Investigation of advanced glycated hemoglobin as an alternative marker for better diagnosis of diabetes

Thesis Submitted to AcSIR
for the Award of the Degree of

Doctor of Philosophy

In

BIOLOGICAL SCIENCES

By

Jagadeeshaprasad M G

10BB15A26045

Under the guidance of

Dr. Mahesh J. Kulkarni (Research Supervisor)

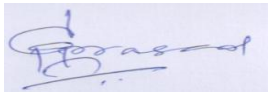
Biochemical Sciences Division
CSIR-National Chemical Laboratory
Pune 411008, India

2018

सीएसआईआर – राष्ट्रीय रासायनिक प्रयोगशाला
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
डॉ. होमी भाभा मार्ग, पुणे - 411 008. भारत
CSIR - NATIONAL CHEMICAL LABORATORY
(Council of Scientific & Industrial Research)
Dr. Homi Bhabha Road, Pune - 411 008. India

CERTIFICATE

This is to certify that the work incorporated in this Ph.D thesis entitled "Investigation of advanced glycated hemoglobin as an alternative marker for better diagnosis of diabetes" submitted by Mr. Jagadeeshaprasad M G to the Academy of Scientific and Innovative Research (AcSIR) in fulfilment of the requirement for the award of the degree of Doctor of Philosophy, embodies original research work under our guidance. We further certify that this work has not been submitted to any other University or institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table, etc., used in the thesis from other sources have been duly cited and acknowledged.



Jagadeeshaprasad M G
(Student)

Dr. Mahesh J. Kulkarni
(Research supervisor)

Date:11.01.2018

Place: Pune

Communications Channels ☎ +91 20 25902000 +91 20 25902600 +91 20 25902222
Fax +91 20 25902601 (Director) +91 20 25902660 (Admin.) +91 20 25902639 (Business Development)
URL : www.ncl-india.org

Dedicated to my Grand mother

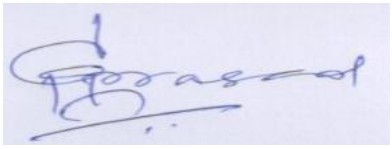
Table of Contents

Contents

Declaration by Research Scholar

I hereby declare that I am the sole author of this thesis entitled "Investigation of advanced glycated hemoglobin as an alternative marker for better diagnosis of diabetes" submitted by me for the degree of Doctor of Philosophy to Academy of Scientific and Innovative Research (AcSIR) and is a record of the research performed by myself with exception to biochemical parameters analysis for clinical subjects. The work in chapter 2 and 3 was performed in National Chemical Laboratory (Pune, MS, India) and the human samples were collected from Chellaram Diabetes Institute (Pune, MS, India), with the help Dr. Unnikrishnan A G. The study was approved by the Institutional Ethical Committee of Chellaram Diabetes Institute, Pune, Ref No: CDIEC/2014/22 and protocol code: CDIEC/R-D/2014/Prj-3 dated 01.12.2014. Written consent was only for participation in the study and the identity of the patients is kept confidential. All the mass spectrometry data analysis was carried out using licensed software except for the Pinpoint^{TM} software, which was provided to us on trial basis by Thermo Scientific (Thermo Fisher Scientific).

This work is original and has not been submitted previously for any higher degree and was supervised by Dr. Mahesh Kulkarni.



Jagadeeshaprasad MG Date: 11th January 2018

Acknowledgement

During my journey as a research fellow, I have come across a world of excitement, happiness, surprises and difficulties. It was not easy to grow without help and make a place for oneself in this journey. This was made possible with the continuous support of several people. First and foremost, I would like to express my sincere and profound gratitude to my research supervisor Dr. Mahesh Kulkarni, who is an endless support with his sustained scientific inputs. He introduced me to the field of proteomics and mass spectrometry. I really admire, the way he handle the scientific and non-scientific things with utmost care. There is no doubt that I have learnt so many things, especially how to analyze and get valuable information even from small things, and how to deal with a problem. I wholeheartedly thank him for new ideas and supported me immensely during my stay at NCL.

I would like to thank Dr. Thulasiram H. V, who introduced me to the research field and for his generous help at various stages of being inspirational and encouraging me to pursue career in research. I have high respect and gratitude for his immense moral support like a family member.

My heartfelt thanks to Dr. Mahesh Kulkarni and Dr. Thulasiram for their valuable time in bringing their output and constant support in shaping my academic career in the perfect manner. It would not have been possible for me to accomplish my thesis without them. I don't have words to thank them for their support which they have extended towards me in my life.

I am thankful to my DAC members Dr. Gurunath Suryavamshi, Dr. Thulasiram H.V. and Dr. Kiran Kulkarni for their valuable advice, help, facilities and encouragement in dealing with scientific and experimental problems during DAC meetings to make this work more meaningful.

I am grateful to the Director, and Head of Department Biochemical Sciences, CSIR-NCL Pune for availing me with research facilities. I am thankful to Indian Council of Medical Research (ICMR), New Delhi for supporting me with Senior Research Fellowship during my research tenure.

I also thank Dr. Unnikrishnan A.G, for supporting us with clinical blood samples. I also extend my thanks to Dr. Shalba Tiwari, Dr. Devi Prathyush and Ms. Nishita for their help in providing clinical samples. I also would like to express my warm

gratitude to the volunteer diabetic and non-diabetic individuals for donating blood samples for this study. I would like to take this opportunity to express my gratitude to Dr. Dhanasekaran Shanmugam and my friends in his lab for providing erythrocytes.

I would like to thank to Dr. Santhakumari B, Dr. Jomon Joseph, Mr. S.S. Deo and Mr. Golap for their support by providing instrument booking, chemicals.

I am extremely grateful to Dr. Arvind M Korwar, Dr. Shweta Bhat and Mr. Vannur Swamy for their incessant guidance and valuable suggestions throughout my Ph.D work. I also thank Ms. Vinashya V. for her ideas, support and enormous contribution in experiments to complete my PhD work and immense constant moral support.I have greatly benefited from discussions with Vinashya, a best friend and researcher.

I don't have words to express my affection Prachi, Rajeshwari and Kedar for always being there to help and share good moments in routine life.

I also address my thanks to all the past and present lab mates. I would like to thank my seniors: Dr. Ashok Chougle, Dr. Sandeep Golegaonkar, Dr. Suresh K.K, Dr. Hemangi Bhonsle, Dr Sneha Bansode and Dr. Yogesh Kolekar. I am grateful to all my lab members Arati, Akshay, Gouri, Rashmi, Rubina, Shakuntala, Reema, Sharda, Amrita, Yugendra, Piyush, Shrikanth, Santhosh, Arvind, Shabda and all other lab members as in one or other way they have made my journey at NCL more meaningful, beautiful and filled with memorable moments. My deepest thanks to Ramesha H.J, who was with me in every aspect of my master's and doctoral degree life as a good friend. I express my heartfelt thanks to Dr. Sheon Mary for her dedicated involvement repairing and maintaining mass spectrometry.

My sincere gratitude to Venkataramaiah H.N and his family for their immense support like family members. I express my obedient thanks to Mrs. Malathi Thulasiram family for providing me homely atmosphere during my stay at NCL and definitely I miss festival celebration, food, suggestions with them. In addition, I extended special thanks Mrs. Savita Kulkarni and family, Mrs. Geeta Pimprikar, Mr. Venkata Subramani and family.

I am extremely grateful to my teachers and professors Shivanna,Chandrappa, Prasad, Sadhashivaiah, Mudlugiriyappa, Dr. Satisha G.J, Dr. Niranjan P, Dr. Ravindra, Dr. Rajesh, Dr. Shivayogi Neelagund, Mrs. Shylaja, for always being inspirational and

encouraging during my educational days to pursue career. I am also thankful to my friends for lifetime who were always there in all my good and bad times.

Last but not least, I am indebted to my parents and sister who have encouraged and helped me at every stages of my life and longed to see this achievement come true. My hard-working parents have sacrificed their lives for me and provided unconditional love and care. The immense support, love, motivation and judicious guidance from my father, my mother and grandmother (Ajji) has brought me to this stage.

There are a number of people who have supported me in many aspects during my PhD life. I am grateful to all of them from the bottom of my heart. Above all, I owe it all to God for granting me the wisdom, health, luck and strength to undertake this research task and enabling me to its completion.

Jagadeeshaprasad M G

List of Tables

List of Figures

List of Accompanying Material

All the supplemental data is provided along with this thesis in the form of soft copy in CD attached to the last page.

Supplemental data 1: The list of all glycated peptides of hemoglobin and their corresponding glycated fragment ions and their MS/MS annotations. (.pdf) (Chapter 2)

Supplemental data 2:Two way ANOVA followed by Bonferonnis posttests for fold change in AUCs of β-N-1-Val of hemoglobin. (.pdf) (Chapter 2)

Supplemental data 3:Pearson's correlations analysis of all modified peptides with fasting blood glucose, postprandial blood glucose, HbA1c, serum triglyceride, LDL, HDL, cholesterol, VLDL, creatinine and microalbuminuria in clinical subjects. (.xlsx) (Chapter 2)

Supplemental data 4:Glycated peptides of hemoglobin were identified, characterized by using IDA approaches and used for SWATH-MS based quantification. (.xlsx) (Chapter 3)

Supplemental data 5:Glycated peptides of hemoglobin spectra's. (.pdf) (Chapter 3)

Supplemental data 6:Area under curve for the glycated peptides of hemoglobin (A), serum albumin (B), and clinical plasma albumin (C). (.xlsx) (Chapter 3)

Supplemental data 7:Representative extraction of chromatograph, area under curve with respective glycated peptide spectra's of hemoglobin, serum albumin, and human serum albumin. (.pdf) (Chapter 3)

Supplemental data 8: - Glycated peptides of serum albumin were identified, characterized by using IDA approaches and used for SWATH-MS based quantification. (.xlsx) (Chapter 3)

Supplemental data 9:Glycated peptides of serum albumin spectra's.(.pdf) (Chapter 3)

Supplemental data 10:Carboxymethyl-lysine modified peptide spectr's of serum albumin. (.pdf) (Chapter 3)

Supplemental data 11:Acetyl-lysine modified peptide spectr's of serum albumin. (pdf) (Chapter 3)

Supplemental data 12:Glycated peptides of human serum albumin were identified, characterized by using IDA approaches and used for SWATH-MS based quantification (.xlsx) (Chapter 3)

Supplemental data 13: Glycated peptides of human serum albumin spectra's. (.pdf) (Chapter 3)

Supplemental data 14:Plasma fructosamine, serum albumin, HbA1c and plasma protein concentrations in individual clinical subjects (.xlsx) (Chapter 3)

Definitions/Abbreviations

AcSA:	N-epsilon-(acetyl) lysine serum albumin
ACN:	Acetonitrile
ADA:	American Diabetes Association
AGEs:	Advanced glycated end products
AMG:	Aminoguanidine
ANOVA:	Analysis of Variance
AUC:	Area under curve
BCG:	Bromocresolgreen
BPI:	Base peak intensity
BSA:	Bovine serum albumin
^{o}C:	Degree Celsius
C:	Control
CMV:	N1-carboxymethyl Valine-β-Hb (CMV-β-Hb)
CMSA:	N-epsilon-(carboxymethyl) lysine serum albumin
CEL:	N-epsilon-(carboxyethyl) lysine
CML:	N-epsilon-(carboxymethyl) lysine
D:	Diabetes
DFV:	N1-deoxyfructosyl Valine-β-Hb (DFV-β-Hb)
EDTA:	Ethylenediamminetetraacetate
ESI-MS:	Electro spray ionisation mass spectrometry
g:	Relative centrifugal force
g,mg,µg:	Gram, milligram, microgram
GLUT1:	Glucose Transporter protein 1
Hb:	Hemoglobin
HbA1c:	Glycation haemoglobin
HDL:	High density lipoprotein
HRMS:	High resolution mass spectrometry
HSA:	Human Serum Albumin
mM, M:	Millimolar, molar
IDA:	Information dependent acquisition
LDL:	Low density lipoprotein
MS:	Mass spectrometry

PCD:	Poorly controlled diabetes
PD:	Pre-diabetes
SA:	Serum albumin
RAF:	Relative albumin fructosamine
RPMI:	Roswell Park Memorial Institute medium
SD:	Standard deviation
SEM:	Scanning electron microscope
SWATH:	Sequential window acquisition of all theoretical mass spectra
TIC:	Total ion chromatogram
VLDL:	Very low density lipoprotein

Preface

Diabetes mellitus is a major and growing health problem in most of the countries including India. Poorly managed diabetes leads to variety of complications including blindness, amputations, kidney failure and neurodegenerative disorders. Diagnosis and management of diabetes heavily relies on detection of levels of HbA1c which is considered as a gold standard for assessing the glycemic status over preceding 90-120 days. However, HbA1c has certain limitation in terms of technical quantitation as well as its ability to predict diabetic complications. Several previous studies have suggested Advanced Glycation End products (AGEs) are the leading cause of development of diabetic complications. Therefore, in this thesis AGE modified peptides of hemoglobin are identified, characterized and quantified by using high resolution accurate mass spectrometer. In addition, the thesis deals with the factors that regulate HbA1c level. The influence of one such factor, serum albumin on HbA1c is studied in great detail using in vitro erythrocyte culture and mass spectrometry. The findings of this study are extended to the clinical settings.

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Diabetes

Diabetes mellitus is a major growing non-communicable metabolic disorder in most countries and its patient population increasing drastically(1). According to the recent report from the International Diabetes Federation (IDF), which estimated around 366 million people were diabetic in 2011, and this rate is expected to rise to 552 million in the year of 2030 in worldwide (2). It is a chronic metabolic disorder characterized by elevated level of glucose, where the body cannot produce enough insulin or effectively use it or both, leading to persistent hyperglycemia (3). The prolonged hyperglycemia leads to the development of diabetes associated complications such as retinopathy, nephropathy, neuropathy, cerebrovascular and cardiovascular diseases (4, 5).

1.2 Classification of Diabetes

Diabetes mellitus is mainly classified into two types based on insulin dependence (3).

1.2.1 Type 1 diabetes

Type 1 diabetes is also called juvenile-onset diabetes (insulin-dependent diabetes), in which pancreatic islet β cells are destroyed by autoimmune response leading to decrease of endogenous insulin production that in turn causes elevated levels of glucose in the blood and urine (6-9). Mostly type 1 diabetes occurs in children and it accounts of about 5 to 10 % of all the diabetes worldwide (10, 11). Autoimmune destruction of islet β cells results from multiple genetic predispositions combined with environmental factors such as viral attack, milk, Vitamin D deficiency, poor exposure to sunlight. Type 1 diabetes is diagnosed by testing the presence of one or more autoimmune markers such as auto-antibodies for islet proteins, blood glucose measurement and HbA1c (6, 12).

1.2.2 Type 2 diabetes

Type 2 diabetes is also called as adult onset diabetes (non-insulin-dependent diabetes) characterized by insulin resistance, pancreatic β cells dysfunction, and relatively less insulin deficiency or combination of all these leads to elevated blood glucose levels. It is the most common form of disorder that accounts for about 90-95% of all cases of diabetes (10-13). Type 2 diabetic patients are mostly obese. The obesity is the causal factor for the insulin resistance. In some case type 2 diabetic patients are not obese by traditional weight criteria but they have higher fat content distributed in their body mostly in the abdominal region. The risk of development of type 2 diabetes increases with age, obesity, and lack of physical activity (14-16). Type 2 diabetes is diagnosed by blood glucose measurement such as fasting blood glucose, postprandial blood glucose and HbA1c (17).

1.2.3 Other types of diabetes

A recent study suggests that malnutrition may be a causal factor for several types of diabetes, such type of diabetes is called Malnutrition–related diabetes mellitus (MRDM). Gestational diabetes mellitus (GDM), is yet another type of diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes (1, 10-13).

1.3 Current diabetes diagnostic tests

Regular assessment and precise measurement of glycemic status is a crucial part of optimal care for individuals with diabetes. Glycemic biomarkers are important tools used to determine whether an individual's metabolic control has been maintained within the target range, but most importantly, they are used as surrogates to estimate and reduce the risk of chronic diabetes complications (18, 19). According to American Diabetes Association (ADA) guidelines, early diagnosis and monitoring of diabetes after treatment are crucial for preventing or delaying the onset of long term diabetes associated complications (20). Diabetes diagnostic and prognostic strategies are mainly based on HbA1c measurement or plasma glucose measurement, either the fasting plasma glucose (FPG) and postprandial plasma glucose (PPG) or oral glucose

tolerance test (OGTT). These tests are recommended by Diabetes Control and Complications Trial (DCCT), World Health Organisation (WHO), International Diabetes Federation (IDF), and American Diabetes Association (ADA) (21, 22).

1.3.1 Fasting blood glucose (FBG)

The glucose level in the blood after fasting for at least 8 hours is termed as FBG. FBG levels, for normal individual vary from 70 to 99 mg/dL (3.9 to 5.5 mmol/L), for pre-diabetes (impaired fasting glucose) from 100 to 125 mg/dL (5.6 to 6.9 mmol/L) and for diabetes 126 mg/dL (7.0 mmol/L) and above glucose levels are considered (20).

1.3.2 Postprandial blood glucose (PPG)

PPG is defined as blood glucose level measured after 2 hours of meal in the individuals fasting between 8-16 hours. The glucose levels are for normal person less than 140 mg/dL (7.8 mmol/L), for pre-diabetes (impaired glucose tolerance) from 140 to 199 mg/dL (7.8 to 11.1 mmol/L) and for diabetes equal to or greater than 200 mg/dL (11.1 mmol/L) (20).

1.3.3 Oral glucose tolerance test (OGTT)

The person is asked to fast overnight (at least 8 hours, but not more than 16 hours) then the fasting plasma glucose is measured. Post fasting, the person receives 75 g of glucose orally, after 2 hours of the glucose intake, blood glucose is measured. The standard glucose levels are: for normal person less than 140 mg/dL (7.8 mmol/L), for pre-diabetes (impaired glucose tolerance) from 140 to 199 mg/dL (7.8 to 11.1 mmol/L) and for diabetes Equal to or greater than 200 mg/dL (11.1 mmol/L)(20).

1.3.4 Glycated hemoglobin (HbA1c)

Glucose reacts non-enzymatically with NH_2-terminal valine of the β-chain hemoglobin to form glycated hemoglobin (HbA1c) (23, 24).HbA1c is a measure of the degree to which hemoglobin is glycated in erythrocytes and is expressed as a percentage of total hemoglobin concentration. It reflects the exposure of erythrocytes to glucose in an irreversible time and concentration dependent manner. Depending on

the measurement method used, HbA1c ranges from 3 to 6.5 % of total hemoglobin in normal individuals to as high as 15 % in individuals with diabetes (22-24).

1.3.5 Urine Tests

The measurement of urinary glucose levels reflects average blood glucose during the period of urine collection and that of single excretion. Urine glucose test is based on colorimetric reaction, occurs among ketone bodies and nitroprusside (Sodium nitroferricyanide), and it is semi-quantitative measurement method of ketone bodies over 4-24 hrs (21). The presence of urine ketone bodies may indicate impending or even established ketoacidosis, a condition that requires immediate medical attention. It is recommended that all people with diabetes test their urine for ketones during acute illness or stress, when blood glucose levels are consistently 300 mg/dl (16.7 mmol/l), during pregnancy, or when any symptoms of ketoacidosis, such as nausea, vomiting, or abdominal pain, are present. In diabetic condition the ketoacidosis, the ratio of hydroxybutyrate to acetoacetate, may increase up to 6:1 or greater. The presence of ketone levels in urine sample represents the urine glucose levels (25).

1.3.6 Other tests

In addition to the above mentioned regular tests, the annual measurement of albuminuria is recommended to determine the rate of disease progression especially in diabetic associated nephropathy condition (26). Also in some cases insulin and islet cell antibodies are measured only after standardizing the potential clinical studies. Similarly insulin and C-peptide concentrations also accounting for prospective studies in order to consider it as a diagnostic marker for diabetes (27).

1.4 Diabetes and Glycation

Hyperglycemic condition in diabetes exposes proteins to elevated glucose levels causing non-enzymatic reaction between glucose and proteins, called as glycation (28). For the first time, Louis Camille Maillard described non-enzymatic glycosylation or glycation. Maillard observed intense browning colour upon heating of mixtures of amino acids and sugars with the generation of carbon dioxide (29, 30),

hence the reaction is also referred as Maillard reaction. Further studies described that during Maillard reaction dicarbonyl compounds are formed and their rearrangement is solely responsible for the aroma, taste and the brown colour appearance of thermally processed food (31-34).Further in 1920, Mario Amadori observed that the condensation reaction between free amine group of aromatic amino acids and carbonyl group of glucose led to the generation of an unstable glucose-aniline Schiff base, which is subsequently converted to the more stable anilinedeoxy-fructose derivative. By the year 1950s it was recognised that Maillard reaction was not only limited to aromatic amines but could occur also with aliphatic amines. The conversion process named as "Amadori rearrangement" and the process product amino-deoxy-ketoses was named as "Amadori products". Formation of the Schiff base from carbonyl group of sugar and free amines of proteins (N-terminal, ε-amine of Lysine and η-amine of Arginine) is fast reaction; while the conversion to the Amadori product is relatively slower, thus the Amadori products tend to accumulate on proteins (29, 31, 33-35).

Louis Camille Maillard postulated that the glycation reaction between sugars and amino acids could occur ubiquitously at physiological conditions (37^{o} C) with sufficient incubation time, the analogous reaction products are ultimately formed as those obtained by strong heating. For the first time this association between glycated proteins and chronic hyperglycemia was observed in 1968 with the discovery of an iso-form of human hemoglobin in the erythrocytes of diabetic patients (24). This variant of hemoglobin, which was later named as HbA1c, glucose reacts non-enzymatically with the N-terminal valine residue of the β-chain, generating the glycated form of hemoglobin. Currently, the percentage of HbA1c is used as an important biomarker to assess the average blood glucose levels for the diagnosis and treatment of diabetes (20,27). Decades of investigation suggest that, not only hemoglobin but also a large number of proteins undergoes glycation under hyperglycaemic conditions, which are prone to glycation for their entire lifespan (36). It is worth emphasizing that, although most of the attention has been on long-lived proteins like hemoglobin, even proteins with a rapid turnover like serum albumin, transferrin, insulin are more prone to glycation (37, 38).

Glycation is a chemical modification of proteins occurs between free amino group of protein and carbonyl group of glucose leads to the formation of unstable reversible

product Schiff's base. This is the first step of glycation reaction. Further Schiff's base converts into stable reversible Amadori product. The Amadori product bearing proteins usually is referred as glycated proteins. Later, the Amadori product undergoes series of oxidation, reduction, dehydration and fragmentation reactions leading to a variety of dicarbonyl compounds like methylglyoxal, glyoxal, glucosone, 3-deoxyglucosone (3-DG) and so on (39,40). During these processes glycated proteins are converted into more stable advanced glycated end products (AGEs). The bi-products like methylglyoxal, glyoxal etc. are highly reactive compounds than sugar and acts as propagators of glycation reaction which directly leads to the formation of advanced glycated end products (AGEs) (41).The detail mechanism involved in the glycation reaction is illustrated in **Figure 1.1.**

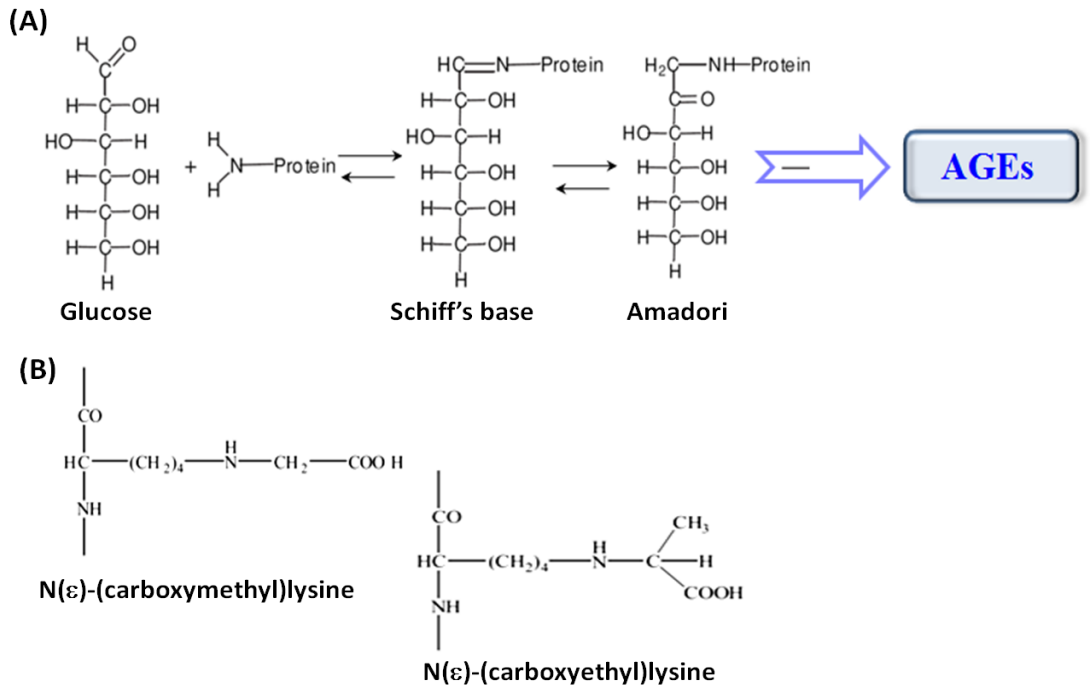


Figure 1.1 Chemistry of protein glycation

(A) Free amino group of proteins reacts with carbonyl group of glucose by non-enzymatic process and production of early and late glycation product (Advanced glycated end products (AGEs)). (B) Different types of advanced glycated end products (AGEs).

Different type of AGEs are characterized and reported by mass spectrometric analysis. The different types of AGEs are held specific to mass increment to specific amino acid like cross-linking of AGEs are specific only to arginine residues. The

specific mass increment and glycation specific amino acid residues are listed in **Table 1.1**. Previous reports suggests that AGEs-modified proteins losses its native protein structure and function (42-45). Additionally, AGEs-modified proteins can bind to receptors of AGE (RAGE) on the surface of many cell types such as endothelial cells, muscle cells, macrophages, lymphocytes, mesangial cells, etc. AGE-RAGE interaction triggers the intracellular ROS generation and pro-inflammatory signals, which are associated with progression of diabetic complications (46-48). For example, glycated albumin interacts with endothelial cells RAGE receptor that leads to the activation of NFκB and RAGE (49). Activation of NFkB leads to over expression of pro-inflammatory cytokines like IL-6 and TNFα, thus increases the inflammatory responses (50-53). Furthermore, studies revealed that glycated albumin, leading to increased serine phosphorylation of IRS receptors, leading to the suppression of insulin signalling and consequently lead to the development of insulin resistance (54,55). Glycation of lipoproteins (apolipoprotein-A and apolipoprotein-B) affects the transportation of lipids especially in diabetes (56). Disturbed lipid transportation is one of the causal factors for development of retinopathy and cardiovascular diseases (57-59). Glycated albumin interacts with pericytes RAGE induces ROS formation in the retina, contributing to their reduced survival and breakdown of the blood-retina barrier, and ultimately leads to diabetic retinopathy by vision loss (60-63). Besides, it has been revealed that glycated albumin induces apoptosis and increased VEGF expression in mesangial cells leading to increased vascular permeability, hyper-filtration and proteinuria. These are the symptoms that are associated with diabetic nephropathy (35, 64-66). As the glycation is implicated in the development of diabetic complications, it has gained more attention in measurement of long lived glycated proteins like hemoglobin (HbA1c) for the diagnosis and treatment of diabetes (38, 67-69).

Table 1.1 Glycation modifications with mass increment for different AGEs at specific amino acid site

Sl. No.	Abbreviations	Modification	Mass increment (Da)	Modification amino acid site
1	FL	Fructosyl lysine	162.0528	K or R
2	CEL	N-ε-carboxyethyl lysine	72.0211	K
3	CML	N-ε-carboxymethyl lysine	58.0055	K
4	PYRRALINE	Pyrraline	108.0211	K
5	FL-$2H_2O$	Fructosyl lysine-$2H_2O$	126.0317	K or R
6	IMIDAZOLONE-A	Imidazolone-A	144.0300	K
7	IMIDAZOLONE-B	Imidazolone-B	142.0266	K
8	ARGPYR	Argpyrimidine	80.0262	K
9	MG-H1	N-ε-(5-hydro-5-methyl-4-imdazolon-2-yl) ornithine	54.0106	R
10	G-H1	N-ε-(5-hydro-4-imdazolon-2-yl) ornithine	39.9949	R
11	AFGP	1-alkyl-2-formyl-3,4-glycosyl-pyrrole	270.0740	K
12	MOLD	2-ammonio-6[1-(5-ammonio-6-oxido-6-oxohexyl)-4-methylimidazolium-3-yl]hexanoate	49.0078	R
13	CROSSLINE	crossline	252.1100	K

1.5 The discovery of HbA1c

1.5.1 History of HbA1c measurement evolution

First Pauling and his colleagues in 1949 showed the heterogeneity of hemoglobin in sickle cell hemoglobin by deviating migration from electric field (70). Then Kunkel and his colleague identified minor hemoglobin variants by ion exchange chromatography in normal adult hemoglobin (71). Allen demonstrated that hemoglobin A1a, hemoglobin A1b, hemoglobin A1c (HbA1c), hemoglobin A1d, and hemoglobin A1e, eluted fast and hemoglobin A0 eluted slowly by ion exchange chromatography (72). Rahbar and colleagues observed that fast moving hemoglobin A1c (HbA1c), were elevated in erythrocytes of diabetes patients (24, 73-76) and the elevated levels of HbA1c were well correlated with mean glucose in addition to diabetic complications (77, 78). In reality, the HbA1c was not abnormal, but was a normal fraction of hemoglobin A1 but it's found to be higher in diabetic patients' blood analyzed by hemoglobin electrophoresis (73, 74). The same observation of increased HbA1c was confirmed by other research group until the mid-1970s, in addition, HbA1c level was higher in hyperglycemia patients (77, 79, 80). By the mid1970s it became more or less clear that HbA1c is a result of addition of glucose to HbA1 and as a result there was an association between HbA1c and glucose levels over the preceding 90 to 120 days (77). By the early 1980s, HbA1c measurement became widely accepted as diagnosis criteria to assess the glucose status in the diabetes individuals (81). HbA1c reflects average plasma glucose of preceding 3-4 months (82). Moreover, HbA1c can be measured at anytime throughout the day and does not required to be under fasting before measurement. These properties have gained an advantage to measure HbA1c for assessing glycemic status (22, 67, 83-85).

1.5.2 Chemistry of HbA1c

Chemically HbA1c is N-1-(deoxyfructosyl) valine (DFV) β-hemoglobin (β-Hb), a widely used diagnostic marker in diabetes (67, 84, 85). Diagnosis and management of diabetes heavily relies on detection of levels of HbA1c [N-1-deoxyfructosyl) valine (DFV) β-hemoglobin], which is considered as a gold standard for assessing the glycemic status and it is believed to provide glycemic status of preceding 90-120 days (20, 27, 86-89). The rate of HbA1c formation is directly proportional to the ambient

glucose concentration exposed to hemoglobin during its lifespan in circulation (90-92).

1.5.3 Methods of HbA1c analysis

From many years, clinical assessment of glycemic status is based on measurement of hemoglobin glycation (HbA1c) (93). The levels of HbA1c comprises all of the information required for managing glycemic levels and its associated complications in diabetic subjects; reducing the levels of HbA1c considering as a tempting approach. There are two major methods available to monitor the glycated hemoglobin based on charge state and structural characteristics from native hemoglobin. The charge based methods such as cation-exchange chromatography, iso-electric focusing and gel electrophoresis and structural characteristics methods like affinity chromatography and immunoassay are currently used (94-96). Most of these methods quantify HbA1c, defined as HbA1 iso-form with glucose attached to the NH_2-terminal Valine of one or both chains(23). Other methods (boronate affinity) quantify "total glycated hemoglobin," which includes both HbA1c and other G-Hb adducts (e.g., glucose-lysine adducts and glucose chain NH_2-terminal valine adducts). Results of methods using different assay principles show excellent correlation, but there are no convincing data to show that any one method or analyte is clinically superior to any other (97-100).

1.5.4 Working principle of HbA1c analysis methods

We would like to emphasize some of the key issues about HbA1c measurement that are related to analytical techniques and wide variety of influencing factors on HbA1c levels. Currently the measurement and quantification of glycated hemoglobin (HbA1c) are based on ion exchange, phenyl boronate affinity or antibody based chromatography methods in routine diagnostics (94-96). Glucose is primarily present in a cyclic form; however, this form is in chemical equilibrium with a little fraction of acyclic glucose which is chemically active because of aldehyde group. The aldehyde group reacts with amino groups of the side chains of protein in a non-enzymatic manner called as glycation. The glycation takes place in two steps: In the first, reversible step, glucose forms a Schiff base (aldimine) with an amino group in the

protein. In the later, irreversible step, the aldimine becomes a ketoamine called as Advanced Glycation End products (AGEs), which is stable and remains for lifetime of the protein (28, 101). In principal, all free amino groups of the side chains of proteins can become glycated; however, couple of factors show important effects on the glycation rate and its end products: (a) Protein concentration, (b) the steric accessibility of the different side chain containing free amino groups, (c) concentration of glucose and it's by products such as methylglyoxal, glyoxal etc. in the compartment, and (d) the lifespan of the protein.

Hemoglobin, the predominant protein in red blood cells, is incessantly exposed to glucose in the blood (102). Therefore, hemoglobin always is glycated. For the first time Samuel Rahbar and co-workers observed a thin extra band on electrophoresis gels, representing glycated hemoglobin (24, 73, 74, 76). At first, the name HbA1c was just given to a protein band in hemoglobin electrophoresis, and subsequently it was confirmed that it consists of a mixture of hemoglobin molecules that are glycated at different side chain amino positions. Presuming that the rate of glycation is proportional to the concentration of hemoglobin and that the accessibility of glucose to free side chain amino groups of hemoglobin and red blood cells life span are constant, the concentration of HbA1c is influenced only by glucose concentration in terms of percentage. Therefore, HbA1c would be the ideal surrogate for the blood glucose concentration over the average lifespan of red blood cells.

In context of small HbA1c peak quantification in comparison to the large HbA0 peak is difficult to perform using gel electrophoresis, as a result advanced technologies have been developed such as phenyl boronate affinity, ion exchange chromatography and immunoassay methods. In ion exchange chromatography HbA1c is separated based on net charge from HbA0 due to its charge difference (94). In phenyl boronate affinity chromatography based techniques where glycated hemoglobin is bound to immobilized *m*-amino phenyl boronic acid to separate it from unmodified haemoglobin. Boronic acid binds to the *cis*-diol retaining group of glycated hemoglobin (deoxy-frucosylated hemoglobin) on resin is the underlying mechanism (95). Hemoglobin molecules which do not contain the *cis*-diol group also co-exist but they are not accounted in the analysis of glycated hemoglobin by this method (103). Immunoassays use antibodies against glycation of N-terminal Valine of residue of β-chain containing tetrapeptide or hexapeptide hemoglobin. Identification and quantification mainly depends on the antibodies and enzymatic detection. It involves

the detection of glycation of N-terminal valine residue tetra-peptide or hexa-peptide group contained in the β-chain of the hemoglobin using specific antibodies (96). It is important to note that certain laboratory methods may give inaccurate results in the presence of hemoglobin variants such as Sickle cell hemoglobin, Hemoglobin variants D and E also cause a similar change in the net charge as that of the glycated hemoglobin (AGE-HbA) molecule or by and large the antibodies are raised against only deoxy-frucosylated valine including tetra-peptide or hexa-peptide of β-chain of the hemoglobin, and therefore as deoxy-frucosylation is an early product of glycation it can undergo and finally make AGEs. In addition highly reactive dicarbonyls can directly modify the Valine residue and other glycation residues can also get glycated (104-107).Previous studies suggest that the formation of HbA1c is slowly reversible, and for a given glucose concentration the HbA1c content in red blood cells ultimately reaches an equilibrium value (108). This suggests that HbA1c does not exactly correlate with the glucose levels in diabetes. In context of HbA1c accuracy, several studies report the interference of other chemical modifications and hemoglobin variants during its measurements as well as physiological and patho-physiological factors without being related to glycemia (97,109). Also tribal and cultural subject groups showed significant differences among serum glucose concentration with HbA1c (110,111). Variations in HbA1c is observed among diverse racial groups, while mean plasma glucose levels do not vary between these groups. These studies suggest that there exists a biological premise for the variability across racial groups with respect to HbA1C measurement (112,113). HbA1c levels also have been inversely correlated to levels of Vitamin D, but yet validation for the relation between HbA1c and vitamin D is unclear (114). Also, some research studies suggest that HbA1c differs based on β-pancreatic cell function and insulin resistance (115). Thus can interfere in separation of HbA1c and may lead to over estimation. It is also important to understand the mechanism of glycation reaction and influencing factors in diabetes to avoid ambiguity about the HbA1c measurement and considering all these drawbacks needed for optimum diabetes control and especially for use of HbA1c to diagnose diabetes.

