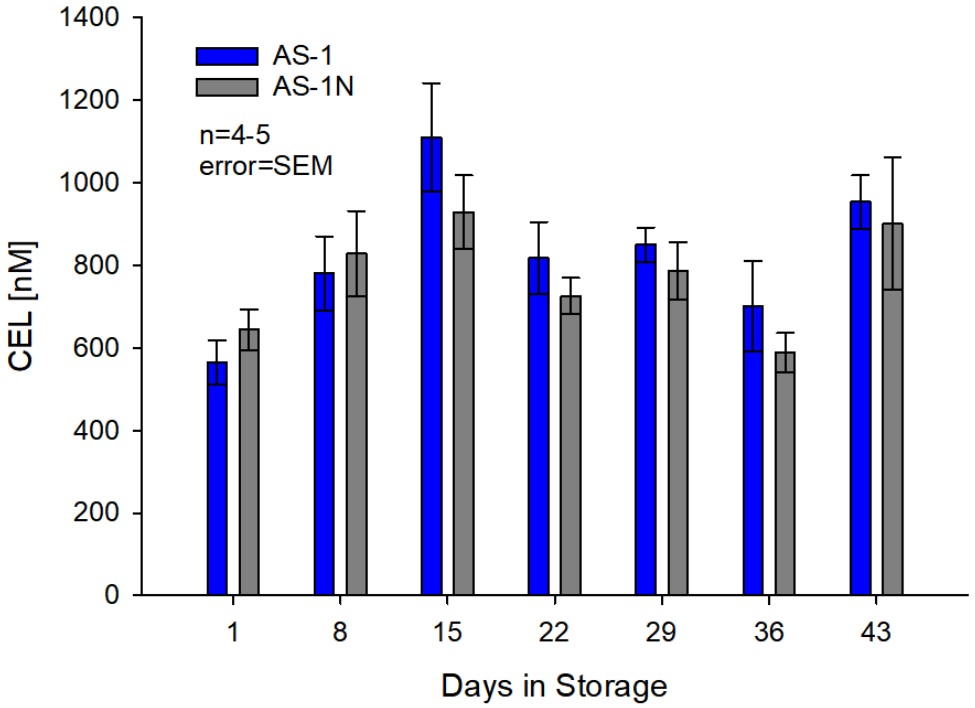

Figure 3.13. Carboxyethyl lysine (CEL) concentration [nM] of RBC solutions in blood storage bags over 43 days. There are marginal statistically significant differences between AS-1 and AS-1N glucose concentration in storage solutions over 43 days. p=0.052, n=4-5, error=SEM.

A repeated measures two-way ANOVA testing revealed that storage time was a significant factor in CEL formation for AS-1 stored RBCs, $F(6,18)=3.717$, $p<0.05$. For AS-1, Bonferroni tests revealed significant differences between days, visualized results are displayed below in **Figure 3.14**. There was a not significant effect of time on CEL for AS-1N, $F(6,18)=2.292$, $p=0.081$. These results indicate that CEL formation was altered with progression of time for AS-1 on storage days 1, 15, and 43.

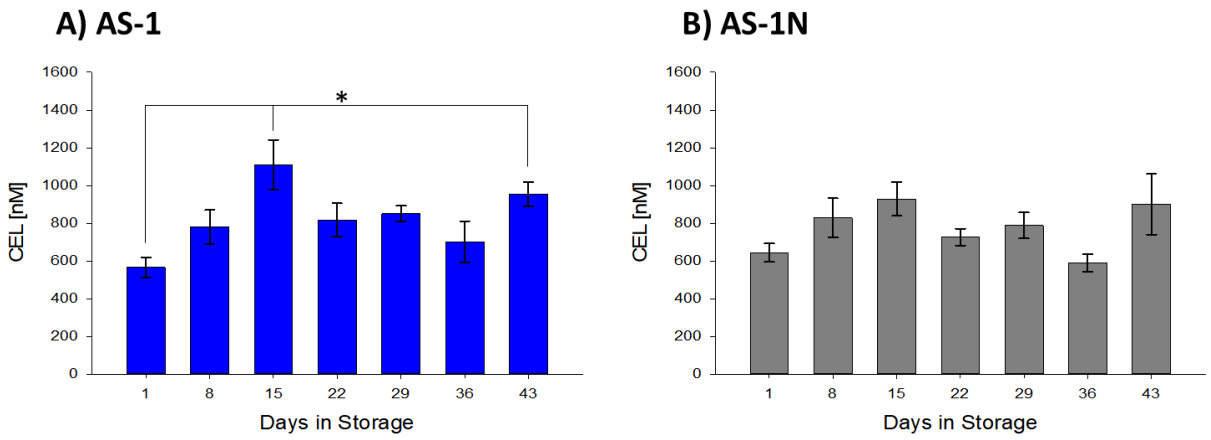

Figure 3.14. CEL [nM] for both storage solutions over 43 days. **A.** AS-1 solution had differences between day 1 and day 15, and day 1 and day 43. **B.** AS-1N solution had no differences between any days in storage. *p<0.05, n=4-5, error=SEM.

3.3.7 Lysine Over 43 Days

The data in **Figure 3.15** represented the total lysine on the RBC membranes. For membrane proteins that contained lysine, there were no significant differences found between samples in AS-1 and AS-1N (p=0.250). There were also no significant interaction effects between solutions and days in storage as seen in **Figure 3.15.** These results indicate that non-glycated lysine formation on RBC membrane was not altered with variance in solution.

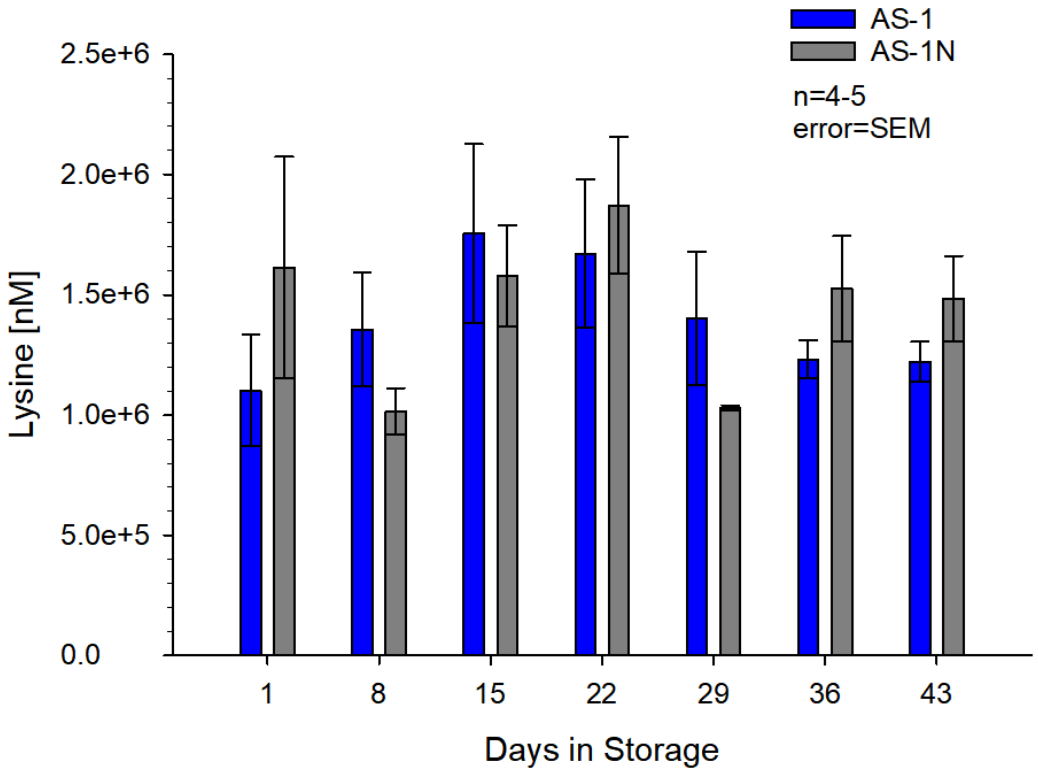

Figure 3.15. Total protein-bound lysine concentration [nM] of RBC solutions in blood storage bags over 43 days. There is no statistically significant difference between AS-1 and AS-1N glucose concentration in storage solutions over 43 days (p=0.250). n=4-5, error=SEM.

Overall, there was limited to no change in total lysine concentration over 43 days. This result can be seen as there was no significant effect of time on lysine for AS-1 $F(6,6)=2.625$, $p=0.133$. Also, there was no significant effect of time for AS-1 $F(6,12)=1.117$, $p=0.408$. Both visualized results are displayed below in **Figure 3.16**. These results indicate that lysine formation was not altered with progression of time for AS-1 and AS-1N on various storage days.

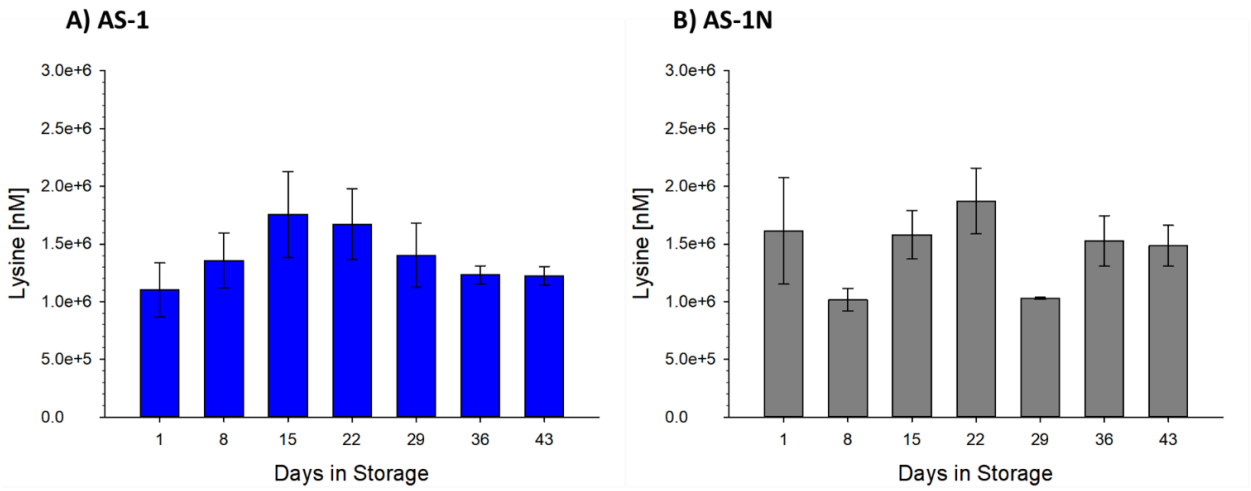

Figure 3.16. Lysine [nM] for both storage solutions over 43 days showed no statically significant differences. **A)** AS-1 solution had no differences between any of the days. **B)** AS-1N solution had no differences between any of the days. n=4-5, error=SEM.

3.3.8 Total AGEs Over 43 Days

For total AGEs (CML and CEL combined), there were no significant differences found between samples in AS-1 and AS-1N ($p=0.107$). Furthermore, although the total AGEs clearly increased as a function of storage duration, there was not a large difference in AGE formation from day 1 to day 43. There were also no significant interaction effects between solutions and days in storage as seen in **Figure 3.17.** These results indicate that total AGEs formation on RBC membrane was not altered with variance in solution. However, an increase in AGEs (relative to day 1 of storage) were measured after 3 weeks of storage

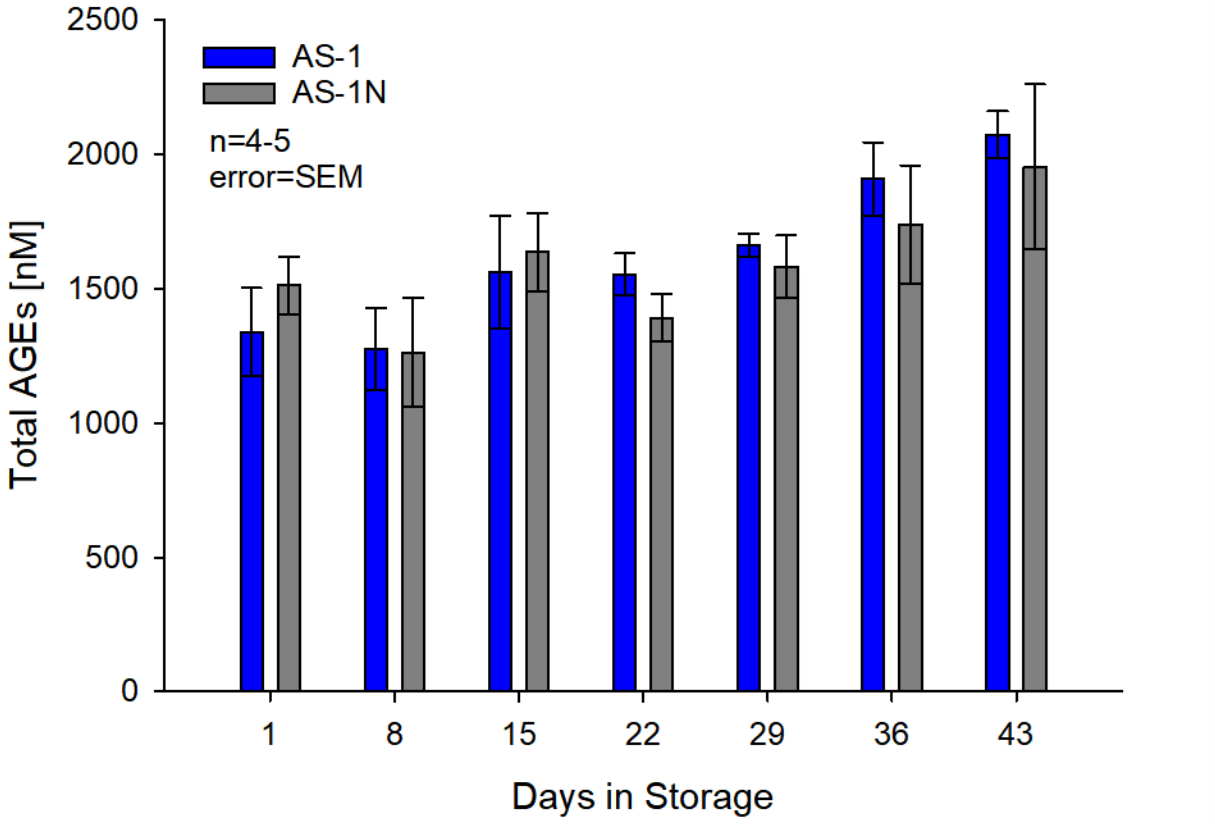

Figure 3.17. Total AGEs (CML + CEL) [nM] of RBC solutions in blood storage bags over 43 days. There is no statistically significant difference between AS-1 and AS-1N glucose concentration in storage solutions over 43 days. p=0.107, n=4-5, error=SEM.

There was a significant effect of time on total AGEs for AS-1, $F(6,12)=4.741$, $p<0.05$. For AS-1, Bonferroni tests revealed significant differences between days, visualized results are displayed below in **Figure 3.18**. There was a not significant effect of time on total AGEs for AS-1N, $F(6,18)=1.475$, $p=0.242$. These results indicate that total AGE formation was altered with progression of time for AS-1 days 8, 15, 29, and 43.

