REGULATION OF GLYCATION IN DIABETES: IDENTIFICATION AND CHARACTERIZATION OF GLYCATED PROTEINS

A THESIS SUBMITTED
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In
BIOTECHNOLOGY

By
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RESEARCH GUIDE

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CERTIFICATE

Certified that the work in the Ph.D. thesis entitled ‘Regulation of glycation in diabetes: Identification and characterization of glycated proteins’ submitted for the award of Ph.D degree by Ms. Hemangi Santaji Bhonsle was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

Date: Dr. Mahesh J. Kulkarni

Place: Pune (Research Guide)

DECLARATION

This is to state that the Ph.D research work entitled “Regulation of glycation in diabetes: Identification and characterization of glycated proteins” has not been submitted in parts or full to any other university for the Doctorate degree by me. This is the original work undertaken by me under the guidance of Dr. M J. Kulkarni at the CSIR-National Chemical Laboratory, Pune, India.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Date: Hemangi S. Bhonsle

Place: Pune

Dedicated to My Respected
Father, Mother
&
My Adorable Sister Poonam...

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Hemangi S. Bhonsle

LIST OF ABBREVIATIONS

ACN	:	Acetonitrile
BCG	:	Bromocresolgreen
BPI	:	Base peak intensity
CBB	:	Coommassie brilliant blue
CHAPS	:	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CBB-R250	:	Coommassie brilliant blue R250
C_7BzO	:	3-(4-Heptyl)phenyl-3-hydroxy-propyl-dimethylammonio- sulfobetaine
Da, kDa	:	Dalton, Kilodalton
DAB	:	2, 4-Diaminobutyric Acid
DTT	:	1, 4-dithio-D-threitol
EDTA	:	Ethylenediamminetetraacetate
ESI MS	:	Electro spray ionisation mass spectrometry
g	:	Relative centrifugal force
g, mg, µg, ng, kg	:	Gram, milligram, microgram, nanogram, kilogram
GFP	:	Glu1-fibrinopeptide B
HbA1c	:	Glycated hemoglobin
HCl	:	Hydrochloric acid
HDL	:	High density lipoprotein
HDMS	:	High definition mass spectrometry
H&E	:	Hematoxylin and eosin
HSA	:	Human serum albumin
IAA	:	Iodoacetamide
IgG	:	Immunoglobin G
IPG	:	Immobilized pH gradient

$LCMS^E$	:	Liquid chromatography-MS at elevated energy
L, dL, ml, µl	:	Litre, decilitre millilitre, microlitre
MALDI MS	:	Matrix associated laser desorption ionisation MS
MS	:	Mass-spectrometry
MS^E	:	MS at elevated energy
NH_4HCO_3	:	Ammonium bicarbonate
PAS	:	Periodide acid staining
PLGS	:	Protein Lynx Global Software
PVDF	:	Polyvinylidene fluoride
SD	:	Standard deviation
STZ	:	Streptozotocin
TBS	:	Tris buffered saline
TBS-T	:	Tris buffered saline tween 20
TIC	:	Total ion chromatogram
V, kV	:	Volt, kilovolt
Δ M	:	Increase in mass
2DE	:	Two dimensional gel electrophoresis
°C	:	Degree Celsius

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THESIS ABSTRACT

The blood glucose is not directly related to the microvascular diabetic complication as previously thought, but highly reactive molecules including 3-DG, methyl glyoxal and AGEs (Advanced Glycation End Products). These heterogeneous molecules are implicated in the pathogenesis of diabetic complications leading to acute health problems. Further, AGEs covalently interact with proteins causing structural and functional alterations. The primary targets of such alterations are plasma proteins due to prolonged exposure to elevated glucose levels. Therefore, association of AGEs in regulation of diabetes and diabetic complications was mechanistically studied by identifying and characterizing glycated proteins using a combination of 2DE, Western blot and Mass spectrometric approaches.

'Factors influencing glycation reaction *in vitro*'

Glycation, a non-enzymatic reaction between glucose and protein is the primary cause of diabetic complications. Albumin, the most abundant plasma protein undergoes glycation both in vivo and in vitro. The influence of albumin on glycation of less abundant proteins has not been addressed. For the first time, we show that albumin competitively inhibits the glycation of less abundant proteins, suggesting that at least in the initial stages of diabetes, albumin may protect other proteins from glycation. Also, glycation is known to be restricted to certain proteins. Previous studies report that glycation is dependent mainly on protein structure and its turnover. The role of molecular mass of protein and protein abundance in determining glycation was addressed in this study. Large molecular mass proteins such as IgG, HSA, and BSA, upon glycation showed higher increase in mass compared to small molecular mass proteins such as papain, apomyoglobin and insulin. Also, the extent of glycation was found to be more in the HSA, BSA and IgG compared to papain, apomyoglobin and insulin. This study combined with previous study on albumin glycation suggests that, in addition to the protein structure and turnover, the molecular mass of protein as well as protein abundance determines the glycation.

‘Association of albumin levels with plasma protein glycation and HbA1c in diabetes’

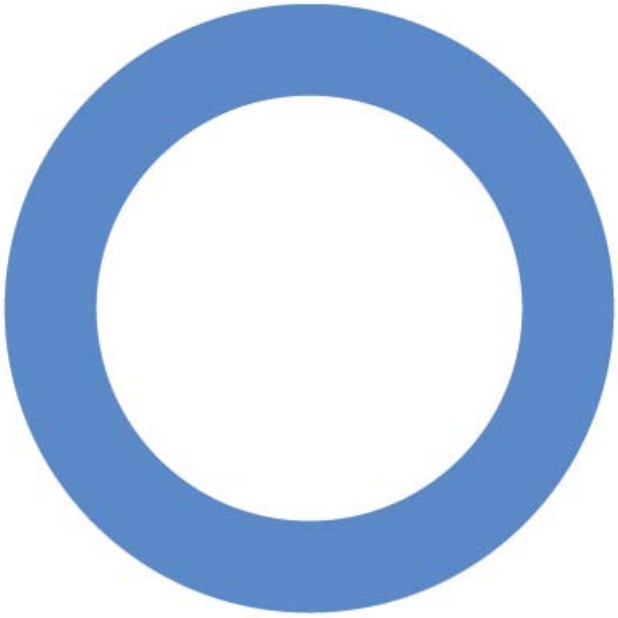
Albumin is one of the most abundant plasma proteins and heavily glycated in diabetes. In this study we have addressed whether variation in the albumin levels influences glycation of plasma proteins and HbA1c. The study was performed in three systems (1) streptozotocin (STZ) induced diabetic mice plasma (2) diabetic clinical plasma (3) *in vitro* glycated plasma. Diabetic mice and clinical plasma samples were categorized as diabetic high albumin plasma (DHAP) and diabetic low albumin plasma (DLAP) based on their albumin levels. While for *in vitro* experiment, two albumin levels, high albumin plasma (HAP) and low albumin plasma (LAP) were created by differential depletion of plasma albumin. Protein glycation was studied by using a combination of two dimensional electrophoresis (2DE), western blotting and LC-MS^E. Identification of glycation modification was achieved by using “zoom in” approach by performing targeted database search. In both mice and clinical experiments, increased plasma protein glycation was observed in DLAP than DHAP. Additionally, plasma albumin levels were negatively correlated with HbA1c. *In vitro* experiments with differential depletion of albumin mechanistically showed that the low albumin levels are associated with increased plasma protein glycation, and albumin competes for glycation with other plasma protein.

‘Proteomic study reveals down regulation of Apolipoprotein A1 in plasma of poorly controlled diabetes’

Differential protein expression in diabetic plasma sample was studied by a combination of proteomic and western blot approaches. Plasma samples were categorized depending on HbA1c levels as non diabetic (ND) with HbA1c >5.8%, controlled diabetic (CD) with HbA1c 7-8 % and poorly controlled diabetic (PCD) with HbA1c > 8%. Ten plasma samples from each group were used for proteomic studies involving 2DE and $LCMS^E$. Amongst six differentially expressed proteins in diabetes, the down-regulation of apolipoprotein a1 was more prominent in poorly controlled diabetes. Down regulation of apolipoprotein A1 could be a potential early marker of diabetic complications.

CHAPTER I

“INTRODUCTION”



I.1. Diabetes

I.1.1. Definition

Diabetes is a group of metabolic disorders characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Expert Committee, 2003). The characteristic symptoms of diabetes mellitus include thirst, polyuria, blurring of vision, and weight loss. Ketoacidosis is the most severe forms of diabetes, a condition developed resulting into semiconsiousness and ineffective treatment might ultimately lead to coma death. Often the symptoms of diabetes are not severe or may be absent and therefore hyperglycemia which sufficiently causes pathological and functional changes might be present for a long time even before the diagnosis of diabetes is achieved. Prolonged elevated glucose in diabetes mellitus results into progressive development of the specific complications. These complications include retinopathy leading to eye damage with potential blindness, nephropathy, a primary cause for renal failure and neuropathy with possible risk of foot ulcers, amputation, neuropathic arthritis and sexual dysfunction. Increased risk of cardiovascular, peripheral vascular and cerebrovascular diseases is associated with people affected by diabetes. The involvement of several pathogenic processes including destruction of the β-cells of the pancreas with consequent insulin deficiency and resistance to insulin action has known to be major factors in the development of diabetes. Further, due to deficient action of insulin and insulin insensitivity on target cells results in abnormalities of carbohydrate, fat and protein metabolism (Alberti et al., 1998).

I.1.2. Classification of Diabetes

Diabetes mellitus is represented by a heterogeneous group of disorders. Some distinct diabetic phenotypes can be characterized in terms of specific etiology or pathogenesis, but in many cases etiological and pathogenetic classification has become difficult due to overlapping phenotypes (Leslie, 1997). Broadly, diabetes mellitus can be classified into three main types of diabetes: (a) type 1 diabetes which results from failure in insulin secretion, (b) type 2 diabetes which is a result of insulin

resistance and (c) gestational diabetes which is observed among pregnant women, who never had diabetes before but have a high blood glucose level during pregnancy.

I.1.2.1. Type 1 Diabetes

Type 1 diabetes was formerly known as insulin-dependent diabetes mellitus (IDDM). Both adults and children were known to be affected by type 1 diabetes but, traditionally the later termed "juvenile diabetes" or "childhood-onset diabetes" as it represents a majority of the diabetes cases in children. Type 1 diabetes mellitus is characterized by destruction of the β-cells of the islets of the pancreas, with consequent insulin deficiency and is often associated with elevated blood glucose levels and symptoms including polyuria, polydipsia, and unexplained weight loss. Type 1 diabetes can be further classified as idiopathic or immune-mediated. Idiopathic refers to rare forms of diabetes with unknown cause. The majority of type 1 diabetes is of the immune-mediated nature, where β-cell loss is through T-cell mediated autoimmune attack destroying the cells in the pancreas that produce insulin (Rother, 2007). Further, the autoantibodies for glutamic acid carboxylase (anti-GAD) serve as autoimmune marker in 85- 90 % type 1 diabetes (Verge et al., 1996). The key symptoms of type 1 diabetes include high blood glucose levels, frequent urination, blurred vision, unusual thirst, extreme hunger, irritability and nausea, extreme hunger but loss of weight, nausea and vomiting, extreme weakness and fatigue.

I.1.2.2. Type 2 Diabetes

Type 2 diabetes was formerly known as non-insulin-dependent diabetes mellitus (NIDDM), adult-onset diabetes or obesity-related diabetes. The cause of type 2 diabetes has been often insulin resistance which may be combined with relatively reduced insulin secretion. Although patients with type 2 diabetes may have insulin levels that appear normal, however insulin levels always are relatively low compared to the elevated plasma glucose levels (Ward et al., 1984). The insulin resistance developed in type 2 diabetes is mainly because of defective insulin receptor. However, the involvement of specific defects in the insulin receptor still remains unclear. Type 2 diabetes accounts for 90 percent amongst all the types of diabetes. In the early stage of type 2 diabetes, the predominant abnormality is reduced

insulin sensitivity. At this stage hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or maintain blood glucose levels. Many people with type 2 diabetes have none of the usual symptoms and therefore remain oblivious to the problem for years, until complications begin to appear.

I.1.2.3. Gestational Diabetes

Gestational diabetes mellitus (GDM) also referred as "type 3 diabetes" resembles type 2 diabetes in several respects, involving a combination of relatively inadequate insulin secretion and responsiveness. About 5% women suffer from gestational diabetes during the pregnancies but after delivery it has been observed that the diabetic condition may improve or disappear. Thirty to forty percent of women experiencing gestational diabetes develop type 2 diabetes within five to ten years. Gestational diabetes is fully treatable only if critical medical supervision including glucose monitoring and insulin therapy is achieved effectively throughout the pregnancy (Homko and Khandelwal, 1996). A study conducted in 2008 among American women has shown an increased number of preexisting diabetes in pregnant women. In fact the rate of diabetes in expectant mothers has been doubled in the past 6 years (Lawrence et al., 2008). The two major factors that are strongly associated with the onset of gestational diabetes are weight gain and the production of hormone resistin, responsible for altering the insulin function and signaling (Saldana et al., 2006; Kuzmicki et al., 2009). Several risks to the newborn baby including macrosomia (high birth weight), congenital cardiac and central nervous system anomalies, and skeletal muscle malformations might be developed by the women having gestational diabetes (Ben-Haroush et al., 2009).

I.1.2.4. Other types of Diabetes Mellitus

Besides the above mentioned three major types of diabetes, other forms of diabetes mellitus have been also observed and are classified as given below.

(a) Pre-diabetes: Pre-diabetes also known as impaired glucose regulation which includes impaired glucose tolerance (IGT) and impaired glucose fasting (IGF). Impaired glucose regulation is a metabolic condition that occurs when blood glucose

levels are higher than normal but not high enough for a diagnosis of type 2 diabetes. IGT and IGF are well characterized pre-diabetic conditions and about half of such individual's progress to type 2 diabetes over their lifetime (Gerstein et al., 2007).

(b) Cystic fibrosis related diabetes mellitus (CFRD): Pancreatic viscous secretion is altered by abnormal functioning of the chloride channels during cystic fibrosis thereby causing obstruction of the exocrine pancreas. This mechanism might lead to the destruction of the islets in turn leading to loss of β-cells (Moran, 1991). Further, the hepatic insulin resistance with elevated glucose production is associated with the development of CFRD (Hardin, 1999).

(c) Steroid induced diabetes mellitus: Glucocorticoids are employed for the therapy for bowel diseases, alcoholic and autoimmune hepatitis, and after liver transplantation. The excess or high doses of glucocorticoids results in insulin resistance developing into steroid induced diabetes mellitus. Prolonged treatment with glucocorticoid alters glucose metabolism resulting in hyperinsulinemia and increased cardiovascular risk (Kern et al., 1999).

(d) Latent autoimmune diabetes of adults (LADA): Latent autoimmune diabetes of adults also known as "type 1.5 diabetes" is a condition in which patients belongs to type 1 diabetes; although the autoimmune destruction of their islets β-cells develops often slowly (Tuomi et al., 1993; Zimmet et al., 1999). However, the processes of β-cell destruction in LADA patients are quite different, which can be discriminated by both the number of islet antibodies and the titer of glutamic acid decarboxylase autoantibody (GADA). Adults with LADA are frequently misdiagnosed as type 2 diabetes, based on age rather than etiology.

(e) Malnutrition-modulated diabetes mellitus (MMDM): It was previously known as protein-deficient diabetes mellitus (PDDM). MMDM develops over a background of chronic malnutrition from childhood. MMDM patients are extremely lean and require high doses of insulin for good glycemic control (Samal et al., 2002).

I.1.3. History of Diabetes

The history of diabetes in over 3000 years old referring to the only holograph, Ebers Papyrus, which was written around 1500 BC (McFarlane et al., 1997). Ebers Papyrus was excavated in 1862 AD from ancient grave in Thebus, Egypt that describes the reference of diabetes as "too great emptying of the urine" and recommending a treatment of a liquid extract of bones, grain, grit, wheat. Nearly at the same time, Indian physicians also developed a clinical test for diabetes by observing the urine from diabetic people that attracted ants and flies and named it as "madhumeha" or "honey urine". Approximately 1370 years later, for the first time the term "diabetes" which in Greek means "to pass through" was used by Appollonius of Mephis. In the second century a Greek physician, Aretaeus of Cappadocia distinguished between diabetes mellitus and diabetes incipidus. His writings gave a detailed account of diabetes, "*Diabetes is a dreadful affliction, not very frequent among men, being a melting down of the flesh and limbs into urine. The patients never stop making water and the flow is incessant, like the opening of the aqueducts. Life is short, unpleasant and painful. If for a while they abstain from drinking, their mouths become parched and their bodies dry, the patients are affected by nausea, restlessness and burning thirst, and within a short time they expire*" (Zajac et al., 2002). The classification of diabetes mellitus was first time reported by two Indian ancient physicians Sushruta and Charaka in the 5th century AD that differentiated diabetes as, thin diabetics who developed diabetes at the young age and heavier diabetics, who had a late onset and lived for a long time (Papaspyros, 1964). Subsequently in the late 18th century John Rollo, Surgeon General to British Army, added the term "mellitus" to diabetes in order to distinguish it from incipidus (Rollo, 1797). In 1869, Paul Langerhans discovered that the human pancreas contained two kinds of cells, although he was unaware of their function. Some 20 years later, Josef von Mering and Oskar Minkowski found that diabetes could be induced in dogs by removing their pancreases, suggesting that this organ was somehow central to the regulation of blood sugar. In 1921, two Canadians, Frederick Banting and Charles Best, under the supervision of Professor John Macleod at the University of Toronto, injected pancreatic extract to a 14 year old boy, Leonard Thompson and observed blood glucose drop from 520mg/dl to 120 mg/dl. It was then; this anti-diabetic

substance was named as ‘insulin’ for which Banting and Macleod were awarded with a Nobel Prize for discovery of Insulin in 1923.

I.1.4. Global Burden of Diabetes

The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity (Wild et al., 2004). As a primary source of information, the numerical estimates and projections for the frequency of diabetes in all countries were assembled by the World Health Organization (WHO) Ad Hoc Diabetes Reporting Group (King et al., 1998). The preliminary observations have estimated that globally, the number of people suffering from diabetes will rise from 151 million in the year 2000 (Amos et al., 1997) to 300 million by 2025 (King et al., 1998).

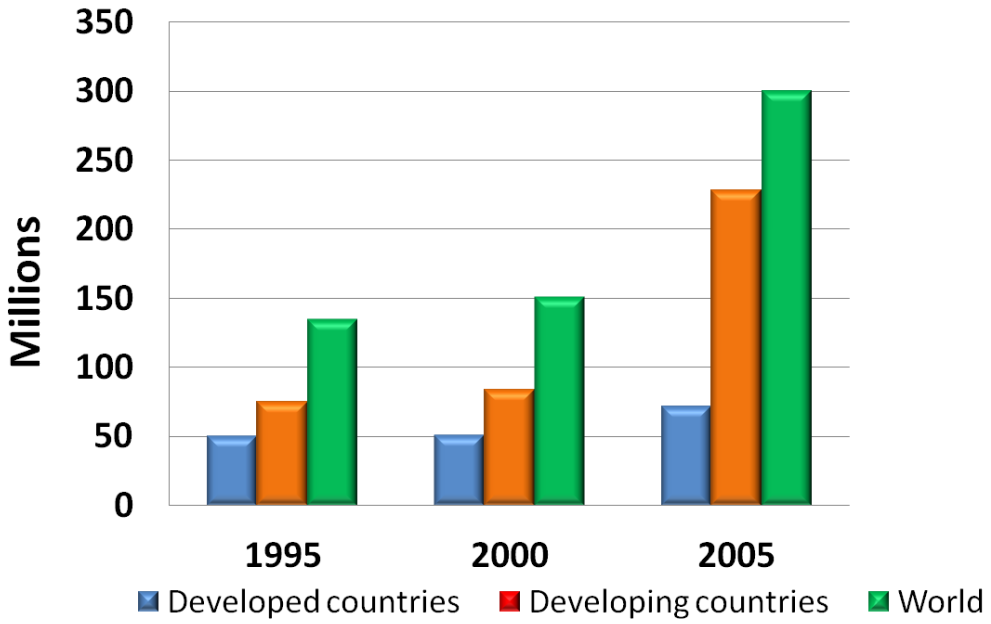


Figure 1. 1: Number of people with diabetes in the adult population by region and year 1995, 2000 and 2025. *Adapted from King et al., 1998.*

The top three countries for estimated number of adults with diabetes between 2000 and 2025 are India, China, and the U.S.A. with India alone has 31 million diabetic people at present. The number might increase to 79 million by 2025 which is more than double (Wild et al., 2004). The major part of this numerical increase will

occur in developing countries. There will be a 42% increase in the number of diabetes adults in the developed countries and about 170% increase, in the developing countries. Thus, by the year 2025, >75% of people with diabetes will reside in developing countries. In developing countries, the majority of people with diabetes fall in the range of 45-64 years compared to the developed countries, where it is above 65 years of age (King et al., 1998).

Table 1. 1: List of countries with the highest numbers of estimated cases of diabetes for 2000 and 2030. (*Table adapted from Wild et al., 2004)*

	2000		2030	
Ranking	Country	People with diabetes (millions)	Country	People with diabetes (millions)
1	India	31.7	India	79.4
2	China	20.8	China	42.3
3	U.S.A.	17.7	U.S.A.	30.3
4	Indonesia	8.4	Indonesia	21.3
5	Japan	6.8	Japan	13.9
6	Pakistan	5.2	Pakistan	11.3
7	Russian Federation	4.6	Russian Federation	11.1
8	Brazil	4.6	Brazil	8.9
9	Italy	4.3	Italy	7.8
10	Bangladesh	3.2	Bangladesh	6.7

The global prevalence of diabetes in adults was estimated to be 4.0% in 1995 and may probably rise to 5.4% by the year 2025. Prevalence of diabetes is higher in developing countries than in developing countries. The prevalence of diabetes is higher in men than women, but there are more women with diabetes than men, especially in developed countries (Wild et al., 2004) and diabetes will be increasingly concentrated in urban areas. Therefore diabetes in adults is now a global health problem, and populations of developing countries, minority groups, and disadvantaged communities in industrialized countries now face the greatest risk (King et al., 1993). Hence it is important to quantify the prevalence of diabetes and the number of people affected by diabetes which will allow rational planning and proper allocation of required resources.

I.1.5. Etiology of Diabetes

I.1.5.1. Obesity

Various etiological factors are responsible for the development of diabetes including obesity. Populations with the highest rate of diabetes, such as the Pima and Nauruans also have very high rates of obesity (Zimmet, 1982). Studies that have been carried for decades together suggest the increasing likelihood of development of diabetic complications according to the obesity levels. A study among women demonstrated that with increasing body mass index (BMI), the risk of developing diabetes increases (Colditz et al., 1990) and women with an average weight having a normal range of BMI were at increased risk of diabetes than does a lower BMI. Apart from BMI, waist to hip ratio (WHR) and waist circumference (WC) is also measured to determine the incidence of diabetes. In the health professional's follow-up studies over 27,000 men higher WC, WHR, and BMI predicted development of diabetes over 13 years. The cumulative proportions of type 2 diabetes cases identified according to the medians of BMI (≥24. 8), WC (≥94 cm), and WHR (≥0. 94) were 82.5%, 83.6%, and 74.1%, respectively and were 7.2 times higher than the recommended cut-offs (Wang et al., 2005).

I.1.5.2. Physical Activity and Exercise

Contracting skeletal muscle takes up more glucose from the blood during the physical activity. This effect is partly mediated by adrenaline and is positively associated with insulin sensitivity (Richter et al., 1982) which replenishes the glycogen storage thereby improving the carbohydrate metabolism during exercise. In addition, exercise has benefits in contributing to weight loss thereby influencing the development of diabetes. In a study of US male physicians, vigorous activity undertaken at least once a week led to a relatively less risk of developing type 2 diabetes, in comparison to those exercising less frequently and the effect was strongest in the most obese. (Manson et al., 1992). A similar study in 85,000 women also resulted in reducing the incidence of diabetes with regular vigorous exercise (Manson et al., 1991). Additionally, several cross-sectional studies have related the amount of time spent on watching television to the risk of having impaired glucose

tolerance and the relation has found to be significant (Dunstan et al., 2004). Indeed, the relationship appears to be stronger for television viewing time than they are for time spent undertaking physical exercise. Therefore, physical activity plays an important role in delaying or prevention of development of type 2 diabetes directly by improving insulin sensitivity and reducing insulin resistance, and indirectly by the beneficial changes in body mass and body composition (Boule et al., 2001; Hamman et al., 2006; Kay and Fiatarone, 2006).

I.1.5.3. Dietary Intake

Diet plays a very important role in the development of diabetes. However it has been remarkably difficult to pin down the precise dietary constituents that are the key players. Several studies have been carried out to understand the importance of dietary intake in the development of diabetes. A study among approximately 1300 cases was undertaken by van Dam et al., 2002 in Boston, USA. Two major dietary patterns were developed including a "prudent diet" characterized by higher consumption of vegetables, fruit, fish, poultry and whole grains and a "western diet" characterized by higher consumption of red meat, processed meat, french fries, high-fat dairy products, refined grains, sweets and desserts. Relative risk after consumption of these diets was studied over a period of twelve years. The Western dietary pattern showed increased risk of diabetes compared to the prudent dietary pattern suggesting the association of higher dietary glycemic index with elevated risk of diabetes (van Dam et al., 2002). In addition, another study performed by Harvard School of Public Health, the high intake of red meat, low-fiber bread and cereal, dried beans, fried potatoes, tomato vegetables, eggs, cheese, and cottage cheese and low intake of wine were positively associated with markers of inflammation plasminogen activator inhibitor-1 (PAI-1) and fibrinogen that have been associated with risk of development of type 2 diabetes (Liese et al., 2009). A recent study has shown that regular consumption of white rice is associated with an increased risk of type 2 diabetes whereas replacement of white rice with brown rice or other whole grains lowers the risk of type 2 diabetes (Sun et al., 2010). The higher intake of saturated and trans fat is associated with type 2 diabetes by adversely affecting glucose metabolism and insulin resistance, (Vessby et al., 1994; Hu et al., 2001) whereas higher intake of unsaturated

fats appear to be protective by lowering the incidence of diabetes by 25 % (Salmerón et al., 2001). Another prospective study has shown higher consumption of butter, potatoes and whole milk is associated with the increased risk of type 2 diabetes while higher consumption of fruits and vegetable was associated with the reduced risk of type 2 diabetes (Montonen et al., 2005) suggesting the possible mechanism of insoluble fiber in consistently improving insulin sensitivity and thereby decreasing the risk of the same (Salmeron et al., 1997; Meyer et al., 2000). Furthermore, large observational studies have suggested an association between low vitamin D status or low vitamin D intake and increased incidence of type 2 diabetes (Knekt et al., 2008; Pittas et al., 2006). The suggested mechanisms are attributed to vitamin D deficiency which may contribute to β-cell dysfunction, insulin resistance and inflammation that may result in type 2 diabetes. The effect of dietary habits in all these studies has been shown to be independent of BMI change.

I.1.5.4. Hypertension

Previous prospective and case control studies have shown that progression in hypertension as an independent predictor of type 2 diabetes. A relationship of blood pressure (BP) and BP progression in the subsequent development of type 2 diabetes was shown among initially healthy women (Conen et al., 2007). The association between type 2 diabetes and hypertension has been attributed to several possible factors including endothelial dysfunction, a common pathophysiological pathway. Studies have shown the markers of endothelial dysfunction E-selectin, Intercellular Adhesion Molecule 1 (ICAM-1), and Vascular cell adhesion protein 1 (VCAM-1) were associated with the onset of diabetes (Meigs et al., 2004), and endothelial dysfunction is closely related to blood pressure and hypertension (Meigs et al., 2006). Markers of inflammation such as C-reactive protein have been consistently related to incidence of type 2 diabetes (Hu et al., 2004) and increased blood pressure levels (Blake et al., 2003), suggesting, inflammation might be another explanatory factor for the association between blood pressure, the metabolic syndrome and incidence of type 2 diabetes (Ridker et al., 2003). Further, insulin resistance could be another potential link between blood pressure levels and the incidence of type 2 diabetes (Ferrannini et al., 1987). A relationship between hypertension and type 2 diabetes was further

strengthened by a recent randomized clinical trial showing a 14% reduction of risk of diabetes in subjects with impaired glucose tolerance and cardiovascular disease after administering valsartan, arenin-angiotensin system blocker, over a period of 5 years (NAVIGATOR study group et al., 2010).

I.1.5.5. Smoking

Active smoking is associated with an increased risk of type 2 diabetes (Willi et al., 2007; Sairenchi et al., 2004). The risk of developing type 2 diabetes is 2.1 times more in smokers consuming more than 20 cigarettes per day when compared with non-smokers. Therefore cigarette smoking is an independent and a modifiable determinant of type 2 diabetes mellitus (Manson et al., 2000). Further, prolonged smoking results into development of insulin resistance and inadequate compensatory insulin secretion response (Attvall et al., 1993; Facchini et al., 1992). This effect could be due to a consequence of nicotinic components of cigarette smoke on β-cells of the pancreas as which is known to be associated with chronic pancreatitis and pancreatic cancer (Talamini et al., 1999). Although cigarette smoking predicts incident of type 2 diabetes, but smoking cessation is associated with weight gain and leads to higher short-term risk (Willi et al., 2007; Yeh et al., 2010). Therefore, for smokers who are at a risk for diabetes, smoking cessation should be paired off with certain schemes involving early detection and prevention of diabetes. However, a follow up over a longer period of time have shown a reduction in risk of development of type 2 diabetes after smoking cessation (Wannamethee et al., 2001).

I.1.5.6. Ethnicity

According to the 2000 census data ethnic minorities constitute approximately 25% of the overall population of the United States (Anonymous, 2012). For example, in comparison with the prevalence rate of diabetes in white Americans, the relative increase in the prevalence of type 2 diabetes is 10 fold greater in certain Native American ethnic groups (Burke et al., 1999; Flegal et al., 1991). In the last fifteen years the increase in prevalence of diabetes has been high (about 68%) among Asians (McBean et al., 2004). The prevalence of diabetes among urban population in India is 20% compared to rural population which is about 10% and is mainly attributed to

sedentary lifestyles among the urban population (Ramachandran, et al., 1999). Diabetes prevalence substantially observed among ethnic groups could be attributed to negligence for care along with several other socioeconomic factors aggravating the unequal burden of diabetes among minority populations.

I.1.5.7. Family History

Risk factors for type 2 diabetes are well established and include underlying genetic susceptibility which is reflected by family history. Genetic components play an important role in pathogenesis of type 2 diabetes (Amini and Janghorban, 2007). Diabetic family history among first degree relatives confers an increased risk of developing type 2 diabetes and the risk is even greater when both parents are diabetic (Bjornholt et al., 2000; Ma et al., 2008). Both maternal and paternal transmission of diabetes significantly contributes and influences the risk of developing diabetes in offsprings. Among Indian population, there is increased risk of developing diabetes increases two- to fourfold for an individual with a positive family history of diabetes (Padaki et al., 2011). Very high risk for abnormal glucose homeostasis among offspring with young age-of-onset maternal diabetes is observed (Meigs et al., 2000). Therefore, globally evaluation of family history could be a promising new public health tool to fight against the growing epidemic of diabetes.

I.1.5.8. Genetics

Over a past decade, there has been a spectacular change in the capacity to identify common genetic variants that contribute to the development of diabetes (Swapan and Stevan, 2006). Gene variants including transcription factor 7-like 2 (TCF7L2) (Grant et al., 2006), peroxisome proliferator-activated receptor gamma (PPARG) (Altshuler et al., 2000), fat mass and obesity-associated protein (FTO) (Frayling et al., 2007), KATP channel subunits Kir6.2 (KCNJ11) (Gloyn et al., 2003b), NOTCH2, wolframin (WFS1) (Saxena et al., 2007), CDK5 regulatory subunit associated protein 1-like 11(CDKAL1), insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2), solute carrier family 30 member 8 (SLC30A8), juxtaposed with another zinc finger protein 1 (JAZF1) and hematopoietically-expressed homeobox protein (HHEX) has significantly associated with the risk of type 2 diabetes. These

eight genes are also associated with impaired β-cell function and are independent of other clinical risk factors (Lyssenko et al., 2008a). Most of these gene variants are responsible for reducing insulin sensitivity and insulin secretion among most of the diabetes cases (Elbein et al., 2000; Gerich et al., 1998). The expression of TCF7L2 the transcription factors 7-like 2, is the locus with the highest risk of type 2 diabetes amongst all the variants (Lyssenko et al., 2008b) corresponding to approximately 25% of risk, due to an average single allele frequency 18-30% in Northern Europeans (Cauchi et al., 2008). Through various efforts, approximately 20 common variants are now robustly implicated in type 2 diabetes susceptibility (Prokopenko et al., 2008). According to James Neel's 'thrifty gene' hypothesis, postulating that, such genes allow efficient food utilization, fat deposition and rapid weight gain in times of plenty, in preparation for famine, thereby making the gene-bearer to survive in a subsequent famine (Neel, 1962). Such genes would be advantageous for traditional human lifestyle, but they would lead to obesity and diabetes in the modern world when the same individuals lack physical activity. Apart from genes predisposing to type 2 diabetes, further investigation is required to define the causative variants thereby understanding the basis of disease mechanisms which is known to affect the pancreatic β-cell function leading to the development of type 2 diabetes.

I.1.5.9. Sociocultural Factors

Apart from the biomedical risk factors that are involved in the development of diabetes, sociocultural factors have also known to play a significant role. The impacts of urbanization have changed many traditional lifestyles into modern lifestyle by altering the dietary habits of the population and therefore inclining towards the development of type 2 diabetes. In a recent study of the Cambodian population, the prevalence of diabetes was twice in the semi urban community as compared to the rural community (King et al., 2005). Another Chennai Urban Rural Epidemiology Study which screened 26,000 individuals, the prevalence of diabetes among the urban Indian population was more than the rural counterparts. Also, a cross-sectional population survey undertaken by Diabetes India group has revealed that the prevalence of diabetes in urban population is higher compared to rural population (Sadikot et al., 2004). A negative correlation has been observed between

socioeconomic status and type 2 diabetes population from United Kingdom. However, the increased prevalence of diabetes is associated with people living in the most deprived areas (Connolly et al., 2000). The possible explanation for this observation could be lack of information about healthy food and complete dietary food practice due to deprived access to health information. On the contrary, in developing countries the impact of poverty has known to decrease the prevalence of diabetes. A study involving south India and China, the higher income groups were twice as likely to have diabetes as the lower income group (Ramachandran et al., 2002). In the developing countries, poor people are involved in manual work and therefore have limited chance to develop diabetes. Moreover, rural population practice traditional food, which comprises of more fruit and vegetables helping in lowering the risk of development of diabetes than the wealthier population with ready access to westernized food (Ekoé et al., 2008).

I.1.6. Screening Detection and Diagnosis of Diabetes

Diabetes mellitus is a common and serious disease that has widely affected the global population where about 30% of the affected population is unaware of being affected (Centres for Disease Control and Prevention, 1997; Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). During clinical diagnosis of diabetes, the individual is frequently observed with several risk factors for macro as well as microvascular complications (Harris and Modam, 1994; Whiteakar et al., 1997). Most of the times the onset of diabetes may occur about 9-12 years before the diagnosis were made by the medical practitioner (Harris et al., 1992). Therefore early detection of type 2 diabetes through screening may be an appropriate public health strategy to prevent or delay diabetic complications and improve health outcomes (Pauker, 1993). A major distinction between diagnostic testing and screening is elucidated in Table 2. When an individual exhibits symptoms or signs of the disease, diagnostic tests are performed whereas the purpose of screening is to differentiate an asymptomatic individual at high risk of an individual at low risk for diabetes (Engelgau et al., 2000).

Table 1. 2: Proposed criteria for screening and diagnosis of diabetes. FPG, fasting plasma glucose; A1c, Glycated hemoglobin; RPG, random plasma glucose; OGTT, Oral Glucose Tolerance Test. *(Table adapted from Saudek et al., 2008)*

Screening	Diagnosis
FPG ≥ 100 mg/dl	FPG≥ 126 mg/dl
HbA1c > 6.0%	HbA1c > 6.5%
RPG ≥ 130 mg/dl	RPG ≥ 200 mg/dl
If a screening result is negative, screen again in 3 years	2-h OGTT ≥ 200 mg/dl
If a screening result is positive but below the diagnostic threshold, perform another test for diagnosis, using different method	Diagnosis requires confirmation unless unequivocal symptoms of hyperglycemia are present
If a screening result is above the diagnostic threshold but the second test does not reach threshold, test again in 1 year	Diagnosis based on HbA1crequires confirmation using glucose dependant test (FPG or OGTT) or, if HbA1c is ≥ 7 %, by a second HbA1c ≥ 6.5%

The World Health Organization (WHO) recommends following revised criteria for diagnosis diabetes mellitus:

1. Fasting plasma glucose (FPG) concentration is greater than or equal to 7 mmol/L (140mg/dL).
2. Postprandial 2-h plasma glucose concentration is greater than or equal to 11.1 mmol/L (200mg/dL) during an oral glucose tolerance test (OGTT) (Alberti et al., 1998).
3. Symptoms of diabetes and a casual (i.e., regardless of the time of the preceding meal) plasma glucose concentration greater than 11.1 mmol/L (200 mg/dL).
4. Hemoglobin A1c (HbA1c) greater than 6.5% (48 mmol/mol).

If one of the above criteria is met, confirmation of the test is required by repeating the test on subsequent days. In 2009, the International Expert Committee (The International Expert Committee, 2009), which comprises of members appointed by the American Diabetes Association (ADA), the European Association for the Study of Diabetes (EASD), and the International Federation of Diabetes (IDF), recommended that diabetes can be diagnosed by measurement of HbA1c, which reflects long-term blood glucose concentrations. The ADA and the WHO have certified the use of HbA1c for diagnosis of diabetes (American diabetes association,

2010). Moreover, the annual testing for albuminuria is recommended by all major guidelines for diabetic patients where the level of urine albumin level (corresponding to 30mg albumin/g of creatinine) is used for determining the rate of disease progression in diabetes particularly in end-stage renal disease (Sacks et al., 2011). Apart from the all above parameters, measurement of insulin and islet cell antibodies from the blood can be used for diagnosis of diabetes only after standardizing the prospective clinical studies. Similarly, evaluation of insulin and C-peptide concentration in blood and ketone bodies in urine also requires further prospective studies in order to consider as a diagnostic marker for diabetes.

I.2. Diabetes and Glycation

Diabetes is characterized by hyperglycemia having plasma glucose levels more than 200 mg/dl. Prolonged exposure of plasma proteins to the elevated blood glucose has been observed in diabetic patients with poor glycemic control (Austin et al., 1987). Several plasma proteins including hemoglobin, serum albumin and transferrin have shown glycation, a post translational modification (PTM) caused by non-enzymatic reaction between glucose and protein (Ulrich and Cerami, 2001). Glycation is a chemical modification of proteins where condensation between the carbonyl group of glucose and free amino group of protein leads to the formation of the Schiff's base. This is the first of step glycation reaction, the formation of the Schiff base from sugar and amine is relatively fast and highly reversible (Ulrich and Cerami, 2001). The next step involves conversion of thermodynamically unstable Schiff's base into a stable reversible Amadori product. Proteins bearing Amadori product are referred as glycated proteins. Finally, the Amadori product undergoes a series of dehydration and fragmentation reactions and results into a variety of carbonyl compounds including methylglyoxal, glyoxal, glucosones, 3-deoxyglucosone (3-DG) and so on (Thornalley et al., 1999). These carbonyl compounds are more reactive than the original sugar and act as propagators of reaction leading to the formation of advanced glycation end products (AGEs). The mechanisms involved in glycation and the formation of AGEs are illustrated in Fig 1.2.

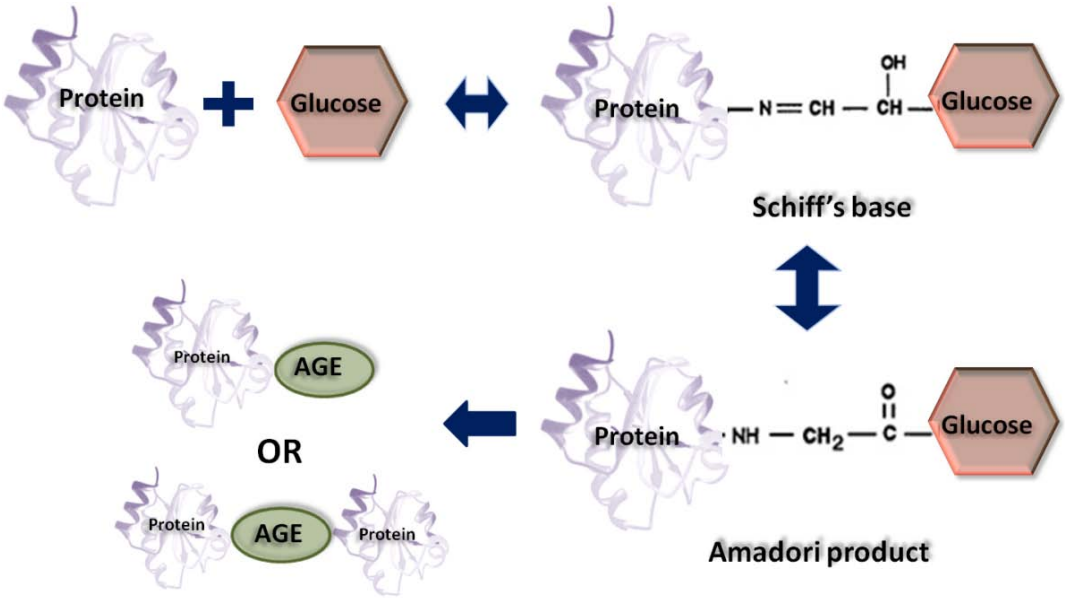


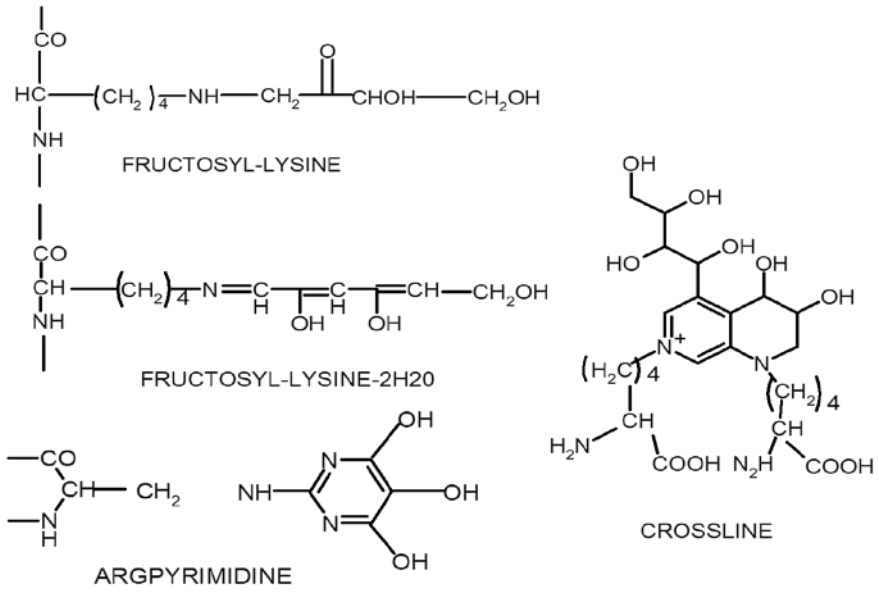
Figure 1. 2: Mechanism of formation of advanced glycation end products (AGEs).

Glycation reaction preferentially takes place at epsilon amino groups of amino acids including lysine, arginine and histidine; however, crosslinking of AGEs with protein is more specific at arginine (Münch et al., 1999). Different modifications have been reported on glycated proteins and have been characterized by mass spectrometric analysis. For example, as shown in the Table 1.3 glycation modification at lysine residue shows increase in mass of 162.0258 Da. Several other modifications like carboxymethyllysine (CML), carboxyethyllysine (CEL) and pyrraline have been reported in the previous studies (Fig 1.3) (Wa et al., 2007; Thornalley., 1990; Biemel et al., 2001). Protein glycation has strongly decreased the activity of hexokinase and glucose-6-phosphate dehydrogenase (Kiho et al., 1996). The key antioxidant enzymes, glutathione peroxidase and glutathione reductase are inactivated by glycation reaction (Vander, et al. 1997; Niwa and Tsukushi, 2001). Further, AGEs have known to interact with the amino groups of several other proteins including collagen thus resulting in the formation of protein cross-links (Reddy, 2004).

Quantitative screening of a comprehensive range of AGEs, have shown that intracellular proteins are heavily glycated than extracellular proteins due to higher amount of intracellular AGEs (Thornalley et al., 2003b). The probable reason of

increase in intracellular AGEs is the auto oxidation of glucose resulting in the formation of either glyoxal or deoxyglucosone or methylglyoxal (Wells-Knecht et al., 1995; Thornalley et al., 1999). Three major mechanisms are known for the damage of the target cells due to production of AGEs. 1) AGE modification of intracellular proteins alters their function, 2) the extracellular matrix components and 3) AGE modified plasma protein binds to the receptor for AGE (RAGE) thereby inducing receptor-mediated production of reactive oxygen species (ROS) (Brownlee, 2001).

AGEs are substantive contributors to the progression of diabetic complications. If the glycation site of a protein occurs close to the protein's active sites or if stereochemical configuration is disturbed, then the protein function is known to be altered (Taylor and Agius., 1988). The involvement of several glycated proteins has been implicated in the development of glycation induced complications in diabetes. Glycated fibrin has shown lesser susceptibility for fibrin digestion (Brownlee et al., 1983), thereby resulting into permanent vascular occlusion. Glycation of several lipoproteins has affected the lipid transport in diabetes for example; glycated apolipoprotein B causes a reduction of affinity for the low density lipoprotein receptor (Kesaniemi et al., 1983). Similarly glycation of apolipoprotein A 1 altered its structure thereby adversely affecting the reverse cholesterol transport in diabetes, thus, developing a risk for cardiovascular diseases (Nobecourt et al., 2007). The collagen glycation results in increased intramolecular cross-links causing the decreased small joint mobility of longstanding diabetes (Schnider and Kohn, 1982).