1.5.5 Limitation of HbA1c analysis

The measurement of glycated hemoglobin (HbA1c) is considered as the most prominent, gold standard biomarker for assessing the glycemic status of people with diabetes and for treatment. However, the measurement of HbA1c to diagnose individuals exposed for the development of diabetes has been controversial (69,116,117). American Diabetes Association and European Association created the International Expert Committee to study the usefulness of HbA1c testing, committee concluded that HbA1c testing is effective to identify individual's diabetes (10). However, the committee pointed the limitations of HbA1c testing, such as inconsistencies in correlating the HbA1c measurement with fasting plasma glucose levels, the overall cost, and availability of the test. In addition, the committee found that the HbA1c tests do not accurately and precisely diagnose diabetes unlike other tests. The strength of HbA1c measurement is that it correlates very well with average blood glucose levels over preceding 3-4 months, compared to other tests like fasting plasma glucose and oral glucose tolerance levels (118). Furthermore, the HbA1c levels correlate closely and helps in the prediction of diabetes associated complications such as the likelihood in the process of diabetic nephropathy and retinopathy (119). Some of the studies also suggest that for the prediction of diabetes can be achieved by measuring HbA1c with oral glucose tolerance levels (120-122).

1.6 Proteomics: A Tool to study protein glycation

Proteomics is the systematic and comprehensive approach to study various aspects of protein expression, post-translational modification, interactions, organization and biological function at a global level (123). Proteomics heavily relies upon achievements of genomics, and provides gene products possible 'blueprint' (124) but protein expression might differ according to the type of cell and time (125-127). The proteins are first effectors of biological functions; also they may undergo protein post translational modifications (PTMs). Protein PTMs are the chemical events that convert an amino acid residue of protein into a non-standard amino acid residue by the addition of chemical groups. The protein chemical modification may affect the folding, function and stability of long-lived proteins (128, 129). The identification of protein and their PTMs are fundamental to elucidate the intricate process of cellular

events. Protein PTMs determines the activity state of some proteins, enzymes, protein turnover and interaction with other proteins (130). Given the protein PTMs have crucial role in cellular physiology and disease patho-physiology. The analysis of these modified proteins is a very challenging task because of their complexity and variability in their modifications. A vast variety of analytical techniques are currently being used to analyse the protein modifications and each technique has its own advantages and limitations (131).

Techniques such as two-dimensional electrophoresis, western blots, fluorescence, and competitive ELISA give overall quantitative information about a protein modification. The best analytical methods available to quantify chemical adducts is mass spectrometric detection and quantification using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). In this regard, in detail proteomics analysis by Mass spectrometry (MS) gives an insight about the cellular function and helps in understanding of diseased states of cells (131-134).

1.6.1 Mass spectrometry as a tool for protein post translational modification analysis

During last one decade, mass spectrometry based proteomics has emphatically proven to be a powerful tool for discovery and quantification of protein post translational modifications (PTMs) (135,136). Mass spectrometry is highly sensitive, high throughput, and robust, compared to or even better than traditional approaches for qualitative and quantitative approach for the identification and in detail characterizes the post translational modifications of proteins (135,137). The attachment of chemical group to the proteins causes covalent modifications and affects the molecular weight by increasing total mass of the proteins. The increment mass of PTMs in detail can be determined and characterised only by using mass spectrometric method. To achieve the identification of PTMs by mass spectrometric studies, there are several important steps to follow; such as most important preliminary step is sample preparation, in which proteins needs to be solubilise in an appropriate buffer and followed by proteolytic digestion using a specific protease enzyme; second step is liquid chromatographic separation, to reduce the complexity of peptide mixture; and finally the mass spectrometric analysis that provides the modified peptides information with high accuracy and high sensitivity by revealing the increment of mass of PTMs at

individual amino acid residue level. Mass spectrometric analysis will give an idea about even the amino acid residues which are getting modified along with the extent of modification (138, 139). It must be emphasized that no present technique other than mass spectrometry can provide an exhaustive identification and quantitative information of modified sites from modified proteins.

1.6.2 Quantification of PTMs by mass spectrometry approaches

Previously label-free mass spectrometry approach has been used for the characterization and quantification of post translational modification (140). Label free analysis approach holds several advantages, such as high throughput, low cost, simple methodology and allows quantification even the low intense modified peptides (141,142). Label-free quantification mass spectrometry approach relies on the precursor ion scan (MS1) for quantification and those that are based on product ion scans (MS/MS) for quantification (142,143). The peptide area/intensity is directly compared with other samples of peptide intensity of the same m/z and retention time extracted (142,143).Data independent mass spectrometry acquisition allows the isolation and fragmentation in a defined mass range window with less noise ratio and high sensitivity and specificity (144,145).

The extensive role of mass spectrometry in PTMs identification and quantification can be attributed by adopting new ionization techniques, high resolution and sensitivity and with accurate mass analyser for example Q-Exactive Orbitrap high resolution accurate mass spectrometry (HR/AM-MS) instruments. Commonly, mass spectrometry is an analytical technique used to determine the molecular weight and their abundance, to elucidate structure of ionized molecules at the gaseous phase. Typical mass spectrometry based approach consists of ionization; analyte converts into an ion form in an ion source and mass analyser separates ions based on their mass-to-charge (m/z) ratio, detection of the ions in a detector, and generation of a mass spectrums of ions. Tandem mass spectrometers provide the additional option that is selection of a specific ion from the precursor ion survey (MS-survey scan (MS1)) and fragmenting it in order to obtain fragment ions information (MS2) (145,146). The high resolving power, high sensitivity and mass accuracy are most essential requirements for the unambiguous PTMs identification and quantification in

mass spectrometer. Determination of accurate mass is a mandatory criteria for the proper identity of PTMs in the peptides (136). The main aim of PTMs analysis is to calculate their abundance in order to understand the cellular processes (136). Modified mass bearing peptides relative quantification is mainly based on number of spectral counts and methods based peak area calculation at the precursor ion and followed by fragment ions levels monitoring seem to be the more accurate and reliable method (147, 148).

1.7 Genesis and organization of thesis

Poorly controlled diabetes leads to variety of complications including blindness, amputations, kidney failure and neurodegenerative disorders. Several studies have demonstrated that the formation of Advanced Glycation End products (AGEs) is the leading cause of development of diabetic complications. Thus glycemic control becomes critical in order to prevent such complications. Diagnosis and management of diabetes heavily relies on detection of levels of HbA1c [N-1-(deoxyfructosyl) valine (DFV) β-hemoglobin], which is considered as a gold standard for assessing the glycemic status over preceding 90-120 days. In normal individuals, HbA1c ranges from 3 to 6.5 % and up to 15 % in poorly controlled diabetes. However, previous studies have suggested that HbA1c is slowly reversible, and for a given glucose concentration it eventually reaches equilibrium. In addition, precise quantification of HbA1c using available methods in routine diagnostics, such as ion exchange, phenyl boronate affinity or antibody based chromatography, is technically challenging. Apart from these factors, human serum albumin (HSA) has been known to influence HbA1c; however it has not been given much attention perhaps due to its abundance in the plasma. Hence, we analyzed advanced glycation modifications of hemoglobin for discovering alternative diagnostics in diabetes using parallel reaction monitoring (PRM) mass spectrometry approach. In this pursuit, for the first time, we have comprehensively characterized and quantified DFV/DFL, CMV/CML and CEV/CEL peptides of hemoglobin. The major findings of one of the study is N-1-β-valine undergoes carboxyethylation; and carboxymethylation is the predominant modification of N-1-β-valine-Hb. Furthermore, CMV and CEV peptides correlate very well with micro-albuminuria. Additionally, we have unequivocally demonstrated in erythrocyte culture that serum albumin levels influence hemoglobin glycation, i.e.

higher levels of serum albumin reduces hemoglobin glycation and vice versa. Under conditions of low serum albumin levels, there was increased serum albumin glycation which perhaps decreased its ability to reduce hemoglobin glycation. This was substantiated by treatment of glycated serum albumin i.e. CMSA, which failed to reduce hemoglobin glycation, instead it increased hemoglobin glycation, as well as caused alteration in erythrocyte structure and membrane permeability, which was clearly established by scanning electron microscopy and increased hemolysis respectively. The inability of CMSA to reduce hemoglobin glycation was due to lack of availability of free lysine residues, which otherwise competitively inhibited the glycation of hemoglobin. This observation was corroborated by modifying lysine residues with acetylation. For the first, we demonstrate that modification of lysine residues of albumin impairs its ability to inhibit hemoglobin glycation. Furthermore, correlation studies between HbA1c and serum albumin or RAF supported our in vitro experimental finding that albumin abundance and its glycation status determine hemoglobin glycation in erythrocyte culture.

Major objectives of the thesis

- To identify, characterize and quantify Advanced glycated hemoglobin (AGE-Hb) for development of alternative markers for diabetes
- To understand the role of albumin and its glycation status in regulating hemoglobin glycation

Thesis is organized in the following manner

Chapter 1. Introduction

Chapter 2. *In-vitro* synthesis, characterization and quantification of advanced glycated hemoglobin in clinical subjects

Chapter 3. Understanding the role of serum albumin and its glycation status on hemoglobin glycation in both *in-vitro* erythrocytes culture and clinical subjects

Chapter 4. Conclusion and future direction

Chapter 2

In-vitro synthesis, characterization and quantification of advanced glycated hemoglobin in clinical subjects

Contents of this chapter is published as research article in Clinical proteomics 2016; 13: 7 and filed patent United States Patent Application 20170074888 (appl. no: 15/254539).

Chapter 2 *In-vitro* synthesis, characterization and quantification of advanced glycated hemoglobin in clinical subjects

2.1 Background

Diagnosis and management of diabetes heavily relies on measurement of glycated hemoglobin levels, N-1-(deoxyfructosyl) valine (DFV) β-chain of hemoglobin (HbA1c); considered as a gold standard for assessing the glycemic status over preceding 3 to 4 months (92). In healthy individuals, HbA1c ranges from 3 to 6.5 % and can go up to 15 % in diabetic subjects (20, 27, 86, 87, 149-152). However, previous studies have suggested that the HbA1c is slowly reversible, and for a given glucose concentration the HbA1c ultimately reaches equilibrium (108). Furthermore precise quantification of HbA1c using currently available methods in routine diagnostics, such as ion exchange (94), phenyl bornate affinity (95), or antibody based chromatography (96), are technically challenging. For example ion exchange based measurement of HbA1c is influenced by other chemical modifications like glutathiolation (153). Whereas phenyl boronate affinity chromatography invariably detects the *cis*-diol groups of glycated hemoglobin not limited to deoxy fructoslylated valine but also includes other amino acids like lysine or arginine. Thus, with currently available techniques, overestimation of HbA1c is one of the plausible outcomes if lysine or arginine residues are glycated. In case of antibody based affinity chromatography method, lack of specificity of the antibody against only DFV peptide poses serious problems.

In the light of current limitations in the measurement of HbA1c, we explored the chemistry of HbA1c. HbA1c is chemically N-1-(deoxyfructosyl) valine (DFV) β-chain of hemoglobin, an early and reversible glycated product formed by non-enzymatic reaction with glucose, can eventually undergo relatively stable advanced glycation modifications such as CMV and CEV during the lifespan of erythrocytes. Therefore, it is believed that measurement of HbA1c provides glycemic status over preceding 90-120 days. In fact, elevated levels of CMV-Hemoglobin were observed in diabetic nephropathic and oxidative stress condition by immunoassay (154, 155). However, its usefulness in diagnosis of diabetes has been overlooked. Advanced mass

spectrometry based quantitative approaches like MRM/PRM may provide better view of glycation status of hemoglobin (156-158). Hence, we explored the existence of advanced glycation modifications (CM and CE) that can provide a better alternative diagnosis and management of diabetes. This chapter describes the characterization and quantification of advanced glycated hemoglobin using HR/AM mass spectrometry in both *in-vitro* and clinical subjects. In this pursuit, for the first time, we have comprehensively characterized and quantified DFV/DFL, CMV/CML and CEV/CEL peptides of hemoglobin. This study demonstrates for the first time that N-1-(carboxymethyl)valine β- chain of hemoglobin (CMV) the predominant modifications and N-1-(carboxyethyl)valine β- chain of hemoglobin (CEV) first time identified and quantified.. Furthermore, CMV and CEV peptides correlate well with fasting glucose, postprandial glucose, and micro-albuminuria. Thus, we proposed that CMV-β-chain of hemoglobin and CEV-β-chain of hemoglobin could provide better insight of glycemic status with severity of diabetes.

2.2 Material and Methods

2.2.1 Materials

All the chemicals used throughout the experiments were procured from Sigma-Aldrich (Sigma-Aldrich, MO, USA). Mass spectrometric grade solvents like acetonitrile (ACN) and water were procured from J T. Baker (J T. Baker, PA, USA). Rapigest was procured from Waters (Waters Corporation, MA, USA). Membrane cut off filters were procured from Millipore (Millipore, MA, USA).

2.2.2 Clinical sample collection

The study was carried out at CSIR-National Chemical Laboratory (CSIR-NCL), Pune in collaboration with Chellaram Diabetes Institute (CDI), Pune. Ethical Committee of Chellaram Diabetes Institute has approved this proposal for investigation. Clinical subject's samples were collected randomly with informed consent, and grouped according to standard guidelines of World Health Organization (WHO) into four groups based on the fasting blood glucose level (FBS), Post-prandial blood glucose level and percent of HbA1c. The groups were healthy control, pre-diabetic, controlled

diabetic, and poorly controlled diabetic patients. The number of clinical subjects considered for the pilot study was statistically acceptable.

2.2.2.1 Inclusion criteria for clinical samples collection

i. All patient's age was between 35 to 75
ii. Controlled Diabetes patients (HbA1c less than 7.5 %; fasting blood glucose 126 mg/dl).
iii. Poorly controlled diabetes patients (HbA1c more than 9.0 %; fasting blood glucose more than 200 mg/dl).
iv. Pre-diabetic patients (HbA1c between 6.0-6.5 %; fasting blood glucose 100-125 mg/dl).
v. Healthy Control (HbA1c less than 5.5 %; fasting blood glucose less than 100 mg/dl).
vi. Pre-diabetic subject (HbA1c between 6.0-6.5 %) with one or more risk factors for developing type 2 diabetes mellitus; BMI > 23kg/m², history of gestational diabetes mellitus (GDM), history of big baby > 4.0 kg, history of type 2 diabetes mellitus in first degree relatives, hypertension (>140/90), hyperlipidaemia and dyslipidaemia (HDL < 0.9 or Triglyceride > 2.3 mmol/L).
vii. Total hemoglobin level should be normal (12-16 g/dl).

2.2.2.2 Exclusion criteria for clinical samples collection

i. Diabetes patients should not be suffering from hemolytic anemia (The decreased red cell survival in hemolytic anemia may lower HbA1c and AGE-HbA and hence will lead to erroneous measurements).
ii. Diabetes patients should not be suffering hemoglobinopathies and renal failure.
iii. History (past 1 year) of hypoglycaemia and use of medication or pharmacotherapy (oral or injectable medication approved by the FDA for type 2 diabetes), used for any condition (e.g. pre-diabetes, diabetes, polycystic ovarian syndrome).
iv. Pregnancy or pregnancy-related condition within three months.

v. History of endocrinopathy (hyperparathyroidism, nephrolithiasis or hypercalcemia)
vi. Blood loss or blood transfusion within two months.
vii. Severe heart, liver or kidney disease or cancer.

2.2.2.3 Collection, processing and storage of clinical subject samples

The laboratory staff estimated the biochemical parameter that has been blind to the identity of the clinical subjects. Blood samples were collected under aseptic conditions in vials, appropriately labelled with code numbers. The blood samples were collected from diabetic subjects with prior intimation following the standard guidelines of the ethical committee (Chellaram Diabetes Institute, Pune). The oral glucose tolerance test (OGTT) was performed prior to the blood sample collection. Plasma glucose levels were measured before (fasting) and 2-hours after meal (postprandial). Blood sample was collected from subjects in heparin-coated capillary tubes containing aprotinin using retro-orbital vascular plexus. The blood samples were kept at 4° C for 15 min and centrifuged at 3000 rpm for 15 minutes to separate plasma from blood cells. The erythrocytes was washed twice with saline buffer and then stored at -80° C until further use. Blood samples were collected immediately after measuring the blood glucose and HbA1c to carry out the proteomic experiments. The descriptive characters and biochemical parameters of the study subjects are provided in **Table 2.1**. Based on the glucose levels and HbA1c clinical subjects classified into four groups viz. healthy control, pre-diabetes, diabetes and poorly controlled diabetes.

Table 2.1 Clinical characteristics of participating subjects

Continuous variables are expressed as mean ± standard deviation (SD) and categorical variables as indicated. Fasting blood glucose; Postprandial blood glucose; HbA1c: Glycated Hemoglobin A; LDL: Low density lipoprotein; HDL: High density lipoprotein; VLDL: Very low-density lipoprotein; ALT: Alanine amino transferase; SGPT: Serum Glutamic Pyruvic Transaminase; AST: Aspartate Aminotransferase; SGOT: Serum Glutamic Oxaloacetic Transaminase; and MIC: Micro-albuminuria.

Characteristics	**Control (n=9)**	**Pre-diabetes (n=10)**	**Diabetes (n=10)**	**Poorly Controlled Diabetes (n=10)**
Age (years)	48.10 ± 18.6	60.20 ± 12.4	54.9 ± 14.3	56.1 ± 13.6
Sex	7(M) and 2 (F)	5(M) and 5(F)	3 (M) and 7(F)	9(M) and 1 (F)
Fasting blood glucose (mmol/L)	5.1 ± 0.3	5.9 ± 0.6	7.6 ± 1.6	14.5 ± 2.1
Postprandial blood glucose (mmol/L)	5.8 ± 1.0	9.4 ± 1.2	15.0 ± 1.6	23.6 ± 3.3
HbA1c (%)	4.94 ± 0.22	5.96 ± 0.25	7.46 ± 0.619	9.99 ± 1.49
HbA1c (mmol/mol)	30 ± 0.86	42 ± 0.75	58 ± 2.00	86 ± 8.33
Hemoglobin (g/dl)	14.21 ± 1.28	12.86 ± 1.44	12.74 ± 1.66	14.50 ± 1.33
Serum triglyceride (mmol/L)	77.40 ±26.80	118.61 ± 49.49	123.09 ± 37.68	120.20 ± 29.10
Serum LDL (mmol/L)	119.20 ± 25.69	129.23 ± 22.21	131.63 ± 42.29	161.30 ± 25.38
Serum HDL (mmol/L)	48.20 ±24.18	41.61 ± 7.43	33.00 ± 5.15	30.90 ± 4.17
Serum Cholesterol (mmol/L)	170.60 ± 32.60	183.15 ± 30.98	194.36 ± 36.63	209.10 ± 27.66
Serum VLDL (mmol/L)	12.30 ± 4.3	22.60 ± 14.50	20.20 ± 5.90	18.97 ± 4.59
Creatinine (µmol/L)	0.94 ± 0.05	0.85 ± 0.15	0.84 ± 0.15	0.93 ± 0.11
ALT or SGPT (units/liter)	18.40 ± 4.28	17.50 ± 4.29	17.6 ± 5.54	18.8 ± 4.54
AST or SGOP (units/liter)	18.60 ± 7.27	20.38 ± 4.69	17.2 ± 2.79	18.7 ± 2.49
MIC (mg/dl)	0.54 ± 0.05	0.69 ± 0.21	0.72±0.24	1.1 ± 0.94

2.2.3 Study workflow

This study deals with characterization and quantification of deoxy-fructosylation, carboxymethylation and carboxyethylation modifications involving N-terminal valine residue and lysine residues of hemoglobin peptides from 39 clinical samples. The overview of the study design is described in **Figure 2.1.**

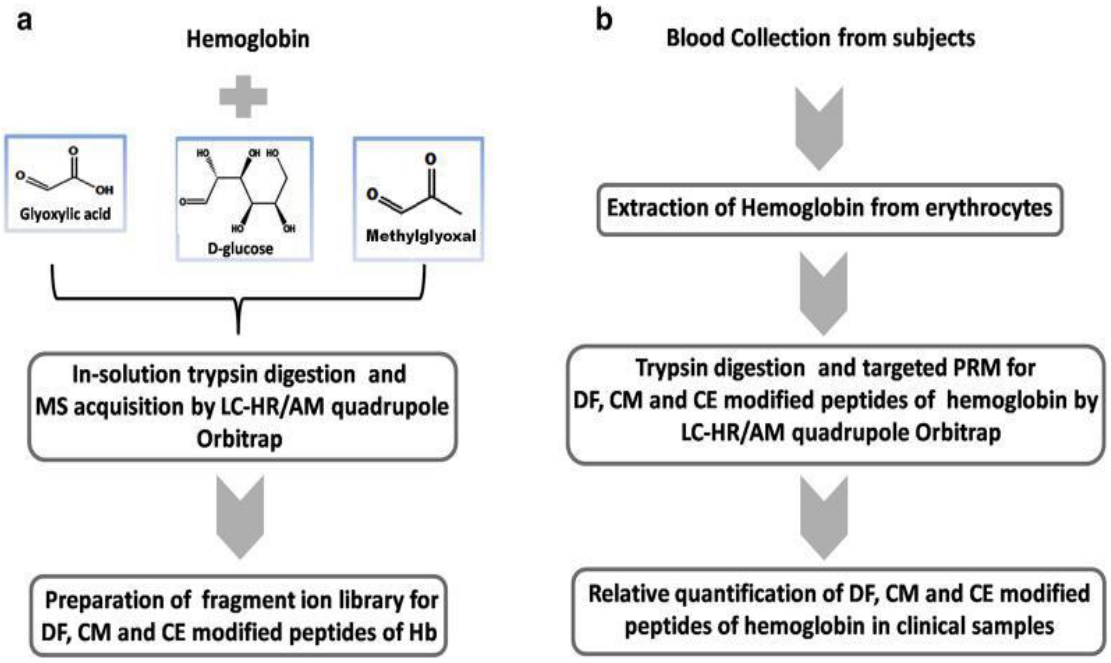


Figure 2.1 Describes the overview of experimental design

(A) *In-vitro* synthesis of DF, CM and CE modified hemoglobin and fragment ion library preparation for glycated peptides of hemoglobin, (B) Relative quantification of glycated peptides of hemoglobin in clinical subjects.

2.2.3.1 Hemoglobin extraction from human blood samples

Hemoglobin was extracted from erythrocyte pellet as described earlier with few changes (159). 40 µl of 0.1 % Rapigest (in 50 mM ammonium bicarbonate) was added to 25 µl of erythrocyte pellet and vortexed intermittently to lysed the cells for 30 minutes at 4° C. Further, cell lysate was centrifuged at 17000 rpm for 30 minutes and protein in the supernatant was collected and protein estimation was performed using Bio-Rad Bradford kit (Bio-Rad Laboratories, CA).

2.2.3.2 *In-vitro* synthesis of AGE modified hemoglobin

2.2.3.2.1. Synthesis of carboxymethyl and carboxyethyl modified hemoglobin

Hemoglobin (10 mg) and sodium cyanoborohydride (0.05 M) were dissolved in 0.2 M phosphate buffer (pH 7.4), to which glyoxylic acid (0.045 M) or methyl glyoxal (0.05 M) were added and final volume was made up to 1 ml with 0.2 M phosphate buffer.

The reaction mixtures were incubated at 37° C for 24 hours under sterile and dark conditions **(Figure 2.2 and Figure 2.3)** (148, 160, 161).

2.2.3.2.2. Synthesis of heterogeneous AGE modified hemoglobin

Hemoglobin (10 mg) was incubated with glucose (0.5 M) in 0.2 M phosphate buffer (pH 7.4, 1 ml) and sample was incubated at 37° C for 15 days under sterile and dark conditions **(Figure 2.2 and Figure 2.3)** (148, 160, 161).

2.2.3.2.3. Control hemoglobin

Hemoglobin (10 mg) was incubated in 0.2 M phosphate buffer (pH 7.4, 1 ml) and was incubated at 37° C for 15 days under sterile and dark conditions.

Prior to proteolytic digestion, hemoglobin and modified hemoglobin samples were passed through 3 kD cut-off filters to remove excess of salts and modifying agents against 0.2 M phosphate buffer. The amount of protein in the sample was estimated using Bio-Rad Bradford kit (Bio-Rad Laboratories, CA).

2.2.4 In-solution tryptic digestion of *in-vitro* modified hemoglobin and clinical samples of hemoglobin

50 µg of *in-vitro* modified hemoglobin and hemoglobin from clinical samples were diluted with 0.1 % Rapigest containing 50 mM ammonium bicarbonate buffer (pH-8.3). Samples were denatured at 80^{o} C for 15 minutes. Denatured hemoglobin samples were reduced with 100 mM of dithiothritol at 60^{o} C for 15 minutes and alkylated using 200 mM of iodoacetamide for 30 minutes at room temperature in a dark condition. After reduction and alkylation protein samples were subjected to proteolytic digestion using an enzyme, trypsin (1:50) for 16 to 18 hours at 37^{o} C. The digestion was stopped by addition of 99% formic acid and incubated at 37^{o} C for 15 to 20 minutes followed by centrifugation at 12500 rpm for 15 minutes. The supernatant containing peptides were collected and used for the LC-MS/MS analysis.

2.2.5 Liquid chromatography followed by Data dependent mass spectrometry acquisition

The analysis of data acquired in data dependent manner was performed with Proteome Discover software (PD 1.4.0.288, Thermo Fisher Scientific). Sequest-HT was used as search engine for peptide identification against hemoglobin protein database (UniProt id: P68871 and P69905). Peptides and fragment mass tolerance was set at 10 ppm and 500 ppm respectively. Deoxyfructosylation (DM +162.0211 Da) (159), carboxymethylation (CMV +58.0055 Da), and carboxyethylation (CE +72.0211 Da) was given as variable modifications at N-terminal valine and lysine residues. The false positive rate was set to 1 % in the processing workflow. Glycated peptides were manually validated for accurate mass shift in precursor ion due to modification, and presence of fragments retaining modification. Subsequently, fragment ion library was generated for DFV/DFL, CMV/CML, and CEV/CEL modified hemoglobin peptides **(Figure 2.2 and Figure 2.3).**

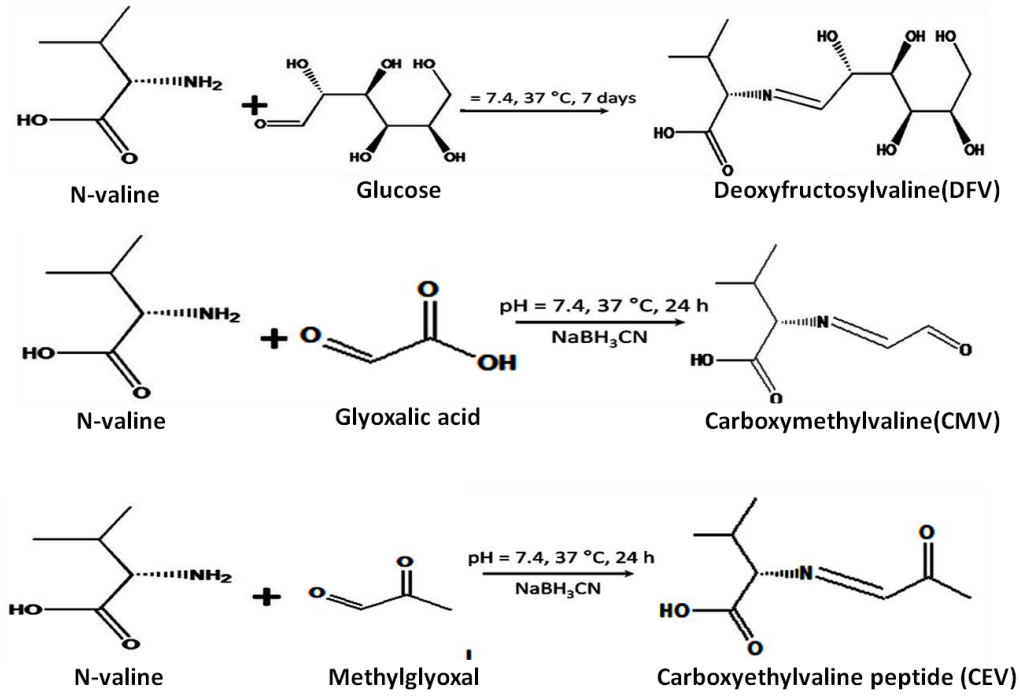


Figure 2.2 Schematic representation of synthesis of glycated hemoglobin

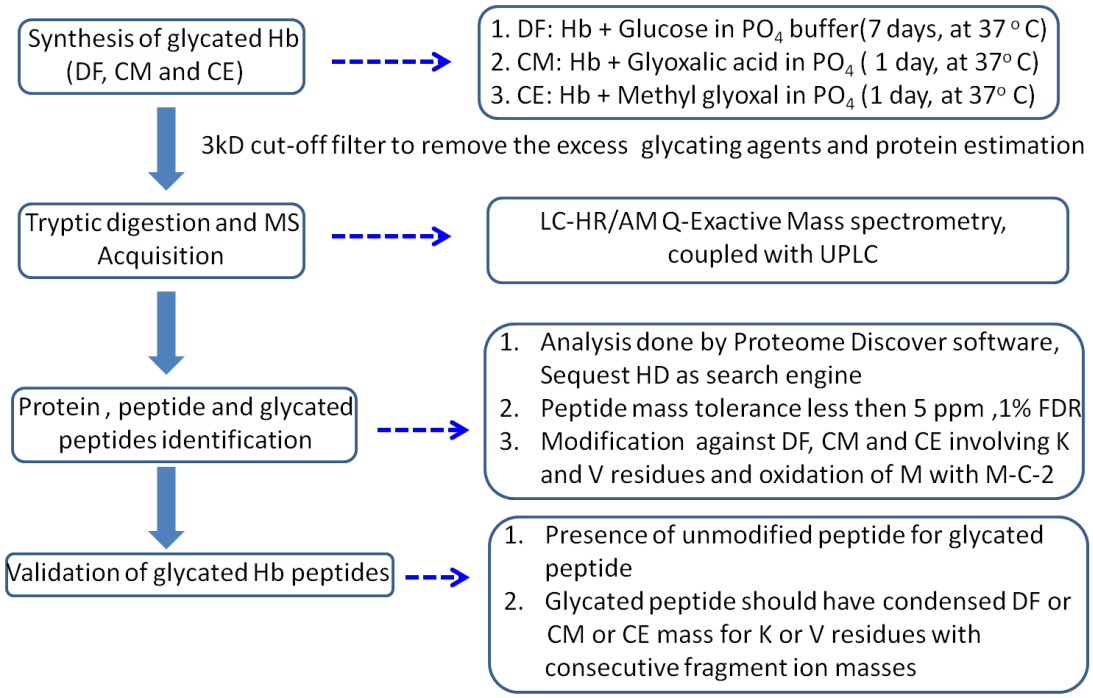


Figure 2.3 Detailed experimental procedure for the synthesis of glycated hemoglobin and construction of fragment ion library for glycated peptides of hemoglobin

2.2.6 Relative quantification of advanced glycated hemoglobin peptides by targeted mass spectrometry approach

We have used parallel reaction monitoring (PRM) which is a targeted mass spectrometry approach for the quantification glycated peptides of haemoglobin. A total of 42 m/z corresponding to DFV/DFL, CMV/CML and CEV/CEL modified peptides observed *in-vitro* were used for PRM analysis in clinical samples (Additional file 2 and **Table 2.2).** PRM was performed at a resolution of 17,500, isolation window of 2 Da, target AGC value of $2e^5$, maximum injection time of 150 ms, MSX count 1, and isolation offset m/z 0.5 Da. Selected precursor peptides (m/z) fragmentation was performed by high energy collision induced dissociation (HCD) of 28 eV and MS/MS scans were acquired with starting mass of m/z 50 to 1999 Da.

All raw files obtained from PRM acquisition were analyzed by Pinpoint (version 1.4.0 Thermo Fisher Scientific). Ion chromatograms were extracted with a precursor level (MS) mass tolerance of 10 ppm and chromatogram peak width for expected m/z mass retention time 3 min. The composite of MS/MS spectrum of each targeted peptide was reconstructed from the area under curve (AUCs) of all the transitions for the peptide.

The AUC of each target peptide was calculated based on the co-elution profiles of all the fragment ions. The AUCs of each individual PRM transition were then summed to obtain AUCs at peptide level (162).

2.2.7 Statistical analysis

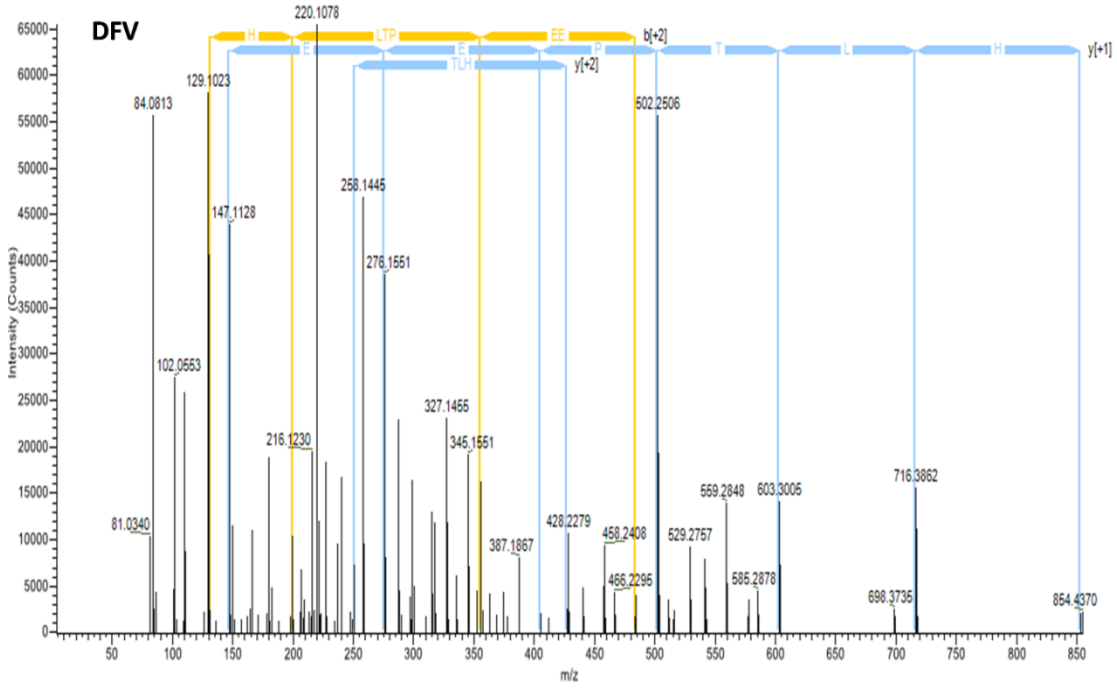
PRM analysis was performed for all the individual clinical samples in technical triplicates. Graph-pad Prism (version 5.01) was used for the statistical significance analysis. Two-way ANOVA followed by Tukey's test and Bonferroni post tests were performed for the AUCs of quantified glycated peptides and their fold change in pre-diabetes, diabetes and poorly controlled diabetes. The AUCs of the glycated hemoglobin peptides were correlated with clinical parameters like blood glucose, HbA1c, micro-albuminuria, etc. by performing Pearson's correlation test. For all the represented data p values significance as follows *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$.