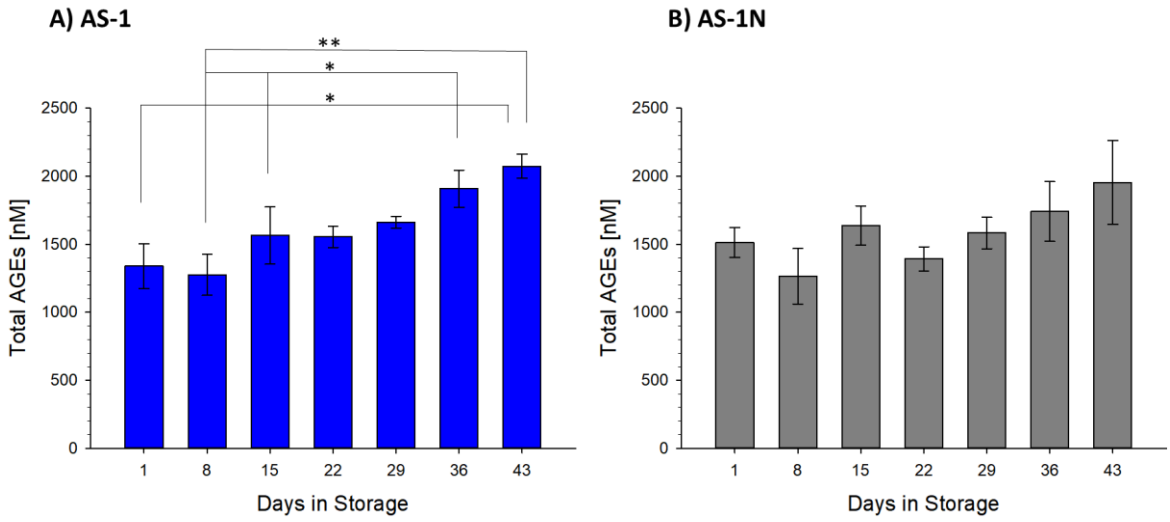

Figure 3.18. Total AGEs [nM] (CML and CEL combined) for both storage solutions over 43 days. **A.** AS-1 solution had differences between day 1 and day 43, and day 8 and day 15, day 8 and day 36, and day 8 and day 43. **B.** AS-1N solution had no differences between any days in storage. *p<0.05, **p<0.01, n=4-5, error=SEM.

3.3.9 Free Reduced Glutathione AS-1 vs AS-1N Over 43 Days

For free reduced glutathione (GSH), there were significant differences found between samples in AS-1 and samples in AS-1N for both solution types and days in storage as seen in **Figure 3.19**. Solution types as a category in the two-way repeated measures ANOVA had significant differences as follows: $F(6,18)=94.406$, $p<0.001$. Days in storage had significant differences as follows: $F(6,18)=14.236$, $p<0.001$. There was no significant interaction between solutions and days in storage $F(6,18)=2.149$, $p=0.133$. These results indicate that free GSH formation was altered with variance in solution.

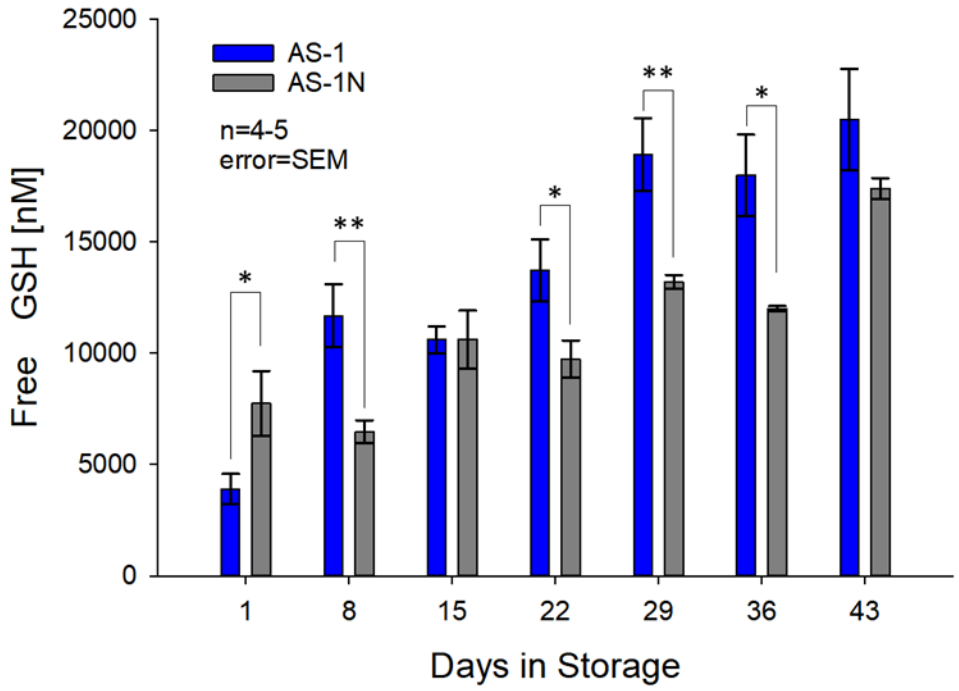

Figure 3.19. Free Glutathione (GSH) [nM] of RBC solutions in blood storage bags over 43 days. There is a statistically significant difference between AS-1 and AS-1N glucose concentration in storage solutions over 43 days. *p<0.05, **p<0.01, n=4-5, error=SEM.

There was a significant effect of time on free GSH for AS-1, $F(6,18)=12.681$, $p<0.05$. For AS-1, Bonferroni tests revealed significant differences between days, visualized results are displayed below in **Figure 3.20**. There was a significant effect of time on GSH for AS-1N, $F(6,18)=8.507$, $p<0.001$. $R=0.8286$. For AS-1N, Bonferroni tests revealed significant differences between days, visualized results are displayed below in **Figure 3.20**. These results indicate that free GSH formation was altered with progression of time.

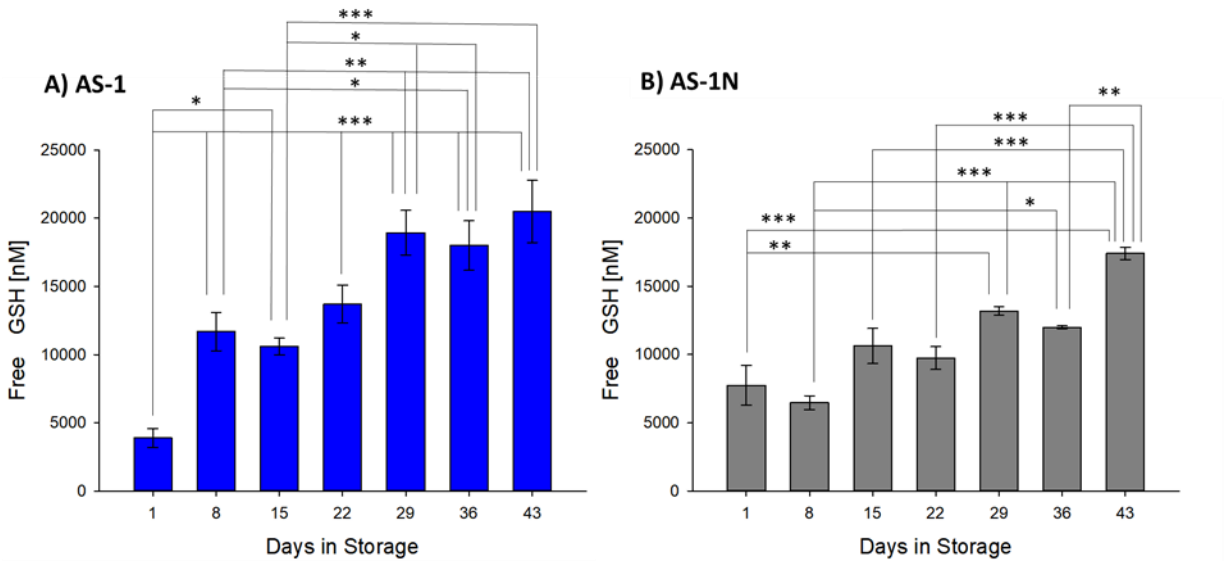

Figure 3.20. Free Reduced Glutathione (GSH) [nM] of RBC solutions in blood storage bags over 43 days. There is a statistically significant difference between AS-1 and AS-1N glucose concentration in storage solutions over 43 days. *p<0.05, **p<0.01, ***p<0.001, n=4-5, error=SEM.

3.4 Discussion

In blood transfusion practices, there is limited knowledge concerning the effects of the high glucose storage solution on RBCs, specifically referring to RBC lesions, deformability, and other adverse effects. AGEs have been reported in various samples, but most reports concerning the glycation in food resulting in higher CML and CEL. There are limited reports of detecting and quantifying CML, CEL, and Lysine in human stored ERY samples. Furthermore, following the method development in chapter 2 in two different storage conditions (hyperglycemic and normoglycemic), AGEs were quantified each week. The sample preparation that enabled adequate separation and quantification of these AGEs over 43 days allows for a closer examination of glycation trends on the RBC membrane. These features, along with comparing other blood banking studies from previous Spence group members, such as metabolic and physical adverse effects

(adhesion, sorbitol, GSH, deformability) give a larger picture of the effect of a high glucose blood storage condition and a possible alternative in future *in vivo* applications.

The automatic "feeding" application allows for customizability and glucose regulation. Even though the septum cap tubes were not successful due to faulty manufacturing and high cell lysis, the idea of continuous feeding in a normoglycemic environment was scaled up from the previous smaller tubes that were successful in determining ATP release and deformability. The novel use of utilizing smaller veterinarian blood bags for storage with an automated system to deliver a glucose solution was successful. Therefore, it is possible to implement this system for larger transfusion bags with confidence before moving to implementing it *in vivo.*

Previously in chapter 2, the change in AGEs and lysine were measured for method validation on freshly drawn blood. Results indicated that there was no statistical difference in the types of storage solutions. However, it was expanded to 43 days and explored to determine how time can contribute to AGE and glutathione formation. There was a spike for both solutions on day 15 which correlates to literature reports of 2-week AGE formation importance.[60,66–68] Furthermore, the data showed that the RBCs stored in AS-1 experience more changes than AS-1N in CML, CEL and total AGEs over the course of 43 days in storage. This finding is important because when examining the two different types of solutions, only AS-1 shows overall changes over time, which confirms the hypothesis mentioned in chapter 2. There was no relationship between increasing time and lysine concentration for both AS-1 and AS-1N. Furthermore, the data showed that the RBCs stored in AS-1 did not experience more changes than AS-1N in lysine concentration over the course of 43 days in storage. This finding is important because it signifies the overall RBC membrane non-glycated proteins did not change.

When further analyzing the CML and CEL data from day 1 to day 43, there are statistically significant increases in the AGEs from day 1 to day 43 ($p<0.01$). However, there are no differences between the solutions on either day (**Figure 3.21**). Interestingly, for CML, AS-1 had a 56.5% increase while AS-1N had a 21.1% increase, which is approximately half of AS-1 percent increase. Similarly, for CEL, AS-1 had a 68.7% increase while AS-1N had a 39.9% increase, again approximately half. Although the two solutions are not statistically different to each other, the surprising percentage differences, and statistical increase from day 1 to 43 show AGE formation is time-dependent and increasing.

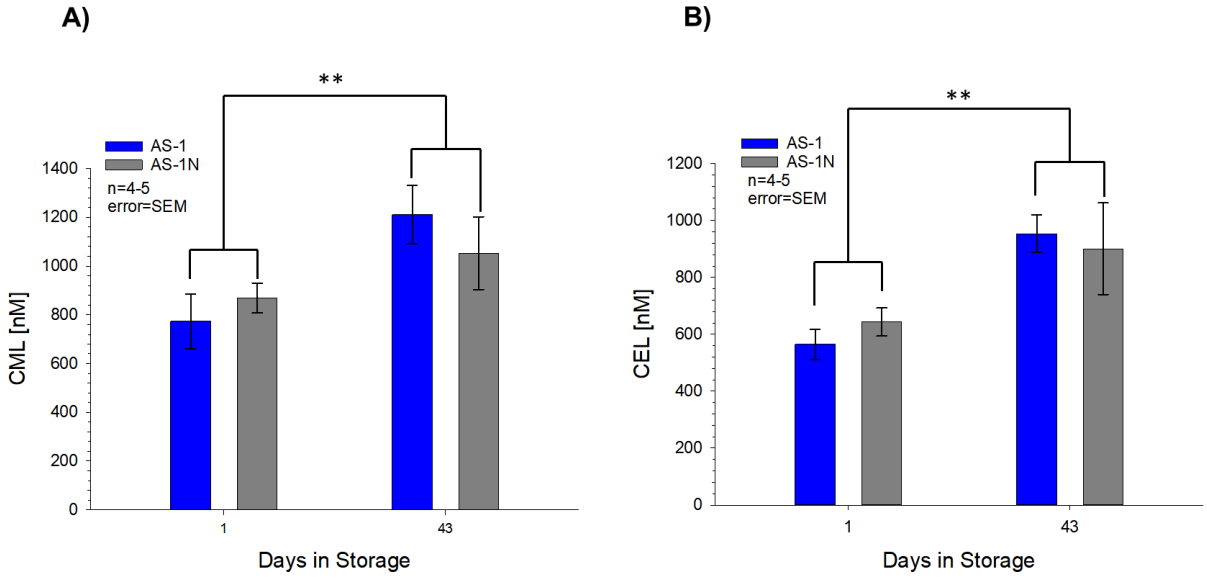

Figure 3.21. CML and CEL day 1 versus 43 for AS-1 and AS-1N. **A.** CML Day 1 AS-1 (773.7 nM ± 112.9) and day 43 (1210.9 nM ± 119.7) and AS-1N day 1 (869.2 nM ± 61.5) and day 43 (1052.8 nM ± 149.0). **B.** CEL day 1 AS-1 (565.2 nM ± 53.2) and day 43 (953.7 nM ± 65.3) and AS-1N day 1 (643.9 nM ± 49.5) and day 43 (901.0 nM ± 160.8). **p<0.01, n=4-5, error=SEM. Image borrowed from Skrajewski-Schuler, L. et. al.

Additionally, to individual AGEs interpreted data, the total AGEs/lysine results show a clear increasing trend over the 43-day blood storage period (**Figure 3.22**). The results are

statistically significant between categories $F(6,18)=17.591$, $p<0.05$. Also, there were statistically significant results between day 43 relative to days 1, 8, 15, and 22 ($p<0.05$). The data specifically shows after day 15 a shift in total AGE/lysine formation, which is indicative of the current literature surround 2-week irreversible AGE formation.[60,66] Again, there is a 53.7% percent increase in total AGE/lysine for AS-1 (day 1 $1.11\text{x}10^{-3} \pm 2.58\text{x}10^{-4}$ and day 43 $1.71\text{x}10^{-3} \pm 6.45\text{x}10^{-5}$). In comparison, AS-1N had a 48.7% percent increase (day 1 $1.02\text{x}10^{-3} \pm 3.06\text{x}10^{-4}$ and day 43 $1.52\text{x}10^{-3} \pm 7.65\text{x}10^{-5}$). Finally, both AS-1 ($F(6,20)=3.911$, $p<0.01$)) and AS-1N ($F(6,8)=7.139$, $p<0.001$)) categories had a significant effect on days in storage. Thus, proving the hypothesis that storage solutions would alter total AGEs/lysine concentration over 43 days.

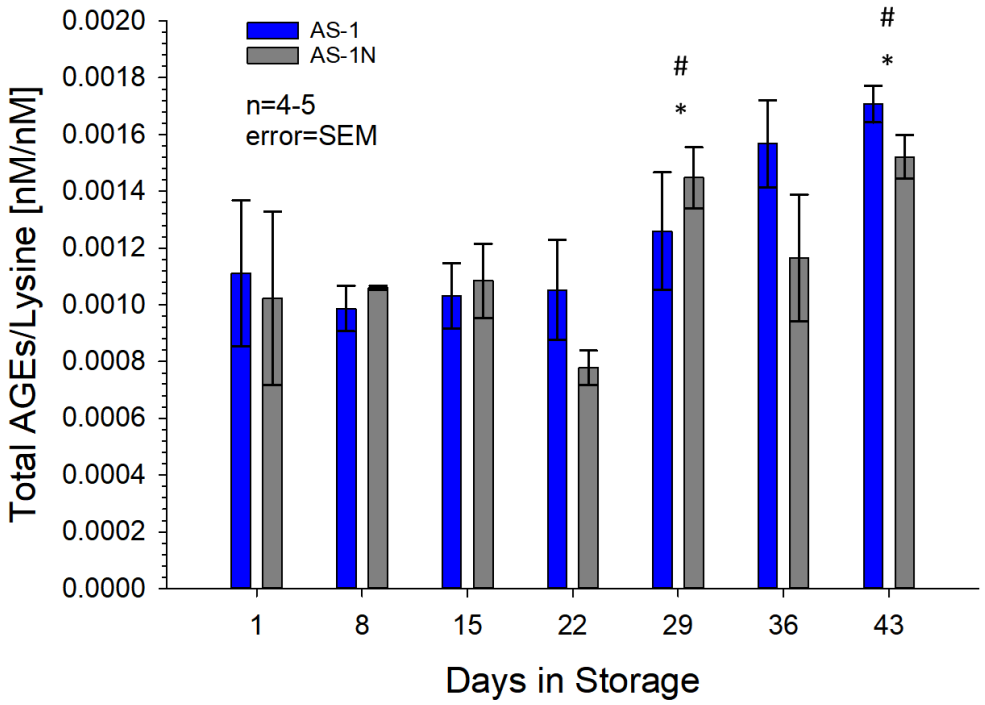

Figure 3.22. Total AGEs/Lysine over 43 days for AS-1 and AS-1N. There was a significant effect of category on results which resulted in determining that AS-1 had a significant effect on specific days in storage ($p<0.05$). AS-1N did have a significant effect on specific days in storage ($p<0.05$) Overall, the solutions did influence the total AGEs/lysine concentration over time. *$p<0.05$ relative to day 1, #$p<0.01$ relative to day 22, n=4-5, error=SEM. Image borrowed from Skrajewski-Schuler, L. et. al. (*in review*).