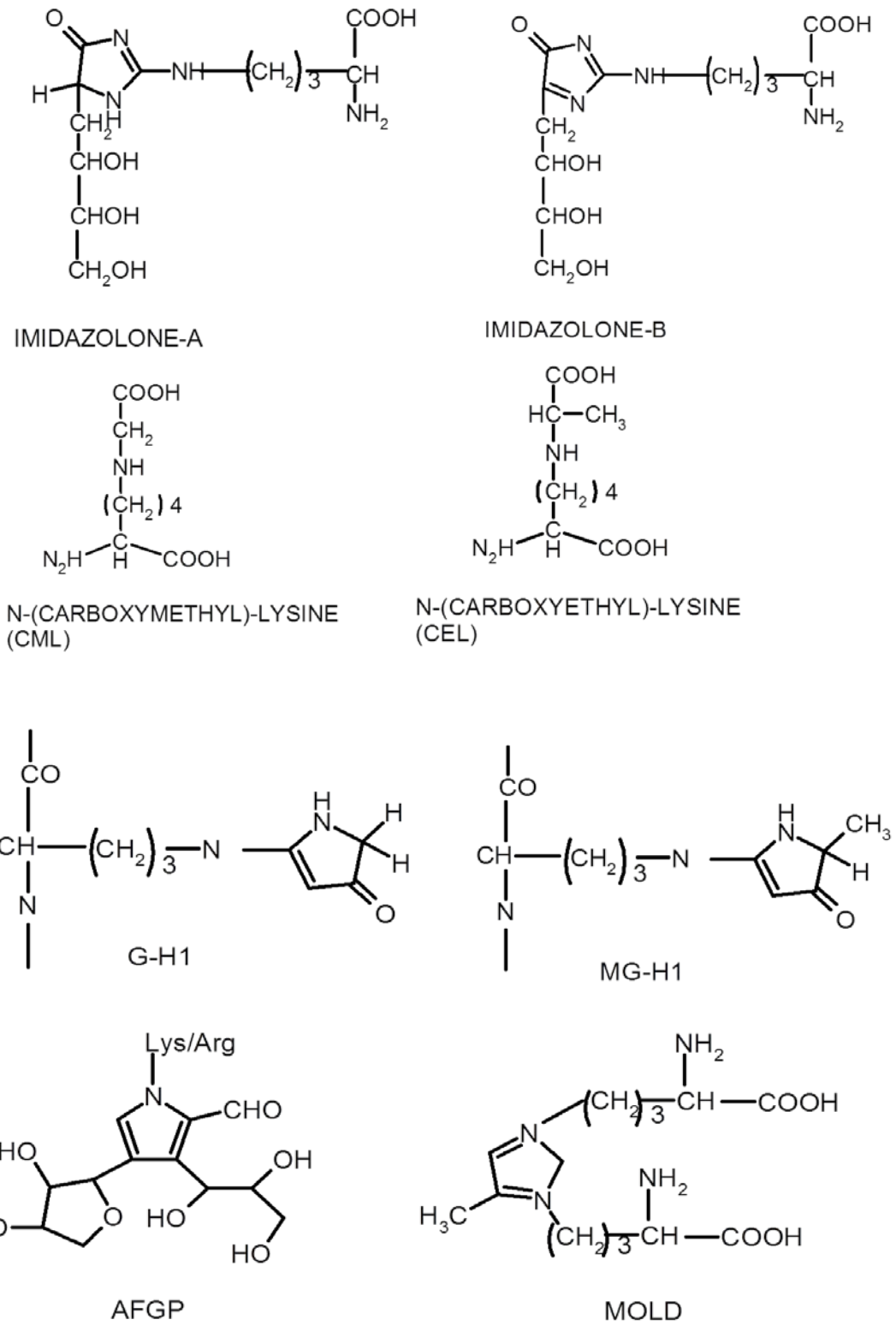


Figure 1. 3: Structural formulae of different Advanced Glycation End products (AGEs). *Modified from Wa et al., (2007); Lapolla et al., (2006); Biemel et al., (2001)*

Table 1. 3: Glycation modifications with mass increase (ΔM) for different AGEs.

S. No	Abbreviations	Modification	ΔM (Da)
1	FL	Fructosyl-lysine	162.0528
2	CEL	N_{ε}-carboxyethyl-lysine	72.0211
3	CML	N_{ε}-carboxymethyl-lysine	58.0055
4	PYRRALINE	Pyrraline	108.0211
5	FL-$2H_2O$	Fructosyl-lysine- $2H_2O$	126.0317
6	IMIDAZOLONE-A	Imidazolone-A	144.0300
7	IMIDAZOLONE-B	Imidazolone-B	142.0266
8	ARGPYR	Argpyrimidine	80.0262
9	MG-H1	Nε-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine	54.0106
10	G-H1	Nε-(5-hydro-4-imidazolon-2-yl)ornithine	39.9949
11	AFGP	1-alkyl-2-formyl-3,4-glycosyl-pyrrole	270.0740
12	MOLD	2-ammonio-6-[1-(5-ammonio-6-oxido-6-oxohexyl)-4-methylimidazolium-3-yl]hexanoate	49.0078
13	CROSSLINE	Crossline	252.1100

A recent study shows glycation of two central extracellular matrix (ECM) proteins laminin and fibronectin associated with neuronal regeneration are directly linked to the failure of axonal regeneration and are therefore involved in the development of diabetic neuropathy (Duran-Jimenez et al., 2009). Glycated albumin is known to inhibit hepatic uptake of glycoproteins (Summerfield et al., 1982) and is also responsible for stimulating renal extracellular matrix production thereby associating with the development of diabetic nephropathy (Chen et al., 2000). The levels of AGEs are correlated with initiation and progression of diabetic retinopathy which is associated with retinal capillary basement membrane thickening, blood retinal barrier dysfunction and loss of pericytes (Stitt et al., 2000).

As glycation is implicated in the development of diabetic complications by structural and functional modification of several proteins there is substantial need to understand the mechanism of protein glycation and their implication in developing diabetic complications.

I.3. Glycation and Plasma Proteome

I.3.1. Plasma Proteome

Blood plasma is not only an exceptional primary clinical specimen but is also the most complex and deepest version human proteome, containing other tissue proteomes as subsets. It possesses rich information concerning the overall pathophysiology of the patient as is in close association with the body. Blood plasma represents an organ or tissue status in health and disease condition due to the presence of significant fractions of tissue-derived proteins as a part of plasma proteome (Zhang et al., 2007). Plasma proteome comprises of true plasma proteins that carry out their functions in the circulation, tissue leakage proteins which are released into plasma as a result of cell death or damage and immunoglobulin class of proteins with tremendous sequence heterogeneity involved in generating their functional specificities (Anderson and Anderson, 2002). There is a large difficulty to obtain other body fluids or tissue sections involving painful and risky invasive procedure in comparison with blood plasma which is readily accessible. Moreover, blood plasma is in circulation throughout the body suggesting that plasma proteome may serve as a treasure trove of protein biomarkers (Issaq et al., 2007). Diagnosis of the involving vascular, renal, retinal, and neural complications in diabetes is quite possible, due to the presence of tissue leakage proteins in plasma of diabetic patients. In addition, the accumulation of glycated proteins has been strongly implicated in the progression of glycation induces diabetic complications which itself is a prerequisite for understanding plasma protein glycation in greater detail.

I.3.2. Dynamic Range of Plasma Proteome

The complexity and dynamic range of specific protein concentration in human plasma spans greater than ten orders of magnitude. This reduces the ability to discover highly specific tissue-derived biomarkers which are present in very low concentration (about ng/ml) (Whiteaker et al., 2007). Twenty two proteins including albumin, IgG, IgA, haptoglobin, alpha-1-antitrypsin and transferrin constitute about 99% of the protein total plasma concentration, while the remaining 1% considered being at low abundant proteins (Tirumalai et al., 2003). It is within this 1% of the plasma proteome

that many tissue secreted proteins are present and are dominated and masked by high abundant proteins.

Although reverse phase LC-MS/MS analysis would certainly be helpful in identifying low abundant proteins but may not be sufficient enough to gain comprehensive coverage of the low abundant plasma proteins. Therefore, effective characterization of plasma proteins requires several methods involving removal of the high abundance proteins prior to downstream analysis.

I.3.3. Abundant Protein Depletion of Plasma

Up to 10,000 proteins might be present in plasma with varying concentrations from millimolar to femtomolar amount (Issaq et al., 2007). Albumin, the most abundant protein and twenty one other high abundant proteins in plasma represent a major challenge for the proper detection of other low abundant proteins (Fig 1.4). For this reason, it is important to unmask the low abundant proteins by various approaches. Albumin can be depleted from plasma by using a dye based approach, Cibracon blue, which has higher affinity towards albumin (Zolotarjova et al., 2005). Even though abundant protein depletion reduces the dynamic range of the plasma proteome by about 2-3 orders of magnitude, the difference between medium-abundant and low abundant plasma proteins is still in the range of 7-8 orders of magnitude and beyond the dynamic range of current proteomic technologies (Linke et al., 2007).

Therefore exploring the plasma proteome in depth still remains a difficult task as we are unaware about the proteins that are below the tip of the iceberg. This technical difficulty can be overcome by using a multiple affinity removal system (MARS) an immunodepletion column for removal of six high abundant proteins in plasma (Dardé et al., 2007). Similar antibody based approaches involving immunoaffinity columns are more specific in removing either 12 (IgY-based affinity LC column, Agilent) or 20 (Prot20, Sigma) most abundant plasma proteins (Linke et al., 2007), thus facilitating in-depth proteomics analysis. In addition to these immunoaffinity columns, development of the ProteoMiner technology which includes hexapeptide ligand library further serves in capturing the 'hidden proteome' thereby equalizing the plasma protein concentration (Righetti et al., 2006).

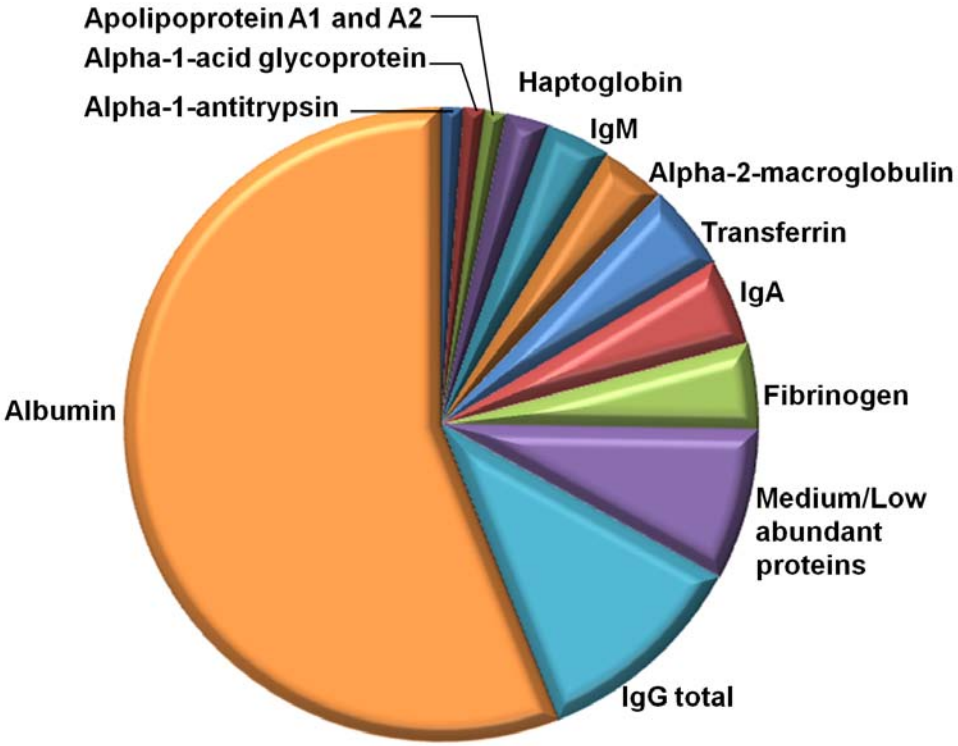


Figure 1. 4: Plasma proteome. Albumin accounts for over more than 50% of the plasma proteome with IgG is the second most abundant.

I.4. Proteomics: A Tool for Studying of Plasma Protein Glycation

Proteomics is the systematic and comprehensive study of the diverse properties of proteins in a parallel manner with the aim of providing detailed descriptions of the structure, function and control of biological systems in health and disease (Patterson and Abersold., 2003). There is a substantial need for studying the human proteome because the genome is more or less constant, but the protein expression might differ according to cell location and time (Dhingra et al., 2005). Proteins are virtually the effectors of biological functions, but protein expression not only depends on the levels of the corresponding mRNA but also on a host of translational controls and regulated degradation (Gygi et al., 1999). Although transcriptome analysis might help in identification of differentially expressed genes, such techniques fail to detect post-translational modifications of proteins, which are significantly crucial in determining specific cellular process (Ohtsubo et al., 2005). In this regard, proteomics is mandatory for the better understanding of cellular function thereby elucidating molecular mechanisms in health and disease. Proteomics

preliminary aims in the simultaneous measurement of levels of large numbers of proteins obtained from complex biological samples (Sundsten and Ortsater, 2008) and initially pursued with proteome separation by two dimensional gel electrophoresis (2DE), protein identification MS followed by immunoblotting for protein validation. However, the technology was not efficient enough to deal with analysis of PTMs. Therefore, biological mass spectrometry (MS), the technological basis of most current proteomics studies, was launched with the development of two major ionization techniques involving the electrospray and Matrix associated laser desorption ionization (MALDI) for identification of protein and characterization of PTMs (Cox and Mann, 2007; Lapolla et al., 1998).

I.4.1. Proteomics and PTM analysis

Previously identification of PTMs used to be achieved by Edman degradation, amino acid analysis, isotopic labelling, or immunochemistry. During the last one and half decade mass spectrometry based proteomics has emphatically become a formidable tool for detecting post translational modifications which of course could not be predicted from genomic information alone. Mass spectrometry has several advantages for PTMs characterization, including (i) very high sensitivity; (ii) ability to identify the exact site of PTM; (iii) discovery of novel PTMs; and (iv) capability to identify PTMs in complex protein mixtures (Cox and Mann, 2007). With the advent of new techniques, such as electron transfer dissociation (ETD), which preserves modifications better than collision dissociation (CID) due to differences in the manner of fragmentation, the analysis of ‘difficult’ PTMs has been significantly improved (Mitchell et al., 2010). Applied to disease proteomics, the latest mass spectrometric techniques involving MALDI-MS and nano-ESI-LC-MS, have proved highly effective in shedding light on the glycation induced PTMs in diabetes (Lapolla et al., 2011). The use of mass spectrometry based proteomics in revealing the post translational modification in diabetic complications including glycation will expand substantially, particularly to meet the need for better diagnostics and to shorten the path for developing effective therapy.

I.4.2. Strategies for Characterizing Plasma Protein Glycation

Protein glycation modifications can be characterized by using various separation methods including ion-exchange chromatography (Al-Abed et al., 1999), capillary electrophoresis (Fayle et al., 2001), and boronate affinity chromatography (Brownlee et al., 1980) followed by mass spectrometry. However, the factors listed below have contributed to the lack of sensitivity and selectivity in the methods for identification and quantitation of glycated proteins (1) the low concentration of glycated proteins; (2) the modification of enzymatic digestion patterns; (3) the low ionization efficiency of glycated peptides, and (4) the lack of software including tools to identify glycation modifications (Priego Capote and Sanchez Capote., 2009). Proteomic analysis of glycated proteins involves three major steps including sample preparation followed by protein separation with one dimensional or two dimensional gel electrophoresis, enzymatic hydrolysis of proteins, chromatographic separation of the resulting peptides followed by MS analysis and finally data processing involving the database search. However, these conventional proteomics protocols have been scarcely used in the analysis of glycated proteins due to two main reasons. The first reason is low concentration of glycated proteins and secondly the information about the preferred glycation sites for each protein could be lost during conventional enzymatic digestion of proteins. For this reason, it becomes mandatory to enrich the glycated proteins by using affinity columns, and liquid chromatography techniques.

Boronic acid chromatography (BAC) involves esterification between boronic acid and 1, 2- cis-diol compounds (possessing two hydroxyl groups of the diol on adjacent carbon atoms) under alkaline conditions which is particularly observed mainly at the glucose moiety in the case of protein glycation. Therefore, BAC is selective for enrichment of non-enzymatically glycated proteins (Li et al., 2001). Identification of glycated proteins can be achieved by phenylboronate affinity chromatography coupled with reversed- phase LC-MS/MS and utilizing data-dependent tandem mass spectrometry with alternating electron transfer dissociation (ETD) and collision induced dissociation (CID) MS/MS (Zhang et al., 2007a). However, by using ETD as the fragmentation mode the glycated peptides can be identified with higher confidence as the fragment containing the labile Amadori

product is kept intact during the fragmentation process thereby proving to be an efficient approach for analysis of glycated proteins (Syka et al., 2004). A specific modulation in boronic acid known as methacrylamido phenylboronic acid has been successfully used in SDS-PAGE for analysis of glycated proteins. 1% methacrylamido phenylboronate acrylamide gel electrophoresis (mP-AGE) shows retention of d-gluconolactone modified proteins and the method is highly selective for glycated proteins (Morais et al., 2010).

Mass spectrometric analysis has shown the development of the following methods for the characterization of glycation modification of glycated proteins. A reverse phase liquid chromatography method followed by a neutral loss scan method consists of two segments for the screening of the glycated peptides. First segment involving identification of glycated peptides based on the neutral loss of 162 Da while the second segment involving selection of glycated peptide as parent ion and further fragmentation at higher collision energy to break the peptide bonds which reveals the amino acid sequences and the sites of glycation (Gadgil et al., 2007).

Metz and co-workers proposed several approaches for the characterization of glycated proteins which are primarily based on bottom-up work flows (Zhang et al., 2008a; Zhang et al., 2008b; Zhang et al., 2009; Zhang et al., 2011). Nevertheless, these approaches have been focused on only qualitative analysis. Therefore, for quantitative analysis of glycated proteins a differential labelling of proteins with isotopically labelled sugars (13C-sugars), named glycation isotopic labelling (GIL) has been evolved. Because of the chemoselectivity of GIL, only preferential glycation targets are labelled followed by analysis of these non-enzymatic glycation sites in the human plasma proteome (Priego-Capote et al., 2010).

I.5. Genesis and Organization of Thesis

An increased blood plasma glucose level is the hallmark of diabetes resulting into plasma protein glycation. The involvement of protein glycation in the pathogenesis of diabetic complications has evoked a special medical interest in preventing the glycation of proteins in human. In this regard, it has become equally important in gaining knowledge about plasma protein glycation and its regulation *in*

vivo. For addressing this question, preliminary studies in determining the factors influencing glycation reaction were performed. Further, albumin constitutes about 50% of plasma proteins and is one of the heavily glycated protein due to its abundance. Therefore any variation in albumin levels may change the stoichiometry of plasma protein glycation. To understand the role of albumin mediated glycation regulation, a systematic and comprehensive plasma proteome analysis of diabetic and non-diabetic subjects were performed. In addition, the differential expression of plasma proteins in non-diabetes, controlled diabetes and poorly controlled diabetes contributing in the development of diabetic complications has been discussed.

Major objectives of the thesis are as follows

- To study various factors influencing the glycation reaction *in vitro*
- To study the association of albumin in the regulation of glycation *in vitro* and *in vivo.*
- To study the differential expression of plasma proteins in poorly controlled diabetes

This thesis is organized and presented in the following manner

- Chapter I: Introduction
- Chapter II: Factors influencing glycation reaction *in vitro*
- Chapter III: Association of albumin levels with plasma protein glycation and HbA1c in diabetes
- Chapter IV: Proteomic study reveals down regulation of apolipoprotein A1 in plasma of poorly controlled diabetes
- Summary and Future Perspectives
- Bibliography
- Appendix I & II (List of glycation modification and their MS MS annotations)

CHAPTER II

"FACTORS INFLUENCING GLYCATION REACTION *IN VITRO*"

II.1. Introduction

Protein glycation is a nonenzymatic interaction between reducing sugars including D-glucose, ribose or fructose and proteins (Neglia et al., 1983). The first step of reactions is the interaction between ε-amino group of lysine and glucose (Maillard, 1916), giving rise to an unstable Shift base derivative of the protein which slowly isomerizes to the more stable ketoamine adduct via the Amadori rearrangement (Gottschalk, et al.,1972; Thorpe and Baynes., 1982). Further cyclization processes lead to intermediate species responsible for protein cross-linking reactions. The ultimate step of such reaction pattern leads to the formation of heterogeneous molecules called “Advanced Glycation End Products" (AGEs) (Reynolds, 1965).

As diabetes is characterized by chronic hyperglycemia, the uncontrolled hyperglycemia may lead to the development of vascular complications including retinopathy, nephropathy, and neuropathy (Brownlee et al., 1984). Many studies have suggested that the formation of AGEs (Advanced Glycation End products) as an important factor associated with the etiology of diabetic complications (Thornalley, 2003a; Goldin et al., 2006). Glycation reaction with lysine and arginine amino acid in protein triggers a cascade of events leading to formation AGEs, which results in alteration of protein structure and function (Thornalley et al., 2003b). Glycation has affected biological activity of many proteins including insulin (Hunter et al., 2003), crystallin (Abraham et al., 2008), glucose-6-phosphate dehydrogenase (Ganea and Harding, 1994), aldehyde reductase (Takahashi et al., 1995), glutathione reductase (Blakytny et al., 1992), Cu-Zn superoxide dismutase (Arai et al., 1987; Oda et al., 1994), HDL (Hendrick et al., 2000), both IgM and IgG (Menini et al., 1992; Lapolla et al., 2000a). Protein glycation has been demonstrated successfully both *in vitro* and *in vivo* in several model proteins including haemoglobin, Bovine serum albumin (BSA), Human serum albumin (HSA), Immunoglobin G (IgG), collagen, insulin etc., and the glycation extent of these proteins is known to differ from protein to protein (Lapolla et al., 2006a). The glycation extent of high molecular mass proteins is more in comparison with low molecular mass proteins. Since, glycation is a non-enzymatic reaction, theoretically all proteins should undergo glycation; but several studies

suggest that certain proteins are more prone to undergo glycation than others depending on the surface-accessible reactive site (Quan et al., 1999). For instance, glycoxidative modifications have been shown to be targeted only to a restricted set of proteins of human peripheral blood lymphocytes (Poggioli et al., 2002). Additionally, 3-deoxyglucosone (3-DG) mediated glycation results in the loss of the activity of the specific enzymes associated with glucose metabolism (Kiho et al., 1996). The specificity of glycation reaction was further strengthened by a recent study showing the intermediate filament vimentin as the major target for the AGE modification in primary human fibroblasts (Kueper et al., 2007). Vimentin glycation is neither based on a slow turnover nor an extremely high intracellular expression level, but remarkably based on its structural properties (Kueper et al., 2007). However, protein structure may not be the sole factor in determining the specificity of glycation. As reported earlier, HbA1c (glycated hemoglobin), the marker of long term diabetes is abundant and has a slow turnover (Krishnamurti and Steffes, 2001). Thus, protein abundance and slow turnover may be equally important factors that may influence glycation reaction. This chapter aims to understand the influence of protein molecular mass and protein abundance on glycation reaction *in vitro*. Glycation studies were performed with proteins varying in molecular masses ranging from 5 kDa to 150 kDa. These proteins include IgG, HSA, BSA, apomyoglobin, papain and insulin. Further, the glycation extent in these proteins was monitored by MALDI-MS analysis.

II.2. Materials and Methods

II.2.1. Chemicals

Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), Apomyoglobin, Papain, Carbonic anhydrase and Glucose were procured from Sigma-Aldrich (St. Louis, USA), Immunoglobin G (mass standards) from Applied Biosystems (CA, USA), and Insulin (Biocon Ltd, India) from local Pharmacist.

II.2.2. *In vitro* Glycation Experiments

To determine the influence of protein molecular mass on glycation reaction, *in vitro* glycation assay was performed. 10 mg/ml HSA (0.15mM), BSA (0.15mM), and papain (0.42mM), 2mg/ml of insulin (0.34mM) and 1mg/ml of apomyoglobin

(0.05mM) and IgG (0.006mM) were used. All protein samples were prepared in double distilled water. The *in vitro* glycation of proteins was performed by incubating equal volume (50µl) of protein with 1M glucose solution dissolved in 0.2M phosphate buffer, at 37°C for either 3, 6 or 10 days.

To study the influence of protein abundance factor on glycation reaction, different concentrations of albumin (0, 2, 20, and 100mg/ml) and insulin (2 mg/ ml) were prepared in 0.2M phosphate buffer (pH 7.4). 10 µl of each protein was used for *in vitro* glycation assay. 0.5M of glucose in 0.2M phosphate buffer pH 7.4 was added to various ratios of albumin and insulin (0: 1, 1:1, 10:1, and 50:1) and then incubated at 37°C for 7 days. The final concentration of insulin was kept constant (1 mg/ml), while the albumin concentrations varied to 0, 1, 10 and 50 mg/ml. Similarly, 10 µl of apomyoglobin was mixed with 10 µl of albumin to have a final concentration of 0, 25, 50 and 100 mg/ml of albumin. The *in vitro* glycation assay was performed in 1:1 ratio of glucose to protein.

II.2.3. Sample Preparation for Mass Spectrometric Analysis

5 µl of the *in vitro* glycation assay mixture was mixed with 45 µl of freshly prepared sinapinic acid (30% acetonitrile, 0.1% trifluoroacetic acid) and loaded onto the stainless steel MALDI plate by dried-droplet method and incubated for 10 min at 37°C.

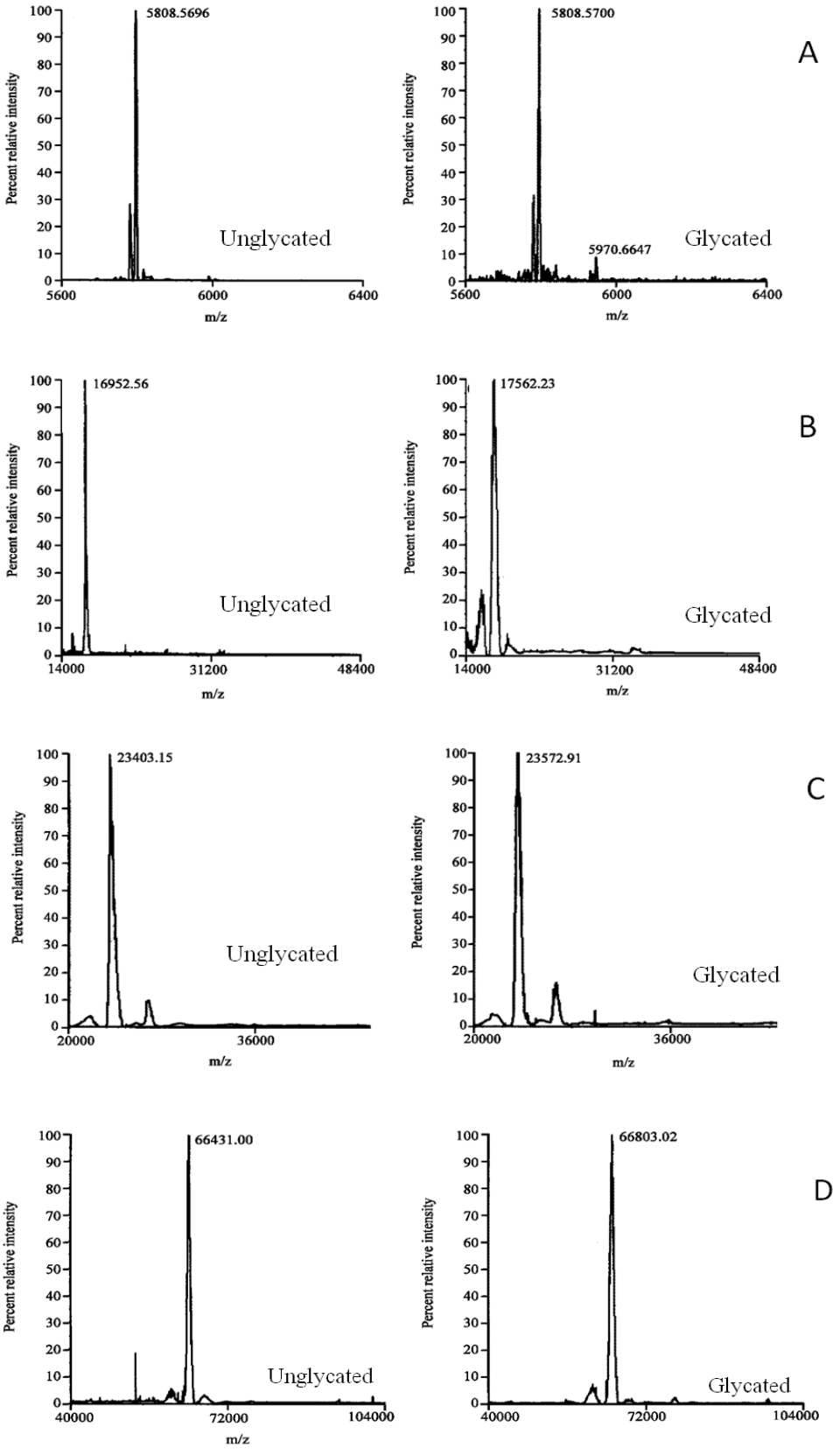
II.2.4. MALDI MS Analysis

The mass spectral analysis was performed on Voyager-DE-STR MALDI-TOF MS (Applied Biosystems, California, USA). A 337nm pulsed nitrogen laser was used for desorption and ionization. An Aquiris 2GHz dizitiger board was used for all experiments. MALDI analysis of insulin, apomyoglobin, papain, HSA, BSA and IgG were done using different instrumental settings. For insulin analysis, spectra were acquired in the range of 2500 Da to 7500 Da, on a positive reflector mode with the following settings: an accelerating voltage 25kV, grid voltage 68.5% of accelerating voltage, delayed ion extraction time of 350 ns, low mass ion gate was set to 500 Da. For apomyoglobin analysis, spectra were acquired in the range of 1000 Da to 25000 Da, on a positive linear mode with the following settings: an accelerating voltage

25kV, grid voltage 93.2% of accelerating voltage, delayed ion extraction time of 1100 ns, low mass ion gate was set to 1000 Da. For HSA, BSA, papain and IgG analysis, spectra were acquired in the mass range of 10 kDa to 200 kDa, on a positive linear mode with the following settings: an accelerating voltage 25kV, grid voltage 92% of accelerating voltage, delayed ion extraction time of 1500 ns, low mass ion gate was set to 4500 Da. For all spectral acquisition, the laser power was set just above the ion generation threshold to obtain peaks with the highest possible signal to noise ratio. All spectra were acquired with 50 shots in three replications. The spectra were processed for advanced base line correction, noise removal and mass calibration.

II.3. Results and Discussion

Glycation occurs at ε-amino group amino acids including lysine or arginine (Zhao et al., 1997). Protein structure and vicinal amino acids to lysine have known to determine the specificity of glycation reaction (Menella et al., 2006; Kueper et al., 2007). However, several other factors also contribute to the specificity of glycation reaction. For example, a study on hemoglobin suggested that site specificity of glycation is attributed to the protein microenvironment (Nacharaju and Acharya, 1992). In addition, the effect of the buffer system and water activity on the specificity and the rate of glycation has been previously studied (Watkins, et al., 1987; Wu et al., 1980). Therefore, to understand the influence of protein molecular mass on glycation extent, several model proteins of varying mass including insulin, apomyoglobin, papain, HSA, BSA and IgG were used for *in vitro* glycation studies. The mass of control and glycated proteins after performing MALDI-MS analysis has been shown in Fig 2.1. The increase in molecular mass (ΔM) upon glycation was observed in all the six proteins. The extent of glycation for low molecular mass proteins such as insulin, apomyoglobin and papain was lower compared to the high molecular mass proteins such as BSA, HSA and IgG.


A
5808.5696
Unglycated
5808.5700
5970.6647
Glycated
B
16952.56
Unglycated
17562.23
Glycated
C
23403.15
Unglycated
23572.91
Glycated
D
66431.00
Unglycated
66803.02
Glycated
Percent relative intensity
m/z

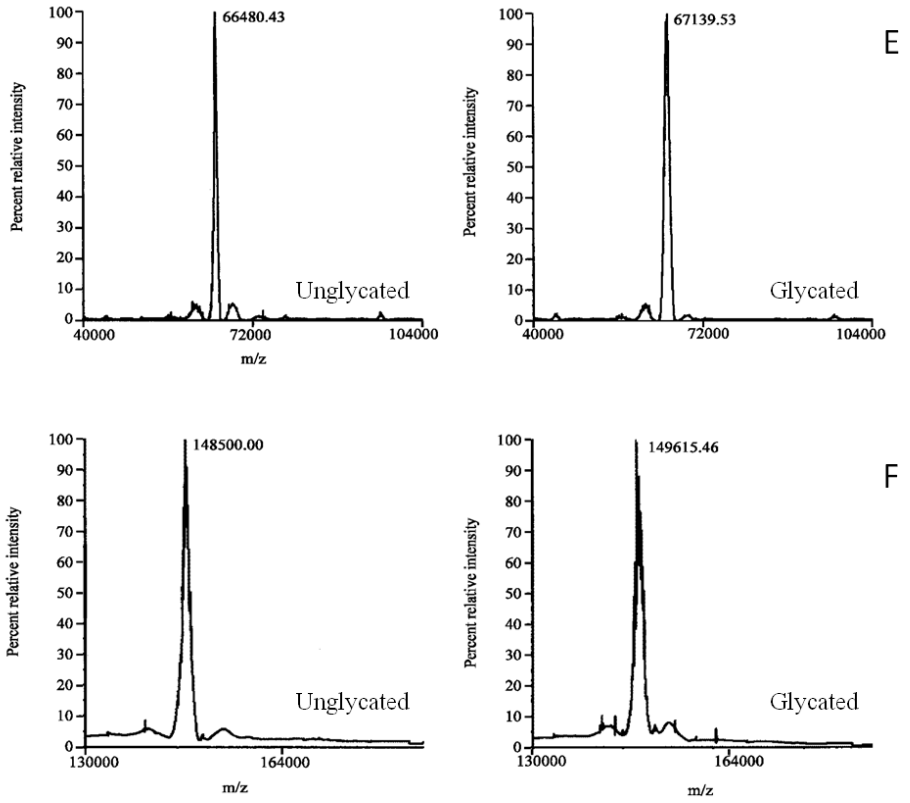


Figure 2. 1: MALDI- MS analysis of *in vitro* glycated proteins (A) Insulin (B) Apomyoglobin (C) Papain (D) BSA (E) HSA (F) IgG.

The increase in mass was analyzed for different proteins during glycation reaction (Fig 2.2). ΔM was less for low molecular mass proteins such as insulin and papain on three different days of glycation whereas for high molecular mass proteins including BSA, HSA and IgG, ΔM increased with increase in time of glycation reaction. A positive correlation between ΔM and the molecular mass of proteins on three different days of glycation was observed as shown in Fig 2.3.

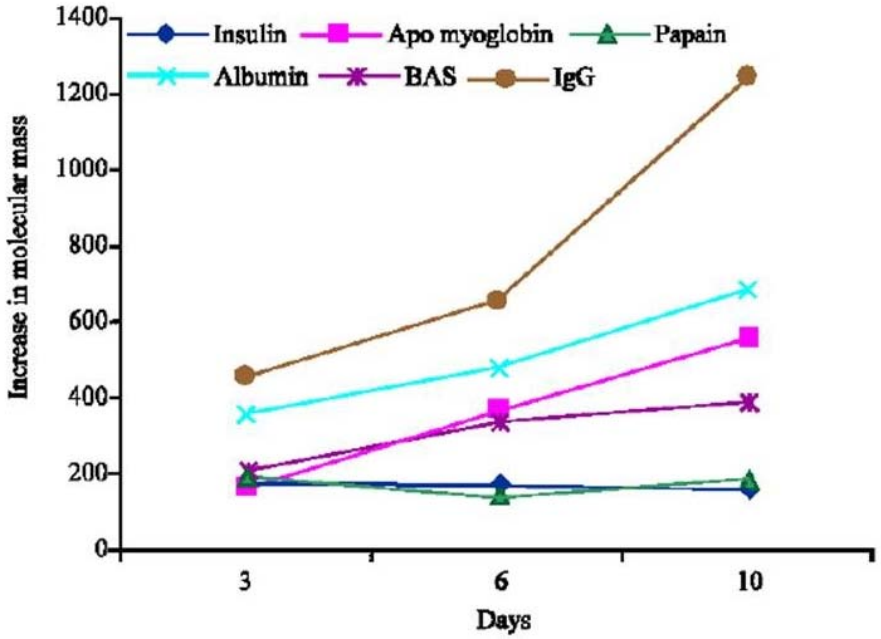


Figure 2. 2: Increase in mass of six proteins on 3rd, 6th and 10th day of glycation reaction monitored by MALDI-MS.

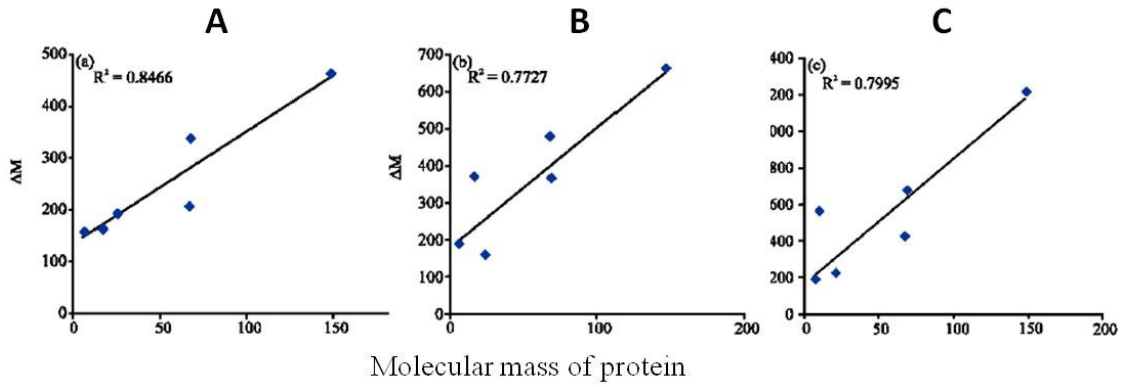


Figure 2. 3: Relationship between protein molecular mass and increase in protein mass (A) day 3 (B) day 6 (C) day 10.

High molecular proteins have more number of lysine and arginine residues; therefore it is obvious to observe an increase in the extent of glycation in larger proteins compared to smaller proteins Fig 2.2. In a recent study, it was observed that the HSA, a large molecular mass protein with 59 lysine residues, is heavily glycated *in vivo* with 31 glycation sites identified from 38 unique glycated peptides (Zhang et al., 2008a). One of the major reasons of HSA being highly glycated protein is by the

virtue of more number of lysine residues (Garlick eand Mazer, 1983; Iberg and Flukiger, 1986). Another possible reason for increased glycation of HSA could be the nature of amino acid present near the lysine residue. It was demonstrated earlier that, the vicinal amino acids including isoleucine, leucine, phenylalanine strongly increases lysine reactivity towards the different carbohydrates (Menella et al., 2006). Therefore, as BSA, HSA and IgG are high molecular mass proteins with more number of lysine and arginine residues facilitating the higher extent of glycation than the low molecular mass protein like insulin and apomyoglobin suggesting the protein molecular mass influences the glycation reaction.

Albumin, the high molecular mass protein is also present in abundance and might compete with low abundant proteins for glycation. For understanding the competitive glycation reaction among proteins, *in vitro* glycation assay of albumin and insulin in a ratio of 0:1, 1:1, 10:1, and 50:1 was performed. The glycated protein peaks showed an increase in mass of 162 Da with MALDI-MS analysis. The relative intensity of a glycated peak (5970 Da) of insulin was maximum at lowest albumin to insulin ratio (0:2) (Fig 2.4A). Further the relative intensity of a glycated peak of insulin decreased with increasing concentration of albumin (Fig 2.4B-C). Albumin inhibited the insulin glycation by getting itself glycated (Fig 2.5A and 2.5B).

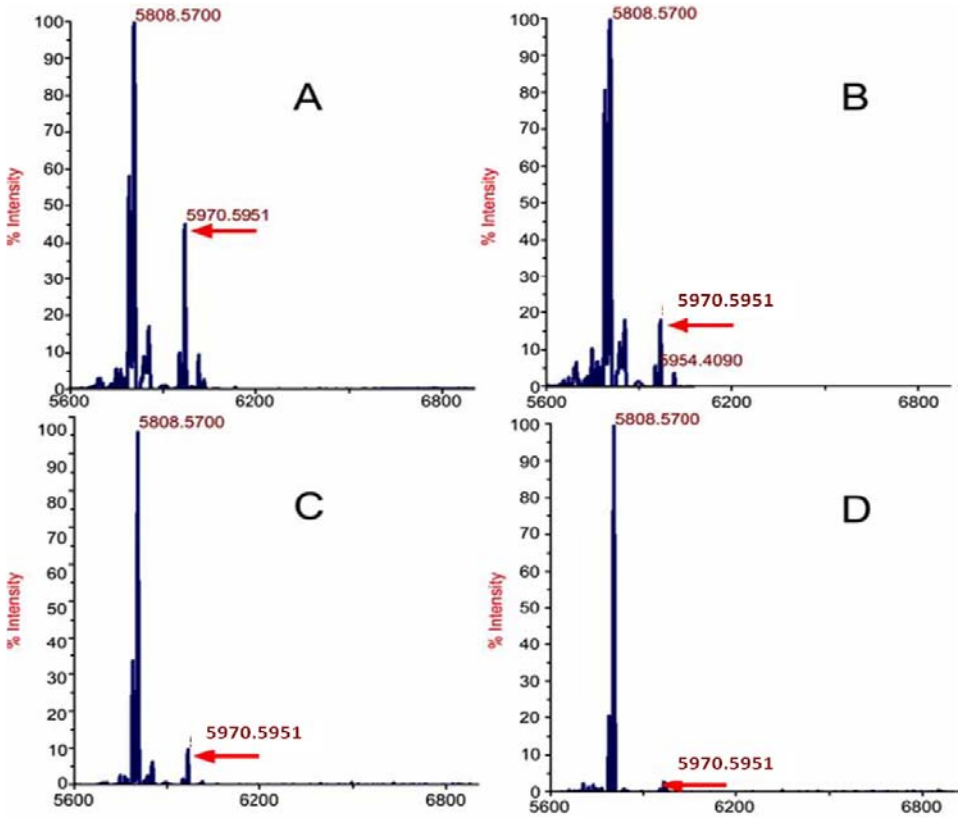


Figure 2. 4: Influence of albumin concentration on insulin glycation (A) 0 mg/ml albumin, (B) 1 mg/ml albumin, (C) 10 mg/ml albumin, (D) 50mg/ml albumin. These spectra were acquired on a positive reflector mode by MALDI-MS analysis.

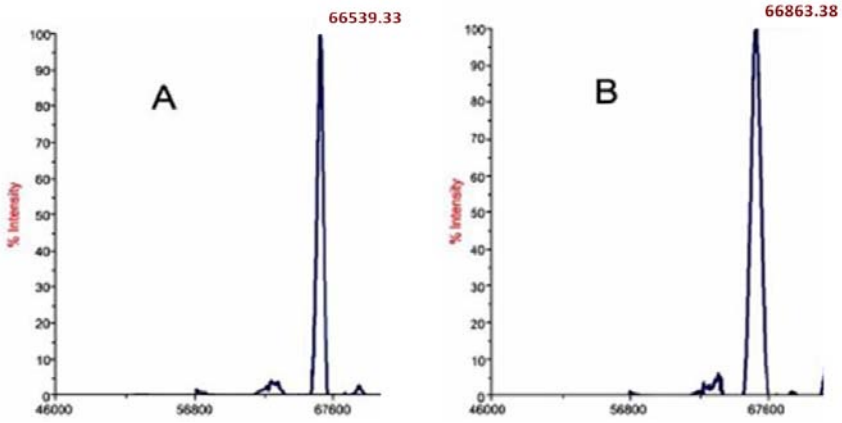


Figure 2. 5: MALDI-MS analysis of (A) unglycated albumin (B) glycated albumin.

Insulin was then replaced with apomyoglobin (0.5 mg/ ml) in order to see whether albumin inhibits the glycation of other proteins. Apomyoglobin shows unglycated peak at 16952.44 Da and three glycated peaks at 17114.88 Da, 17276.59

Da, 17438.29 Da respectively, suggesting that at least it has three sites of glycation (Fig 2.6). The intensity of the glycated peaks decreased with increasing albumin concentration. The relationship between the albumin concentration and ratio of absolute intensity of glycated to unglycated peaks showed negative co-relation (Fig 2.7). Inhibition of glycation by albumin was more pronounced in second and third glycated peaks of apomyoglobin than the first glycated peak (Fig 2.7), suggesting that albumin also effects the extent of protein glycation.