2.3 Results and discussion

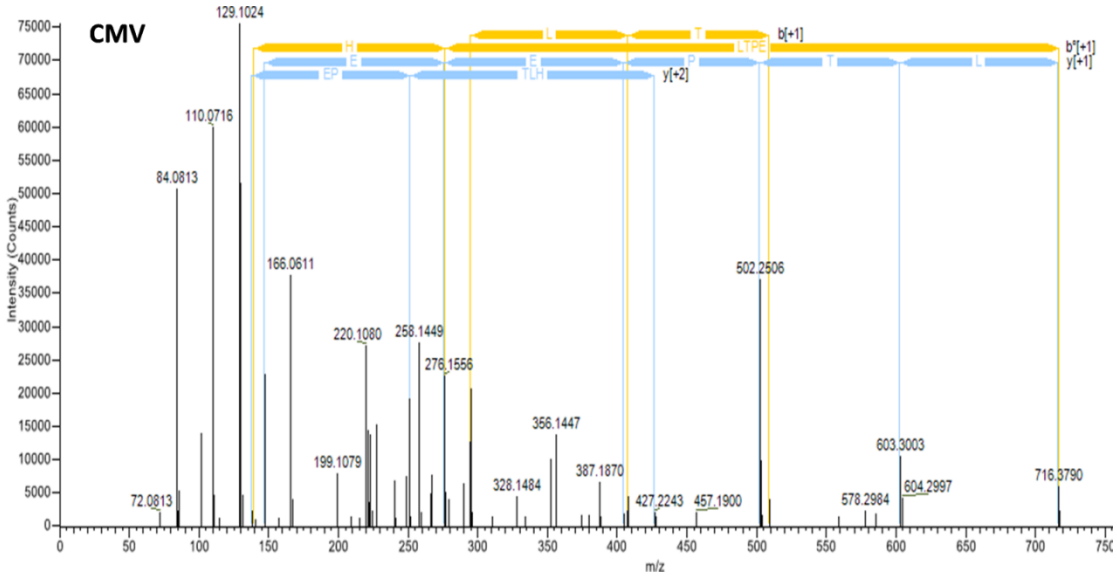
2.3.1 Construction of fragment ion library for in-vitro synthesized glycated hemoglobin peptides

Quantification of peptides by targeted mass spectrometry approaches like MRM and PRM heavily relies on fragment ion or spectral library(148, 162). Therefore, we constructed fragment ion library for synthetically glycated peptides of hemoglobin. Synthetically modified DFV/DFL, CMV/CML and CEV/CEL modified peptides of hemoglobin were studied by data dependent LC–MS/MS analysis. Initially the advanced glycated peptides identified by Proteome Discoverer were analyzed for exact mass increment of 162.0211 Da (DFV/DFL), 58.0055 Da (CMV/CML) and 72.0211 Da (CEV/CEL) in the precursor ions and modified mass retaining fragment ions. Subsequently, the MS/MS spectra were manually inspected for presence of modified fragment ions as like described in the previous study(148). For example, the N-terminal peptide of β-Hb "VHLTPEEK" was interrogated for deoxyfructosyl (DF), carboxymethyl (CM), and carboxyethyl (CE) modifications involving N-1-valine. The precursor mass of unmodified "VHLTPEEK" was 952.5078 Da and m/z: 476.76 Da, while V_{DF}HLTPEEK (precursor mass 1114.5607 Da and m/z: 557.7840 Da),

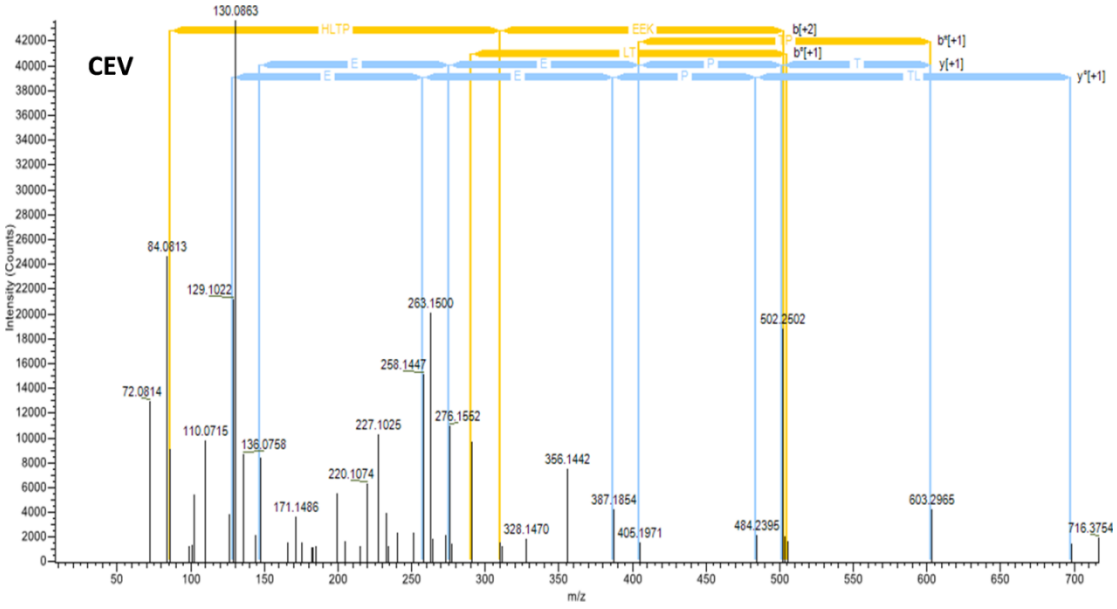
V_{CM}HLTPEEK (precursor mass: 1010.5116 Da and m/z: 505.75946 Da), and V_{CE}HLTPEEK (precursor mass: 1024.5297 Da and m/z: 512.7684 Da) peptides showed mass increment of 162.0211, 58.0055, 72.0211 Da respectively. Further, these peptides were manually inspected for presence of modified fragment ions (e.g. Carboxymethyl modified V*HLTPEEK, m/z-505.75 Da, b_2+ 295.1400 Da, b_3+ 408.2241 Da, b_4+ 509.2718 Da) and were considered to be truly modified if the MS/MS spectrum showed presence of corresponding modified fragment ions. The peptides were considered to be truly modified by the presence of b ions retaining modification, and unmodified y ions. The annotated MS/MS spectra for V_{DF}HLTPEEK, V_{CM}HLTPEEK and V_{CE}HLTPEEK are provided in **(Figure 2.4 A, B C).** The list of all glycated hemoglobin peptides and their corresponding modified fragment ions are listed in **Table 2.2**



A) Deoxyfructosyl modification at N-terminal Valine, V*HLTPEEK (m/z: 557.7840 Da, MH+: 1114.5607 Da



B) Carboxymethyl modification at N-terminal Valine, V*HLTPEEK (m/z: 505.75946 Da, MH+: 1010.51164 Da



C) Carboxyethyl modification at N-terminal Valine, V*HLTPEEK (m/z: 512.7684 Da and MH+: 1024.5297 Da

Figure 2.4 The annotated MS/MS spectra for β-hemoglobin at N-terminal valine involving glycated peptide

Table 2.2 Fragment ion library for glycated hemoglobin peptides.

Detailed information of glucose (A), glyoxylic acid (B) and methylglyoxal (C) derived N-Valine and lysine peptides of Hb and their corresponding modified fragment ions. DFV/DFL: N-1-deoxyfructosyl-Valine/Lysine; CMV/CML: N-1-carboxymethyl-Valine/Lysine; CEV/CEL: N-1-carboxyethyl-Valine/Lysine; CS: Charge state; XC: Xcorr; MC: Miss cleavage; and Mod: Modification. Static modification: Carbamidomethyl (57.02146 Da), CS-Charge state, XC- Xcorr, MC-Missed cleavage

Sl.N	Modsite	Peptide start-end	Peptide sequence	Peptide MH+ Da	Monoisotopic m/z Da (mmu/ppm)	CS	MC	Gly Mod	Signature fragment ions		
Glucose induced glycation modifications											
Alpha chain of Hemoglobin											
1	K7 and K 11	1 to 17	VLSPADK*TNVK*AAWGK	1904.99453	477.00409 Da (-0.49 mmu/-1.02 ppm),	4	1	DFL and CML	$y^{+}6$ 718.38829	$y^{+}7$ 817.45671	$y^{2+}11$ 719.36467
2	K 7	1 to 11	VLSPADK*TNVK	1229.67131	615.33929 Da (-1.17 mmu/-1.9 ppm),	2	1	CML	$y^{+}5$ 647.37229	$y^{+}6$ 762.39924	$y^{+}7$ 833.43636
3	K 16	12 to 31	AAWGK*VGAHAGEYGAEALER	2205.04978	735.68811 Da (-2.47 mmu/-3.36 ppm),	3	1	DFL	$b^{+}7$ 832.41999	$b^{+}11$ 1168.57461	$y^{2+}16$ 910.44472
4	K 16	12 to 31	AAWGK*VGAHAGEYGAEALER	2101.01455	526.00909 Da (+1.18 mmu/+2.24 ppm),	4	1	CML	$b^{+}6$ 671.35118	$b^{+}8$ 799.40977	$b^{+}9$ 936.46868
5	K 61	61 to 90	K*VADALTNAVAHVDDMPNALSALSDLHAHK	3182.58649	637.32312 Da (-0.71 mmu/-1.11 ppm),	5	1	CML	$b^{+}2$ 286.17615	$b^{+}3$ 357.21327	$b^{+}4$ 472.24022
6	K 90	62 to 92	VADALTNAVAHVDDMPNALSALSDLHAHK*LR	3427.71986	686.34979 Da (-1.55 mmu/-2.26 ppm)	5	1	DFL	$y^{+}4$ 715.40974	$y^{+}5$ 786.44686	$y^{+}6$ 923.50577
7	K 90	61 to 92	KVADALTNAVAHVDDMPNALSALSDLHAHK*LR	3555.81860	593.47583 Da (-0.66 mmu/-1.12 ppm),	6	2	DFL	$y^{+}3$ 578.35083	$y^{+}6$ 923.50577	$y^{2+}16$ 953.01310
8	K 139	100 to 141	LLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSK*YR	4635.47875	773.41919 Da (-3.7 mmu/-4.78 ppm),	6	2	CABD and CML	$y^{+}3$ 524.28273	$y^{+}5$ 712.36244	$y^{+}6$ 825.44651

Beta chain of Hemoglobin											
9	V 1	1 to 8	V*HLTPEEK	1114.56072	557.78400 Da (-0.99 mmu/-1.77 ppm)	2	0	DFV	$b^{2+}1$ 131.56790	$b^{2+}2$ 200.09735	$b^{+}3$ 512.27150
10	V 1	1 to 8	V*HLTPEEK	1010.51164	505.75946 Da (-1.85 mmu/-3.66 ppm)	2	0	CMV	$b^{+}2$ 295.14009	$b^{+}3$ 408.22416	$b^{+}4$ 509.27184
11	K 17	9 to 30	SAVTALWGK*VNVDEVGGEALGR	2390.22086	797.41180 Da (+0.31 mmu/+0.39 ppm)	3	1	DFL	$b^{3+}9$ 359.52562	$b^{3+}11$ 430.56273	$b^{3+}14$ 544.94206
12	K 17	9 to 30	SAVTALWGK*VNVDEVGGEALGR	2286.17484	762.72980 Da (+0.75 mmu/+0.99 ppm)	3	1	CML	$y^{+}15$ 1557.78682	$b^{3+}9$ 324.84317	$b^{3+}15$ 543.28242
13	K 144	133 to 146	VVAGVANALAHK*YH	1507.79827	754.40277 Da (-1.69 mmu/-2.24 ppm),	2	1	CML	$y^{+}3$ 505.24052	$y^{+}4$ 642.29943	$y^{+}5$ 713.33655
Glyoxal induced glycation modifications											
Alpha Chain of Hemoglobin											
1	K 40 and K 56	32 to 60	MFLSFPTTK*TYFPHFDLSHGSAQVK*GHGK	3381.6435	677.13452 Da (+1.46 mmu/+2.15 ppm)	5	1	CML and CML	$y^{+}5$ 584.31511	$y^{+}6$ 683.38353	$y^{+}8$ 882.47923
2	K 40, K 56 and K 60	32 to 60	MFLSFPTTK*TYFPHFDLSHGSAQVK*GHGK*	3439.6412	688.73407 Da (-0.09 mmu/-0.14 ppm)	5	2	CML CML and CML	$y^{+}5$ 642.32059	$y^{+}6$ 741.38901	$y^{+}7$ 869.44759
3	K 61 and K 90	61 to 92	K*VADALTNAVAHVDDMPNALSALSDLHAHK*LR	3509.7777	878.19989 Da (-0.75 mmu/-0.85 ppm)	4	2	CML and CML	$b^{+}2$ 286.17615	$b^{+}3$ 357.21327	$b^{+}4$ 472.24022
									$y^{+}3$ 474.30348	$y^{+}4$ 611.36239	$y^{+}5$ 682.39951
4	K 61 and K 90	61 to 92	K*VADALTNAVAHVDDMPNALSALSDLHAHK*LR	3525.7761	705.96106 Da (+0.11 mmu/+0.16 ppm)	5	2	CML, OXD and CML	$b^{+}2$ 286.17615	$b^{+}3$ 357.21327	$b^{+}4$ 472.24022
									$y^{+}3$ 474.30348	$y^{+}4$ 611.36239	$y^{+}5$ 682.39951

5	K 90	62 to 92	VADALTNAV AHVDDMPNA LSALSDLHAH K*LR	3323.6833	665.54248 Da (+0.61 mmu/+0.91 ppm)	5	1	CML	y^+3 474.30348	y^+4 611.36239	y^+5 682.39951
6	K 90	61 to 92	KVADALTNA VAHVDDMPN ALSALSDLHA HK*LR	3451.7783	691.16150 Da (+0.63 mmu/+0.91 ppm)	5	2	CML	y^+3 474.30348	y^+4 611.36239	y^+5 682.39951
7	K 90	62 to 92	VADALTNAV AHVDDMPNA LSALSDLHAH K*LR	3339.6729	668.74042 Da (-0.44 mmu/-0.66 ppm)	5	1	OXD and CML	y^+3 474.30348	y^+4 611.36239	y^+5 682.39951
8	K 139	128 to 141	FLASVSTVLTS K*YR	1629.8869	543.96716 Da (+0.75 mmu/+1.37 ppm)	3	1	CML	y^+3 524.28273	y^+4 611.31476	y^+5 712.36244
9	K 139	100 to 141	LLSHCLLVTL AAHLPAEFTP AVHASLDKFL ASVSTVLTSK *YR	4635.4970	773.42224 Da (-0.64 mmu/-0.83 ppm)	6	2	CAB D and CML	y^+3 524.28273	y^+4 611.31476	y^+5 712.36244
Beta Chain of Hemoglobin											
10	V 1	1 to 8	V*HLTPEEK	1010.5151	505.76123 Da (-0.08 mmu/-0.15 ppm)	2	0	CMV	b^+1 158.081	b^+2 295.140	b^+3 408.224
11	V 1 and K 8	1 to 17	V*HLTPEEK*S AVTALWGK	1982.0245	661.34637 Da (+0.52 mmu/+0.78 ppm)	3	1	CMV and CML	b^+2 295.14009	b^+3 408.22416	b^+4 509.27184
12	V 1, K 8 and K 17	1 to 30	V*HLTPEEK*S AVTALWGK* VNVDEVGGE ALGR	3335.6835	1112.56604 Da (+2.67 mmu/+2.4 ppm)	3	2	CMV, CML and CML	b^+2 295.14009	b^+8 1050.51025	y^+14 1500.76535
13	V 1, K 8 and K 17	1 to 30	V*HLTPEEK*S AVTALWGK* VNVDEVGGE ALGR	3349.6951	1117.23657 Da (+1.32 mmu/+1.18 ppm)	3	2	CEV, CML and CML	b^+2 309.15574	b^+10 1222.59505	y^+14 1500.76535
14	K 59	41 to 61	FFESFGDLSTP DAVMGNPK* VK	2360.1113	787.37531 Da (-0.08 mmu/-0.11 ppm)	3	1	OXDN and CML	y^+7 847.43427	y^+8 946.50269	y^+9 1017.53981
15	K 59	41 to	FFESFGDLSTP	2811.3238	703.58643 Da	4	2	OXDN	y^+8	y^+10	y^+11

	and K 61	65	DAVMGNPK* VK*AHGK		(-1.42 mmu/-2.02 ppm)			, CML and CML	980.55240	1151.61680	1298.65221
16	K 59 and K 61	41 to 61	FFESFGDLSTP DAVMGNPK* VK*	2418.1188	806.71112 Da (+0.57 mmu/+0.71 ppm),	3	1	OXD CML and CML	y^+3 490.28716	y^+4 587.33993	y^+7 905.43974
17	K 65 and K 66	62 to 82	AHGK*K*VLG AFSDGLAHLD NLK	2307.2118	577.55841 Da (+0.63 mmu/+1.1 ppm)	4	2	CML and CML	b^+5 638.32567	b^+6 737.39409	b^+9 978.53675
18	K 66 and K 82	66 to 95	K*VLGAFSDG LAHLDNLK*G TFATLSELHC DK	3373.6704	675.53992 Da (-0.59 mmu/-0.88 ppm)	5	2	CML, CML and CABD	b^+4 456.28169	b^+5 527.31881	b^+6 674.38723
19	K 120	105 to 132	LLGNVLVCVL AHHFGK*EFT PPVQAAYQK	3194.6810	1065.56519 Da (-0.35 mmu/-0.33 ppm),	3	1	CABD And CML	y^+14 1621.82211	y^+15 1768.89053	y^+16 1905.94944
20	K 144	133 to 146	VVAGVANAL AHK*YH	1507.8036	503.27274 Da (+0.67 mmu/+1.34 ppm)	3	1	CML	y^+3 505.24052	y^+4 642.29943	y^+5 713.33655
Methyl glyoxal induced glycation modifications											
Alpha Chain of Hemoglobin											
1	K 61	61 to 90	K*VADALTNA VAHVDDMPN ALSALSDLHA HK	3196.5965	640.12512 Da (-1.84 mmu/-2.87 ppm)	5	1	CEL	b^+2 300.19180	b^+3 486.25587	b^+4 557.29299
2	K 11	8 to 16	TNVK*AAWG K	1046.5608	523.78406 Da (-1.07 mmu/-2.04 ppm)	2	1	CEL	b^+4 515.28241	b^+5 586.31953	–
3	K 90	62 to 92	VADALTNAV AHVDDMPNA LSALSDLHAH K*LR	3337.6939	668.34460 Da (-0.4 mmu/-0.6 ppm)	5	1	CEL	y^+3 488.31913	y^+4 625.37804	y^+5 696.41516

4	K 90	61 to 92	KVADALTNAVAHVDDMPNALSALSDLHAHK*LR	3465.7984	693.96552 Da (+1.52 mmu/+2.19 ppm)	4	2	CEL	y^+3 488.31913	y^+4 625.37804	y^+5 696.41516
5	K 139	128 to 141	FLASVSTVLTSK*YR	1643.9034	548.63934 Da (+1.04 mmu/+1.9 ppm),	3	1	CEL	y^+4 625.33041	y^+5 726.37809	y^+6 839.46216
Beta chain of Hemoglobin											
6	V 1	1 to 8	V*HLTPEEK	1024.5297	512.76849 Da (-0.64 mmu/-1.25 ppm)	2	0	CEV	b^+1 172.096	b^+2 309.155	–
7	V 1 and K 8	1 to 17	V*HLTPEEK*SAVTALWGK	1996.0387	499.76514 Da (+0.01 mmu/+0.02 ppm)	3	1	CEV And CML	b^+2 309.15574	b^+3 422.23981	b^+4 523.28749
8	K 59	41 to 61	FFESFGDLSTPDAVMGNPK*VK	2374.1282	792.04761 Da (+0.33 mmu/+0.42 ppm)	3	1	OXD and CEL	y^+4 543.35010	y^+6 714.41450	y^+7 861.44992
9	K 144	133 to 146	VVAGVANALAHK*YH	1521.8168	507.94379 Da (-0.16 mmu/-0.32 ppm)	3	1	CEL	y^+3 519.25617	y^+4 656.31508	y^+5 727.35220

A total of 42 glycated hemoglobin peptides **(Table 2.2.)** were identified and characterized in data dependent (DDA) analysis. Glyoxylic acid modified hemoglobin had 20 glycated hemoglobin peptides, 13 glycated hemoglobin peptides of glucose modified, and 9 of methylglyoxal modified glycated hemoglobin peptides. Irrespective of the glycating agents used, total 26 peptides were observed to be carboxymethylated. Together these glycated peptides involved 19 sites, 9 from α-chain of hemoglobin and 10 from β-chain of hemoglobin **(Table 2.3.)**. In case of glucose modified hemoglobin, apart from deoxy-fructosylation of N-1-β-valine, it was interesting to observe carboxymethylation and carboxyethylation of N-1-β-valine and other lysine residues.

2.3.2 Relative quantifications of glycated hemoglobin peptides by targeted (PRM) mass spectrometry approach

Using precursor ion information from data dependent analysis, DFV/DFL, CMV/CML, and CEV/CEL modified hemoglobin peptides from **Table 2.2.**were used for the relative quantification performed by targeted (PRM) mass spectrometry approach followed by data analysis using PINPOINT^{TM} software in clinical samples. Both b and y ions from all the transitions were used to generate the composite MS/MS spectrum for each targeted peptide. The co-eluted fragment ions of the glycated precursor were reconfirmed for the presence of modification retained fragment ions observed in the synthetically glycated hemoglobin. For relative quantification of glycated hemoglobin peptides, the total AUCs of corresponding common co-eluted fragment ions observed in all replication and groups were used. The list of modified peptides and their corresponding fragment ions used for quantification is mentioned in **Table 2.3**. Total 26 glycated peptides (15 from α-chain hemoglobin and 11 from β-chain hemoglobin) were identified and quantified in clinical samples **(Table 2.3, Figure 2.5 A and B).** The relative fold change in AUCs was calculated for all the glycated peptides across different clinical conditions and represented in **Figure 2.5 A and B.** A total of 13 and 9 peptides of α-chain hemoglobin and β-chain hemoglobin respectively are depicted in **Figure 2.5 A and B.** were significantly elevated in poorly controlled diabetes. Glycated peptides of α-chain hemoglobin i) sequence: K*(CM)VADALTNAVAHVDDM*(Oxd)PNALSALSDLHAHK*(CM)LR, m/z-705.96 Da, site-K61 and K90; ii) sequence: K*(CM)VADALTNAVAHVDDMPNALSALSDLHAHK, m/z-640.12Da, site-K61; and β-chain hemoglobin iii) sequence: V*(CM)HLTPEEK*(CM)SAVTALWGK*(CM)VNVDEVGGEALGR, m/z: 1112.56 Da, site-V1, K8 and K17 and iv) FFESFGDLSTPDAVM*(Oxd)GNPK*(CEL)VK, m/z: 792.04 Da, site- K61 showed significant increase in all the diabetic conditions. Thus, these sites perhaps can be considered as glycation sensitive sites in the hemoglobin.

Table 2.3 The list of glycated hemoglobin peptides and their corresponding fragment ions used for relative quantification in clinical samples

Sl.No.	Mod site	Peptide sequence	Peptide MH+ Da	Monoisotopic m/z Da (mmu/ppm)	CS	Mod	Fragment ions used for quantification	
Alpha chain of Hemoglobin								
1	K7 and K 11	VLSPADKTNVK*AAWGK	1741.0936	436.491 Da (-0.49 mmu/-1.02 ppm),	4	CML	204.133 (y^+2)	390.2130 (y^+3)
2	K 7	VLSPADK*TNVK	1229.67131	615.33929 Da (-1.17 mmu/-1.9 ppm),	2	CML	213.1592 (b^+2)	300.1912 (b^+3)
3	K 16	AAWGK*VGAHAGEYGAEALER	2205.04978	735.68811 Da (-2.47 mmu/-3.36 ppm),	3	DFL	329.1602 (b^+3)	386.1817 (b^+4)
4	K 16	AAWGK*VGAHAGEYGAEALER	2101.01455	526.00909 Da (+1.18 mmu/+2.24 ppm),	4	CML	386.1817 (b^+4)	572.2821 (b^+5)
5	K 61	K*VADALTNAVAHVDDMPNALSALSDLHAHK	3182.58649	637.32312 Da (-0.71 mmu/-1.11 ppm),	5	CML	286.1755 (b^+2)	357.2127 (b^+3)
6	K 90	VADALTNAVAHVDDMPNALSALSDLHAHK*LR	3427.71986	686.34979 Da (-1.55 mmu/-2.26 ppm)	5	DFL	578.3185 (y^+3)	715.3774 (y^+4)
7	K 90	KVADALTNAVAHVDDMPNALSALSDLHAHK*LR	3555.81860	593.47583 Da (-0.66 mmu/-1.12 ppm),	6	DFL	578.3185 (y^+3)	715.3774 (y^+4)
8	K 40 and K 56	MFLSFPTTK*TYFPHFDLSHGSAQVK*GHGK	3381.6435	677.13452 Da (+1.46 mmu/+2.15 ppm)	5	CML and CML	626.3001 (b^+5)	811.4415 (y^+7)
9	K 61 and K 90	K*VADALTNAVAHVDDMPNALSALSDLHAHK*LR	3525.7761	705.96106 Da (+0.11 mmu/+0.16 ppm)	5	CML, OXD and CML	175.118 (y^+1)	
10	K 90	VADALTNAVAHVDDMPNALSALSDLHAHK*LR	3323.6833	665.54248 Da (+0.61 mmu/+0.91 ppm)	5	CML	932.541 (y^+7)	1047.568 (y^+8)

11	K 139	FLASVSTVLTSK*YR	1629.8869	543.96716 Da (+0.75 mmu/+1.37 ppm)	3	CML	175.1184 (y^+1)	611.3147 (y^+4)
12	K 11	TNVK*AAWGK	1046.5608	523.78406 Da (-1.07 mmu/-2.04 ppm)	2	CEL	515.2817 (b^+4)	586.3189 (b^+5)
13	K 90	VADALTNAVAHVDDMPNALSALSDLHAHK*LR	3337.6939	668.34460 Da (-0.4 mmu/-0.6 ppm)	5	CEL	833.4734 (y^+6)	1061.5844 (y^+8)
14	K 90	KVADALTNAVAHVDDMPNALSALSDLHAHK*LR	3465.7984	693.96552 Da (+1.52 mmu/+2.19 ppm)	4	CEL	625.3774 (y^+4)	833.4734 (y^+6)
15	K 139	FLASVSTVLTSK*YR	1643.9034	548.63934 Da (+1.04 mmu/+1.9 ppm),	3	CEL	175.1184 (y^+1)	938.5299 (y^+7)
Beta chain of Hemoglobin								
16	V 1	V*HLTPEEK	1114.56072	557.78400 Da (-0.99 mmu/-1.77 ppm)	2	DFV	1095.511 (b^+8)	853.4408 (y^+7)
17	V 1	V*HLTPEEK	1010.51164	505.75946 Da (-1.85 mmu/-3.66 ppm)	2	CMV	295.1317 (b^+2)	605.3162 (b^+5)
18	V 1 and K 8	V*HLTPEEK*SAVTALWGK	1982.0245	661.34637 Da (+0.52 mmu/+0.78 ppm)	3	CMV and CML	295.1395 (b^+2)	147.1122 (y^+1)
19	V 1, K 8 and K 17	V*HLTPEEK*SAVTALWGK*VNVDEVGGEALGR	3335.6835	1112.56604 Da (+2.67 mmu/+2.4 ppm)	3	CMV, CML and CML	295.1395 (b^+2)	659.3465 (y^+7)
20	K 59 and K 61	FFESFGDLSTPDAVMGNPK*VK*AHGK	2811.3238	703.58643 Da (-1.42 mmu/-2.02 ppm)	4	OXDN, CML and CML	511.2181 (b^+4)	658.2866 (b^+5)

21	K 66 and K 82	K*VLGAFSDGLAHLDNLK*GTFATLSELHCDK	3373.6704	675.53992 Da (-0.59 mmu/-0.88 ppm)	5	CML, CML and CABD	187.1071 (b^+1)	286.1755 (b^+2)
22	K 120	LLGNVLVCVLAHHFGK*EFTPPVQAAYQK	3194.6810	1065.56519 Da (-0.35 mmu/-0.33 ppm),	3	CABD And CML	284.1963 (b^+3)	497.3076 (b^+5)
23	V 1	V*HLTPEEK	1024.5297	512.76849 Da (-0.64 mmu/-1.25 ppm)	2	CEV	522.2790 (b^+4)	276.1548 (y^+2)
24	K 59	FFESFGDLSTPDAVMGNPK*VK	2374.1282	792.04761 Da (+0.33 mmu/+0.42 ppm)	3	OXD and CEL	658.2866 (b^+5)	715.3080 (b^+6)
25	K 144	VVAGVANALAHK*YH	1521.8168	507.94379 Da (-0.16 mmu/-0.32 ppm)	3	CEL	199.1435 (b^+2)	
26	K 144	VVAGVANALAHK*YH	1507.79827	754.40277 Da (-1.69 mmu/-2.24 ppm),	2	CML	505.2399 (y^+3)	826.4201 (y^+6)

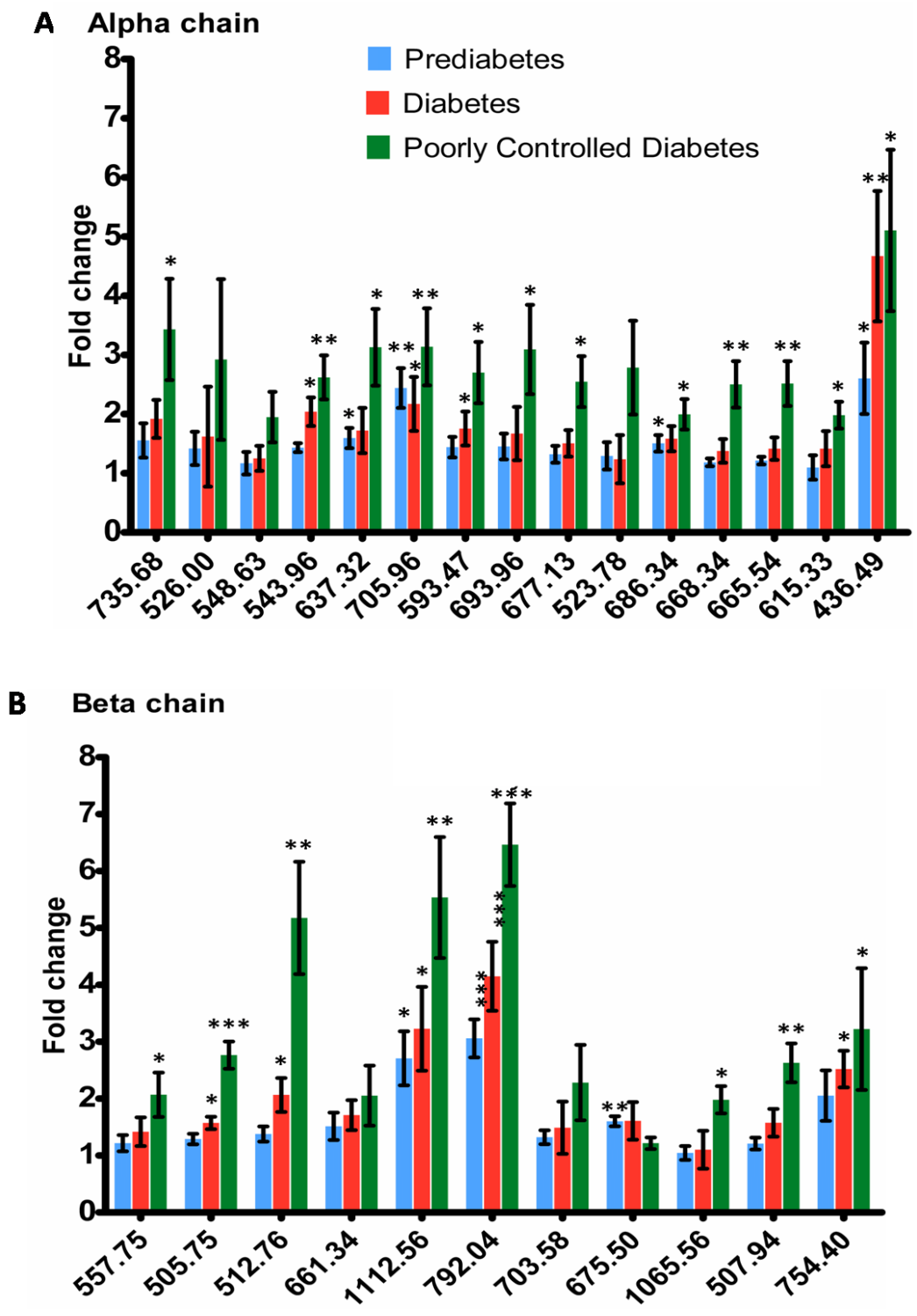


Figure 2.5 Relative fold change in AUCs for glycated peptide ions (m/z in Da).

A) α-chain of hemoglobin and B) β-chain of hemoglobin peptides among diabetes conditions viz. pre-diabetes, diabetes and poorly controlled diabetes with respect to healthy control. Statistical analysis was performed by Two-way ANOVA followed by Tukey's test. PD-Prediabetes, D-Diabetes and PCD-Poorly controlled diabetes. (*$p<0.05$, **$p<0.005$, and ***$p<0.0005$).

2.3.3 Relative quantification of β-chain N-1-valine involving glycated peptides

In the context of diagnostics of diabetes, DFV-N1-β-chain of hemoglobin (HbA1c) is quantified. Therefore, here we have emphatically discussed the quantification of advanced glycation of peptides involving β-N-1-valine. **Figure 2.6** displays the $Log_{(10)}$ values of average TIC, and average AUCs of CMV, CEV and DFV peptides, indicating that there was no major variation in TIC across different samples, although the AUCs of CMV, CEV and DFV increased with severity of diabetes. Subsequently, quantification of CMV, DFV and CEV peptides was performed by PINPOINT software analysis. CMV was found to be the predominant modification of β-N-1-valine followed by DFV and CEV in all the subjects **(Figure 2.7 and Figure 2.8).** However, β-N-1-valine involving carboxyethylation peptide showed higher fold change in AUCs followed by CMV and DFV, in all diabetic conditions compared to healthy controls **Figure 2.9**. Apart from V*(CM)HLTPEEK (m/z-505.75 Da), an addition, glycated peptide with two missed cleavages V*(CM)HLTPEEK*(CM)SAVTALWGK*(CM)VNVDEVGGEALGR (m/z-1112.56 Da) involving CMV modification was found to be significantly elevated in all the diabetic conditions. This analysis suggests that CMV, not DFV is the predominant modification of β-N-1-valine of hemoglobin.

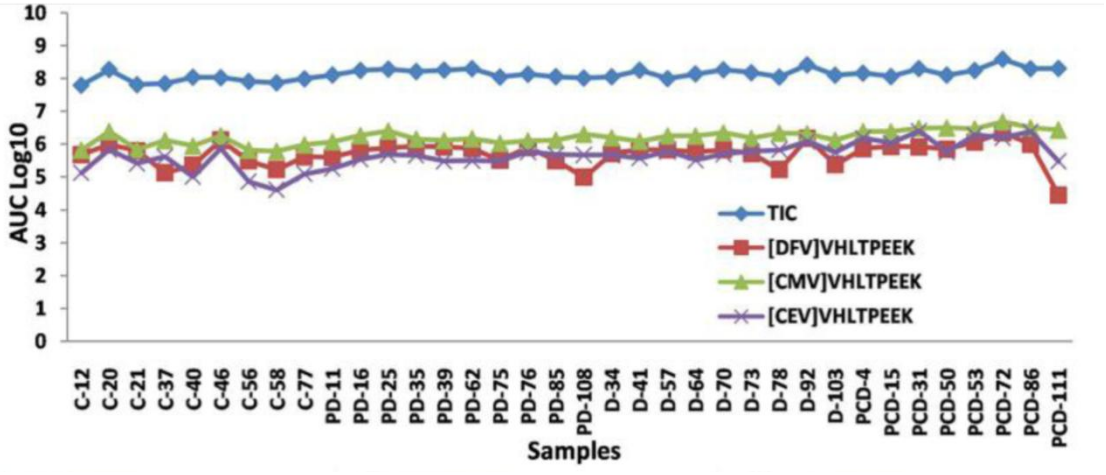


Figure 2.6 Displays the Log (10) values of average of TIC and average of AUCs of CMV, CEV and DFV peptides.

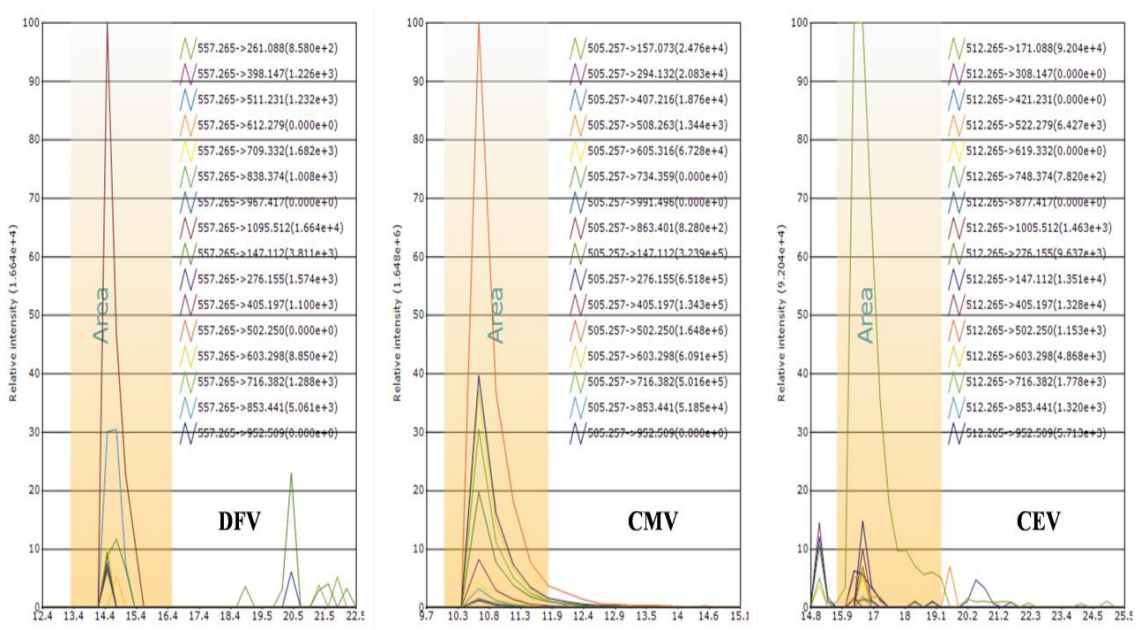


Figure 2.7 Displays representative spectra depicting area under curves (AUCs) for co-eluted fragment ions of the modified β-hemoglobin peptide after analysis using PINPOINT software.

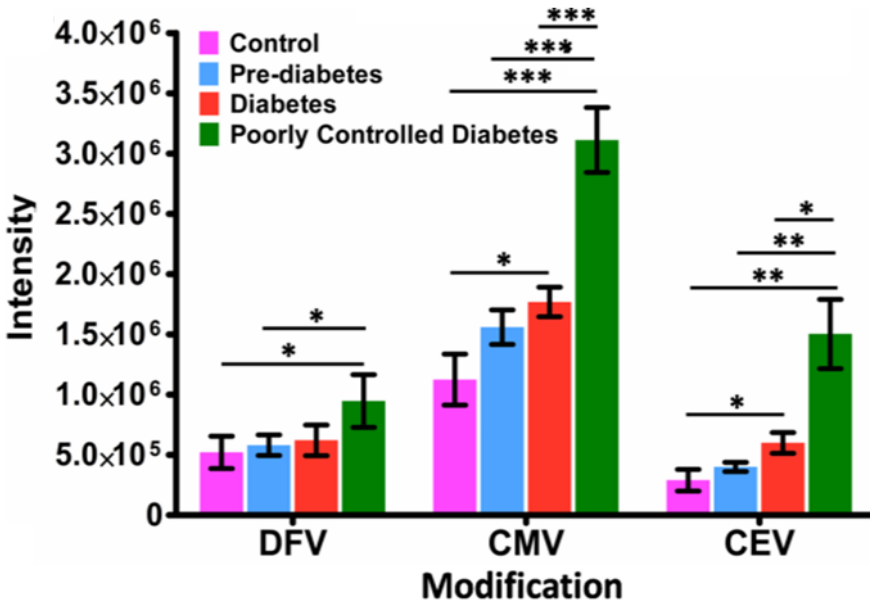


Figure 2.8 Relative quantification of DFV, CMV and CEV peptides of β-hemoglobin by PRM.