Few studies and reports have shown the adverse complications that exist after transfusion, often around the 2-week mark of blood storage.[60,66–68] Specifically, from the Spence group with published ATP and deformability data, such as in Liu, Y. et al.[37] It is hypothesized that at day 8, adverse effects to RBCs start to become irreversible and the damage to the cells is permanent by day 15.[37] These findings could be due to the increased AGEs forming around this time marker and thus the quality of the RBCs dramatically changes with the increased glycation. It is still unknown whether the RBCs irreversible AGEs formed would be unable to recover after transfusion, but the time frame that the RBCs are in storage relative to when transfusion occurs is important to note. The weekly blood storage studies that involved normoglycemic conditions with lower glucose feeding and sample removal are novel. Thus, it will require more time, donors, samples, and studies to understand more about the blood storage timepoints and important timeframes for AGEs and RBCs.

These results provide a platform for new blood transfusion research, in discovering how normoglycemic storage solutions as well as storage time can alleviate microvascular complications attributed with increased AGEs and oxidative stress.[69,70] While AGEs can form relatively quickly under certain experimental conditions or in specific tissues with short-lived proteins, it is not common in the literature for significant AGE formation to occur within just 2 weeks RBCs under normal physiological conditions. AGE accumulation is more often a gradual and chronic process linked to long-term exposure to high blood glucose levels. So, by examining the trend over 43 days, it is clear there is an increase in overall AGE formation after 2 weeks up to 43 days. This study can be expanded on in future efforts with longer storage time, more sample types, and different biomarkers to determine the overall effectiveness of a normoglycemic blood storage.

GSH levels were measured as an indirect example of cellular toxicity due to the changing levels of oxidative stress. Previous reports indicate that a reduction in glutathione peroxidase activity and thus free GSH refers to increased levels of oxidation.[42,61] In this study, there is a statistically significant increase in GSH in AS-1 compared to AS-1N. Also, there is a clear increase in free GSH levels over time for both solutions. Therefore, the amount of GSH quantified from the stored blood bags were affected by both time and solution time in the opposite trend that was hypothesized. The data shows that the RBCs stored in AS-1 at statistically increased levels of GSH to suggests there are an opposite correlation between increased oxidative stress and GSH production than expected, especially after 15 days of storage.

It may seem counterintuitive that GSH, an antioxidant molecule, can be present at high levels while still being associated with oxidative stress. One theory is in response to oxidative stress, cells can upregulate the synthesis of GSH to counteract the increased levels of reactive oxygen species (ROS) and free radicals.[62,71] This is a protective mechanism, and it leads to higher intracellular GSH concentrations.[71] Even though GSH production may be elevated, the rate of GSH consumption due to the excessive presence of ROS can exceed its synthesis.[62] In other words, the cellular antioxidant defense system may be overwhelmed, leading to a net decrease in the active reduced form of GSH.[64] In cases of chronic or severe oxidative stress, GSSG levels may rise significantly, even as GSH is produced in response to the ongoing stress.[42,55] Therefore, in the presence of oxidative stress, the balance between GSH and GSSG may shift toward a more oxidized state, despite the overall high levels of GSH (**Figure 3.21.)**.[63]

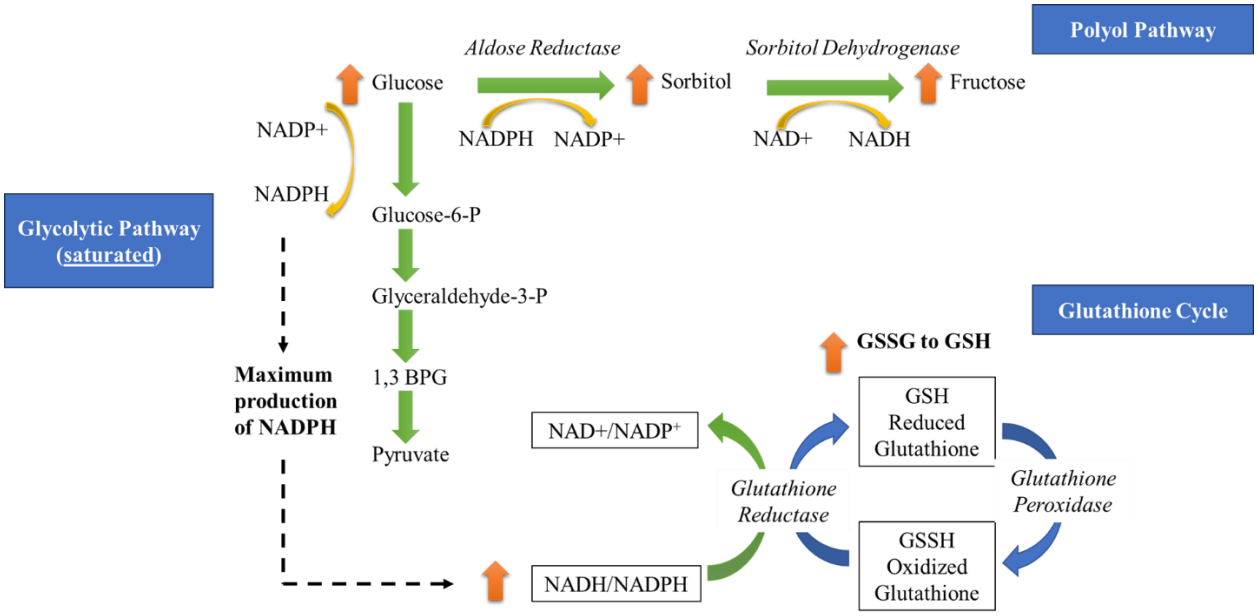

Figure 3.21. Possible hypothesis for increased in GSH production. Hyperglycemia induces the glycolytic pathway to become saturated, which results in the maximum amount of NADPH production possible. Therefore, increasing the NADPH utilization to convert GSSG to GSH, increasing GSH levels. Increased glucose levels can also trigger the activation of the polyol pathway, leading to increased sorbitol production. The conversion of glucose to sorbitol in the polyol pathway consumes NADPH, resulting in its depletion. But NAD+ is also converted to NADH, which increases the production of this cofactor, and thus cancels out the NADPH used to make sorbitol.

Another hypothesis is in certain pathological conditions or environmental exposures (such as *in vitro* experiments), the rate of ROS generation can outpace the cell's ability to neutralize them, even with increased GSH production.[55-57] This results in a situation where GSH is present but unable to completely counteract the oxidative stress.[56,57,63]

In summary, the presence of high GSH levels in the context of oxidative stress does not necessarily mean that GSH is fully effective at neutralizing the stress. Instead, high GSH could reflect an adaptive response by the cell to the increased oxidative challenge.[58,63,64] To assess the actual impact of oxidative stress and the effectiveness of GSH, it is important to consider the GSH/GSSG ratio and the balance between antioxidants and pro-oxidants within the cellular environment.[61–63] Thus, another assay kit with the ability to measure either the ratio in its entirety

or compare the results from the reduced and oxidized components would be a clear next step. Or an alternative theory could be as simple as this phenomenon is due to matrix effects.[42] The biological matrix in the sample and the assay kit constituents could alter the results. If the matrix theory is the case, then instead of pRBCs, another blood component, such as serum or plasma or separating the pRBCs from the additive solution mix in the bag could be another option to examine. These theories of explaining high GSH levels in hypothesized increased oxidative stress environments (i.e., hyperglycemic blood storage) will also be used to explain T1D and control results in chapter 4. The study of AGEs in transfusion medicine is an evolving field with potential implications for the quality and safety of blood products and their impact on recipient health. These studies can be used to show the biomarkers in this chapter are important and can be used in future clinical applications to learn more about *in vivo* samples.

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Chapter 4: Potential Biomarkers for T1D Patient Screening in C-peptide Therapy

4.1 Introduction

4.1.1 Background

According to the 2022 Center for Disease Control and Prevention (CDC) National Diabetes Statistics Report, 28.7 million Americans have been diagnosed with diabetes.[1] The two main forms of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D).[2,3] Approximately 1.4 million Americans are diagnosed with diabetes every year, with about 5% of these diagnoses being T1D.[2,3] T1D is a chronic autoimmune disorder characterized by the destruction of insulin-producing β-cells in the pancreas, leading to an absolute deficiency of insulin.[4] Insulin is a hormone that plays a crucial role in regulating blood glucose levels by facilitating the uptake of glucose into certain cells for energy production or storage.[5,6] Without sufficient insulin, glucose accumulates in the bloodstream, leading to hyperglycemia and downstream severe health consequences.[5] T1D is often treated with exogenous insulin taken several times a day to control blood glucose levels.[7] In T2D, the body becomes resistant to the effects of insulin, leading to a relative deficiency of insulin.[8] Although the pancreas may still produce insulin, the body's cells are less responsive to its action, resulting in elevated blood glucose levels.[8] T2D is often managed through lifestyle changes, oral medications, and, in some cases, insulin therapy.[9]

Even when well controlled, and especially when not, diabetes can have major long-term health ramifications. Many of these complications can be attributed to poor blood flow and decreased oxygen delivery to various parts of the body, resulting in localized hypoxia.[10] Diabetic retinopathy is defined as hypoxia that can contribute to the development and progression of complications affecting the eyes, leading to extreme vision impairment or blindness in approximately 11% of people with T1D.[10,11] Hypoxia in the kidneys may play a role in the

development and progression of diabetic nephropathy, a condition characterized by kidney damage in diabetes.[10] Another complication is neuropathy, which is nerve damage leading to sensory and motor deficits in individuals with diabetes.[10] Cardiovascular disease (CVD) causes more deaths in people with diabetes than any other complication, with a 1 – 3 fold increase in men and a 2 – 5 fold increase in women for risk of CVD morbidity and mortality compared to healthy adults.[12]

4.1.2 β-Cells, Insulin, and Glucose Transporters

The exact cause of T1D is not fully understood, but it is believed to result from a complex interplay of certain genetic markers, autoantibodies, and environmental triggers.[4] This endocrine disorder is an autoimmune attack that results in a significant reduction in or complete loss of insulin secretion.[4,13] With the loss of insulin-secreting β-cells, there is a lack of insulin to facilitate glucose uptake by insulin-dependent cells, resulting in hyperglycemia.[4] Hyperglycemia can lead to a range of acute and chronic complications, including diabetic ketoacidosis in the short term and microvascular and macrovascular complications in the long term.[13,14] Blood glucose, also known as blood sugar, refers to the concentration of glucose present in the bloodstream.[6] Glucose is the primary source of energy for the body's cells and is essential for various physiological processes, as previously discussed in chapter 1.[14]

Insulin is a hormone[5,6] that plays a crucial role in regulating blood glucose levels.[5,6] As blood glucose levels rise, the entering glucose changes ratios of adenosine triphosphate to adenosine diphosphate (ATP/ADP). This changing ratio stimulates specific mechanisms that result in the vesicles docking at the cell membrane and releasing its contents, which is insulin into the bloodstream.[8] Insulin acts as a "key" that unlocks the body's cells, allowing glucose to enter and be used as energy.[15] Insulin binds to receptors on the cell surface, triggering a series of intracellular

processes that facilitate the transport of glucose from the bloodstream into the insulin-dependent cells via the glucose transporter (**Figure 4.1**).[6,15,16]

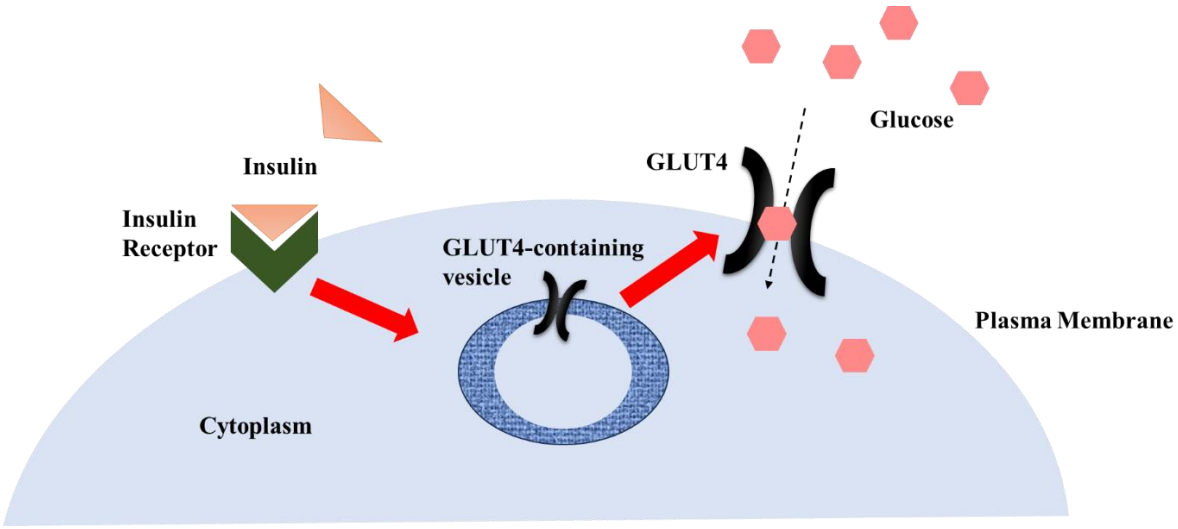

Figure 4.1. The mechanism for insulin-responding glucose transporter, GLUT4. On the membrane surface, insulin binds to the insulin receptor and triggers an intracellular response to facilitate the GLUT4- containing vesicle to move to the cell surface. The GLUT4 vesicle fuses with the plasma membrane and allows glucose to enter the cell.[16,19,21]

Glucose transporter 4 (GLUT4) is a member of the glucose transporter family.[16] It is a protein responsible for transporting glucose into cells and is predominantly found in insulin-sensitive tissues, such as muscle cells and adipose (fat) tissue.[17–19] The main feature that distinguishes GLUT4 from other glucose transporters, like GLUT1, is its regulation by insulin.[18] Under normal physiological conditions, GLUT4 is mostly sequestered inside specialized vesicles within the cell, and only a small portion is present on the cell surface.[19] However, when insulin levels rise in response to increased blood glucose levels after a meal, GLUT4 is translocated to the cell membrane, allowing more glucose to enter the cell.[20,21] This insulin-mediated translocation of GLUT4 to the cell surface is a crucial step in glucose uptake and utilization by insulin-sensitive tissues.[17,20,21] Impairments in GLUT4 function or insulin signaling can lead to insulin resistance, a

condition where insulin's ability to promote glucose uptake is diminished.[19,21–23] Once the excess glucose moves into the cell, it needs to be processed for energy and storage. Glucagon helps promote the conversion of glucose into glycogen, a storage form of glucose, in the liver and muscles.[16]

Glucose transporter 1 (GLUT1) is a protein that plays a critical role in facilitating the transport of glucose across insulin-independent cell membranes.[24,25] GLUT1 is another member of the glucose transporter family, which includes several other transporters responsible for glucose uptake in different tissues and cell types throughout the body, as previously discussed.[23,25] The GLUT1 protein is primarily expressed in red blood cells (RBCs), the blood-brain barrier, and tissues with a high demand for glucose, such as the brain and placenta.[22,24,26] Its primary function is to transport glucose molecules from the extracellular fluid into the cells, where glucose is utilized for energy production or other metabolic processes.[25] The RBC membrane has many different proteins, 5-10% of which are GLUT1, making this hydrophobic protein essential for providing glucose for energy production and protects against insulin-resistant glucose uptake from oxidative stress.[24]