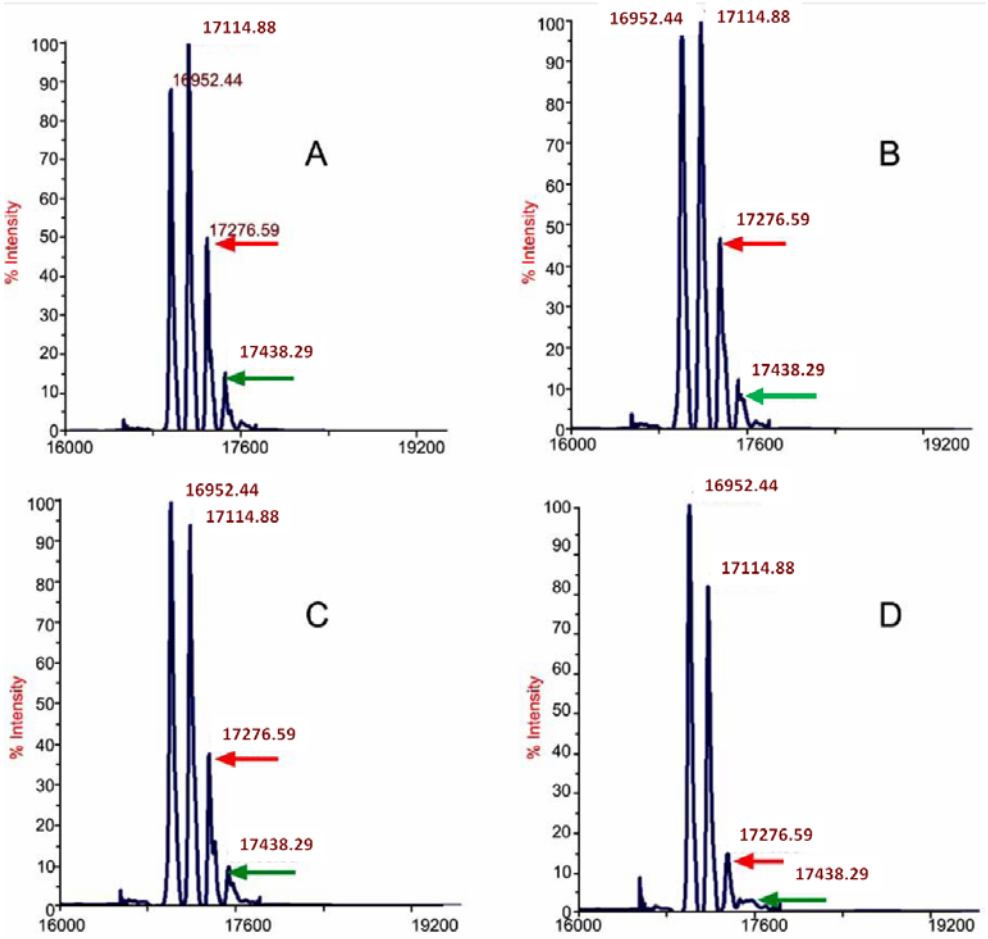


Figure 2. 6: Effect of albumin concentration on apomyoglobin glycation (A) 0 mg/ml albumin, (B) 5 mg/ml albumin, (C) 25 mg/ml albumin (D) 50 mg/ml albumin. These spectra were acquired on a positive reflector mode by MALDI- MS analysis. Glycated peaks are labeled.

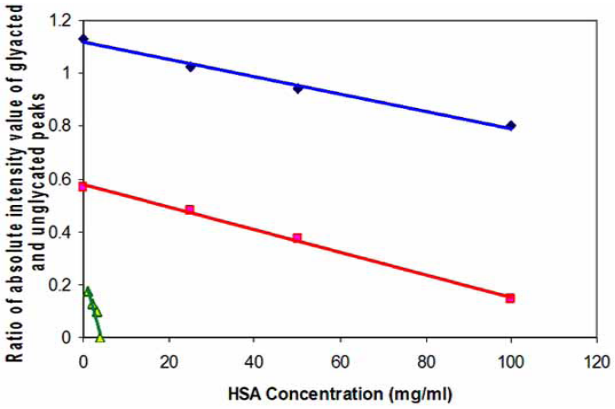


Figure 2. 7: Relationship between ratio of absolute intensity value of glycated and unglycated peaks of apomyoglobin and albumin (HSA) concentration. Blue, red and green curve indicates first, second and third glycated peaks respectively.

Competitive inhibition of insulin glycation was also observed by replacing albumin with 2mg/ml of carbonic anhydrase (Fig 2.8A and 2.8B) and papain (Fig 2.8C and 2.8D).

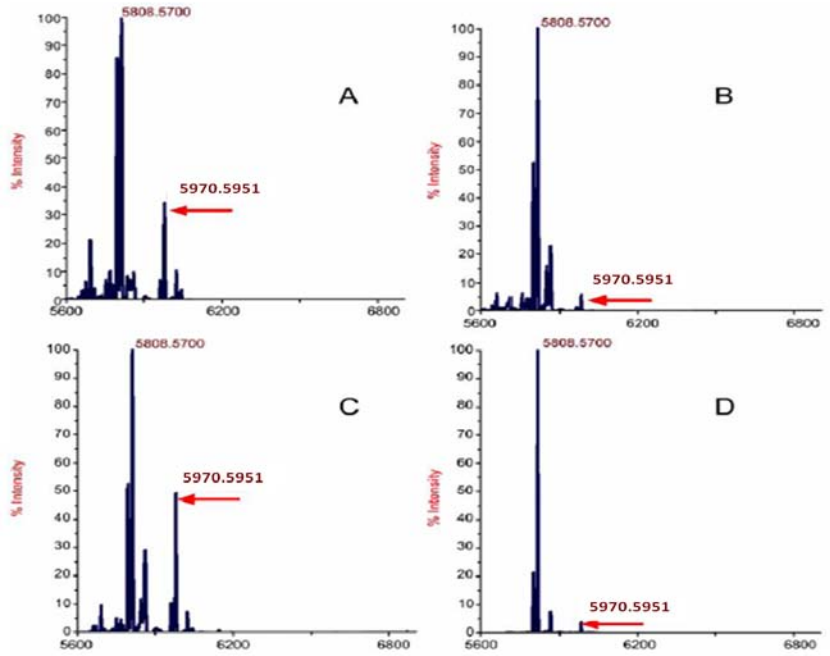


Figure 2. 8: Effect of carbonic anhydrase (CA) and papain on insulin glycation (A) 0 mg/ml CA, (B) 2mg/ml CA, (C) 0 mg/ml papain, (D) 2mg/ml papain. Spectra were acquired on a positive reflector mode by MALDI- MS analysis. Glycated peaks are labeled.

At higher concentration, both carbonic anhydrase and papain were degraded and the degraded products were interfering in MALDI analysis of insulin. Therefore, protein turnover is also an important factor in determining glycation. In this regard, our *in vitro* experiments strongly suggest that albumin may also have a similar role *in vivo* in protecting glycation of less abundant proteins. Additionally, previous studies have shown role of glycated albumin in the pathogenesis of diabetic complications by increasing gene expression of interleukin (IL-8) and nitric oxide synthase, as well as increased protein expression of alpha-1 (IV) collagen and fibronectin, the predominant constituents of the expanded extracellular matrix seen in diabetes (Cohen, 2003). However, it has been observed that the patients with hypoalbuminemia ascribed to malnutrition are more prone to develop vascular complications in diabetes (Kaysen, 2001) and is a known predictor of vascular morbidity (Suliman et al., 2003). Similar effects are ascertained with hemoglobin, which is the most abundant protein in the blood. Low hemoglobin level in diabetic patients has been associated with an increased risk of micro vascular complications, cardiovascular disease and end stage renal disease (ESRD). Even in mild anemia, Hb (<13.8 g/dL) increases the risk for progression to ESRD and correction of the same improves performance and quality of life in diabetic patients (Thomas, 2007; Mohanram et al., 2004). These studies suggest that hypoalbuminemia and anemia are strongly associated with diabetic complications.

As mentioned earlier, glycation has affected biological functions of several proteins; however, the functions of albumin and hemoglobin are not easily altered due to their high concentration and slow turnover. These studies combined with above results support our hypothesis that high abundant proteins prevent complications by inhibiting glycation of less abundant proteins. At least in the initial stages of diabetes high abundant proteins like albumin and hemoglobin may protect less abundant proteins from the adverse effects of glycation. In the absence of these proteins the effect of glycation might have been more severe. Therefore, in conclusion both the molecular mass of protein as well as protein abundance is important factors that determine glycation reaction *in vitro* in addition to the protein structure and its turnover.

CHAPTER III

"ASSOCIATION OF ALBUMIN LEVELS WITH PLASMA PROTEIN GLYCATION AND HbA1c IN DIABETES"

III.1. Introduction

Diabetes is characterized by elevated levels of blood plasma glucose, which in turn modify the proteins by a non-enzymatic reaction called glycation (Brownlee, 2001). Protein glycation leads to formation of heterogeneous fluorescent molecules 'Advanced Glycation End products' (AGEs), which modify the conformation and function of proteins (Hunter et al., 2003). Furthermore, AGEs interact with receptor for AGEs (RAGE), triggering cascade of events leading to reactive oxygen species (ROS) generation and pro-inflammatory pathways (Hofmann et al., 1999). Accumulation of AGEs has been found to be accelerated in diabetes contributing in the pathogenesis of diabetic complications.

Blood plasma proteins are the primary target of glycation as they are directly exposed to higher glucose concentrations in plasma (Austin et al., 1987). Various plasma proteins including human serum albumin (HSA) and immunoglobulin G (IgG) were the first few proteins to be characterized as glycated proteins (Lapolla et al., 2000a; Lapolla et al., 2006). Amongst plasma proteins, albumin is one of the heavily glycated protein, due to its abundance, relatively longer half life time (21 days), and has more number of lysine and arginine residues, the hotspots of glycation (Philippe and Bourdon, 2011). Albumin constitutes about 50% of plasma proteins and any variation in levels of albumin may change the stoichiometry of plasma protein glycation. Albumin levels in plasma are determined by its expression, secretion and catabolism. Various factors including diet (Caso et al., 2000), lifestyle, inflammation (Liao et al., 1986), disease (Nicholson et al., 2000), drugs etc. affect either of these processes leading to altered levels of albumin in plasma. For example, albumin synthesis and secretion has been reported to be decreased in diabetic rats, which was attributed to insulin deficiency, as replenishment of insulin in diabetic rats restored the albumin levels to that of normal rats (Peavy et al., 1978; Jefferson et al., 1983). Therefore, it is expected that albumin levels may vary in diabetes. Patients with decreased albumin levels ascribed to malnutrition were more prone to develop vascular complications in diabetes (Kaysen et al., 2001). In our previous study with *in vitro* experiments, it was observed that higher levels of albumin were associated with decreased glycation of insulin and apomyoglobin, which were used as a model for low

abundant proteins and vice versa (Bhonsle et al., 2008). It is likely that albumin may have a similar role in inhibiting glycation of other plasma proteins *in vivo*. The fact that hypoalbuminemia ascribed to malnutrition increases the risk of diabetic complication, coupled with our previous study of competitive glycation inhibition by albumin strengthened our hypothesis that albumin regulates the plasma protein glycation *in vivo* as well. This chapter, study particularly shows that how a variation in plasma albumin level in diabetes influences the glycation of other plasma proteins and HbA1c by using two dimensional gel electrophoresis (2DE), western blot analysis and LC-MS^E approach.

III.2. Materials and Methods

All chemicals were procured from Sigma-Aldrich (St. Louis, USA) otherwise are mentioned.

III.2.1. Establishment of Diabetic Mice Model

The animals selected for the experiment were male BALB/c strain of mice with average body weight of 23 g. Streptozotocin (STZ) induced diabetic mice model was developed by using injecting 45mg/kg body weight of the animal STZ solution was prepared in 0.05M Sodium citrate buffer, pH 4.5. Prior to injections animals were fasted for 4 hours. Area near the mid ventral line is cleaned with an alcohol swab. Using 1 ml insulin syringe with 26 gauge needle about 200 µl STZ solutions were injected. Intraperitoneal injections were carried out and the mice were monitored for 15 minutes after which, they were placed into their respective cage. Similar STZ doses were followed for five consecutive days. Another group of male BALB/c strain of mice were injected with 50mM citrate buffer served as control. The induction of diabetes was confirmed after 15 days by measuring the blood glucose levels with a glucometer (Bayer, Germany).

III.2.2. Histopathology Studies

Kidney and liver tissues from 10 experimental animals and 10 control animals were collected and fixed in 10% paraformaldehyde for 24 h at 4º C. The processing

was carried out at Chitale Pathology Laboratory, Pune, followed by Hematoxylin and Eosin (H& E) staining and Peroxide Acid Schiff's base (PAS) staining.

III.2.3 Collection of Plasma Samples

III.2.3.1 Mice Plasma

Blood samples from 20 experimental and 10 control mice were collected after two months of STZ injection. Collected blood was immediately analyzed for blood glucose and HbA1c by using ' in2it^{TM,} analyzer (Bio-Rad, CA, USA). Animals having blood glucose level above 6.12 ± 1.7 mmol /L were considered as diabetic animals and therefore selected for further studies. Plasma was obtained by EDTA treatment, which was then centrifuged at 1500 g for 15 min and the supernatant was stored at -80° C until further use.

III.2.3.2 Clinical Plasma

Blood samples were collected from diabetic patients through an informed consent from Maharashtra Medical Research Society (MMRS), Joshi Hospital approved by the Institutional Ethics Committee. Blood plasma was collected from 60 diabetic patients and 7 non diabetic control subjects. Glucose and HbA1c levels were determined.

III.2.4. Pooling of Diabetic Plasma Samples

Both mice and clinical plasma samples were analyzed for fructosamine and plasma albumin levels by using nitroblue tetrazolium colorimetric method (Fructosamine, Merck, NJ, USA) and Bromocresolgreen (BCG) method, (Innoline^{TM}Albumin, Merck, NJ, USA), respectively according to the manufacturer's instructions. Based on plasma albumin levels, diabetic plasma was grouped into two groups; Diabetic-High Albumin Plasma (DHAP) and Diabetic-Low Albumin Plasma (DLAP). The non-diabetic plasma had relatively higher levels of albumin than diabetic plasma. To create sufficient material for further proteomic analysis, seven representative samples from each group were pooled.

III.2.5. Plasma Protein Sample Preparation

Abundant plasma proteins were depleted either by ProteoPrep® Blue Albumin & IgG Depletion Kit (PROTBA) Sigma-Aldrich (St. Louis, USA) for two dimensional electrophoresis or by Proteominer (Bio-Rad, CA, USA) for LC-MS^E analysis, as per manufacturer's instruction.

III.2.5.1. Depletion of Plasma Proteins using PROTBA^{TM} kit

In order to deplete the high-abundant proteins like albumin and IgG, from plasma, the PROTBA were used. 30 µl plasma sample was processed using 500 µl of blue slurry, and the depletion procedure was performed at room temperature according to manufacturer's instructions. The depleted plasma sample was then used for performing two dimensional gel electrophoresis after protein estimation.

III.2.5.2. Depletion of Plasma Protein using ProteoMiner^{TM} kit

The low abundant proteins in the plasma were enriched by the ProteoMiner kit according to the manufacturer's instructions. 200 µl of plasma having protein concentration of 10 mg was used for enriching low abundant plasma proteins. The ProteoMiner beads consist of a unique hexapeptide ligand library, which limits binding capacity of abundant proteins thereby allowing the enrichment of medium to low abundance proteins. Final protein concentration was estimated and the enriched plasma samples were then used for LC-MS^E analysis. Protein concentration was estimated by using Quick Bradford protein assay kit (Bio-Rad, CA, USA).

III.2.6. 2DE, Western Blotting and Image Analysis

III.2.6.1. First Dimension - Isoelectrofocussing

In the first dimension, proteins were separated by Isoelectrofocussing (IEF) by using Immoboline pH gradient (IPG) strips (nonlinear gradient pH 4-7, 7 cm, BioRad, CA, USA). 150 µg of protein was solubilized in 125 µl rehydration buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 70 mM DTT, 0.2% C_7BzO, 1 µl ampholytes(pH 3-10). IPG strips were passively rehydrated overnight at room temperature. IEF was performed using the PROTEAN IEF Cell (BioRad, CA, USA) at 20°C in three steps,

at 250 V for 30 min, slow ramping; followed 4000 V for 2.5 hour, linear ramping; and finally 10000 Volt-hour with rapid ramping.

III.2.6.2. Second Dimension - SDS PAGE

After performing IEF, IPG strips were equilibrated in 0.375 mM Tris/ HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS and 2% DTT or 2.5% iodoacetamide for reduction and alkylation of proteins respectively. Then the IPG strips were transferred on to the top of 12% SDS-PAGE gel and overlayed with 0.5% low melting agarose. Resolved proteins were visualized by CBB-R250 staining or by western blotting.

III.2.6.3. Western Blot Analysis

Proteins were transferred onto polyvinylidenefluoride (PVDF) membrane and blocked overnight with 5% skimmed milk at 4°C. Blocked membranes were then incubated with goat anti-AGE antibody (Millipore, MA, USA) in 1:3000 dilutions for 1 hour at room temperature. Membranes were then washed once with TBS-T (0.05% tween 20) for 3 mins followed by two washes with TBS (3 minutes each). Washed membranes were then incubated with 1:5000 dilution of rabbit anti-goat secondary antibody for 30 minutes at room temperature. Membranes were washed again as mentioned above and were then incubated with streptavidin conjugated horseradish peroxidase with a dilution of 1:5000 for 15 minutes. Immunodetection on washed membranes was then performed by incubating membranes by using SIGMAFAST^{TM} DAB substrate. Stained gels and developed blots images were acquired by using calibrated densitometer (GS 800 Bio-Rad, CA, USA). Image analysis was performed using PDQuest Advanced software (Bio-Rad, CA, USA).

III.2.7. Trypsin Digestion

III.2.7.1. In-gel Trypsin Digestion

Spots were excised and destained by using 50% ACN/50 mM ammonium bicarbonate buffer. After destaining, gel pieces were dehydrated by using 100% acetonitrile (ACN) followed by reduction of proteins with 10 mM DTT for 30 minutes at 56º C. Proteins were then alkylated with 55 mM of iodoacetamide for 45

minutes at room temperature in dark. Gel pieces were washed twice with 50% ACN/50 mM ammonium bicarbonate followed by dehydration using 100% ACN. To the dehydrated gel pieces trypsin was added in 1:10 (trypsin: protein) ratio and were incubated overnight at 37°C. Tryptically digested peptides were extracted in 50% ACN containing 5% formic acid. Extracted peptides were vacuum concentrated and were then reconstituted in 5 µl of 0.1% formic acid in 3% ACN.

III.2.7.2. In-solution Trypsin Digestion

10 µg of protein was solubilised in 10 µl 50 mM NH_4HCO_3 containing 0.1% RapiGest SF (Waters Corporation, MA, USA) to enhance proteolytic cleavage. Proteins were reduced with 100 mM DTT at 56^{o}C for 15 minutes. Reduced protein was then alkylated with 200 mM iodoacetamide in the dark for 15 minutes. The proteins were digested overnight with trypsin at 37°C. The reaction was stopped by adding 2 µl of formic acid.

III.2.8. *In vitro* Glycation of Plasma Proteins

Non diabetic plasma was differentially depleted by Cibacron-Blue, so as to achieve two concentrations (45 g/L and 35 g/L) of albumin. *In vitro* glycation of depleted plasma was performed by incubating with 0.5 M of glucose prepared in 0.2 M phosphate buffer pH 7.4 at 37^{o}C for 7 days. Glucose was removed by dialyzing the sample against 50 mM Tris-HCl. The sample obtained was further processed for 2DE followed by western blot analysis and LCMS^{E} as described in section III.2.6 and III.2.9.

III.2.9. LC-MS^{E} Analysis

Two micro-litre digested peptides with final concentration of 100 ng/ µL was analyzed by using nanoACQUITY UPLC online coupled to SYNAPT HDMS system (MS^{E}) (Waters Corporation, USA) as described by Cheng et al., 2009 The separation was performed on a BEH 130 C18 1.7 µM × 75 µM × 150 mm Peptide Separation Technology column at 40°C. Mobile phase A was aqueous 0.1% formic acid and B was 0.1% formic acid in acetonitrile. The protein digests were eluted with a 60-min gradient (0-50% B) and a 90-min gradient (0-50% B). The flow rate was 300nl/min.

An auxiliary pump was used to spray a solution of 500 femto mole / μL Glu1-fibrinopeptide B (GFP) in 50 % ACN containing 0.1% formic acid for mass accuracy reference (lockmass channel), with a flow rate of 500nl/min. MS acquisition was operated in the positive ion V-mode with scan time of 1 second. An alternating low-energy (collision cell energy 3 V) and elevated energy (collision cell energy ramped from 15 to 40 V) was used to obtain the precursor ions (MS) and their fragmentation ions (MS^E), respectively. A capillary voltage of 3.2 kV, source temperature of 80°C and cone voltage of 32 V were maintained during the analyses. Sampling of the lock spray channel was performed every 30s. The system was tuned for a minimum resolution of 10000 and calibrated using a 500 fmol/μl GFP infusion.

III.2.10. Protein Identification, Database Search and PTM Analysis

After MS^E acquisition, data was analyzed by using Protein Lynx Global Server software (PLGS. Version 2.4. Waters Corporation, Milford, MA, USA). For protein identification, processed samples were searched against UniProt human database or mouse database. After the protein identification, glycation modification sites were identified by subjecting by targeted search using PLGS. Search criteria included fixed and variable modifications as carbamidomethylation and oxidation (M) respectively. Additionally, variable glycation modification involving lysine specific residue were Fructosyl- lysine-$2H_20$ (FL-$2H_2O$) (+126.0317Da) Carboxymethyllysine (CML) (+58.0055 Da); Carboxyethyllysine (CEL) (+72.0211Da); Pyrraline (+108.0211Da); involving arginine specific residue were Imidazolone-B (+142.03); Argpyrimidine (80.0262Da); Nε-(5-hydro-5-methyl-4-imidazolon-2-yl) ornithine (MG-H1) (+54.0106 Da); Nε-(5-hydro-4- imidazolon-2-yl) ornithine (G-H1) (+39.9949Da) and involving both lysine and arginine residues were 1-Alkyl-2-formyl-3,4-glycosyl-pyrrole (AFGP) (+270.074Da); fructosyl lysine (+162.02); Imidazolone-A (144.03Da); methylglyoxal lysine dimer (MOLD) (49.0078Da); Methylglyoxal-derived imidazolium cross-link (MODIC) (36.0179Da) and Crossline (252.11Da) were included in the search (Nakayama et al., 2004; Wa et al., 2007). The PLGS identified glycated peptides were manually validated with the following criteria. a) all glycated peptides (identified by PLGS) should have a corresponding unglycated peptide with/without missed cleavage in MS^E analysis. b) all glycated

peptides should have a minimum of 5 fragment ions matching with the sequence d) all glycated peptides should exist at least in two replications.

III.2.11. Statistical Analysis

All experiments were performed in triplicates. Statistical analysis was performed by student's *t* test. Data are expressed as means ± SD and *P* value < 0.05 was considered as statistically significant.

III.3. Results and Discussion

Albumin is one of the most abundant plasma proteins and is heavily glycated in diabetes. In this study, we have addressed whether variation in the albumin levels influence glycation of plasma proteins and HbA1c. The study was performed in three systems: (1) streptozotocin (STZ)-induced diabetic mice plasma, (2) diabetic clinical plasma, and (3) *in vitro* glycated plasma. *In vivo* studies included STZ induced diabetic mice models and the induction of diabetes was confirmed by performing histopathological studies of tissue sections. PAS positive inclusions, intercapillary mesengial sclerosis and glomerular basement thickening which are the known manifestations of the onset of diabetes were observed in the kidney sections (Fig 3.1.B-D). In addition, inflammatory infiltration and fibrous band formation were observed mice liver tissue after subjecting to H&E staining (Fig 3.1F-H).

After confirming the onset of diabetes, blood samples from STZ induced diabetic mice was collected and plasma glucose, HbA1c, fructosamine and albumin levels were estimated. In both animal and clinical experiments, the glucose, HbA1c and fructosamine levels were significantly higher in diabetes than their corresponding control. Further, albumin levels were found to be significantly higher in non-diabetic plasma than in diabetic plasma. Depending on the albumin levels, diabetic plasma samples were further categorized into two groups, diabetic high albumin plasma DHAP and DLAP in both mice and clinical samples (Table 3.1). Variation in albumin levels and its influence on fructosamine was studied in both mice and clinical plasma samples (Table 3.1).

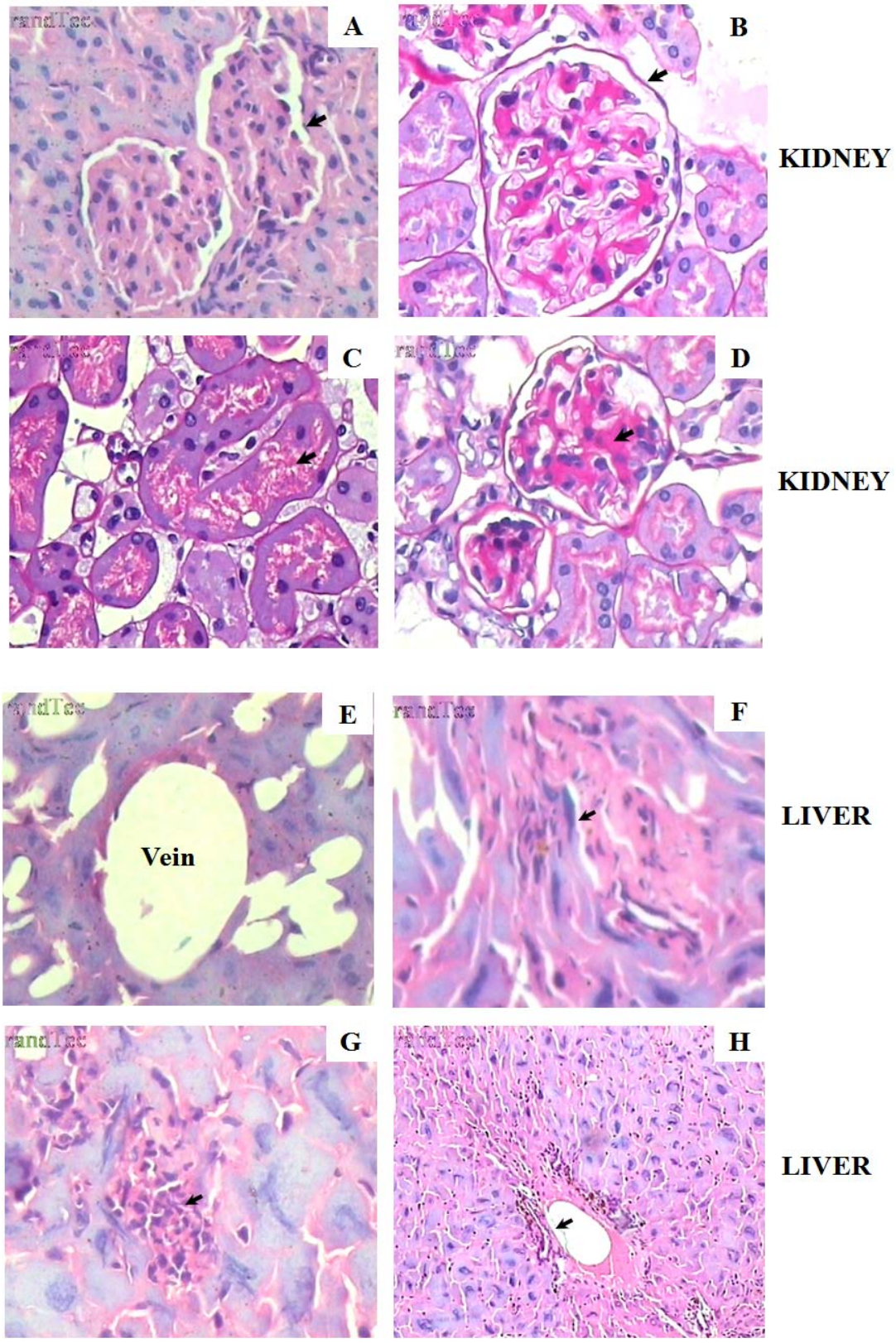


Figure 3. 1: Histopathology of kidney and liver tissue of Balb/C mice. A-D, kidney sections; E-I, liver sections. (A) H&E staining showing normal glomerulus, (B) PAS

staining showing glomerulus basement membrane thickening, (C) PAS positive inclusions, (D) Intercapillary mesengial sclerosis, (E) H&E staining with healthy liver tissue, (F) H&E staining fibrosis with inflammatory cells, G) Inflammatory infiltration, H) Fibrous band formation (200x).

Table 3. 1: Characteristics of plasma samples used for 2DE, western blot, and LC-MS^E analysis

Samples	Fasting plasma glucose (mmol/L)	HbA1c (%)	Albumin (g/L)	Fructosamine (µmol/L)	No. of glycated proteins identified by $LCMS^E$
Mice					
ND (n=9)	4.23±1.67	6.4±0.08	45.54±2.8	168.31±57.72	6
DHAP (n=9)	6.12±1.76*	8.8±0.86*	34.73±1.5*	341.51±65.97*	15
DLAP (n=9)	6.31±2.54*†	10.2±0.67*†	26.12±1.7*†	417.22±111.37*†	20
Clinical					
ND (n=10)	4.31±0.49	5.2±0.17	60.61±3.24	186.38±6.51	24
DHAP (n=7)	6.85±1.75*	8.4±0.9*	51.33±1.43*	369.67±16.93*	32
DLAP (n=7)	6.60±0.91*†	10.5±1.6*†	44.11±4.31*†	348.12 ±49.77*	36

Fructosamine level was higher in DLAP than DHAP in mice. However, in clinical plasma, though the levels of fructosamine were higher in diabetes, there was no significant difference between DLAP and DHAP. Fructosamine, the first product of glycation reaction is eventually converted to heterogeneous AGEs. The formation of AGEs is accelerated by various factors including auto-oxidation of glucose, age, oxidative stress etc. (Brownlee, 2001). Alternatively, they are enzymatically deglycated by fructosamine-3-kinase and chemically transglycated (Szwergold et al., 2005). Moreover, the fructosamine level in the clinical samples may be affected by the medication and glycemic control. ND, DLAP, DHAP and *in vitro* glycated plasma samples were processed as shown in Fig 3.2 for protein identification. A representative base peak intensity (BPI) chromatogram and total ion count (TIC)

chromatogram of diabetic plasma sample acquired by nanoACQUITY Ultra Performance LC (Waters Corporation, Milford, USA) respectively is depicted in Fig 3.3A and 3.3B respectively.

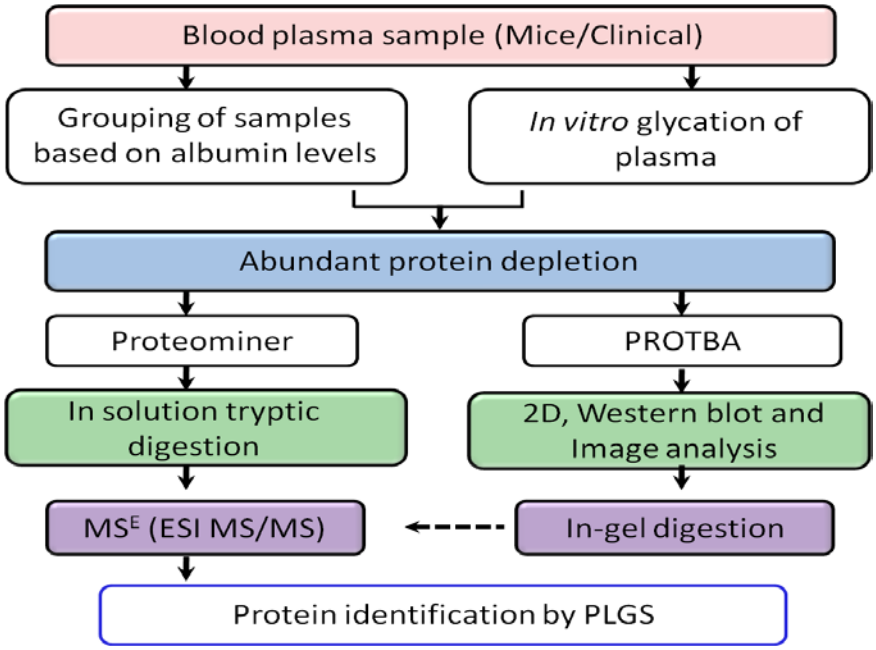
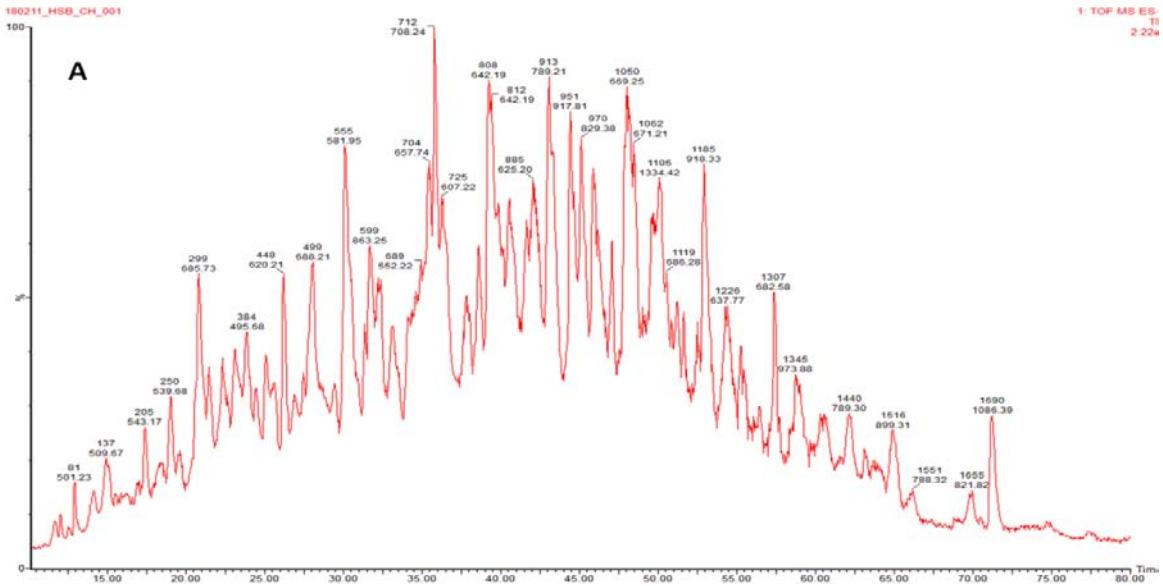


Figure 3. 2: Scheme depicting glycated plasma protein identification from non diabetic and diabetic samples. Pooled samples from mice, clinical patients and *in vitro* glycated were processed and analyzed as outlined in the chart.



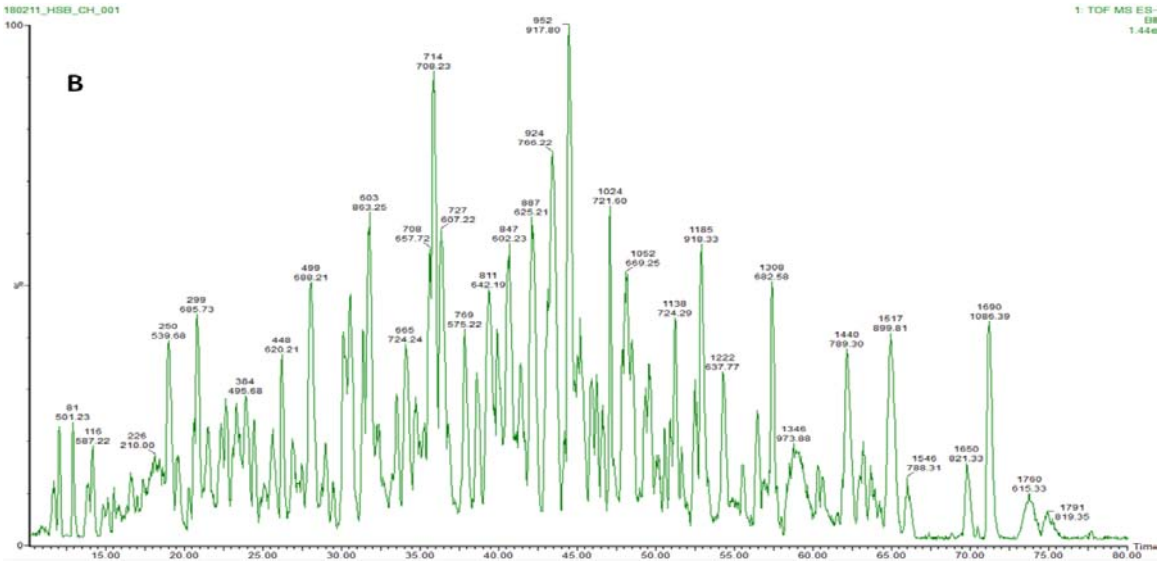


Figure 3. 3: Representative (A) total ion count (TIC) chromatogram and (B) base peak intensity (BPI) chromatogram of clinical diabetic plasma using nano-LC-ESI-MS.

A total number of 53 proteins in mice plasma and 73 proteins in clinical plasma were found to be glycated as shown in Fig 3.4. In both mice and clinical plasma samples, the number of glycated proteins was more in diabetes than the corresponding controls. However, in diabetic group, the number of glycated proteins was more in DLAP than DHAP in both mice and clinical plasma as shown in Table 3.1. The identified glycated proteins from mice and clinical plasma are listed in Table 3.2A and 3.2B respectively. Many of these identified proteins have been previously reported to be glycated in the diabetic plasma (Austin et al., 1987; Jaleel et al., 2005; Zhang et al., 2008; Zhang et al., 2011).

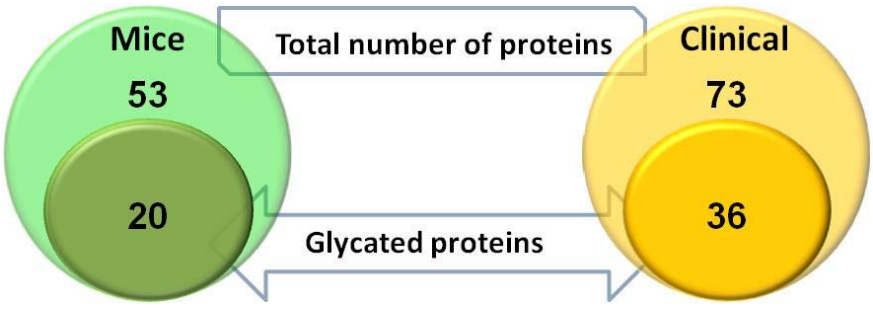


Figure 3. 4: Total number of proteins and glycated proteins identified by LCMS^{E} in both mice and clinical plasma.

Table 3. 2a: Glycated protein identification by LC-MS^E analysis from mice plasma samples

S.No	Accession number	Protein name	mW (Da)	pI (pH)	PLGS Score	Coverage (%)
1	P33622	Apolipoprotein C III	10975	4.4	1893.18	38.38
2	Q9QWK4	CD5 antigen like	38810	4.8	82.47	13.35
3	Q08879	Fibulin 1	77980	4.8	37.19	03.69
4	Q8VCM7	Fibrinogen γ chain	49359	5.4	114.35	09.17
5	P16301	Phosphatidylcholine sterol acyltransferase	49733	6.0	165.28	08.68
6	P46412	Glutathione peroxidase 3	25257	8.4	507.68	21.24
7	O70362	Phosphatidylinositol glycan specific phospholipase D	93196	6.7	257.50	17.68
8	P09813	Apolipoprotein A II	11311	7.2	1841.35	25.49
9	Q00623	Apolipoprotein A I	30568	5.5	6285.93	80.30
10	P06728	Apolipoprotein A IV	45001	5.3	252.58	25.82
11	P41317	Mannose binding protein C	25940	4.7	407.04	21.72
12	Q06890	Clusterin	51622	5.3	769.97	37.28
13	P52430	Serum paraoxonase arylesterase 1	39540	4.9	831.65	18.31
14	O88947	Coagulation factor X	53983	5.3	142.07	12.06
15	Q8K0E8	Fibrinogen β chain	54717	6.7	156.05	16.84
16	P98064	Mannan binding lectin serine protease 1	79916	5.2	142.03	15.77
17	Q61147	Ceruloplasmin	121074	5.4	116.08	18.38
18	P01027	Complement C3	186364	6.3	106.22	12.03
19	P08226	Apolipoprotein E	35844	5.4	727.02	37.30
20	P07724	Serum albumin	68647	5.6	1605.27	58.22

Table 3. 2b: Glycated protein identification by LC-MS^E analysis from clinical plasma samples

S. No	Accession number	Protein name	mW (Da)	pI (pH)	PLGS Score	Coverage (%)
1	P02671	Fibrinogen α chain	94914	5.6	1018.56	18.94
2	P02675	Fibrinogen β chain	55892	8.3	3553.24	47.86
3	P04217	α1B glycoprotein	54238	5.5	13670.25	50.30
4	P02787	Serotransferrin	76999	6.8	11988.39	58.88
5	P02679	Fibrinogen γ chain	51478	5.2	3187.94	31.79

6	P02790	Hemopexin	51643	6.6	7692.64	37.45
7	P04004	Vitronectin	54271	5.4	271.80	13.39
8	P02647	Apolipoprotein A I	30758	5.4	1290.93	32.21
9	P00738	Haptoglobin	45176	6.1	3442.24	48.52
10	P01042	Kininogen 1	71912	6.3	996.06	25.93
11	P01023	α2 macroglobulin	163187	6.0	2739.88	36.70
12	P02652	Apolipoprotein A II OS	11167	6.6	2783.40	58.00
13	P06727	Apolipoprotein A IV	45371	5.1	409.64	33.84
14	P19652	α1 acid glycoprotein 2	23587	4.8	115.67	14.93
15	P01019	Angiotensinogen	53120	5.8	190.82	25.76
16	P02749	β2 glycoprotein 1	38272	7.9	799.18	12.46
17	P00450	Ceruloplasmin	122127	5.3	717.42	25.26
18	P01024	Complement C3	187029	6.0	909.55	19.48
19	P01009	α1 antitrypsin	46707	5.2	1130.88	34.93
20	P02774	Vitamin D binding protein	52929	5.2	7865.02	51.05
21	P02768	Serum albumin	69321	5.9	6080.81	56.98
22	P43652	Afamin	69024	5.5	200.19	04.84
23	P02760	Protein AMBP	38973	5.9	702.33	18.18
24	O14791	Apolipoprotein L1	43946	5.5	175.72	17.09
25	P00751	Complement factor B	85478	6.7	294.16	17.41
26	P0C0L5	Complement C4 B	192671	6.7	878.92	10.21
27	P02748	Complement component C9	63132	5.3	619.47	16.10
28	P02765	α2 HS glycoprotein	39299	5.3	1445.69	33.52
29	P06396	Gelsolin	85644	5.8	371.93	07.42
30	P69905	Hemoglobin subunit alpha	15247	9.2	5153.95	55.63
31	P68871	Hemoglobin subunit beta	15988	6.9	9606.11	67.35
32	P01876	Ig α1 chain C region	37630	6.1	5543.72	55.24
33	Q14624	Inter α trypsin inhibitor heavy chain H4	103293	6.5	199.53	15.16
34	Q96PD5	N acetylmuramoyl L alanine amidase	62177	7.3	236.03	22.22
35	P00747	Plasminogen	90510	6.9	171.75	12.84
36	Q9Y5J7	Mitochondrial import inner membrane translocase subunit Tim9	10371	7.0	110.05	39.33

Table 3. 2c: Glycated protein identification by LCMS^E analysis from *in vitro* glycated plasma samples

S. No	Accession number	Protein name	mW (Da)	pI (pH)	PLGS Score	Coverage (%)
1	P02675	Fibrinogen β chain	55892	8.3	802.42	20.98
2	P69905	Hemoglobin subunit alpha	15247	9.2	6164.65	66.90
3	P02671	Fibrinogen α chain	94914	5.6	206.09	11.09
4	P01023	α 2 macroglobulin	163187	6.0	6190.42	33.79
5	P01042	Kininogen 1	71912	6.3	149.19	07.14
6	P02774	Vitamin D binding protein	52929	5.2	6154.85	56.96
7	P19652	α 1 acid glycoprotein 2	23587	4.8	2099.59	27.36
8	P01008	Antithrombin III	52568	6.3	110.45	06.68
9	P02787	Serotransferrin	77013	6.8	27780.8	61.17
10	P05155	Plasma protease C1 inhibitor	55119	6.1	1057.66	10.80
11	P20742	Pregnancy zone protein	163759	5.9	452.08	05.74
12	P04004	Vitronectin	54271	5.4	753.31	15.48
13	P01024	Complement C3	187029	6.0	180.45	07.46
14	P00738	Haptoglobin	45176	6.1	3027.81	40.64
15	P10909	Clusterin	52461	5.8	280.00	20.04
16	P01009	α 1 antitrypsin	46707	5.2	11382.4	39.47
17	P06727	Apolipoprotein A IV	45371	5.1	1932.92	41.92
18	P02768	Serum albumin	69321	5.9	6137.23	53.04
19	P0C0L4	Complement C4 A	192649	6.6	171.62	08.08
20	P02749	β 2 glycoprotein	38272	7.9	799.18	12.46
21	Q9BUY7	EF hand domain containing protein C14	19212	9.0	62.40	14.11
22	P02679	Fibrinogen gamma chain	51478	5.2	3696.71	40.62
23	P01700	Ig lambda chain V I region	11888	9.2	203.21	21.43
24	Q9UIK5	Tomoregulin 2	41400	4.8	142.22	29.68
25	P01876	Ig α 1 chain C region	37630	6.1	14276.8	47.88
26	P68871	Hemoglobin subunit β	15988	6.9	15028.1	82.31
27	P25311	Zinc α 2 glycoprotein	34237	5.6	62.05	09.06
28	O43866	CD5 antigen like	38062	5.1	70.13	11.82
29	P00450	Ceruloplasmin	122127	5.3	5274.55	26.57
30	P00751	Complement factor B	85478	6.7	85.26	7.59
31	P01019	Angiotensinogen	53120	5.8	1394.78	25.77
32	P01591	Immunoglobulin J chain	18086	4.9	2430.29	38.36

33	P01857	Ig γ 1 chain C region	36083	8.2	2385.65	24.24
34	P01877	Ig α 2 chain C region	36503	5.7	9317.15	36.76
35	P02750	Leucine rich α 2 glycoprotein	38154	6.5	949.81	27.95
36	P04217	Alpha 1B glycoprotein	54219	5.5	5957.79	33.13
37	P04220	Ig μ heavy chain disease protein	43030	5.0	3581.68	25.32
38	P08697	α 2 antiplasmin	54531	5.8	87.61	08.55
39	P20848	Putative α1 antitrypsin related protein	47860	7.9	1312.19	07.38
40	P43251	Biotinidase	61093	5.8	072.40	07.73
41	Q06033	Inter α trypsin inhibitor heavy chain H3	99786	5.4	45.29	05.62
42	Q96II8	Leucine rich repeat and calponin homology domain containing protein 3	86030	6.2	60.42	03.86
43	Q9NZL4	Hsp70 binding protein	39448	5.0	59.55	17.96
44	Q9Y4I1	Myosin V	215267	8.5	87.74	03.56
45	A2IDD5	Coiled coil domain containing protein 78	48491	8.2	66.66	05.48

Further, characterization of glycation modifications in glycated proteins was performed either by using comprehensive mice protein database (UniProt) for mice plasma samples or by comprehensive human protein database (UniProt) for clinical samples. But, the number of glycation modifications identified in glycated proteins in both mice and clinical plasma samples was found to be very low. The probable reason for the scarce number of glycation modifications could be (i) low concentration of modified peptides as they were not enriched, (ii) the glycated peptides were analyzed by MS^E, a data independent acquisition method, where all the peptides were fragmented, without precursor ions selection, (iii) inefficiency of algorithm to identify PTMs using large database. This technical difficulty was overcome by using 'zoom in' approach where a targeted protein database search was carried out for identification of glycation modifications from both mice and clinical plasma samples. This kind of approach was used in the InsPecT (Interpretation of Spectra with PT modifications) algorithm that uses peptide sequence tags as efficient filters to reduce the size of the database by a few orders of magnitude while retaining the correct peptide with very high probability (Tanner et al., 2005). Furthermore, identification of proteins by reducing database size is already in practice. For example, reducing the database by restricting certain parameters like taxonomy, allows searches to be limited to entries

from a particular species or groups of species. This ensures the correct protein identification and the hit list contains entries only from the selected species. Therefore, by using 'zoom in' approach the number of glycation modifications in glycated proteins increased significantly as described in Fig 3.5

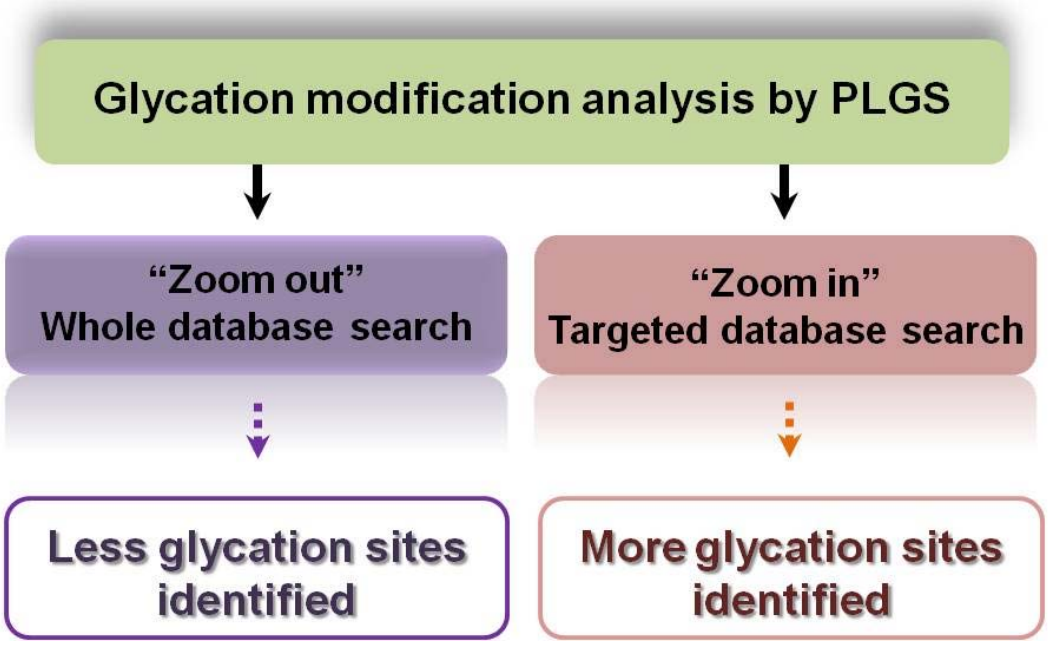


Figure 3. 5: Targeted database search for analysis of glycation modification in glycated proteins.