Y- axis represents the intensity for each of the peptide. Statistical analysis was performed by One-way ANOVA followed by Tukey's test. Number of Asterisk represents statistical significance at $p<0.0001$ (indicated by ***), at $p<0.001$ (indicated by **). Clinical samples

are represented as C- control, PD-prediabetes, D-diabetes and PCD- poorly controlled diabetes.

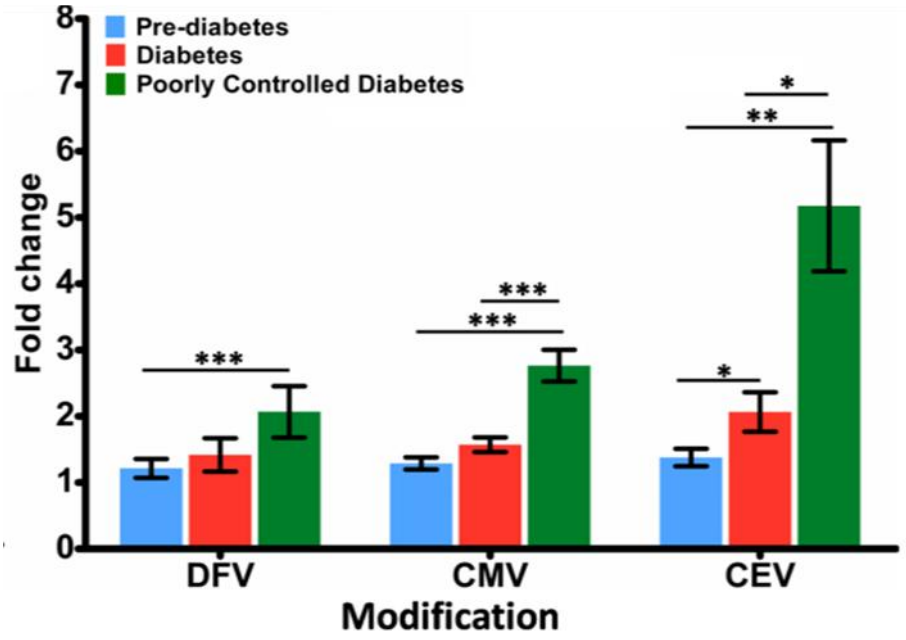


Figure 2.9 Relative fold change in AUC for DFV, CMV and CEV peptides of β-hemoglobin by PRM.

Statistical analysis was performed by Two-way ANOVA followed by Tukey's test. Clinical groups are represented as C-control, PD-Prediabetes, D-Diabetes and PCD-Poorly controlled diabetes. (*p<0.05, **p<0.005, and ***p<0.0005).

Two-way ANOVA followed by Bonferroni post test suggested the level of CMV peptide was significantly higher than DFV or CEV peptides in all the samples irrespective of clinical condition. Further the levels of these peptides across different condition of diabetes were compared by One-way ANOVA-Tukey's Test. The levels of CMV peptide were highest in poorly controlled diabetes followed by diabetes, pre-diabetes and healthy control. There was a significant difference in the levels of CMV peptide between PCD and pre-diabetes or healthy controls **(Figure 2.9).** However, the levels of DFV and CEV peptides did not differ significantly across different clinical conditions. Although in earlier studies, have reported that CMV-hemoglobin was more prevalent in diabetic patients than in healthy subjects, and was proposed to be a valuable marker for the progression of diabetic nephropathy, and oxidative stress(155), this study reports that CMV is the predominant modification of of hemoglobin in all the clinical conditions **(Figure 2.8).** Furthermore, β-N-1-valine

involving carboxyethylation (CEV) of hemoglobin peptide showed higher fold change in AUCs followed by CMV and DFV, in all diabetic conditions compared to healthy controls **(Figure 2.9)** suggesting its usefulness as marker in the early stages of diabetes. Thus, monitoring CMV and CEV, along with DFV could be useful to assess the glycemic status in diabetes.

2.3.4 Correlation of glycated hemoglobin peptides with biochemical parameters

We proceeded further by correlating the levels of the modified peptides with clinical parameters by using Pearson's correlation method **(Table 2.4 and Supplemental data 3)** However, in the context of HbA1c, glycated peptides involving β-N-1-valine are discussed here. All three DFV, CMV and CEV peptides showed significant positive correlation with each other. However, CMV and CEV peptides showed significant correlation with HbA1c (r=0.729*** and r=0.549***). Interestingly, the CMV peptide also showed a relatively better correlation with micro-albuminuria (r=0.305) and a negative correlation with HDL (r=-0.362), although both were statistically not significant. This study suggests that CMV and CEV are better correlated with severity of diabetes.

Table 2.4 Correlations of glycated peptides of hemoglobin with biochemical parameters by Pearson's correlation method.

Sl. No.	Chain, glycation site andtype of modification	precursor m/z (Da)	HbA1c	HDL	MIC
1	**α-K-11-CML**	436.491	0.569***	-0.351	0.126
2	**α-K-90-CML**	665.54	0.546***	- 0.326	- 0.0284
3	**α-K-90-CEL**	668.34	0.554	- 0.338	- 0.0323
4	**β-V-1-DFV**	557.75	0.283*	- 0.317	0.132
5	**β-V-1-CMV**	505.75	0.729***	- 0.362	0.305

6	**β-V-1-CEV**	512.75	0.549***	- 0.332	0.089
7	**β-V-1-CMV,K-8-CML and K-17**	1112.56	0.540***	- 0.383	0.093
8	**β-K-61-CEL**	792.04	0.692***	- 0.375	0.225
9	**β-K-144-CML**	754.4	0.650***	- 0.320	0.229

2.4 Conclusion

Proteins undergo heterogeneous glycation modification during dynamic advanced glycation reactions(39). Generally lysine residues of protein reacts with glucose to form fructosyl lysine, which eventually undergo structural rearrangement involving fragmentation and glyoxidation to form AGEs like carboxymethyl lysine (CML) and carboxyethyl lysine (CEL). The occurrence of CML and CEL is accelerated in hyperglycemic conditions. Especially long lived proteins are known to have elevated levels of CML modifications(52). Therefore, these AGE modifications of proteins may reflect the glycemic status over a long time. Despite this fact, HbA1c i. e. DFV-β-Hb, an early glycated product is used for diagnosis of diabetes. Furthermore, the turnover of hemoglobin is 120 days, it is expected that DFV-β-Hb is the first and reversible modification of glycation, to undergo stable AGE modifications like carboxymethylation and carboxyethylation **Figure 2.10.**

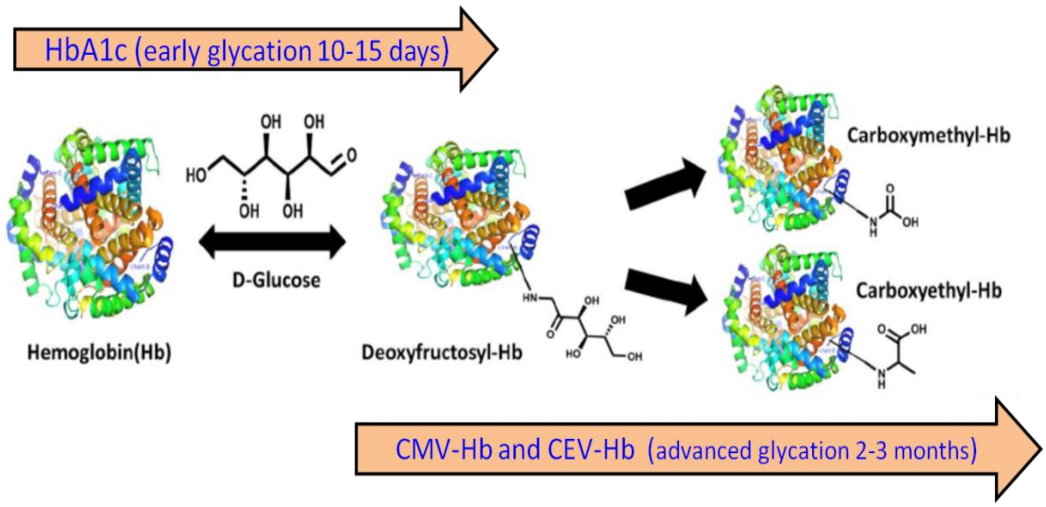


Figure 2.10 Probable mechanism of formation of carboxymethyl and carboxyethyl hemoglobin during dynamic glycation reaction.

Moreover, elevated levels of dicarbonyl such as glyoxal and methylglyoxal can also cause carboxymethylation and carboxyethylation (34, 39, 155). Our observation of CMV, but not DFV as abundant peptide supports the hypothesis that hemoglobin undergoes advanced glycation modifications. This is the first study of this kind that describes detailed characterization and quantification of these peptides. Since CM and CE modification are associated with diabetic complications, quantification of CMV and CEV peptides may hence be more meaningful in the evaluation of glycemic status in diabetes (154, 163). In addition, CMV and CEV show better correlation with severity of diabetes. Therefore, it is important to quantify these modifications along with HbA1c measurement. This discovery of CMV-β-Hb as a predominant modification may change the diagnostic practice of HbA1c measurement and thus management of diabetes.

Chapter 3

Understanding the role of serum albumin and its glyation status on hemoglobin glycation in both *in-vitro* erythrocytes culture and clinical subjects

Contents of this chapter is under minor revision as research article in Molecular & Cellular Proteomics (MCP/2017/000374R1 dated 04.01.2018).

Chapter 3 Understanding the role of serum albumin and its glyation status on hemoglobin glycation in both *in-vitro* erythrocytes culture and clinical subjects

3.1 Background

Poorly managed diabetes leads to the development of associated with diabetic complications such as nephropathy, retinopathy, neuropathy and cardiovascular diseases (3, 11, 35, 41, 46, 47, 60, 61, 63, 64, 66, 163-165). Therefore, well controlled glycemic status is important to prevent diabetic associated complications (10, 11). Currently, glycemic status monitored by measurement of blood glucose levels but it provides instantaneous level, however varies throughout the day depending upon the diet, physical activity and anti-diabetic medicine (9, 88, 89, 109, 166).In view of considering the limitations in the measurement of blood glucose, an International Expert Committee recommended measurement of glycated hemoglobin (HbA1c or A1c), as it is possible to deduce average glucose by measuring the extent of hemoglobin glycation during preceding 90 to 120 days, the during the lifespan of erythrocytes (89, 92, 97-100, 109, 111, 153, 167).Later, The HbA1c measurement was subsequently adopted by the World Health Organization (WHO). Furthermore, the usefulness of HbA1c measurement used for the prediction of diabetic complications was also established by Diabetes Control and Complications Trial (DCCT) in type 1 diabetes (168) and the UK Prospective Diabetes Study (UKPDS) in type 2 diabetes (169). Thus, HbA1c measurement has considered as a gold standard for assessment of glycemic status in clinical practice. However, quite a few number of studies also debate the usefulness of HbA1c measurement, because of technical challenges in precise quantification of N1-deoxyfructosyl valine-β-Hb (DFV-β-Hb) substance (HbA1c) by routinely used laboratory methods, such as ion exchange chromatography (94), and phenyl-boronate affinity chromatography (95) as well as presence of other clinical conditions like anaemia, blood loss, splenomegaly, and iron deficiency affect HbA1c levels (97).

Apart from above mentioned factors, human serum albumin (HSA) has been known to influence HbA1c levels, however it has not been given much attention perhaps due to its abundance among the plasma proteins, although it has been well established that

low albumin levels were negatively associated with HbA1c in a large diabetic cohort study (n=4,158) (170). The similar association was also observed in Asian Indian diabetic subjects (n=610) (171).Furthermore, *in-vitro* study demonstrated that serum albumin competitively protects glycation of other proteins such as insulin and apomyoglobin (172). Additionally, low serum albumin levels were associated with increased plasma protein glycation including albumin, fibrinogen, apolipoprotein, heptaglobin etc., as well as HbA1c in streptozotocin induced diabetic mice model and in clinical subjects (36, 173). Moreover, diabetic patients with low serum albumin levels are at a risk of development of cardiovascular complications (174).And hence, it was proposed that maintaining serum albumin levels in diabetes would be helpful to reduce the accumulation of advanced glycation end products and delay the onset of diabetic complications (175). Despite the great clinical significance of serum albumin in diabetes, it is not a routinely quantified in clinical diagnostics. While, glycation of serum albumin and plasma proteins is quantified as total plasma fructosamine, which is used to predict HbA1c. Discordance has been observed between predicted HbA1c and the observed HbA1c, which is termed as glycation gap, suggesting that HbA1c does not correlate to the fructosamine levels (176). Since serum albumin is the most abundant plasma protein, it is plausible that its glycation status could influence in prediction of HbA1c using plasma fructosamine content. Moreover, plasma albumin may act as a principal target for glycation compare to hemoglobin, because it is free circulated protein in plasma; and in addition, it has large number of lysine and arginine residues, which are glucose binding residues. Whereas, hemoglobin is an intracellular protein and for its glycation requires glucose to be transported into erythrocytes mediated through transporter i.e. Glucose Transporter protein 1 (GLUT1). Therefore, we hypothesize that glycation of serum albumin precedes hemoglobin glycation and hence serum albumin concentration and its glycation status influences hemoglobin glycation or HbA1c levels. To prove the hypothesis, we have mechanistically demonstrated that serum albumin levels and its glycation status indeed influence hemoglobin glycation in *in-vitro* erythrocyte culture. Furthermore, *in-vitro* erythrocyte culture results corroborated in clinical subjects between serum albumin, relative plasma albumin fructosamine and HbA1c.

3.2 Materials and methods

3.2.1 Reagents

Bovine serum albumins (BSA), glyoxylic acid, sodium cyanoborohydride, dialysis membrane, penstrep were procured from Sigma-Aldrich, MO, USA. MS grade solvents such as acetonitrile and water were obtained from J T. Baker, PA, USA. Rapigest-SF was procured from Waters Corporation, MA, USA. Membrane cut-off filters of 3 kDa were procured Millipore, MA, USA. RPMI-1640 media from GIBCO Invitrogen. All other chemicals and reagents of analytical grade were procured from Sigma Aldrich unless otherwise stated.

3.2.2 Study design and clinical details

To understand the role of serum albumin and its glycation status on hemoglobin glycation, we have cultured erythrocytes to evaluate the hemoglobin glycation in presence of serum albumin and carboxymethylated serum albumin. The overview of the current study is depicted in **Figure 3.1.**

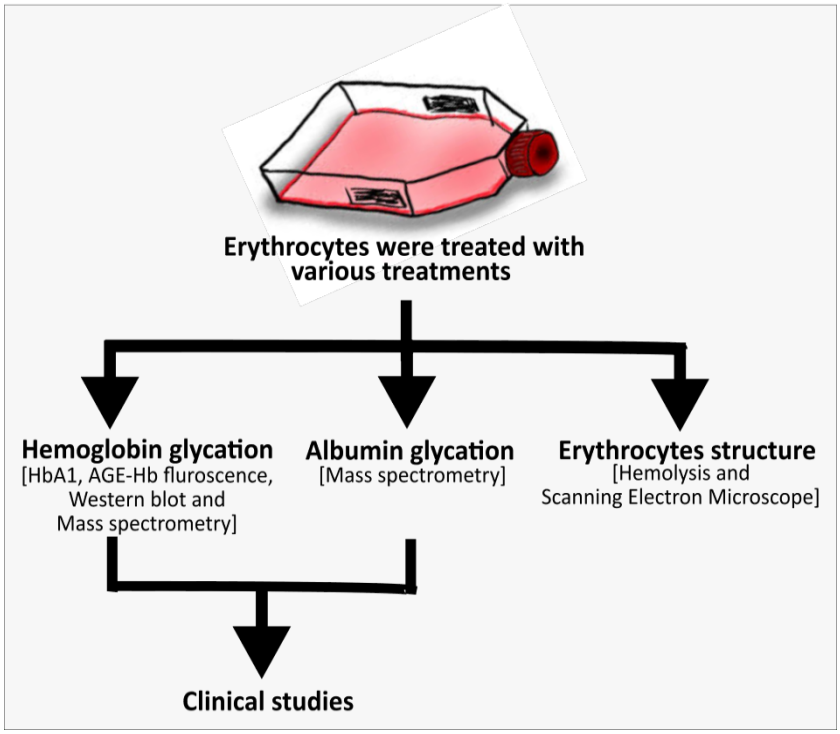


Figure 3.1 Overview of the complete study design.

The study was conducted to understand the role of albumin and its glycation on glycated hemoglobin by using both In-vitro erythrocyte culture and clinical samples.

3.2.3 Synthesis of CML modified serum albumin (CMSA) and acetylated serum albumin (AcSA)

Human serum albumin (HSA) and BSA share a considerable amount of homology in their amino acid sequence and structure (175)and therefore, in all our experiments BSA was used in place of HSA. The CMSA was synthesized as described earlier (148, 177). Briefly, glyoxalic acid (45 mM), BSA (50 mg/ml) and sodium cyanoborohydride (150 mM) were dissolved in 100 mM phosphate buffer (pH 7.4), and incubated for 24 hours in the dark condition at 37^{o} C. While AcSA was synthesized as described previously (178). Briefly, BSA (50 mg/ml) in 100 mM phosphate buffer (pH 7.4) and 10 mM acetyl salicylic acid (ASA) were incubated for 24 hours in dark condition at 37^{o} C. And for unmodified serum albumin, BSA was dissolved in 100 mM phosphate buffer (pH 7.4) and incubated for 24 hours in dark condition at 37^{o} C. After completion of incubation, both modified and unmodified serum albumin was extensively dialysed, followed by ultra-filtration with 3 kDa cut-off membrane filters to remove the free glyoxalic acid/aspirin/salts. The synthesis of CMSA and AcSA were confirmed by liquid chromatography followed by mass spectrometry (LC-MS/MS) analysis. Detailed procedure is described below.

3.2.4 Erythrocytes culture

Erythrocytes (approximately 4 $X10^{9}$ cells/ml) were maintained in RPMI media (RPMI 1640 media, 28mM HEPES buffer, 1X Penstrep) containing either low glucose (5.2 mmol/l) or high glucose (15.7 mmol/l). For different experiments, erythrocytes were treated with various treatments such as aminoguanidine (AMG) 10 mM; unmodified serum albumin (SA) or carboxymethylated serum albumin (CMSA) (15, 20 or 25 mg/ml); Acetylated serum albumin (AcSA) (25 mg/ml), or cytochalasin B with / without CMSA (25 mg/ml), control and diabetic plasma (25 mg/ml plasma protein), and were maintained at 37^{o} C in 5 % CO_2 incubator for 7 days (179-181).

3.2.5 Measurement of hemoglobin glycation

Hemoglobin glycation was monitored by measuring HbA1c, AGE-Hb fluorescence, AGE-Hb by western blotting with anti-CML antibody and mass spectrometry. The detailed procedure is given below.

3.2.5.1 HbA1c analysis

For the measurement of HbA1c, erythrocytes were centrifuged at 1500 × g for 15 min at room temperature to separate the media. Using erythrocyte pellet, HbA1c was measured by Nycocard HbA1c analyser as per the manufacturer's instruction.

3.2.5.2 AGE-Hb fluorescence analysis

Hemoglobin was isolated from 50 µl of erythrocyte pellet by vortexing with a buffer (7 M Urea, 2 M thiourea and 1 DTT in 100 mM ammonium bicarbonate buffer pH 8.3) followed by incubation on ice for 30 min. Thereafter, lysed erythrocytes were centrifuged at 12000 x g for 30 min at 4 ^{o}C. The supernatant containing hemoglobin was estimated for protein concentration by Bradford's method. 1.5 mg of hemoglobin was used for the measurement of AGE-hemoglobin fluorescence with excitation/ emission wavelength at 308/345 nm respectively by using Varioskan flash multimode plate reader (Thermo scientific) (182).

3.2.5.3 CML-Hb analysis by Western blot analysis

SDS-PAGE followed by Western blotting was performed with 5 µg of protein. Hemoglobin was reduced and denatured by dissolving in Laemmli buffer (5 % β-mercaptoethanol and 10 % SDS) and separated on 12% SDS-PAGE. Then the proteins from the gel were transferred onto PVDF membrane. For the confirmation of protein transfer ponceau staining was performed and the stain was removed by washing thrice with 1X PBS. Membrane was blocked by using 5% skimmed milk (HiMedia, India) in PBS overnight at 4^{o} C. Then membrane was probed with anti-carboxymethyl (1:2000) antibody (Abcam, Cambridge, UK) for 2 hours at room temperature. Following washing with PBS-T and PBS, the membrane was incubated with secondary antibody conjugated to HRP(1:2500) antibody (Bangalore Genei,

India) for 1 hour at room temperature. Bands were detected by chemiluminescence using the WesternBright^{TM} Quantum Western blotting detection kit (Biorad) as per the manufacturer's instructions.

3.2.5.4 Sample preparation for mass spectrometric analysis

100 µg of protein either hemoglobin isolated from erythrocytes, or serum albumin treated to erythrocytes/ clinical plasma, or CMSA, AcSA, were diluted to 100 µl with 50 mM ammonium bicarbonate buffer containing 0.1 % Rapigest. Proteins were denatured at 80^{o} C for 15 minutes, subsequently reduced with dithiothritol (100mM) at 60^{o}C for 15 minutes and alkylated with iodoacetamide (200 mM) at room temperature in dark condition. Proteins were digested with 2 µg of proteomic grade trypsin (Sigma-Aldrich) at 37° C for 14-16 hours. Proteolytic digestion was stopped by addition of concentrated HCl. Digested peptides were desalted by using C_{18} Ziptips (Millipore, Billerica, MA) and concentrated in a vacuum concentrator. Peptides were reconstituted in 3 % ACN containing 0.1 % formic acid.

3.2.5.5 Mass spectrometry analysis

3.2.5.5.1 Characterisation of CMSA and AcSA by LC-MS/MS analysis

1.5 µg peptides of CMSA or AcSA were loaded onto reverse-phase column (150 x 2.1 mm, 1.9µm) and separated on an UPLC (Accela 1250, Thermo Fisher Scientific) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) at a flow rate of 350 µL/min for a duration of 45 min, using 5 gradient segments (2–40 % A for 35 min, 40–98 % A for 2 min, held at 98 % A for 2 min, 98–2 % A for 2 min and held at 2 % A for 4 min) . Solvent A was ACN with 0.1 % formic acid and solvent B was aqueous with 0.1% formic acid. The mass spectra of eluted peptides were acquired in a data dependent manner using Hybrid quadruple Q-Exactive Orbitrap mass spectrometer in a positive acquisition mode. The precursor mass range of 350-2000 m/z (resolution of 70000 with AGC target 1e^{6}) and scan time of 120 msec. MS acquisition was performed at precursor's ion selection width of 2 m/z, under fill ratio of 0.3 % and with dynamic exclusion time of 15 sec. The peptides fragmentation was achieved by high energy collision induced dissociation (HCD)

with 28 eV and fragment ions resolution of 17500 (MS/MS at m/z 400) with AGC target $1e^5$ (MS/MS).

Acquired mass spectral data was analyzed by Proteome Discover software (PD 1.4.0.288, Thermo Fisher Scientific). Sequest-HT was used as a search engine for peptide identification against protein database of bovine serum albumin (UniProt IDs: P02769). Peptide and fragment mass tolerance was set at 10 ppm and 0.6 Da respectively. For carboxymethylation [(CML) mass increment of + 58.0055 Da] and acetylation [mass increment of + 42.0105 Da] at lysine residue was given as variable modifications(148, 178). Carboxymethylated and acetylated peptides were manually validated for accurate mass shift in precursor ion due to modification, and presence of fragments ions retaining modification, as described earlier(148).

3.2.5.5.2 Quantification of glycated peptides of hemoglobin and serum albumin by SWATH-MS

Mass spectral acquisition of glycated peptides of hemoglobin or serum albumin was performed in by using micro LC 200 (Eksigent; Dublin, CA) coupled Triple- ToF 5600 (SCIEX) in a high sensitivity mode. For the development of spectral library, mass spectral acquisition was done in an information dependent manner (IDA) and subsequently SWATH-MS was performed for relative quantification of glycated peptides. Peptides (3 µg) directly injected onto a Agilent C_{18}-RP HPLC column (100×0.3mm, 3µm, 120 Å) and then separated using a 95-min gradient of 3 % to 40 % mobile phase (Mobile phase A: 100 % water with 0.1 % (v/v) formic acid, mobile Phase B: 100 % acetonitrile with 0.1 % (v/v) formic acid) at a flow rate of 8 µL/min. Mass spectrometry parameters were set as follows; mass range from 400–1250 Da with an accumulation time 0.25 ms at MS level and 50-1999 Da with an accumulation time 0.01 ms at MS/MS level. Fragmentation of peptides was done using rolling collision energy of 28 eV.

For relative quantification, hemoglobin or serum albumin peptides were individually analyzed by SWATH-MS mode in technical triplicates. Briefly, peptide (1 µg) was directly injected onto a Agilent C_{18}-RP HPLC column (100×0.3mm, 3µm, 120 Å) and then separated as mentioned in IDA data. In SWATH-MS mode, the instrument was specifically optimized, the quadrupole settings for the selection of precursor ion selection windows m/z of 25 Da widewith a 0.5 Da window overlap, a set of 34 overlapping windows was constructed covering the precursor mass range of 400–1250

Da. SWATH MS/MS spectra were collected from 50 to 1999 Da. The rolling collision energy was optimized for each window with a spread of 25 eV. Dwell time was used 70 ms for all fragment-ion scans in high-sensitivity mode, and for each SWATH-MS cycle acquire in high resolution mode for 100 ms resulting in a duty cycle of 3.4.
For the identification of glycated peptides of hemoglobin or albumin peptides list from IDA acquisitions, the spectral data was analyzed by using Protein Pilot software (SCIEX) with respective database by selecting post-translational modification option. Identified glycated peptides were manually verified for accurate precursor mass and presence of modified fragment ions. Post SWATH-MS, the glycated peptides were quantified by extracting by extracting precursor mass with an error of 0.001 Da and by retention time shift of ±1 minute and the presence of fragment ions were manually inspected. Considering all these parameters glycated peptide intensities were obtained and were normalized with average total ion counts for quantification.

3.2.6 Hemolysis assay

Hemolysis was measured spectrometrically by using plate reader Varioskan flash multimode plate reader (Thermo scientific). Erythrocytes were centrifuged (1500 x g, 10 min, at room temperature) and supernatant containing hemoglobin was spectrometrically measured at 540 nm. As a positive control erythrocytes were treated with 1 % SDS to achieve 100 % hemolysis. The percentage of hemolysis was calculated using the formula (%) = (sample O.D/SDS treated O.D) × 100 (181).

3.2.7 Scanning electron microscope (SEM) analysis

Diluted erythrocyte pellet (1:1000) was transferred on clean glass cover slip with 1% (v/v) gluteraldehyde in Dulbecco's phosphate buffered saline (DPBS) with a pH of 7.4 until dry. The erythrocytes were rinsed thrice with DPBS and cells were dehydrated serially with 30 %, 50 %, 70 %, 90 % and 100 % ethanol. The fixed erythrocytes were stored at 4^{o}C until used for SEM analysis. Gold coating was done before electron micrographs were captured by using Carl Ziess Sigma SEM instrument.

3.2.8 Clinical study

The blood samples were collected from individual volunteers at Chellaram Diabetes Institute, Pune with an approval of their Institutional Ethics Committee and written consent, according to standard guidelines of American Diabetes Association (ADA). This study involved 75 clinical subjects. After blood withdrawal, biochemical parameters such as fasting plasma glucose (FPG), Post-prandial glucose, Hemoglobin, HbA1c, serum lipid profile for each subject was measured **(Table 3.1).** Subjects were divided into three groups based on glucose and HbA1c measurements. Groups: 1) 25 controls [mean age 53.37 ± 11.27 years]; 2) 25 pre-diabetes subjects [mean age 56.8 ± 8.78 years]; and 3) 25 controlled diabetes subjects [mean age 58.74 ± 8.83 years]. Remaining blood collected was used for preparation of plasma using Blood Collection Tubes with K_2EDTA (BD Vacutainer™ Plastic, Thermo Fisher Scientific).

Table 3.1: Clinical characteristics of participating subjects.

Continuous variables are expressed as mean ± SD and categorical variables as indicated. Fasting blood glucose; Postprandial blood glucose; HbA1c: Glycated Hemoglobin A; LDL: Low density lipoprotein; HDL: High density lipoprotein; VLDL: Very low-density lipoprotein; ALT: Alanine amino transferase; SGPT: Serum Glutamic Pyruvic Transaminase; AST: Aspartate Aminotransferase; SGOT: Serum Glutamic Oxaloacetic Transaminase; and MIC: Micro-albuminuria.

Sl. No.	Characteristics	Control	Pre-diabetes	Diabetes
1	Age (years)	46.2 ± 17 (n=25)	57.8 ± 13.5 (n=25)	57.4 ± 12.7 (n=25)
2	Fasting blood glucose (mmol/L)	5.1 ± 0.3 (n=25)	5.8 ± 0.6 (n=25)	9.9 ± 2.3 (n=25)
3	Postprandial blood glucose (mmol/L)	6.1 ± 1.2 (n=25)	8.7 ± 3.1 (n=25)	16.2 ± 4.2 (n=25)
4	HbA1c (%)	5.1 ± 0.2 (n=25)	5.8 ± 0.2 (n=25)	8.0 ± 1.3 (n=25)

5	HbA1c (mmol/mol)	32.2 ± 1.0 (n=25)	39.9 ± 1.0 (n=25)	63.9 ± 6.5 (n=25)
6	Hemoglobin (g/dl)	12.8 ± 2.1 (n=25)	12.2 ± 1.6 (n=25)	13.1 ± 1.7 (n=25)
7	Total RBC	4.5 ± 0.4 (n=25)	4.3 ± 0.6 (n=25)	4.8 ± 0.8 (n=25)
8	RBC Distribution	14.1 ± 1.6 (n=25)	14.1 ± 1.4 (n=25)	14.6 ± 2.1 (n=25)
9	Mean Corpuscular HB Conc	33.7 ± 1.0 (n=25)	33.6 ± 1.1 (n=25)	33.4 ± 1.0 (n=25)
10	Serum triglyceride (mmol/L)	72.8 ± 29.4 (n=10)	112.3 ± 40.0 (n=14)	119.0 ± 40.9 (n=15)
11	Serum LDL (mmol/L)	115.6 ± 31.8 (n=10)	120.7 ± 21.6 (n=14)	139.0 ± 35.8 (n=15)
12	Serum HDL (mmol/L)	50.8 ±17.5 (n=10)	43.2 ± 10.4 (n=14)	36.9 ± 8.4 (n=15)
13	Serum Cholesterol (mmol/L)	168.6 ± 39.9 (n=10)	173.4 ± 27.6 (n=14)	180.9 ± 41.4 (n=15)
14	Serum VLDL (mmol/L)	17.1 ± 13.8 (n=10)	18.0 ± 6.4 (n=14)	27.9 ± 7.1 (n=15)
15	Creatinine (µmol/L)	0.8 ± 0.1 (n=10)	0.8 ± 0.1 (n=14)	0.8 ± 0.1 (n=15)
16	Estimated GFR	97 ± 16.3 (n=10)	77.7 ± 17.2 (n=14)	89.5 ± 16.1 (n=15)
17	ALT or SGPT (units/litre)	18.0 ± 3.8 (n=10)	17.6 ± 5.2 (n=14)	16.4 ± 6.0 (n=15)

18	AST or SGOP (units/litre)	18.0 ± 6.5 (n=10)	21.4 ± 6.5 (n=14)	16.6 ± 4.9 (n=15)
19	MIC (mg/dl)	0.8 ± 0.3 (n=10)	0.7 ± 0.2 (n=14)	0.9 ± 0.3 (n=15)

3.2.9 Total plasma protein quantification

Plasma samples were diluted 10 fold with milli-Q water and protein concentration was determined by using Bradford protein assay kit (Bio-Rad Protein Assay: Bradford) (183).

3.2.10 Plasma albumin estimation

Plasma albumin was estimated by using a Bromocresol Green based colorimetric method. Bromo-cresol green interacts with albumin to form a chromophore, which was measured at 520 nm spectrophotometrically (170).

3.2.11 Estimation of plasma fructosamine and calculation of relative albumin fructosamine (RAF)

Plasma proteins fructosamine measured using a colorimetric fructosamine assay (Merck, NJ). This colorimetric assay is based on the ability of ketoamines to reduce nitrotetrazolium blue (NBT) to formazan formation in an alkaline solution. The rate of formazan formation is directly proportional to the concentration of plasma proteins fructosamine (170). The Relative albumin fructosamine (RAF) calculated by deducing from the ratio of albumin to total protein concentration.

3.2.12 Statistical analysis

All the statistical analyses were performed using GraphPad Prism Software (GraphPad Software, Inc., USA). p values were calculated and the level of significance was set at $p<0.05$. The correlation between different variables was estimated using Pearson's correlation coefficient was examined with an ANOVA model.