During fasting, the liver produces glucose by synthesizing glucose from lactate and amino acids (gluconeogenesis).[27] Post-meal, insulin is released from the pancreas and works with glucagon to maintain normal glucose concentration in the bloodstream.[27] Glucose gets deposited into the liver, which becomes glycogen to be stored in the liver and muscles while blood glucose levels rise.[27] When blood glucose levels decrease, the stored glycogen is broken down back into glucose (glycogenolysis) to maintain blood glucose levels within a normal range (euglycemia).[27] Insulin helps to suppress the liver's production of glucose.[28] By reducing glucose production, insulin helps maintain stable blood glucose levels.[27]

When insulin is released from the β-cells, equimolar concentrations of C-peptide are also released in addition to high levels of zinc (Zn^{2+}) **(Figure 4.2)**.[29] C-peptide is an important by-product of insulin production, being cleaved from proinsulin before the formation of insulin hexamers.[30] The production and function of C-peptide will be discussed later in this introduction. Insulin, Zn^{2+}, and C-peptide are stored within the β-cell granule until release, which allows for blood glucose regulation.[29,30]

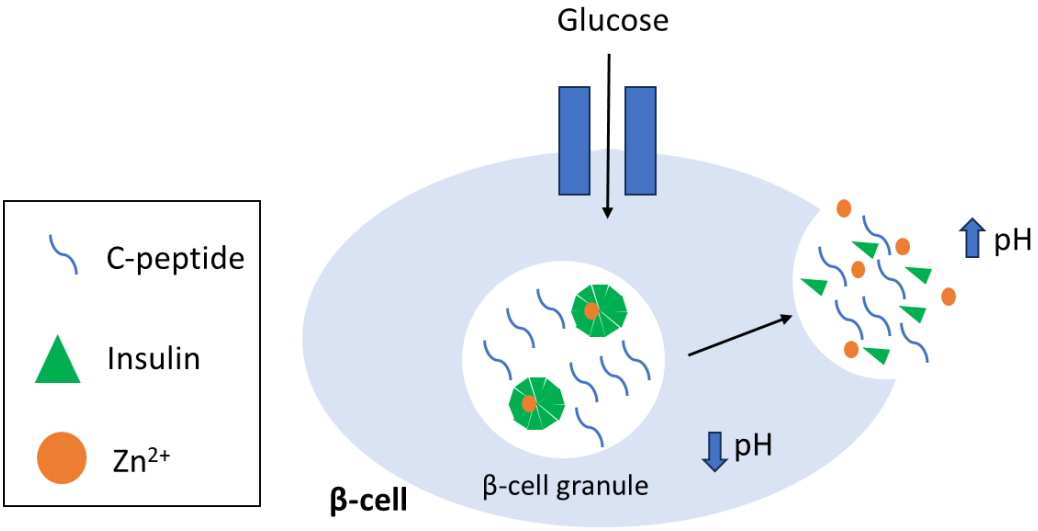

Figure 4.2. Pancreatic β-cell. The islets of Langerhans are clusters of cells (β-cells) in the pancreas that produce and subsequently release insulin to regulate glucose levels in the bloodstream. The pH inside the β-cell granule is approximately 5.5, which forces insulin to remain a crystalline hexamer unit with two Zn^{2+} ions because it is insoluble below pH 7.0. The slightly acidic pH inside of β-cell granule maintains the packing and storage of insulin and Zn^{2+}. Also, at 5.5 pH, C-peptide is protonated and therefore does not bind to Zn^{2+}. [29,30,57]

In individuals with T1D, the immune system mistakenly attacks and destroys the insulin-producing β-cells in the pancreas.[4,13,31] As a result, the production of insulin is significantly reduced or absent, leading to an absolute deficiency of insulin.[4,6] Without sufficient insulin, glucose cannot enter the insulin-dependent cells effectively, and blood glucose levels rise.[5,6] This

condition requires lifelong insulin therapy to provide the body with the necessary insulin for glucose regulation.[4,12,13]

4.1.3 Therapeutics for Diabetes

The mainstay of T1D management is insulin therapy, which involves the administration of replacement of endogenous insulin to maintain blood glucose levels within a target range.[32] It has a half-life of 2-3 minutes, so it must be administered throughout the day.[6] Insulin can be administered through injections or insulin pumps, mimicking the physiological insulin secretion pattern.[33,34] Insulin pump therapy is an alternative to multiple daily injections, providing continuous subcutaneous insulin infusion.[7,35] The pump delivers a basal rate of insulin to maintain steady glucose levels and allows for bolus doses to correct spikes in high blood sugar, such as those that may occur after a meal.[35]

Various types of insulin are available, categorized based on their onset, peak, and duration of action. The four major types of insulin include rapid-acting, short-acting, intermediate-acting, and long-acting.[35,36] Rapid-acting insulin starts working within 15 minutes, efficacy peaks within 1 to 2 hours, and lasts for 3 to 4 hours.[7,36] Short-acting insulin begins working within 30 minutes, peaks in 2 to 3 hours, and lasts for 4 to 6 hours.[7,36] Intermediate-acting insulin takes effect in 2 to 4 hours, peaks in 4 to 12 hours, and lasts for up to 18 hours.[7,36] Long-acting insulin has a gradual onset, has no pronounced peak, and lasts for approximately 24 hours.[7,36] Insulin therapy allows individuals to mimic the natural insulin secretion pattern to effectively regulate blood glucose levels.[33] However, despite significant advancements in insulin formulations and delivery methods, achieving optimal glycemic control remains a challenge.

Various innovative therapeutic methods are being explored to improve T1D management and potentially offer a cure. Some of these include β-cell replacement therapies, such as islet cell

transplantation and stem cell-derived β-cells, with the aim of restoring endogenous insulin production.[37,38] Immunotherapies and immune modulation strategies are also being investigated to prevent or halt the autoimmune destruction of β-cells.[39]

Pramlintide is an injectable medication that can be used in addition to insulin therapy to help control post-meal blood sugar spikes.[40] It slows the movement of food through the stomach and can help reduce after-meal glucose fluctuations.[40] Some medications, such as metformin, have been explored as adjunctive therapies in T2D.[41] While they are not a substitute for insulin, they may provide additional benefits in certain cases, such as improving insulin sensitivity and reducing insulin requirements.[5]

While not a medication, continuous glucose monitoring (CGM) systems are essential tools for individuals with T1D.[42] CGMs uses a small sensor inserted under the skin to measure interstitial fluid glucose levels continuously.[42] Real-time data is sent to a receiver or smartphone, enabling users to monitor glucose trends and make informed decisions about insulin dosing and lifestyle adjustments.[42] It is essential for individuals with T1D to work closely with their healthcare team to tailor their treatment plan, which may include a combination of insulin therapy, CGM, and other medications to achieve optimal glycemic control and minimize the risk of complications. The specific treatment plan will vary based on individual needs, lifestyle, and overall health.

4.1.4 Biomarkers: HbA1c, C-peptide, and Glutathione

Various methods are used to monitor blood glucose levels and assess the overall glycemic control in individuals with T1D. Additionally, several biomarkers have been explored for the early diagnosis of T1D and to predict the risk of developing the condition.

Glycated hemoglobin (HbA1c) is a valuable biomarker used to assess long-term glycemic control and is considered a standard of care for people with diabetes.[43,44] The HbA1c test result is expressed as a percentage of glycated hemoglobin and reflects the average blood glucose levels

over the past 2-3 months.[43] Higher HbA1c levels indicate poor glycemic control and an increased risk of developing diabetes-related complications.[43] For instance, HbA1c below 5.7% is considered normal range, between 5.7% and 6.4% indicates prediabetes, and above 6.4% is considered diabetes.[43,45] As RBCs have a lifespan of approximately 120 days, the HbA1c test is described as a better, long term representation of the glucose environment.[43] However, it does not reflect short-term glucose variability or daily fluctuation in blood sugar;[46] both aspects are also important for diabetes management.

For individuals with diabetes, the HbA1c test is routinely used to monitor the effectiveness of diabetes management strategies, including lifestyle changes, medication, and insulin therapy.[43] It helps healthcare providers adjust treatment plans to achieve optimal glycemic control. Maintaining HbA1c levels within target range is associated with a reduced risk of developing diabetes-related complications, especially for the onset of T2D.[47] The process of glycation can occur throughout the body and can affect a wide range of proteins, impacting their structure and function.[48] This process has been identified as a major cause of metabolic diseases and increases the rate of the aging process.[48] Over time, the accumulation of advanced glycation end products (AGEs) can lead to tissue damage and contribute to the complications associated with chronic conditions like diabetes, macular degeneration, heart disease, chronic inflammation, and tissue damage.[49,50] Managing blood glucose levels effectively can help reduce the formation of AGEs and minimize their detrimental effects for people with T1D.[48,49,51]

Another biomarker that is used in the diagnosis and treatment of diabetes is C-peptide, a byproduct of insulin production used to measure levels of endogenous insulin secretion.[52,53] C-peptide measurement can help evaluate pancreas function in individuals with diabetes or other pancreatic disorders.[53] Low C-peptide levels may indicate impaired β-cell function or insulin

deficiency (normal fasting range 0.9-1.8 ng/mL).[54,55] In T1D, fasting plasma C-peptide levels are greatly reduced due to the destruction of β-cells (less than 0.2 nM).[53,54] Measuring C-peptide levels can help distinguish between T1D and T2D and assess residual β-cell function.[54–56] C-peptide, also known as connecting peptide, is a 31 amino-acid peptide that plays a crucial role in the production of insulin in the body.[52,55] It is formed in the β-cells of the pancreas[54,58] during the process of insulin synthesis when proinsulin is cleaved into insulin and C-peptide.[29,52,55,57] Proinsulin consists of three parts: an A-chain, a B-chain, and C-peptide.[55,57] Proinsulin folds into mature insulin and C-peptide is cleaved off all during the secretory vesicle packaging stage. Upon secretion into the bloodstream, the insulin hexametric crystal with Zn^{2+} breaks apart into insulin monomers. C-peptide is also secreted to the bloodstream during this process (**Figure 4.3)**.[30,53,55]

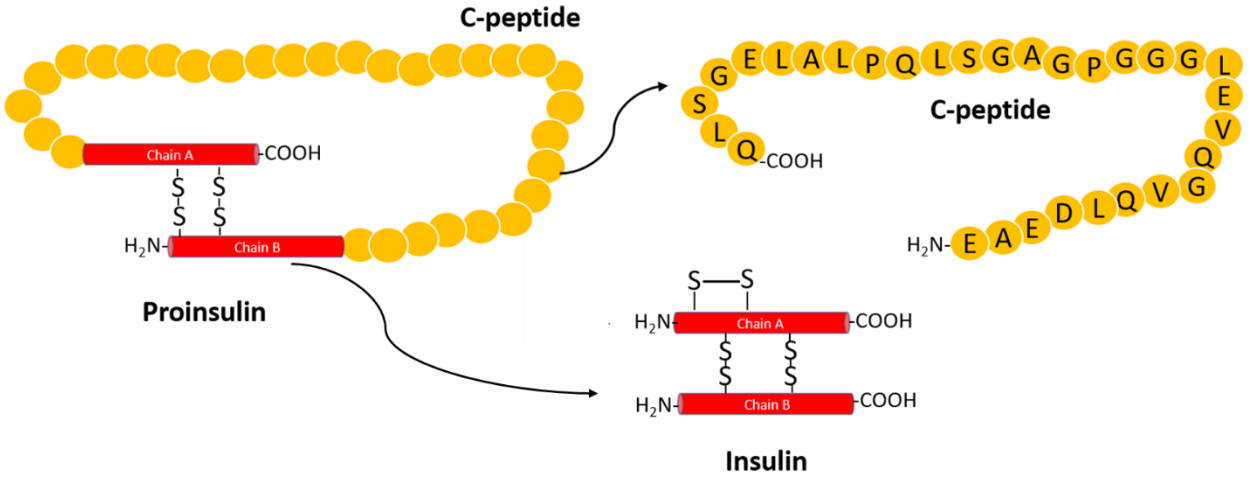

Figure 4.3. Proinsulin cleaved to produce insulin and C-peptide. Proinsulin is an 86 amino acid molecule consisting of an A and B chain containing insulin, with 3 disulfide bridges, and C-peptide. During cleavage and subsequent release, proinsulin is cleaved into insulin, a 51 amino acid hormone, and C-peptide, which is a peptide with 31 amino acids. This process is catalyzed in response to high levels of glucose in the bloodstream.[29,55]

These endoproteases consist of type I proprotein convertase 1 and 3 (PC1/PC3) that cleave the B-chain and C-peptide and type II proprotein convertase 2 (PC2) that cleaves the C-peptide and the A-chain.[58,59] The measurement of C-peptide in the blood provides valuable information about the amount of insulin being produced by the pancreas. Unlike insulin, C-peptide has a longer half-life (30 minutes) in the bloodstream when compared to insulin (6 minutes).[60] This characteristic makes C-peptide a useful marker for assessing endogenous insulin secretion,[60] and thus, C-peptide levels are used to differentiate between T1D and T2D.[52,55,56]

For individuals with diabetes receiving exogenous insulin therapy, measuring C-peptide levels facilitates the determination of the cause of hypoglycemia (low blood sugar) in certain situations.[52,54] For instance, elevated C-peptide levels during hypoglycemia may indicate an insulinoma, a rare tumor of the pancreas that produces excessive insulin.[61] Overall, C-peptide measurement is a valuable tool in diabetes management, helping to assess pancreatic function and differentiate between types of diabetes.[52–54] It provides insight into the body's endogenous insulin production and enables informed decisions regarding diabetes treatment and care.

Glutathione is a crucial antioxidant and plays an essential role in cellular defense against oxidative stress, as discussed in chapter 3. In the context of T1D, oxidative stress is considered a contributing factor in the pathogenesis and progression of the disease.[62,63] Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them with antioxidants.[62,64,65] This imbalance can lead to damage of cellular components including proteins, lipids, and deoxyribonucleic acid (DNA), ultimately contributing to the development of various T1D complications.[63,66–69]

Several studies have explored the role of glutathione in T1D, focusing on its potential impact on β-cell function, immune regulation, and oxidative stress management.[70–74] Glutathione

has been shown to modulate immune responses and regulate the activity of immune cells involved in the pathogenesis of T1D.[75–77] As an essential antioxidant, glutathione plays a crucial role in neutralizing ROS and preventing oxidative damage to cells, including β-cells.[78,79] Maintaining sufficient levels of glutathione may help reduce oxidative stress and its detrimental effects on β-cell function and overall pancreatic health.[79] Given its antioxidant and immune-modulating properties, glutathione has been investigated as a potential therapeutic target for T1D.[78] Strategies to study glutathione levels or its antioxidant capacity may offer more in-depth knowledge concerning therapeutic benefits for T1D and its complications. However, it is essential to note that while glutathione shows promise as a potential biomarker for studying T1D, research in this area is still ongoing. The complexities of the pathogenesis of T1D and the interplay of oxidative stress and hyperglycemic environments warrants further investigation to fully understand the role of glutathione and its potential implications for T1D complication progression and management.

4.1.5 Previous Studies, Increased Glycation, and T1D AGEs

In diabetes, elevated blood glucose levels contribute to increased glycation reactions, which can be seen in studies involving increased glycated albumin (gHSA) in diabetic plasma. The Spence group has studied the amount of glycation through glycated albumin levels (**Figure 4.4**) as well as the associated albumin (HSA) binding to T1D RBCs, hypothesizing the increased glycation could be the cause of lower downstream C-peptide binding to the cells.