A characteristic MS/MS annotation of glycation modification, Carboxyethyllysine (CEL) with the increase in mass of 72.0154 Da is depicted in Fig 3.6. A similar trend as that of glycated proteins was observed for glycation modified sites. The number of glycation modifications identified in glycated protein was higher in DLAP than in DHAP and non-diabetic control in both mice and clinical plasma (Fig. 3.7A and 3.7B).

Moreover, 2DE-western blot analysis with anti-AGE antibodies indicated an increase in the extent of glycation in diabetes than control. Glycated proteins (Haptoglobin, Vitamin D binding protein, Apolipoprotein A IV, and Fibrinogen gamma) that were common to mice, clinical and *in vitro* glycated plasma are represented in Fig 3.8.

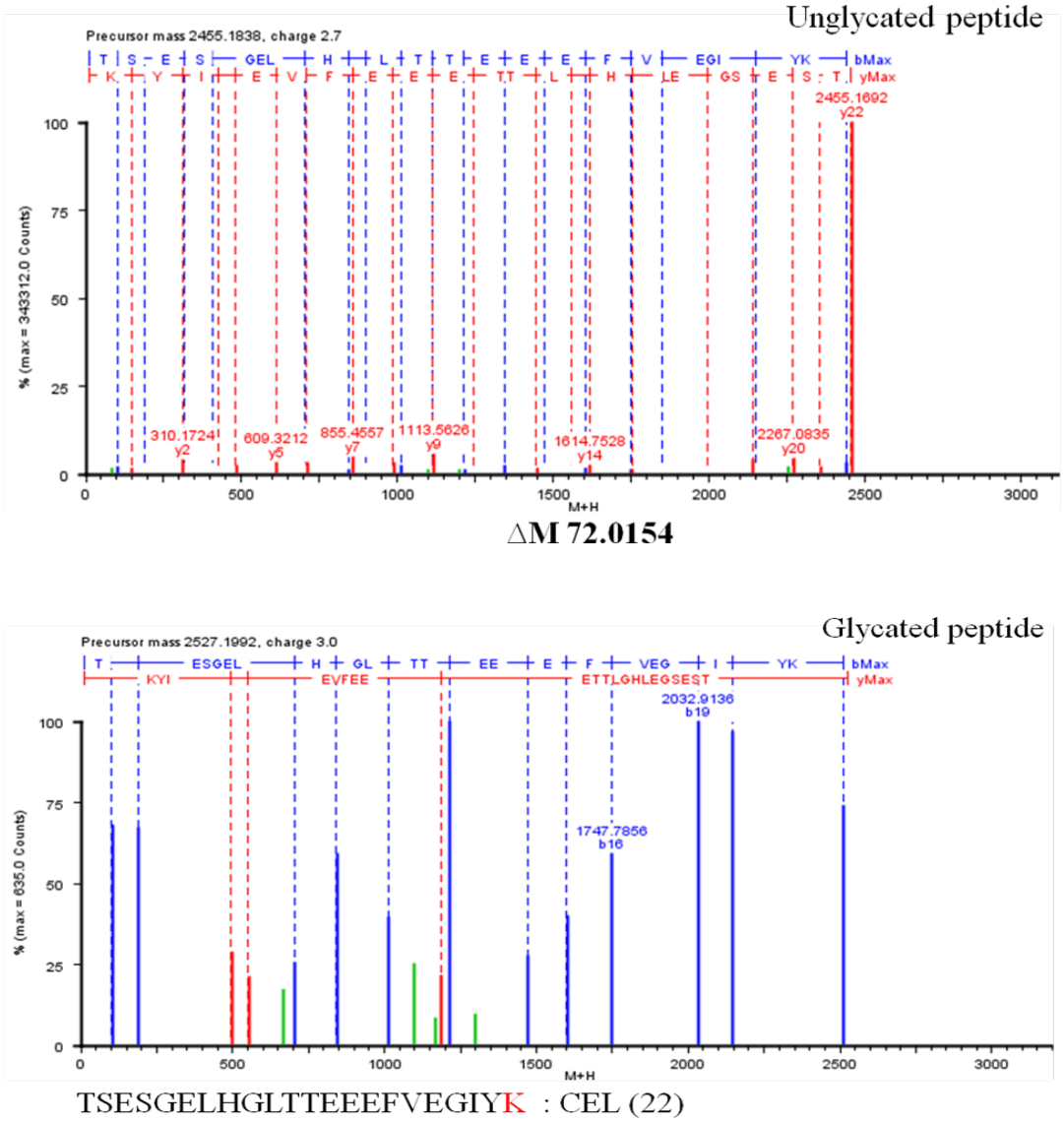


Figure 3. 6: A representative MS/MS annotation of glycation modification of carboxyethyllysine (CEL) showing an increase in mass of 72.0154 Da.

The extent of glycation was higher in plasma proteins of the DLAP than in DHAP as revealed by densitometry. These proteins also had more number of glycation sites in LC-MS^{E} experiment as shown in Fig 3.7. The logical explanation for the differential glycation of plasma proteins could be the differential exposure of these proteins to glucose due to variation in albumin levels thereby affecting the stoichiometry of plasma protein glycation.

Additionally, albumin levels showed a negative correlation with glycated hemoglobin HbA1c in both mice and clinical experiments (Fig 3.9A and 3.9B). With increasing albumin level the HbA1c was found to be decreased. This was also quite evident in a previous study with 4158 participants, where it was shown that more than 50% of the patients with lower albumin had higher HbA1c (> 8 %), where as HbA1c was lower among patients with high albumin levels (Rodriguez-Segade et al., 2005).

This study suggested that variation in albumin levels also influenced differential glycation of HbA1c. Possibly similar blood glucose level might lead to different HbA1c depending upon the albumin content in the plasma. Further, HbA1c levels have been known to be influenced by various factors including glycemic status over a period of 8 weeks, erythrocyte life span, anaemia, hemodialysis etc (Jeffcoate et al., 2004). Therefore, sometimes HbA1c may not indicate the precise glycemic control. Alternatively, glycated albumin has been suggested to be a better marker for short term glycemic control (Philippe and Bourdon, 2011). Additionally, low serum albumin in diabetes was also associated with increased cardiovascular disease (Folsom et al., 1995). As well as hypoalbuminemic patients associated with malnutrition were more prone to develop diabetic complications (Folsom et al., 1995; Kaysen et al., 2001).

To establish the fact that variation in albumin levels influences glycation of plasma proteins, control human plasma samples were differentially depleted for albumin and categorised into two groups; high albumin plasma (HAP) and low albumin plasma (LAP) with albumin levels of 45g/L and 35 g/L, respectively, and were *in vitro* glycated. LC-MS^E analysis of these plasma samples resulted in identification of more number of glycated proteins as shown in Table 1, as well as the glycation modifications in the identified proteins were higher in LAP than HAP (Fig 3.7C).

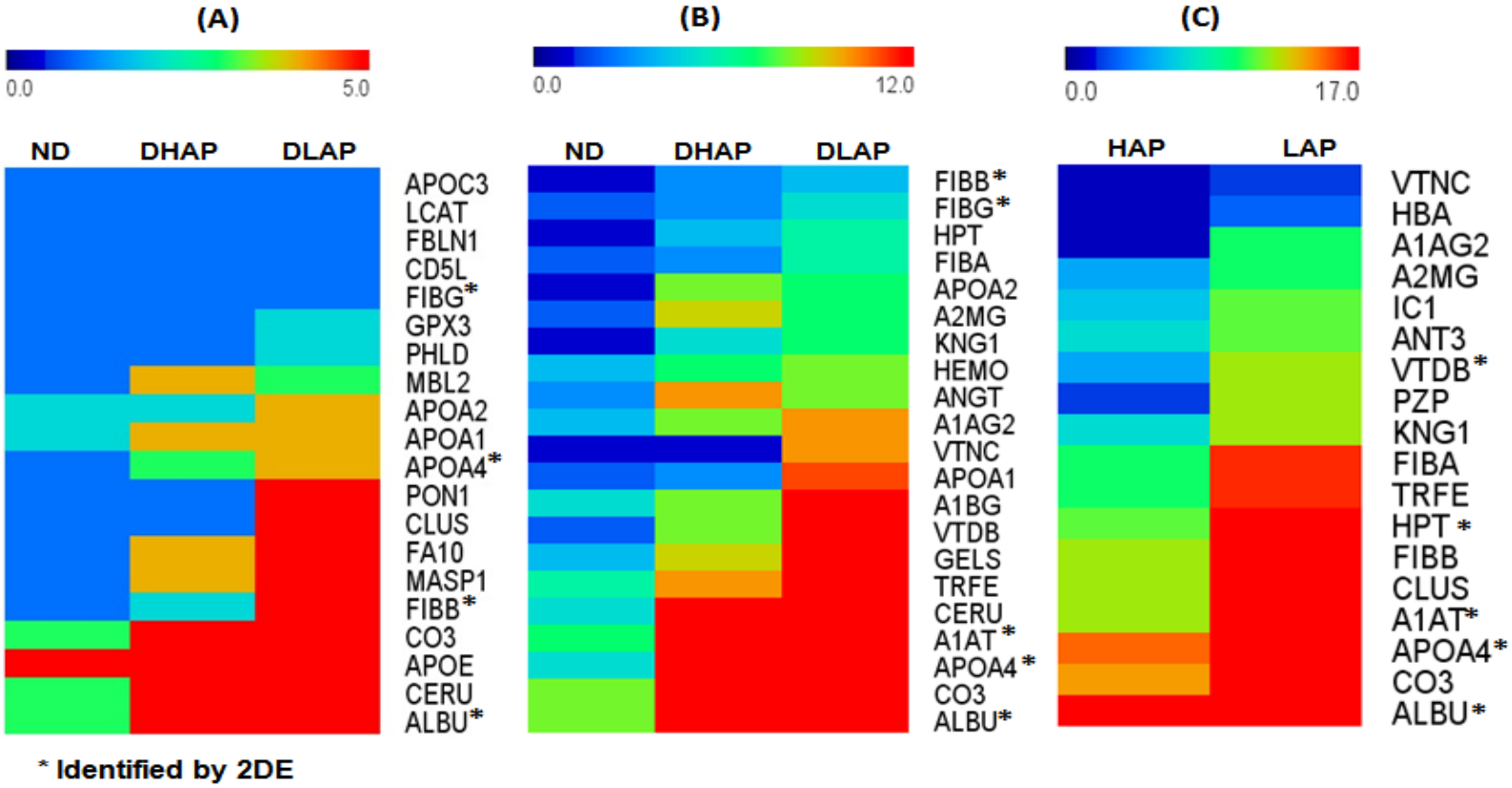


Figure 3. 7: Heat map analysis of glycation modification sites on glycated proteins of (A) mice plasma (B) clinical plasma (C) *in vitro* glycated plasma. The analysis shows increased numbers of glycation modification in DLAP than in DHAP and ND plasma. The expanded form of abbreviated proteins is listed in Table 3.2.

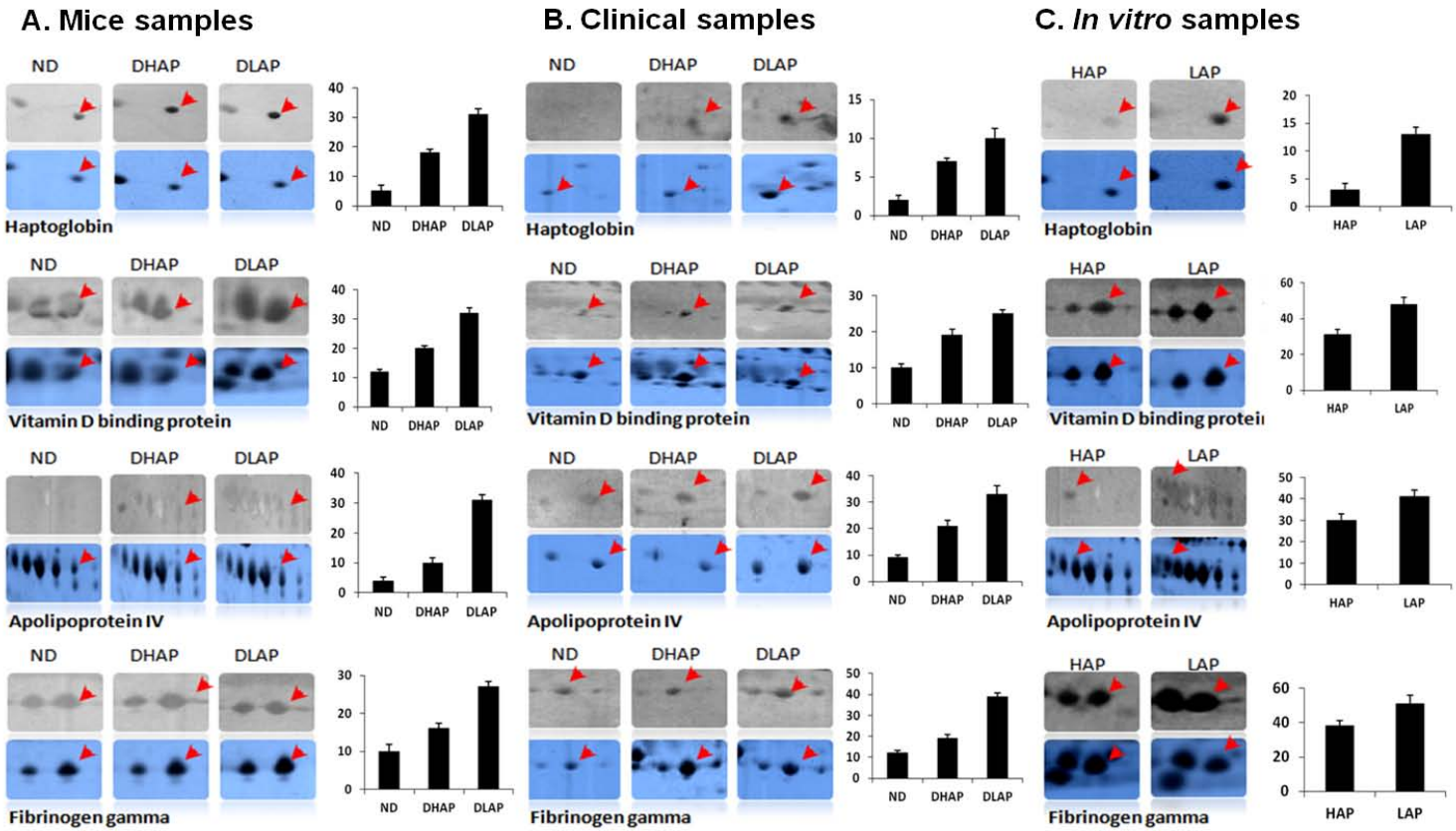


Figure 3. 8: 2DE and western blot analysis of glycated proteins (A) mice plasma (B) clinical plasma (C) *in vitro* glycated plasma. Abundant plasma proteins were depleted and separated by 2DE. Glycated proteins were visualized by western blotting using anti-AGE antibody.

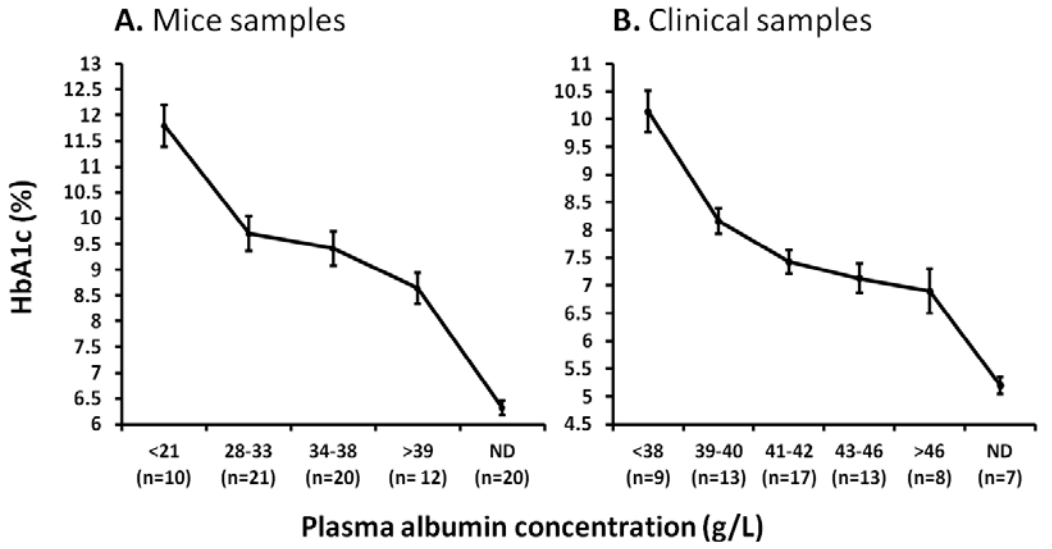


Figure 3. 9: Co-relation between albumin and HbA1c levels. (A) Mice plasma [n= 83, diabetic (63) and 20 non diabetic (20)], (B) Clinical plasma [n= 67, diabetic (60) and non diabetic (7)]. Albumin and HbA1c levels were estimated from non diabetic and diabetic plasma. A negative correlation was observed where, HbA1c levels were found to be decreased with increased albumin concentration. Vertical lines indicate SE.

The same trend was also reflected in 2DE-western blot analysis. These results conclusively prove that the variation in albumin levels influences the glycation of plasma proteins. Furthermore, albumin competes for glycation with other plasma proteins, as it itself gets more glycated than the other proteins as revealed by a number of glycation modified sites (Fig 3.7C). Our previous study also revealed that, albumin competitively inhibited the glycation of insulin, which was used as a model for low abundant proteins (Bhonsle et al., 2008). The probable mechanism by which albumin can inhibit or regulate the glycation of insulin, has been mechanistically illustrated in Fig 3.10. At low albumin levels, the relative intensity of glycated insulin was about 50%, while the intensity of glycated insulin decreased with higher albumin levels. These results are justified as albumin gets glycated competitively due its abundance and protects insulin from getting glycated.

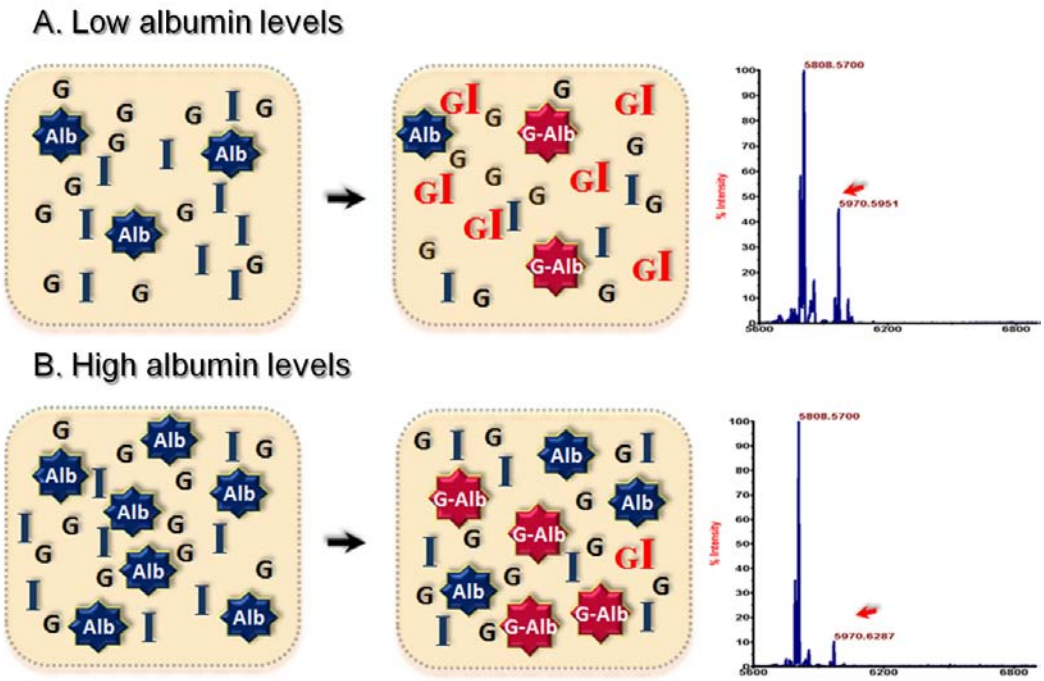


Figure 3. 10: Mechanism of glycation regulation by albumin. Insulin (model protein) was glaciated *in vitro* by incubating with glucose in the presence of either low or high levels of albumin. (A) At low albumin levels, the relative intensity of glycated insulin was higher and (B) At high albumin levels, the relative intensity of glycated insulin was decreased as albumin competes for glycation due to its abundance. I-insulin; Alb-albumin; G-glucose; GI-glycated insulin; G-Alb-glycated albumin.

In conclusion, our systematic and comprehensive studies emphasises that albumin regulates glycation of low abundant proteins both *in vitro* as well as *in vivo*. All these evidences reinforce the fact that low levels of albumin are associated with increased plasma protein glycation and HbA1c, elucidating the importance of albumin levels in the regulation of glycation of plasma proteins and HbA1c. Perhaps, maintaining near normal levels of albumin in diabetes may be helpful thereby protecting plasma proteins from the adverse effects of glycation. However, corroboration with clinical trials is a further requisite. Thus, lower levels of albumin in diabetes could be a risk factor for glycation induced complications.

CHAPTER IV

"PROTEOMIC STUDY REVEALS DOWN REGULATION OF APOLIPOPROTEIN A1 IN PLASMA OF POORLY CONTROLLED DIABETES"

IV.1. Introduction

The human plasma proteome analysis has the potential to ease disease diagnosis and therapeutic monitoring (Anderson and Anderson, 2002; Anderson, 2010). Plasma protein biomarkers are useful for diagnosis and prognosis of many diseases including diabetes. Inability to utilize blood glucose is the hallmark of diabetes leading to the development of complications including neuropathy, retinopathy, nephropathy and atherosclerosis (Taylor and Agius, 1988). Development of these complications is 2.5 times higher in patients with long-term poorly controlled glycemic levels than controlled glycemic levels (Chase et al., 1989). Further, on the basis of HbA1c (glycated haemoglobin) levels diabetic subjects can be categorized as controlled diabetics (CD) and poorly controlled diabetes (PCD) with HbA1c levels up to 8% and more than 8%, respectively, of total haemoglobin (Takahashi, 2007).

To understand molecular mechanisms of pathophysiology of diabetic complications, many studies have utilized proteomic approaches and have been reviewed in great detail (Sundsten and Ortsater, 2009) Pathology of diabetic complications is associated with increased generation of Reactive Oxygen Species (ROS) resulting in oxidative, glycoxidative and carbonyl stress (Brownlee, 2001). Advanced Glycation End products (AGEs) upon engagement with the receptor for AGE (RAGE) induce the generation of ROS and activation of transcription factor NF-kB causing changes in gene expression (Giacco and Brownlee, 2010).

AGEs are also known to affect the activity of several plasma proteins. For example, about fifty percent enzyme activity of asparte aminotransferase was inactivated as a result of glycation (Bousova et al., 2005). Similarly impaired activity of glycated alpha-1-antitrypsin was observed in diabetes thereby leading to protease-antiprotease imbalance (Hashemi et al., 2007). Glycated transferrin showed deterioration of antioxidant capacity in diabetic patients (Mohammad et al., 2010). Additionally, glycoxidative modification leads to protein aggregation resulting in protein instability. In order to prevent serious metabolic disturbances caused by accumulation of glycoxidatively modified proteins, these proteins are further degraded by the proteasomal system (Jung and Grune., 2008).

Glycoxidative modification of protein results in the elicitation of autoantibodies against several diabetic plasma proteins. These proteins include albumin, insulin, carbonic anhydrase and heat shock proteins, thereby resulting in their decreased levels in diabetic plasma (Winter and Schatz, 2011). However, any variation in insulin levels affects insulin regulated protein synthesis of several proteins. For example, decreased insulin synthesis and insulin resistance in diabetes affects gene expression of albumin and fibrinogen (Peavy et al., 1978; Tessari et al., 2006). All these factors contribute to the differential protein expression. It is possible that to compensate altered protein functions and protein loss; there could be enhanced or altered gene expression thereby resulting in varying levels of proteins in diabetes. Previous studies have reported differential expression of various proteins like alpha-1 antitrypsin, fibrinogen, vitamin D binding protein, complement C3 and apolipoprotein in diabetes (Blanton et al., 2011; Ceriello, 1997; Engstrom et al., 2005; Lapolla et al., 2008). However, it is important to study the differential protein expression in poorly controlled diabetes to understand the pathophysiology associated with the development of diabetic complications. Therefore, in this study for the first time we have analyzed differential protein expression in plasma of controlled and poorly controlled diabetic subjects by using proteomic methods, and validated by western and dot blot analysis.

IV.2. Materials and Methods

All chemicals were procured from Sigma-Aldrich (St. Louis, USA) otherwise mentioned. Antibodies for, fibrinogen, haptoglobin, vitamin D binding protein, alpha-1-antitrypsin, and apolipoprotein A1 (Apo A1) were procured from Abcam, UK.

IV.2.1. Clinical Plasma Sample Collection

Blood samples were collected from diabetic patients through an informed consent from Maharashtra Medical Research Society (MMRS) and approved by the Joshi Hospital Ethics Committee. Fasting blood glucose and HbA1c levels were determined by using a glucometer (Bayer, Germany) and 'in2itTM' analyzer (Bio-Rad, CA, USA) respectively. Plasma was obtained by EDTA treatment, which was then centrifuged at 1500 *g* for 15 min and the supernatant was stored at -80°C until

further use. Serum creatinine, serum HDL, serum triglycerides and serum cholesterol were estimated using diagnostic kits that were procured from Agappe Diagnostics (Switzerland).

IV.2.2. Plasma Sample Preparation

Based on HbA1c levels, plasma samples were grouped into non-diabetic (<6.4%), controlled diabetic (7-8 %) and poorly controlled diabetes (8.8-12.3 %). Ten representative plasma samples with equal volume from each group were pooled and were used for proteomic analysis. In order to remove the high-abundant proteins like Albumin and Immunoglobin G from plasma, the ProteoPrep® Blue albumin & IgG Depletion Kit, Sigma-Aldrich (St. Louis, USA),were used. 30 µl plasma sample was processed using 500 µl of blue slurry, and the depletion procedure was performed at room temperature according to manufacturer's instructions. Protein concentration of the depleted plasma sample was determined by using a quick start Bradford protein assay kit (Bio-Rad, CA, USA).

IV.2.3. 2DE, Western Blot and Image Analysis

IV.2.3.1. First Dimension- Isoelectrofocussing (IEF)

In the first dimension, proteins were separated by IEF with precast IPG strips (nonlinear gradient pH 4-7, 7 cm, BioRad). 150 µg of protein was solubilized in 125 µl rehydration buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 70 mM DTT, 0.5% C7BzO, 1 µl ampholytes pH 3-10. IPG strips were passively rehydrated overnight at room temperature. IEF was performed using the PROTEAN IEF Cell (BioRad, CA, USA) at 20°C with a constant power (50 µA/ IPG-strip) at 250 V for 30 min, slow ramping; followed 4000 V for 2.5 hour, linear ramping; and finally 10000 Volt-hour with linear ramping.

IV.2.3.2. Second Dimension- SDS PAGE

After performing IEF, IPG strips were equilibrated in 0.375 mM Tris/ HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS and 2% DTT for 15 min, washed with 0.375 mM Tris/HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, and 2.5%

iodoacetamide for another 15 min. Then the IPG strip was transferred to the top of 12% SDS-PAGE gel, and overlayed with 0.5% low melting agarose. SDS-PAGE was carried out at constant voltage of 70 V for initial 10min followed by 110V/ IPG-strip for the rest of the time. Resolved proteins were visualized by CBB-R250 staining or by western blotting.

IV.2.3.3. Western Blot Analysis

Proteins were transferred onto PVDF membrane and blocked overnight at 4°C using 5% skimmed milk in TBS. Blocked membranes were then incubated with goat anti-AGE antibody (Millipore, MA) in 1:3000 dilutions for 1 hour at room temperature. Membranes were then washed once with TBS-T (0.05% tween 20) for 3 mins followed by two washes with TBS 3 minutes each. Washed membranes are then incubated with 1:5000 dilution of rabbit anti-goat secondary antibody for 30 minutes at room temperature. Membranes were washed again as mentioned above and were then incubated with streptavidin conjugated horseradish peroxidase with a dilution of 1:5000 for 15 minutes.

Immunodetection on washed membranes was then performed by incubating membranes by using SIGMAFAST^{TM} DAB substrate. Stained gels and developed blots were acquired by the calibrated densitometer (GS 800 Bio-Rad, CA, USA).

IV.2.3.4. Image Analysis

Image analysis was performed using PDQuest Advanced software version 8.0.1 (Bio-Rad, CA, USA). Acquired images were cropped and auto scaled using advanced crop settings. For all the cropped images same pre-processing parameters were used, including background correction which uses the ‘‘floating ball’’ method and the streak removal filter. After pre-processing by using spot detection wizard, total number of spots on the gel was determined. The unmatched spots were manually matched using manual spot editing tools. The spots showing fold change of more or less than 1.5 were considered as differentially expressed proteins.

IV.2.4. Trypsin Digestion

Spots were excised and destained using 50% ACN/50 mM ammonium bicarbonate. After destaining, gel pieces were dehydrated using 100% ACN followed by reduction of proteins with 10 mM DTT for 30 minutes at 56º C. Proteins were then alkylated with 55 mM of iodoacetamide for 45 minutes at room temperature in dark. Gel pieces were washed twice with 50% ACN/50 mM ammonium bicarbonate followed by dehydration using 100% ACN. To the dehydrated gel pieces trypsin was added in 1:10 (trypsin to protein ratio) and was incubated overnight at 37°C. Tryptically digested peptides were extracted with 5% formic acid in 50% ACN. Extracted peptides were vacuum concentrated and were then reconstituted in 5 µl of 0.1% formic acid in 3% ACN.

IV.2.5. LC-MS^E Analysis and Protein Identification

Two micro-liter digested peptides with final concentration of 100 ng/ µl was analyzed by using nanoACQUITY UPLC online coupled to the SYNAPT HDMS system (MS^E) (Waters Corporation, USA) as described by Cheng et al., 2009. The separation was performed on a BEH 130 C18 1.7 µM × 75 µM × 150 mm Peptide Separation Technology column at 40°C. Mobile phase A was aqueous 0.1% formic acid and B was 0.1% formic acid in acetonitrile. The protein digests were eluted with a 60-min gradient (0-50% B) and a 90-min gradient (0-50% B). The flow rate was 300nl/min. An auxiliary pump was used to spray a solution of 500 femto mole/µl Glu1-fibrinopeptide B (GFP) in 50% ACN containing 0.1% formic acid for mass accuracy reference (lockmass channel), with a flow rate of 500nl/min. MS acquisition was operated in the positive ion V-mode with scan time of 1 second. An alternating low-energy (collision cell energy 3 V) and elevated energy (collision cell energy ramped from 15 to 40 V) was used to obtain the precursor ions (MS) and their fragmentation data (MS^E), respectively. A capillary voltage of 3.2 kV, source temperature of 80°C and cone voltage of 32 V were maintained during the analyses. Sampling of the lock spray channel was performed every 30s. The system was tuned for a minimum resolution of 10000 and calibrated using a 500 fmol/µl GFP infusion.

After MSE analysis, data was analyzed by using Protein Lynx Global Server software (PLGS. Version 2.4. Waters Corporation, Milford, MA, USA). For protein identification, processed samples were searched against the UniProt human database containing 44,987 protein entries. Search criteria included fixed and variable modifications as carbamidomethylation and oxidation (M) respectively.

IV.2.6. Dot Blot Analysis

Ten plasma samples each from non-diabetic, controlled diabetic and poorly controlled diabetic patients were diluted with PBS in 1:1000 dilutions. 2 µl of the diluted sample was spotted onto nitrocellulose membranes. The membrane was air dried and blocked for two hours with 5% skimmed milk prepared in TBS at 37 °C. The membrane was then incubated with anti-Apo A1 antibody with a dilution of 1:7000 for one hour at room temperature. The membrane was washed twice with (0.05% tween 20) followed by incubation with biotinylated secondary antibody for 30 minutes. The membrane was washed twice with TBS-T followed by incubation with streptavidin conjugated horseradish peroxidase for 15 min at room temperature. Immunodetection was performed by SIGMAFAST^{TM} DAB substrate.

IV.2.7. Statistical Analysis

All experiments were performed in triplicates. Statistical analysis was performed by student's *t* test. Data are expressed as means ± SD. *P* value < 0.05 was considered as statistically significant.

IV.3. Results and Discussion

Diabetic plasma protein differential expression studies help in better understanding of pathophysiology of diabetic complications. Many such studies have been reported earlier with a comparison of ND and diabetic plasma samples (Sundsten and Ortsater, 2009). However, the pathophysiology of diabetic complication is better reflected in PCD than CD. In view of this, the differential plasma protein expression was studied in CD and PCD clinical plasma samples after monitoring fasting plasma glucose, HbA1c levels, serum creatinine, High Density Lipoproteins (HDL), triglycerides and cholesterol levels (Table 4.1).

Table 4. 1: Parameters evaluated in ten controlled, ten poorly controlled diabetic patients and ten normal controls for 2DE, western blot, and LC-MS^E analysis. Data expressed are in mean ± SD; * P< 0.005 when compared with ND; †P< 0.05 when compared with CD.

Subjects (n= 10)	**Fasting plasma glucose (mg/dl)**	**HbA1c (%)**	**Serum creatinine (mg/dl)**	**HDL (mg/dl)**	**Triglycerides (mg/dl)**	**Cholesterol (mg/dl)**
Non diabetic **(ND)**	87.9 ± 11.7	5.5 ± 0.3	0.7 ± 0.05	50.6 ± 5.6	91 ± 22.6	145.1 ± 15.4
Controlled diabetic **(CD)**	92.8 ± 11.55*	7.2 ± 0.6*	0.8 ± 0.02*	42.7 ± 6.2*	116.3 ± 19.2*	146 .0 ± 14.0
Poorly controlled diabetic **(PCD)**	187.3 ± 23.7†	9.9 ± 1.0†	1.1 ± 0.17†	33 ± 2.1†	219.3 ± 16.8†	200 .0 ± 30.3

Proteins were separated by using 2DE followed by staining with CBB-R250 as shown in Fig 4.1. PDQuest analysis disclosed six spots that were differentially expressed. The spots were excised and tryptically digested followed by protein identification by LCMS^E. Differentially expressed proteins are enlisted in Table 4.2.

The densitometric analysis of 2DE gels revealed upregulation of fibrinogen and haptoglobin and downregulation of vitamin D binding protein, alpha-1-antitrypsin, transthyretin and Apo A1 in diabetic plasma compared to non-diabetic plasma samples.

In diabetes, fibrinogen is upregulated to compensate protein loss in peritoneal dialysis fluid (Prinsen et al., 2003). Further, the elevated levels of fibrinogen are associated with lower platelet inhibition in patients with cardiovascular disease (Ang et al., 2008). Haptoglobin, by binding to hemoglobin prevents the loss of iron and parenchymal injury mediated by hemoglobin during vascular hemolysis (Langlios and Delanghe, 1996).

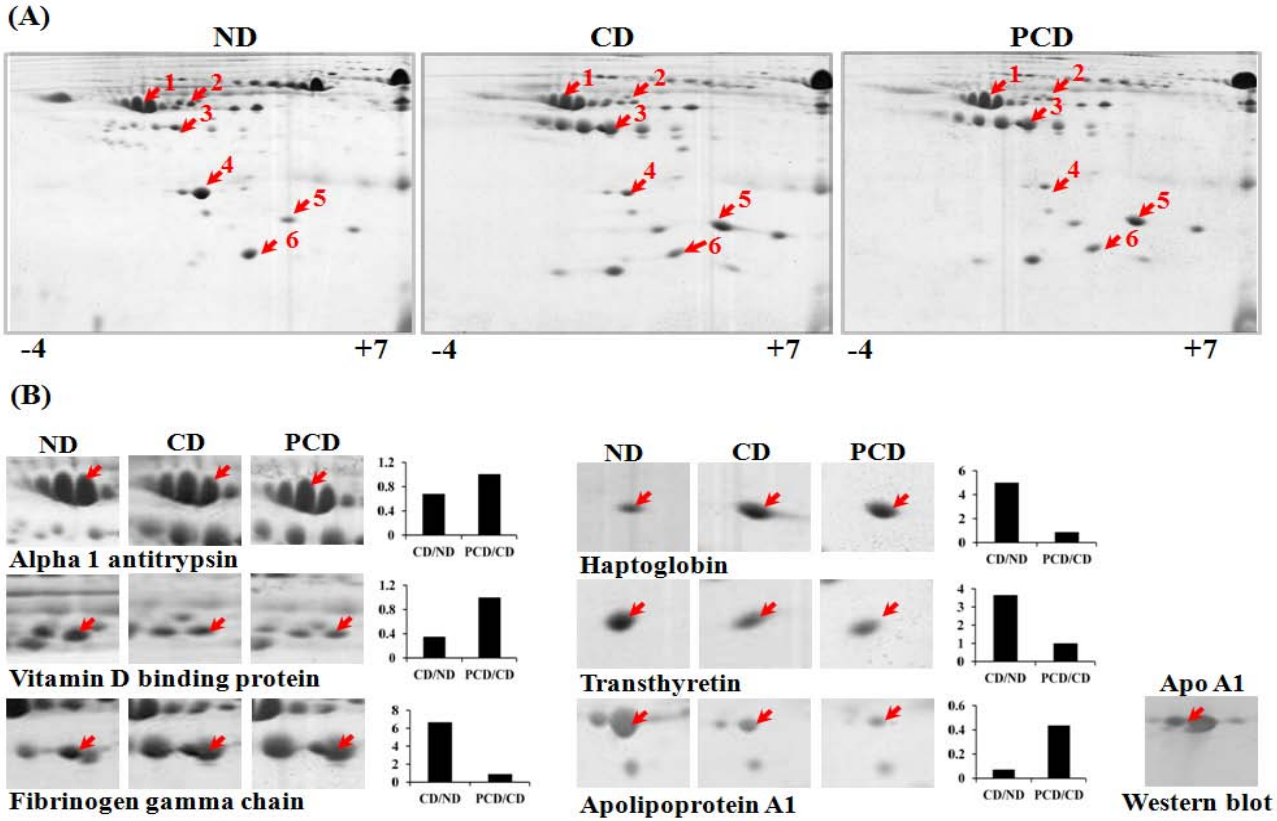


Figure 4. 1: Differential protein expression study by 2DE. (A) 2DE analysis of non diabetic (ND), controlled diabetic (CD) and poorly controlled diabetic (PCD) clinical plasma samples. (B) Densitometric analysis of differentially expressed proteins from ND, CD and PCD.

Table 4. 2: Protein identification and fold expression in diabetic and non diabetic controls by using 2DE and LC-MS^{E} analysis. [a] - Non diabetic [b] - Controlled diabetic [c] - Poorly controlled diabetes. **$**- P value was found to statistically significant, $P < 0.05$.

Spot No.	Protein Name	Accession number	mW (Da)	pI (pH)	PLGS Score	Coverage (%)	Fold change (CD^{b} /ND^{a})	Fold change (PCD^{c} /CD^{b})
1	Alpha 1 antitrypsin	P01009	46707	5.24	1130.88	34.92	0.78	1.05
2	Vitamin D binding protein	P02774	52929	5.23	7865.01	51.05	0.77	1.04
3	Fibrinogen gamma chain	P02679	51478	5.23	3187.94	31.78	1.87	1.08
4	Apolipoprotein A 1	P02647	30758	5.43	1290.93	32.20	0.5	0.6
5	Haptoglobin	P00738	25176	6.11	3442.24	48.52	1.64	1.03
6	Transthyretin	P02766	15877	5.39	9106.519	45.83	0.68	1.02

Haptoglobin polymorphism in human is implicated in inflammatory diseases including infections, atherosclerosis, and autoimmune disorders (Langlios et al., 1996). Additionally, haptoglobin is marker for adiposity, virus-induced autoimmune diabetes and provides protection against the development of diabetic vascular complications (Chiellini et al., 2004; Kruger et al., 2010; Levy et al., 2000). However, in this study, there was no significant change in the levels of haptoglobin in CD and PCD was observed (Fig 4.1). Vitamin D-binding protein (VDBP) is a multifunctional plasma protein associated with bone development, actin scavenger system, binding of fatty acids and immune and inflammatory responses (White and Coke, 2000; Gomme and Bertolini, 2004). Urinary loss of VDBP in type 1 diabetic patients with albuminuria is quite clear (Thrailkill et al., 2010) and its downregulation is associated with the pathogenesis of type 1 diabetes (Blanton et al., 2011). The association of the reduced trypsin inhibitory capacity of diabetic plasma is due to the non-enzymatic glycation of alpha-1-anti-trypsin (Hashemi et al., 2007) and our previous study has shown the increased extent of glycated alpha-1-anti-trypsin in both diabetic clinical and mice plasma (Bhonsle et al., 2012). It was interesting to observe the prominent down regulation of apo A1 in PCD than CD as shown in Fig 4.1.B. Further Apo A1 identification by MS^E was validated by western blot analysis. Additionally, this result was supported by dot-blot analysis of 10 each clinical plasma sample from ND, CD and PCD using Apo A1 antibody as shown in Fig 4.2.