3.3 Results

3.3.1 Establishment of erythrocytes as an in vitro model for studying glycation

To establish erythrocytes as *in vitro* model for studying glycation, they were maintained in RPMI media containing either normal glucose (5.2 mM) or high glucose (15.7 mM) with or without aminoguanidine (AMG) for 7 days. Hemoglobin glycation was monitored by measuring HbA1c, AGE-Hb fluorescence, and western blotting with anti-CML antibody. HbA1c was about 6.1 % in erythrocytes maintained in normal or relatively low glucose (5.2 mM), whereas it increased to about 10.6% in erythrocytes maintained in high glucose (15.7 mM). Aminoguanidine (AMG), a well know glycation inhibitor was used to study whether its treatment reduces hemoglobin glycation. As expected AMG reduced HbA1c to 6.8 % when compared to erythrocytes maintained in high glucose **(Figure 3.2 (A)).** The non-enzymatic reaction between glucose and protein leads to formation of both fluorescent and non-fluorescent advanced glycation end products (AGEs) (184). Therefore, fluorescent AGEs were quantified by measuring AGE-Hb fluorescence, and amongst non-fluorescent AGEs, carboxymethyl lysine (CML) is the predominant AGE, and hence CML modification of Hb was monitored by anti-CML western blotting (185). Both AGE-Hb fluorescence and CML modification of Hb showed a similar trend as that of HbA1c **(Figure 3.2 (B) and Figure 3.2 (C) respectively).**

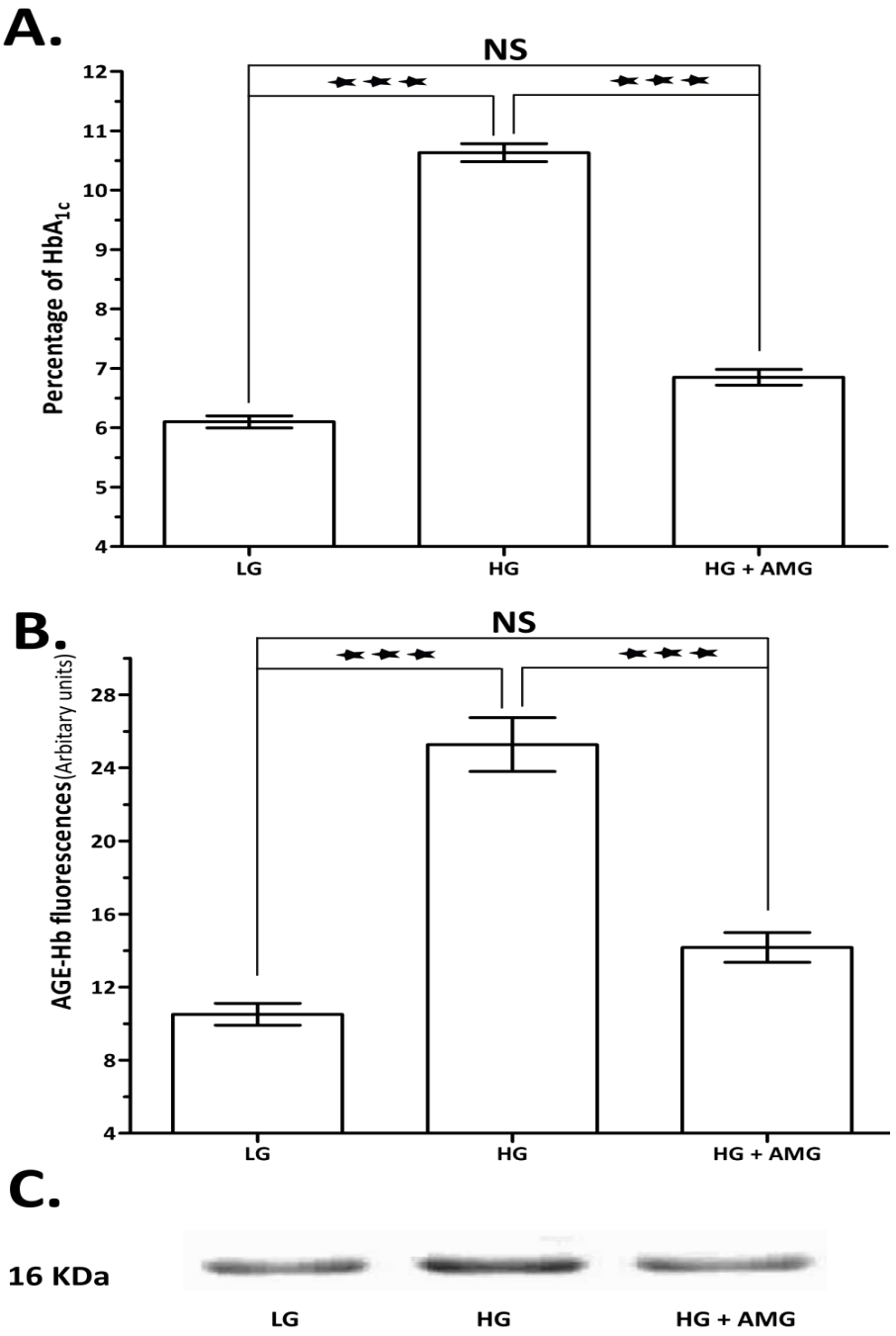


Figure 3.2 Establishment of erythrocytes as an *in-vitro* model for studying glycation

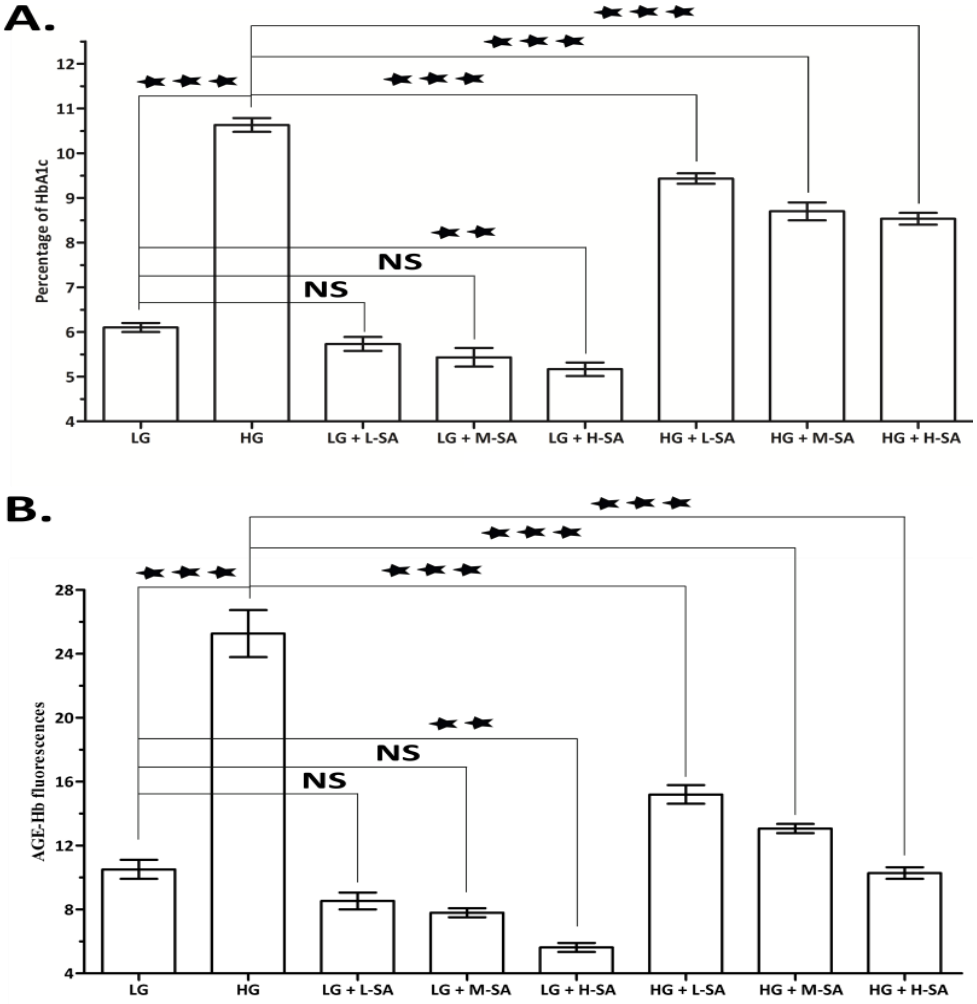
(A) Glycation of hemoglobin was measured by HbA1c analysis. Bar graph depicting HbA1c levels in low glucose, high glucose and high glucose treated with AMG from erythrocytes. HbA1c was increased in erythrocytes maintained in high glucose than low glucose and AMG treatment reduced HbA1c in erythrocytes maintained in high glucose to almost similar to that of erythrocytes maintained in low glucose. The values represent mean and standard deviation. The significance calculated by two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (B) Advanced glycation end product (AGE)

modification of hemoglobin monitored by fluorescence spectrometry by using an excitation and emission wavelengths of 308 nm and 345 nm respectively. Bar graph depicts AGE-Hb fluorescence values in low glucose, high glucose and high glucose with AMG treated. Erythrocytes maintained in high glucose showed increased AGE-Hb fluorescence than that of erythrocytes maintained in high glucose with AMG treatment or low glucose. The values represents mean with standard error. The significance calculated by two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (C) Western blot analysis was performed for hemoglobin isolated from erythrocyte culture by using anti-carboxymethyl (CML) antibody. The intensity of bands indicate that CML modification of hemoglobin was more in erythrocytes maintained in high glucose as compared to erythrocytes maintained in low glucose or erythrocytes maintained in high glucose with AMG treatment.

3.3.2 Unmodified serum albumin protects hemoglobin glycation

Previous studies have shown that low albumin is associated with increased HbA1c (36, 170, 171). In order to find out the mechanistic insight into this association, erythrocytes were maintained at varying levels of serum albumin (15 mg/ ml, 20 mg /ml and 25 mg/ml) in both low and high glucose condition. Glycation of erythrocytes was monitored by HbA1c, AGE-Hb fluorescence, Western blotting with anti-CML antibody, and mass spectrometric quantification of glycated peptides of hemoglobin. Serum albumin reduced HbA1c in a concentration dependent manner. Higher level of serum albumin (25 mg/ ml) reduced HbA1c significantly as compared to lower serum albumin (20 mg/ml of 15 mg/ ml) or no serum albumin in high glucose conditions. However, in low glucose condition only serum albumin concentration of 25 mg/ml reduced the HbA1c significantly **(Figure 3.3 A).** In addition serum albumin (USA) reduced advanced glycation of hemoglobin in a concentration dependent manner as measured by AGE-Hb fluorescence, in both low and high glucose condition **(Figure 3.3 B).** This trend was also observed in western blotting with anti-CML antibody **(Figure 3.3 C).** Furthermore, the effect of albumin on hemoglobin glycation was characterized and quantified by using information dependent acquisition (IDA) and targeted SWATH-MS approach respectively. By using IDA approaches, a total of 16 glycated peptides of hemoglobin were characterized **(Supplemental data 4, Supplemental data 6, Supplemental data 7, and additionally, modified peptide spectra of hemoglobin are listed in Supplemental data 5).** SWATH-MS, a label free quantitative approach allowed targeted extraction and quantification of low

abundant glycated peptides post acquisition. Amongst 16 glycated peptides, only 4 glycated peptides viz. V_{CM}HLTPEEK ($V^{CM}{}_1$--K_8β-chain), V_{DF}HLTPEEK ($V^{DF}{}_1$--K_8β-chain), AGVANALAHK$_{CM}$YH (A_{135}--$K^{CM}{}_{144}YH_{146}$ β-chain), and VVAGVANALAHK$_{CM}$YH (V_{133}--$K^{CM}{}_{144}YH_{146}$ β-chain) were consistently present in all the mass spectrometric acquisitions, and therefore these peptides were selected for targeted quantification **(Figure 3.3 D).** In general V_{CM}HLTPEEK, a carboxymethylated N-terminal Valine containing peptide of β subunit of Hb was found to be most intense glycated peptide, as reported earlier (106, 186).The intensities of these glycated peptides were higher in erythrocytes grown in high glucose condition as compared to that of those grown in low glucose condition. Furthermore, in presence of serum albumin, the intensities of these glycated peptides were reduced in a concentration dependent manner **(Figure 3.3).** All these data strengthens the fact that presence of serum albumin in erythrocyte culture reduces the glycation of hemoglobin.



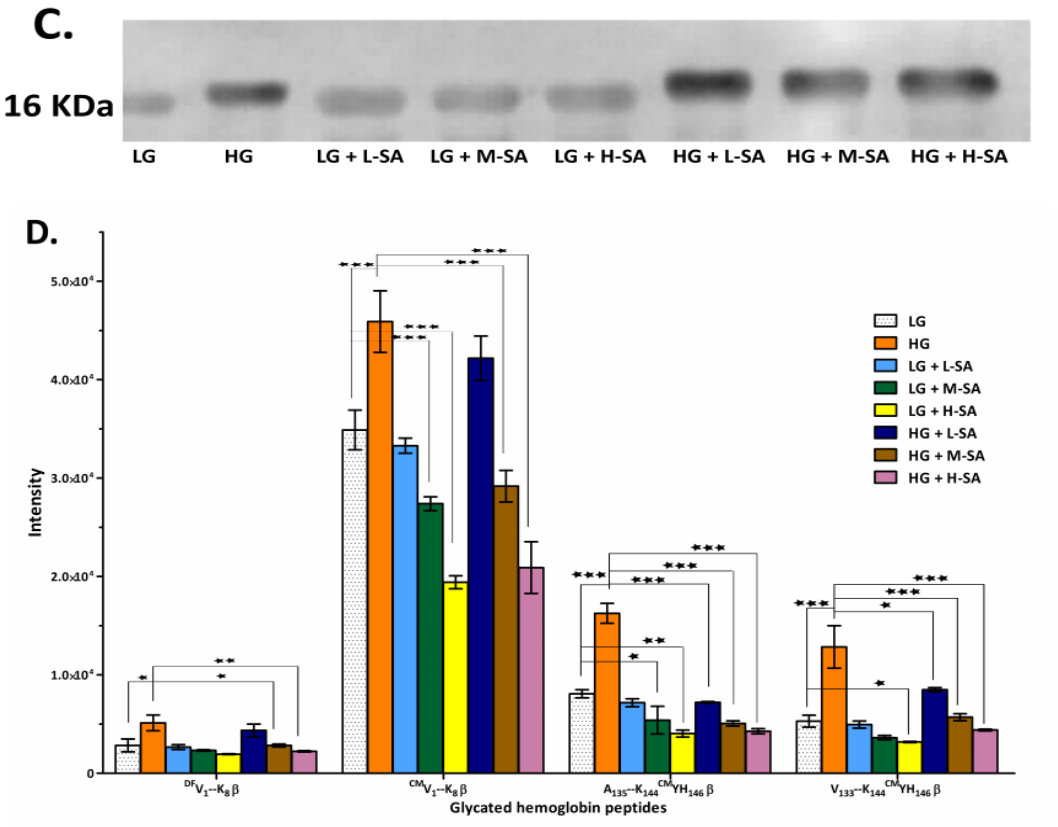


Figure 3.3 Influence of varying levels (15 mg/ ml, 20 mg /ml and 25 mg/ml)of serum albumin on glycation of hemoglobin

(A) Bar graph depicts HbA1c levels measured by using Nycocard HbA1c analyser. Serum albumin reduced HbA1c in a concentration dependent manner. Higher level of serum albumin (25 mg/ ml) reduced HbA1c significantly as compared to lower serum albumin (20 mg/ml of 15 mg/ ml) or no serum albumin in high glucose conditions. The values represents mean with standard deviation. The significance calculated by two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (B) Bar graph shows AGE-Hb fluorescence, which was monitored by using excitation and emission wavelengths 308 nm and 345 nm respectively. The values represents mean with standard error. The significance calculated by two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (C) Western blot analysis was performed for hemoglobin isolated from erythrocyte culture by using anti-carboxymethyl (CML) antibody. CML modification of hemoglobin showed similar trend as that of HbA1c and AGE-Hb fluorescence. (D) Glycated hemoglobin peptide analysis was performed by using targeted SWATH-MS analysis to validate HbA1c analysis, AGE-Hb fluorescence and Western blot analysis. Representative glycated peptides V_{CM}HLTPEEK (V_{CM}1--K8β-chain), V_{DF}HLTPEEK (V_{DF}1--K8β-chain), AGVANALAHK$_{CM}$YH (A135--K$_{CM}$144YH146 β-chain), and VVAGVANALAHK$_{CM}$YH

(V133--K_{CM}144YH146 β-chain) were used for their relative quantification. The peptide intensities were normalized with average total ion count of all individual runs. The intensities of glycated peptides were higher in erythrocytes maintained in high glucose condition as compared to that of those maintained in low glucose condition. In addition upon serum albumin treatment, the intensities of these glycated peptides were reduced in a concentration dependent manner. For example V_{CM}HLTPEEK ($_{CM}$V1-K8 of β-Hb) peptide was found to be the most predominant glycated peptides among all the other glycated peptides of hemoglobin and in addition upon serum albumin treatment reduced the intensity of glycated peptides in a concentration dependent manner. More or less remaining all glycated peptides showed similar trend as that of (CMV1-K8 of β-Hb) peptide. The statistical significance was calculated by using two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.3.3 Low serum albumin concentration in erythrocyte culture was associated with increase in its glycation

Unmodified serum albumin reduced the hemoglobin glycation in a concentration dependent manner in erythrocyte culture suggesting that it may competitively inhibit the glycation, by itself getting glycated, since it has more number of freely accessible lysine and arginine residues. To test this possibility, we analysed glycation of serum albumin by using mass spectrometry. A total of 25 glycated peptides were identified by IDA approach, and 7 of them were consistently present in all the acquisitions (Supplemental data 8, Supplemental data 6, Supplemental data 7, and additionally, modified peptide spectra of serum albumin are listed in supplemental data 9). The lysine residues involved in glycation of these 6 peptides were reported to be glycation sensitive in previous studies(148, 187). These seven peptides include AEFVEVTK$_{DF}$LVTDLTK (A_{249}--K^{DF}_{256}--K_{263}), FK$_{CM}$DLGEEHFK ($F_{35}K^{CM}_{36}$--K_{44}), K$_{CM}$QTALVELLK (K^{CM}_{548}--K_{557}), K$_{DF}$QTALVELLK (K^{DF}_{548}--K_{557}), K$_{CM}$VPQVSTPTLVEVSR (K^{CM}_{437}--R_{451}) and LSQK$_{CM}$FPK (L_{242}--K^{CM}_{245}--K_{248}) were consistently present in all the mass spectrometric acquisitions, and therefore these peptides were selected for targeted quantification. As expected the intensity of glycated peptides was observed to be higher in high glucose than low glucose condition. Furthermore, the intensity of glycated peptides was observed to be more in lower serum albumin concentration and vice versa **(Figure 3.4).** This suggests that (i) at a higher concentration of serum albumin, relatively more numbers of lysine and /or

arginine are accessible for modification, and possibly not all of them get modified thus lead to lower extent of glycation; (ii) whereas at a lower concentration of serum albumin, relatively lesser number of lysine and/ or arginine residues are accessible for modification and possibly majority of them get modified, thus higher extent of glycation. Hence, at a lower serum albumin concentration due to increase in its own glycation, it may lose its ability to protect glycation of other proteins. Conversely, a higher concentration of serum albumin, due to its lesser glycation, may have a better ability to protect glycation of other proteins such as hemoglobin.

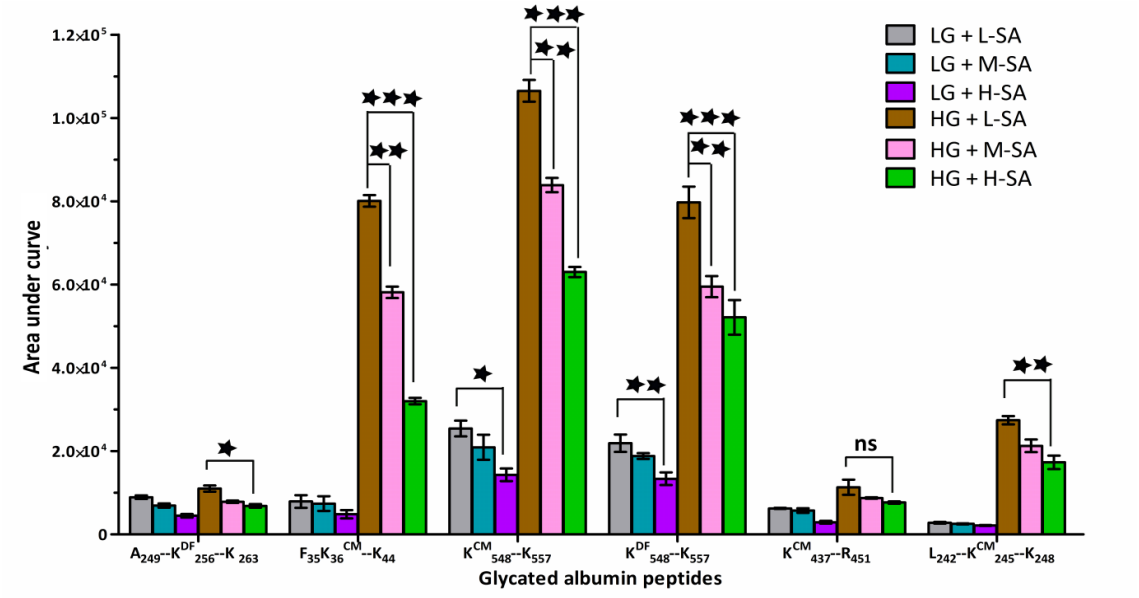


Figure 3.4 Analysis of serum albumin glycation collected from erythrocyte culture

Representative glycated peptides AEFVEVT**K**^{DF}LVTDLTK (A_{249}--**K**$^{DF}_{256}$--K_{263}), F**K**^{CM}DLGEEHFK (F_{35}**K**$^{CM}_{36}$--K_{44}), **K**^{CM}QTALVELLK (**K**$^{CM}_{548}$--K_{557}), **K**^{DF}QTALVELLK (**K**$^{DF}_{548}$--K_{557}), **K**^{CM}VPQVSTPTLVEVSR (**K**$^{CM}_{437}$--R_{451}) and LSQ**K**^{CM}FPK (L_{242}--**K**$^{CM}_{245}$--K_{248}) were used for their relative quantification. The bar graph represents normalized AUC's of glycated peptides of serum albumin with respect to concentration of glucose and serum albumin respectively. The area under curve (AUC) of glycated peptides was observed to be higher in high glucose condition, as well as in lower serum albumin concentration and vice versa respectively. The statistical significance was calculated by using two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.3.4 N(ε)-(carboxymethyl)lysine (CML) modified serum albumin (CMSA) increases HbA1c and distorts erythrocyte structure

To corroborate the role of serum albumin levels and its glycation status, we have modified the serum albumin with glyoxylic acid to obtain CMSA and evaluated its ability to protect glycation of hemoglobin. Formation of CMSA was characterized mass spectrometrically, representative N (ε)-(carboxymethyl) lysine (CML) modified serum albumin peptide annotated spectra **Figure 3.5.** A total of 25 CML modified peptides of serum albumin were identified and characterized **(Table 3.2** and additionally, carboxymethllysine modified peptide spectra of serum albumin are depicted in **Supplemental data 10).**.

Table 3.2 List carboxymethyl modified serum albumin peptides identified by mass spectrometry.

Carboxymethylation sites are bolden and underlined; and Carbamidomethyl are italic.

Sl. No.	**Sequence**	**Modifications**	**MH+ [Da]**	**ΔM [ppm]**	**Charge [z]**
1	T*C*VADESHAG*C* E**K**SLHTLFGDE L*C*K	C2-Carbamidomethyl, C11-Carbamidomethyl, K13-CML, C24-Carbamidomethyl	2922.2837	4.58	3
2	SLHTLFGDEL*C* **K**VASLR	C11-Carbamidomethyl , K12- Carboxymethyl	2004.0197	-1.11	3
3	L**K**HLVDEPQNL IK	K2- Carboxymethyl	1604.9058	+ 1.15	3
4	L**K**HLVDEPQNL I**K**QN*C*DQFEK	K2-Carboxymethyl, K13-Carboxymethyl, C16-Carbamidomethyl	2712.3463	+ 6.04	4
5	LVNELTEFA**K**T *C*VADESHAG*C*E K	K10-Carboxymethyl, C12-Carbamidomethyl, C21-Carbamidomethyl	2666.2122	+1.83	3

6	L*C*VLHE**K**TPVS E**K**VTK	C2-Carbamidomethyl; K7-Carboxymethyl; K13-Carboxymethyl	1984.0414	-0.28	3
7	TPVSE**K**VT**K***CC* TESLVNR	K6-Carboxymethyl, K9-Carboxymethyl, C10-Carbamidomethyl, C11-Carbamidomethyl	2224.0599	+0.66	3
8	VH**K**E*CC*HGDLL E*C*ADDRADLAK	K3-Carboxymethyl, C5-Carbamidomethyl, C6-Carbamidomethyl, C13-Carbamidomethyl	2670.1730	+0.89	3
9	RP*C*FSALTPDET YVP**K**AFDEK	C3-Carbamidomethyl, K16-Carboxymethyl	2529.2062	+3.75	4
10	L*C*VLHE**K**TPVS EK	C2-Carbamidomethyl, K7-Carboxymethyl	1597.8250	-0.3	2
11	TCVADESHAG*C* E**K**SLHTLFGDE L*C***K**VASLR	C2-Carbamidomethyl; C11-Carbamidomethyl; K13-Carboxymethyl; C24-Carbamidomethyl; K25-Carboxymethyl	3506.6058	+2.06	4
12	LA**K**EYEATLEE *CC*A**K**DDPHA*C* YSTVFDK	K3-Carboxymethyl, C12-Carbamidomethyl, C13-Carbamidomethyl, K15-Carboxymethyl, C21-Carbamidomethyl	3466.4740	-0.6	4
13	**K**VPQVSTPTLV EVSR	K1-Carboxymethyl	1697.94925	+3.51	3
14	YTR**K**VPQVSTP TLVEVSR	K4-Carboxymethyl	2118.1519	- 1.64	3
15	**K**FWG**K**YLYEIA R	K1-Carboxymethyl, K5-Carboxymethyl	1689.8695	+3.53	3
16	QI**KK**QTALVEL LK	K3-Carboxymethyl, K4-Carboxymethyl	1627.9696	+ 4.1	3
17	AEFVEVT**K**LVT DLTK	K8-Carboxymethyl	1750.9540	+3.82	3
18	HLVDEPQNLI**K** QN*C*DQFEK	K11-Carboxymethyl,C14-Carbamidomethyl	2413.1476	+0.97	3

19	*C*ASIQ**K**FGER	C1-Carbamidomethyl, K6-Carboxymethyl	1253.5938	-0.44	2
20	H**K**P**K**ATEEQL**K**TVMENFVAFVDK	K2-Carboxymethyl. K4-Carboxymethyl, K11-Carboxymethyl	2863.4259	+2.64	4
21	NYQEA**K**DAFLGSFLYEYSR	K6-Carboxymethyl	2359.0922	+1.87	3
22	LA**K**EYEATLEE*CC*AK	K3-Carboxymethyl, C12-Carbamidomethyl, C13-Carbamidomethyl	1872.8364	+0.5	3
23	GA*C*LLP**K**IETMR	C3-Carbamidomethyl, K7-Carboxymethyl	1446.7476	+2.21	2
24	**K**QTALVELLK	K1-Carboxymethyl	1200.7212	+1.13	3
25	KFWG**K**YLYEIAR	K5-Carboxymethyl	1631.8604	+1.44	3

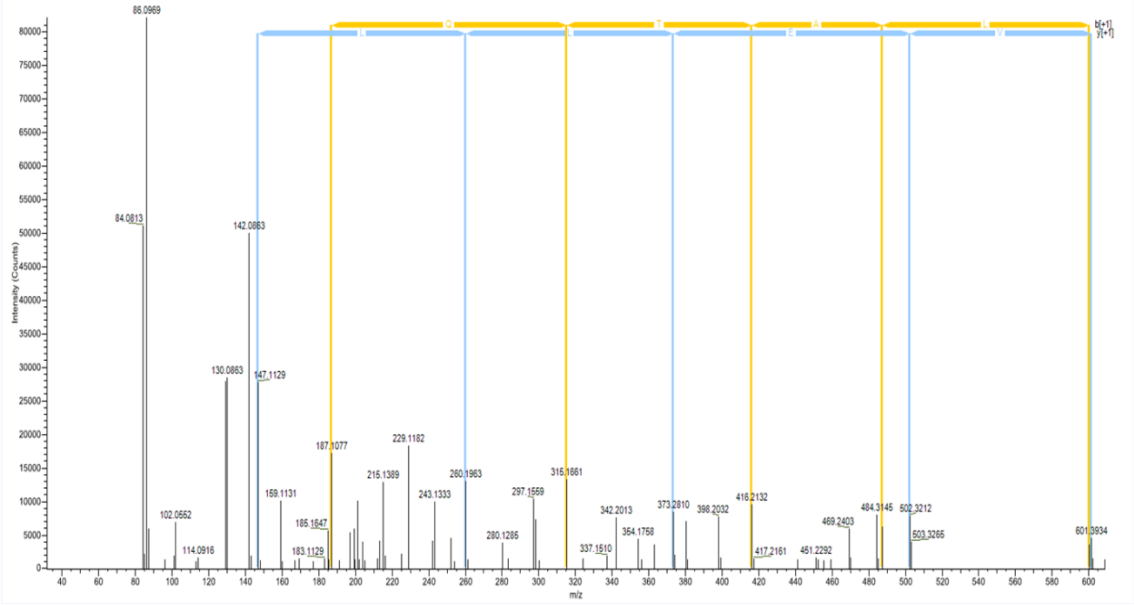
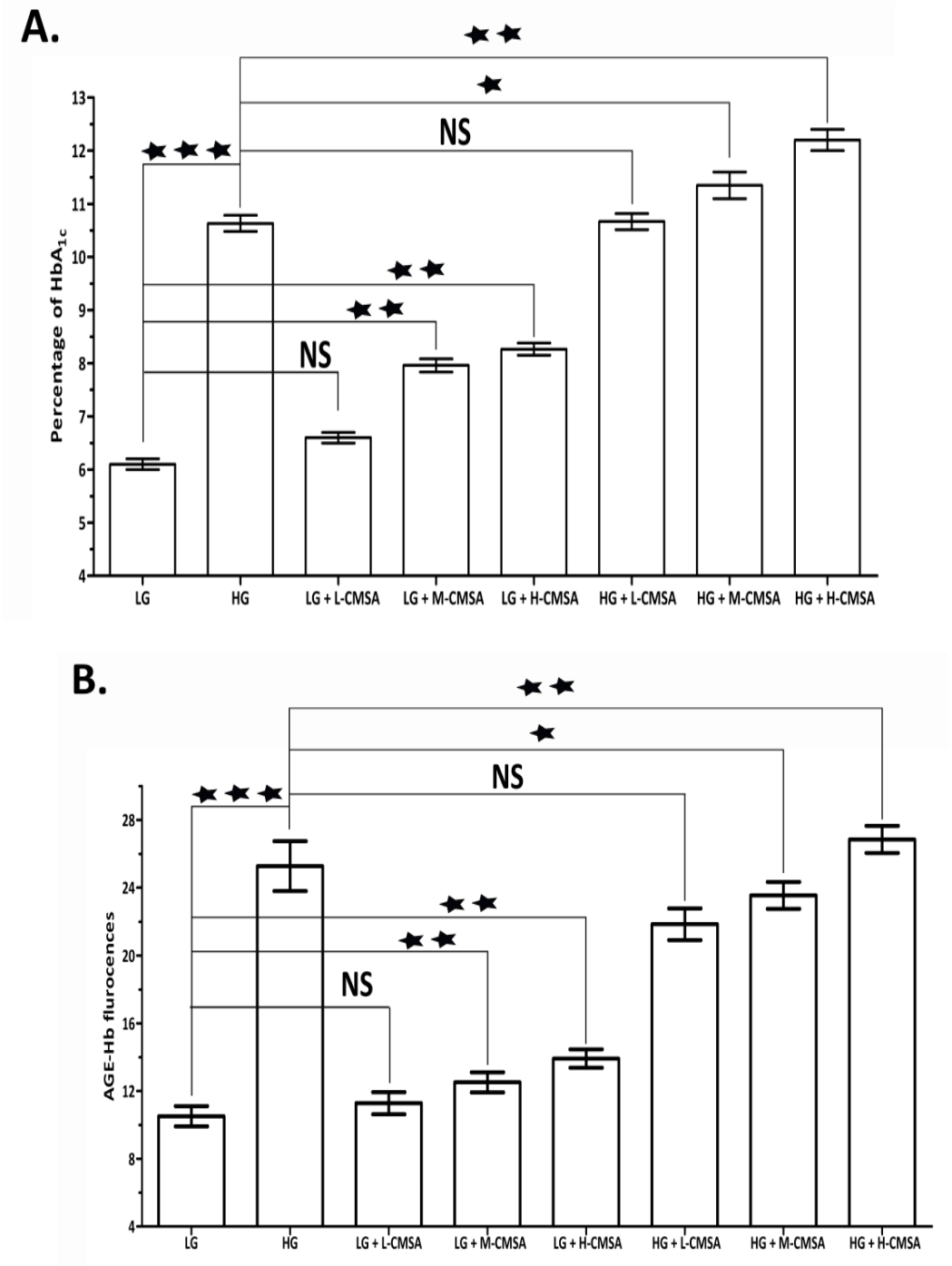


Figure 3.5 A representative N (ε)-(carboxymethyl) lysine (CML) modified serum albumin peptide annotated spectra

Carboxymethyl modified peptide: **K**QTALVELLK, m/z: 400.9119 Da; MH+: 1200.7212 Da; XCorr: 2.55

Erythrocytes maintained in presence of various concentration of CMSA in low and high glucose concentration were evaluated for the hemoglobin glycation by various

methods. Unlike serum albumin, CMSA failed to protect hemoglobin glycation as measured by HbA1c, AGE-Hb fluorescence, Western blotting with anti-CML antibody, and mass spectrometry based quantification of glycated peptides of hemoglobin at both low and high glucose concentrations **(Figure 3.6 A, B, C, and D)** (Supplemental data 6, Supplemental data 7**).**



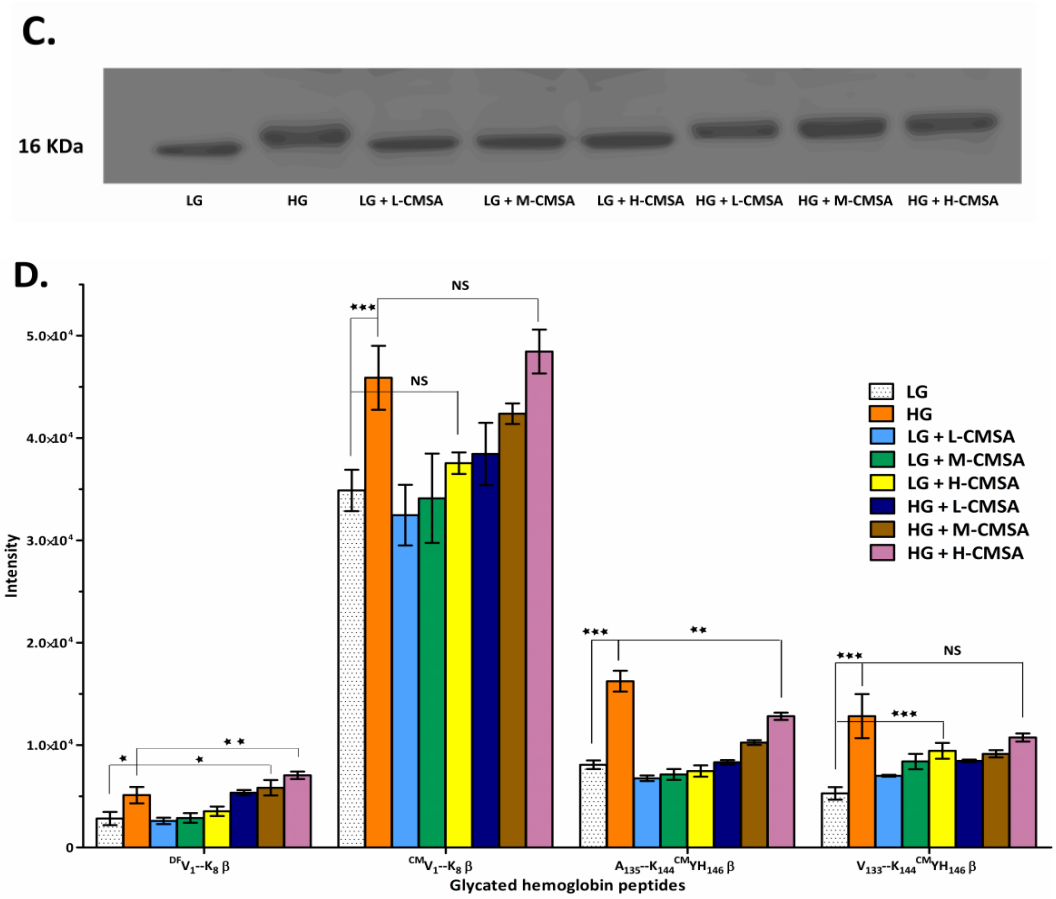


Figure 3.6 Effect of varying levels (15 mg/ ml, 20 mg /ml and 25 mg/ml)of N(ε)-(carboxymethyl)lysine (CML) modified serum album (CMSA) on hemoglobin glycation.

(A) Bar graph represents HbA1c levels measured by using Nycocard HbA1c analyser. CMSA increases HbA1c levels in a concentration dependent manner. Higher level of CMSA (25 mg/ ml) increases HbA1c significantly as compared to lower serum albumin (20 mg/ml of 15 mg/ ml) or no CMSA in high glucose conditions. The values represents mean with standard deviation. The significance calculated by two way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (B) Bar graph represents AGE-Hb fluorescence, which was monitored by using excitation and emission wavelengths 308 nm and 345 nm respectively. The results were found to be similar to HbA1c data. The values represents mean with standard error. The significance calculated by two way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (C) Western blotting analysis of hemoglobin extracted from erythrocyte culture was performed by using anti-carboxymethyl antibody. The band intensity of the glycated hemoglobin was higher in high glucose with 25 mg/ml CMSA treatment compare to low glucose with 25 mg/ml CMSA treatment. (D) Glycated hemoglobin peptide analysis was performed by using targeted SWATH-MS analysis to validate HbA1c analysis, AGE-Hb fluorescence and Western blot

analysis. Representative glycated peptides V_{CM}HLTPEEK (V_{CM}^{1}--K^{8}β-chain), V_{DF}HLTPEEK (V_{DF}^{1}--K^{8}β-chain), AGVANALAHK$_{CM}$YH (A^{135}--K_{CM}^{144}YH146 β-chain), and VVAGVANALAHK$_{CM}$YH (V^{133}--$K_{CM}^{144}YH^{146}$ β-chain) were used for their relative quantification. Glycated hemoglobin peptide intensities were normalized with average total ion count of all individual runs. The intensities of glycated peptides were higher in erythrocytes maintained in high glucose condition as compared to that of those maintained in low glucose condition. In addition upon CMSA treatment, the intensities of these glycated peptides were increased in a concentration dependent manner. The statistical significance was calculated by using two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

Although a more or less similar trend was observed in AGE-Hb fluorescence, western blotting and mass spectrometry **(Figure 3.6),** a strikingly higher HbA1c was observed with CMSA treatment, as compared to only low or high glucose treatment **(Figure 3.6 A).** This observation was quite intriguing, as it was difficult to explain the increase in HbA1c by CMSA treatment.

HbA1c can increase due to increase in intracellular glucose concentration, which is possible due to increase in glucose transport through GLUT1 or due to alteration in the membrane permeability of the erythrocytes. Therefore, we further investigated the CMSA induced increase in HbA1c by inhibiting glucose transport through GLUT1 using cytochalasin B. Cytochalasin B treatment to erythrocytes maintained in high glucose media reduced HbA1c similar to that of erythrocytes maintained in low glucose media. However, combination of cytochalasin B and CMSA treatment to erythrocytes maintained in high glucose media increased the HbA1c as compared to only cytochalasin B treatment, albeit it was lower as compared to erythrocytes maintained only in high glucose media. Nevertheless CMSA increased HbA1c even in presence of a glucose transport inhibitor suggesting that CMSA may alter the membrane permeability of erythrocytes **(Figure 3.7).**

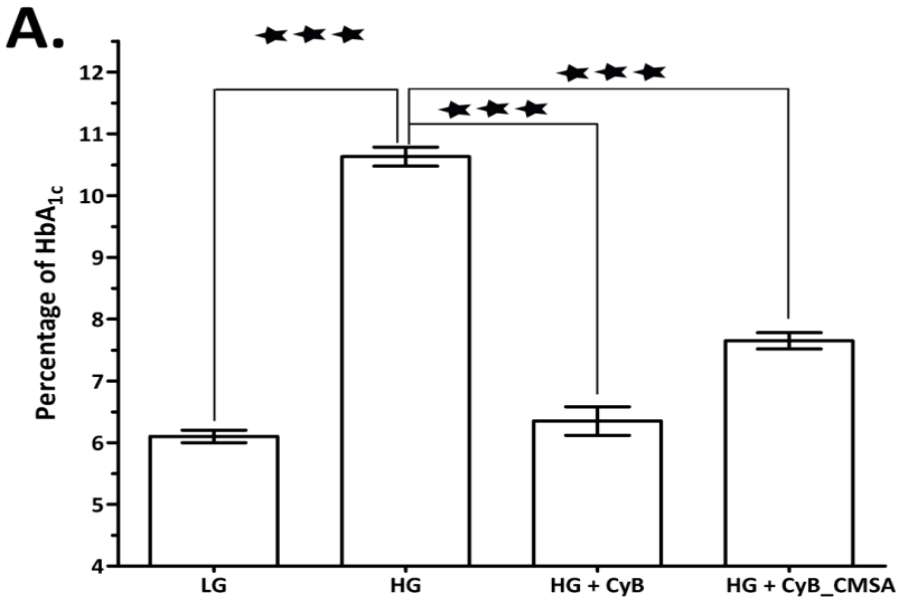


Figure 3.7 Effect of cytochalasin B, a glucose transport 1 (GLUT1) inhibitor on hemoglobin glycation.