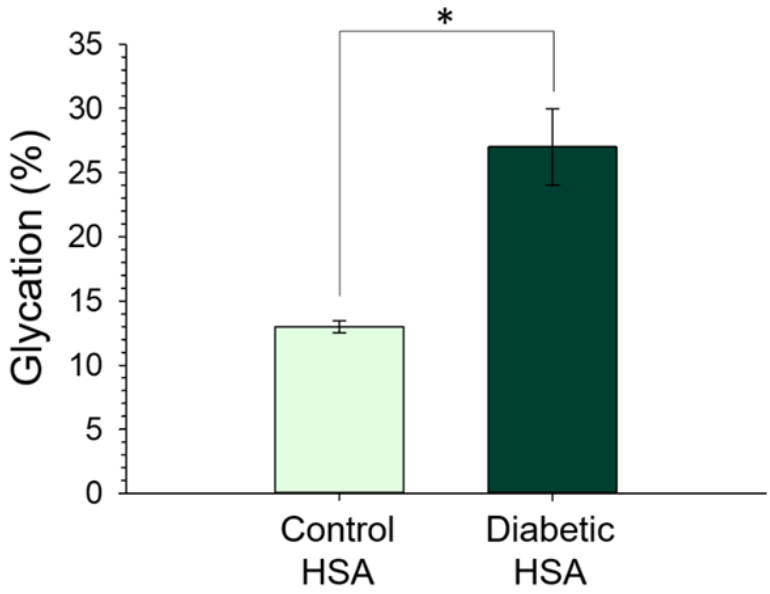

Figure 4.4. Differences in glycation percent for control and diabetic HSA isolated from plasma. Control HSA isolated from healthy control plasma had an average glycation percentage of 13 ± 0.5%. HSA isolated from plasma of people with diabetes had an average glycation percentage of 27 ± 3.0% (n=3 control, n=5 diabetic, *p<0.05, error =SEM). Borrowed from Jacobs, M. et al.[122]

Previous T1D and T2D studies by the Spence group have primarily focused on the importance of RBC-derived ATP release, C-peptide binding to RBCs, and glycation percentages of the C-peptide carrier, albumin. These studies motivate our current and ongoing research areas relating to connecting increased protein glycation and various downstream vascular issues associated with less C-peptide/albumin/Zn^{2+}.[80,81] Also, prior work determined GLUT1 RBC membrane levels of control and T1D patients to examine the role of hyperglycemic conditions on glucose regulation.[81] It is important to note that AGEs have been linked to increased diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, and cardiovascular complications in diabetes.[82,83] While AGEs have been measured in T1D subjects, a direct correlation between RBC, AGEs, HbA1c%, glycated albumin levels, and overall RBC function, including the RBCs ability to bind C-peptide, is without precedent.

Managing blood glucose levels and reducing the formation of AGEs may help mitigate the risk of diabetes-related complications.[50,84] Individuals with T1D have higher levels of overall AGEs compared to control subjects, as previously discussed.[85,86] AGEs can undergo further reactions, leading to the formation of more complex structures, cross-linking proteins, and causing tissue damage.[82] These AGEs play a significant role in the development of microvascular and macrovascular complications in T1D.[85,87] For example, AGEs contribute to endothelial dysfunction, oxidative stress, and inflammation.[75,88–91] In the ongoing research for the pathogenesis of diabetes-associated complications, AGEs have been implicated through their interaction with cell receptors. Specifically, the receptor for AGEs (RAGE), which activates various pro-inflammatory and pro-fibrotic signaling pathways.[65,87,89,91,92]

Given the detrimental effects of AGEs in T1D, various therapeutic strategies targeting AGE formation and accumulation have been explored. These include the use of anti-AGE agents, such as aminoguanidine and pyridoxamine, which inhibit AGE formation.[93,94] Additionally, dietary interventions aimed at reducing sugar intake and the use of antioxidants have been investigated to mitigate AGE-induced oxidative stress in T1D and T2D.[88,95–98] Circulating AGEs and their specific metabolites have emerged as potential biomarkers for assessing the risk of diabetes complications and disease progression in individuals with T1D and T2D.[67,83,85,99] Nε-carboxymethyl-lysine (CML) and Nε-carboxyethyl-lysine (CEL) have been lightly studied as potential biomarkers to predict the onset and severity of diabetic complications, but there is no cohesive method or in-depth understanding of how it can be related to HbA1c levels or other known biomarkers.[67,100–105] Thus, this gap in the literature is a potential avenue to explore utilizing the previous method developed in chapter 2, explored with various *in vitro* glycation in chapter 3, and future study ideas that will be discussed in chapter 5.

Understanding the mechanisms of AGE formation and overall changes in protein glycation can aid in understanding C-peptide binding to RBCs in T1D. C-peptide has been known to be a viable link in diabetic drug therapy, and the clinical trial in 2011 with Cebix explored this possibility.[106,107] In this trial, 250 participants were injected with mono-pegylated C-peptide therapy, Ersatta, or a saline-solution placebo.[106,107] The study lasted 12 months to determine the effects of C-peptide drug therapy on people with T1D, specifically targeting neuropathy complications.[106–108] The trial unfortunately failed in phase IIb, as the results concluded that the C-peptide drug therapy did not significantly improve the sensory nerve conduction in people with T1D when compared to the control group.[106–108] Even though the clinical trial failure was never publicly discussed, there are several factors that can be attributed to why this drug failed.[107,108]

One of the major theories involves the hyperglycemic conditions caused by excess glycation of RBCs and proteins. Glycation is known to alter the transport, binding, and function of RBCs and albumin,[109,110] which can contribute to why adding C-peptide to patients was not successful. If the overall glycation of proteins and cells were too high due to prolonged hyperglycemia, the people with T1D would not have significant improvement. It is theorized that C-peptide binds to albumin and albumin carries the C-peptide to the RBCs and albumin binds to the cell.[109,110] If albumin was too glycated and/or the RBCs had too many AGEs on the membrane, it could inhibit C-peptide, albumin, and RBC binding. Therefore, additional research involving the levels of protein and RBC glycation is important to understand more about C-peptide drug therapies.

One avenue of furthering the C-peptide drug trial research is implementing a biomarker screening process. Hypothetically, if a T1D patient has high HbA1c%, gHSA, AGEs, and exhibiting oxidative stress (GSH), this person would not be a good candidate for a C-peptide drug

trial. This is because theoretically the subjects would have too much glycated proteins to fully benefit from adding C-peptide exogenously, and C-peptide might not be delivered to the RBCs, and therefore not work properly. Therefore, the Spence group proposes using the methods in chapter 2 to explore RBC samples from people with T1D and controls to determine if HbA1c%, AGEs, and GSH could be useful for future patient screenings for a C-peptide drug trial. Further research is warranted to identify novel therapeutic targets and validate AGEs as reliable biomarkers for diabetic complications, which will be explored in the methodology of this chapter.

4.2 Methods

4.2.1 RBC collection and purification

Whole blood was collected via venipuncture from healthy consenting adults into 3 mL 3.2% sodium citrate blood collection tubes (Fisher Scientific, Hampton, NH). For each blood draw, 10 µL was removed to complete HbA1c and glucose analysis. Whole blood tubes were then centrifuged at 2000*g* for 10 minutes, the plasma and buffy coat were removed by aspiration, and packed RBCs (pRBCs) were kept in the tubes. The hematocrit was determined based on the pRBCs remaining for acid hydrolysis and thiol assay preparation. The drawing of blood followed a protocol approved by the Institutional Review Board of Michigan State University. Blood was obtained from healthy humans and informed consent was obtained from all volunteers. All record keeping complied with Health Insurance Portability and Accountability Act regulations.

4.2.2 Sample Preparation

All sample preparation and detection of the prepared RBCs was prepared in a manner identical to those reported in **2.2.5, 2.2.6, 2.2.7,** and **3.2.6**.

4.2.3 Data Analysis

Calibration plots of analyte/IS (internal standard) peak area ratio versus CML, CEL, and lysine concentrations were constructed, and a linear regression was used for all analytes. The peak area ratio of sample versus associated IS was used to produce a response to determine the concentrations from the calibration line. Free GSH results were collected from the Thiol Assay kit results and a linear regression was used for standard and analyte quantification. SigmaPlot (Systat Software Inc) was used to plot all results, GraphPad (Dotmatics) T-test Calculator (Unpaired T-Test) was used to perform statistical testing for free GSH, and R software (version 4.2.2) was used to perform statistical testing with the "rstatix" package (v0.7.2; Kassambara, A. 2023) for CML, CEL, total AGEs, lysine, and total AGEs/lysine.

ANOVA tests for each analyte (CML, CEL, Total AGEs, Lysine, Total AGEs/Lysine) were conducted to examine for differences between sample types. The T1D and control samples were treated as a between-subjects dependent variable. Prior to conducting the ANOVA test, the assumptions of normality and homogeneity of variances were assessed using Shapiro-Wilk and Levene's tests, respectively. If these assumptions were met, the ANOVA test was performed to determine which specific samples exhibited statistically significant differences on the analytes studied.

4.3 Results

4.3.1 Blood Glucose

There was a statistically significant difference of sample type on blood glucose concentration, and visualized results are displayed below in **Figure 4.5**. The unpaired T-test revealed T1D samples to have significantly higher blood glucose (mM) levels than the control samples ($p<0.01$). These results indicate that blood glucose concentration was altered with T1D

samples (8.03 mM ± 0.09) versus control samples (4.54 nM ± 0.23). T1D group resulted in approximately 28% higher blood glucose concentration than controls.

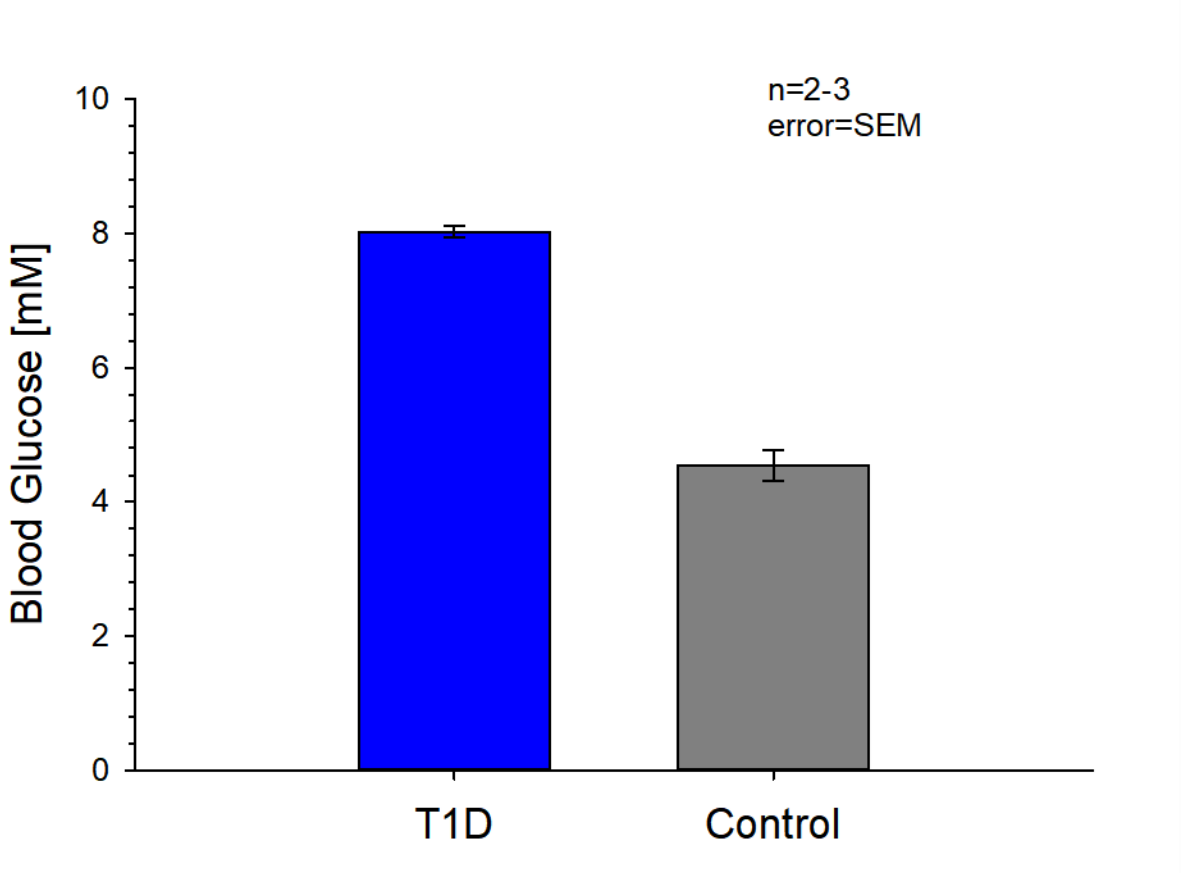

Figure 4.5. Blood Glucose (mM) quantification for both T1D and control samples. There were statistically significant differences between T1D (8.03 mM ± 0.09) and control (4.54 nM ± 0.23) **p<0.01, n=2-3, error=SEM.

4.3.2 HbA1c%

There was no statistically significant difference of sample type on HbA1c% concentration (**Figure 4.6)**. The unpaired T-test revealed T1D samples do not have significantly higher HbA1c% levels than the control samples (p=0.796). These results indicate that HbA1c% was not altered with T1D samples (6.10% ± 0.30) versus control samples (5.27% ± 0.18). There was only a 13.6% increase in HbA1c% for T1D compared to control samples.

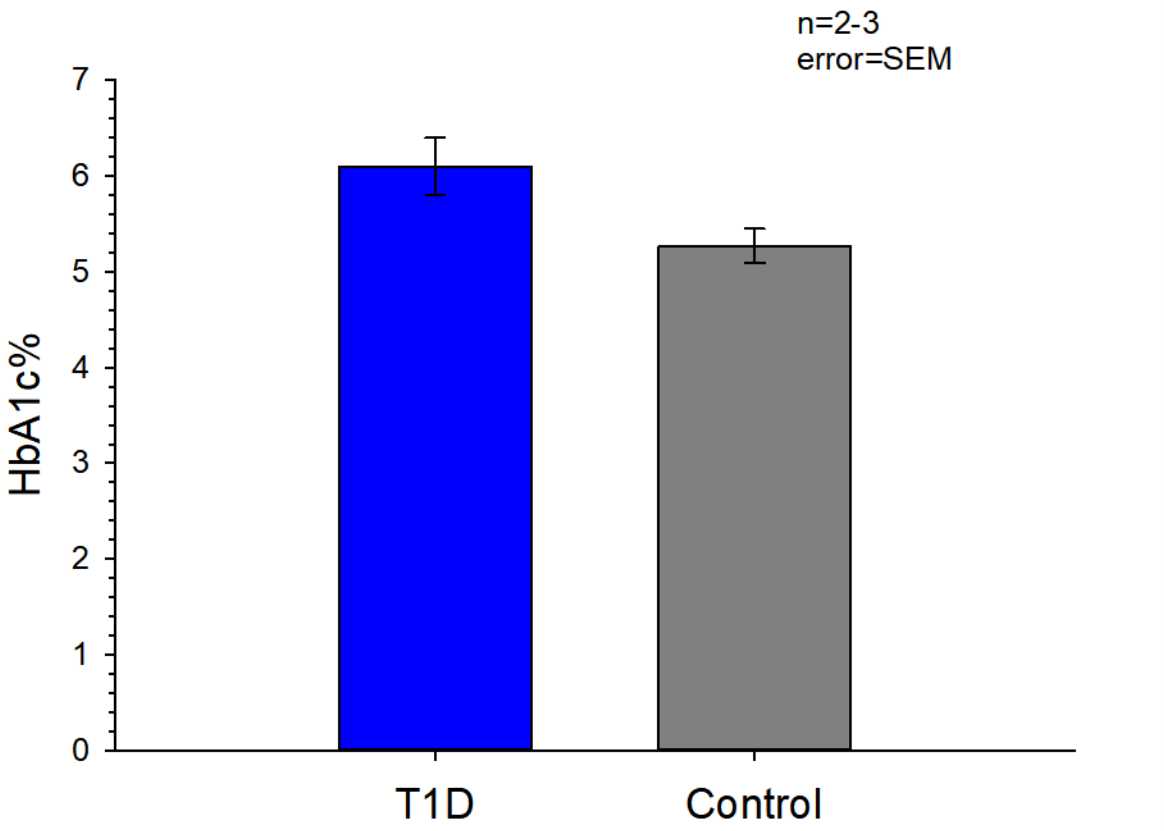

Figure 4.6. HbA1c% quantification for both T1D and control samples. There were no statistically significant differences between T1D (6.10% ± 0.30) and control (5.27% ± 0.18) p=0.0796, n=2-3, error=SEM.

4.3.3 CML

There was a statistically significant difference of sample type on CML, (**Figure 4.7)**. The ANOVA revealed T1D samples to have significantly higher CML (nM) levels than the control samples ($F(1,6)=7.729$, $p<0.05$). These results indicate that CML formation was altered with T1D samples (101.8 nM ± 2.2) versus control samples (60.8 nM ± 14.6). There was an increase in CML concentration that resulted in a 25% higher concentration in T1D versus controls.