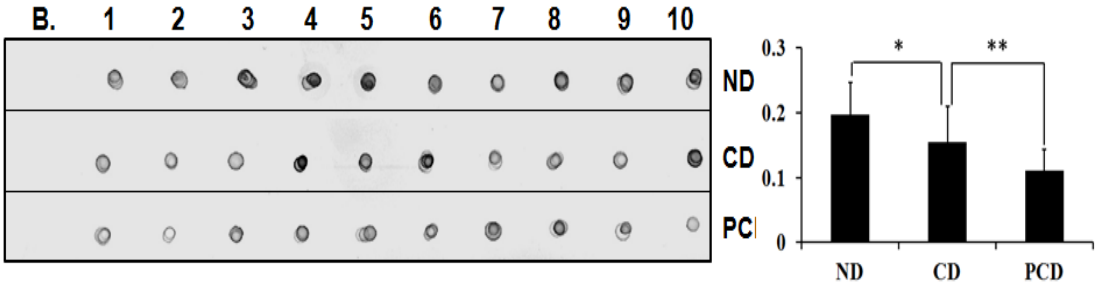


Figure 4. 2: Dot blot analysis of ten ND, CD and PCD samples using anti-Apo A1 antibody. B- Represents a blank.

Lower levels of Apo A1 are associated with the development of diabetic vascular complications mediated by the reverse cholesterol transport system (Quintao et al., 2000). The decreased levels of Apo A1 in PCD could be due to several reasons

including autoantibodies against Apo A1 (Vuilleumier et al., 2010; Montecucco et al., 2011); elevated levels of inflammatory molecules (Haas et al., 2003) and insulin resistance (Mooradian et al., 2004). Apo B 100/Apo A1 ratio represents the balance between atherogenic and anti-atherogenic particles and is a better parameter for the prediction of cardiovascular risk than the lipids, lipoproteins, and lipid ratios (Mallick et al., 2011). Low levels of HDL were found to be negatively correlated with HbA1c and triglycerides in PCD. However, the levels of total cholesterol were not found to be significant. A recent study has shown that Apo B 100/Apo A1 ratio was increased in poorly controlled diabetes plasma than the controlled diabetes plasma (Wagner and Ordonez-Llanos, 2002) suggesting the contribution of lower levels of Apo A1 to this increased ratio. In this study, elevated levels of serum creatinine were observed in poorly controlled diabetes, suggesting Apo A1 levels were inversely proportional to serum creatinine levels. As increased serum creatinine levels are implicated in improper kidney functioning, these results support the previous work, where lower plasma HDL-Cholesterol levels were associated with a greater incidence of chronic kidney disease (Zoppini et al., 2009). Therefore, lower levels of Apo A1 in diabetes could be associated with increased risk of cardiovascular disease and chronic kidney disease.

The 2DE analysis along with validation with dot blot strongly suggests the down regulation of Apo A1, which may serve as an early marker for diabetic complications unlike microalbuminuria which is known to be one of the late markers of diabetic complications.

“SUMMARY AND FUTURE PERSPECTIVES”

V. Summary and Future Perspectives

Uncontrolled hyperglycemia is associated with the development of complications in diabetes. One of the primary causes of diabetic complication is glycation, a non-enzymatic reaction between glucose and protein. Glycation is implicated in the pathogenesis of diabetes and is principally involved in aggravating diabetic complications. Serum albumin, the most abundant plasma protein undergoes glycation and albumin level is decreased in diabetes due to insulin deficiency. Therefore, this study was performed to understand the role of albumin in regulation of glycation i.e., whether decreased albumin levels are responsible for affecting the stoichiometry of plasma protein glycation. The study was performed in three systems (1) streptozotocin (STZ) induced diabetic mice plasma (2) diabetic clinical plasma (3) *in vitro* glycated plasma. Protein glycation was studied by using a combination of two dimensional electrophoresis (2DE), western blotting and LC-MS^E. In both mice and clinical experiments, increased plasma protein glycation was associated with low levels of albumin. Additionally, plasma albumin levels were negatively correlated with HbA1c. *In vitro* glycated plasma experiments with differential depletion of albumin mechanistically showed that the low albumin levels were associated with increased plasma protein glycation. In this study, for the first time it was addressed that variation in the albumin levels influences glycation of plasma proteins and HbA1c. Further, it was also shown that albumin competitively inhibits the glycation of low abundant proteins *in vitro* by using insulin as a model protein. These studies have suggested that at least in the initial stages of diabetes, albumin may protect other proteins from glycation. Therefore, reduced albumin in diabetes is a risk factor for glycation induced complications.

In addition, the influence of protein molecular mass on glycation of proteins was studied by using MALDI MS analysis with model proteins including insulin, apomyoglobin, papain, BSA, HSA and IgG. The study revealed that high molecular weight proteins were more prone to get glycated than the low molecular weight proteins suggesting the role of high molecular weight proteins like IgG and HSA in protecting the glycation of low molecular weight proteins.

Elevated blood glucose levels in diabetes have resulted in glycoxidative modification of plasma proteins. Moreover, decreased insulin synthesis has known to affect gene expression of albumin and fibrinogen contributing to the differential protein expression. Differential protein expression in diabetic plasma sample was studied by a combination of proteomic and western blot approaches. Plasma samples were categorized depending on HbA1c levels as non diabetic (ND) with HbA1c >5.8%, controlled diabetic (CD) with HbA1c 7-8 % and poorly controlled diabetic (PCD) with HbA1c > 8%. Six proteins including alpha-1-antitrypsin, vitamin D binding protein, fibrinogen gamma chain, haptoglobin, transthyretin and apolipoprotein A 1 were differentially expressed in diabetic plasma. Amongst six differentially expressed proteins in diabetes, the down-regulation of apolipoprotein A1 was more prominent in poorly controlled diabetes. The 2DE analysis along with validation with dot blot strongly suggested the down regulation of Apo A1 may serve as an early marker for diabetic complications unlike microalbuminuria which is known to be one of the late markers of diabetic complications.

Low albumin levels in diabetic plasma are associated with increased plasma protein glycation. In addition, apolipoprotein A1 was found to be downregulated and is also glycated in poorly controlled diabetes. However the study has to be extended to a larger population for considering them as diagnostic markers for early diabetic complications. Further, maintaining normal or near normal levels of albumin in diabetes may help in reducing the glycation associated complications. Intervention with medicine or nutraceuticals that help in maintaining albumin levels may delay the onset of diabetic complications.

“BIBILIOGRAPHY”

Abraham EC, Huaqian J, Aziz A, Kumarasamy A, Datta P (2008) Role of the specifically targeted lysine residues in the glycation dependent loss of chaperone activity of alpha A- and alpha B-crystallins. Mol Cell Biochem 310: 235-239.

Al-Abed Y, Mitsuhashi T, Li H, Lawson JA, FitzGerald GA, Founds H et al., (1999) Inhibition of advanced glycation endproduct formation by acetaldehyde: role in the cardioprotective effect of ethanol. Proc Natl Acad Sci U S A 96: 2385-2390.

Alberti KG, Zimmet PZ (1998) 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 15: 539-553.

Altshuler D, Hirschhorn JN, Klannemark M, Lindgren CM, Vohl MC, Nemesh J et al., (2000) The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. Nat Genet 26:76-80.

American Diabetes Association. Standards of medical care in diabetes- 2010 (2010) Diabetes 33: S11-S61.

Amini M Janghorban M (2007) Diabetes and impaired glucose regulation in first degree relatives of patients with type 2 diabetes in Isfahan, Iran: Prevalence and risk factors. Rev Diabet Stud 4: 169-176.

Amos AF, McCarty DJ, Zimmet P (1997) The rising global burden of diabetes and its complications: estimates and projections to the year 2010. Diabet Med 14: S1-85.

Anderson NL (2010) The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum. Clin Chem 56: 177-185.

Anderson NL, Anderson NG (2002) The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 1: 845-867.

Ang L, Palakodeti V, Khalid A, Tsimikas S, Idrees Z, Tran P et al., (2008) Elevated plasma fibrinogen and diabetes mellitus are associated with lower inhibition of platelet reactivity with clopidogrel. J Am Coll Cardiol 52: 1052-1059.

Anonymous 2012, http://www.census.gov/compendia/statab/cats/population/estimates_and_projections_by_age_sex_raceethnicity.html, accesses on April 10th, 2012.

Arai K, Maguchi S, Fuji S, Ishibashi H, Oikawa K, Taniguchi N (1987) Glycation and inactivation of human Cu-Zn superoxidase dismutase. J Biol Chem 262: 16969-16972.

Attvall S, Fowelin J, Lager I, Von SH, Smith U (1993) Smoking induces insulin resistance- a potential link with the insulin resistance syndrome. J Intern Med 233: 327-332.

Austin GE, Mullins RH, Morin LG (1987) Non-enzymatic glycation of individual plasma proteins in normoglycemic and hyperglycaemic patients. Clin Chem 33: 2220-2224.

Ben-Haroush A, Hadar E, Chen R, Hod M, Yogev Y (2009) Maternal obesity is a major risk factor for large-for-gestational-infants in pregnancies complicated by gestational diabetes. Arch Gynecol Obstet. 279: 539-543.

Bhonsle HS, Korwar AM, Kote SS, Golegaonkar SB, Chougale AD, Shaik ML, Dhande NL, Giri AP, Shelgikar KM, Boppana R, Kulkarni MJ (2012) Low plasma albumin levels are associated with increased plasma protein glycation and HbA1c in diabetes. J Proteome Res 11: 1391-1396.

Bhonsle HS, Singh SK, Srivastava G, Boppana R, Kulkarni MJ (2008) Albumin competitively inhibits glycation of less abundant proteins. Protein Peptide Lett 15: 663-667.

Biemel KM, Bühler HP, Reihl O, Lederer MO (2001) Identification and quantitative evaluation of the lysine-arginine crosslinks GODIC, MODIC, DODIC, and glucosepan in foods. Nahrung 45: 210-214.

Bjornholt JV, Erikssen G, Liestol K, Jervell J, Thaulow E, Erikssen J (2000) Type 2 diabetes and maternal family history: an impact beyond slow glucose removal rate and fasting hyperglycemia in low-risk individuals? Results from 22.5 years of follow-up of healthy nondiabetic men. Diabetes Care 23: 1255-1259.

Blake GJ, Rifai N, Buring JE, Ridker PM (2003) Blood pressure, C-reactive protein, and risk of future cardiovascular events. Circulation 108: 2993-2999.

Blanton D, Han Z, Bierschenk L, Linga-Reddy MV, Wang H, Clare-Salzler M et al., (2011) Reduced serum vitamin D-binding protein levels are associated with type 1 diabetes. Diabetes 60: 2566-2570.

Boule NG, Haddad E, Kenny GP, Wells GA, Sigal RJ (2001) Effects of exercise on glycemic control and body mass in type 2 diabetes mellitus: a meta-analysis of controlled clinical trials. JAMA 286: 1218-1227.

Bousova I, Bakala H, Chudacek R, Palicka V, Drsata J (2005) Glycation-induced inactivation of aspartate aminotransferase, effect of uric acid. Mol Cell Biochem 278: 85-92.

Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414: 813-820.

Brownlee M, Vlassara H, Cerami A (1980) Measurement of glycosylated amino acids and peptides from urine of diabetic patients using affinity chromatography. Diabetes 29: 1044-1047.

Brownlee M, Vlassara H, Cerami A (1983) Nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by plasmin. Diabetes 32: 680-684.

Brownlee M, Vlassara H, Cerami A (1984) Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann Intern Med 101: 527-537.

Burke JP, Williams K, Gaskill SP, Hazuda HP, Haffner SM, Stern MP (1999) Rapid rise in the incidence of type 2 diabetes from 1987 to 1996: results from the San Antonio Heart Study. Arch Intern Med 159: 1450-1456.

Caso G, Scalfi C, Marra M, Covino A, Muscaritoli M, McNurlan MA et al., (2000) Albumin synthesis is diminished in men consuming a predominantly vegetarian diet. J. Nutri 130: 528-533.

Cauchi S, Froguel P (2008) TCF7L2 genetic defect and type 2 diabetes. Curr Diab Rep; 8: 149-155.

Centers for Disease Control and Prevention. National diabetes awareness month, November 1997 (1997) Morb Mortal Wkly Rep 46:1013.

Ceriello A. Fibrinogen and diabetes mellitus: is it time for intervention trials? Diabetologia 1997; 40: 731-734.

Chase HP, Jackson WE, Hoops SL, Cockerham RS, Archer PG, O'Brien D (1989) Glucose control and the renal and retinal complications of insulin-dependent diabetes. JAMA 261: 1155-1160.

Chen S, Cohen MP, Ziyadeh FN (2000) Amadori-glycated albumin in diabetic nephropathy: pathophysiologic connections. Kidney Int Suppl 77: S40-S44.

Cheng FY, Blackburn K, Lin YM, Goshe MB, Williamson JD (2009) Absolute protein quantification by LC/MS(E) for global analysis of salicylic acid-induced plant protein secretion responses. J Proteome Res 8: 82-93.

Chiellini C, Santini F, Marsili A, Berti P, Bertacca A, Pelosini C et al., (2004) Serum haptoglobin: a novel marker of adiposity in humans. J Clin Endocrinol Metab 89: 2678-2683.

Cohen MP (2003) Intervention strategies to prevent pathogenetic effects of glycated albumin. Arch Biochem Biophy 419: 25-30.

Colditz GA, Willett WC, Stampfer MJ, Manson JE, Hennekens CH, Arky RA, Speizer FE (1990) Weight as a risk factor for clinical diabetes in women. Am J Epidemiol 132: 501-513.

Conen D, Ridker PM, Mora S, Buring JE, Glynn RJ (2007) Blood pressure and risk of developing type 2 diabetes mellitus: the Women's Health Study. Eur Heart J 28: 2937-2943.

Connolly V, Unwin N, Sherriff P, Bilous R, Kelly W (2000) Diabetes prevalence and socioeconomic status: a population based study showing increased prevalence of type 2 diabetes mellitus in deprived areas. J Epidemiol Community Health 54: 173-177.

Cox J, Mann M (2007) Is proteomics the new genomics? Cell 130:395-398.

Dardé VM, Barderas MG, Vivanco F (2007) Depletion of high-abundance proteins in plasma by immunoaffinity subtraction for two-dimensional difference gel electrophoresis analysis. Methods Mol Biol 357:351-364.

Dunstan DW, Salmon J, Owen N, Armstrong T, Zimmet PZ, Welborn TA; Aus Diab Steering Committee (2004) Physical activity and television viewing in relation to risk of undiagnosed abnormal glucose metabolism in adults. Diabetes Care 27: 2603-2609.

Duran-Jimenez B, Dobler D, Moffatt S, Rabbani N, Streuli CH, Thornalley PJ, et al., (2009) Advanced glycation end products in extracellular matrix proteins contribute to the failure of sensory nerve regeneration in diabetes. Diabetes 58: 2893-903.

Dhingra V, Gupta M, Andacht T, Fu ZF (2005) New frontiers in proteomics research: a perspective. Int J Pharm 299: 1-18.

Ekoé JM, Rewers M, Williams R, Zimmet P (2008) The Epidemiology of Diabetes Mellitus. In: Epidemiology of metabolic syndrome. 2nd ed. John Wiley & Sons Pp 31-50.

Elbein SC, Wegner K, Kahn SE (2000) Reduced beta-cell compensation to the insulin resistance associated with obesity in members of caucasian familial type 2 diabetic kind reds. Diabetes Care 23: 221-227.

Engelgau MM, Narayan KM, Herman WH (2000) Screening for type 2 diabetes. Diabetes Care 23: 1563-1580.

Engstrom G, Hedblad B, Eriksson KF, Janzon L, Lindgarde F (2005) Complement C3 Is a Risk Factor for the Development of Diabetes. A Population-Based Cohort Study. Diabetes 54: 570-575.

Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2003) Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care Suppl 1:S5-S20.

Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997) Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 20: 1183-1197.

Facchini FS, Hollenbeck CB, Jeppesen J, Chen YD, Reaven GM (1992) Insulin resistance and cigarette smoking. Lancet 339: 1128-1130.

Fayle SE, Healy JP, Brown PA, Reid EA, Gerrard JA, Ames JM (2001) Novel approaches to the analysis of the Maillard reaction of proteins. Electrophoresis 22: 1518-1525.

Ferrannini E, Buzzigoli G, Bonadonna R, Giorico MA, Oleggini M, Graziadei L, et al., (1987) Insulin resistance in essential hypertension. N Engl J Med 317: 350-7.

Flegal KM, Ezzati TM, Harris MI, Haynes SG, Juarez RZ, Knowler WC, et al., (1991) Prevalence of diabetes in Mexican Americans, Cubans, and Puerto Ricans from the Hispanic Health and Nutrition Examination Survey, 1982-1984. Diabetes Care 14: 628-638.

Folsom AR, Ma J, Eckfeldt JH, Nieto FJ, Metcalf PA, Barnes RW (1995) Low serum albumin. Association with diabetes mellitus and other cardiovascular risk factors but not with prevalent cardiovascular disease or carotid artery intima-media thickness. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. Ann Epidemiol 3: 86-191.

Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, et al., (2007) A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 316: 889-894.

Gadgil HS, Bondarenko PV, Treuheit MJ, Ren D (2007) Screening and sequencing of glycated proteins by neutral loss scan LC/MS/MS method. Anal Chem 7: 5991-5999.

Ganea E, Harding JJ (1994) Inactivation of glucose-6-phosphate dehydrogenase by glycation. Biochem Soc Trans 22: 445S.

Garlick RL, Mazer JS (1983) The principal site of nonenzymatic glycosylation of human serum albumin in vivo. J Biol Chem 258: 6142-6146.

Gerich JE (1998) The genetic basis of type 2 diabetes mellitus: impaired insulin secretion versus impaired insulin sensitivity. Endocr Rev 19: 491-503.

Gerstein HC, Santaguida P, Raina P, Morrison KM, Balion C, Hunt D, et al., (2007) Annual incidence and relative risk of diabetes in people with various categories of dysglycemia: a systematic overview and meta-analysis of prospective studies. Diabetes Res Clin Pract 78: 305-312.

Giacco F, Brownlee M (2010) Oxidative stress and diabetic complications. Circ Res 107: 1058-1070.

Gloyn AL (2003a) The search for type 2 diabetes genes. Ageing Res Rev 2: 111-127.

Gloyn AL, Weedon MN, Owen KR, Turner MJ, Knight BA, Hitman G et al., (2003b) Large-scale association studies of variants in genes encoding the pancreatic beta-cell KATP channel subunits Kir6.2(KCNJ11) and SUR1 (ABCC8) confirm that the KCNJ11 E23K variant is associated with type 2 diabetes. Diabetes 52: 568-572.

Goldin A, Beckman JA, Schmidt AM, Creager MA (2006) Advanced glycation end products: sparking the development of diabetic vascular injury. Circulation 114: 597-605.

Gomme PT, Bertolini J (2004) Therapeutic potential of vitamin D-binding protein. Trends Biotechnol 22: 340-345.

Gottschalk, A. (1972) In: The Glycoproteins (Gottschalk, A., ed) Part A, Elsevier Publishing Co., New York pp. 141-157.

Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Sainz J et al., (2006) Stefansson K.Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat Genet 38: 320-323.

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19: 1720-1730.

Haas MJ, Horani M, Mreyoud A, Plummer B, Wong NC, Mooradian AD (2003) Suppression of apolipoprotein AI gene expression in HepG2 cells by TNF alpha and IL-1beta. Biochim Biophys Acta 1623: 120-128.

Hamman RF, Wing RR, Edelstein SL, Lachin JM, Bray GA, Delahanty L et al., (2006) Effect of weight loss with lifestyle intervention on risk of diabetes. Diabetes Care 29: 2102-2107.

Hardin DS, LeBlanc A, Para L, Seilheimer DK (1999) Hepatic insulin resistance and defects in substrate utilization in cystic fibrosis. Diabetes 48: 1082-1087.

Harris MI, Klein R, Welborn TA, Knuiman MW (1992) Onset of NIDDM occurs at least 4-7 yr before clinical diagnosis. Diabetes Care 15: 815-819.

Harris MI, Modan M (1994) Screening for NIDDM: why is there no national program? Diabetes Care 17: 440-444.

Hashemi M, Naderi M, Rashidi H, Ghavami S (2007) Impaired activity of serum alpha-1-antitrypsin in diabetes mellitus. Diabetes Res Clin Pract 75: 246-248.

Hendrick CC, Thorpe SR, Fu M-X, Harper CM, Yoo J, Kim S-M et al., (2000) Glycation impairs high density lipoprotein function. Diabetologia 43: 312-320.

Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y et al., (1999) RAGE mediates a novel proinflammatory axis: the cell surface receptor for S100/calgranulin polypeptides. Cell 97: 889–901.

Homko CJ, Khandelwal M (1996) Glucose monitoring and insulin therapy during pregnancy. Obstet Gynecol Clin North Am 23: 47-74.

Hu FB, Meigs JB, Li TY, Rifai N, Manson JE (2004) Inflammatory markers and risk of developing type 2 diabetes in women. Diabetes 53: 693-700.

Hu FB, van Dam RM, Liu S (2001) Diet and risk of Type II diabetes: the role of types of fat and carbohydrate. Diabetologia 44: 805-817.

Hunter SJ, Boyd AC, O'Harte FP, McKillop AM, Wiggam MI, Mooney MH et al., (2003) Demonstration of glycated insulin in human diabetic plasma and decreased

biological activity assessed by euglycemic-hyperinsulinemic clamp technique in humans. Diabetes 52: 492-498.

Hunter SJ, Boyd AC, O'Harte FP, McKillop AM, Wiggam MI, Mooney MH et al., (2003) Demonstration of glycated insulin in human diabetic plasma and decreased biological activity assessed by euglycemic-hyperinsulinemic clamp technique in humans. Diabetes 52: 492-498.

Iberg N, Flückiger R (1986) Nonenzymatic glycosylation of albumin in vivo. Identification of multiple glycosylated sites. J Biol Chem 261: 13542-13545.

Issaq HJ, Xiao Z, Veenstra TD (2007) Serum and plasma proteomics. Chem Rev 107: 3601-3620.

Jaleel A, Halvatsiotis P, Williamson B, Juhasz P, Martin S, Nair KS (2005) Identification of amadori-modified plasma proteins in type 2 diabetes and the effect of short-term intensive insulin treatment. Diabetes Care 28: 645-652.

Jefferson LS, Liao WS, Peavy DE, Miller TB, Appel MC, Taylor JM (1983) Diabetes-induced alterations in liver protein synthesis. J Biol Chem 258: 1369-1375.

Jung T, Grune T (2008) The proteasome and its role in the degradation of oxidized proteins. IUBMB Life 2008; 11: 743-752.

Kay SJ, Fiatarone Singh MA (2006) The influence of physical activity on abdominal fat: a systematic review of the literature. Obes Rev 7: 183-200.

Kaysen GA (2001) The microinflammatory state in uremia: causes and potential consequences. J Am Soc Nephrol 12: 1549-1557.

Kern W, Stange EF, Fehm HL, Klein HH (1999) Glucocorticoid-induced diabetes mellitus in gastrointestinal diseases. Z Gastroenterol 1: 36-42.

Kesaniemi YA, Witztum JL, Steinbrecher UP (1983) Receptor-mediated catabolism of low density lipoprotein in man. Quantitation using glucosylated low density lipoprotein. J Clin Invest 71: 950-959.

Kiho T, Asahi T, Usui S, Matsunaga T, Ukai S (1996) Effect of 3-deoxyglucosone on the activities of enzymes responsible for glucose metabolism in mouse liver. Biol Pharm Bull 19: 1106-1108.

King H, Aubert RE, Herman WH (1998) Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. Diabetes Care 21: 1414-31.

King H, Keuky L, Seng S, Khun T, Roglic G, Pinget M (2005) Diabetes and associated disorders in Cambodia: two epidemiological surveys. Lancet 366: 1633-1639.

King H, Rewers M (1993) Global estimates for prevalence of diabetes mellitus and impaired glucose tolerance in adults. WHO Ad Hoc Diabetes Reporting Group. Diabetes Care16: 157-177.

Knekt P, Laaksonen M, Mattila C, Härkänen T, Marniemi J, Heliövaara M et al., (2008) Serum vitamin D and subsequent occurrence of type 2 diabetes. Epidemiology 19: 666-671.

Krishnamurti U, Steffes MW (2001) Glycohemoglobin: a primary predictor of the development or reversal of complications of diabetes mellitus. Clin Chem 47: 1157-1165.

Kruger AJ, Yang C, Tam SW, Hinerfeld D, Evans JE, Green KM et al., (2010) Haptoglobin as an early serum biomarker of virus-induced autoimmune type 1 diabetes in biobreeding diabetes resistant and LEW1WR1 rats. Exp Biol Med (Maywood) 235: 1328-1337.

Kueper T, Grune T, Prahl S, Lenz H, Welge V, Biernoth T et al., (2007) Vimentin is the specific target in skin glycation. Structural prerequisites, functional consequences, and role in skin aging. J Biol Chem 282: 23427-23436.

Kuzmicki M, Telejko B, Szamatowicz J, Zonenberg A, Nikolajuk A, Kretowski A, et al., (2009) High resistin and interleukin-6 levels are associated with gestational diabetes mellitus. Gynecol Endocrinol. 25: 258-263.

Langlois MR, Delanghe JR (1996) Biological and clinical significance of haptoglobin polymorphism in humans. Clin Chem 42: 1589-1600.

Lapolla A, Brioschi M, Banfi C, Tremoli E, Bonfante L, Cristoni S et al., (2008) On the search for glycated lipoprotein ApoA-I in the plasma of diabetic and nephropathic patients. J Mass Spectrom 43: 74-78.

Lapolla A, Fedele D, Garbeglio M, Martano L, Tonani R, Seraglia R et al., (2000b) Matrix-assisted laser desorption/ionization mass spectrometry, enzymatic digestion, and molecular modeling in the study of nonenzymatic glycation of IgG. J Am Soc Mass Spectrom 11: 153-159.

Lapolla A, Fedele D, Plebani M, Garbeglio M, Seraglia R, D'Alpaos M et al., (1998) Direct evaluation of glycated and glycooxidated globins by matrix assisted laser desorption ionization mass spectrometry. Rapid Commun Mass Spectrom 13: 8-14.

Lapolla A, Fedele D, Seraglia R, Traldi P (2006b) The role of mass spectrometry in the study of non-enzymatic protein glycation in diabetes: an update. Mass Spectrom Rev 25: 775-797.

Lapolla A, Fedele D, Traldi P (2000a) The role of mass spectrometry in the study of non-enzymatic protein glycation in diabetes. Mass Spectrom Rev 19: 279-304.

Lapolla A, Porcu S, Traldi P (2011) Some views on proteomics in diabetes. Clin Chem Lab Med 49: 943-957.

Lawrence JM, Contreras R, Chen W, Sacks DA (2008) Trends in the prevalence of preexisting diabetes and gestational diabetes mellitus among a racially/ethnically diverse population of pregnant women, 1999-2005. Diabetes Care 31: 899-904.

Leslie RDG (1997) In: Leslie RDG (eds) Molecular pathogenesis of diabetes mellitus. Frontiers of hormone research. Karger pp 2-23.

Li YC, Pfu¨ller U, Larsson EL, Jungvid H, Galaev IY, Mattiasson B (2001) Separation of mistletoe lectins based on the degree of glycosylation using boronate affinity chromatography. J Chromatogr A 925: 115-121.

Levy AP, Roguin A, Hochberg I, Herer P, Marsh S, Nakhoul FM et al., (2000) Haptoglobin phenotype and vascular complications in patients with diabetes. N Engl J Med 343: 969-970.

Liao WSL, Jefferson LS, Taylor JM (1986) Changes in plasma albumin concentration, synthesis rate, and mRNA level during acute inflammation. Am J Physiol Cell Physiol 251: C928-C934.

Liese AD, Weis KE, Schulz M, Tooze JA (2009) Food intake patterns associated with incident type 2 diabetes: the Insulin Resistance Atherosclerosis Study. Diabetes Care 32: 263-268.

Linke T, Doraiswamy S, Harrison EH (2007) Rat plasma proteomics: effects of abundant protein depletion on proteomic analysis. Chromatogr B Analyt Technol Biomed Life Sci 849: 273-281.

Lyssenko V (2008b) The transcription factor 7-like 2 gene and increased risk of type 2 diabetes: an update. Curr Opin Clin Nutr Metab Care 11: 385-392.

Lyssenko V, Jonsson A, Almgren P, Pulizzi N, Isomaa B, Tuomi T et al., (2008a) Clinical risk factors, DNA variants, and the development of type 2 diabetes. N Engl J Med 359: 2220-2232.

Ma XJ, Jia WP, Hu C, Zhou J, Lu HJ, Zhang R et al., (2008) Genetic characteristics of familial type 2 diabetes pedigrees: a preliminary analysis of 4468 persons from 715 pedigrees. Zhonghua Yi Xue Za Zhi 88: 2541-2543.

Maillard LC (1916) Synthè des materies huniques par action des acid aminès sur le sucres reducteurs. Ann Chim Ser 9: 258-268.

Mallick KA, Joshi MR, Bhat PG (2011) A study on Apo B100/Apo A-I ratio in uncontrolled type 2 diabetes mellitus. IAJBPT 2: 379-384.

Manson JE, Ajani UA, Liu S, Nathan DM, Hennekens CH (2000) A prospective study of cigarette smoking and the incidence of diabetes mellitus among US male physicians. Am J Med 109: 538-542.

Manson JE, Nathan DM, Krolewski AS, Stampfer MJ, Willett WC, Hennekens CH (1992) A prospective study of exercise and incidence of diabetes among US male physicians JAMA 268: 63-67.

Manson JE, Rimm EB, Stampfer MJ, Colditz GA, Willett WC, Krolewski AS et al., (1991) Physical activity and incidence of non-insulin-dependent diabetes mellitus in women. Lancet 338: 774-778.

McBean AM, Li S, Gilbertson DT, Collins AJ (2004) Differences in diabetes prevalence, incidence, and mortality among the elderly of four racial/ethnic groups: whites, blacks, hispanics, and asians. Diabetes Care 27: 2317-2324.

McFarlane IA, Bliss M, Jackson JGL, Williams G (1997) The history of Diabetes mellitus. In: Textbook of diabetes, 2nd edn (eds Pickup J and Williams G) Blackwell Science, London, Pp. 1.1-1.21.

Meigs JB, Cupples LA, Wilson PW (2000) Parental transmission of type 2 diabetes: the Framingham Offspring Study. Diabetes 49: 2201-2207.

Meigs JB, Hu FB, Rifai N, Manson JE (2004) Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. JAMA 291: 1978-1986.

Meigs JB, O'donnell CJ, Tofler GH, Benjamin EJ, Fox CS, Lipinska I et al., (2006) Hemostatic markers of endothelial dysfunction and risk of incident type 2 diabetes: The Framingham Offspring Study. Diabetes 55: 530-537.

Menini T, Gugliucci A, Stahl AJ (1992) Polyclonal immunoglobulin M: location of glycation sites. Clin Chim Acta 213: 23-35.

Mennella C, Visciano M, Napolitano A, Castillo MDD, Fogliano V (2006) Glycation of lysine-containing dipeptides. J Peptide Sci 12: 291-296.

Meyer KA, Kushi LH, Jacobs DR, Jr., Slavin J, Sellers TA, Folsom AR (2000) Carbohydrates, dietary fiber, and incident type 2 diabetes in older women. Am J Clin Nutr 71: 921-930.

Mitchell P (2010) Proteomics retrenches. Nat Biotechnol 28: 665-670.

Mohammad TG, Mojtaba R, Mohsen R (2010) Study of Nonenzymatic Glycation of Transferrin and its Effect on Iron-Binding Antioxidant Capacity. Iranian J Basic Med Sci 13: 194-199.

Mohanram A, Zhang Z, Shahinfar S, Keane WF, Brenner BM, Toto RD (2004) Anemia and end-stage renal disease in patients with type 2 diabetes and nephropathy. Kidney Int 66: 1131-1138.

Montecucco F, Vuilleumier N, Pagano S, Lenglet S, Bertolotto M, Braunersreuther V et al., (2011) Anti-Apolipoprotein A-1 auto-antibodies are active mediators of atherosclerotic plaque vulnerability. Eur Heart J 32: 412-421.

Montonen J, Knekt P, Härkänen T, Järvinen R, Heliövaara M, Aromaa A et al., (2005) Dietary patterns and the incidence of type 2 diabetes. Am J Epidemiol 161: 219-227.

Mooradian AD, Haas MJ, Wong NC (2004) Transcriptional control of apolipoprotein A-I gene expression in diabetes. Diabetes 53: 513-520.

Morais MP, Mackay JD, Bhamra SK, Buchanan JG, James TD, Fossey JS et al., (2010) Analysis of protein glycation using phenylboronate acrylamide gel electrophoresis. Proteomics10: 48-58.

Münch G, Schicktanz D, Behme A, Gerlach M, Riederer P, Palm D et al., (1999) Amino acid specificity of glycation and protein-AGE crosslinking reactivities determined with a dipeptide SPOT library. Nat Biotechnol 17: 1006-1010.

Nacharaju P, Acharya AS (1992) Amadori rearrangement potential of haemoglobin at its glycation sites is dependent on the three dimensional structure of protein. Biochemistry 31: 12673-12679.

Nakayama M, Yoshimura K, Maruyama Y, Numata M, Hosoya T, Izumi G (2004) Possible involvement of cross-linking advanced glycation endproducts in long-term CAPD peritoneal degeneration. Nephrol Dial Transplant 19: 1664-1665.

NAVIGATOR Study Group, McMurray JJ et al., (2010) Effect of valsartan on the incidence of diabetes and cardiovascular events. N Engl J Med 362: 1477-1490.

Neel, J (1962) Diabetes Mellitus: A "Thrifty" Genotype Rendered Detrimental by "Progress"? Am J Hum Genet 14: 353-362.

Neglia CI, Cohen HJ, Garber AR, Ellis PD, Thorpe SR, Baynes JW (1983)13C NMR investigation of nonenzymatic glucosylation of protein. Model studies using RNase A. J Biol Chem 258: 14279-14283.

Nicholson JP Wolmarns MR Park GR (2000) The role of albumin in critical illness. Brit J Anaesth 85: 599-610.

Niwa T, Tsukushi S (2001) 3-deoxyglucosone and AGEs in uremic complications: inactivation of glutathione peroxidase by 3-deoxyglucosone. Kidney Int Suppl 78: S37-41.

Nobecourt E, Davies MJ, Brown BE, Curtiss LK, Bonnet DJ, Charlton F et al., (2007) The impact of glycation on apolipoprotein A-I structure and its ability to activate lecithin:cholesterol acyltransferase. Diabetologia 50: 643-653.

Oda A, Bannai C, Yamaoka T, Katori T, Matsushima T, Yamashita K (1994) Inactivation of Cu, Zn-superoxide dismutase by in vitro glycosylation and in erythrocytes of diabetic patients Horm Metab Res 26: 1-4.

Ohtsubo K, Takamatsu S, Minowa MT, Yoshida A, Takeuchi M, Marth JD (2005) Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes. Cell 123: 1307-1321.

Padaki S, Vijayakrishna K, Dambal A, Ankad R, Manjula R, Surekharani C et al., (2011) Anthropometry and physical fitness in individuals with family history of type-2 diabetes mellitus: A comparative study. Indian J Endocrinol Metab 5: 327-330.

Papaspyros NS (1964) The History of Diabetes Mellitus, 2nd edn, Thieme medical and Scientific Publishers, Stuttgart.

Patterson SD, Aebersold RH (2003) Proteomics: the first decade and beyond. Nat Genet 33 Suppl: 311-323.

Pauker SG (1993) Deciding about screening. Ann Intern Med 118: 901-902.

Peavy DE, Taylor JM, Jefferson LS (1978) Correlation of albumin production rates and albumin mRNA levels in livers of normal, diabetic, and insulin-treated diabetic rats. Proc Natl Acad Sci U S A 75: 5879-5883.

Philippe R, Bourdon E (2011) The glycation of albumin: structural and functional impacts. Biochimie 93: 645-658.

Pittas AG, Dawson-Hughes B, Li T, Van Dam RM, Willett WC, Manson JE et al., (2006) Vitamin D and calcium intake in relation to type 2 diabetes in women. Diabetes Care 29: 650-656.

Poggioli S, Bakala H, Friguet, B (2002) Age-related increase of protein glycation in peripheral blood lymphocytes is restricted to preferential target proteins Exp Gerontol 37: 1207-1215.

Priego Capote F, Sanchez JC (2009) Strategies for proteomic analysis of non-enzymatically glycated proteins. Mass Spectrom Rev 28: 135-46.

Priego-Capote F, Scherl A, Müller M, Waridel P, Lisacek F, Sanchez JC (2010) Glycation isotopic labeling with 13C-reducing sugars for quantitative analysis of glycated proteins in human plasma. Mol Cell Proteomics 9: 579-592.

Prinsen BH, Rabelink TJ, Beutler JJ, Kaysen GA, De Boer J, Boer WH et al., (2003) Increased albumin and fibrinogen synthesis rate in patients with chronic renal failure Kidney Int 64: 1495-1504.

Prokopenko I, McCarthy MI, Lindgren CM (2008) Type 2 diabetes: new genes, new understanding. Trends Genet 24: 613-621.

Quan CP, Wu S, Dasovich N, Hsu C, Patapoff T, Canova-Davis E (1999) Susceptibility of rhDNase I to glycation in the dry-powder state. Anal Chem 71: 4445-4454.

Quintao EC, Medina WL, Passarelli M (2000) Reverse cholesterol transport in diabetes mellitus. Diabetes Metab Res Rev 16: 237-250.

Ramachandran A, Snehalatha C, Latha E, Manoharan M, Vijay V(1999) Impacts of urbanisation on the lifestyle and on the prevalence of diabetes in native Asian Indian population. Diabetes Res Clin Pract 44: 207-213.

Ramachandran A, Snehalatha C, Vijay V, King H (2002) Impact of poverty on the prevalence of diabetes and its complications in urban southern India. Diabet Med. 19: 130-135.

Reddy GK (2004) Cross-linking in collagen by nonenzymatic glycation increases the matrix stiffness in rabbit achilles tendon. Exp Diabesity Res5: 143-153.

Reynolds TM (1965) Chemistry of non-enzymatic browning. Adv Food Res 14: 167-283.

Richter EA, Garetto LP, Goodman MN, Ruderman NB (1982) Muscle glucose metabolism following exercise in the rat: increased sensitivity to insulin. J Clin Invest 69: 785-793.

Ridker PM, Buring JE, Cook NR, Rifai N (2003) C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. Circulation 107: 391-397.

Righetti PG, Boschetti E, Lomas L, Citterio A (2006) Protein Equalizer Technology: the quest for a "democratic proteome". Proteomics 6: 3980-3992.

Rodriguez-Segade S, Rodriguez R, Mayan D, Camina F (2005) Plasma albumin concentration is a predictor of HbA1c among type 2 diabetic patients, independently of fasting plasma glucose and fructosamine. Diabetes care 28: 437-439.

Rollo J (1797) An account of two cases of diabetes mellitus with remarks as they arose during the progress of the cure, C. Dilly, London.

Rother KI (2007) Diabetes treatment--bridging the divide. N Engl J Med Apr 356: 1499-1501.

Sacks DB, Arnold M, Bakris GL, Bruns DE, Horvath AR, Kirkman MS et al., (2011) National Academy of Clinical Biochemistry; Evidence-Based Laboratory Medicine Committee of the American Association for Clinical Chemistry. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. Diabetes Care. 34: e61-99.

Sadikot SM, Nigam A, Das S, Bajaj S, Zargar AH, Prasannakumar KM et al., (2004) Diabetes India The burden of diabetes and impaired glucose tolerance in India using the WHO 1999 criteria: prevalence of diabetes in India study (PODIS). Diabetes Res Clin Pract. 66: 301-307.

Sairenchi T, Iso H, Nishimura A, Hosoda T, Irie F, Saito Y et al., (2004) Cigarette smoking and risk of type 2 diabetes mellitus among middle-aged and elderly Japanese men and women. Am J Epidemiol 160: 158-162.

Saldana TM, Siega-Riz AM, Adair LS, Suchindran C (2006) The relationship between pregnancy weight gain and glucose tolerance status among black and white women in central North Carolina. Am J Obstet Gynecol 195: 1629-1635.

Salmerón J, Ascherio A, Rimm EB, Colditz GA, Spiegelman D, Jenkins DJ et al., (1997) Dietary fiber, glycemic load, and risk of NIDDM in men. Diabetes Care 20: 545-550.

Salmerón J, Hu FB, Manson JE, Stampfer MJ, Colditz GA, Rimm EB, Willett WC (2001) Dietary fat intake and risk of type 2 diabetes in women. Am J Clin Nutr Jun; 73: 1019-1026.

Samal KC, Kanungo A, Sanjeevi CB (2002) Clinicoepidemiological and biochemical profile of malnutrition-modulated diabetes mellitus. Ann N Y Acad Sci. 958: 131-137.

Saudek CD, Herman WH, Sacks DB, Bergenstal RM, Edelman D, Davidson MB (2008) A new look at screening and diagnosing diabetes mellitus. J Clin Endocrinol Metab. 93: 2447-2453.

Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H et al., (2007) Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. Science 316: 1331-1336.

Schnider SL, Kohn RR (1982) Effects of age and diabetes mellitus on the solubility of collagen from human skin, tracheal cartilage and dura mater. Exp Gerontol 17: 185-194.

Stitt AW, Bhaduri T, McMullen CB, Gardiner TA, Archer DB (2000) Advanced glycation end products induce blood-retinal barrier dysfunction in normoglycemic rats. Mol Cell Biol Res Commun 3: 380-388.

Suliman ME, Stenvinkel P, Bárány P, Heimbürger O, Anderstam B, Lindholm B (2003) Hyperhomocysteinemia and its relationship to cardiovascular disease in ESRD: influence of hypoalbuminemia, malnutrition, inflammation, and diabetes mellitus. Am J Kidney Dis 41: S89-95.

Summerfield JA, Vergalla J, Jones EA (1982) Modulation of a glycoprotein recognition system on rat hepatic endothelial cells by glucose and diabetes mellitus. Clin Invest 69: 1337-1347.

Sun Q, Spiegelman D, van Dam RM, Holmes MD, Malik VS, Willett WC et al., (2010) White rice, brown rice, and risk of type 2 diabetes in US men and women. Arch Intern Med 170: 961-969.

Sundsten T, Ortsäter H (2009) Proteomics in diabetes research. Mol Cell Endocrinol 297: 93-103.

Swapan Kumar Das, Steven C Elbein (2006) The genetic basis of type 2 diabetes. Cellscience 2: 100-131.

Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc Natl Acad Sci USA 101: 9528-9533.

Szwergold BS, Howell SK, Beisswenger PJ (2005) Transglycation-A Potential New Mechanism for Deglycation of Schiff's Bases. Ann. Ny. Acad. Sci. 1043, 845-864.

Takahashi M, Lu Y, Myint T, Fujii J, Wada Y, Taniguchi N (1995) In vivo glycation of aldehyde reductase, a major 3-deoxyglucosone reducing enzyme: identification of glycation sites. Biochemistry 34: 1433-1438.

Takahashi S, Uchino H, Shimizu T, Kanazawa A, Tamura Y, Sakai K, et al., (2007) Comparison of glycated albumin (GA) and glycated hemoglobin (HbA1c) in type 2 diabetic patients: usefulness of GA for evaluation of short-term changes in glycemic control. Endocr J 54: 139-144.

Talamini G, Bassi C, Falconi M, Sartori N, Salvia R, Rigo L, et al., (1999) Alcohol and smoking as risk factors in chronic pancreatitis and pancreatic cancer. Dig Dis Sci 44: 1303-1311.

Tanner S, Shu H, Frank H, Wang L, Zandi E, Mumby M, et al.,(2005) InsPect: identification of post translationally modified peptides from tandem mass spectra. Anal. Chem 77: 4626-4639.

Taylor R, Agius L (1988) The biochemistry of diabetes. Biochem J 250: 625-640.

Tessari P, Kiwanuka E, Millioni R, Vettore M, Puricelli L, Zanetti M, et al., (2006) Albumin and fibrinogen synthesis and insulin effect in type 2 diabetic patients with normoalbuminuria. Diabetes Care 29: 323-328.

The International Expert Committee (2009) International Expert Committee report on the role of the A1c assay in the diagnosis of diabetes. Diabetes Care 32: 1327-1334.

Thomas MC (2007) Anemia in diabetes: marker or mediator of microvascular disease? Nat Clin Pract Nephrol 3: 20-30.

Thornalley PJ (1990) The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem J 269: 1-11.

Thornalley PJ (2003a) Glyoxalase I: structure, function and a critical role in the enzymatic defence against glycation. Biochem Soc Trans 31: 1343-1248.

Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, et al., (2003b) Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 375: 581-592.

Thornalley PJ, Langborg A, Minhas HS (1999) Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J 344: 109-116.

Thorpe SR and Baynes JW (1982) Glycosylated proteins. In: The Glycoconjugates Horowitz, M. I., ed Vol. 3, Academic Press, New York pp. 113-132.

Thrailkill KM, Jo CH, Cockrell GE, Moreau CS, Fowlkes JL (2010) Enhanced excretion of vitamin D binding protein in type 1 diabetes: a role in vitamin D deficiency? J Clin Endocrinol Metab 96: 142-149.

Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD (2003) Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2: 1096-1103.

Tull ES, Roseman JM. Diabetes in African Americans. In: Diabetes In America. 2nd ed. Bethesda, MD: National Diabetes Data Group, NIH; 1995: 613-629.

Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR (1993) Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. Diabetes 42: 359-362.

Ulrich P, Cerami A (2001) Protein glycation, diabetes, and aging. Recent Prog Horm Res 56: 1-21.

van Dam RM, Rimm EB, Willett WC, Stampfer MJ, Hu FB (2002) Dietary patterns and risk for type 2 diabetes mellitus in U.S. men. Ann Intern Med. 136: 201-209.

Vander Jagt D L, Hunsaker L A, Vander Jagt T J, et al (1997) Inactivation of glutathione reductase by 4-hydroxynonenal and other endogenous aldehydes. Biochem Pharmacol 53: 1133-1140.

Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, et al., (1996) Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. Diabetes 45: 926-933.

Vessby B, Aro A, Skarfors E, Berglund L, Salminen I, Lithell H (1994) The risk to develop NIDDM is related to the fatty acid composition of the serum cholesterol esters. Diabetes 43: 1353-1357.