Bar graph represents HbA1c level. Erythrocytes maintained in high glucose with cytochalasin B showed a significant reduction in HbA1c compared to that of those maintained in high glucose. While erythrocytes treated with a combination of cytochalasin B and CMSA treatment showed significantly higher HbA1c as compared to only cytochalasin B treatment. The values represents mean with standard deviation. The significance calculated by two way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

Next we monitored the effect of CMSA on membrane permeability as reflected by hemolysis and studied the erythrocyte structure by using scanning electron microscope (SEM). Erythrocytes maintained in high glucose media were treated with either serum albumin or CMSA, and evaluated for hemolysis and changes in membrane structure. Erythrocytes maintained in high glucose condition showed increased hemolysis as compared to those maintained in low glucose. Treatment of unmodified serum albumin to erythrocytes maintained in high glucose media reduced the hemolysis as compared to those maintained on only high glucose media **(Figure 3.8 A).** Whereas CMSA treatment aggravated the hemolysis of erythrocytes maintained in high glucose media **(Figure 3.8 B).** Furthermore, we studied the erythrocyte structure by using SEM. Erythrocytes maintained in low glucose media were appeared to be round/discoidal structure and while those maintained in high glucose media displayed distorted structure with spikes on their surface, as well as

they were cross-linked. CMSA treatment to erythrocytes maintained in high glucose aggravated the distortion of erythrocyte structure, while unmodified serum albumin treatment to erythrocytes maintained in high glucose restored the structure to an extent similar to those grown in low glucose **(Figure 3.8 B).**

These evidences suggest that the CMSA induced increase in HbA1c may be through altered membrane permeability due to increased hemolysis, and by distorting the erythrocytes structure. A previous study has also demonstrated that hyperglycemia and AGE-albumin causes distortion of hemoglobin structure (188).

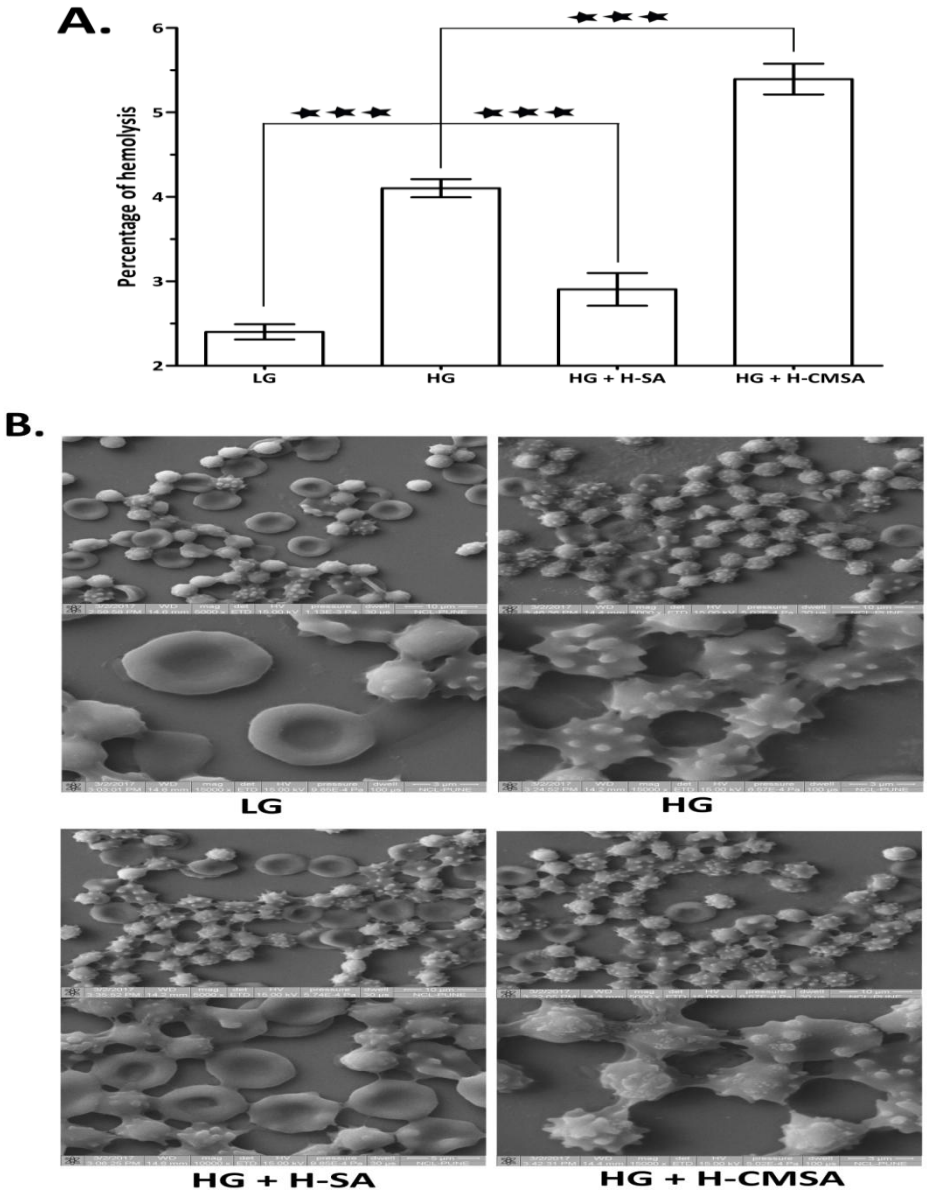


Figure 3.8 CMSA affected erythrocytes structure and membrane permeability.

(A) The bar graph depicts the percent hemolysis. Erythrocytes maintained in high glucose media with CMSA showed significant increase in hemolysis compared to those maintained only with high glucose media. Whereas SA treatment reduced the hemolysis. The percent hemolysis significance calculated by two way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). (B) The erythrocyte morphological structure studied by using SEM. Scanning electron micrographs of erythrocytes maintained in low glucose media were appeared to be round / discoid structure and those that were maintained in high glucose displayed distorted structure with spikes on their surface, as well as they were cross-linked. Erythrocytes in presence of high glucose with CMSA aggravated the distortion of erythrocyte structure, while unmodified serum albumin treatment to erythrocytes maintained in high glucose restored the structure similar to those grown in low glucose.

3.3.5 Modification of lysine residues of albumin impairs it ability to protect hemoglobin glycation

The above results suggest that modification of lysine residues of albumin may impair its ability to protect hemoglobin glycation, as treatment of CMSA led to increase in HbA1 and affected the erythrocyte structure. In order to examine further that lysine modification affects its ability to protect hemoglobin glycation, lysine residues of serum albumin were acetylated with acetyl salicylic acid (ASA) (aspirin). Acetylation of lysine residue was confirmed mass spectrometrically. A total of 24 acetylated peptides with 24 lysine were involved in modification. **(Figure 3.9 Table 3.3** and additionally, acetyl modified peptide spectra's of serum albumin are listed in **Supplemental data 11).** Like CMSA, acetylated serum albumin (AcSA) failed to protect hemoglobin glycation of erythrocytes maintained at high glucose concentration, as measured by HbA1c, AGE-Hb fluorescence, hemolysis, and western blotting with anti-CML antibody **(Figure 3.10 A, B, C and D).** These results unequivocally suggest that serum albumin needs to maintain lysine residues in unmodified state to protect the glycation of other proteins such as hemoglobin.

Table 3.3 List acetyl modified serum albumin peptides identified by mass spectrometry

Acetylation sites are bolden and underlined; and Carbamidomethyl are italic.

Sl. No.	Sequence	Modifications	MH+ (Da)	ΔM (ppm)	Charge (z)
1	L**K**HLVDEPQNLIK	K2-Acetyl	1588.906	- 0.16	2
2	ATEEQL**K**TVMENFVAFVDK	K7-Acetyl	2241.125	- 6.23	3
3	L**K**PDPNTL*C*DEFKADEK	K2-Acetyl, C9-Carbamidomethyl	2061.982	+ 1.20	3
4	LA**K**EYEATLEE*CC*AK	K3-Acetyl,C12-Carbamidomethyl, C13-Carbamidomethyl	1856.842	- 0.75	3
5	H**K**PKATEEQLK	K2-Acetyl	1350.737	- 0.43	3
6	GA*C*LLP**K**IETMR	C3-Carbamidomethyl, K7-Acetyl	1430.749	- 0.30	2
7	LFTFHADI*C*TLPDTE**K**QIK	C9-Carbamidomethyl, K16-Acetyl	2319.170	+ 0.21	3
8	VT**K***CC*TESLVNR	K3-Acetyl,C4-Carbamidomethyl, C5-Carbamidomethyl	1508.721	- 0.73	2
9	L**K**PDPNTL*C*DEFK	K2-Acetyl, C9-Carbamidomethyl	1618.781	- 1.60	2

10	*C*ASIQ**K**FGER	C1-Carbamidomethyl, K6-Acetyl	1237.600	+ 0.66	2
11	**K**QTALVELLK	K1-Acetyl	1184.726	- 1.29	2
12	DTH**K**SEIAHR	K4-Acetyl	1235.612	- 0.34	3
13	L*C*VLHE**K**TPVSEK	C2-Carbamidomethyl, K7-Acetyl	1581.829	- 0.94	3
14	LKE*CC*D**K**PLLEK	C4-Carbamidomethyl, C5-Carbamidomethyl, K7-Acetyl	1574.792	- 0.18	2
15	*CC*T**K**PESER	C1-Carbamidomethyl, C2-Carbamidomethyl, K4-Acetyl	1208.503	- 0.11	2
16	FP**K**AEFVEVTK	K3-Acetyl	1336.716	- 0.89	2
17	SLG**K**VGTR	K4-Acetyl	859.500	- 0.54	2
18	F**K**DLGEEHFK	K2-Acetyl	1291.632	+ 0.30	3
19	AL**K**AWSVAR	K3-Acetyl	1043.601	- 1.36	2
20	KFWG**K**YLYEIAR	K5-Acetyl	1615.865	+ 1.40	3
21	LVTDLT**K**VHK	K7-Acetyl	1195.705	+ 0.49	3

22	**K**VPQVSTPTLVEVSR	K1-Acetyl	1681.951	- 1.42	3
23	TPVSE**K**VTK	K6-Acetyl	1030.578	-0.01	2
24	E*CC*D**K**PLLEK	C2-Carbamidomethyl, C3-Carbamidomethyl, K5-Acetyl	1333.611	-0.73	2

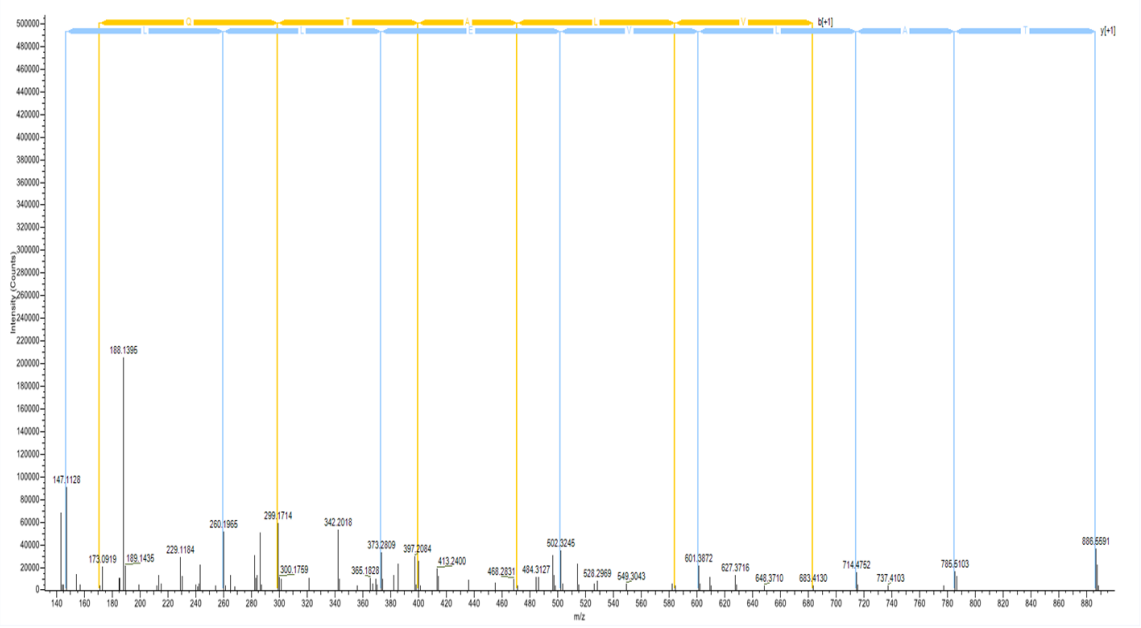


Figure 3.9 A representative N (ε)-(acetyl) lysine modified serum albumin (AcSA) peptide annotated spectra

Acetylated peptide: **K**QTALVELLK; m/z: 592.8664 Da; MH+: 1184.7256 Da; Xcorr: 3.03.

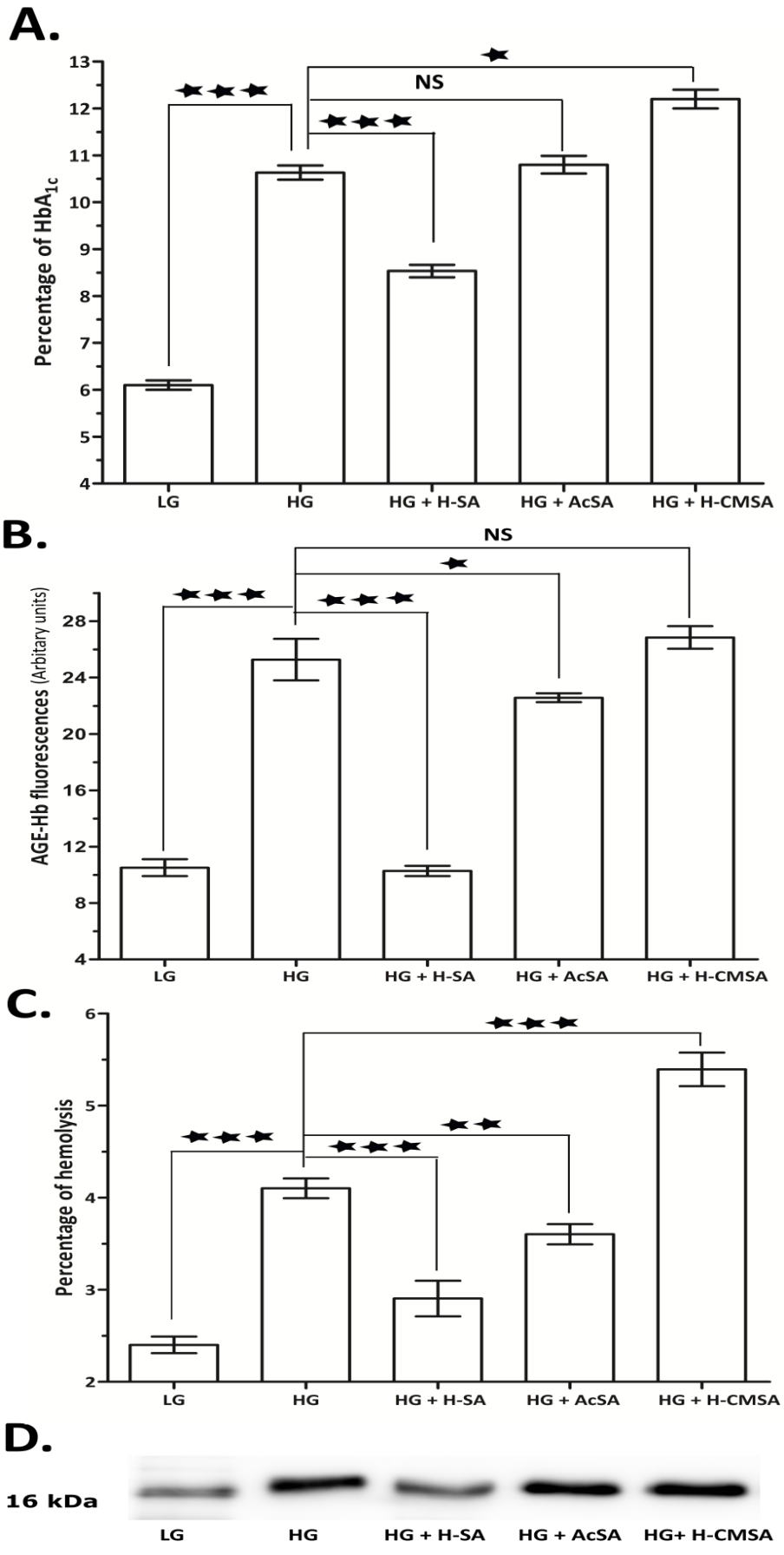

A.
Percentage of HbA1c
NS
LG
HG
HG + H-SA
HG + AcSA
HG + H-CMSA
B.
AGE-Hb fluorescences (Arbitary units)
NS
LG
HG
HG + H-SA
HG + AcSA
HG + H-CMSA
C.
Percentage of hemolysis
LG
HG
HG + H-SA
HG + AcSA
HG + H-CMSA
D.
16 kDa
LG
HG
HG + H-SA
HG + AcSA
HG+ H-CMSA

Figure 3.10 Modification of lysine residues of serum albumin impairs it ability to protect hemoglobin glycation.

Erythrocytes were maintained in high **glucose (HG)**media either with/without AcSA (25mg/ml represented as **H-AcSA**) or CMSA (25mg/ml represented **as H-CMSA**). **(A)** Bar graph represents HbA1c level. Unlike unmodified serum albumin, treatment of AcSA failed to reduce HbA1c in the erythrocytes maintained in high glucose. **(B, C& D)** In AGE-Hb fluorescence, percent of hemolysis and Western blot analysis was performed for hemoglobin isolated from erythrocyte culture by using anti-carboxymethyl (CML) antibody showed similar trend as that of HbA1c data. The values of HbA1c, AGE-Hb fluorescence and percent of hemolysis represent mean with standard deviation. The significance calculated by two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.3.6 Diabetic plasma has reduced ability to protect hemoglobin glycation

Further, we have examined the effect of clinical plasma from healthy and diabetic subjects on hemoglobin glycation in erythrocyte culture. Human serum albumin (HSA) is the most abundant plasma protein and preferentially glycated in diabetes owing to its abundance, as well as due to large number lysine and arginine residues accessible for glycation(175).Therefore, the effect of clinical plasma on hemoglobin glycation can be attributed to mainly albumin and its glycation, although the effect of glucose, other metabolites and protein cannot be ruled out. Individual plasma obtained from healthy control and diabetic subjects was analysed for fasting and post prandial blood glucose, HbA1c, plasma fructosamine, and albumin levels **(Table 3.4).** Three individual plasma samples from healthy control and diabetic subjects were pooled based on fasting and post-prandial blood glucose and HbA1c. The mean serum albumin levels were relatively more in healthy control plasma (48.5 $\pm$ 3.6 g/L) than the diabetic plasma (30.5 $\pm$ 4.7 g/L). While the mean plasma fructosamine in healthy control was (217.56 $\pm$ 14.7 µmol /L) where as in diabetes, it was 410.4 $\pm$ 52.6 µmol /L. Further glycation status of albumin was characterized and quantified by IDA and SWATH-MS. Previously reported glycation sensitive peptides viz. AEFAEVSK$_{DF}$LVTDLTK (A_{250}--K^{DF}_{257}--K^{264}), ATKEQLK$_{DF}$AVMDDFAAFVEK (A_{563}--K^{DF}_{569}--K_{581}), EQLK$_{DF}$AVMDDFAAFVEK (E_{567}--K^{DF}_{569}--K_{581}), FK$_{DF}$DLGEENFK ($F_{35}K^{DF}_{36}$--K_{44}), K$_{DF}$LVAASQAALGL (K^{DF}_{598}--L_{609}), K$_{CM}$QTALVELVK (K^{CM}_{549}--K_{558}), K$_{DF}$QTALVELVK (K^{DF}_{549}--K_{558}), K$_{DF}$VPQVSTPTLVEVSR (K^{DF}_{438}--R_{452}) and K$_{CM}$VPQVSTPTLVEVSR (K^{CM}_{438}--R_{452}) were used for quantification **(Figure 3.11) (Supplemental data 6,**

Supplemental data 7, Supplemental data12 and additionally, modified peptide spectra's of human serum albumin are listed in **Supplemental data 13)** (148, 187). The intensities of all the studied glycated peptides were higher in diabetic plasma than healthy control plasma.

Table 3.4 Characterisation of glycation status of healthy control and diabetic plasma used for erythrocytes experiment

Sl. No.	Characteristics	Control patients (n=3)	Diabetic patients (n=3)
1	Fasting glucose (mmol/L)	4.8 ± 0.2	10.8 ± 2.2
2	Postprandial glucose (mmol/L)	4.9 ± 0.1	22.3 ± 2.0
3	$HbA1_C$ (%)	5.0 ± 0.1	9.6 ± 0.1
4	HbA1c (mmol/mol)	31 ± 1.0	81 ± 0.9
5	Plasma fructosamine (μmol/L)	217.56 ± 14.7	410.4 ± 52.6
6	Plasma protein concentration (g/L)	81.3 ± 2.5	79.0 ± 1.7
7	Plasma albumin concentration (g/L)	48.5 ± 3.6	30.5 ± 4.7

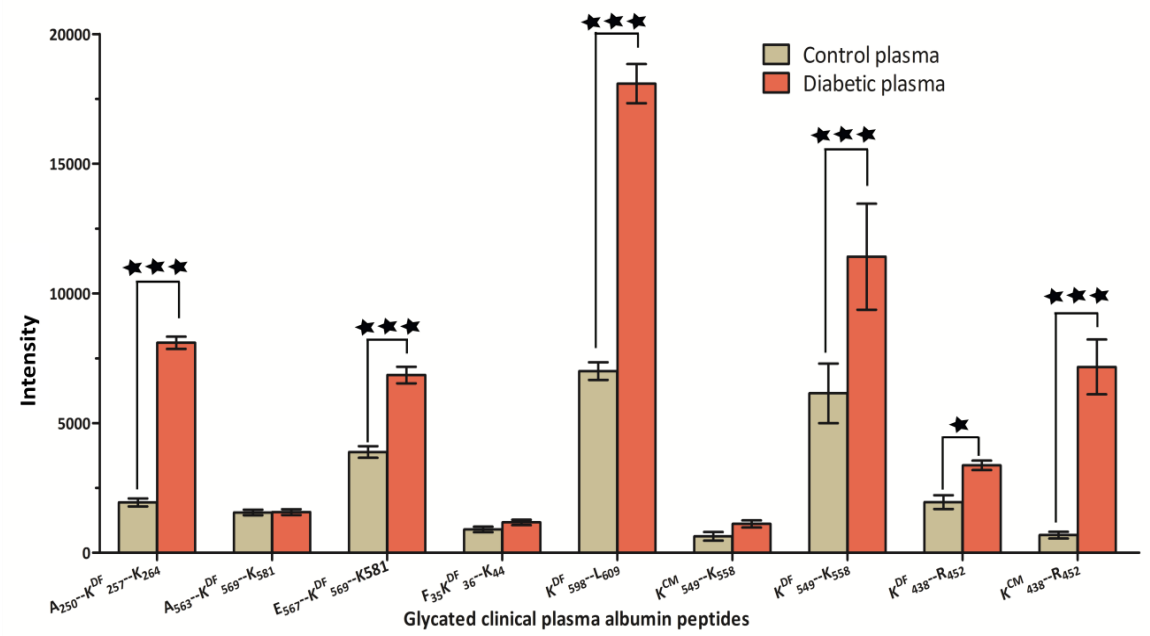


Figure 3.11 Glycated serum albumin peptides analysis in clinical subjects using SWATH-MS approach.

Bar graph represents the relative quantification of AGE-modified peptides in serum albumin from clinical plasma samples. Glycated serum albumin peptide intensities were normalized with average total ion count of all individual runs. Representative glycated peptides AEFAEVSK$_{DF}$LVTDLTK (A_{250}--K^{DF}_{257}--K^{264}), ATKEQLK$_{DF}$AVMDDFAAFVEK (A_{563}--K^{DF}_{569}--K_{581}), EQLK$_{DF}$AVMDDFAAFVEK (E_{567}--K^{DF}_{569}--K_{581}), FK$_{DF}$DLGEENFK ($F_{35}K^{DF}_{36}$--K_{44}), K$_{DF}$LVAASQAALGL (K^{DF}_{598}--L_{609}), K$_{CM}$QTALVELVK (K^{CM}_{549}--K_{558}), K$_{DF}$QTALVELVK (K^{DF}_{549}--K_{558}), K$_{DF}$VPQVSTPTLVEVSR (K^{DF}_{438}--R_{452}) and K$_{CM}$VPQVSTPTLVEVSR (K^{CM}_{438}--R_{452}) were used for their relative quantification. The most of these glycated peptides were higher in diabetic plasma as compared to control plasma and in addition these were reported as glycation sensitive residue containing peptides in previous studies. The statistical significance was calculated by using two-way ANOVA analysis (ns-no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

Further treatment of healthy control plasma to erythrocytes maintained in high glucose condition reduced HbA1c. Where diabetes plasma displayed reduced ability to reduce HbA1c. Although both control and diabetic plasma reduced the hemoglobin glycation of erythrocytes maintained in high glucose condition, as measured by HbA1c, AGE-Hb fluorescence and western blotting **(Figure 3.12 A, B and D),** the extent of decrease in hemoglobin glycation was more with the treatment of control plasma than the diabetic plasma. This data support the fact that the albumin levels and

its glycation status influence hemoglobin glycation, since albumin levels were relatively low in pooled diabetic plasma, while its glycation was relatively higher **(Table 3.4).**

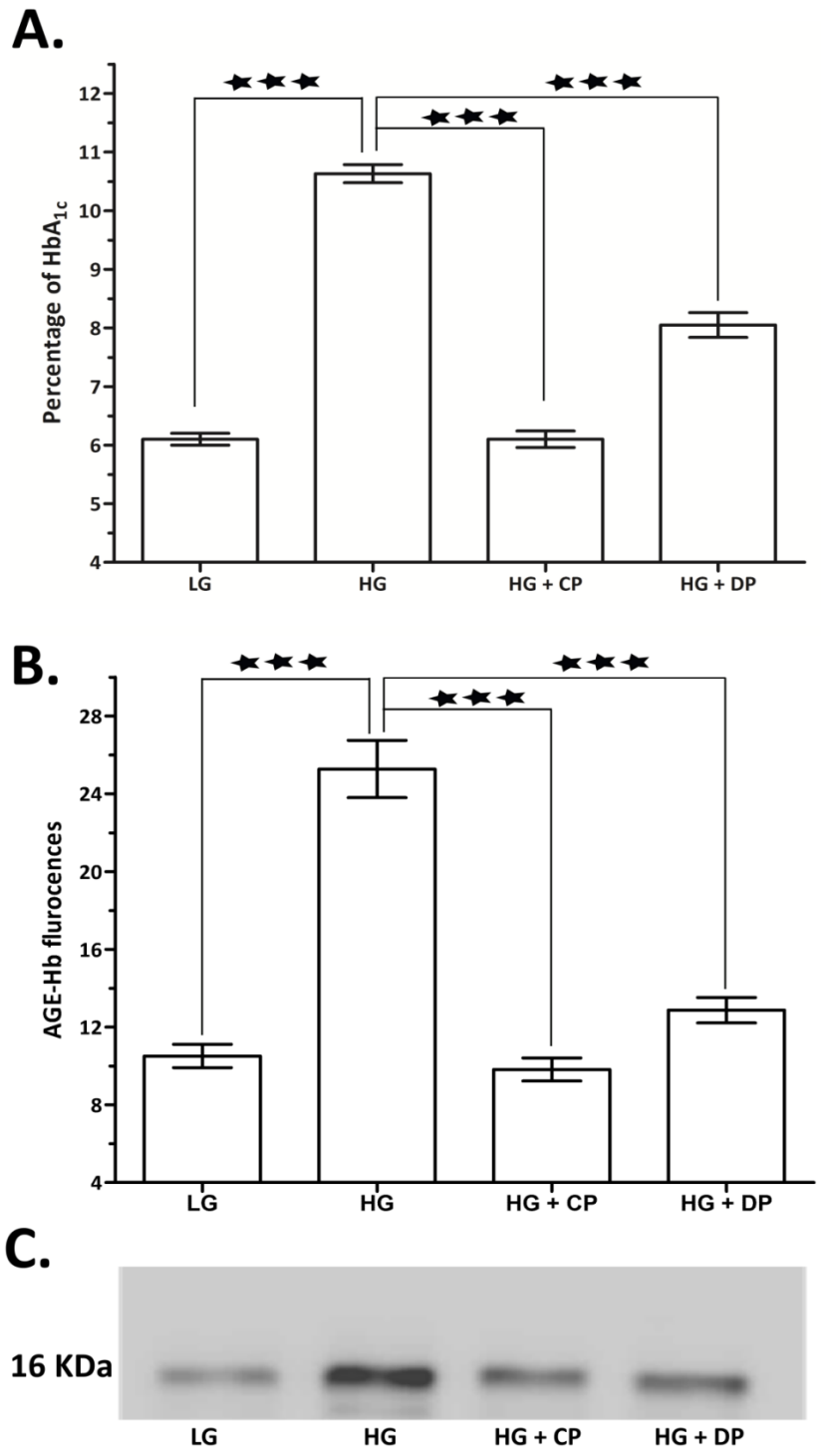


Figure 3.12 Effect of clinical plasma samples addition on hemglobin glycation.

(A) Bar graph depicts HbA1c values. Erythrocytes maintained in presence of high glucose with healthy control plasma treatment showed significant decrease in HbA1c as compared to

erythrocytes maintained only in high glucose media. Whereas treatment of erythrocytes with diabetic plasma resulted in increase in HbA1c compared to erythrocytes treated with healthy control plasma treatment. (B & C) In AGE-Hb fluorescence and Western blot with anti-carboxymethyl antibody showed similar trend as that of HbA1c data. The values of HbA1c and AGE-Hb fluorescence represents mean with standard deviation. The significance calculated by two-way ANOVA analysis (ns- no significance ($p > 0.05$),*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.3.7 HSA and HSA fructosamine (RAF) are negatively and positively associated with HbA1c respectively

Next we investigated whether the results of our in vitro experiments with erythrocyte culture can be extended to clinical settings. A total of 75 blood plasma collected from 25 subjects each of healthy control, pre-diabetes and diabetes were analysed for various biochemical parameters such as fasting and postprandial blood glucose, HbA1c, albumin levels, fructosamine etc**. (Table 3.1, Table 3.4 and Supplemental data 14).** The average HbA1c in healthy control was 5.1 ± 0.2 %, where in pre-diabetes and diabetes was 5.9 ± 0.2 % and 7.4 ± 0.9 % respectively. While the serum albumin levels were more in healthy control (46.49 ± 2.7 g/L) then pre-diabetes (42.1 ± 2.4 g/L) and diabetes (39.1 ± 3.0 g/L). Quantification of plasma fructosamine revealed that it was maximum (570.3 ± 85.6 µmol/L) in diabetes followed by pre-diabetes (457.2 ± 34.6 µmol/L) and health control (304.1 ± 37.6 µmol/L) **(Table 3.4).**

As plasma fructosamine reflects the total plasma protein glycation, a relative albumin fructosamine (RAF), which is contribution of albumin to plasma fructosamine, was deduced from the ratio of albumin to total protein concentration. The RAF levels showed similar trend as that of plasma fructosamine. Furthermore, we performed Pearson's correlation between HbA1c and serum albumin level or plasma fructosamine or RAF respectively **(Figure 3.13 A, B and C).** HbA1c and serum albumin level showed a significant negative correlation (r=-0.6584, n=75), as observed in previous studies (36, 170, 171). While plasma fructosamine (r=0.7357) and RAF (r=0.6718) showed positive correlation with HbA1c. The coefficient of correlation between plasma fructosamine and RAF was more or less same suggesting that RAF contributes predominantly to plasma fructosamine. Although it has been well established that plasma fructosamine is positively associated with HbA1c in

many previous studies, we would like to hypothesize that RAF could determine HbA1c outcome since it contributes predominantly to plasma fructosamine.

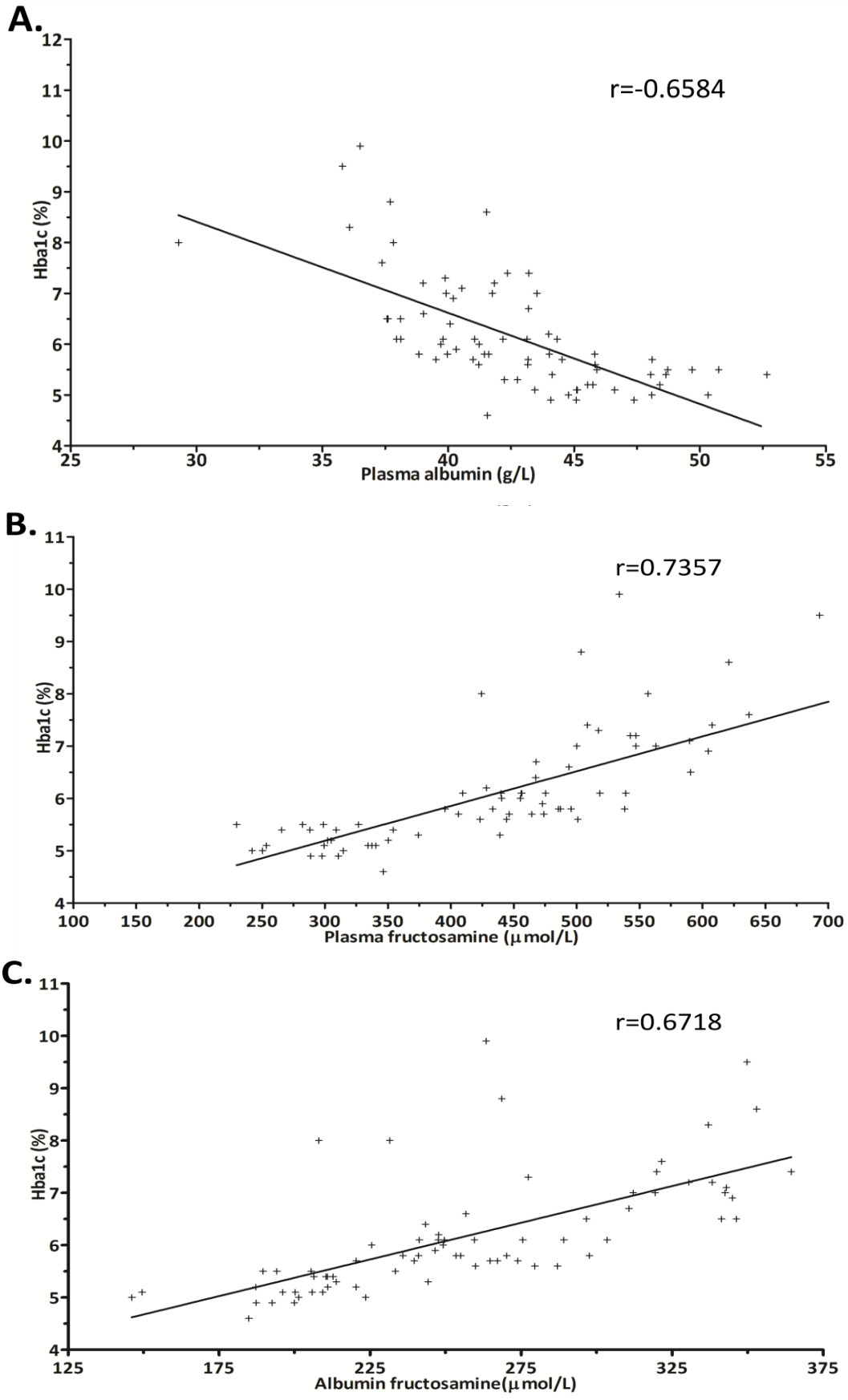


Figure 3.13 Pearson's correlation analysis HbA1c and serum albumin level or plasma fructosamine or relative albumin fructosamine (RAF).

(A) The correlation analysis showed significant negative correlation between HbA1c and serum albumin level (r=-0.6584 and p value (one-tail) <0.0001). (B) The correlation analysis showed significant positive correlation between HbA1c and plasma fructosamine level (r=0.7357 and p value (one-tail) <0.0001), and (C) The correlation analysis showed significant positive correlation between HbA1c and plasma fructosamine level (r=0.6718 and p value (one-tail) <0.0001).