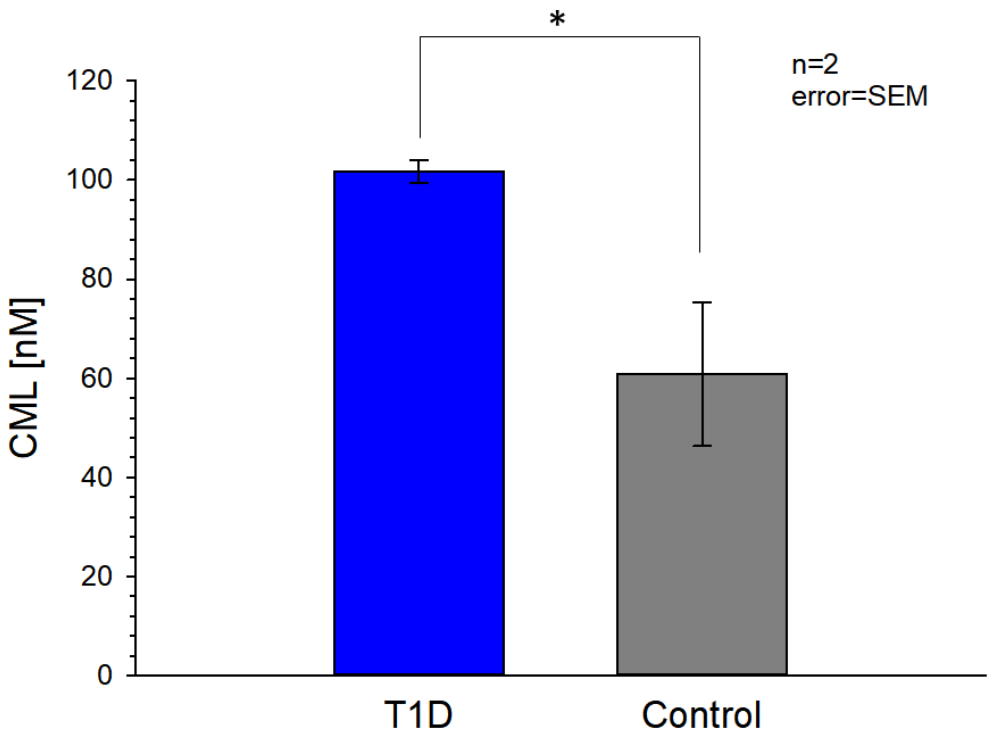

Figure 4.7. CML (nM) quantification for both T1D and control samples. There were statistically significant differences between T1D (101.8 nM ± 2.2) and control (60.8 nM ± 14.6) *p<0.05, n=2, error=SEM.

4.3.4 CEL

There was not a statistically significant difference of sample type on CEL, (**Figure 4.8)** ($F(1,6)=0.765$, $p=0.415$). These results indicate that CEL formation was not altered with T1D samples versus control samples.

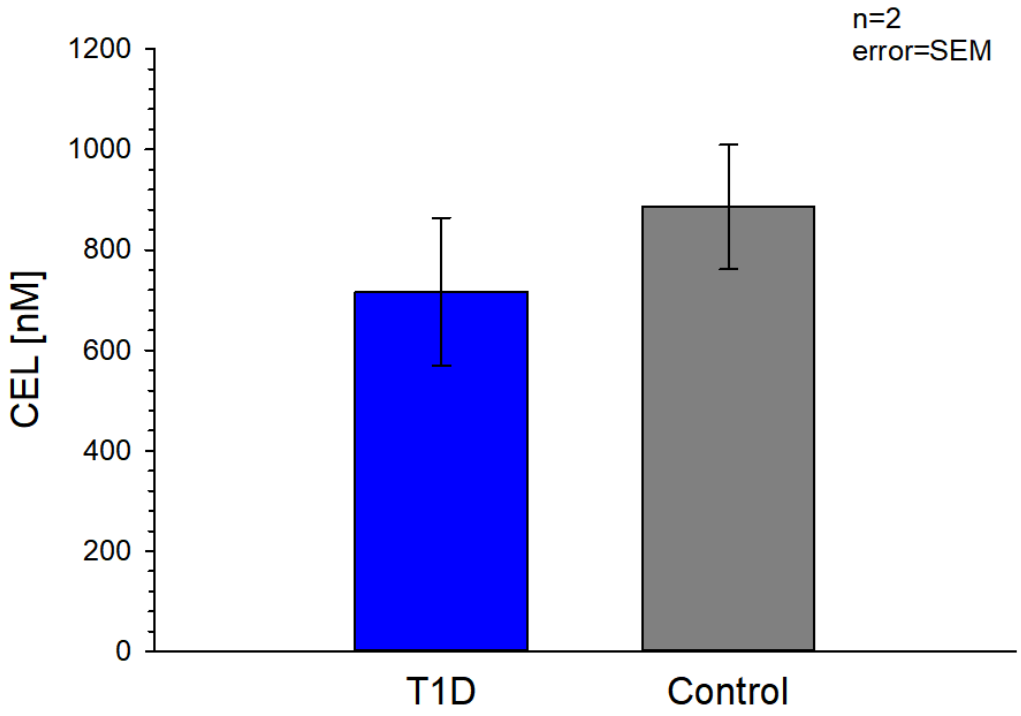

Figure 4.8. CEL (nM) quantification for both T1D and control samples. There were no statistically significant differences between T1D (716.6 nM ± 147.7) and control (885.2 nM ± 123.8) p=0.415, n=2, error=SEM.

4.3.5 Total AGEs

There was not a statistically significant difference of sample type on total AGEs, (**Figure 4.9)** (F(1,6)=0.407, p=0.547). These results indicate that total AGE formation was not altered with T1D samples versus control samples.

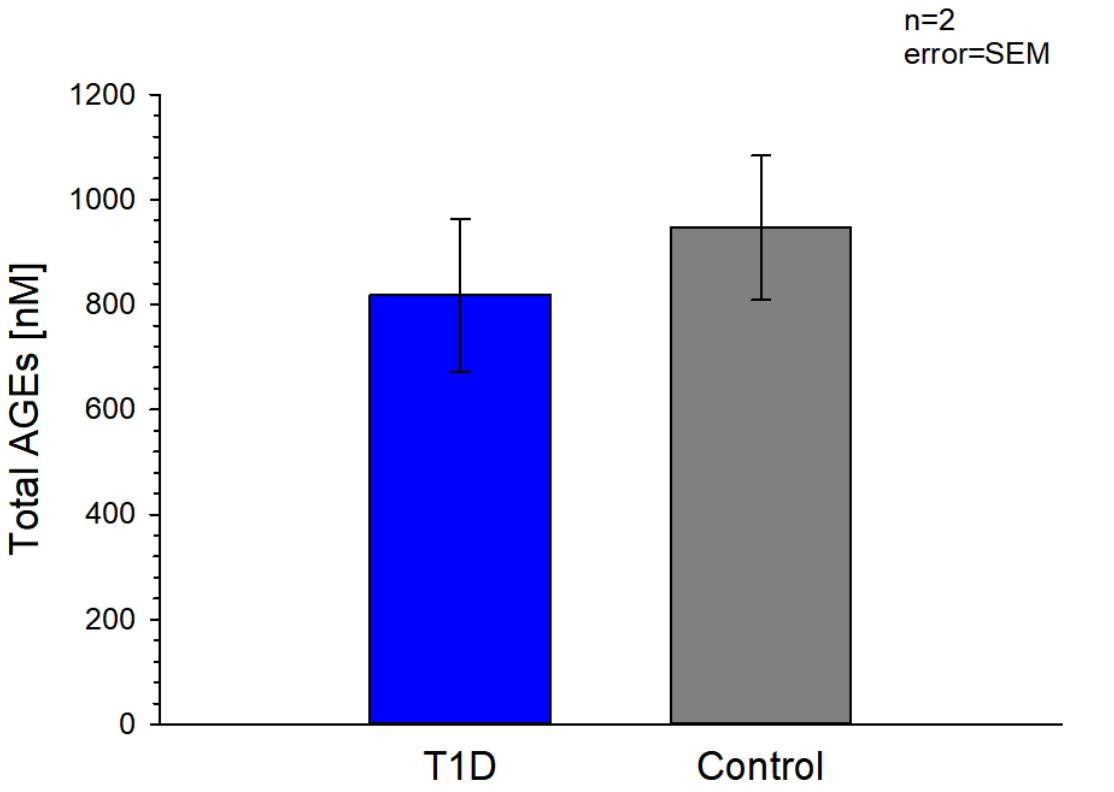

Figure 4.9. Total AGEs (nM) quantification for both T1D and control samples. There were no statistically significant differences between T1D (818.4 nM ± 145.5) and control (946.0 nM ± 137.4) p=0.415, n=2, error=SEM.

4.3.6 Lysine

There was a statistically significant difference of sample type on lysine, showing a 17.6% increase in lysine concentration in T1D samples versus control samples (**Figure 4.10)**. The ANOVA revealed T1D samples to have significantly higher lysine (nM) levels than the control samples ($F(1,6)=15.025$, $p<0.01$). These results indicate that lysine formation was altered with T1D samples versus control samples.

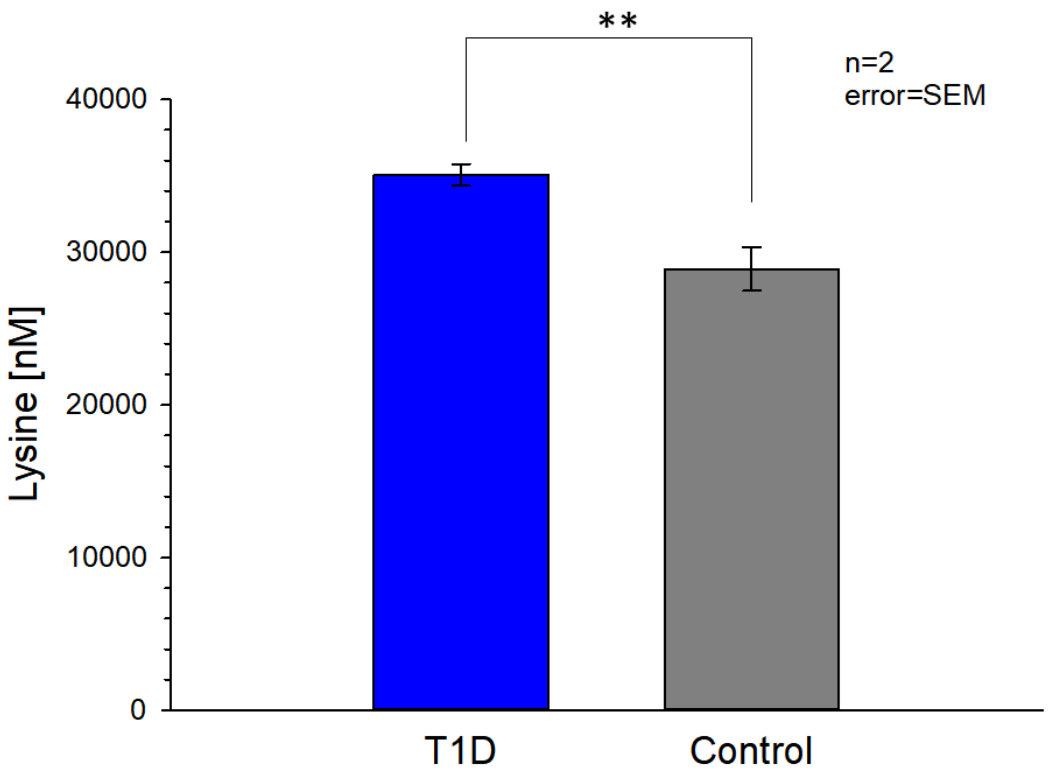

Figure 4.10. Lysine (nM) quantification for both T1D and control samples. There were statistically significant differences between T1D (35070.6 ± 721.2) and control (28888.6 nM ± 1422.5) **p<0.01, n=2, error=SEM.

4.3.7 Free Glutathione Assay

There was not a statistically significant difference of sample type on free GSH, and visualized results are displayed below in **Figure 4.11**. However, it is trending toward significance as there is an 18.1% increase in GSH concentration than control samples. The unpaired T-test revealed T1D samples do not have significantly higher free GSH (nM) levels (24211.8 nM ± 2544.8) than the control samples (19831.3 nM ± 2698.2) (p=0.3696). These results indicate that free GSH was not altered with T1D samples versus control samples.

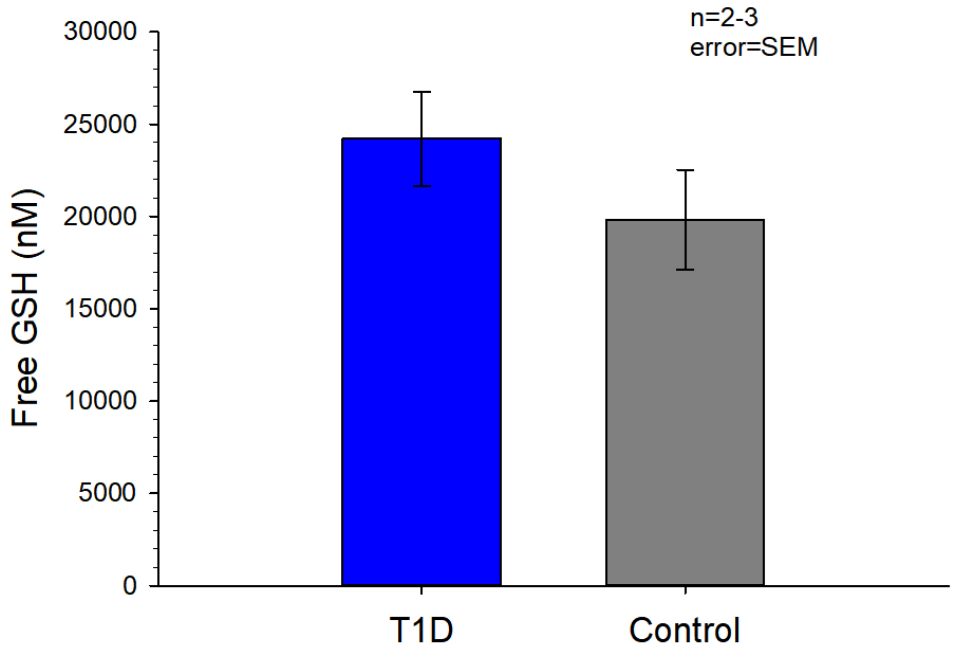

Figure 4.11. Free GSH (nM) quantification for both T1D and control samples. There were no statistically significant differences between T1D (24211.8 nM ± 2544.8) and control (19831.3 nM ± 2698.2) p=0.3696, n=2-3, error=SEM.

4.3.8 Relationship Between HbA1c% and Potential Biomarkers

To further understand the relationship between HbA1c% and potential biomarkers, the results are visualized in **Figure 4.12** and **Figure 4.13** showing total AGEs and Free GSH both have a positive linear relationship with HbA1c%. Total AGEs have a strong positive linear relationship with HbA1c%, R^2=0.902 and free GSH has a moderately strong linear relationship with HbA1c%, R^2=0.6779.

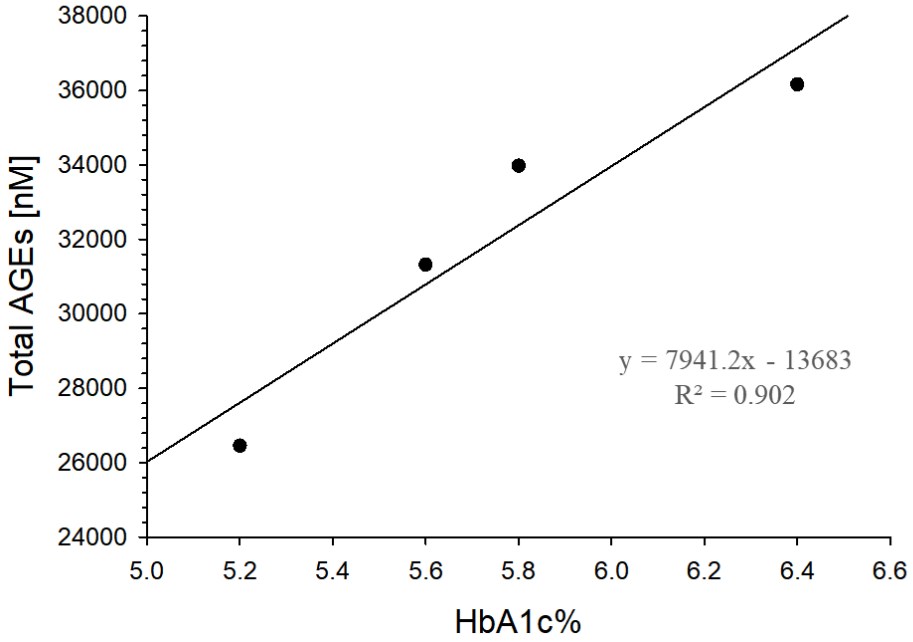

Figure 4.12. Correlation between HbA1c% and total AGEs. HbA1c% has a strong positive linear relationship with total AGEs, R^2=0.902, n=4.