Vuilleumier N, Bas S, Pagano S, Montecucco F, Guerne PA, Finckh A, et al., (2010) Anti-apolipoprotein A-1 IgG predicts major cardiovascular events in patients with rheumatoid arthritis. Arthritis Rheum 62: 2640-2650.

Wa C, Cerny RL, Clarke WA, Hage DS (2007) Characterization of glycation adducts on human serum albumin by matrix assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chim Acta 385: 48-60.

Wagner AM, Ordonez-Llanos J (2002) Apolipoproteins and prediction of fatal myocardial infarction. Lancet 359: 1863-1864.

Wang Y, Rimm EB, Stampfer MJ, Willett WC, Hu FB (2005) Comparison of abdominal adiposity and overall obesity in predicting risk of type 2 diabetes among men. Am J Clin Nutr 81: 555-563.

Wannamethee SG, Shaper AG, Perry IJ (2001) Smoking as a modifiable risk factor for type 2 diabetes in middle-aged men. Diabetes Care 24: 1590-1595.

Ward WK, Bolgiano DC, McKnight B, Halter JB, Porte D Jr (1984) Diminished B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. J Clin Invest 74: 1318-1328.

Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW (1995) Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. Biochemistry 34: 3702-3709.

White P, Cooke N (2000) The multifunctional properties and characteristics of vitamin D-binding protein. Trends Endocrinol Metab 11: 320-327.

Whiteaker JR, Zhang H, Eng JK, Fang R, Piening BD, Feng LC, et al., (1997) High fasting glucose as a predictor of total and cardiovascular disease (CVD) mortality in patients with NIDDM. Diabetes 46: 137A.

Whiteaker JR, Zhang H, Eng JK, Fang R, Piening BD, Feng LC, et al., (2007) Head-to-head comparison of serum fractionation techniques. J Proteome Res 6: 828-836.

Wild S, Roglic G, Green A, Sicree R, King H (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004 27: 1047-1053.

Willi C, Bodenmann P, Ghali WA, Faris PD, Cornuz J (2007) Active smoking and the risk of type 2 diabetes: a systematic review and met-analysis. JAMA 298: 2654-2664.

Winter WE, Schatz DA (2011) Autoimmune markers in diabetes. Clin Chem 57:168-175.

Yeh HC, Duncan BB, Schmidt MIs, Wang NY, Brancati FL (2010) Smoking, smoking cessation, and risk for type 2 diabetes mellitus. Ann Intern Med 152:10-17.

Zajac J, Shrestha A, Poretsky L (2002) The main events in the history of diabetes mellitus. In: Poretsky L (eds). Principles of diabetes mellitus. Kluwer Academic publishers, Dordrecht, The Netherlands. Pp. 19-38.

Zhao HR, Nagaraj RH, Abraham EEC (1997) The role of α and ε-amino groups in the glycation-mediated cross-linking of γB-crystallin. Study of three site-directed mutants. J Biol Chem 272: 14465-14469.

Zhang H, Liu AY, Loriaux P, Wollscheid B, Zhou Y, Watts JD, et al., (2007) Mass spectrometric detection of tissue proteins in plasma. Mol Cell Proteomics 6: 64-71.

Zhang Q, Ames JM, Smith RD, Baynes JW, Metz TO (2009) A perspective on the Maillard reaction and the analysis of protein glycation by mass spectrometry: probing the pathogenesis of chronic disease. J Proteome Res 8: 754-769.

Zhang Q, Monroe ME, Schepmoes AA, Clauss TR, Gritsenko MA, Meng D, et al., (2011) Comprehensive identification of glycated peptides and their glycation motifs in plasma and erythrocytes of control and diabetic subjects. J Proteome Res 10: 3076-3088.

Zhang Q, Petyuk VA, Schepmoes AA, Orton DJ, Monroe ME, Yang F, et al.,(2008b) Analysis of non-enzymatically glycated peptides: neutral-loss-triggered MS(3) versus multi-stage activation of tandem mass spectrometry. Rapid Commun. Mass Spectrom. 22: 3027-3034.

Zhang Q, Tang N, Brock JW, Mottaz HM, Ames JM, Baynes JW, et al., (2007) Enrichment and analysis of nonenzymatically glycated peptides: boronate affinity chromatography coupled with electron-transfer dissociation mass spectrometry. J Proteome Res 6: 2323-2330.

Zhang Q, Tang N, Schepmoes AA, Phillips LS, Smith RD, Metz TO (2008a) Proteomic profiling of nonenzymatically glycated proteins in human plasma and erythrocyte membranes. J Proteome Res 7: 2025-2032.

Zhang Y, Cocklin RR, Bidasee KR, Wang M (2003) Rapid determination of advanced glycation end products of proteins using MALDI-TOF-MS and PERL script peptide searching algorithm. J Biomol Tech 14: 224-230.

Zimmet P (1982) Type 2 (non-insulin-dependent) diabetes-an epidemiological overview. Diabetologia 22: 399-411.

Zimmet P, Turner R, McCarty D, Rowley M, Mackay I (1999) Crucial points at diagnosis. Type 2 diabetes or slow type 1 diabetes. Diabetes Care 22: B59-64.

Zolotarjova N, Martosella J, Nicol G, Bailey J, Boyes BE, Barrett WC (2005) Differences among techniques for high-abundant protein depletion. Proteomics 5: 3304-3313.

Zoppini G, Targher G, Chonchol M, Perrone F, Lippi G, Muggeo M (2009) Higher HDL cholesterol levels are associated with a lower incidence of chronic kidney disease in patients with type 2 diabetes. Nutr Metab Cardiovasc Dis 19: 580-586.

APPENDIX I

Table A. 1. Identification of protein glycation modifications from mice plasma. ND-Non-Diabetic; DHAP- Diabetic High Albumin Plasma; DLAP- Diabetic Low Albumin Plasma; UG- Un-Glycated peptide; G- Glycated peptide.

Protein	Precursor MH+ (Da)	PLGS Score	Sequence	Modifications	Intensity
ND (ALB)					
UG	1681.848	7.90	(R)LSQTFPNADFAEITK(L)		213095
G	1730.842	6.23	(R)LSQTFPNADFAEITK(L)	MOLD (15)	3398
UG	1443.639	8.81	(K)YICENQDSISSK(L)	Carbamidomethyl C (3)	886321
G	1515.65	6.70	(K)YICENQDSISSK(L)	Carbamidomethyl C (3), CEL (12)	1975
UG	2599.325	8.01	(K)QNCELFEQLGEYKFQNALLVR(Y)	Carbamidomethyl C (3)	100298
G	2707.316	7.00	(K)QNCELFEQLGEYKFQNALLVR(Y)	Carbamidomethyl C (3), PYRRALINE (13)	155979
DHAP (ALB)					
UG	1681.848	7.90	(R)LSQTFPNADFAEITK(L)		213095
G	1730.843	6.56	(R)LSQTFPNADFAEITK(L)	MOLD (15)	7856
UG	1840.945	7.37	(K)EQLKAVMDDFAAFVEK(C)		16425
G	1938.943	6.79	(K)EQLKAVMDDFAAFVEK(C)	MOLD (4), MOLD (16)	49294
UG	1443.639	8.64	(K)YICENQDSISSK(L)	Carbamidomethyl C (3)	2240528
G	1479.657	6.91	(K)YICENQDSISSK(L)	Carbamidomethyl C (3), MODIC (12)	14280
UG	1499.64	7.75	(K)ADDKETCFAEEGK(K)	Carbamidomethyl C (7)	88066
G	1751.677	7.06	(K)ADDKETCFAEEGK(K)	PYRRALINE (4), Carbamidomethyl C (7), IMIDAZOLONE-A (13)	14559
UG	1941.946	6.87	(R)ADLAKYICENQDSISSK(L)	Carbamidomethyl C (8)	5349
G	2471.143	6.34	(R)ADLAKYICENQDSISSKLK(E)	IMIDAZOLONE-A (5), Carbamidomethyl C (8), IMIDAZOLONE-A (17)	6581
DLAP (ALB)					
UG	1439.782	8.15	(K)APQVSTPTLVEAAR(N)		201837
G	1511.809	6.35	(K)APQVSTPTLVEAAR(N)	MG-DH (14)	2511
UG	1681.848	7.90	(R)LSQTFPNADFAEITK(L)		213095
G	1730.843	6.56	(R)LSQTFPNADFAEITK(L)	MOLD (15)	7856
UG	1932.048	8.57	(K)SLHTLFGDKLCTVATLR(E)	Carbamidomethyl C (11)	776429
G	1990.063	7.41	(K)SLHTLFGDKLCTVATLR(E)	CML (9), Carbamidomethyl C (11)	39723
UG	2636.238	8.20	(K)QEPERNECFLQHKDDNPNLP R(L)	Carbamidomethyl C (8)	1109805
G	2784.254	6.71	(K)QEPERNECFLQHKDDNPNLP R(L)	GH1 (5), Carbamidomethyl C (8), PYRL (13)	11455
UG	2674.332	8.49	(K)RMPCAEDYLSVVLNQLCVLHE K(T)	Carbamidomethyl C (4), Carbamidomethyl C (17)	629442
G	2746.359	7.36	(K)RMPCAEDYLSVVLNQLCVLHE K(T)	CEL (1), Carbamidomethyl C (4),	36196

				Carbamidomethyl C (17)	
UG	2674.332	8.49	(K)RMPCAEDYLSVVLNQLCVLHE K(T)	Carbamidomethyl C (4), Carbamidomethyl C (17)	629442
G	2777.354	6.67	(K)RMPCAEDYLSVVLNQLCVLHE K(T)	MOLD (1), Carbamidomethyl C (4), Carbamidomethyl C (17), MGH1 (22)	14235
UG	2545.2	6.87	(K)EFNAETFTFHADICTLSEKER(Q)	Carbamidomethyl C (14)	67984
G	2585.169	7.23	(K)EFNAETFTFHADICTLSEKER(Q)	Carbamidomethyl C (14), GH1 (21)	89289
UG	4152.94	6.65	(K)SHCIAEVENDEMPADLPSLAAD FVESKDVCKNYAEAK(D)	Carbamidomethyl C (3), Carbamidomethyl C (30)	13282
G	4318.914	6.73	(K)SHCIAEVENDEMPADLPSLAAD FVESKDVCKNYAEAK(D)	Carbamidomethyl C (3), CML (27), Carbamidomethyl C (30), CEL (31), MODIC (37)	1401
UG	2052.207	6.68	(K)KVPQVSTPTLVEVSRNLGK(V)		2555
G	2626.318	6.70	(K)KVPQVSTPTLVEVSRNLGK(V)	AFGP (1), IMIDAZOLONE (15), Glycation KR (19)	768721
ND (APO A1)					
UG	1981.997	6.33	(R)TQLAPHSEQMRESLAQR(L)		4023
G	2061.996	6.24	(R)TQLAPHSEQMRESLAQR(L)	ARGPYR (17)	7756
DHAP (APO A1)					
UG	1467.78	7.81	(K)VKDFANVYVDAVK(D)		26873
G	1701.836	6.57	(K)VKDFANVYVDAVK(D)	PYRRALINE (2), FL-2H2O (13)	4819
UG	1530.751	6.00	(K)SNPTLNEYHTRAK(T)		4968
G	1782.895	6.05	(K)SNPTLNEYHTRAK(T)	CROSSLINE (13)	12226
UG	1981.997	6.33	(R)TQLAPHSEQMRESLAQR(L)		4023
G	2061.992	6.55	(R)TQLAPHSEQMRESLAQR(L)	ARGPYR (11)	17885
DLAP (APO A1)					
UG	1790.958	6.66	(K)TKAQSVIDKASETLTAQ(-)		12362
G	1862.983	6.49	(K)TKAQSVIDKASETLTAQ(-)	CEL (9)	7083
UG	1981.997	6.33	(R)TQLAPHSEQMRESLAQR(L)		4023
G	2062.003	6.17	(R)TQLAPHSEQMRESLAQR(L)	ARGPYR (11)	3039
UG	2149.03	6.49	(K)ETDWVRQEMNKDLEEVK(Q)		1146
G	2203.031	6.69	(K)ETDWVRQEMNKDLEEVK(Q)	MG-H1 (6)	10250
UG	1530.751	6.00	(K)SNPTLNEYHTRAK(T)		4968
G	1570.752	6.19	(K)SNPTLNEYHTRAK(T)	G-H1 (11)	11861
ND (APO A2)					
UG	1218.67	6.87	(K)AKTSEIQSQVK(A)		13243
G	2073.014	5.94	(K)AKTSEIQSQVKAYFEK(T)	CEL (11), IMIDAZOLONE-A (16)	5951
DHAP (APO A2)					
UG	1218.668	7.45	(K)AKTSEIQSQVK(A)		13385
G	1362.708	6.38	(K)AKTSEIQSQVK(A)	CEL (2), CEL (11)	31439

DLAP (APO A2)					
UG	1218.668	7.15	(K)AKTSEIQSQVK(A)		13260
G	1362.704	6.44	(K)AKTSEIQSQVK(A)	FL-1H2O (11)	41207
UG	1657.816	5.31	(K)TSEIQSQVKAYFEK(T)		2343
G	1751.877	6.55	(K)TSEIQSQVKAYFEK(T)	CML (9), MODIC (14)	1158
ND (APO A4)					
DHAP (APO A4)					
UG	1060.553	5.17	(K)KNAEELQTK(V)		8196
G	1118.554	5.72	(K)KNAEELQTK(V)	CML (9)	64003
UG	1447.679	6.15	(K)LGDASTYADGVHNK(L)		4118
G	1505.698	5.37	(K)LGDASTYADGVHNK(L)	CML (14)	2672
UG	1623.832	5.57	(K)TDVTQQLSTLFQDK(L)		4025
G	1681.854	6.96	(K)TDVTQQLSTLFQDK(L)	CML (14)	200079
DLAP (APO A4)					
UG	1312.708	6.52	(K)NLAPLVEDVQSK(V)		20525
G	1348.7	5.05	(K)NLAPLVEDVQSK(V)	MODIC (12)	2469
UG	1623.832	5.57	(K)TDVTQQLSTLFQDK(L)		4025
G	1681.849	5.84	(K)TDVTQQLSTLFQDK(L)	CML (14)	80638
UG	1443.799	5.32	(K)ATIDQNLEDLRR(S)		3887
G	1603.821	5.63	(K)ATIDQNLEDLRR(S)	ARGPYR (11), ARGPYR (12)	27226
ND (APO E)					
DHAP (APO E)					
UG	1170.656	6.67	(R)LGKEVQAAQAR(L)		3257
G	1332.703	5.57	(R)LGKEVQAAQAR(L)	GLYCATION (3)	3096
UG	1727.865	5.75	(K)KELEEQLGPVAEETR(A)		49800
G	1763.903	5.74	(K)KELEEQLGPVAEETR(A)	MODIC (1)	1891
UG	1727.865	5.75	(K)KELEEQLGPVAEETR(A)		3923
G	1853.914	6.17	(K)KELEEQLGPVAEETR(A)	FL-2H2O (1)	78846
UG	1239.673	5.97	(R)TANLGAGAAQPLR(D)		25790
G	2478.204	5.11	(R)TANLGAGAAQPLRDRAQAFGDR(I)	IMIDAZOLONE-B (13), G-H1 (15), G-H1 (22)	1630
DLAP (APO E)					
UG	1510.81	6.18	(R)TANLGAGAAQPLRDR(A)		9528
G	1546.821	5.45	(R)TANLGAGAAQPLRDR(A)	MODIC (15)	3284
UG	1510.81	6.18	(R)TANLGAGAAQPLRDR(A)		9528
G	1618.813	5.28	(R)TANLGAGAAQPLRDR(A)	MG-DH (13), MODIC (15)	10333
UG	1510.81	6.18	(R)TANLGAGAAQPLRDR(A)		9528
G	1694.816	5.26	(R)TANLGAGAAQPLRDR(A)	G-H1 (13), IMIDAZOLONE-A (15)	8537
UG	1727.865	5.75	(K)KELEEQLGPVAEETR(A)		3923

G	1807.903	5.45	(K)KELEEQLGPVAEETR(A)	ARGPYR (15)	5223
UG	1510.81	6.18	(R)TANLGAGAAQPLRDR(A)		9528
G	1816.862	4.70	(R)TANLGAGAAQPLRDR(A)	AFGP (13), MODIC (15)	1364
UG	1727.865	5.75	(K)KELEEQLGPVAEETR(A)		3923
G	1853.917	5.60	(K)KELEEQLGPVAEETR(A)	FL-2H2O (1)	69681
UG	1743.84	5.70	(R)NEVHTMLGQSTEEIR(A)		3247
G	2385.06	5.06	(R)NEVHTMLGQSTEEIRAR(L)	AFGP (15), IMIDAZOLONE-A (17)	2361
UG	1510.81	6.18	(R)TANLGAGAAQPLRDR(A)		9528
G	2440.159	5.24	(R)TANLGAGAAQPLRDRAQAFGDR(I)	G-H1 (13), IMIDAZOLONE-A (15)	9635
ND (APO C3)					
DHAP (APO C3)					
UG	2341.126	6.59	(R)AEEVEGSLLLGSVQGYMEQASK(T)	Oxidation M (17)	1553
G	2383.123	6.04	(R)AEEVEGSLLLGSVQGYMEQASK(T)	CML (22)	5947
DLAP (APO C3)					
UG	2341.126	6.59	(R)AEEVEGSLLLGSVQGYMEQASK(T)	Oxidation M (17)	1553
G	2383.132	5.98	(R)AEEVEGSLLLGSVQGYMEQASK(T)	CML (22)	6150
ND (CD5L)					
DHAP (CD5L)					
DLAP (CD5L)					
UG	1290.62	3.99	(R)GASYQPPASEQR(V)		2365
G	1370.646	4.58	(R)GASYQPPASEQR(V)	ARGPYR (12)	15285
ND (CLUS)					
DHAP (CLUS)					
UG	1335.691	7.34	(R)ASGIIDTLFQDR(F)		13668
G	1393.735	5.76	(R)ASGIIDTLFQDR(F)	CML (12)	5359
DLAP (CLUS)					
UG	1231.694	6.75	(K)SLLNSLEEAKK(K)		6349
G	1357.715	5.58	(K)SLLNSLEEAKK(K)	FL-2H2O (10)	2655
UG	1401.737	6.12	(R)QELNDSLQVAER(L)		1070
G	1473.728	5.87	(R)QELNDSLQVAER(L)	MG-DH (12)	70129
UG	1841.975	7.03	(R)LTEQYKELLQSFQSK(M)		15844
G	1967.999	5.62	(R)LTEQYKELLQSFQSK(M)	FL-2H2O (6)	1229
UG	2290.074	6.28	(K)CQEILSVDCSTNNPAQANLR(Q)	Carbamidomethyl C (1), Carbamidomethyl C (9)	5261
G	3752.756	5.62	(K)CQEILSVDCSTNNPAQANLRQELNDSLQVAER(L)	Carbamidomethyl C (1), Carbamidomethyl C (9), G-H1 (20), G-H1 (32)	14112
ND (FA10)					
DHAP (FA10)					
UG	2061.085	4.69	(K)NTYYVTGIVSWGEGCARK(G)	Carbamidomethyl C (15)	1641

G	2096.984	5.72	(K)NTYYVTGIVSWGEGCARK(G)	Carbamidomethyl C (15), MODIC (18)	7356
UG	2695.327	5.00	(K)DWAESTLMTQKTGIVSGFGR THEK(G)	Oxidation M (8)	2099
G	3165.468	5.05	(K)DWAESTLMTQKTGIVSGFGRTHEK(G)	PYRRALINE (11), CROSSLINE (20), FL-2H2O (24)	8523
DLAP (FA10)					
UG	2061.085	4.69	(K)NTYYVTGIVSWGEGCARK(G)	Carbamidomethyl C (15)	1641
G	2119.029	4.86	(K)NTYYVTGIVSWGEGCARK(G)	Carbamidomethyl C (15), CML (18)	3782
UG	1324.604	5.57	(K)SCISTAPFPCGK(I)	Carbamidomethyl C (2), Carbamidomethyl C (10)	9671
G	2153.031	5.25	(K)SCISTAPFPCGKITTGRR(K)	Carbamidomethyl C (2), Carbamidomethyl C (10), IMIDAZOLONE-A (17)	12636
UG	2695.327	5.00	(K)DWAESTLMTQKTGIVSGFGRTHEK(G)	Oxidation M (8)	2099
G	3165.499	4.84	(K)DWAESTLMTQKTGIVSGFGRTHEK(G)	PYRRALINE (11), CROSSLINE (20), FL-2H2O (24)	10113
UG	3234.593	4.91	(K)TPITFRMNVAPACLPQKDWAESTLM TQK(T)	Carbamidomethyl C (13)	4055
G	3283.62	5.33	(K)TPITFRMNVAPACLPQKDWAESTLM TQK(T)	Carbamidomethyl C (13), MOLD (28)	5867
ND (CO3)					
UG	1946.018	4.72	(K)RPQDAKNTMILEICTR(Y)	Carbamidomethyl C (14)	1061
G	2148.043	5.40	(K)RPQDAKNTMILEICTR(Y)	CML (6), Carbamidomethyl C (14), IMIDAZOLONE-A (16)	11807
DHAP (CO3)					
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1687.821	5.52	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7), CML (9), MOLD (13)	24763
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1899.872	5.19	(K)AKDQLTCNKFDLK(V)	GLYCATION (2), Carbamidomethyl C (7), PYRRALINE (9), MOLD (13)	1704
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
	1940.916	4.77	(K)AKDQLTCNKFDLK(V)	GLYCATION (2), Carbamidomethyl C (7), CEL (9), FL-2H2O (13)	1290
DLAP (CO3)					
UG	872.4637	6.10	(K)QLANGVDR(Y)		1097
G	926.4591	5.04	(K)QLANGVDR(Y)	MG-H1 (8)	1221
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1243.557	5.80	(K)AKDQLTCNK(F)	CML (2), Carbamidomethyl C (7), PYRRALINE (9)	3396
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1687.821	5.52	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7), CML (9), MOLD (13)	24763
UG	1597.856	4.19	(R)TLDPERLGREGVQK(E)		6570
G	1691.88	6.39	(R)TLDPERLGREGVQK(E)	MG-H1 (6), G-H1 (9)	1265
UG	1618.847	4.80	(R)TKKQELSEAEQATR(T)		8125
G	1708.861	5.76	(R)TKKQELSEAEQATR(T)	MODIC (3), MG-H1 (14)	46412
UG	1896.022	4.97	(R)NKFVTVQATFGTQVVEK(V)		1534

G	1994.019	5.68	(R)NKFVTVQATFGTQVVEK(V)	MOLD (2), MOLD (17)	18750
UG	1923.996	4.56	(K)DSITTWEILAVSMSDKK(G)		2080
G	2338.145	5.71	(K)DSITTWEILAVSMSDKK(G)	GLYCATION (16), CROSSLINE (17)	1165
ND (FIBB)					
UG	3072.62	4.96	(K)SSIAELNNNIQSVSDTSSVTFQYLTLLK(D)		1586
G	3234.593	5.22	(K)SSIAELNNNIQSVSDTSSVTFQYLTLLK(D)	GLYCATION (28)	4055
DHAP (FIBB)					
UG	1618.799	6.11	(R)TPCTVSCNIPVVSGK(E)	Carbamidomethyl C (3), Carbamidomethyl C (7)	16553
G	1676.798	5.59	(R)TPCTVSCNIPVVSGK(E)	Carbamidomethyl C (3), Carbamidomethyl C (7), CML (15)	15364
UG	2152.078	5.06	(K)YKGTAGNALMDGASQLVGENR(T)		1093
G	2210.055	5.44	(K)YKGTAGNALMDGASQLVGENR(T)	CML (2)	16767
DLAP (FIBB)					
UG	1364.709	5.38	(K)GFGNIATNEDAKK(Y)		4017
G	1584.758	6.02	(K)GFGNIATNEDAKK(Y)	GLYCATION (12),CML (13)	5699
UG	1902.002	6.42	(R)LYIDETVNDNIPLNLR(V)		4325
G	1956.016	5.10	(R)LYIDETVNDNIPLNLR(V)	MG-H1 (16)	6713
UG	2327.106	5.07	(K)AHYGGFTVQNEASKYQVSVNK(Y)		1559
G	2421.19	5.13	(K)AHYGGFTVQNEASKYQVSVNK(Y)	CML (14), MODIC (21)	9554
ND (FBLN1)					
DHAP (FBLN1)					
UG	2132.042	4.67	(K)ARENSDFVQGNGADLQDPAK(I)		1311
G	2186.007	4.72	(K)ARENSDFVQGNGADLQDPAK(I)	MG-H1 (2)	1505
DLAP (FBLN1)					
UG	2132	4.70	(K)ARENSDFVQGNGADLQDPAK(I)		2694
G	2186.017	4.52	(K)ARENSDFVQGNGADLQDPAK(I)	MG-H1 (2)	3115
ND (GPX3)					
DHAP (GPX3)					
DLAP (GPX3)					
UG	1789.883	5.99	(R)TTVSNVKMDILSYMR(R)	Oxidation M (8), Oxidation M (14)	10151
G	1811.905	5.70	(R)TTVSNVKMDILSYMR(R)	MG-H1 (15)	3884
UG	1789.883	5.99	(R)TTVSNVKMDILSYMR(R)	Oxidation M (8), Oxidation M (14)	10151
G	1829.915	6.06	(R)TTVSNVKMDILSYMR(R)	CML(15)	60715
ND (MASP1)					
DHAP (MASP1)					
UG	1357.725	4.67	(K)DWIQRITGVRN(-)		2051
G	1397.732	5.52	(K)DWIQRITGVRN(-)	G-H1 (10)	2753
UG	1650.814	4.77	(R)TGTITSPDYPNPYPK(S)		6324
G	1794.841	5.26	(R)TGTITSPDYPNPYPK(S)	IMIDAZOLONE-A (15)	1144

UG	2126.099	4.51	(R)DQWYLVGVVSWGEDCGKK(D)	Carbamidomethyl C (15)	2940
G	2450.136	5.05	(R)DQWYLVGVVSWGEDCGKK(D)	Carbamidomethyl C (15), CEL (17), CROSSLINE (18)	3435
DLAP (MASP1)					
UG	1357.734	3.72	(K)DWIQRITGVRN(-)		1370
G	1681.847	4.73	(K)DWIQRITGVRN(-)	MG-DH (5), CROSSLINE (10)	87671
UG	1650.814	4.77	(R)TGTITSPDYPNPYPK(S)		6324
G	1794.846	5.67	(R)TGTITSPDYPNPYPK(S)	IMIDAZOLONE-A (15)	2154
UG	1622.806	3.62	(K)VTKDMICAGEKEGGK(D)	Carbamidomethyl C (7)	1900
G	1797.82	5.38	(K)VTKDMICAGEKEGGK(D)	Carbamidomethyl C (7), FL-2H2O (11), MOLD (15)	2622
UG	2082.033	4.31	(K)VLKDNEVMDTFQIECLK(D)	Carbamidomethyl C (15)	1639
G	2140.014	4.92	(K)VLKDNEVMDTFQIECLK(D)	Carbamidomethyl C (15), CML (17)	3765
UG	1365.669	4.70	(R)LSYRAAGNECPK(L)	Carbamidomethyl C (10)	6185
G	1401.663	5.26	(R)LSYRAAGNECPK(L)	Carbamidomethyl C (10), MODIC (12)	5163
ND (MBL2)					
DHAP (MBL2)					
UG	1323.621	6.45	(R)VEGSFEDLTGNR(V)		13980
G	1690.805	5.86	(R)VEGSFEDLTGNRVR(Y)	G-H1 (12), MG-DH (14)	18227
UG	1749.923	6.23	(R)VKALCSEFQGSVATPR(N)	Carbamidomethyl C (5)	2253
G	1807.912	6.21	(R)VKALCSEFQGSVATPR(N)	Carbamidomethyl C (5), CML (16)	3537
DLAP (MBL2)					
UG	1323.621	6.45	(R)VEGSFEDLTGNR(V)		13980
G	1780.832	6.59	(R)VEGSFEDLTGNRVR(Y)	GLYCATION (12), G-H1 (14)	5461
UG	1749.896	5.59	(R)VKALCSEFQGSVATPR(N)	Carbamidomethyl C (5)	35055
G	2037.997	6.25	(R)VKALCSEFQGSVATPR(N)	IMIDAZOLONE-A (2), Carbamidomethyl C (5),IMIDAZOLONE-A (16)	11297
ND (PHLD)					
DHAP (PHLD)					
DLAP (PHLD)					
UG	1648.763	5.09	(R)ENYPLPWEKDTEK(L)		4716
G	1949.909	4.91	(R)ENYPLPWEKDTEK(L)	MOLD (9), CROSSLINE (13)	8095
ND (LCAT)					
DHAP (LCAT)					
DLAP (LCAT)					
UG	1569.754	5.40	(R)SIGGEVFIDFTKEK(D)		4187
G	1627.833	5.58	(R)SIGGEVFIDFTKEK(D)	CML (12)	1198

Table A. 2. Identification of protein glycation modifications from clinical plasma. ND- Non-Diabetic; DHAP- Diabetic High Albumin Plasma; DLAP- Diabetic Low Albumin Plasma; UG- Un-Glycated peptide; G- Glycated peptide.

Protein	Precursor MH+ (Da)	Score	Sequence	Modifications	Intensity
ND (ALB)					
UG	2316.122	7.16	(K)NYAEAKDVFLGMFLYEYAR(R)	Oxidation M (12)	78446
G	2840.275	6.77	(K)NYAEAKDVFLGMFLYEYAR(R)	AFGP (6), AFGP (19)	21717
UG	2052.207	6.68	(K)KVPQVSTPTLVEVSRNLGK(V)		2555
G	2626.318	6.70	(K)KVPQVSTPTLVEVSRNLGK(V)	AFGP (1), IMIDAZOLONE (15), Glycation KR (19)	768721
UG	1840.917	8.18	(K)EQLKAVMDDFAAFVEK(C)		137152
G	1898.963	6.73	(K)EQLKAVMDDFAAFVEK(C)	CML (4)	1266993
UG	2650.264	7.87	(R)LVRPEVDVMCTAFHDNEETFLK(K)	Carbamidomethyl C (10)	103492
G	2776.314	6.44	(R)LVRPEVDVMCTAFHDNEETFLK(K)	Carbamidomethyl C (10), FL-2h2o (22)	1362
UG	2650.264	7.87	(R)LVRPEVDVMCTAFHDNEETFLK(K)	Carbamidomethyl C (10)	103492
G	3172.524	6.70	(R)LVRPEVDVMCTAFHDNEETFLKK(Y)	Carbamidomethyl C (10), IMIDAZOLONE (22), CROSSLINE (23)	7494
DHAP (ALB)					
UG	1840.945	7.37	(K)EQLKAVMDDFAAFVEK(C)		16425
G	1938.943	6.79	(K)EQLKAVMDDFAAFVEK(C)	MOLD (4), MOLD (16)	49294
UG	1443.639	8.81	(K)YICENQDSISSK(L)	Carbamidomethyl C (3)	886321
G	1515.65	6.70	(K)YICENQDSISSK(L)	Carbamidomethyl C (3), CEL (12)	1975
UG	2599.325	8.01	(K)QNCELFEQLGEYKFQNALLVR(Y)	Carbamidomethyl C (3)	100298
G	2707.316	7.00	(K)QNCELFEQLGEYKFQNALLVR(Y)	Carbamidomethyl C (3), PYRRALINE (13)	155979
UG	1840.945	7.37	(K)EQLKAVMDDFAAFVEK(C)		16425
G	1966.937	6.60	(K)EQLKAVMDDFAAFVEK(C)	FL-2H2O (16)	9112
UG	1736.911	6.21	(R)LSQRFPKAEFAEVSK(L)		3380
G	1875.94	6.38	(R)LSQRFPKAEFAEVSK(L)	MODIC (4), MGH1 (7), MOLD (15)	1695877
UG	2636.238	8.20	(K)QEPERNECFLQHKDDNPNLPR(L)	Carbamidomethyl C (8)	1109805
G	2784.254	6.71	(K)QEPERNECFLQHKDDNPNLPR(L)	GH1 (5), Carbamidomethyl C (8), PYRL (13)	11455
UG	2052.207	6.68	(K)KVPQVSTPTLVEVSRNLGK(V)		2555
G	2626.318	6.70	(K)KVPQVSTPTLVEVSRNLGK(V)	AFGP (1), IMIDAZOLONE (15), Glycation KR (19)	768721
UG	2650.264	7.87	(R)LVRPEVDVMCTAFHDNEETFLK(K)	Carbamidomethyl C (10)	103492
G	3172.524	6.70	(R)LVRPEVDVMCTAFHDNEETFLKK(Y)	Carbamidomethyl C (10), IMIDAZOLONE (22), CROSSLINE (23)	7494
UG	1650.894	8.83	(K)AEFAEVSKLVTDLTK(V)		750954
G	1708.943	7.04	(K)AEFAEVSKLVTDLTK(V)	CML (8)	11363
DLAP (ALB)					

UG	1443.639	8.64	(K)YICENQDSISSK(L)	Carbamidomethyl C (3)	2240528
G	1479.657	6.91	(K)YICENQDSISSK(L)	Carbamidomethyl C (3), MODIC (12)	14280
UG	1499.64	7.75	(K)ADDKETCFAEEGK(K)	Carbamidomethyl C (7)	88066
G	1751.677	7.06	(K)ADDKETCFAEEGK(K)	PYRRALINE (4), Carbamidomethyl C (7), IMIDAZOLONE-A (13)	14559
UG	1941.946	6.87	(R)ADLAKYICENQDSISSK(L)	Carbamidomethyl C (8)	5349
G	2471.143	6.34	(R)ADLAKYICENQDSISSKLK(E)	IMIDAZOLONE-A (5), Carbamidomethyl C (8), IMIDAZOLONE-A (17)	6581
UG	2300.108	6.51	(K)NYAEAKDVFLGMFLYEYAR(R)		13251
G	2586.199	6.37	(K)NYAEAKDVFLGMFLYEYARR(H)	CML (6), MODIC (19), MODIC (20)	5869
UG	2636.238	8.22	(K)QEPERNECFLQHKDDNPNLPR(L)	Carbamidomethyl C (8)	1109549
G	2756.244	6.53	(K)QEPERNECFLQHKDDNPNLPR(L)	ARGPYR (5), Carbamidomethyl C (8), G-H1 (21)	168016
UG	3355.607	7.25	(K)LVTDLTKVHTECCHGDLLECAD DRADLAK(Y)	Carbamidomethyl C (12), Carbamidomethyl C (13), Carbamidomethyl C (20)	101272
G	3427.592	6.67	(K)LVTDLTKVHTECCHGDLLECADDRA DLAK(Y)	Carbamidomethyl C (12), Carbamidomethyl C (13), Carbamidomethyl C (20), MG-DH (24)	92597
UG	1932.048	8.57	(K)SLHTLFGDKLCTVATLR(E)	Carbamidomethyl C (11)	776429
G	1990.063	7.41	(K)SLHTLFGDKLCTVATLR(E)	CML (9), Carbamidomethyl C (11)	39723
UG	2636.238	8.20	(K)QEPERNECFLQHKDDNPNLPR(L)	Carbamidomethyl C (8)	1109805
G	2784.254	6.71	(K)QEPERNECFLQHKDDNPNLPR(L)	GH1 (5), Carbamidomethyl C (8), PYRL (13)	11455
UG	2674.332	8.49	(K)RMPCAEDYLSVVLNQLCVLHEK(T)	Carbamidomethyl C (4), Carbamidomethyl C (17)	629442
G	2746.359	7.36	(K)RMPCAEDYLSVVLNQLCVLHEK(T)	CEL (1), Carbamidomethyl C (4), Carbamidomethyl C (17)	36196
UG	2674.332	8.49	(K)RMPCAEDYLSVVLNQLCVLHEK(T)	Carbamidomethyl C (4), Carbamidomethyl C (17)	629442
G	2777.354	6.67	(K)RMPCAEDYLSVVLNQLCVLHEK(T)	MOLD (1), Carbamidomethyl C (4), Carbamidomethyl C (17), MGH1 (22)	14235
UG	2545.2	6.87	(K)EFNAETFTFHADICTLSEKER(Q)	Carbamidomethyl C (14)	67984
G	2585.169	7.23	(K)EFNAETFTFHADICTLSEKER(Q)	Carbamidomethyl C (14), GH1 (21)	89289
UG	4152.94	6.65	(K)SHCIAEVENDEMPADLPSLA ADFVESKDVCKNYAEAK(D)	Carbamidomethyl C (3), Carbamidomethyl C (30)	13282
G	4318.914	6.73	(K)SHCIAEVENDEMPADLPS LAADFVESKDVCKNYAEAK(D)	Carbamidomethyl C (3), CML (27), Carbamidomethyl C (30), CEL (31), MODIC (37)	1401
UG	2316.122	7.16	(K)NYAEAKDVFLGMFLYEYAR(R)	Oxidation M (12)	78446
G	2840.275	6.77	(K)NYAEAKDVFLGMFLYEYAR(R)	AFGP (6), AFGP (19)	21717
UG	2052.207	6.68	(K)KVPQVSTPTLVEVSRNLGK(V)		2555
G	2626.318	6.70	(K)KVPQVSTPTLVEVSRNLGK(V)	AFGP (1), IMIDAZOLONE (15), Glycation KR (19)	768721
ND (A1AG2)					
UG	1671.838	5.35	(K)TEDTIFLREYQTR(Q)		15297
G	1707.827	5.33	(K)TEDTIFLREYQTR(Q)	MODIC (8)	1697

DHAP (A1AG2)					
UG	1671.842	5.15	(K)TEDTIFLREYQTR(Q)		7803
G	1707.827	5.33	(K)TEDTIFLREYQTR(Q)	MODIC (8)	1697
UG	1708.858	5.50	(K)NWGLSVYADKPETTK(E)		81508
G	1888.897	4.73	(K)NWGLSVYADKPETTK(E)	IMIDAZOLONE-A (10), MODIC (15)	1125
DLAP (A1AG2)					
UG	1297.599	4.74	(K)CEPLEKQHEK(E)	Carbamidomethyl C (1)	1224
G	1513.669	5.62	(K)CEPLEKQHEK(E)	Carbamidomethyl C (1), PYRRALINE (6), PYRRALINE (10)	2966
UG	1671.842	5.15	(K)TEDTIFLREYQTR(Q)		7803
G	1707.827	6.17	(K)TEDTIFLREYQTR(Q)	MODIC (13)	2011
UG	1483.725	4.25	(K)SDVVYTDWKKDK(C)		1996
G	1753.794	5.97	(K)SDVVYTDWKKDK(C)	PYRRALINE (9), FL-2H2O (10), MODIC (12)	2759
UG	1708.858	5.50	(K)NWGLSVYADKPETTK(E)		81508
G	1888.897	4.73	(K)NWGLSVYADKPETTK(E)	IMIDAZOLONE-A (10), MODIC (15)	1125
ND (A1BG)					
UG	1770.934	5.45	(R)GEKELLVPRSSTSPDR(I)		1985
G	1850.961	5.17	(R)GEKELLVPRSSTSPDR(I)	ARGPYR (16)	3543
UG	2190.075	4.85	(R)CEGPIPDVTFELLREGETK(A)	Carbamidomethyl C (1)	15260
G	2334.105	5.61	(R)CEGPIPDVTFELLREGETK(A)	Carbamidomethyl C (1), IMIDAZOLONE-A (14)	8714
DHAP (A1BG)					
UG	1723.96	4.87	(R)LELHVDGPPPRPQLR(A)		1582
G	2030.043	5.08	(R)LELHVDGPPPRPQLR(A)	GLYCATION (11), IMIDAZOLONE-A (15)	1704
UG	2190.075	5.44	(R)CEGPIPDVTFELLREGETK(A)	Carbamidomethyl C (1)	2436
G	2334.105	5.61	(R)CEGPIPDVTFELLREGETK(A)	Carbamidomethyl C (1), IMIDAZOLONE-A (14)	8714
DLAP (A1BG)					
UG	1580.785	5.23	(R)CRSGLSTGWTQLSK(L)	Carbamidomethyl C (1)	35597
G	1638.791	5.94	(R)CRSGLSTGWTQLSK(L)	Carbamidomethyl C (1), CML (14)	1993
UG	1580.785	5.23	(R)CRSGLSTGWTQLSK(L)	Carbamidomethyl C (1)	35597
G	1660.811	5.58	(R)CRSGLSTGWTQLSK(L)	Carbamidomethyl C (1), ARGPYR (2)	1000
UG	1580.785	5.23	(R)CRSGLSTGWTQLSK(L)	Carbamidomethyl C (1)	35597
G	1724.806	5.51	(R)CRSGLSTGWTQLSK(L)	Carbamidomethyl C (1), MODIC (2), PYRRALINE (14)	1904
ND (A2MG)					
UG	1901.969	5.36	(K)AIGYLNTGYQRQLNYK(H)		1432
G	2172.063	6.72	(K)AIGYLNTGYQRQLNYK(H)	AFGP (11)	422986
UG	2369.163	5.00	(K)QQNAQGGFSSTQDTVVALHALSK(Y)		4670
G	2549.241	5.75	(K)QQNAQGGFSSTQDTVVALHALSK(Y)	GLYCATION (23)	90740
DHAP (A2MG)					
UG	1017.555	6.11	(K)SLNEEAVKK(D)		1993

G	1125.568	6.29	(K)SLNEEAVKK(D)	PYRRALINE (8)	29029
UG	2210.135	6.23	(K)RTTVMVKNEDSLVFVQTDK(S)		1443
G	2170.047	5.72	(R)TTVMVKNEDSLVFVQTDK(S)	CML (6), CML (18)	3521
DLAP (A2MG)					
UG	1017.557	7.26	(K)SLNEEAVKK(D)		12101
G	1053.551	6.15	(K)SLNEEAVKK(D)	MODIC (8)	1281
UG	1529.682	5.28	(K)TAQEGDHGSHVYTK(A)		1658
G	1781.792	5.31	(K)TAQEGDHGSHVYTK(A)	CROSSLINE (14)	9458
ND (ANGT)					
UG	1964.074	4.35	(K)ANAGKPKDPTFIPAPIQAK(T)		1136
G	2292.163	5.25	(K)ANAGKPKDPTFIPAPIQAK(T)	GLYCATION (5), CML (7), PYRRALINE (19)	2702
DHAP (ANGT)					
UG	1967.028	4.77	(R)VGEVLNSIFFELEADER(E)		1017
G	2129.021	4.51	(R)VGEVLNSIFFELEADER(E)	GLYCATION (17)	5964
DLAP (ANGT)					
UG	1569.793	4.46	(K)RAPQSEMAPAGVSLR(A)		2699
G	1875.919	5.11	(K)RAPQSEMAPAGVSLR(A)	GLYCATION (1), IMIDAZOLONE-A (15)	3383
UG	1938.979	5.16	(K)VEGLTFQQNSLNWMKK(L)	Oxidation M (14)	1403
G	2175.046	5.34	(K)VEGLTFQQNSLNWMKK(L)	FL-2H2O (15), FL-2H2O (16)	17822
UG	1794.806	5.17	(K)VEGLTFQQNSLNWMK(K)		1236
G	2536.275	5.12	(K)VEGLTFQQNSLNWMKKLSPR(T)	Oxidation M (14), IMIDAZOLONE-A (16)	1074
UG	2821.485	4.85	(K)LSNDRIRVGEVLNSIFFELEADER(E)		1661
G	3313.548	5.49	(K)LSNDRIRVGEVLNSIFFELEADER(E)	IMIDAZOLONE-B (5), ARGPYR (7), AFGP (24)	11862
ND (APOA1)					
UG	1534.784	5.52	(R)QEMSKDLEEVKAK(V)		3633
G	1443.653	6.41	(R)QEMSKDLEEVK(A)	PYRRALINE (5)	3522
DHAP (APOA1)					
UG	1129.596	5.46	(R)AELQEGARQK(L)		1234
G	1271.622	5.98	(R)AELQEGARQK(L)	IMIDAZOLONE-B (8)	1312
DLAP (APOA1)					
UG	1534.784	5.52	(R)QEMSKDLEEVKAK(V)		3633
G	1443.655	6.23	(R)QEMSKDLEEVK(A)	PYRRALINE (5)	2261
UG	1534.784	5.52	(R)QEMSKDLEEVKAK(V)		3633
G	1799.825	5.53	(R)QEMSKDLEEVKAK(V)	PYRRALINE (5), PYRRALINE (11), MOLD (13)	2269
UG	1723.945	6.37	(R)QKVEPLRAELQEGAR(Q)		9279
G	1881.886	5.92	(K)VEPLRAELQEGAR(Q)	AFGP (5), IMIDAZOLONE-A (13)	305990
ND (APOA2)					
UG	1626.783	4.54	(K)SPELQAEAKSYFEK(S)		4579
G	1742.798	5.01	(K)SPELQAEAKSYFEK(S)	CML (9), CML (14)	1695