3.4 Discussion

Glycated hemoglobin (HbA1c) is considered as a gold standard for assessment of glycemic status in diabetes. The treatment and management of diabetes majorly depends upon the HbA1c values. Thus it is important to consider the confounding factors that influence HbA1c. Many previous studies have shown that various factor affect HbA1c value. For example age and lifespan of erythrocytes; intracellular glucose in erythrocytes; condition like anaemia, splenomegaly, pregnancy; ethnicity and gender; estimation methods; chemical modification such gluthiolation, advanced glycation like carboxymethylation; anti-glycation drugs like aspirin etc.; iron containing diet and supplements (97). Apart from these factors, plasma albumin level has been shown to negatively associate with HbA1 in large cohort of diabetic subjects (36, 170, 171). The plausible explanation that serum albumin could competitively protects hemoglobin glycation was arrived from previous studies where low serum albumin levels were associated with increased glycation of plasma proteins including insulin, fibrinogen (36, 172, 173). In this study we have unequivocally demonstrated in erythrocyte culture that serum albumin levels influence hemoglobin glycation, i.e. higher levels of serum albumin reduces hemoglobin glycation and vice versa. Under conditions of low serum albumin levels, there was increased serum albumin glycation which perhaps decreased its ability to reduce hemoglobin glycation. This was substantiated by treatment of glycated serum albumin i.e. CMSA which failed to reduce hemoglobin glycation, instead it increased hemoglobin glycation, as well as caused alteration in erythrocyte structure and membrane permeability, which was clearly established by scanning electron microscopy and increased hemolysis respectively. The inability of CMSA to reduce hemoglobin glycation was due to lack

of availability of free lysine residues, which otherwise competitively inhibited the glycation of hemoglobin. This observation was corroborated by modifying lysine residues with acetylation. For the first, we demonstrate that modification of lysine residues of albumin impairs its ability to inhibit hemoglobin glycation. Furthermore, correlation studies between HbA1c and serum albumin or relative albumin fructosamine (RAF) supported our *in-vitro* experimental finding that albumin abundance and its glycation status determine hemoglobin glycation in erythrocyte culture. Therefore, it is quite plausible that albumin glycation precedes hemoglobin glycation *in-vivo*, since albumin is the most abundant protein in circulation with large number of free lysine or arginine residues for accessible for glycation. Even though hemoglobin is 3-4 times more abundant than albumin; it is an intracellular protein and has relatively lesser number of lysine /arginine residues accessible for glycation. Also previous studies have shown that the percent of glycated albumin is relatively higher (control range (10-15%) and diabetic range (18-30%) than the percent glycated hemoglobin, as measured by HbA1c (control 4-6% and diabetic 6.5 to 15%)(151). which supports our hypothesis that albumin glycation precedes hemoglobin glycation, although it is technically challenging to prove this in clinical setting or in animal experiments. *In vivo* glycated albumin can also increase HbA1c via can interacting with receptor for AGE (RAGE), which cause oxidative stress, inflammation, and insulin resistance, forming a vicious cycle (189). Therefore, it is not only important to maintain normal levels of serum albumin in diabetes, but also with minimal glycation **(Figure 3.14).** In conclusion, for the first time we demonstrate the role of albumin and its glycation status regulates the hemoglobin glycation (HbA1c), thus it is important quantify albumin and glycated albumin in conjunction with HbA1c for better management of diabetes.

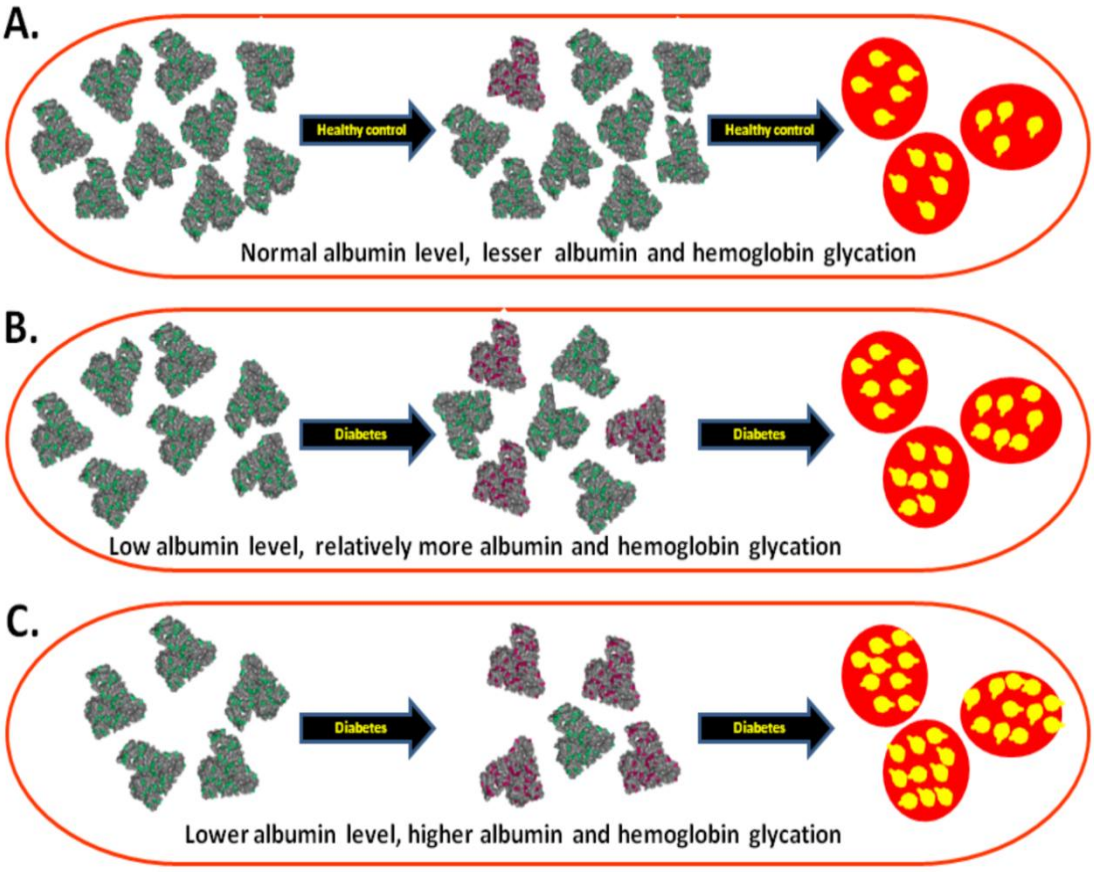


Figure 3.14 Depicts probable mechanism by which serum albumin protects hemoglobin glycation.

Albumin structure depicting unmodified and modified lysine residues green and pink colour respectively. Erythrocytes represented in round red colour and glucose bound to erythrocytes is represented in yellow colour. (A) In Healthy control condition, normal serum albumin levels and lesser glycated albumin shows relatively lesser normal hemoglobin glycation. Figure B; represents diabetes, low serum albumin level and elevated its glycation higher hemoglobin glycation. Serum albumin may preferentially get glycation than hemoglobin, since hemoglobin is inside the erythrocytes, for glucose transportation needs transporter and in addition serum albumin has more lysine and residues as well as its free circulate in blood.

Chapter 4

Summary

Chapter 4 Summary

This thesis mainly deals with identification, characterization and quantification of advanced glycated peptides of hemoglobin for development of novel markers for diabetes. In this study it has been shown that HbA1c (N-1-deoxyfructosyl valine-β-Hb (DFV-Hb), currently used marker for assessment of glycemic status, undergoes advanced glycation modification. We have comprehensively characterized and quantified various advanced glycated peptides of both α and β subunits of hemoglobin. In addition, we have emphatically focused on identification, characterization and quantification of advanced glycated peptides involving N-1-β-valine of hemoglobin i.e. N-1-(carboxymethyl) valine (CMV) and N-1-(carboxyethyl) valine (CEV) β-Hb. For the first time, we have observed that N-1-β-valine undergoes carboxyethylation (CEV); and carboxymethylation (CMV) as a predominant modification of N-1-β-Val in both diabetes and healthy control. CMV and CEV peptides of hemoglobin showed higher fold change in AUCs in all diabetic conditions compared to healthy controls suggesting its usefulness as marker in the early stages of diabetes. Furthermore, CMV and CEV peptides showed significant positive and negative correlation with micro-albuminurea (MIC) and HDL respectively than HbA1c. Therefore, quantification of CMV and CEV peptides of β-Hb along with HbA1c measurement could be useful in the prediction of diabetic complication, treatment and management of diabetes. Above all, both CMV-β-Hb and CEV-β-Hb peptides are more reliable markers and will make a great impact on the existing diagnostic scenario in diabetes in upcoming years.

In another study we have identified a variation in the serum albumin and its glycation status as a strong candidate to introduce inter-individual variability in the rate of hemoglobin glycation formation by *in-vitro* erythrocyte culture experiments and corroborated the results in clinical study. Recognizing this variable has important implications for clinical interpretation. Understanding the underlying mechanism between serum albumin and its glycation status and hemoglobin glycation may have fundamental implications for inter-individual variation. In summary, it is not only important to maintain normal levels of albumin in diabetes, but also with minimal glycation. Therefore, we propose serum albumin level and its glycation status to be quantified in conjunction with hemoglobin glycation measurement for better management of diabetes.

Chapter 5

References

List of References

1. Hu FB. Globalization of Diabetes. The role of diet, lifestyle, and genes.34(6):1249-57. doi: 10.2337/dc11-0442.

2. Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract 2011 Dec;94(3):311-21 doi: 101016/jdiabres201110029 Epub 2011 Nov 12.

3. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998 Jul;15(7):539-53.

4. Hanssen KF, Bangstad HJ, Brinchmann-Hansen O, Dahl-Jorgensen K. Blood glucose control and diabetic microvascular complications: long-term effects of near-normoglycaemia. Diabet Med 1992 Oct;9(8):697-705.

5. Fox CS, Coady S, Sorlie PD, D'Agostino RB, Sr., Pencina MJ, Vasan RS, Meigs JB, Levy D, Savage PJ. Increasing cardiovascular disease burden due to diabetes mellitus: the Framingham Heart Study. Circulation 2007 Mar 27;115(12):1544-50 Epub 2007 Mar 12.

6. Taplin CE, Barker JM. Autoantibodies in type 1 diabetes. Autoimmunity 2008 Feb;41(1):11-8 doi: 101080/08916930701619169.

7. Barker JM. Clinical review: Type 1 diabetes-associated autoimmunity: natural history, genetic associations, and screening. J Clin Endocrinol Metab 2006 Apr;91(4):1210-7 Epub 2006 Jan 10.

8. Taplin CE, Barker JM. Natural evolution, prediction, and prevention of type 1 diabetes in youth. Endocr Res 2008;33(1-2):17-33 doi: 101080/07435800802080328.

9. Lorini R, Alibrandi A, Vitali L, Klersy C, Martinetti M, Betterle C, d'Annunzio G, Bonifacio E. Risk of type 1 diabetes development in children with incidental hyperglycemia: A multicenter Italian study. Diabetes Care 2001 Jul;24(7):1210-6.

10. Standards of Medical Care in Diabetesâ€”2014. Diabetes Care.37(Supplement 1):S14-S80. doi: 10.2337/dc14-S014.

11. Diagnosis and classification of diabetes mellitus. Diabetes Care 2014 Jan;37 Suppl 1:S81-90 doi: 102337/dc14-S081.

12. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care.33(Supplement 1):S62-S9. doi: 10.2337/dc10-S062.

13. Pippitt K, Li M, Gurgle HE. Diabetes Mellitus: Screening and Diagnosis. Am Fam Physician 2016 Jan 15;93(2):103-9.

14. Ryan AS. Exercise in aging: its important role in mortality, obesity and insulin resistance. Aging health 2010 Oct;6(5):551-563.

15. Kriska AM, Saremi A, Hanson RL, Bennett PH, Kobes S, Williams DE, Knowler WC. Physical Activity, Obesity, and the Incidence of Type 2 Diabetes in a High-Risk Population. American Journal of Epidemiology. 2003;158(7):669-75.

16. Defay R, Delcourt C, Ranvier M, Lacroux A, Papoz L, Group PS. Relationships between physical activity, obesity and diabetes mellitus in a French elderly population: the POLA study. International Journal Of Obesity. 2001;25:512.

17. Kuzuya T, Nakagawa S, Satoh J, Kanazawa Y, Iwamoto Y, Kobayashi M, Nanjo K, Sasaki A, Seino Y, Ito C, Shima K, Nonaka K, Kadowaki T. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. Diabetes Res Clin Pract 2002 Jan;55(1):65-85.

18. Modan M, Harris MI. Fasting plasma glucose in screening for NIDDM in the U.S. and Israel. Diabetes Care. 1994;17(5):436-9. Epub 1994/05/01. PubMed PMID: 7741837.

19. Harris MI, Modan M. Screening for NIDDM. Why is there no national program. Diabetes Care. 1994;17(5):440-4. Epub 1994/05/01. PubMed PMID: 8062613.

20. Standards of Medical Care in Diabetes—2010. Diabetes Care. 2010;33(Supplement 1):S11-S61. doi: 10.2337/dc10-S011.

21. Goldstein DE, Little RR, Lorenz RA, Malone JI, Nathan DM, Peterson CM. Tests of glycemia in diabetes. Diabetes Care. 2004;27 Suppl 1:S91-3. Epub 2003/12/25. PubMed PMID: 14693937.

22. Higgins T. HbA1c for screening and diagnosis of diabetes mellitus. Endocrine. 2013;43(2):266-73. Epub 2012/08/22. doi: 10.1007/s12020-012-9768-y. PubMed PMID: 22907625.

23. Sacks DB. Measurement of Hemoglobin A_{1c}. A new twist on the path to harmony. 2012;35(12):2674-80. doi: 10.2337/dc12-1348.

24. Rahbar S. The discovery of glycated hemoglobin: a major event in the study of nonenzymatic chemistry in biological systems. Annals of the New York Academy of Sciences. 2005;1043:9-19. Epub 2005/07/23. doi: 10.1196/annals.1333.002. PubMed PMID: 16037217.

25. Laffel L. Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes/metabolism research and reviews. 1999;15(6):412-26. Epub 2000/01/15. PubMed PMID: 10634967.

26. Foggensteiner L, Mulroy S, Firth J. Management of diabetic nephropathy. Journal of the Royal Society of Medicine. 2001;94(5):210-7. PubMed PMID: 11385086; PMCID: PMC1281451.

27. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care. 2010;33(Suppl 1):S62-9. doi: 10.2337/dc10-S062. PubMed PMID: 20042775; PMCID: PMC2797383.

28. Wautier J-L, Schmidt AM. Protein Glycation. A Firm Link to Endothelial Cell Dysfunction. 2004;95(3):233-8. doi: 10.1161/01.res.0000137876.28454.64.

29. Hellwig M, Henle T. Baking, ageing, diabetes: a short history of the Maillard reaction. Angewandte Chemie (International ed in English). 2014;53(39):10316-29. Epub 2014/07/22. doi: 10.1002/anie.201308808. PubMed PMID: 25044982.

30. Tamanna N, Mahmood N. Food Processing and Maillard Reaction Products: Effect on Human Health and Nutrition. International journal of food science. 2015;2015:526762. Epub 2016/02/24. doi: 10.1155/2015/526762. PubMed PMID: 26904661; PMCID: PMC4745522.

31. Chuyen NV. Maillard reaction and food processing. Application aspects. Advances in experimental medicine and biology. 1998;434:213-35. Epub 1998/05/23. PubMed PMID: 9598202.

32. Hollnagel A, Kroh LW. Degradation of oligosaccharides in nonenzymatic browning by formation of alpha-dicarbonyl compounds via a "peeling off" mechanism. Journal of agricultural and food chemistry. 2000;48(12):6219-26. Epub 2001/04/21. PubMed PMID: 11312795.

33. Henning C, Glomb MA. Pathways of the Maillard reaction under physiological conditions. Glycoconjugate journal. 2016;33(4):499-512. Epub 2016/06/14. doi: 10.1007/s10719-016-9694-y. PubMed PMID: 27291759.

34. Thornalley PJ. Dicarbonyl intermediates in the maillard reaction. Annals of the New York Academy of Sciences. 2005;1043:111-7. Epub 2005/07/23. doi: 10.1196/annals.1333.014. PubMed PMID: 16037229.

35. Carranza K, Veron D, Cercado A, Bautista N, Pozo W, Tufro A, Veron D. Cellular and molecular aspects of diabetic nephropathy; the role of VEGF-A. Nefrologia : publicacion oficial de la Sociedad Espanola Nefrologia. 2015;35(2):131-8. Epub 2015/08/25. doi: 10.1016/j.nefro.2015.05.013. PubMed PMID: 26300505.

36. Bhonsle HS, Korwar AM, Kote SS, Golegaonkar SB, Chougale AD, Shaik ML, Dhande NL, Giri AP, Shelgikar KM, Boppana R, Kulkarni MJ. Low plasma albumin levels are associated with increased plasma protein glycation and HbA1c in diabetes. Journal of proteome research. 2012;11(2):1391-6. Epub 2011/12/21. doi: 10.1021/pr201030m. PubMed PMID: 22181049.

37. Ulrich P, Cerami A. Protein glycation, diabetes, and aging. Recent progress in hormone research. 2001;56:1-21. Epub 2001/03/10. PubMed PMID: 11237208.

38. Welsh KJ, Kirkman MS, Sacks DB. Role of Glycated Proteins in the Diagnosis and Management of Diabetes: Research Gaps and Future Directions. Diabetes Care. 2016;39(8):1299-306. doi: 10.2337/dc15-2727. PubMed PMID: 27457632; PMCID: PMC4955935.

39. Thornalley PJ, Langborg A, Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. The Biochemical journal. 1999;344 Pt 1:109-16. Epub 1999/11/05. PubMed PMID: 10548540; PMCID: PMC1220620.

40. Thornalley PJ, Minhas HS. Rapid hydrolysis and slow alpha,beta-dicarbonyl cleavage of an agent proposed to cleave glucose-derived protein cross-links. Biochemical pharmacology. 1999;57(3):303-7. Epub 1999/01/16. PubMed PMID: 9890557.

41. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005;54(6):1615-25. Epub 2005/05/28. PubMed PMID: 15919781.

42. Taylor R, Agius L. The biochemistry of diabetes. The Biochemical journal. 1988;250(3):625-40. Epub 1988/03/15. PubMed PMID: 3291853; PMCID: PMC1148905.

43. Iannuzzi C, Irace G, Sirangelo I. Differential effects of glycation on protein aggregation and amyloid formation. Frontiers in Molecular Biosciences. 2014;1(9). doi: 10.3389/fmolb.2014.00009.

44. Gomes Ricardo A, Oliveira Luís MA, Silva M, Ascenso C, Quintas A, Costa G, Coelho Ana V, Sousa Silva M, Ferreira António EN, Ponces Freire A, Cordeiro C. Protein glycation in vivo: functional and structural effects on yeast enolase. Biochemical Journal. 2008;416(3):317-26. doi: 10.1042/bj20080632.

45. Luthra M, Balasubramanian D. Nonenzymatic glycation alters protein structure and stability. A study of two eye lens crystallins. The Journal of biological chemistry. 1993;268(24):18119-27. Epub 1993/08/25. PubMed PMID: 8349689.

46. Singh VP, Bali A, Singh N, Jaggi AS. Advanced Glycation End Products and Diabetic Complications. The Korean Journal of Physiology & Pharmacology : Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology. 2014;18(1):1-14. doi: 10.4196/kjpp.2014.18.1.1. PubMed PMID: 24634591; PMCID: PMC3951818.

47. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circulation research. 2010;107(9):1058-70. doi: 10.1161/circresaha.110.223545. PubMed PMID: 21030723; PMCID: PMC2996922.

48. Yamagishi S, Matsui T. Advanced glycation end products, oxidative stress and diabetic nephropathy. Oxidative Medicine and Cellular Longevity. 2010;3(2):101-8. doi: 10.4161/oxim.3.2.4. PubMed PMID: 20716934; PMCID: PMC2952094.

49. Rubenstein DA, Maria Z, Yin W. Glycated Albumin Modulates Endothelial Cell Thrombogenic and Inflammatory Responses. Journal of Diabetes Science and Technology. 2011;5(3):703-13. PubMed PMID: 21722586; PMCID: PMC3192637.

50. Ramasamy R, Yan SF, Schmidt AM. Receptor for AGE (RAGE): signaling mechanisms in the pathogenesis of diabetes and its complications. Annals of the New York Academy of Sciences. 2011;1243:88-102. doi: 10.1111/j.1749-6632.2011.06320.x. PubMed PMID: 22211895; PMCID: PMC4501013.

51. Nowotny K, Jung T, Höhn A, Weber D, Grune T. Advanced Glycation End Products and Oxidative Stress in Type 2 Diabetes Mellitus. Biomolecules. 2015;5(1):194-222. doi: 10.3390/biom5010194. PubMed PMID: 25786107; PMCID: PMC4384119.

52. Stirban A, Gawlowski T, Roden M. Vascular effects of advanced glycation endproducts: Clinical effects and molecular mechanisms. Molecular metabolism. 2014;3(2):94-108. Epub 2014/03/19. doi: 10.1016/j.molmet.2013.11.006. PubMed PMID: 24634815; PMCID: PMC3953708.

53. Byun K, Yoo Y, Son M, Lee J, Jeong GB, Park YM, Salekdeh GH, Lee B. Advanced glycation end-products produced systemically and by macrophages: A common contributor to inflammation and degenerative diseases. Pharmacology & therapeutics. 2017;177:44-55. Epub 2017/02/23. doi: 10.1016/j.pharmthera.2017.02.030. PubMed PMID: 28223234.

54. Boucher J, Kleinridders A, Kahn CR. Insulin Receptor Signaling in Normal and Insulin-Resistant States. Cold Spring Harbor Perspectives in Biology. 2014;6(1). doi: 10.1101/cshperspect.a009191. PubMed PMID: 24384568; PMCID: PMC3941218.

55. Miele C, Riboulet A, Maitan MA, Oriente F, Romano C, Formisano P, Giudicelli J, Beguinot F, Van Obberghen E. Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C alpha-mediated mechanism. The Journal of biological chemistry. 2003;278(48):47376-87. Epub 2003/09/13. doi: 10.1074/jbc.M301088200. PubMed PMID: 12970360.

56. Moro E, Alessandrini P, Zambon C, Pianetti S, Pais M, Cazzolato G, Bon GB. Is glycation of low density lipoproteins in patients with Type 2 diabetes mellitus a LDL pre-oxidative condition? Diabetic medicine : a journal of the British Diabetic Association. 1999;16(8):663-9. Epub 1999/09/07. PubMed PMID: 10477211.

57. Ankit BS, Mathur G, Agrawal RP, Mathur KC. Stronger relationship of serum apolipoprotein A-1 and B with diabetic retinopathy than traditional lipids. Indian Journal of Endocrinology and Metabolism. 2017;21(1):102-5. doi: 10.4103/2230-8210.196030. PubMed PMID: 28217507; PMCID: PMC5240048.

58. Sasongko MB, Wong TY, Nguyen TT, Kawasaki R, Jenkins A, Shaw J, Wang JJ. Serum Apolipoprotein AI and B Are Stronger Biomarkers of Diabetic Retinopathy Than Traditional Lipids. Diabetes Care. 2011;34(2):474-9. doi: 10.2337/dc10-0793.

59. Dai Y, Shen Y, Li QR, Ding FH, Wang XQ, Liu HJ, Yan XX, Wang LJ, Yang K, Wang HB, Chen QJ, Shen WF, Zhang RY, Lu L. Glycated Apolipoprotein A-IV Induces Atherogenesis in Patients With CAD in Type 2 Diabetes. Journal of the American College of Cardiology. 2017;70(16):2006-19. Epub 2017/10/14. doi: 10.1016/j.jacc.2017.08.053. PubMed PMID: 29025558.

60. Sharma Y, Saxena S, Mishra A, Saxena A, Natu SM. Advanced glycation end products and diabetic retinopathy. Journal of Ocular Biology, Diseases, and Informatics. 2012;5(3-4):63-9. doi: 10.1007/s12177-013-9104-7. PubMed PMID: 24596941; PMCID: PMC3709028.

61. Shin ES, Sorenson CM, Sheibani N. Diabetes and Retinal Vascular Dysfunction. Journal of Ophthalmic & Vision Research. 2014;9(3):362-73. doi: 10.4103/2008-322x.143378. PubMed PMID: 25667739; PMCID: PMC4307665.

62. Safi SZ, Qvist R, Kumar S, Batumalaie K, Ismail ISB. Molecular Mechanisms of Diabetic Retinopathy, General Preventive Strategies, and Novel Therapeutic Targets. BioMed Research International. 2014;2014. doi: 10.1155/2014/801269. PubMed PMID: 25105142; PMCID: PMC4106080.

63. Arroba AI, Valverde AM. Modulation of microglia in the retina: new insights into diabetic retinopathy. Acta diabetologica. 2017;54(6):527-33. Epub 2017/03/30. doi: 10.1007/s00592-017-0984-z. PubMed PMID: 28349217.

64. Kolset SO, Reinholt FP, Jenssen T. Diabetic Nephropathy and Extracellular Matrix. Journal of Histochemistry and Cytochemistry. 2012;60(12):976-86. doi: 10.1369/0022155412465073. PubMed PMID: 23103723; PMCID: PMC3527883.

65. Wang S. Role of upstream stimulatory factor 2 in diabetic nephropathy. Frontiers in biology. 2015;10(3):221-9. doi: 10.1007/s11515-015-1359-x. PubMed PMID: 26494984; PMCID: PMC4610405.

66. Chen S, Cohen MP, Ziyadeh FN. Amadori-glycated albumin in diabetic nephropathy: pathophysiologic connections. Kidney international Supplement. 2000;77:S40-4. Epub 2000/09/21. PubMed PMID: 10997689.

67. Saudek CD, Brick JC. The Clinical Use of Hemoglobin A1c. Journal of Diabetes Science and Technology. 2009;3(4):629-34. PubMed PMID: 20144304; PMCID: PMC2769940.

68. Sherwani SI, Khan HA, Ekhzaimy A, Masood A, Sakharkar MK. Significance of HbA1c Test in Diagnosis and Prognosis of Diabetic Patients. Biomarker Insights. 2016;11:95-104. doi: 10.4137/bmi.s38440. PubMed PMID: 27398023; PMCID: PMC4933534.

69. Bonora E, Tuomilehto J. The Pros and Cons of Diagnosing Diabetes With A1C. Diabetes Care. 2011;34(Supplement 2):S184-S90. doi: 10.2337/dc11-s216.

70. Pauling L, Itano HA, et al. Sickle cell anemia a molecular disease. Science (New York, NY). 1949;110(2865):543-8. Epub 1949/11/25. PubMed PMID: 15395398.

71. Kunkel HG, Wallenius G. New hemoglobin in normal adult blood. Science (New York, NY). 1955;122(3163):288. Epub 1955/08/12. PubMed PMID: 13246634.

72. David W. Allen WAS, and Joan Balog. Observations on the Chromatographic Heterogeneity of Normal Adult and Fetal Human Hemoglobin: A Study of the Effects of Crystallization and Chromatography on the Heterogeneity and Isoleucine Content. Journal of the American Chemical Society. 1958:1628-34. doi: 10.1021/ja01540a030.

73. Rahbar S. An abnormal hemoglobin in red cells of diabetics. Clinica chimica acta; international journal of clinical chemistry. 1968;22(2):296-8. Epub 1968/10/01. PubMed PMID: 5687098.

74. Rahbar S, Blumenfeld O, Ranney HM. Studies of an unusual hemoglobin in patients with diabetes mellitus. Biochemical and biophysical research communications. 1969;36(5):838-43. Epub 1969/08/22. PubMed PMID: 5808299.

75. Trivelli LA, Ranney HM, Lai HT. Hemoglobin components in patients with diabetes mellitus. The New England journal of medicine. 1971;284(7):353-7. Epub 1971/02/18. doi: 10.1056/nejm197102182840703. PubMed PMID: 5539916.

76. Gebel E. The Start of Something Good: The Discovery of HbA(1c) and the American Diabetes Association Samuel Rahbar Outstanding Discovery Award. Diabetes Care. 2012;35(12):2429-31. doi: 10.2337/dc12-1763. PubMed PMID: 23173131; PMCID: PMC3507589.

77. Koenig RJ, Peterson CM, Jones RL, Saudek C, Lehrman M, Cerami A. Correlation of glucose regulation and hemoglobin AIc in diabetes mellitus. The New England journal of medicine. 1976;295(8):417-20. Epub 1976/08/19. doi: 10.1056/nejm197608192950804. PubMed PMID: 934240.

78. Nathan DM, Genuth S, Lachin J, Cleary P, Crofford O, Davis M, Rand L, Siebert C. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The New England journal of medicine. 1993;329(14):977-86. Epub 1993/09/30. doi: 10.1056/nejm199309303291401. PubMed PMID: 8366922.

79. Gonen B, Rubenstein A, Rochman H, Tanega SP, Horwitz DL. Haemoglobin A1: An indicator of the metabolic control of diabetic patients. Lancet (London, England). 1977;2(8041):734-7. Epub 1977/10/08. PubMed PMID: 71541.

80. Gabbay KH, Hasty K, Breslow JL, Ellison RC, Bunn HF, Gallop PM. Glycosylated hemoglobins and long-term blood glucose control in diabetes mellitus. The Journal of clinical endocrinology and metabolism. 1977;44(5):859-64. Epub 1977/05/01. doi: 10.1210/jcem-44-5-859. PubMed PMID: 858776.

81. Massi-Benedetti M. Changing targets in the treatment of type 2 diabetes. Current medical research and opinion. 2006;22 Suppl 2:S5-13. Epub 2006/08/18. doi: 10.1185/030079906x112714. PubMed PMID: 16914071.

82. Nathan DM, Kuenen J, Borg R, Zheng H, Schoenfeld D, Heine RJ. Translating the A1C Assay Into Estimated Average Glucose Values. Diabetes Care. 2008;31(8):1473-8. doi: 10.2337/dc08-0545. PubMed PMID: 18540046; PMCID: PMC2742903.

83. Florkowski C. HbA(1c) as a Diagnostic Test for Diabetes Mellitus – Reviewing the Evidence. The Clinical Biochemist Reviews. 2013;34(2):75-83. PubMed PMID: 24151343; PMCID: PMC3799221.

84. International Expert Committee Report on the Role of the A1C Assay in the Diagnosis of Diabetes. Diabetes Care. 2009;32(7):1327-34. doi: 10.2337/dc09-9033.

85. Kim HJ, Choi EY, Park EW, Cheong YS, Lee HY, Kim JH. The Utility of HbA1c as a Diagnostic Criterion of Diabetes. Korean journal of family medicine. 2011;32(7):383-9. Epub 2012/06/30. doi: 10.4082/kjfm.2011.32.7.383. PubMed PMID: 22745876; PMCID: PMC3383149.

86. Hanas R, John G. 2010 consensus statement on the worldwide standardization of the hemoglobin A1c measurement. Diabetic medicine : a journal of the British Diabetic Association. 2010;27(7):737-8. Epub 2010/07/20. doi: 10.1111/j.1464-5491.2010.03033.x. PubMed PMID: 20636952.

87. Braga F, Dolci A, Mosca A, Panteghini M. Biological variability of glycated hemoglobin. Clinica chimica acta; international journal of clinical chemistry. 2010;411(21-22):1606-10. Epub 2010/08/07. doi: 10.1016/j.cca.2010.07.030. PubMed PMID: 20688052.

88. Cohen RM, Franco RS, Joiner CH. Is poor glycemic control associated with reduced red blood cell lifespan? Diabetes Care. 2004;27(4):1013-4. Epub 2004/03/30. PubMed PMID: 15047667.

89. Juraschek SP, Steffes MW, Selvin E. Associations of alternative markers of glycemia with hemoglobin A(1c) and fasting glucose. Clinical chemistry. 2012;58(12):1648-55. Epub 2012/09/29. doi: 10.1373/clinchem.2012.188367. PubMed PMID: 23019309; PMCID: PMC3652236.

90. Makris K, Spanou L. Is There a Relationship between Mean Blood Glucose and Glycated Hemoglobin? Journal of Diabetes Science and Technology. 2011;5(6):1572-83. PubMed PMID: 22226280; PMCID: PMC3262729.

91. Cohen RM, Franco RS, Khera PK, Smith EP, Lindsell CJ, Ciraolo PJ, Palascak MB, Joiner CH. Red cell life span heterogeneity in hematologically normal people is sufficient to alter HbA1c. Blood. 2008;112(10):4284-91. doi: 10.1182/blood-2008-04-154112. PubMed PMID: 18694998; PMCID: PMC2581997.

92. Sikaris K. The correlation of hemoglobin A1c to blood glucose. Journal of Diabetes Science and Technology. 2009;3(3):429-38. Epub 2010/02/11. doi: 10.1177/193229680900300305. PubMed PMID: 20144279; PMCID: PMC2769865.

93. John WG. Glycated haemoglobin analysis. Annals of clinical biochemistry. 1997;34 (Pt 1):17-31. Epub 1997/01/01. doi: 10.1177/000456329703400105. PubMed PMID: 9022885.

94. Jeppsson JO, Jerntorp P, Almer LO, Persson R, Ekberg G, Sundkvist G. Capillary blood on filter paper for determination of HbA1c by ion exchange chromatography. Diabetes Care. 1996;19(2):142-5. Epub 1996/02/01. PubMed PMID: 8718434.

95. Fluckiger R, Woodtli T, Berger W. Quantitation of glycosylated hemoglobin by boronate affinity chromatography. Diabetes. 1984;33(1):73-6. Epub 1984/01/01. PubMed PMID: 6690345.

96. John WG, Gray MR, Bates DL, Beacham JL. Enzyme immunoassay--a new technique for estimating hemoglobin A1c. Clinical chemistry. 1993;39(4):663-6. Epub 1993/04/01. PubMed PMID: 8472363.

97. Radin MS. Pitfalls in Hemoglobin A1c Measurement: When Results may be Misleading. Journal of General Internal Medicine. 2014;29(2):388-94. doi: 10.1007/s11606-013-2595-x. PubMed PMID: 24002631; PMCID: PMC3912281.

98. Turley E, Rodriguez-Capote K, Estey MP, Barakauskas VE, Bailey GR, McKenzie LM, Higgins TN. Erroneous diabetes diagnosis: a case of HbA1c interference. Diabetes Care. 2015;38(10):e154-5. Epub 2015/08/22. doi: 10.2337/dc15-0733. PubMed PMID: 26294665.

99. Adekanmbi J, Higgins T, Rodriguez-Capote K, Thomas D, Winterstein J, Dixon T, Gifford JL, Krause R, Venner AA, Clarke G, Estey MP. Erroneous HbA1c results in a patient with elevated HbC and HbF. Clinica chimica acta; international journal of clinical chemistry. 2016;462:153-7. Epub 2016/10/19. doi: 10.1016/j.cca.2016.09.017. PubMed PMID: 27664335.

100. Weykamp C, Garry John W, Mosca A. A Review of the Challenge in Measuring Hemoglobin A1c. Journal of Diabetes Science and Technology. 2009;3(3):439-45. PubMed PMID: 20144280; PMCID: PMC2769874.

101. Gkogkolou P, Böhm M. Advanced glycation end products: Key players in skin aging? Dermato-endocrinology. 2012;4(3):259-70. doi: 10.4161/derm.22028. PubMed PMID: 23467327; PMCID: PMC3583887.

102. Thom CS, Dickson CF, Gell DA, Weiss MJ. Hemoglobin Variants: Biochemical Properties and Clinical Correlates. Cold Spring Harbor Perspectives in Medicine. 2013;3(3). doi: 10.1101/cshperspect.a011858. PubMed PMID: 23388674; PMCID: PMC3579210.

103. Frantzen F, Grimsrud K, Heggli DE, Sundrehagen E. Protein-boronic acid conjugates and their binding to low-molecular-mass cis-diols and glycated hemoglobin. Journal of chromatography B, Biomedical applications. 1995;670(1):37-45. Epub 1995/08/04. PubMed PMID: 7493083.

104. Makita Z, Vlassara H, Rayfield E, Cartwright K, Friedman E, Rodby R, Cerami A, Bucala R. Hemoglobin-AGE: a circulating marker of advanced glycosylation. Science (New York, NY). 1992;258(5082):651-3. Epub 1992/10/23. PubMed PMID: 1411574.

105. Chen HJ, Chen YC, Hsiao CF, Chen PF. Mass Spectrometric Analysis of Glyoxal and Methylglyoxal-Induced Modifications in Human Hemoglobin from Poorly Controlled Type 2 Diabetes Mellitus Patients. Chemical research in toxicology. 2015;28(12):2377-89. Epub 2015/10/31. doi: 10.1021/acs.chemrestox.5b00380. PubMed PMID: 26517015.

106. Jagadeeshaprasad MG, Batkulwar KB, Meshram NN, Tiwari S, Korwar AM, Unnikrishnan AG, Kulkarni MJ. Targeted quantification of N-1-(carboxymethyl) valine and N-1-(carboxyethyl) valine peptides of beta-hemoglobin for better diagnostics in diabetes. Clinical proteomics. 2016;13:7. Epub 2016/04/01. doi: 10.1186/s12014-016-9108-y. PubMed PMID: 27030792; PMCID: PMC4812615.

107. Kulkarni MJ, Mashanipalya JG, Batkulwar KB, Fernandes M. Method for identifying and quantifying carboxyethyl valine modified haemoglobin. Google Patents; 2017.

108. Dunn PJ, Cole RA, Soeldner JS, Gleason RE, Kwa E, Firoozabadi H, Younger D, Graham CA. Temporal relationship of glycosylated haemoglobin concentrations to glucose control in diabetics. Diabetologia. 1979;17(4):213-20. Epub 1979/10/01. PubMed PMID: 499681.

109. Hinzmann R, Schlaeger C, Tran CT. What do we need beyond hemoglobin A1c to get the complete picture of glycemia in people with diabetes? International journal of medical sciences. 2012;9(8):665-81. Epub 2012/10/12. doi: 10.7150/ijms.4520. PubMed PMID: 23055818; PMCID: PMC3465850.

110. Herman WH, Ma Y, Uwaifo G, Haffner S, Kahn SE, Horton ES, Lachin JM, Montez MG, Brenneman T, Barrett-Connor E. Differences in A1C by race and ethnicity among patients with impaired glucose tolerance in the Diabetes Prevention Program. Diabetes Care. 2007;30(10):2453-7. Epub 2007/05/31. doi: 10.2337/dc06-2003. PubMed PMID: 17536077; PMCID: PMC2373980.