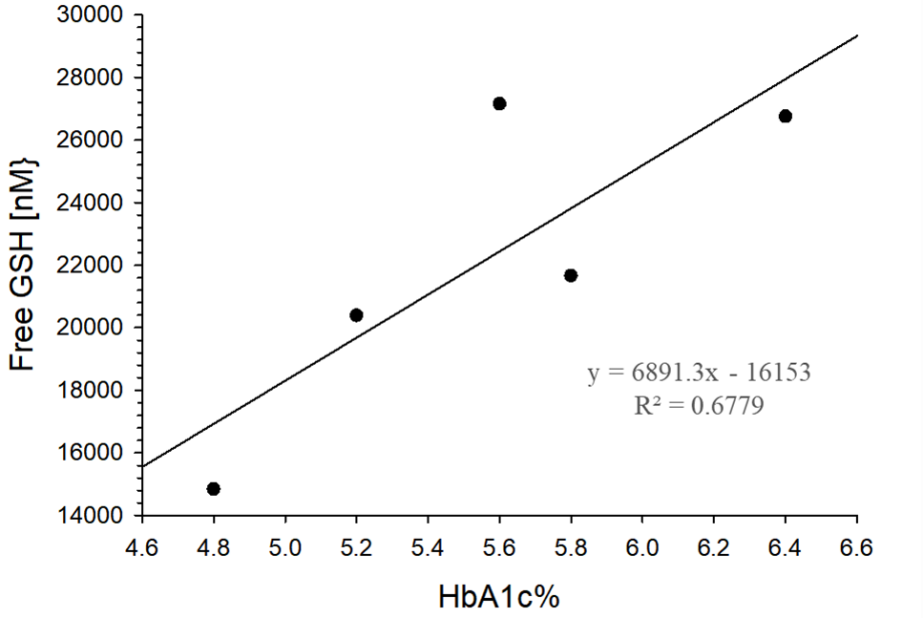

Figure 4.13. Correlation between HbA1c% and free GSH. HbA1c% has a moderately strong positive linear relationship with free GSH, R^2=0.6779, n=4.

4.4 Discussion

Further assessment of blood storage solutions and associated adverse effects related to RBCs promote questions about the effectiveness of these hyperglycemic blood storage solutions. The methods explored in chapters 2 and 3 were used to evaluate T1D samples to explore the connection between C-peptide binding and the adverse effects to RBCs from chronic hyperglycemia. Although there is research showing increased AGEs, decreased GSH, and changes in oxidative stress in T1D patients compared to people without diabetes, it is unclear how HbA1c% relates to these biomarkers.[50,111] Specifically, how AGEs, CML and CEL, as compared to total protein-bound lysine concentration, has yet to be evaluated in RBCs of T1D patients versus controls. Also, it is unclear in the literature whether elevated HbA1c% levels can be attributed to increased AGEs and changes in GSH.[45,112,113] Based on previous Spence group projects[81,109,110,114] and the potential to expand into C-peptide therapy applications, measuring glycation could be an additional biomarker screening process for future drug candidates.

The novel methods described in chapter 2 and 3 were utilized for T1D patients and controls while accounting for blood glucose and HbA1c% levels. The method development in chapter 2 provided a platform for the T1D blood sample research discussed in this chapter. Also, chapter 3 methods and results provide more information about hyperglycemic conditions over 6-weeks, which is a starting point for T1D subjects that often experience hyperglycemic blood glucose for much longer time frames. Overall, these results promote the discovery of how normoglycemic storage solutions as well as reduced storage time could be attributed to research to alleviate microvascular complications in T1D attributed to increased AGEs and oxidative stress.

Blood glucose quantification in **Figure 4.5** shows the varying range of T1D and control donors. There were significant differences between the sample groups as expected, with the T1D

group resulting in approximately 28% higher blood glucose concentration. Unexpectedly, the HbA1c% (**Figure 4.6**) showed less variation in range which did not make the type of sample groups statistically significant. One reason is that the two T1D donors had well controlled blood glucose (lower than 7 mM blood glucose) which resulted in ranges between the prediabetes and diabetes for HbA1c%. Thus, the results attributing the HbA1c% hypothesis could be skewed and therefore will need more participants to fully investigate any relationship between HbA1c% and the biomarkers measured in this chapter. Since HbA1c% reflects 120 days of glycated hemoglobin in circulation,[112] it was important to consider how a donor with elevated levels of glycation would compare to the noted T1D donors for the biomarkers studied. As previously discussed, studying glycated proteins is important to future studies, specifically for the C-peptide binding study. An area of interest is exploring how glycated proteins can affect the binding on C-peptide to RBCs, and the research involving gHSA, HbA1c%, and AGEs can be used to further drug related trials. Specifically, how C-peptide could be administered exogenously with insulin as an alternative therapeutic to help with vascular complications. If there are extensive glycated proteins, the binding could be inhibited due to the hyperglycemic environment and alterations to albumin and/or the RBCs. So, implementing a screening process utilizing the biomarkers previously discussed can aid in clinical trial management.

To better understand the physiological relationship between T1D patients and AGEs, blood was isolated and LC-MS/MS methods were utilized to measure CML, CEL, total AGEs, and protein-bound lysine. There were statistically significant differences between T1D and control CML concentration that resulted in a 25% increase in AGEs in T1D seen in **Figure 4.7**. This result is notable, as it is well known that CML is the most prominent AGE associated with oxidative stress complications. As shown in **Figure 4.8**, there were no significant differences in CEL

concentration between the T1D and control samples. Additionally, in **Figure 4.9**, due to the 6-10-fold increase in concentration of CEL in comparison to CML, there were no statistically significant differences in total AGEs between T1D and control. The results in **Figure 4.10** indicate there was a significant difference in amount of lysine concentration, and there was approximately 10% increase in T1D lysine than in control samples.

To further investigate the effect of diabetic glycation and oxidative stress conditions on free GSH levels, glutathione levels were measured using a commercially available thiol assay kit. The results in **Figure 4.11** did not show statistically significant differences in free GSH concentration between T1D and controls. This increase indicates there could be more to investigate for people with elevated, or close to prediabetic, HbA1c% levels.

The HbA1c% has a similar effect on both total AGEs and free GSH, as the results show different levels of a positive relationship. The results show as HbA1c% increases, and there is strong positive correlation in increasing total AGEs in **Figure 4.12**. Increasing free GSH concentration showed moderately strong correlation with increasing HbA1c% in **Figure 4.13**. Overall, these findings showed good correlation, but the sample size was small. In the interest of exploring more about the relationship between HbA1c% and total AGEs and HbA1c% and GSH, there will need to be more patients and sample testing.

Rising HbA1c% is a known biomarker for type II diabetes,[112] but these results typically are not attributed to indicators of other increased adverse effects to the RBCs. Lower GSH concentration is normally regarded as increased oxidative stress, but as discussed in chapter 3, the typical glycation mechanism could be saturated and forcing the polyol pathway to increase activity.[115,116] Thus, increasing sorbitol production and therefore increased GSH due to the increase in H^+ ions available.[116,117] It is not known through this assay if the GSSG/GSH ratio would change

between the sample types, and this ratio is the key to understanding more about oxidative stress and the effect of increased HbA1c% and hyperglycemic conditions. Hence, obtaining an assay kit that would either test GSSH alone or the GSSG/GSH ratio would be recommended steps to investigate further.

Numerous diseases are associated with elevated blood glucose and HbA1c%, including Alzheimer's Disease (AD).[22] AD is now commonly referred to as type 3 diabetes because of the similarities to increased glycation levels of various proteins, including AGEs.[22] There could be a link to increased glycated proteins and common complications associated with diabetes that could also be found with people with AD.[118] Thus, more experiments involving glycated proteins and T1D patients are necessary to learn more about these connections. Overall, these findings may help explain the pathophysiological role of hyperglycemic environments, oxidative stress, and the progression of T1D, AD, and other diseases associated with increased protein glycation.

The current link between HbA1c% and AGEs is not well understood, but the results shown in this work provide a basis for future applications. It is theorized that utilizing these potential biomarkers to improve patient screening process for future C-peptide drug trials will aid in the overall success of the clinical applications. By understanding more about glycation, the adverse effects of hyperglycemia on proteins and RBCs, and C-peptide binding, future drug trials for people with T1D can be more robust in choosing the best patients.

The research surrounding HbA1c% linking to AGEs, glutathione levels, and other biomarkers for autoimmune or other diseases is scarce, and the understanding medicine with hyperglycemic RBCs can aid in furthering the clinical drug discovery.[119–121] Thus, the research behind understanding more about the link between current hyperglycemic stored RBCs and T1D

RBCs can show how detrimental chronic high glucose can be over time including increasing oxidative stress.[119,120,121]

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Chapter 5: Conclusions and Future Directions

5.1 Blood Banking and Transfusion Medicine Conclusions

Blood transfusions serve as a critical medical intervention for individuals experiencing blood loss resulting from trauma or surgical procedures and those with inadequate red blood cells (RBCs), such as patients with sickle cell disease or anemia.[1–4] The conventional blood storage procedures approved by the Food and Drug Administration (FDA) employ glucose levels approximately six times the healthy range (4-6 mM) to preserve the vitality of RBCs during storage.[5,6] Despite more than six decades of usage, the Spence group has questioned the suitability of high glucose as a preservative due to the accumulation of storage-related abnormalities, referred to as storage lesions.[7,8] These abnormalities have raised questions about their potential contribution to transfusion-related adverse effects, such as transfusion-related acute lung injury (TRALI) and transfusion-associated circulatory overload (TACO), which collectively accounted for a notable percentage of morbidity and mortality between 2014 and 2018, as reported by the FDA.[9]

Furthermore, it is established that neonates and infants are typically administered RBC units that have been in storage for less than seven days, as reports suggest that storage exceeding ~14 days leads to irreversible adverse effects on RBCs.[10–12] These effects encompass reduced adenosine-triphosphate (ATP) and nitric oxide (NO) release, decreased deformability, increased oxidative stress, and other alterations observable after the 14-day mark.[13] It is worth noting that these post-14-day adverse effects account for a significant proportion of transfusion-related complications, surpassing those attributable to RBCs stored for less than 14 days.[13]

Advanced glycation end products (AGEs) have been extensively studied in the context of diet and nutrition but have not received enough attention in relation to RBCs and blood transfusion.[14–16] AGEs form due to elevated glucose levels and have been linked to aging and

metabolic disorders, such as diabetes.[17,18] These storage lesion markers, which include oxidative stress, are associated with increased AGEs and diminished levels of reduced glutathione (GSH).[19] Notably, research investigating blood transfusions and RBC storage in hyperglycemic solutions compared to normoglycemic storage is limited. In this work, the specific AGEs studied were Nε-carboxymethyl-lysine (CML) and Nε-carboxyethyl-lysine (CEL).

Some former members of the Spence laboratory explored normoglycemic storage conditions, which revealed higher ATP levels in solutions with lower glucose content compared to hyperglycemic blood storage.[20,21] These findings underscore the importance of the two-week storage time frame, a period that is theorized to mark the onset of irreversible byproduct formation, including AGEs.[20,21] The potential implications of transitioning from current high-glucose blood storage practices extend to individuals already at risk for transfusion-related complications. For those with diabetes, sickle cell disease, and anemia, the glucose levels in transfused blood may exacerbate vascular complications due to elevated glucose concentrations.[2,22–25]

The primary objective of this dissertation is to investigate the feasibility of utilizing normoglycemic blood storage conditions and assess RBCs using various biomarkers and assays to evaluate the overall effectiveness of a lower glucose storage solution.

5.2 Future Studies for AGE Development and Quantification

In chapter 2, the experiments primarily centered on method development for the detection and quantification of CML, CEL, and lysine on RBCs. These experiments were conducted following the guidelines provided by the International Council for Harmonization (ICH) and the European Medicines Agency (EMA).[26] Initial findings demonstrated strong adherence to these guidelines, especially in terms of matrix effects, limits of detection and quantification (LOD/LOQ), linearity, carryover, and accuracy/precision for both intra- and inter-assay evaluations.

The ultra-high performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) method's selectivity exhibited effective peak separation, and clear retention time correlation was observed with the respective analyte internal standards (IS). However, on day 1 of storage, no statistically significant differences were identified in the AGE analytes between the two solution types. These results signify the success of the novel method, prompting the need for further analysis.

Chapter 3 extended the investigation by exploring the impact of storage time and solution type on AGE formation. The results conclusively demonstrated that both time and solution type contribute to AGE formation. While the data concerning GSH and heightened oxidative stress did not provide absolute clarity, a noticeable trend emerged, emphasizing the importance of considering the potential influence of hyperglycemia on free GSH.

To delve deeper into this phenomenon, a rigorous examination of the oxidized glutathione (GSSG) to reduced glutathione (GSSG:GSH) ratio will be conducted on RBCs stored. Ideally, this analysis will incorporate an automated feeding system to maintain glucose levels within the range of 4-6 mM, even on days 36 and 43, as discussed in chapter 3. Adjusting the glucose solution content on these days is suggested, as it is hypothesized that RBCs may alter the rate of glucose metabolism and uptake during storage. This interconnected relationship among increasing AGEs, GSH levels, and blood glucose concentrations underscores the need for additional samples to further enrich our understanding of this intriguing research field.

To further this research and obtain a more comprehensive understanding of the connection between different levels of hyperglycemia, AGEs, and their impact on RBCs, an experiment could be undertaken by using a range of glucose solutions with varying, incrementally increasing concentrations. This experiment would mimic more closely type I diabetes (T1D) patients with

varying blood glucose levels, some well controlled and others with extreme fluctuations. This could also help with the further understanding of people with prediabetes and how quickly GSH and AGEs can form. The overall object of this experiment would be to assess how variations in glucose concentrations within the storage solution affect AGE formation and GSH in RBCs.

A glucose concentration gradient will be set up and a set of storage solutions with varying glucose concentrations will be prepared. Solutions will include several glucose levels higher than 6 mM (representing hyperglycemia) and a solution with glucose levels within the healthy range (4-6 mM). Sample collection will be performed by obtaining a consistent volume of RBC samples from the same donor source and dividing it between the different glucose gradient bags for automated feeding. The RBC samples will be stored in separate bags, each filled with AS-1N, and kept under controlled storage conditions at 4°C for 42 days. There will be sampling at pre-determined intervals (e.g., daily, weekly, or bi-weekly), to collect RBC samples from each storage solution bag. To determine significance, there will be a comprehensive analysis of AGE formation for each RBC sample type using the previously described sample preparation technique (**2.2.1-2.2.5**) and LC-MS/MS method established in the previous experiments **(2.2.6-2.2.7**). This analysis should include the quantification of CML, CEL, lysine, and other relevant AGEs. Beyond the AGEs determination, additional parameters would include blood glucose and glutathione (both reduced and oxidized) in each RBC sample. Statistical analysis will be important to identify any significant differences in AGE formation, glucose levels, GSH ratios, and oxidative stress markers among the different glucose concentration groups over time.

This experiment will likely reveal how altering glucose concentrations within the storage solutions affects the formation of AGEs in RBCs. It will provide insights into the specific impact of hyperglycemia on AGE accumulation for T1D and fluctuations in blood glucose. It has the

potential to provide connections with variations in reduced and oxidized glutathione levels, as well as oxidative stress. The results may further elucidate the mechanisms underlying these phenomena and contribute to the development of more effective blood storage protocols for various medical conditions.

5.3 Future Studies for SPR Binding Studies

Previous research conducted by some members of the Spence group has explored binding patterns of RBCs from both "healthy" individuals and those with T1D to C-peptide, as depicted in **Figure 5.1**.[27] The divergence in binding behaviors raises an intriguing question: how does glycation and hyperglycemia influence RBC binding? It is established that C-peptide uptake is influenced by two critical factors: the presence of zinc ions (Zn^{2+}) and the presence of albumin.