DHAP (APOA2)					
UG	1626.783	4.54	(K)SPELQAEAKSYFEK(S)		4579
G	1724.799	5.61	(K)SPELQAEAKSYFEK(S)	MOLD (9), MOLD (14)	1904
UG	1626.783	4.54	(K)SPELQAEAKSYFEK(S)		4579
G	1890.935	5.75	(K)SPELQAEAKSYFEKSK(E)	MOLD (14)	2639
UG	1626.783	4.54	(K)SPELQAEAKSYFEK(S)		4579
G	2166.024	6.69	(K)SPELQAEAKSYFEKSK(E)	GLYCATION (9), GLYCATION (16)	1725
UG	1626.783	4.54	(K)SPELQAEAKSYFEK(S)		4579
G	2188.06	5.65	(K)SPELQAEAKSYFEKSK(E)	CML (9), MODIC (14), CROSSLINE (16)	93296
DLAP (APOA2)					
UG	1626.815	4.62	(K)SPELQAEAKSYFEK(S)		1415
G	2003.975	5.19	(K)SPELQAEAKSYFEKSK(E)	GLYCATION (16)	3930
UG	1626.815	4.62	(K)SPELQAEAKSYFEK(S)		1415
G	2220.059	5.50	(K)SPELQAEAKSYFEKSK(E)	FL-2H2O (9), CROSSLINE (16)	21402
UG	2350.12	5.00	(K)EPCVESLVSQYFQTVTDYGK(D)	Carbamidomethyl C (3)	10468
G	3290.51	5.03	(K)EPCVESLVSQYFQTVTDYGKDLMEK(V)	Carbamidomethyl C (3), CEL (20), CROSSLINE (25)	12331
UG	2966.401	5.71	(K)EPCVESLVSQYFQTVTDYGKDLMEK(V)	Carbamidomethyl C (3)	1778
G	3344.538	4.96	(K)EPCVESLVSQYFQTVTDYGKDLMEK(V)	Carbamidomethyl C (3), FL-2H2O (20), CROSSLINE (25)	11868
ND (APOA4)					
UG	1296.661	5.11	(K)GRLTPYADEFK(V)		2460
G	1332.659	4.95	(K)GRLTPYADEFK(V)	MODIC (2)	1554
UG	1341.715	5.25	(K)IGDNLRELQQR(L)		1664
G	1565.777	4.97	(K)IGDNLRELQQR(L)	ARGPYR (6), IMIDAZOLONE-A (11)	1452
DHAP (APOA4)					
UG	1350.653	5.98	(R)SLAPYAQDTQEK(L)		7317
G	1458.68	5.34	(R)SLAPYAQDTQEK(L)	PYRRALINE (12)	20427
UG	1350.653	5.98	(R)SLAPYAQDTQEK(L)		7317
G	1586.78	5.33	(R)RSLAPYAQDTQEK(L)	ARGPYR (1)	148160
UG	1585.855	5.41	(K)LKEEIGKELEELR(A)		1871
G	1891.936	5.14	(K)LKEEIGKELEELR(A)	MODIC (7), AFGP (13)	14324
DLAP (APOA4)					
UG	1258.676	5.60	(K)IDQTVEELRR(S)		9995
G	1338.653	5.48	(K)IDQTVEELRR(S)	G-H1 (9), G-H1 (10)	1492
UG	1506.741	5.35	(R)RSLAPYAQDTQEK(L)		2237
G	1542.761	5.28	(R)RSLAPYAQDTQEK(L)	MODIC (1)	22807
UG	1319.665	6.08	(R)DKVNSFFSTFK(E)		6173
G	1553.731	5.75	(R)DKVNSFFSTFK(E)	GLYCATION (2), CEL (11)	4832
UG	1341.715	5.25	(K)IGDNLRELQQR(L)		1664
G	1755.812	5.37	(K)IGDNLRELQQR(L)	AFGP (6), IMIDAZOLONE-A (11)	15535

ND (APOH)					
UG	1286.655	5.60	(K)VSFFCKNKEK(K)	Carbamidomethyl C (5)	3321
G	1461.687	4.01	(K)VSFFCKNKEK(K)	Carbamidomethyl C (5), FL-2H2O (6), MOLD (8)	1806
UG	1881.888	5.62	(K)NGMLHGDKVSFFCKNK(E)	Carbamidomethyl C (13)	64708
G	1711.763	4.72	(K)NGMLHGDKVSFFCK(N)	MODIC (8), Carbamidomethyl C (13), MODIC (14)	63402
UG	1881.888	5.62	(K)NGMLHGDKVSFFCKNK(E)	Carbamidomethyl C (13)	64708
G	1801.835	4.84	(K)NGMLHGDKVSFFCK(N)	Carbamidomethyl C (13), GLYCATION (14)	1384
DHAP (APOH)					
UG	1881.888	5.62	(K)NGMLHGDKVSFFCKNK(E)	Carbamidomethyl C (13)	64708
G	1711.768	5.03	(K)NGMLHGDKVSFFCK(N)	MODIC (8), Carbamidomethyl C (13), MODIC (14)	178253
UG	1881.888	5.62	(K)NGMLHGDKVSFFCKNK(E)	Carbamidomethyl C (13)	64708
G	2133.974	5.10	(K)NGMLHGDKVSFFCKNK(E)	CEL (8), Carbamidomethyl C (13), CEL (14), PYRRALINE (16)	3340
UG	2016.997	4.57	(K)CTPRVCPFAGILENGAVR(Y)	Carbamidomethyl C (1), Carbamidomethyl C (6)	3482
G	2159.043	5.80	(K)CTPRVCPFAGILENGAVR(Y)	Carbamidomethyl C (1), Carbamidomethyl C (6), IMIDAZOLONE-B (18)	1548
DLAP (APOH)					
UG	1468.787	4.56	(R)VYKPSAGNNSLYR(D)	X	4144
G	1612.781	4.82	(R)VYKPSAGNNSLYR(D)	IMIDAZOLONE-A (13)	4023
UG	1881.888	5.62	(K)NGMLHGDKVSFFCKNK(E)	Carbamidomethyl C (13)	64708
G	1711.761	5.06	(K)NGMLHGDKVSFFCK(N)	MODIC (8), Carbamidomethyl C (13), MODIC (14)	93428
UG	1881.888	5.62	(K)NGMLHGDKVSFFCKNK(E)	Carbamidomethyl C (13)	64708
G	2314.016	5.21	(K)NGMLHGDKVSFFCKNK(E)	IMIDAZOLONE-A (8), Carbamidomethyl C (13), IMIDAZOLONE-A (14), IMIDAZOLONE-A (16)	3054
UG	2974.351	4.49	(K)CPFPSRPDNGFVNYPAKPTLYYKDK(A)	Carbamidomethyl C (1)	2605
G	3329.545	5.04	(K)CPFPSRPDNGFVNYPAKPTLYYKDK(A)	Carbamidomethyl C (1), GLYCATION (17), IMIDAZOLONE-A (23), MOLD (25)	5403
ND (CERU)					
UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1229.629	5.43	(K)GSLHANGRQK(D)	GLYCATION (10)	11009
UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1247.607	5.98	(K)GSLHANGRQK(D)	MODIC (8), IMIDAZOLONE-A (10)	26763
UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1618.801	5.12	(K)GSLHANGRQKDVDK(E)	MODIC (8), CML (10)	1034
DHAP (CERU)					
UG	1219.639	6.01	(K)KDSLDKEKEK(H)		24268
G	1439.706	5.73	(K)KDSLDKEKEK(H)	GLYCATION (6), CML (10)	1133

UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1596.793	5.54	(K)GSLHANGRQKDVDK(E)	MODIC (8), MODIC (14)	1359
UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1600.784	5.51	(K)GSLHANGRQKDVDK(E)	G-H1 (8), MODIC (14)	48016
UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1834.896	4.96	(K)GSLHANGRQKDVDK(E)	CROSSLINE (8), CML (10)	1397
UG	2028.971	5.34	(K)AGLQAFFQVQECNKSSSK(D)	Carbamidomethyl C (12)	1644
G	2159.027	5.60	(K)AGLQAFFQVQECNKSSSK(D)	Carbamidomethyl C (12), CEL (14), CML (18)	1253
UG	2256.179	5.68	(R)KAEEEHLGILGPQLHADVGDK(V)		9180
G	2314.191	4.99	(R)KAEEEHLGILGPQLHADVGDK(V)	CML (1)	2392
UG	2900.482	4.88	(R)KERGPEEEHLGILGPVIWAEVGDTIR(V)		2739
G	2826.471	5.89	(K)ERGPEEEHLGILGPVIWAEVGDTIR(V)	MG-H1 (2)	9006
DLAP (CERU)					
UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1609.804	5.37	(K)GSLHANGRQKDVDK(E)	MODIC (8), MOLD (10)	8773
UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1618.803	5.03	(K)GSLHANGRQKDVDK(E)	MODIC (8), CML (14)	5358
UG	1219.639	6.01	(K)KDSLDKEKEK(H)		24268
G	1705.813	6.10	(K)KDSLDKEKEK(H)	PYRRALINE (1), FL-2H2O (6), CROSSLINE (10)	2539
UG	1524.764	4.93	(K)GSLHANGRQKDVDK(E)		2917
G	1848.884	5.36	(K)GSLHANGRQKDVDK(E)	GLYCATION (8), GLYCATION (14)	205834
UG	1734.899	5.45	(K)LVYREYTDASFTNR(K)		2910
G	2058.963	5.60	(K)LVYREYTDASFTNR(K)	GLYCATION (4), GLYCATION (14)	7406
UG	2256.179	5.68	(R)KAEEEHLGILGPQLHADVGDK(V)		9180
G	2292.151	5.54	(R)KAEEEHLGILGPQLHADVGDK(V)	MODIC (21)	2092
UG	2362.096	5.35	(K)VNKDDEEFIESNKMHAINGR(M)	Oxidation M (14)	2289
G	2598.244	5.51	(K)VNKDDEEFIESNKMHAINGR(M)	CROSSLINE (3)	2695
ND (CO3)					
UG	1598.798	5.11	(R)IPIEDGSGEVVLSRK(V)		8378
G	1850.941	5.26	(R)IPIEDGSGEVVLSRK(V)	IMIDAZOLONE-A (14), PYRRALINE (15)	1794
UG	1946.018	4.72	(K)RPQDAKNTMILEICTR(Y)	Carbamidomethyl C (14)	1061
G	2148.043	5.40	(K)RPQDAKNTMILEICTR(Y)	CML (6), Carbamidomethyl C (14), IMIDAZOLONE-A (16)	11807
UG	1946.018	4.72	(K)RPQDAKNTMILEICTR(Y)	Carbamidomethyl C (14)	1061
G	2170.054	5.42	(K)RPQDAKNTMILEICTR(Y)	ARGPYR (1), Carbamidomethyl C (14), IMIDAZOLONE-A (16)	4897
DHAP (CO3)					
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1940.916	4.77	(K)AKDQLTCNKFDLK(V)	GLYCATION (2), Carbamidomethyl C (7), CEL (9), FL-2H2O (13)	1290

UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1687.823	5.87	(K)AKDQLTCNKFDLK(V)	CML (2), Carbamidomethyl C (7), MOLD (9)	28025
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1899.872	5.19	(K)AKDQLTCNKFDLK(V)	GLYCATION (2), Carbamidomethyl C (7), PYRRALINE (9), MOLD (13)	1704
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1687.821	5.52	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7), CML (9), MOLD (13)	24763
DLAP (CO3)					
UG	872.4637	6.10	(K)QLANGVDR(Y)		1097
G	926.4591	5.04	(K)QLANGVDR(Y)	MG-H1 (8)	1221
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1243.557	5.80	(K)AKDQLTCNK(F)	CML (2), Carbamidomethyl C (7), PYRRALINE (9)	3396
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1687.823	5.87	(K)AKDQLTCNKFDLK(V)	CML (2), Carbamidomethyl C (7), MOLD (9)	28025
UG	1597.856	4.19	(R)TLDPERLGREGVQK(E)		6570
G	1691.88	6.39	(R)TLDPERLGREGVQK(E)	MG-H1 (6), G-H1 (9)	1265
UG	1618.847	4.80	(R)TKKQELSEAEQATR(T)		8125
G	1708.861	5.76	(R)TKKQELSEAEQATR(T)	MODIC (3), MG-H1 (14)	46412
UG	1896.022	4.97	(R)NKFVTVQATFGTQVVEK(V)		1534
G	1994.019	5.68	(R)NKFVTVQATFGTQVVEK(V)	MOLD (2), MOLD (17)	18750
UG	1923.996	4.56	(K)DSITTWEILAVSMSDKK(G)		2080
G	2338.145	5.71	(K)DSITTWEILAVSMSDKK(G)	GLYCATION (16), CROSSLINE (17)	1165
UG	3328.606	5.32	(R)SEETKENEGFTVTAEGKGQGTLSV VTMYHAK(A)		3380
G	3380.603	5.35	(R)SEETKENEGFTVTAEGKGQGTLS VVTMYHAK(A)	MODIC (17), Oxidation M (27)	11451
ND (FIBA)					
UG	1628.784	4.09	(K)VTSGSTTTTRRSCSK(T)	Carbamidomethyl C (13)	1208
G	1246.644	4.20	(K)VTSGSTTTTRR(S)	ARGPYR (10)	1787
DHAP (FIBA)					
UG	1441.786	6.26	(K)MKPVPDLVPGNFK(S)		11453
G	1643.813	4.96	(K)MKPVPDLVPGNFK(S)	CML (2), IMIDAZOLONE-A (13)	7953
DLAP (FIBA)					
UG	1628.789	4.64	(K)VTSGSTTTTRRSCSK(T)	Carbamidomethyl C (13)	6241
G	1664.787	5.16	(K)VTSGSTTTTRRSCSK(T)	MODIC (11), Carbamidomethyl C (13)	5701
UG	1423.768	5.24	(K)EKVTSGSTTTTRR(S)		5719
G	1575.805	5.49	(K)EKVTSGSTTTTRR(S)	CEL (2), ARGPYR (13)	1452
ND (FIBB)					
UG	4083.987	5.39	(R)KAPDAGGCLHADPDLGVLCP TGCQLQEALLQQERPIR(N)	Carbamidomethyl C (8), Carbamidomethyl C (19), Carbamidomethyl C (23)	4465

G	4300.072	5.29	(R)KAPDAGGCLHADPDLGVLCP TGCQLQEALLQQERPIR(N)	CEL (1), Carbamidomethyl C (8), Carbamidomethyl C (19), Carbamidomethyl C (23), IMIDAZOLONE-A (34)	1814
DHAP (FIBB)					
UG	1618.793	6.58	(R)TPCTVSCNIPVVSGK(E)	Carbamidomethyl C (3), Carbamidomethyl C (7)	19010
G	1676.78	5.30	(R)TPCTVSCNIPVVSGK(E)	Carbamidomethyl C (3), Carbamidomethyl C (7), CML (15)	4409
DLAP (FIBB)					
UG	1308.62	6.87	(K)QGFGNVATNTDGK(N)		15736
G	1357.611	5.76	(K)QGFGNVATNTDGK(N)	MOLD (13)	23177
UG	4013.831	5.67	(K)YRGTAGNALMDGASQLM GENRTMTIHNGMFFSTYDR(D)		36320
G	3874.706	5.84	(R)GTAGNALMDGASQLM GENRTMTIHNGMFFSTYDR(D)	IMIDAZOLONE-A (19), MODIC (34)	1544
ND (FIBG)					
UG	3167.555	5.26	(K)KNWIQYKEGFGHLSPTGTT EFWLGNEK(I)		2633
G	3297.568	5.46	(K)KNWIQYKEGFGHLSPTGTTE FWLGNEK(I)	CEL (1), CML (7)	19803
DHAP (FIBG)					
UG	1264.661	5.41	(K)SRKMLEEIMK(Y)		71525
G	1588.746	6.08	(K)SRKMLEEIMK(Y)	MODIC (2), IMIDAZOLONE-A (3), IMIDAZOLONE-A (10)	1385
DLAP (FIBG)					
UG	3167.555	5.26	(K)KNWIQYKEGFGHLSPTGT TEFWLGNEK(I)		2633
G	3255.516	5.69	(K)NWIQYKEGFGHLSPTGT TEFWLGNEK(I)	PYRRALINE (6), PYRRALINE (26)	2195
UG	2494.293	4.96	(K)DCQDIANKGAKQSGLYFIKPLK(A)	Carbamidomethyl C (2)	2065
G	1946.038	6.16	(K)GAKQSGLYFIKPLK(A)	PYRRALINE (3), MODIC (11), CROSSLINE (14)	157671
ND (HPT)					
UG	1850.916	7.86	(R)VMPICLPSKDYAEVGR(V)	Oxidation M (2), Carbamidomethyl C (5)	41712
G	1883.915	5.68	(R)VMPICLPSKDYAEVGR(V)	Carbamidomethyl C (5), MOLD (9)	6613
DHAP (HPT)					
UG	1708.896	7.78	(K)LRTEGDGVYTLNDKK(Q)		30268.63
G	1757.914	6.46	(K)LRTEGDGVYTLNDKK(Q)	MOLD (14)	10663.52
UG	1824.997	5.69	(R)ILGGHLDAKGSFPWQAK(M)		2995.729
G	2185.092	6.35	(R)ILGGHLDAKGSFPWQAK(M)	PYRRALINE (9), CROSSLINE (17)	1468.079
UG	2172.072	7.09	(K)SPVGVQPILNEHTFCAGMSK(Y)	Carbamidomethyl C (15)	50255
G	2334.105	6.46	(K)SPVGVQPILNEHTFCAGMSK(Y)	Carbamidomethyl C (15), GLYCATION (20)	8714
DLAP (HPT)					
UG	1439.668	7.79	(R)TEGDGVYTLNNEK(Q)		145927
G	1511.682	6.44	(R)TEGDGVYTLNNEK(Q)	CEL (13)	5917
UG	1439.668	7.79	(R)TEGDGVYTLNNEK(Q)		145927

G	1555.716	6.94	(R)TEGDGVYTLNDKK(Q)	CML (12), CML (13)	6869.339
UG	1708.896	7.78	(K)LRTEGDGVYTLNDKK(Q)		30268.63
G	1616.782	6.15	(K)LRTEGDGVYTLNDK(K)	MODIC (2)	4994.485
UG	2109.065	6.35	(R)TEGDGVYTLNDKKQWINK(A)		2464.996
G	2225.078	6.57	(R)TEGDGVYTLNDKKQWINK(A)	CML (12), CML (13)	1571.018
ND (GELS)					
UG	980.4911	7.30	(R)VGYVSGWGR(N)		37358
G	1608.8	6.53	(R)VGYVSGWGRNANFK(F)	MG-H1 (9)	3120
UG	980.4911	7.30	(R)VGYVSGWGR(N)		37358
G	1612.789	6.32	(R)VGYVSGWGRNANFK(F)	CML (14)	34889
UG	980.4911	7.30	(R)VGYVSGWGR(N)		37358
G	1716.817	6.25	(R)VGYVSGWGRNANFK(F)	GLYCATION (9)	12858
DHAP (GELS)					
UG	1707.832	7.87	(K)YVMLPVADQDQCIR(H)	Carbamidomethyl C (12)	116192
G	1743.825	6.16	(K)YVMLPVADQDQCIR(H)	Carbamidomethyl C (12), MODIC (14)	7913
UG	1708.861	7.56	(K)LRTEGDGVYTLNNEK(Q)		28849
G	1762.871	6.07	(K)LRTEGDGVYTLNNEK(Q)	MG-H1 (2)	3573
UG	1708.866	7.21	(K)LRTEGDGVYTLNNEK(Q)		8325
G	2562.233	6.20	(K)LRTEGDGVYTLNNEKQWINK(A)	G-H1 (2), IMIDAZOLONE-A (20)	9297
DLAP (GELS)					
UG	1857.933	8.23	(K)AVGDKLPECEAVCGKPK(N)	Carbamidomethyl C (9), Carbamidomethyl C (13)	121666
G	2135.987	7.19	(K)AVGDKLPECEAVCGKPK(N)	GLYCATION (5), Carbamidomethyl C (9), Carbamidomethyl C (13), CML (15), CML (17)	105956
UG	1439.705	7.87	(R)TEGDGVYTLNDKK(Q)		39795
G	2225.077	6.26	(R)TEGDGVYTLNDKKQWINK(A)	CML (12), CML (18)	34404
UG	1439.705	7.87	(R)TEGDGVYTLNDKK(Q)		39795
G	2397.176	6.83	(R)TEGDGVYTLNDKKQWINK(A)	MODIC (12), CROSSLINE (18)	35348
UG	2579.314	5.71	(R)TEGDGVYTLNNEKQWINK AVGDK(L)		1047
G	2817.31	6.44	(R)TEGDGVYTLNNEKQWINKA VGDK(L)	CML (13), IMIDAZOLONE-A (18), MODIC (23)	10476
UG	2579.314	5.71	(R)TEGDGVYTLNNEKQWINKA VGDK(L)		1047
G	2835.35	6.87	(R)TEGDGVYTLNNEKQWINKA VGDK(L)	CEL (13), CML (18), FL-2H2O (23)	20684
ND (HEMO)					
UG	1203.635	5.37	(K)WDRELISER(W)		88333
G	1345.649	5.97	(K)WDRELISER(W)	IMIDAZOLONE-B (9)	1192
UG	1689.839	5.93	(K)ERSWPAVGNCSSALR(W)	Carbamidomethyl C (10)	1966
G	1761.847	5.42	(K)ERSWPAVGNCSSALR(W)	MG-DH (2), Carbamidomethyl C (10)	8716
DHAP (HEMO)					
UG	973.5319	5.92	(R)LWWLDLK(S)		1595
G	1045.565	5.83	(R)LWWLDLK(S)	CEL (7)	31562

UG	1689.839	5.93	(K)ERSWPAVGNCSSALR(W)	Carbamidomethyl C (10)	1966
G	1458.685	5.86	(R)SWPAVGNCSSALR(W)	Carbamidomethyl C (8), MG-H1 (13)	14548
UG	2202.075	5.57	(K)ERSWPAVGNCSSALRWLGR(Y)	Carbamidomethyl C (10)	10423
G	2350.116	5.65	(K)ERSWPAVGNCSSALRWLGR(Y)	G-H1 (2), Carbamidomethyl C (10), MG-DH (15), MODIC (19)	1865
DLAP (HEMO)					
UG	1689.839	5.93	(K)ERSWPAVGNCSSALR(W)	Carbamidomethyl C (10)	1966
G	1458.685	5.86	(R)SWPAVGNCSSALR(W)	Carbamidomethyl C (8), MG-H1 (13)	14548
UG	1445.788	5.66	(K)GDKVWVYPPEKK(E)		3975
G	1575.805	6.34	(K)GDKVWVYPPEKK(E)	CEL (11), CML (12)	1452
UG	3463.551	5.51	(R)GECQAEGVLFFQGDREWF WDLATGTMKER(S)	Carbamidomethyl C (3)	11661
G	3376.525	5.65	(R)GECQAEGVLFFQGDREW FWDLATGTMK(E)	Carbamidomethyl C (3), MODIC (15), GLYCATION (27)	1182
ND (KNG1)					
UG	1706.868	4.70	(K)DAAKAATGECTATVGKR(S)	Carbamidomethyl C (10)	1482
G	1657.765	5.38	(K)DAAKAATGECTATVGK(R)	CML (4), Carbamidomethyl C (10), MOLD (16)	9801
DHAP (KNG1)					
UG	1390.737	4.98	(K)RPPGFSPFRSSR(I)		1036
G	1722.873	5.28	(K)RPPGFSPFRSSR(I)	ARGPYR (1), CROSSLINE (9)	1980
UG	1706.868	4.70	(K)DAAKAATGECTATVGKR(S)	Carbamidomethyl C (10)	1482
G	1786.891	5.07	(K)DAAKAATGECTATVGKR(S)	Carbamidomethyl C (10), ARGPYR (17)	10886
UG	2237.064	5.03	(K)HNLGHGHKHERDQGHGHQR(G)		2988
G	2505.138	5.23	(K)HNLGHGHKHERDQGHGHQR(G)	FL-2H2O (8), IMIDAZOLONE-B (19)	1066
DLAP (KNG1)					
UG	2139.089	5.66	(R)DIPTNSPELEETLTHTITK(L)		3115
G	2175.062	4.36	(R)DIPTNSPELEETLTHTITK(L)	MODIC (19)	1498
UG	2216.105	5.32	(K)CPGRPWKSVSEINPTTQMK(E)	Carbamidomethyl C (1)	3725
G	2270.127	4.20	(K)CPGRPWKSVSEINPTTQMK(E)	Carbamidomethyl C (1), MG-H1 (4)	2888
UG	2237.064	5.03	(K)HNLGHGHKHERDQGHGHQR(G)		2988
G	2505.143	5.15	(K)HNLGHGHKHERDQGHGHQR(G)	FL-2H2O (8), IMIDAZOLONE-B (19)	19097
UG	3139.426	4.94	(R)IGEIKEETTVSPPHTSMAPAQDEERDSGK(E)		1310
G	3283.55	5.18	(R)IGEIKEETTVSPPHTSMAPAQDEERDSGK(E)	CEL (5), CEL (29)	3851
ND (TRFE)					
UG	2573.315	5.69	(K)HQTVPQNTGGKNPDPWAKNLNEK(D)		3815
G	2784.354	6.06	(K)HQTVPQNTGGKNPDPWAKNLNEK(D)	FL-2H2O (11), MOLD (18), MODIC (23)	21399
UG	2698.246	6.15	(K)WCALSHHERLKCDEWSVNSVGK(I)	Carbamidomethyl C (2), Carbamidomethyl C (12)	1563
G	2990.367	7.28	(K)WCALSHHERLKCDEWSVNSVGK(I)	Carbamidomethyl C (2), G-H1 (9), Carbamidomethyl C (12), CROSSLINE (22)	11239

DHAP (TRFE)					
UG	2574.349	6.55	(K)KSCHTAVGRTAGWNIPMGLLYNK(I)	Carbamidomethyl C (3)	3343
G	1721.85	6.29	(R)TAGWNIPMGLLYNK(I)	IMIDAZOLONE-A (14)	2205
UG	1543.826	7.01	(K)SVIPSDGPSVACVKK(A)	Carbamidomethyl C (12)	6295
G	1849.891	5.73	(K)SVIPSDGPSVACVKK(A)	Carbamidomethyl C (12), GLYCATION (14), IMIDAZOLONE-A (15)	4497
UG	1881.888	7.63	(K)ADRDQYELLCLDNTR(K)	Carbamidomethyl C (10)	64708
G	2314.024	6.58	(K)ADRDQYELLCLDNTR(K)	GLYCATION (3), Carbamidomethyl C (10), AFGP (15)	1605
DLAP (TRFE)					
UG	1615.802	6.49	(K)HSTIFENLANKADR(D)		4131
G	1723.824	7.41	(K)HSTIFENLANKADR(D)	PYRRALINE (11)	1113
UG	1562.821	6.04	(K)KSASDLTWDNLKGK(K)		1827
G	1782.867	6.36	(K)KSASDLTWDNLKGK(K)	GLYCATION (12), CML (14)	38947
UG	3025.519	5.89	(R)KPVDEYKDCHLAQVPSHTV VARSMGGK(E)	Carbamidomethyl C (9), Oxidation M (24)	2409
G	2402.154	6.46	(K)DCHLAQVPSHTVVARSMGGK(E)	Carbamidomethyl C (2), CROSSLINE (15)	1942
ND (VTDB)					
UG	1478.663	5.68	(K)DVCDPGNTKVMDK(Y)	Carbamidomethyl C (3)	1775
G	1586.694	6.18	(K)DVCDPGNTKVMDK(Y)	Carbamidomethyl C (3), PYRRALINE (9)	1815
DHAP (VTDB)					
UG	1183.583	5.21	(R)DYEKNKVCK(E)	Carbamidomethyl C (8)	1199
G	1255.601	6.13	(R)DYEKNKVCK(E)	CEL (4), Carbamidomethyl C (8)	3243
UG	1646.816	5.26	(R)VCSQYAAYGEKKSR(L)	Carbamidomethyl C (2)	1458
G	1461.663	6.34	(R)VCSQYAAYGEKK(S)	Carbamidomethyl C (2), CML (11)	75173
UG	1646.816	5.26	(R)VCSQYAAYGEKKSR(L)	Carbamidomethyl C (2)	1458
G	1682.779	5.64	(R)VCSQYAAYGEKKSR(L)	Carbamidomethyl C (2), MODIC (14)	6605
UG	1646.816	5.26	(R)VCSQYAAYGEKKSR(L)	Carbamidomethyl C (2)	1458
G	1781.787	6.14	(R)VCSQYAAYGEKK(S)	Carbamidomethyl C (2), FL-2H2O (11), CROSSLINE (12)	7615
UG	2213.11	5.49	(K)ELPEHTVKLCDNLSTKNSK(F)	Carbamidomethyl C (10)	1350
G	2334.105	5.51	(K)ELPEHTVKLCDNLSTKNSK(F)	MOLD (8), Carbamidomethyl C (10), MODIC (16), MODIC (19)	8714
UG	2213.11	5.49	(K)ELPEHTVKLCDNLSTKNSK(F)	Carbamidomethyl C (10)	1350
G	2663.274	6.58	(K)ELPEHTVKLCDNLSTKNSK(F)	CEL (8), Carbamidomethyl C (10), FL-2H2O (16), CROSSLINE (19)	12278
UG	2974.372	5.77	(K)ELSSFIDKGQELCADYSENTFTEYK(K)	Carbamidomethyl C (13)	6919
G	3244.398	6.04	(K)ELSSFIDKGQELCADYSENTFTEYK(K)	FL-2H2O (8), Carbamidomethyl C (13), IMIDAZOLONE-A (25)	10444
UG	2974.372	5.77	(K)ELSSFIDKGQELCADYSENTFTEYK(K)	Carbamidomethyl C (13)	6919
G	3349.511	6.03	(K)ELSSFIDKGQELCADYSENTFTEYKK(K)	CEL (8), Carbamidomethyl C (13),	425818

				FL-2H2O (25), MOLD (26)	
DLAP (VTDB)					
UG	1279.618	5.60	(K)LCDNLSTKNSK(F)	Carbamidomethyl C (2)	1348
G	1351.643	5.67	(K)LCDNLSTKNSK(F)	Carbamidomethyl C (2), CEL (11)	25531
UG	1646.816	5.26	(R)VCSQYAAYGEKKSR(L)	Carbamidomethyl C (2)	1458
G	1461.663	6.34	(R)VCSQYAAYGEKK(S)	Carbamidomethyl C (2), CML (11)	75173
UG	1694.918	6.42	(R)KFPSGTFEQVSQLVK(E)		16496
G	1856.981	5.91	(R)KFPSGTFEQVSQLVK(E)	GLYCATION (1)	2378
ND (VTNC)					
UG	2029.013	5.02	(K)LIRDVWGIEGPIDAAFTR(I)		1025
G	2171.113	4.96	(K)LIRDVWGIEGPIDAAFTR(I)	IMIDAZOLONE-B (18)	137993
DHAP (VTNC)					
UG	1268.619	5.02	(R)GHSRGRNQNSR(R)		1432
G	1448.651	5.10	(R)GHSRGRNQNSR(R)	IMIDAZOLONE-A (4), MODIC (6)	55415
UG	1268.619	5.02	(R)GHSRGRNQNSR(R)		1432
G	1556.729	5.20	(R)GHSRGRNQNSR(R)	MODIC (4), CROSSLINE (6)	25879
DLAP (VTNC)					
UG	2029.013	5.02	(K)LIRDVWGIEGPIDAAFTR(I)		1025
G	2171.113	4.96	(K)LIRDVWGIEGPIDAAFTR(I)	IMIDAZOLONE-B (18)	137993
UG	3072.416	5.19	(R)MDWLVPATCEPIQSVFFFSGDKYYR(V)	Oxidation M (1), Carbamidomethyl C (9)	7941
G	3344.524	3.88	(R)MDWLVPATCEPIQSVFFFSGDKYYR(V)	Carbamidomethyl C (9), IMIDAZOLONE-A (22), IMIDAZOLONE-A (25)	2073
UG	3057.453	5.16	(R)NISDGFDGIPDNVDAALALPAHSYSGRER(V)		1441
G	3363.517	5.09	(R)NISDGFDGIPDNVDAALALPAHSYSGRER(V)	AFGP (27), MODIC (29)	1053

Table A. 3. Identification of protein glycation modifications from *in vitro* plasma. ND- Non-Diabetic; DHAP- Diabetic High Albumin Plasma; DLAP- Diabetic Low Albumin Plasma; UG- Un-Glycated peptide; G- Glycated peptide.

Protein	Precursor MH+ (Da)	PLGS Score	Sequence	Modifications	Intensity
HAP (ALB)					
UG	1840.945	7.37	(K)EQLKAVMDDFAAFVEK(C)		16425
G	1938.943	6.79	(K)EQLKAVMDDFAAFVEK(C)	MOLD (4), MOLD (16)	49294
UG	1443.639	8.81	(K)YICENQDSISSK(L)	Carbamidomethyl C (3)	886321
G	1515.65	6.70	(K)YICENQDSISSK(L)	Carbamidomethyl C (3), CEL (12)	1975
UG	2599.325	8.01	(K)QNCELFEQLGEYKFQNALLVR(Y)	Carbamidomethyl C (3)	100298
G	2707.316	7.00	(K)QNCELFEQLGEYKFQNALLVR(Y)	Carbamidomethyl C (3), PYRRALINE (13)	155979
UG	1840.945	7.37	(K)EQLKAVMDDFAAFVEK(C)		16425
G	1966.937	6.60	(K)EQLKAVMDDFAAFVEK(C)	FL-2H2O (16)	9112
UG	1736.911	6.21	(R)LSQRFPKAEFAEVSK(L)		3380
G	1875.94	6.38	(R)LSQRFPKAEFAEVSK(L)	MODIC (4), MGH1 (7), MOLD (15)	1695877
UG	2636.238	8.20	(K)QEPERNECFLQHKDDNPNLPR(L)	Carbamidomethyl C (8)	1109805
G	2784.254	6.71	(K)QEPERNECFLQHKDDNPNLPR(L)	GH1 (5), Carbamidomethyl C (8), PYRL (13)	11455
UG	2052.207	6.68	(K)KVPQVSTPTLVEVSRNLGK(V)		2555
G	2626.318	6.70	(K)KVPQVSTPTLVEVSRNLGK(V)	AFGP (1), IMIDAZOLONE (15), Glycation KR (19)	768721
UG	2650.264	7.87	(R)LVRPEVDVMCTAFHDNEETFLK(K)	Carbamidomethyl C (10)	103492
G	3172.524	6.70	(R)LVRPEVDVMCTAFHDNEETFLKK(Y)	Carbamidomethyl C (10), IMIDAZOLONE (22), CROSSLINE (23)	7494
UG	1650.894	8.83	(K)AEFAEVSKLVTDLTK(V)		750954
G	1708.943	7.04	(K)AEFAEVSKLVTDLTK(V)	CML (8)	11363
UG	1840.917	8.18	(K)EQLKAVMDDFAAFVEK(C)		137152
G	2755.229	7.15	(K)EQLKAVMDDFAAFVEKCCK(A)	IMIDAZOLONE (4), CEL (16), Carbamidomethyl C (17), Carbamidomethyl C (18), CROSSLINE (19)	41467
LAP					
UG	1443.639	8.64	(K)YICENQDSISSK(L)	Carbamidomethyl C (3)	2240528
G	1479.657	6.91	(K)YICENQDSISSK(L)	Carbamidomethyl C (3), MODIC (12)	14280
UG	1499.64	7.75	(K)ADDKETCFAEEGK(K)	Carbamidomethyl C (7)	88066
G	1751.677	7.06	(K)ADDKETCFAEEGK(K)	PYRRALINE (4), Carbamidomethyl C (7), IMIDAZOLONE-A (13)	14559
UG	1941.946	6.87	(R)ADLAKYICENQDSISSK(L)	Carbamidomethyl C (8)	5349
G	2471.143	6.34	(R)ADLAKYICENQDSISSKLK(E)	IMIDAZOLONE-A (5), Carbamidomethyl C (8), IMIDAZOLONE-A (17)	6581

UG	2300.108	6.51	(K)NYAEAKDVFLGMFLYEYAR(R)		13251
G	2586.199	6.37	(K)NYAEAKDVFLGMFLYEYARR(H)	CML (6), MODIC (19), MODIC (20)	5869
UG	2636.238	8.22	(K)QEPERNECFLQHKDDNPNLPR(L)	Carbamidomethyl C (8)	1109549
G	2756.244	6.53	(K)QEPERNECFLQHKDDNPNLPR(L)	ARGPYR (5), Carbamidomethyl C (8), G-H1 (21)	168016
UG	3355.607	7.25	(K)LVTDLTKVHTECCHGDLLECAD DRADLAK(Y)	Carbamidomethyl C (12), Carbamidomethyl C (13), Carbamidomethyl C (20)	101272
G	3427.592	6.67	(K)LVTDLTKVHTECCHGDLLE CADDRADLAK(Y)	Carbamidomethyl C (12), Carbamidomethyl C (13), Carbamidomethyl C (20), MG-DH (24)	92597
UG	1932.048	8.57	(K)SLHTLFGDKLCTVATLR(E)	Carbamidomethyl C (11)	776429
G	1990.063	7.41	(K)SLHTLFGDKLCTVATLR(E)	CML (9), Carbamidomethyl C (11)	39723
UG	2636.238	8.20	(K)QEPERNECFLQHKDDNPNLPR(L)	Carbamidomethyl C (8)	1109805
G	2784.254	6.71	(K)QEPERNECFLQHKDDNPNLPR(L)	GH1 (5), Carbamidomethyl C (8), PYRL (13)	11455
UG	2674.332	8.49	(K)RMPCAEDYLSVVLNQLCVLHEK(T)	Carbamidomethyl C (4), Carbamidomethyl C (17)	629442
G	2746.359	7.36	(K)RMPCAEDYLSVVLNQLCVLHEK(T)	CEL (1), Carbamidomethyl C (4), Carbamidomethyl C (17)	36196
UG	2674.332	8.49	(K)RMPCAEDYLSVVLNQLCVLHEK(T)	Carbamidomethyl C (4), Carbamidomethyl C (17)	629442
G	2777.354	6.67	(K)RMPCAEDYLSVVLNQLCVLHEK(T)	MOLD (1), Carbamidomethyl C (4), Carbamidomethyl C (17), MGH1 (22)	14235
UG	2545.2	6.87	(K)EFNAETFTFHADICTLSEKER(Q)	Carbamidomethyl C (14)	67984
G	2585.169	7.23	(K)EFNAETFTFHADICTLSEKER(Q)	Carbamidomethyl C (14), GH1 (21)	89289
UG	4152.94	6.65	(K)SHCIAEVENDEMPADLPSLAADFV ESKDVCKNYAEAK(D)	Carbamidomethyl C (3), Carbamidomethyl C (30)	13282
G	4318.914	6.73	(K)SHCIAEVENDEMPADLPSLAAD FVESKDVCKNYAEAK(D)	Carbamidomethyl C (3), CML (27), Carbamidomethyl C (30), CEL (31), MODIC (37)	1401
UG	2316.122	7.16	(K)NYAEAKDVFLGMFLYEYAR(R)	Oxidation M (12)	78446
G	2840.275	6.77	(K)NYAEAKDVFLGMFLYEYAR(R)	AFGP (6), AFGP (19)	21717
UG	2052.207	6.68	(K)KVPQVSTPTLVEVSRNLGK(V)		2555
G	2626.318	6.70	(K)KVPQVSTPTLVEVSRNLGK(V)	AFGP (1), IMIDAZOLONE (15), Glycation KR (19)	768721
UG	1840.917	8.18	(K)EQLKAVMDDFAAFVEK(C)		137152
G	1898.963	6.73	(K)EQLKAVMDDFAAFVEK(C)	CML (4)	1266993
UG	2650.264	7.87	(R)LVRPEVDVMCTAFHDNEETFLK(K)	Carbamidomethyl C (10)	103492
G	2776.314	6.44	(R)LVRPEVDVMCTAFHDNEETFLK(K)	Carbamidomethyl C (10), FL-2h2o (22)	1362
UG	2650.264	7.87	(R)LVRPEVDVMCTAFHDNEETFLK(K)	Carbamidomethyl C (10)	103492
G	3172.524	6.70	(R)LVRPEVDVMCTAFHDNEETFLKK(Y)	Carbamidomethyl C (10), IMIDAZOLONE (22), CROSSLINE (23)	7494
UG	1650.894	8.83	(K)AEFAEVSKLVTDLTK(V)		750954
G	1708.943	7.04	(K)AEFAEVSKLVTDLTK(V)	CML (8)	11363
UG	4009.775	6.88	(K)VHTECCHGDLLECADDRADLA KYICENQDSISSK(L)	Carbamidomethyl C (5), Carbamidomethyl C (6), Carbamidomethyl C (13), Carbamidomethyl C (25)	10580

G	4103.763	6.77	(K)VHTECCHGDLLECADDRADL AKYICENQDSISSK(L)	Carbamidomethyl C (5), Carbamidomethyl C (6), Carbamidomethyl C (13), MGH1 (22), Carbamidomethyl C (25), GH1 (34)	17272
UG	1941.946	5.00	(R)ADLAKYICENQDSISSK(L)	Carbamidomethyl C (8)	5349
G	2561.245	6.94	(R)ADLAKYICENQDSISSKLK(E)	Carbamidomethyl C (8), FL-2h2o (17), CROSSLINE (19)	465754
UG	1941.946	5.00	(R)ADLAKYICENQDSISSK(L)	Carbamidomethyl C (8)	5349
G	2741.202	6.80	(R)ADLAKYICENQDSISSKLK(E)	AFGP (5), Carbamidomethyl C (8), Glycation (17), FL-2H20 (19)	96429
UG	2316.122	7.16	(K)NYAEAKDVFLGMFLYEYAR(R)	Oxidation M (12)	78446
G	2690.256	6.68	(K)NYAEAKDVFLGMFLYEYARR(H)	Glycation KR (6), CEL (19)	266273
UG	1840.917	8.18	(K)EQLKAVMDDFAAFVEK(C)		137152
G	2539.141	6.86	(K)EQLKAVMDDFAAFVEKCCK(A)	IMIDAZOLONE (4), CEL (16), Carbamidomethyl C (17), Carbamidomethyl C (18), MODIC (19)	12600
UG	1840.917	8.18	(K)EQLKAVMDDFAAFVEK(C)		137152
G	2755.229	7.15	(K)EQLKAVMDDFAAFVEKCCK(A)	IMIDAZOLONE (4), CEL (16), Carbamidomethyl C (17), Carbamidomethyl C (18), CROSSLINE (19)	41467
HAP (A1AG2)					
UG	2081.055	5.70	(K)EQLGEFYEALDCLRIPK(S)	Carbamidomethyl C (12)	81791
G	2225.044	5.35	(K)EQLGEFYEALDCLRIPK(S)	Carbamidomethyl C (12), IMIDAZOLONE-A (17)	2497
LAP (A1AG2)					
UG	1540.732	5.45	(K)DKCEPLEKQHEK(E)	Carbamidomethyl C (3)	238345
G	1706.773	6.87	(K)DKCEPLEKQHEK(E)	Carbamidomethyl C (3), CML (8), PYRRALINE (12)	443090
UG	1708.862	5.75	(K)NWGLSVYADKPETTK(E)		27693
G	1793.876	5.84	(K)NWGLSVYADKPETTK(E)	MOLD (10), MODIC (15)	37169
UG	1708.862	5.75	(K)NWGLSVYADKPETTK(E)		27693
G	1910.893	6.31	(K)NWGLSVYADKPETTK(E)	CML (10), IMIDAZOLONE-A (15)	31757
UG	1708.862	5.75	(K)NWGLSVYADKPETTK(E)		27693
G	2068.985	6.52	(K)NWGLSVYADKPETTK(E)	PYRRALINE (10), CROSSLINE (15)	3905
HAP (A1AT)					
UG	779.4142	6.46	(K)SPLFMGK(V)		1137
G	1671.8771	6.70	(K)SPLFMGKVVNPTQK(-)	FL-2H2O (14)	48719
UG	779.4142	6.46	(K)SPLFMGK(V)		1137
G	1797.9094	6.31	(K)SPLFMGK(V)	PYRRALINE (7), IMIDAZOLONE-A (14)	269701
UG	1944.0507	5.64	(R)SASLHLPKLSITGTYDLK(S)		1012
G	2016.0858	6.42	(R)SASLHLPKLSITGTYDLK(S)	MODIC (8), MODIC (18)	74808
UG	2162.1533	7.59	(K)VFSNGADLSGVTEEAPLKLSK(A)		172983

G	2472.264	6.63	(K)VFSNGADLSGVTEEAPLKLSK(A)	CML (18), CROSSLINE (21)	23521
UG	2314.1099	6.30	(K)KLYHSEAFTVNFGDTEEAKK(Q)		1424
G	2926.3618	6.44	(K)KLYHSEAFTVNFGDTEEAKK(Q)	PYRRALINE (1), CROSSLINE (19), CROSSLINE (20)	37658
LAP (A1AT)					
UG	2162.1533	7.59	(K)VFSNGADLSGVTEEAPLKLSK(A)		172983
G	2364.192	5.94	(K)VFSNGADLSGVTEEAPLKLSK(A)	CML (18), IMIDAZOLONE-A (21)	43687
UG	1479.7235	5.69	(K)QINDYVEKGTQGK(I)		1018
G	1677.795	6.59	(K)QINDYVEKGTQGK(I)	CEL (8), FL-2H2O (13)	3550
UG	779.4103	7.37	(K)SPLFMGK(V)		16347
G	1797.908	6.60	(K)SPLFMGKVVNPTQK(-)	PYRRALINE (7), IMIDAZOLONE-A (14)	417915
UG	1136.5894	7.63	(K)KQINDYVEK(G)		27181
G	1805.8688	7.05	(K)KQINDYVEKGTQGK(I)	FL-2H2O (1), MODIC (9), MODIC (14)	5874
UG	1136.5894	7.63	(K)KQINDYVEK(G)		27181
G	1863.8846	6.25	(K)KQINDYVEKGTQGK(I)	GLYCATION (1), CML (9), MODIC (14)	5359
UG	1136.5894	7.63	(K)KQINDYVEK(G)		27181
G	1967.9485	6.77	(K)KQINDYVEKGTQGK(I)	PYRRALINE (9), CROSSLINE (14)	6745
UG	1944.0507	5.64	(R)SASLHLPKLSITGTYDLK(S)		1012
G	2016.0826	6.73	(R)SASLHLPKLSITGTYDLK(S)	MODIC (8), MODIC (18)	37709
HAP (ANT3)					
UG	1225.614	3.87	(M)YSNVIGTVTSGK(R)		41.8385
G	1333.65	5.08	(M)YSNVIGTVTSGK(R)	PYRRALINE (12)	25.3127
UG	1978.962	4.97	(R)DIPMNPMCIYRSPEKK(A)	Carbamidomethyl C (8)	50.2722
G	2156.97	5.02	(R)DIPMNPMCIYRSPEKK(A)	Carbamidomethyl C (8), IMIDAZOLONE-B (11), MODIC (16)	43.8472
UG	1874.893	4.61	(K)ATEDEGSEQKIPEATNR(R)		38.6799
G	2205.005	4.53	(K)KATEDEGSEQKIPEATNR(R)	CML (11), IMIDAZOLONE-A (18)	51.0746
LAP (ANT3)					
UG	1076.533	4.63	(K)WVSNKTEGR(I)		43.0978
G	1116.534	5.17	(K)WVSNKTEGR(I)	G-H1 (9)	35.9842
UG	1065.522	4.98	(K)SKFSPENTR(K)		24.6644
G	1273.653	5.18	(K)SKFSPENTRK(E)	ARGPYR (9)	35.9419
UG	1065.522	4.98	(K)SKFSPENTR(K)		24.6644
G	1301.638	4.84	(K)SKFSPENTRK(E)	PYRRALINE (10)	45.8568
UG	1225.604	5.11	(M)YSNVIGTVTSGK(R)		42.0386
G	1333.667	4.52	(M)YSNVIGTVTSGK(R)	PYRRALINE (12)	57.4691
UG	1956.956	4.45	(K)ANSRFATTFYQHLADSK(N)		25.2107
G	1992.951	4.79	(K)ANSRFATTFYQHLADSK(N)	MODIC (17)	48.4113
UG	1848.921	5.31	(K)EQLQDMGLVDLFSPEK(S)		64.8591
G	2010.961	5.34	(K)EQLQDMGLVDLFSPEK(S)	GLYCATION (16)	48.5053
UG	1330.656	4.60	(K)ENAEQSRAAINK(W)		36.72