111. Herman WH, Dungan KM, Wolffenbuttel BH, Buse JB, Fahrbach JL, Jiang H, Martin S. Racial and ethnic differences in mean plasma glucose, hemoglobin A1c, and 1,5-anhydroglucitol in over 2000 patients with type 2 diabetes. The Journal of clinical endocrinology and metabolism. 2009;94(5):1689-94. Epub 2009/03/12. doi: 10.1210/jc.2008-1940. PubMed PMID: 19276235.

112. Christensen DL, Witte DR, Kaduka L, Jorgensen ME, Borch-Johnsen K, Mohan V, Shaw JE, Tabak AG, Vistisen D. Moving to an A1C-based diagnosis of diabetes has a different impact on prevalence in different ethnic groups. Diabetes Care. 2010;33(3):580-2. Epub 2009/12/17. doi: 10.2337/dc09-1843. PubMed PMID: 20009099; PMCID: PMC2827511.

113. Cohen RM. A1C: does one size fit all? Diabetes Care. 2007;30(10):2756-8. Epub 2007/09/29. doi: 10.2337/dc07-1301. PubMed PMID: 17901536.

114. Kositsawat J, Freeman VL, Gerber BS, Geraci S. Association of A1C levels with vitamin D status in U.S. adults: data from the National Health and Nutrition Examination Survey. Diabetes Care. 2010;33(6):1236-8. Epub 2010/03/11. doi: 10.2337/dc09-2150. PubMed PMID: 20215453; PMCID: PMC2875430.

115. Clark TA, Heyliger CE, Kopilas M, Edel AL, Junaid A, Aguilar F, Smyth DD, Thliveris JA, Merchant M, Kim HK, Pierce GN. A tea/vanadate decoction delivered orally over 14 months to diabetic rats induces long-term glycemic stability without organ toxicity. Metabolism: clinical and experimental. 2012;61(5):742-53. Epub 2011/12/08. doi: 10.1016/j.metabol.2011.10.007. PubMed PMID: 22146096.

116. Standards of Medical Care in Diabetes—2007. Diabetes Care. 2007;30(suppl 1):S4-S41. doi: 10.2337/dc07-S004.

117. McCarter RJ, Hempe JM, Gomez R, Chalew SA. Biological variation in HbA1c predicts risk of retinopathy and nephropathy in type 1 diabetes. Diabetes Care. 2004;27(6):1259-64. Epub 2004/05/27. PubMed PMID: 15161772.

118. Nathan DM, Turgeon H, Regan S. Relationship between glycated haemoglobin levels and mean glucose levels over time. Diabetologia. 2007;50(11):2239-44. Epub 2007/09/14. doi: 10.1007/s00125-007-0803-0. PubMed PMID: 17851648.

119. Sasongko MB, Wong TY, Donaghue KC, Cheung N, Jenkins AJ, Benitez-Aguirre P, Wang JJ. Retinal arteriolar tortuosity is associated with retinopathy and early kidney dysfunction in type 1 diabetes. American journal of ophthalmology. 2012;153(1):176-83.e1. Epub 2011/09/13. doi: 10.1016/j.ajo.2011.06.005. PubMed PMID: 21907319.

120. Stern MP, Williams K, Haffner SM. Identification of persons at high risk for type 2 diabetes mellitus: do we need the oral glucose tolerance test? Annals of internal medicine. 2002;136(8):575-81. Epub 2002/04/17. PubMed PMID: 11955025.

121. McNeely MJ, Boyko EJ, Leonetti DL, Kahn SE, Fujimoto WY. Comparison of a Clinical Model, the Oral Glucose Tolerance Test, and Fasting Glucose for Prediction of Type 2 Diabetes Risk in Japanese Americans. Diabetes Care. 2003;26(3):758-63. doi: 10.2337/diacare.26.3.758.

122. Tuomilehto J. Point: a glucose tolerance test is important for clinical practice. Diabetes Care. 2002;25(10):1880-2. Epub 2002/09/28. PubMed PMID: 12351496.

123. Patterson SD, Aebersold RH. Proteomics: the first decade and beyond. Nature genetics. 2003;33 Suppl:311-23. Epub 2003/03/01. doi: 10.1038/ng1106. PubMed PMID: 12610541.

124. Tyers M, Mann M. From genomics to proteomics. Nature. 2003;422(6928):193-7. Epub 2003/03/14. doi: 10.1038/nature01510. PubMed PMID: 12634792.

125. Dhingra V, Gupta M, Andacht T, Fu ZF. New frontiers in proteomics research: a perspective. International journal of pharmaceutics. 2005;299(1-2):1-18. Epub 2005/06/28. doi: 10.1016/j.ijpharm.2005.04.010. PubMed PMID: 15979831.

126. Graves PR, Haystead TAJ. Molecular Biologist's Guide to Proteomics. Microbiology and Molecular Biology Reviews. 2002;66(1):39-63. doi: 10.1128/mmbr.66.1.39-63.2002. PubMed PMID: 11875127; PMCID: PMC120780.

127. Pandey A, Mann M. Proteomics to study genes and genomes. Nature. 2000;405(6788):837-46. Epub 2000/06/24. doi: 10.1038/35015709. PubMed PMID: 10866210.

128. Toyama BH. Identification of long-lived proteins reveals exceptional stability of essential cellular structures2013;154(5):971-82. doi: 10.1016/j.cell.2013.07.037. PubMed PMID: 23993091; PMCID: PMC3788602.

129. Shah DD. Biophysical analysis of the effect of chemical modification by 4-oxononenal on the structure, stability, and function of binding immunoglobulin protein (BiP)2017;12(9). doi: 10.1371/journal.pone.0183975. PubMed PMID: 28886061; PMCID: PMC5590874.

130. Sundsten T, Ortsater H. Proteomics in diabetes research. Molecular and cellular endocrinology. 2009;297(1-2):93-103. Epub 2008/07/29. doi: 10.1016/j.mce.2008.06.018. PubMed PMID: 18657591.

131. Lapolla A, Fedele D, Plebani M, Garbeglio M, Seraglia R, D'Alpaos M, Arico CN, Traldi P. Direct evaluation of glycated and glyco-oxidized globins by matrix-assisted laser desorption/ionization mass spectrometry. Rapid communications in mass spectrometry : RCM. 1999;13(1):8-14. Epub 1999/01/28. doi: 10.1002/(sici)1097-0231(19990115)13:1<8::aid-rcm438>3.0.co;2-h. PubMed PMID: 9921686.

132. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422(6928):198-207. Epub 2003/03/14. doi: 10.1038/nature01511. PubMed PMID: 12634793.

133. Yeboah FK, Yaylayan VA. Analysis of glycated proteins by mass spectrometric techniques: qualitative and quantitative aspects. Die Nahrung. 2001;45(3):164-71. Epub 2001/07/18. doi: 10.1002/1521-3803(20010601)45:3<164::aid-food164>3.0.co;2-q. PubMed PMID: 11455782.

134. Parker CE, Pearson TW, Anderson NL, Borchers CH. Mass-spectrometry-based clinical proteomics – a review and prospective. The Analyst. 2010;135(8):1830-8. doi: 10.1039/c0an00105h. PubMed PMID: 20520858; PMCID: PMC2966304.

135. Cox J, Mann M. Is proteomics the new genomics? Cell. 2007;130(3):395-8. Epub 2007/08/19. doi: 10.1016/j.cell.2007.07.032. PubMed PMID: 17693247.

136. Stokes MP, Farnsworth CL, Moritz A, Silva JC, Jia X, Lee KA, Guo A, Polakiewicz RD, Comb MJ. PTMScan direct: identification and quantification of peptides from critical signaling proteins by immunoaffinity enrichment coupled with LC-MS/MS. Molecular & cellular proteomics : MCP. 2012;11(5):187-201. Epub 2012/02/11. doi: 10.1074/mcp.M111.015883. PubMed PMID: 22322096; PMCID: PMC3418847.

137. Mitchell P. Proteomics retrenches. Nature biotechnology. 2010;28(7):665-70. Epub 2010/07/14. doi: 10.1038/nbt0710-665. PubMed PMID: 20622833.

138. Priego Capote F, Sanchez JC. Strategies for proteomic analysis of non-enzymatically glycated proteins. Mass spectrometry reviews. 2009;28(1):135-46. Epub 2008/10/25. doi: 10.1002/mas.20187. PubMed PMID: 18949816.

139. Gu H, Ren JM, Jia X, Levy T, Rikova K, Yang V, Lee KA, Stokes MP, Silva JC. Quantitative Profiling of Post-translational Modifications by Immunoaffinity Enrichment and LC-MS/MS in Cancer Serum without Immunodepletion. Molecular & cellular proteomics : MCP. 2016;15(2):692-702. Epub 2015/12/05. doi: 10.1074/mcp.O115.052266. PubMed PMID: 26635363; PMCID: PMC4739682.

140. Higgs RE, Knierman MD, Gelfanova V, Butler JP, Hale JE. Comprehensive label-free method for the relative quantification of proteins from biological samples. Journal of proteome research. 2005;4(4):1442-50. Epub 2005/08/09. doi: 10.1021/pr050109b. PubMed PMID: 16083298.

141. Silva JC, Gorenstein MV, Li GZ, Vissers JP, Geromanos SJ. Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. Molecular & cellular proteomics : MCP. 2006;5(1):144-56. Epub 2005/10/13. doi: 10.1074/mcp.M500230-MCP200. PubMed PMID: 16219938.

142. Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ, McKenna T, Nold MJ, Richardson K, Young P, Geromanos S. Quantitative proteomic analysis by accurate mass retention time pairs. Analytical chemistry. 2005;77(7):2187-200. Epub 2005/04/02. doi: 10.1021/ac048455k. PubMed PMID: 15801753.

143. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(12):6940-5. Epub 2003/05/29. doi: 10.1073/pnas.0832254100. PubMed PMID: 12771378; PMCID: PMC165809.

144. Venable JD, Dong MQ, Wohlschlegel J, Dillin A, Yates JR. Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. Nature methods. 2004;1(1):39-45. Epub 2005/03/23. doi: 10.1038/nmeth705. PubMed PMID: 15782151.

145. Geromanos SJ, Vissers JP, Silva JC, Dorschel CA, Li GZ, Gorenstein MV, Bateman RH, Langridge JI. The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. Proteomics. 2009;9(6):1683-95. Epub 2009/03/19. doi: 10.1002/pmic.200800562. PubMed PMID: 19294628.

146. Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ. Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. Proteomics. 2009;9(6):1696-719. Epub 2009/03/19. doi: 10.1002/pmic.200800564. PubMed PMID: 19294629.

147. Li Y, Silva JC, Skinner ME, Lombard DB. Mass spectrometry-based detection of protein acetylation. Methods in molecular biology (Clifton, NJ). 2013;1077:81-104. Epub 2013/09/10. doi: 10.1007/978-1-62703-637-5_6. PubMed PMID: 24014401; PMCID: PMC3954751.

148. Korwar AM, Vannuruswamy G, Jagadeeshaprasad MG, Jayaramaiah RH, Bhat S, Regin BS, Ramaswamy S, Giri AP, Mohan V, Balasubramanyam M, Kulkarni MJ. Development of Diagnostic Fragment Ion Library for Glycated Peptides of Human Serum Albumin: Targeted Quantification in Prediabetic, Diabetic, and Microalbuminuria Plasma by Parallel Reaction Monitoring, SWATH, and MSE. Molecular & cellular proteomics : MCP. 2015;14(8):2150-9. Epub 2015/05/30. doi: 10.1074/mcp.M115.050518. PubMed PMID: 26023067; PMCID: PMC4528244.

149. Hu Y, Liu W, Chen Y, Zhang M, Wang L, Zhou H, Wu P, Teng X, Dong Y, Zhou J, Xu H, Zheng J, Li S, Tao T, Hu Y, Jia Y. Combined use of fasting plasma glucose and glycated hemoglobin A1c in the screening of diabetes and impaired glucose tolerance. Acta diabetologica. 2010;47(3):231-6. Epub 2009/09/18. doi: 10.1007/s00592-009-0143-2. PubMed PMID: 19760291.

150. Mohan V, Vijayachandrika V, Gokulakrishnan K, Anjana RM, Ganesan A, Weber MB, Narayan KM. A1C cut points to define various glucose intolerance groups in Asian Indians. Diabetes Care. 2010;33(3):515-9. Epub 2009/11/12. doi: 10.2337/dc09-1694. PubMed PMID: 19903752; PMCID: PMC2827500.

151. van 't Riet E, Alssema M, Rijkelijkhuizen JM, Kostense PJ, Nijpels G, Dekker JM. Relationship between A1C and glucose levels in the general Dutch population: the new Hoorn study. Diabetes Care. 2010;33(1):61-6. Epub 2009/10/08. doi: 10.2337/dc09-0677. PubMed PMID: 19808928; PMCID: PMC2797987.

152. van 't Riet E, Dekker JM, Sun Q, Nijpels G, Hu FB, van Dam RM. Role of adiposity and lifestyle in the relationship between family history of diabetes and 20-year incidence of type 2 diabetes in U.S. women. Diabetes Care. 2010;33(4):763-7. Epub 2010/01/14. doi: 10.2337/dc09-1586. PubMed PMID: 20067970; PMCID: PMC2845024.

153. Li S, Guo CG, Chen L, Yin XY, Wu YX, Fan LY, Fan HZ, Cao CX. Impact of glutathione-HbA1c on HbA1c measurement in diabetes diagnosis via array isoelectric focusing, liquid chromatography, mass spectrometry and ELISA. Talanta. 2013;115:323-8. Epub 2013/09/24. doi: 10.1016/j.talanta.2013.05.040. PubMed PMID: 24054598.

154. Uchimura T, Nakano K, Hashiguchi T, Iwamoto H, Miura K, Yoshimura Y, Hanyu N, Hirata K, Imakuma M, Motomiya Y, Maruyama I. Elevation of N-(carboxymethyl)valine residue in hemoglobin of diabetic patients. Its role in the development of diabetic nephropathy. Diabetes Care. 2001;24(5):891-6. Epub 2001/05/12. PubMed PMID: 11347750.

155. Shimada S, Tanaka Y, Ohmura C, Tamura Y, Shimizu T, Uchino H, Watada H, Hirose T, Nakaniwa T, Miwa S, Kawamori R. N-(carboxymethyl)valine residues in hemoglobin (CMV-Hb) reflect accumulation of oxidative stress in diabetic patients. Diabetes research and clinical practice. 2005;69(3):272-8. Epub 2005/08/16. doi: 10.1016/j.diabres.2005.01.007. PubMed PMID: 16098924.

156. Lapolla A, Porcu S, Traldi P. Some views on proteomics in diabetes. Clinical chemistry and laboratory medicine. 2011;49(6):943-57. Epub 2011/03/18. doi: 10.1515/cclm.2011.151. PubMed PMID: 21410413.

157. Chung LM, Colangelo CM, Zhao H. Data Pre-Processing for Label-Free Multiple Reaction Monitoring (MRM) Experiments. Biology. 2014;3(2):383-402. Epub 2014/06/07. doi: 10.3390/biology3020383. PubMed PMID: 24905083; PMCID: PMC4085614.

158. Tsai TH, Wang M, Ressom HW. Preprocessing and Analysis of LC-MS-Based Proteomic Data. Methods in molecular biology (Clifton, NJ). 2016;1362:63-76. Epub 2015/11/01. doi: 10.1007/978-1-4939-3106-4_3. PubMed PMID: 26519169; PMCID: PMC4838194.

159. Wang SH, Wang TF, Wu CH, Chen SH. In-depth comparative characterization of hemoglobin glycation in normal and diabetic bloods by LC-MSMS. Journal of the American Society for Mass Spectrometry. 2014;25(5):758-66. Epub 2014/03/01. doi: 10.1007/s13361-014-0830-2. PubMed PMID: 24578002.

160. Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC, Elliston K, Stern D, Shaw A. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. The Journal of biological chemistry. 1992;267(21):14998-5004. Epub 1992/07/25. PubMed PMID: 1378843.

161. Ikeda K, Higashi T, Sano H, Jinnouchi Y, Yoshida M, Araki T, Ueda S, Horiuchi S. N (epsilon)-(carboxymethyl)lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. Biochemistry. 1996;35(24):8075-83. Epub 1996/06/18. doi: 10.1021/bi9530550. PubMed PMID: 8672512.

162. Gallien S, Duriez E, Crone C, Kellmann M, Moehring T, Domon B. Targeted Proteomic Quantification on Quadrupole-Orbitrap Mass Spectrometer. Molecular & cellular proteomics : MCP. 2012;11(12):1709-23. doi: 10.1074/mcp.O112.019802. PubMed PMID: 22962056; PMCID: PMC3518128.

163. Sato E, Mori F, Igarashi S, Abiko T, Takeda M, Ishiko S, Yoshida A. Corneal advanced glycation end products increase in patients with proliferative diabetic retinopathy. Diabetes Care. 2001;24(3):479-82. Epub 2001/04/06. PubMed PMID: 11289471.

164. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001;414(6865):813-20. Epub 2001/12/14. doi: 10.1038/414813a. PubMed PMID: 11742414.

165. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet (London, England). 1998;352(9131):837-53. Epub 1998/09/22. PubMed PMID: 9742976.

166. Rodbard D. Continuous Glucose Monitoring: A Review of Successes, Challenges, and Opportunities. Diabetes technology & therapeutics. 2016;18 Suppl 2:S3-s13. Epub 2016/01/20. doi: 10.1089/dia.2015.0417. PubMed PMID: 26784127; PMCID: PMC4717493.

167. Juraschek SP, Steffes MW, Miller ER, 3rd, Selvin E. Alternative markers of hyperglycemia and risk of diabetes. Diabetes Care. 2012;35(11):2265-70. Epub 2012/08/10. doi: 10.2337/dc12-0787. PubMed PMID: 22875225; PMCID: PMC3476908.

168. Kilpatrick ES, Rigby AS, Atkin SL. A1C variability and the risk of microvascular complications in type 1 diabetes: data from the Diabetes Control and Complications Trial. Diabetes Care. 2008;31(11):2198-202. Epub 2008/07/25. doi: 10.2337/dc08-0864. PubMed PMID: 18650371; PMCID: PMC2571045.

169. Nicholas J, Charlton J, Dregan A, Gulliford MC. Recent HbA1c values and mortality risk in type 2 diabetes. population-based case-control study. PLoS ONE. 2013;8(7):e68008. Epub 2013/07/19. doi: 10.1371/journal.pone.0068008. PubMed PMID: 23861845; PMCID: PMC3702542.

170. Rodriguez-Segade S, Rodriguez J, Mayan D, Camina F. Plasma albumin concentration is a predictor of HbA1c among type 2 diabetic patients, independently of fasting plasma glucose and fructosamine. Diabetes Care. 2005;28(2):437-9. Epub 2005/01/29. PubMed PMID: 15677811.

171. Tiwari S, Bothale M, Hasan I, Kulkarni MJ, Sayyad MG, Basu R, Basu A, Unnikrishnan AG. Association between serum albumin and glycated hemoglobin in Asian Indian subjects. Indian Journal of Endocrinology and Metabolism. 2015;19(1):52-5. Epub 2015/01/17. doi: 10.4103/2230-8210.144631. PubMed PMID: 25593826; PMCID: PMC4287780.

172. Bhonsle HS, Singh SK, Srivastava G, Boppana R, Kulkarni MJ. Albumin competitively inhibits glycation of less abundant proteins. Protein and peptide letters. 2008;15(7):663-7. Epub 2008/09/11. PubMed PMID: 18782060.

173. Kielmas M, Szewczuk Z, Stefanowicz P. A study on human serum albumin influence on glycation of fibrinogen. Biochemical and biophysical research communications. 2013;439(1):78-83. Epub 2013/08/21. doi: 10.1016/j.bbrc.2013.08.025. PubMed PMID: 23958299.

174. Arques S, Ambrosi P. Human serum albumin in the clinical syndrome of heart failure. Journal of cardiac failure. 2011;17(6):451-8. Epub 2011/06/01. doi: 10.1016/j.cardfail.2011.02.010. PubMed PMID: 21624732.

175. Bhat S, Jagadeeshaprasad MG, Venkatasubramani V, Kulkarni MJ. Abundance matters: role of albumin in diabetes, a proteomics perspective. Expert review of proteomics. 2017;14(8):677-89. Epub 2017/07/12. doi: 10.1080/14789450.2017.1352473. PubMed PMID: 28689445.

176. Cohen RM, Holmes YR, Chenier TC, Joiner CH. Discordance between HbA1c and fructosamine: evidence for a glycosylation gap and its relation to diabetic nephropathy. Diabetes Care. 2003;26(1):163-7. Epub 2002/12/28. PubMed PMID: 12502674.

177. Vannuruswamy G, Korwar AM, Jagadeeshaprasad MG, Kulkarni MJ. Targeted Quantification of the Glycated Peptides of Human Serum Albumin. Methods in molecular biology (Clifton, NJ). 2017;1619:403-16. Epub 2017/07/05. doi: 10.1007/978-1-4939-7057-5_28. PubMed PMID: 28674900.

178. Garikapati Vannuruswamy MGJ, K. Kashinath, Suresh K. Kesavan, Shweta Bhat, Arvind M. Korwar, Ashok D. Chougale, Ramanamurthy Boppana, D. Srinivasa Reddy, and Mahesh J. Kulkarni. Molecules with O-acetyl group protect protein glycation by acetylating lysine residues. RSC Advances. 2016;6(70):65572-8. doi: 10.1039/C6RA11313C.

179. Ladyzynski P, Foltynski P, Bak MI, Sabalinska S, Krzymien J, Kawiak J. Validation of a hemoglobin A1c model in patients with type 1 and type 2 diabetes and its use to go beyond the averaged relationship of hemoglobin A1c and mean glucose level. Journal of translational medicine. 2014;12:328. Epub 2014/12/11. doi: 10.1186/s12967-014-0328-5. PubMed PMID: 25491199; PMCID: PMC4268801.

180. Ladyzynski P, Wojcicki JM, Bak M, Sabalinska S, Kawiak J, Foltynski P, Krzymien J, Karnafel W. Validation of hemoglobin glycation models using glycemia monitoring in vivo and culturing of erythrocytes in vitro. Annals of biomedical engineering. 2008;36(7):1188-202. Epub 2008/05/02. doi: 10.1007/s10439-008-9508-x. PubMed PMID: 18449641.

181. Viskupicova J, Blaskovic D, Galiniak S, Soszynski M, Bartosz G, Horakova L, Sadowska-Bartosz I. Effect of high glucose concentrations on human erythrocytes in vitro. Redox biology. 2015;5:381-7. Epub 2015/07/05. doi: 10.1016/j.redox.2015.06.011. PubMed PMID: 26141922; PMCID: PMC4506982.

182. Gopalkrishnapillai B, Nadanathangam V, Karmakar N, Anand S, Misra A. Evaluation of autofluorescent property of hemoglobin-advanced glycation end product as a long-term glycemic index of diabetes. Diabetes. 2003;52(4):1041-6. Epub 2003/03/29. PubMed PMID: 12663478.

183. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976;72:248-54. Epub 1976/05/07. PubMed PMID: 942051.

184. Yanagisawa K, Makita Z, Shiroshita K, Ueda T, Fusegawa T, Kuwajima S, Takeuchi M, Koike T. Specific fluorescence assay for advanced glycation end products in blood and urine of diabetic patients. Metabolism: clinical and experimental. 1998;47(11):1348-53. Epub 1998/11/24. PubMed PMID: 9826211.

185. Bartakova V, Kollarova R, Kuricova K, Sebekova K, Belobradkova J, Kankova K. Serum carboxymethyl-lysine, a dominant advanced glycation end product, is increased in women with gestational diabetes mellitus. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia. 2016;160(1):70-5. Epub 2015/11/27. doi: 10.5507/bp.2015.045. PubMed PMID: 26607295.

186. Carlsson H, Tornqvist M. Strategy for identifying unknown hemoglobin adducts using adductome LC-MS/MS data: Identification of adducts corresponding to acrylic acid, glyoxal, methylglyoxal, and 1-octen-3-one. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2016;92:94-103. Epub 2016/04/06. doi: 10.1016/j.fct.2016.03.028. PubMed PMID: 27046699.

187. Zhang M, Xu W, Deng Y. A new strategy for early diagnosis of type 2 diabetes by standard-free, label-free LC-MS/MS quantification of glycated peptides. Diabetes. 2013;62(11):3936-42. Epub 2013/07/31. doi: 10.2337/db13-0347. PubMed PMID: 23894188; PMCID: PMC3806625.

188. Awasthi S, Gayathiri SK, Ramya R, Duraichelvan R, Dhason A, Saraswathi NT. Advanced Glycation-Modified Human Serum Albumin Evokes Alterations in Membrane and Eryptosis in Erythrocytes. Applied biochemistry and biotechnology. 2015;177(5):1013-24. Epub 2015/08/16. doi: 10.1007/s12010-015-1793-x. PubMed PMID: 26276445.

189. Kim KJ, Lee BW. The roles of glycated albumin as intermediate glycation index and pathogenic protein. Diabetes & metabolism journal. 2012;36(2):98-107. Epub 2012/04/28. doi: 10.4093/dmj.2012.36.2.98. PubMed PMID: 22540045; PMCID: PMC3335903.

Jagadeeshaprasad et al. Clin Proteom (2016) 13:7
DOI 10.1186/s12014-016-9108-y

Clinical Proteomics

RESEARCH Open Access

Targeted quantification of *N*-1-(carboxymethyl) valine and *N*-1-(carboxyethyl) valine peptides of β-hemoglobin for better diagnostics in diabetes

Mashanipalya G. Jagadeeshaprasad[1,2], Kedar B. Batkulwar[1,2], Nishita N. Meshram[3], Shalbha Tiwari[3], Arvind M. Korwar[1], Ambika G. Unnikrishnan[3] and Mahesh J. Kulkarni[1,2*]

Abstract

Background: *N*-1-(Deoxyfructosyl) valine (DFV) β-hemoglobin (β-Hb), commonly referred as HbA1c, is widely used diagnostic marker in diabetes, believed to provide glycemic status of preceding 90–120 days. However, the turnover of hemoglobin is about 120 days, the DFV-β-Hb, an early and reversible glycation product eventually may undergo irreversible advanced glycation modifications such as carboxymethylation or carboxyethylation. Hence quantification of *N*-1-(carboxymethyl) valine (CMV) and *N*-1-(carboxyethyl) valine (CEV) peptides of β-Hb would be useful in assessing actual glycemic status.

Results: Fragment ion library for synthetically glycated peptides of hemoglobin was generated by using high resolution–accurate mass spectrometry (HR/AM). Using parallel reaction monitoring, deoxyfructosylated, carboxymethylated and carboxyethylated peptides of hemoglobin were quantified in clinical samples from healthy control, pre-diabetes, diabetes and poorly controlled diabetes. For the first time, we report *N*-1-β-valine undergoes carboxyethylation and mass spectrometric quantification of CMV and CEV peptides of β-hemoglobin. Carboxymethylation was found to be the most abundant modification of *N*-1-β-valine. Both CMV-β-Hb and CEV-β-Hb peptides showed better correlation with severity of diabetes in terms of fasting glucose, postprandial glucose and microalbuminuria.

Conclusions: This study reports carboxymethylation as a predominant modification of *N*-1-β-valine of Hb, and quantification of CMV-β-Hb and CEV-β-Hb could be useful parameter for assessing the severity of diabetes.

Keywords: Diabetes, Diagnosis, Glycation, Glycemic control, HbA1c, Mass spectrometry

Background

Poorly controlled diabetes leads to variety of complications including blindness, amputations and, kidney failure [1]. Thus, glycemic control is crucial in management of diabetes [2]. Diagnosis and management of diabetes heavily relies on detection of levels of HbA1c [*N*-1-(deoxyfructosyl) valine (DFV) β-hemoglobin], which is considered as a gold standard for assessing the glycemic status over preceding 90–120 days [3, 4]. In normal individuals, HbA1c ranges from 3 to 6.5 % and up to 15 % in poorly controlled diabetes [5]. However, previous studies have suggested that the HbA1c is slowly reversible, and for a given glucose concentration it eventually reaches equilibrium [6]. Furthermore precise quantification of HbA1c using available methods in routine diagnostics, such as ion exchange [7], phenylboronate affinity

*Correspondence: mj.kulkarni@ncl.res.in
[1] Proteomics Facility, Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune 411008, India
Full list of author information is available at the end of the article

US 20170074888A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2017/0074888 A1**

Kulkarni et al. (43) **Pub. Date: Mar. 16, 2017**

(54) **METHOD FOR IDENTIFYING AND QUANTIFYING CARBOXYETHYL VALINE MODIFIED HAEMOGLOBIN**

(71) Applicant: **Council of Scientific & Industrial Research**, New Delhi (IN)

(72) Inventors: **Mahesh Jagdishrao Kulkarni**, Pune (IN); **Jagadeeshaprasad Guddadarangaiah Mashanipalya**, Pune (IN); **Kedar Balaji Batkulwar**, Pune (IN); **Moneesha Fernandes**, Pune (IN)

(73) Assignee: **Council of Scientific & Industrial Research**, New Delhi (IN)

(21) Appl. No.: **15/254,539**

(22) Filed: **Sep. 1, 2016**

(30) **Foreign Application Priority Data**

Sep. 1, 2015 (IN) 2730/DEL/2015

Publication Classification

(51) **Int. Cl.**
G01N 33/68 (2006.01)

(52) **U.S. Cl.**
CPC ***G01N 33/6848*** (2013.01)

(57) **ABSTRACT**

The present invention relates to a method for identification and quantification of carboxyethylated valine modified haemoglobin to assess the extent of diabetic complications.

JAGADEESHAPRASAD M G
Address: c/o Dr. Mahesh J. Kulkarni
Proteomics and Mass Spectrometry Group
Biochemical Sciences Division, CSIR-National Chemical Laboratory
Pune - 411008, India.
Date of Birth: April 11,1988
Nationality: Indian
Email ID: jagadeeshaprasad@gmail.com
Phone no.: +91-9822039264

Education

2015-Present	PhD in Life Sciences, CSIR-National Chemical Laboratory, Pune, India
2011-2014	Project Fellow at Proteomics and Mass Spectrometry Facility, CSIR-National Chemical Laboratory, Pune, India.
2009-2011	Masters in Biochemistry, Kuvempu University,Shimoga, Karnataka, India
2006-2009	Bachelors in Zoology, Biochemistry and Microbiology, Government Science College, Tumkur University, Tumkuru, Karnataka, India

Research Experience

2011-present	Project fellow and Doctoral fellow CSIR-National Chemical Laboratory, Pune, India Advisor: Dr.Mahesh J. Kulkarni Project: Repositioning of drugs for novel uses and understanding the mechanism of drug toxicity by chemical proteomics approach and Advanced glycated hemoglobin for better diagnosis of diabetes.

Skills and Techniques

Proteomic techniques: one and two dimensional electrophoresis, Western blot, ELISA, functional assay and Immunoprecipitation

- Mass spectrometry based techniques: label and label free proteome identification and quantification and protein and PTMs identification, characterization and quantification
- Protein digestion techniques and experimental handled difeerent mass spectrometers such as Q-Exactive Orbitrap, Waters-SYNAPT HDMS (LC-QTOF) and AB SCIEX TOF/TOF 5800 system and worked with different LC-MS based acquisition methods PRM, HR-MRM, MSE, IDA, DDA and SWATH for proteomic research.
- For protein identification, and quantification worked with different vendor based proteomic data analysis software (Proteome Discoverer, ProteinPilot, Protein Lynx Global Server (PLGS), Pinpoint, Skyline, Peakview and Markerview) Animal handling license: small animals

(mice and rat)

- Microbial and animal cell culture, growth and maintenance of bacterial and erythrocyte cultures.
- Metabolomics: Untargeted metabolite identification and quantification
- Molecular biology techniques: DNA and RNA isolation, PCR, qPCR, gene cloning and transformation studies

Awards and Fellowship

- **PSI Travel Award** at the 9th Annual Meeting of Proteomics Society, India and 'International Conference on Proteomics in Health and Disease' organized by Institute of Life Sciences, Bhuvaneshwar during November 30- December 02, 2017
- **NCL-RF Agnimitra Memorial Best Poster Award 2016** in Biological Sciences for poster entitled "Targeted quantification of advanced glycated peptides of hemoglobin: Carboxymethylation is the predominant modification of β- valine of β-hemoglobin." as a part of Science Day Celebrations held at CSIR-NCL, Pune, India on 25th& 26th February 2016
- **Certificate of Merit for the excellence in performance** during the Targeted Proteomics Workshop and International Symposium, December 10th- 14th 2015, IIT Bombay, Mumbai, India
- Indian council of Medical Research - Senior Research Fellowship for the period of 2015-2017

Conferences

- 9th Annual Meeting of Proteomics Society, India and 'International Conference on Proteomics in Health and Disease' organized by Institute of Life Sciences, Bhuvaneshwar during November 30- December 02, 2017
- Participated in the Targeted Proteomics Workshop & International Symposium - TPWIS -2015 held on 10-14, December 2015, at IIT Bombay, India
- Participated in the Practical Proteomics Course conducted by University of WARWICK, UK held on September 24-27, 2013 at Waters India, Bangalore
- 6th Annual meeting of the Proteomics Society of India PSI, 2014, Bombay, India.
- 4th Annual meeting of the Proteomics Society of India PSI, 2012, Pune, India.

Patent and Publications

1 **Jagadeeshaprasad MG,** Batkulwar KB, Kulkarni MJ. "Method for identifying and quantifying carboxyethyl valine modified haemoglobin". **United States Patent Application Publication, US2017/0074888 A1**

2 **Jagadeeshaprasad MG,** Vinashya V, Unnikrishnan AG, Kulkarni MJ."Low abundance of albumin enhances its glycation and impairs its ability to protect against hemoglobin glycation: A mechanistic study using erythrocyte culture". (**under revision Mol Cell Proteomics**).

3 Bhat S, **Jagadeeshaprasad MG,** Vinashya V, Kulkarni MJ. Abundance matters: Role of albumin in diabetes, a proteomics perspective. **Expert Rev Proteomics. 2017 Jul 13:1-13.**

4 Vannuruswamy G, Korwar AM, **Jagadeeshaprasad MG,** Kulkarni MJ. Targeted Quantification of the Glycated Peptides of Human Serum Albumin. **Methods Mol Biol. 2017; 1619:403-416.**

5 Gajbhiyea A, Dabhia R, Taunk K, **Jagadeeshaprasad MG**, Rapole S. "Multipronged quantitative proteomics reveals serum proteome alterations in breast cancer intrinsic subtypes". **J Proteomics. 2017;1 63:1-13.**

6 Kazi RS, Banarjee RM, Deshmukh AB, Patil GV, **Jagadeeshaprasad MG**, Kulkarni MJ. "Glycation inhibitors extend yeast Chronological lifespan by reducing advanced glycation end products and by back regulation of proteins involved in mitochondrial respiration." **J Proteomics. 2017; 156:104-112.**

7 Vannuruswamy G[#],**Jagadeeshaprasad MG** [#], K. Kashinath[#], Kesavan SK, Bhat S, Korwar AM, Chougale AD, Boppana R, Reddy DS, Kulkarni MJ. "Molecules with O-acetyl, not N-acetyl group, protect protein glycation by acetylating lysine residues." **RSC Advances.2016;6:70.** # represents equal first author contribution

8 Bhat S, **Jagadeeshaprasad MG**, Patil YR, Shaikh ML, Regin BS, Mohan V, Giri AP, Balasubramanyam M, Boppana R, Kulkarni MJ. "Proteomics insight reveals elevated levels of albumin in circulating immune complexes in diabetic plasma." **Mol Cell Proteomics. 2016;15:2011-20.**

9 **Jagadeeshaprasad MG**, Batkulwar KB, Meshram NN, Tiwari S, Korwar AM, Unnikrishnan AG, Kulkarni MJ. "Targeted quantification of N-1-(carboxymethyl) valine and N-1-(carboxyethyl) valine peptides of β-hemoglobin for better diagnostics in diabetes." **Clin Proteomics. 2016;13:7.**

10 Korwar AM, Vannuruswamy G, **Jagadeeshaprasad MG,** Jayaramaiah RH, Bhat S, Regin BS, Ramaswamy S, Giri AP, Mohan V, Balasubramanyam M, Kulkarni MJ. "Development of diagnostic fragment ion library for glycated peptides of human serum albumin:

targeted quantification in prediabetic, diabetic and microalbuminuria plasma by PRM,SWATH and MS^E." **Mol Cell Proteomics. 2015;14:2150-9.**

11 Golegaonkar S, Tabrez SS, Pandit A, Sethurathinam S, **Jagadeeshaprasad MG**, Bansode S, Sampathkumar SG, Kulkarni MJ, Mukhopadhyay A. "Rifampicin reduces advanced glycation end products and activates DAF-16 to increase lifespan in Caenorhabditis elegans." **Aging Cell. 2015;14:463-73.**

12 Mukherjee S, **Jagadeeshaprasad MG**, Banerjee T, Ghosh SK, Biswas M, Dutta S, Kulkarni MJ, Pattari S, Bandyopadhyay A. "Proteomic analysis of human plasma in chronic rheumatic mitral stenosis reveals proteins involved in the complement and coagulation cascade." **Clin Proteomics. 2014;11:35.**

13 Kesavan SK, Bhat S, Golegaonkar SB, **Jagadeeshaprasad MG**, Deshmukh AB, Patil HS, Bhosale SD, Shaikh ML, Thulasiram HV, Boppana R, Kulkarni MJ. "Proteome wide reduction in AGE modification in streptozotocin induced diabetic mice by hydralazine mediated transglycation." **Sci Rep. 2013; 3:2941.**