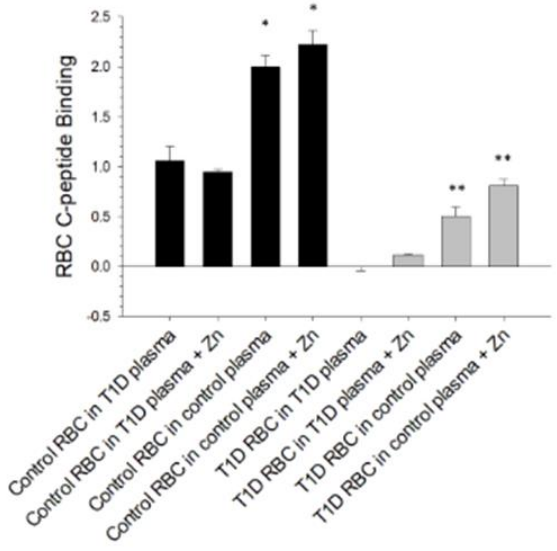

Figure 5.1. RBC C-peptide binding to control and T1D samples. There is a significant increase in C-peptide binding uptake in control RBCs than in T1D. Additionally, the samples in control plasma have more binding than those in T1D plasma (*p≤0.05, **p≤0.05, n≥7, error=SEM). Borrowed from Janes, T.[27]

Notably, the albumin employed in these binding experiments is non-glycated. However, the results derived from investigations involving increased levels of glycated albumin reveal distinct trends, specifically decreased ATP release and a higher equilibrium dissociation constant (Kd), as visually represented in **Figure 5.2**.[28]

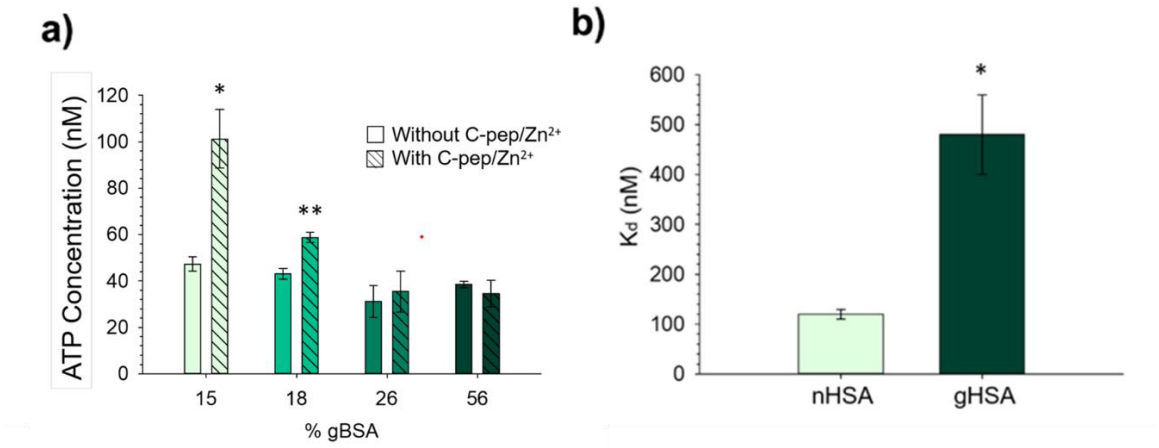

Figure 5.2. RBC C-peptide binding to control and T1D samples. A. Glycation percentages ranged from 15% to 56% and there was an overall decreasing trend of ATP release. There were significant differences between with and without C-peptide at 15% glycation (*p<0.05) and 18% (p**<0.05) (n=3-6, error=SEM). B. There was significantly higher Kd from gHSA sample than the nHSA sample which resulted in a 2.3x increase (n=5-6, error=SD, *p<0.05). Borrowed from Jacobs, M.[28]

It is worth noting that the extent of albumin glycation in plasma has been recorded to range between (12-16%) for individuals without diabetes and 2-5x higher for those with T1D.[29,30] While various binding experiments have been conducted using different quantities of glycated albumin, direct measurements for C-peptide and Zn^{2+} binding to determine overall binding strength differences have not been performed.[28]

To delve deeper into this notion of binding strength, an instrumental approach that can be employed is surface plasmon resonance (SPR).[31] Preliminary experiments were conducted to investigate the binding of C-peptide to albumin, both with and without the presence of zinc ions,

and under varying degrees of glycation. These experiments measured the response units (RU) of biotinylated albumin (EZ-link Sulfo-NHS-LC Biotinylation Kit, Thermo Fisher Scientific, Waltham, MA), which was immobilized on the Octet streptavidin in dextran hydrogel (SADH) chip (Sartorius, Bohemia, New York). A solution containing varying concentrations of C-peptide was then flowed over the chip.

In an ideal scenario, the C-peptide RU would reach a level that signifies strong binding, utilizing refractive index changes as an indicator of C-peptide binding to albumin and not the chip surface. In **Figure 5.3,** there was some evidence of binding to albumin, and this evidence intensified with increasing concentrations of C-peptide. However, the RU levels remained low, making it uncertain whether the binding was significant enough to determine a notable Kd.

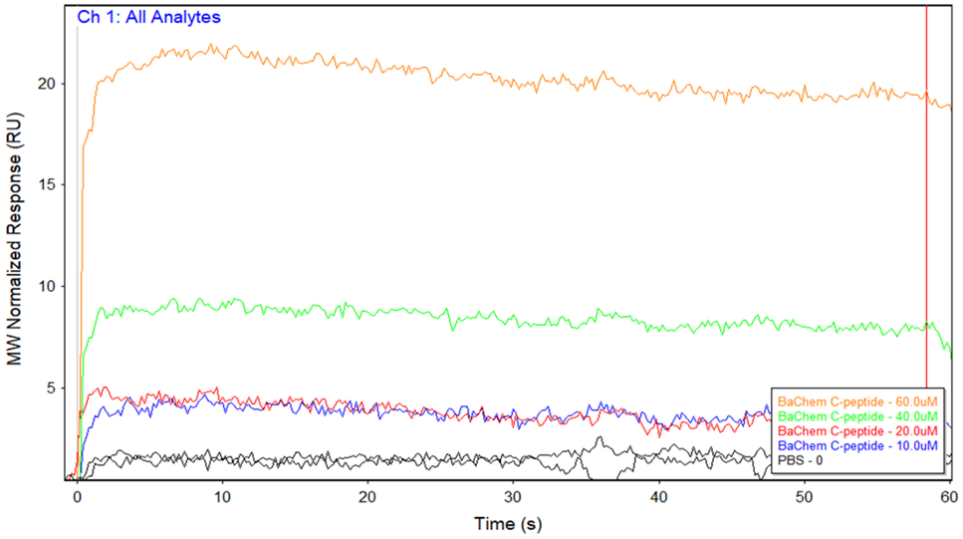

Figure 5.3. SPR data from biotinylated albumin and C-peptide analyte. There were increasing concentrations of C-peptide (10-60 µM) flowed over the biotinylated albumin on the SADH chip. The PBS had low RU (1-3), but the highest C-peptide also showed relatively low RY (20-23). The overall difference between the blank and analyte should be much greater than 20 RU in order to determine significant specific binding.

The low RU values could be attributed to the substantial size disparity between albumin (a much larger protein) and C-peptide (a considerably smaller peptide). Considering these challenges, the experiment was repeated with commercially available biotinylated C-peptide and varying concentrations of normally glycated albumin flowed over the chip. Unfortunately, the experiment yielded high non-specific binding, and the RU values were remarkably similar to high concentrations of albumin, rendering it unsuccessful. To reduce non-specific interactions, 0.05% (v/v) Tween-20 was added to PBS, but this resulted in an exceedingly high blank PBS RU, signifying no notable binding between C-peptide and albumin, rendering the experiment once again unsuccessful.

In scientific literature, experiments often employ low quantities of albumin to mitigate non-specific binding in protein studies.[31,32] However, since the analyte in this case is albumin itself, this approach is not viable. Additionally, altering the biotinylated peptide and flowing it over a larger protein did not yield an increased RU level. Consequently, these experiments demand further investigation and refinement to gain a more comprehensive understanding of C-peptide and glycated albumin binding across varying concentrations.

Insights derived from these binding studies hold significant potential for advancing blood banking practices, particularly the development of a new "rejuvenation" solution by introducing C-peptide to current blood storage bags, as discussed in section **5.5**.

5.4 Future Studies for Glycation Binding and T1D Samples

In Chapter 4, samples from individuals with T1D were compared to "healthy" controls in terms of AGEs, GSH, hemoglobin A1c (HbA1c%), and blood glucose levels. While no statistically significant differences were observed in HbA1c%, CEL, total AGEs, and GSH, the limited sample size hindered a comprehensive evaluation of the results. Consequently, further research requires

an expanded donor pool, with at least six donors, to draw more conclusive insights into the relationship between T1D and the associated AGEs and GSH levels.

Given the established knowledge that hyperglycemia induces increased protein glycation, the next step involves conducting an experiment that builds upon previous binding studies while simultaneously measuring AGEs, GSH, HbA1c%, and blood glucose levels.[33,34] Since HbA1c% cannot be relied on for diagnosing diabetes in patients with recent blood transfusions, it is important to evaluate why this is the case based on the process of transfusion.[35] This initiative aims to ensure the accurate assessment of glycation levels for potential use in a screening process for a C-peptide therapy drug.

An intriguing avenue for research lies in comparing albumin glycation data for individuals with T1D and assessing the rate of formation relative to AGEs, GSH, and HbA1c%. To perform this experiment, T1D and control donors would provide a blood sample, it would be processed similar to methods described in 4.2.1, and the sample will be aliquoted to study the biomarkers previous discussed (HbA1c%, blood glucose, AGEs, and GSH) and C-peptide binding to RBCs. Then, linear regression will be used to explore relationship strength of HbA1c% and C-peptide binding. Therefore, creating a robust screening process for T1D patients for use in the future C-peptide clinical drug trial.

5.5 Future Studies for Blood Storage Studies

A novel experimental concept involves the addition of C-peptide to blood storage bags, with a focus on studying the binding and AGE qualities. Several questions must be addressed to inform the execution of this experiment. These include determining the appropriate C-peptide concentration (in solution, with albumin and zinc), deciding whether it will be administered at the beginning, continuously fed throughout the trial, or introduced at the end. Additionally,

understanding how RBCs change over time in comparison to control samples without C-peptide (AS-1 and AS-1N) is crucial. The purpose of this proposed experiment is to explore healthier ways to store the blood, not to prolong shelf-life, although that could be another avenue of research in the future.

To systematically address these inquiries, the following approach is proposed. Firstly, it is advisable to conduct binding experiments involving the progressive glycation of albumin, utilizing SPR methods as detailed in section **5.3**. The aim here is to determine the rate of albumin glycation over the 42-day storage period. It is already known that albumin is glycated quicker than RBCs, which produces about 4.5 times more gHSA than HbA1c% when blood glucose concentrations increase dramatically.[35] In this experiment, albumin will be added to both AS-1 and AS-1N stored blood samples and removed weekly for the evaluation of non-glycated bovine serum albumin (nBSA) and glycated bovine serum albumin (gBSA) levels. The same techniques employed in chapter 2, specifically acid hydrolysis, and LC-MS/MS, will be utilized to analyze the samples.

Once the average glycation level of albumin is established, the binding studies can be expanded upon by conducting SPR experiments to identify the optimal C-peptide concentration for preliminary studies. In one scenario, C-peptide, along with equal-molar concentration of Zn^{2+} and commercially available bovine serum albumin (BSA), can be introduced into a blood storage bag containing AS-1, with a parallel control bag devoid of the C-peptide solution. This process should be repeated at multiple time points: at the beginning, halfway through the storage period, and at the final time point.

After the initial assessment determines the ideal timing for adding the C-peptide solution based on albumin glycation, a comprehensive evaluation of RBCs can be conducted. This assessment

will encompass the analysis of AGEs, sorbitol changes, C-peptide binding, GSH, ATP levels, deformability, and various other assays to investigate potential adverse effects on RBCs.

The goal of these experiments is to not only demonstrate the benefits of normoglycemic storage conditions but also to underscore the advantages of introducing C-peptide/BSA/Zn^{2+} to rejuvenate stored blood for improved transfusion medicine. This multifaceted approach holds the potential to enhance our understanding of blood storage dynamics and its impact on RBC quality.

5.6 Future *In Vitro* Experiments to Clinical Trials Advancement

Clinical trial experiments involving blood banking hold a paramount significance for individuals affected by diabetes and sickle cell anemia.[2,23–25,36] These medical conditions pose unique challenges and complications that necessitate tailored solutions for effective management and treatment. People with sickle cell anemia often require frequent blood transfusions to alleviate anemia, manage complications, and reduce the risk of stroke.[22,37] Blood banking and clinical trial experiments are indispensable for this patient group, as the quality and compatibility of blood are of paramount importance.

The availability of a safe and reliable blood supply is crucial for individuals with diabetes, as they may require frequent blood transfusions for various reasons, such as surgical interventions, acute episodes, or chronic complications.[25,36] Clinical trial experiments within the realm of blood banking are instrumental in optimizing the transfusion process for patients with diabetes. These experiments can help determine the ideal storage conditions for blood, ensuring that it remains safe and effective for transfusion, even when it needs to be stored for extended periods.

One of the primary concerns for individuals with diabetes or sickle cell disease is the potential impact of hyperglycemia and AGEs in stored blood, as previously discussed. Through clinical trials and experiments, it becomes possible to investigate how different storage conditions,

additives, and interventions affect the quality and safety of blood intended for transfusion. Understanding the intricate relationship between blood storage and glycation can lead to the development of tailored solutions that minimize the risks associated with transfusions in individuals with diabetes. This knowledge also paves the way for innovations like "rejuvenation" solutions, incorporating substances like C-peptide to enhance the therapeutic benefits of transfused blood.

Furthermore, advancements in blood banking can lead to the development of novel therapies, offering the potential for a cure or long-term disease management.[38,39] Clinical trials in this field are pivotal in testing these innovative treatments, which hold the promise of transforming the lives of individuals at-risk.

Clinical trial experiments within the domain of blood banking are pivotal to enhancing the safety, efficacy, and quality of blood transfusions, addressing unique challenges, and ultimately improving the outcomes and quality of life for patients grappling with various medical conditions. As medical research continues to advance, these trials are indispensable steps toward providing tailored solutions and improving the overall healthcare landscape for various patient populations.

When designing a clinical trial for testing the effectiveness of blood transfusions, there would need to be willing patients to be studied over several weeks to evaluate the new RBCs circulating. Recruiting participants to get blood transfusion and divided into two cohorts: AS-1 and AS-1N subjects. First, the individuals will be below 60 years old and they will be transfused with 3 units (equal to about 3 pints) of blood from the stored blood bags prior to 14 days in storage.[40,41] The second set of experiments would include participants who receive blood transfusion after 14 days of blood being stored. Each participant would have their blood glucose levels recorded at hourly intervals on days 1, 2, 3, and then weekly testing post-transfusion, until 120 days. Also, samples

will be removed from each patient during these glucose checks for an overall panel of tests, including HbA1c%, ATP release, and C-peptide binding.

In conclusion, blood banking and *in vitro* research is integral to the success of transfusion medicine clinical trials. They provide the necessary infrastructure for collecting and preserving blood samples, conducting preliminary safety and efficacy assessments, and developing essential assays for patient monitoring. These practices ultimately contribute to the safe and effective development of new treatments and therapies for various medical conditions.

Blood banking has been a cornerstone of modern medicine for decades, providing a vital lifeline to countless patients in need of transfusions. However, it is striking how few significant advancements have been made in this critical field during this time. In a world that has seen remarkable progress in medical science, it is time to turn the attention to enhancing the methods of blood storage and preservation. One promising avenue for improvement lies in the monitoring of AGEs and other storage lesions. Detecting and managing AGEs in blood banked units could have a profound impact on the health of patients receiving transfusions, and a critical screening technique for future diabetic drug discoveries. Additionally, addressing the storage lesion markers, which can occur during prolonged storage and lead to decreased blood cell viability, is equally crucial. By developing better methods to store blood, such as utilizing automatic and sterile feeding techniques for innovative storage solutions, as discussed in this dissertation, it can ensure the preservation of blood components is optimized. These advancements would not only benefit patients in dire need of transfusions but would also enhance the overall quality and availability of blood for diverse patient populations, marking a new era in the history of blood banking.

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