G	1994.013	5.13	(K)ENAEQSRAAINKWVSNK(T)	MOLD (17)	34.3078
UG	1874.893	4.61	(K)ATEDEGSEQKIPEATNR(R)		38.6799
G	2355.071	4.55	(K)ATEDEGSEQKIPEATNRR(V)	GLYCATION (17), GLYCATION (18)	55.3414
HAP (APOA4)					
UG	2193.091	5.40	(R)ENADSLQASLRPHADELKAK(I)		1365
G	2337.162	5.44	(R)ENADSLQASLRPHADELKAK(I)	IMIDAZOLONE-A (20)	514890
UG	2362.257	5.19	(R)VLRENADSLQASLRPHADELK(A)		3556
G	2758.368	5.69	(R)VLRENADSLQASLRPHADELK(A)	IMIDAZOLONE-A (3), CROSSLINE (14)	1333
LAP (APOA4)					
UG	1350.656	6.85	(R)SLAPYAQDTQEK(L)		17349
G	1408.654	5.94	(R)SLAPYAQDTQEK(L)	CML (12)	2597
UG	1287.665	6.33	(R)TQVNTQAEQLR(R)		5238
G	1429.703	5.73	(R)TQVNTQAEQLR(R)	IMIDAZOLONE-B (11)	2670
UG	1319.665	6.70	(R)DKVNSFFSTFK(E)		14278
G	1539.718	6.40	(R)DKVNSFFSTFK(E)	GLYCATION (2), CML (11)	40769
UG	1535.756	5.20	(K)LGEVNTYAGDLQKK(L)		3940
G	1593.818	6.22	(K)LGEVNTYAGDLQKK(L)	CML (13)	10211
UG	1319.665	6.70	(R)DKVNSFFSTFK(E)		14278
G	1612.789	5.96	(R)DKVNSFFSTFKEK(E)	MODIC (2)	34889
UG	1535.756	5.20	(K)LGEVNTYAGDLQKK(L)		3940
G	1629.822	6.80	(K)LGEVNTYAGDLQKK(L)	CML (13), MODIC (14)	137153
UG	1535.756	5.20	(K)LGEVNTYAGDLQKK(L)		3940
G	1661.849	6.01	(K)LGEVNTYAGDLQKK(L)	FL-2H2O (13)	3843
UG	1319.665	6.70	(R)DKVNSFFSTFK(E)		14278
G	1778.843	6.45	(R)DKVNSFFSTFKEK(E)	CML (2), IMIDAZOLONE-A (13)	4424
UG	1548.827	5.68	(R)RQLTPYAQRMER(V)		3583
G	1858.876	5.66	(R)RQLTPYAQRMER(V)	G-H1 (1), AFGP (9)	1185
UG	2193.145	5.43	(R)ENADSLQASLRPHADELKAK(I)		1299
G	2337.159	6.20	(R)ENADSLQASLRPHADELKAK(I)	MODIC (11), PYRRALINE (20)	85807
UG	2401.247	5.44	(R)TQVNTQAEQLRRQLTPYAQR(M)		1063
G	2599.325	6.18	(R)TQVNTQAEQLRRQLTPYAQR(M)	GLYCATION (11), MODIC (12)	1165
HAP (CLUS)					
UG	1075.586	4.79	(K)KEDALNETR(E)		2524
G	1111.542	5.72	(K)KEDALNETR(E)	MODIC (1)	4039
UG	1421.748	5.26	(R)KNPKFMETVAEK(A)		14162
G	1457.74	5.28	(R)KNPKFMETVAEK(A)	MODIC (12)	29956
UG	1245.703	5.28	(K)TLLSNLEEAKK(K)		1401
G	1530.838	5.36	(K)TLLSNLEEAKKK(K)	PYRRALINE (10), MOLD (11)	10470
UG	1342.671	4.52	(R)EIRHNSTGCLR(M)	Carbamidomethyl C (9)	1150
G	1743.825	5.32	(R)EIRHNSTGCLRMK(D)	Carbamidomethyl C (9), IMIDAZOLONE-B (11)	7913

UG	1599.84	4.93	(R)KYNELLKSYQWK(M)		2710
G	1797.9	5.91	(R)KYNELLKSYQWK(M)	GLYCATION (1), MODIC (12)	280642
UG	1762.874	4.65	(K)EDALNETRESETKLK(E)		6684
G	1834.916	5.55	(K)EDALNETRESETKLK(E)	CEL (13)	98351
UG	1575.863	5.00	(K)YVNKEIQNAVNGVK(Q)		53132
G	2043.095	5.36	(K)YVNKEIQNAVNGVKQIK(T)	MOLD (4), MOLD (14)	2201
LAP (CLUS)					
UG	947.472	4.86	(K)EDALNETR(E)		2915
G	1109.49	5.50	(K)EDALNETR(E)	GLYCATION (8)	1708
UG	1075.586	4.79	(K)KEDALNETR(E)		2524
G	1111.54	4.88	(K)KEDALNETR(E)	MODIC (9)	6197
UG	1342.671	4.52	(R)EIRHNSTGCLR(M)	Carbamidomethyl C (9)	1150
G	1677.78	4.92	(R)EIRHNSTGCLRMK(D)	G-H1 (3), Carbamidomethyl C (9), MODIC (11)	2464
UG	1342.671	4.52	(R)EIRHNSTGCLR(M)	Carbamidomethyl C (9)	1150
G	1743.83	5.34	(R)EIRHNSTGCLRMK(D)	Carbamidomethyl C (9), IMIDAZOLONE-B (11)	31470
UG	1599.84	4.93	(R)KYNELLKSYQWK(M)		2710
G	1743.87	6.06	(R)KYNELLKSYQWK(M)	PYRRALINE (1), MODIC (7)	155082
UG	1599.84	4.93	(R)KYNELLKSYQWK(M)		2710
G	1797.90	5.56	(R)KYNELLKSYQWK(M)	GLYCATION (1), MODIC (7)	409078
UG	1599.84	4.93	(R)KYNELLKSYQWK(M)		2710
G	1833.89	4.97	(R)KYNELLKSYQWK(M)	PYRRALINE (7), FL-2H2O (12)	12087
UG	2551.184	4.67	(R)FFTREPQDTYHYLPFSLPHR(R)		5231
G	3999.85	4.87	(R)FFTREPQDTYHYLPFSLPHRRPHFFFPK(S)	IMIDAZOLONE-B (20), IMIDAZOLONE-B (21), PYRRALINE (28)	151551
HAP (CO3)					
UG	1598.798	5.11	(R)IPIEDGSGEVVLSRK(V)		8378
G	1850.941	5.26	(R)IPIEDGSGEVVLSRK(V)	IMIDAZOLONE-A (14), PYRRALINE (15)	1794
UG	1946.018	4.72	(K)RPQDAKNTMILEICTR(Y)	Carbamidomethyl C (14)	1061
G	2148.043	5.40	(K)RPQDAKNTMILEICTR(Y)	CML (6), Carbamidomethyl C (14), IMIDAZOLONE-A (16)	11807
LAP (CO3)					
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1687.823	5.87	(K)AKDQLTCNKFDLK(V)	CML (2), Carbamidomethyl C (7), MOLD (9)	28025
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1899.872	5.19	(K)AKDQLTCNKFDLK(V)	GLYCATION (2), Carbamidomethyl C (7), PYRRALINE (9), MOLD (13)	1704
UG	1580.799	5.14	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	35597
G	1940.916	4.77	(K)AKDQLTCNKFDLK(V)	GLYCATION (2), Carbamidomethyl C (7), CEL (9), FL-2H2O (13)	1290

UG	1580.796	4.23	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	3314
G	1778.844	4.77	(K)AKDQLTCNKFDLK(V)	CEL (2), Carbamidomethyl C (7), FL-2H2O (13)	4549
UG	1580.795	3.97	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7)	4295
G	1940.952	4.73	(K)AKDQLTCNKFDLK(V)	Carbamidomethyl C (7), PYRRALINE (9), CROSSLINE (13)	10115
HAP (FIBB)					
UG	4083.987	5.39	(R)KAPDAGGCLHADPDLGVLCPT GCQLQEALLQQERPIR(N)	Carbamidomethyl C (8), Carbamidomethyl C (19), Carbamidomethyl C (23)	4465
G	4300.072	5.29	(R)KAPDAGGCLHADPDLGVLCP TGCQLQEALLQQERPIR(N)	CEL (1), Carbamidomethyl C (8), Carbamidomethyl C (19), Carbamidomethyl C (23), IMIDAZOLONE-A (34)	1814
LAP (FIBB)					
UG	1618.793	6.58	(R)TPCTVSCNIPVVSGK(E)	Carbamidomethyl C (3), Carbamidomethyl C (7)	19010
G	1676.78	5.30	(R)TPCTVSCNIPVVSGK(E)	Carbamidomethyl C (3), Carbamidomethyl C (7), CML (15)	4409
UG	1308.62	6.87	(K)QGFGNVATNTDGK(N)		15736
G	1357.611	5.76	(K)QGFGNVATNTDGK(N)	MOLD (13)	23177
UG	4013.831	5.67	(K)YRGTAGNALMDGASQL MGENRTMTIHNGMFFSTYDR(D)		36320
G	3874.706	5.84	(R)GTAGNALMDGASQLMGEN RTMTIHNGMFFSTYDR(D)	IMIDAZOLONE-A (19), MODIC (34)	1544
HAP (FIBA)					
UG	1423.768	5.24	(K)EKVTSGSTTTTRR(S)		5719
G	1575.805	5.49	(K)EKVTSGSTTTTRR(S)	CEL (2), ARGPYR (13)	1452
LAP (FIBA)					
UG	1628.784	4.09	(K)VTSGSTTTTRRSCSK(T)	Carbamidomethyl C (13)	1208
G	1246.644	4.20	(K)VTSGSTTTTRR(S)	ARGPYR (10)	1787
UG	1441.786	6.26	(K)MKPVPDLVPGNFK(S)		11453
G	1643.813	4.96	(K)MKPVPDLVPGNFK(S)	CML (2), IMIDAZOLONE-A (13)	7953
UG	1628.789	4.64	(K)VTSGSTTTTRRSCSK(T)	Carbamidomethyl C (13)	6241
G	1664.787	5.16	(K)VTSGSTTTTRRSCSK(T)	MODIC (11), Carbamidomethyl C (13)	5701
UG	1423.768	5.24	(K)EKVTSGSTTTTRR(S)		5719
G	1575.805	5.49	(K)EKVTSGSTTTTRR(S)	CEL (2), ARGPYR (13)	1452
HAP (HPT)					
UG	980.4911	7.30	(R)VGYVSGWGR(N)		37358
G	1608.8	6.53	(R)VGYVSGWGRNANFK(F)	MG-H1 (9)	3120
UG	980.4911	7.30	(R)VGYVSGWGR(N)		37358
G	1612.789	6.32	(R)VGYVSGWGRNANFK(F)	CML (14)	34889
UG	980.4911	7.30	(R)VGYVSGWGR(N)		37358
G	1716.817	6.25	(R)VGYVSGWGRNANFK(F)	GLYCATION (9)	12858
UG	1707.832	7.87	(K)YVMLPVADQDQCIR(H)	Carbamidomethyl C (12)	116192
G	1743.825	6.16	(K)YVMLPVADQDQCIR(H)	Carbamidomethyl C (12), MODIC (14)	7913

UG	1708.861	7.56	(K)LRTEGDGVYTLNNEK(Q)		28849
G	1762.871	6.07	(K)LRTEGDGVYTLNNEK(Q)	MG-H1 (2)	3573
UG	1708.866	7.21	(K)LRTEGDGVYTLNNEK(Q)		8325
G	2562.233	6.20	(K)LRTEGDGVYTLNNEKQWINK(A)	G-H1 (2), IMIDAZOLONE-A (20)	9297
LAP (HPT)					
UG	1857.933	8.23	(K)AVGDKLPECEAVCGKPK(N)	Carbamidomethyl C (9), Carbamidomethyl C (13)	121666
G	2135.987	7.19	(K)AVGDKLPECEAVCGKPK(N)	GLYCATION (5), Carbamidomethyl C (9), Carbamidomethyl C (13), CML (15), CML (17)	105956
UG	1439.705	7.87	(R)TEGDGVYTLNDKK(Q)		39795
G	2225.077	6.26	(R)TEGDGVYTLNDKKQWINK(A)	CML (12), CML (18)	34404
UG	1439.705	7.87	(R)TEGDGVYTLNDKK(Q)		39795
G	2397.176	6.83	(R)TEGDGVYTLNDKKQWINK(A)	MODIC (12), CROSSLINE (18)	35348
UG	2579.314	5.71	(R)TEGDGVYTLNNEKQWINK AVGDK(L)		1047
G	2817.31	6.44	(R)TEGDGVYTLNNEKQWINK AVGDK(L)	CML (13), IMIDAZOLONE-A (18), MODIC (23)	10476
UG	2579.314	5.71	(R)TEGDGVYTLNNEKQWINKA VGDK(L)		1047
G	2835.35	6.87	(R)TEGDGVYTLNNEKQWINKA VGDK(L)	CEL (13), CML (18), FL-2H2O (23)	20684
HAP (HBA)					
UG	1815.989	5.39	(-)MVLSPADKTNVKAAWGK(V)		1551
G	2081.054	6.36	(-)MVLSPADKTNVKAAWGK(V)	CEL (8), IMIDAZOLONE-A (12), MOLD (17)	82943
LAP (HBA)					
UG	1815.989	5.39	(-)MVLSPADKTNVKAAWGK(V)		1551
G	2081.054	6.36	(-)MVLSPADKTNVKAAWGK(V)	CEL (8), IMIDAZOLONE-A (12), MOLD (17)	82943
UG	3124.602	6.45	(K)KVADALTNAVAHVDDMPNA LSALSDLHAHK(L)		5326
G	3537.776	5.81	(K)KVADALTNAVAHVDDMPN ALSALSDLHAHKLR(V)	PYRRALINE (1), MODIC (30)	13859
HAP (KNG1)					
UG	1706.868	4.70	(K)DAAKAATGECTATVGKR(S)	Carbamidomethyl C (10)	1482
G	1657.765	5.38	(K)DAAKAATGECTATVGK(R)	CML (4), Carbamidomethyl C (10), MOLD (16)	9801
UG	1390.737	4.98	(K)RPPGFSPFRSSR(I)		1036
G	1722.873	5.28	(K)RPPGFSPFRSSR(I)	ARGPYR (1), CROSSLINE (9)	1980
LAP (KNG1)					
UG	2139.089	5.66	(R)DIPTNSPELEETLTHTITK(L)		3115
G	2175.062	4.36	(R)DIPTNSPELEETLTHTITK(L)	MODIC (19)	1498
UG	2216.105	5.32	(K)CPGRPWKSVSEINPTTQMK(E)	Carbamidomethyl C (1)	3725
G	2270.127	4.20	(K)CPGRPWKSVSEINPTTQMK(E)	Carbamidomethyl C (1), MG-H1 (4)	2888
UG	2237.064	5.03	(K)HNLGHGHKHERDQGHGHQR(G)		2988
G	2505.143	5.15	(K)HNLGHGHKHERDQGHGHQR(G)	FL-2H2O (8),	19097

				IMIDAZOLONE-B (19)	
UG	3139.426	4.94	(R)IGEIKEETTVSPPHTSMAPAQ DEERDSGK(E)		1310
G	3283.55	5.18	(R)IGEIKEETTVSPPHTSMAPAQ DEERDSGK(E)	CEL (5), CEL (29)	3851
UG	1706.868	4.70	(K)DAAKAATGECTATVGKR(S)	Carbamidomethyl C (10)	1482
G	1657.765	5.38	(K)DAAKAATGECTATVGK(R)	CML (4), Carbamidomethyl C (10), MOLD (16)	9801
HAP (IC1)					
UG	1347.7	4.89	(K)NSVIKVPMMNSK(K)		48081
G	1405.73	5.45	(K)NSVIKVPMMNSK(K)	CML (5)	13210
UG	1347.7	4.89	(K)NSVIKVPMMNSK(K)		48081
G	1511.803	5.57	(K)NSVIKVPMMNSKK(Y)	MODIC (13)	11774
UG	1347.7	4.89	(K)NSVIKVPMMNSK(K)		48081
G	1857.92	5.31	(K)NSVIKVPMMNSKK(Y)	GLYCATION (5), GLYCATION (12), CML (13)	7619
LAP (IC1)					
UG	1347.7	4.89	(K)NSVIKVPMMNSK(K)		48081
G	1509.758	5.64	(K)NSVIKVPMMNSK(K)	GLYCATION (5)	187639
UG	1347.7	4.89	(K)NSVIKVPMMNSK(K)		48081
G	1591.811	5.42	(K)NSVIKVPMMNSKK(Y)	CML (5), CML (13)	6089
UG	1886.971	5.16	(K)HRLEDMEQALSPSVFK(A)		3887
G	1940.952	5.63	(K)HRLEDMEQALSPSVFK(A)	MG-H1 (2)	10115
UG	2549.145	5.24	(R)ASSNPNATSSSSQDPESLQDRGEGK(V)		2479
G	2657.132	5.48	(R)ASSNPNATSSSSQDPESLQDRGEGK(V)	PYRRALINE (25)	4005
UG	1218.594	5.89	(K)DFTCVHQALK(G)	Carbamidomethyl C (4)	12145
G	2058.972	5.26	(K)DFTCVHQALKGFTTK(G)	Carbamidomethyl C (4), GLYCATION (10), IMIDAZOLONE-A (15)	81047
UG	1826.992	6.41	(K)GVTSVSQIFHSPDLAIR(D)		25959
G	2825.404	5.09	(K)GVTSVSQIFHSPDLAIRDTFVNASR(T)	MG-H1 (17), MG-H1 (25)	2285
HAP (PZP)					
UG	1571.823	5.01	(K)QNQNREILNSLDK(E)		1646
G	1629.821	5.64	(K)QNQNREILNSLDK(E)	CML (13)	138074
UG	1622.825	4.80	(R)NALFCLESAWNVAK(E)	Carbamidomethyl C (5)	30870
G	1748.827	4.52	(R)NALFCLESAWNVAK(E)	Carbamidomethyl C (5), FL-2H2O (14)	1146
LAP (PZP)					
UG	1602.798	4.93	(R)IREEGTDLEVTANR(I)		1393
G	1764.851	5.10	(R)IREEGTDLEVTANR(I)	GLYCATION (14)	25328
UG	1333.694	4.59	(R)EEGTDLEVTANR(I)		2391
G	2498.265	5.34	(R)EEGTDLEVTANRISEITNIVSK(L)	ARGPYR (12)	42867
UG	1602.798	4.93	(R)IREEGTDLEVTANR(I)		1393
G	3119.534	4.37	(R)IREEGTDLEVTANRISEITNIVSK(L)	MODIC (2), CROSSLINE (14), IMIDAZOLONE-A (24)	2953
UG	2994.483	5.23	(R)ENRSLFTDLVAEKDLFHCVSFTLPR(I)	Carbamidomethyl C (18)	4843

G	3282.576	5.26	(R)ENRSLFTDLVAEKDLFHCVSFTLPR(I)	IMIDAZOLONE-A (3), Carbamidomethyl C (18), IMIDAZOLONE-A (25)	38685
UG	1602.798	4.93	(R)IREEGTDLEVTANR(I)		1393
G	1762.862	5.40	(R)IREEGTDLEVTANR(I)	ARGPYR (2), ARGPYR (14)	5797
HAP (TRFE)					
UG	2573.315	5.69	(K)HQTVPQNTGGKNPDPWAKNLNEK(D)		3815
G	2784.354	6.06	(K)HQTVPQNTGGKNPDPWAKNLNEK(D)	FL-2H2O (11), MOLD (18), MODIC (23)	21399
UG	2698.246	6.15	(K)WCALSHHERLKCDEWSVNSVGK(I)	Carbamidomethyl C (2), Carbamidomethyl C (12)	1563
G	2990.367	7.28	(K)WCALSHHERLKCDEWSVNSVGK(I)	Carbamidomethyl C (2), G-H1 (9), Carbamidomethyl C (12), CROSSLINE (22)	11239
LAP (TRFE)					
UG	3025.519	5.89	(R)KPVDEYKDCHLAQVPSHTVVARS MGGK(E)	Carbamidomethyl C (9), Oxidation M (24)	2409
G	2402.154	6.46	(K)DCHLAQVPSHTVVARSMGGK(E)	Carbamidomethyl C (2), CROSSLINE (15)	1942
UG	2574.349	6.55	(K)KSCHTAVGRTAGWNIPMGLLYNK(I)	Carbamidomethyl C (3)	3343
G	1721.85	6.29	(R)TAGWNIPMGLLYNK(I)	IMIDAZOLONE-A (14)	2205
UG	1562.821	6.04	(K)KSASDLTWDNLKGK(K)		1827
G	1863.877	6.60	(K)KSASDLTWDNLKGK(K)	PYRRALINE (1), IMIDAZOLONE-A (12), MOLD (14)	10722
UG	1881.888	7.63	(K)ADRDQYELLCLDNTR(K)	Carbamidomethyl C (10)	64708
G	2314.024	6.58	(K)ADRDQYELLCLDNTR(K)	GLYCATION (3), Carbamidomethyl C (10), AFGP (15)	1605
UG	1562.821	6.04	(K)KSASDLTWDNLKGK(K)		1827
G	1782.867	6.36	(K)KSASDLTWDNLKGK(K)	GLYCATION (12), CML (14)	38947
HAP (VTDB)					
UG	1403.714	5.30	(R)VCSQYAAYGEKK(S)	Carbamidomethyl C (2)	2685
G	1439.667	6.27	(R)VCSQYAAYGEKK(S)	Carbamidomethyl C (2), MODIC (11)	125950
UG	1883.922	5.15	(K)ELPEHTVKLCDNLSTK(N)	Carbamidomethyl C (10)	1974
G	2135.987	5.99	(K)ELPEHTVKLCDNLSTK(N)	PYRRALINE (8), Carbamidomethyl C (10), IMIDAZOLONE-A (16)	51791
LAP (VTDB)					
UG	3102.458	6.10	(K)ELSSFIDKGQELCADYSENTF TEYKK(K)	Carbamidomethyl C (13)	11001
G	3262.411	6.01	(K)ELSSFIDKGQELCADYSENTF TEYK(K)	GLYCATION (8), Carbamidomethyl C (13), FL-2H2O (25)	40058
UG	1403.649	5.84	(R)VCSQYAAYGEKK(S)	Carbamidomethyl C (2)	2562
G	1461.667	6.74	(R)VCSQYAAYGEKK(S)	Carbamidomethyl C (2), CML (11)	94059
UG	1883.922	5.15	(K)ELPEHTVKLCDNLSTK(N)	Carbamidomethyl C (10)	1974
G	2135.992	6.46	(K)ELPEHTVKLCDNLSTK(N)	PYRRALINE (8), Carbamidomethyl C (10), IMIDAZOLONE-A (16)	103947
UG	3102.457	6.43	(K)ELSSFIDKGQELCADYSENTF TEYKK(K)	Carbamidomethyl C (13)	11705
G	3349.465	6.34	(K)ELSSFIDKGQELCADYSENTF TEYKK(K)	CEL (8), Carbamidomethyl C (13),	399359

				FL-2H2O (25), MOLD (26)	
HAP (VTNC)					
UG	1268.619	5.02	(R)GHSRGRNQNSR(R)		1432
G	1448.651	5.10	(R)GHSRGRNQNSR(R)	IMIDAZOLONE-A (4), MODIC (6)	55415
UG	1268.619	5.02	(R)GHSRGRNQNSR(R)		1432
G	1556.729	5.20	(R)GHSRGRNQNSR(R)	MODIC (4), CROSSLINE (6)	25879
LAP (VTNC)					
UG	2149.037	4.57	(R)INCQGKTYLFKGSQYWR(F)	Carbamidomethyl C (3)	1786
G	2207.067	5.03	(R)INCQGKTYLFKGSQYWR(F)	Carbamidomethyl C (3), CML (11)	7614
UG	2852.346	4.84	(K)TYLFKGSQYWRFEDGVLDPDYPR(N)		1583
G	2978.399	4.64	(K)TYLFKGSQYWRFEDGVLDPDYPR(N)	FL-2H2O (5)	2393
UG	2029.013	5.02	(K)LIRDVWGIEGPIDAAFTR(I)		1025
G	2171.113	4.96	(K)LIRDVWGIEGPIDAAFTR(I)	IMIDAZOLONE-B (18)	137993
UG	3072.416	5.19	(R)MDWLVPATCEPIQSVFFFSGDKYYR(V)	Oxidation M (1), Carbamidomethyl C (9)	7941
G	3344.524	3.88	(R)MDWLVPATCEPIQSVFFFSGDKYYR(V)	Carbamidomethyl C (9), IMIDAZOLONE-A (22), IMIDAZOLONE-A (25)	2073
UG	3057.453	5.16	(R)NISDGFDGIPDNVDAALALPAHSYSGRER(V)		1441
G	3363.517	5.09	(R)NISDGFDGIPDNVDAALALPAHSYSGRER(V)	AFGP (27), MODIC (29)	1053
UG	1666.781	5.84	(R)DWHGVPGQVDAAMAGR(I)		6933
G	1706.773	5.24	(R)DWHGVPGQVDAAMAGR(I)	G-H1 (16)	33370
UG	1268.619	5.02	(R)GHSRGRNQNSR(R)		1432
G	1308.620	5.22	(R)GHSRGRNQNSR(R)	G-H1 (4)	75252

APPENDIX 2

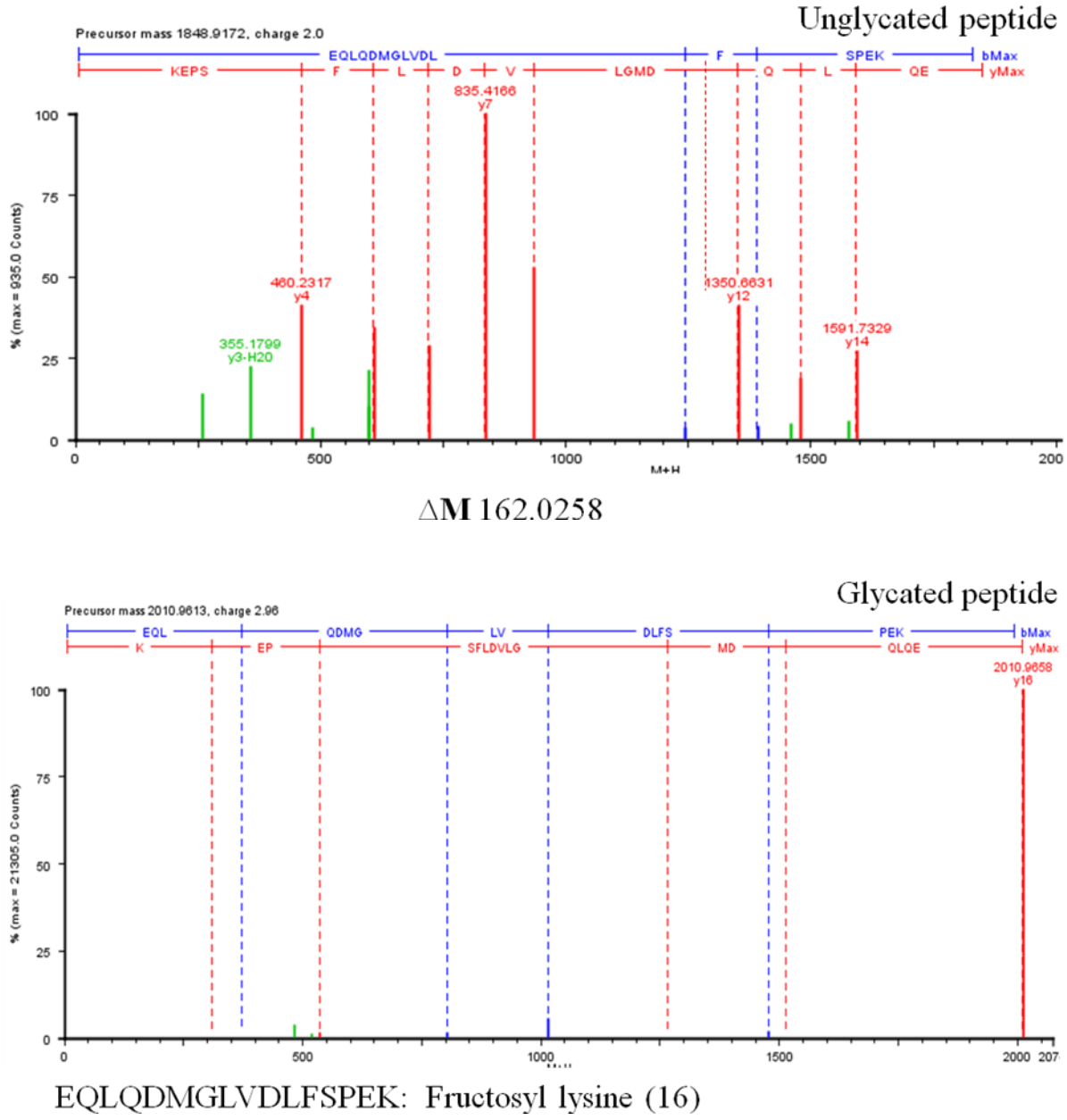


Figure A1. MS/MS annotation of Fructosyl lysine modification

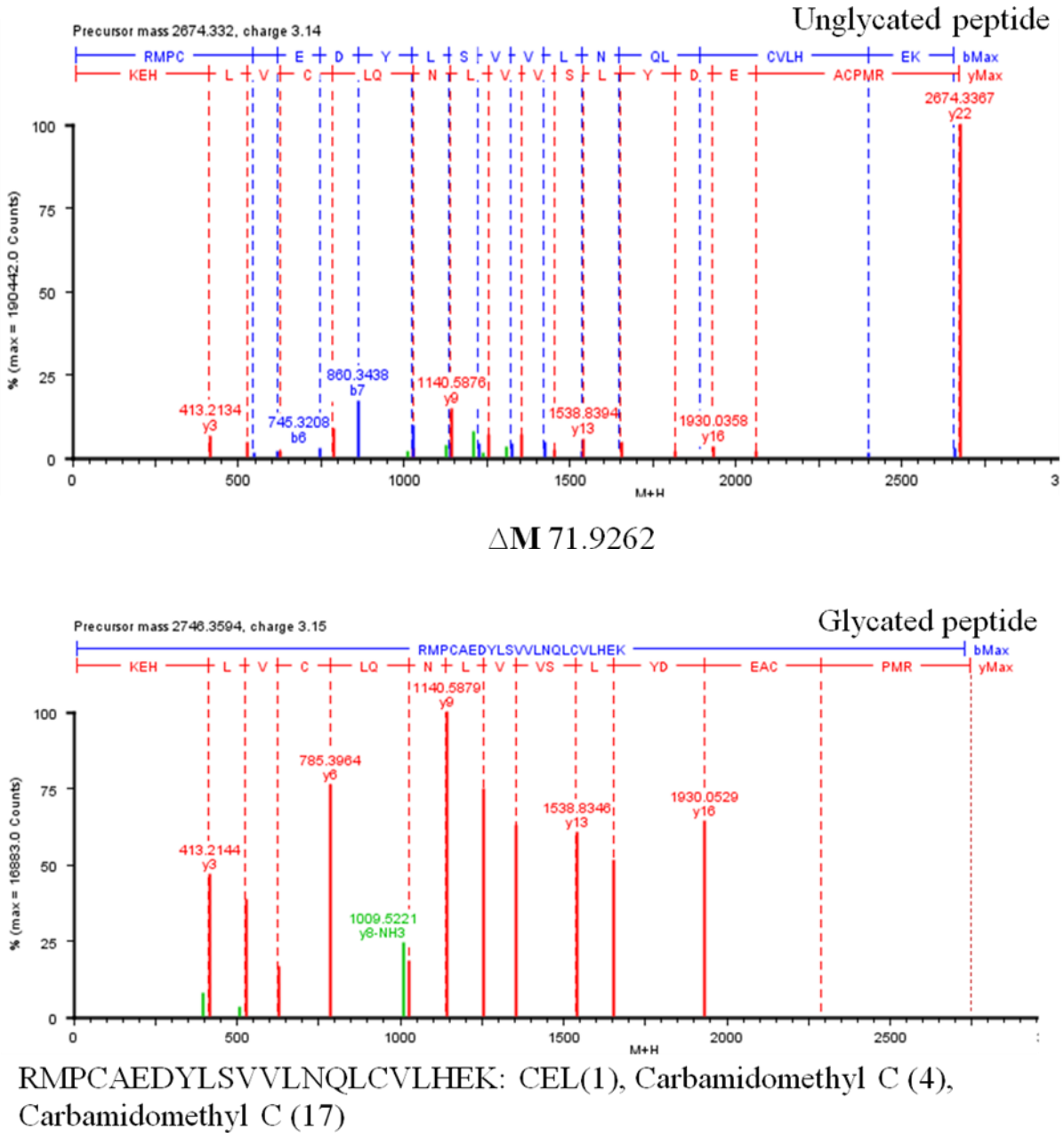


Figure A2. MS/MS annotation of CEL modification

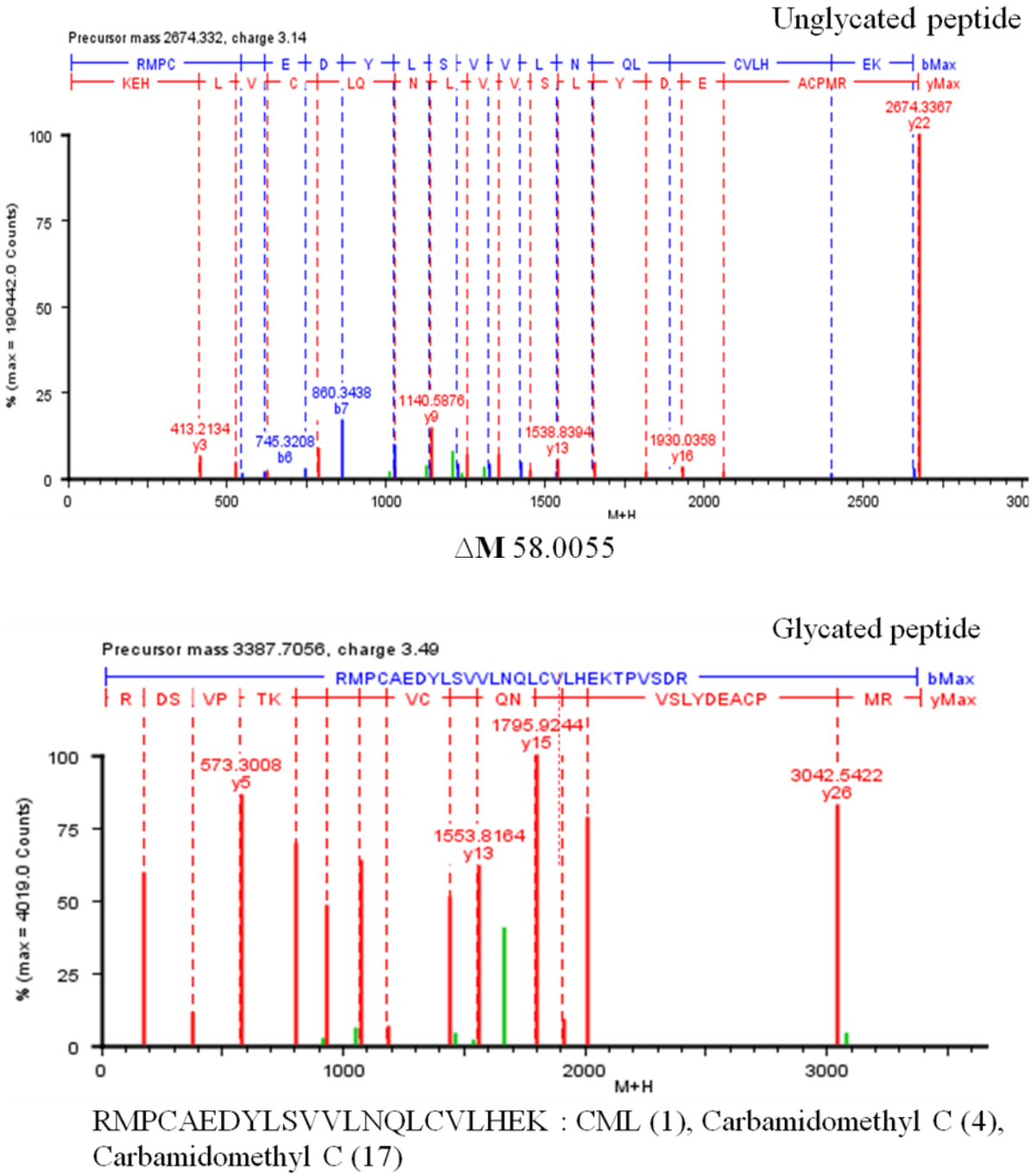


Figure A3. MS/MS annotation of CML modification

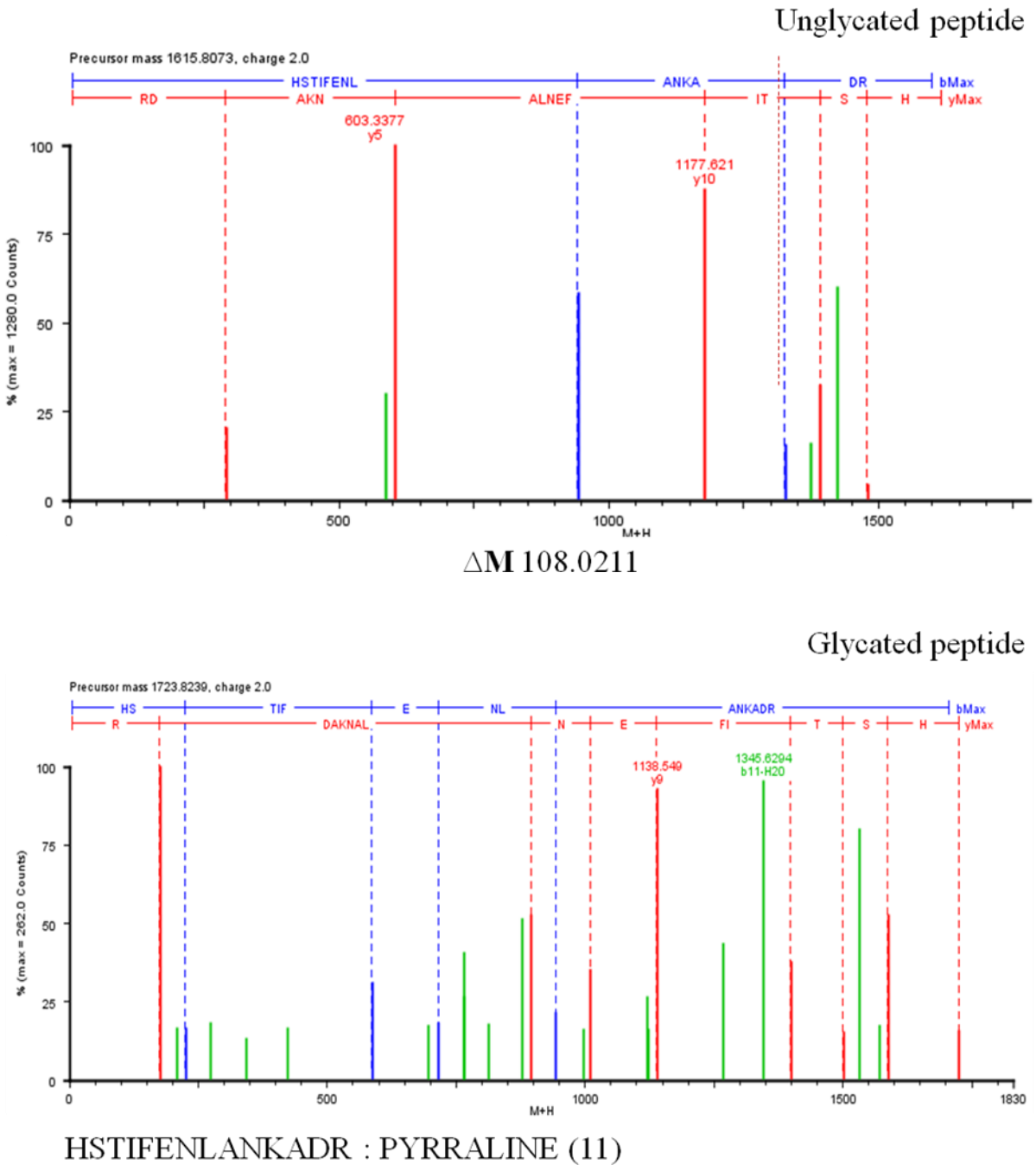


Figure A4. MS/MS annotation of Pyrraline modification

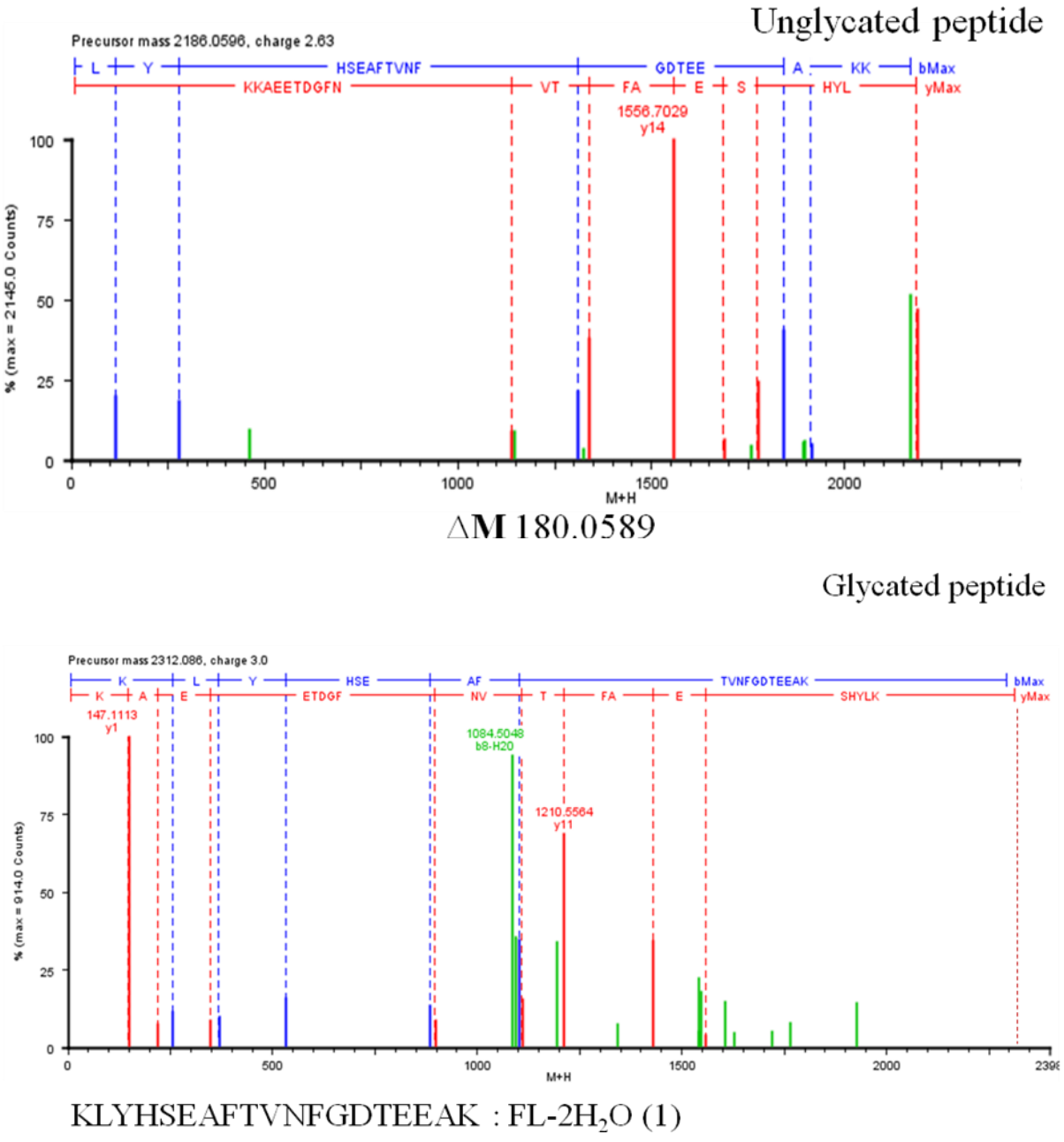


Figure A5. MS/MS annotation of FL-$2H_2O$ modification

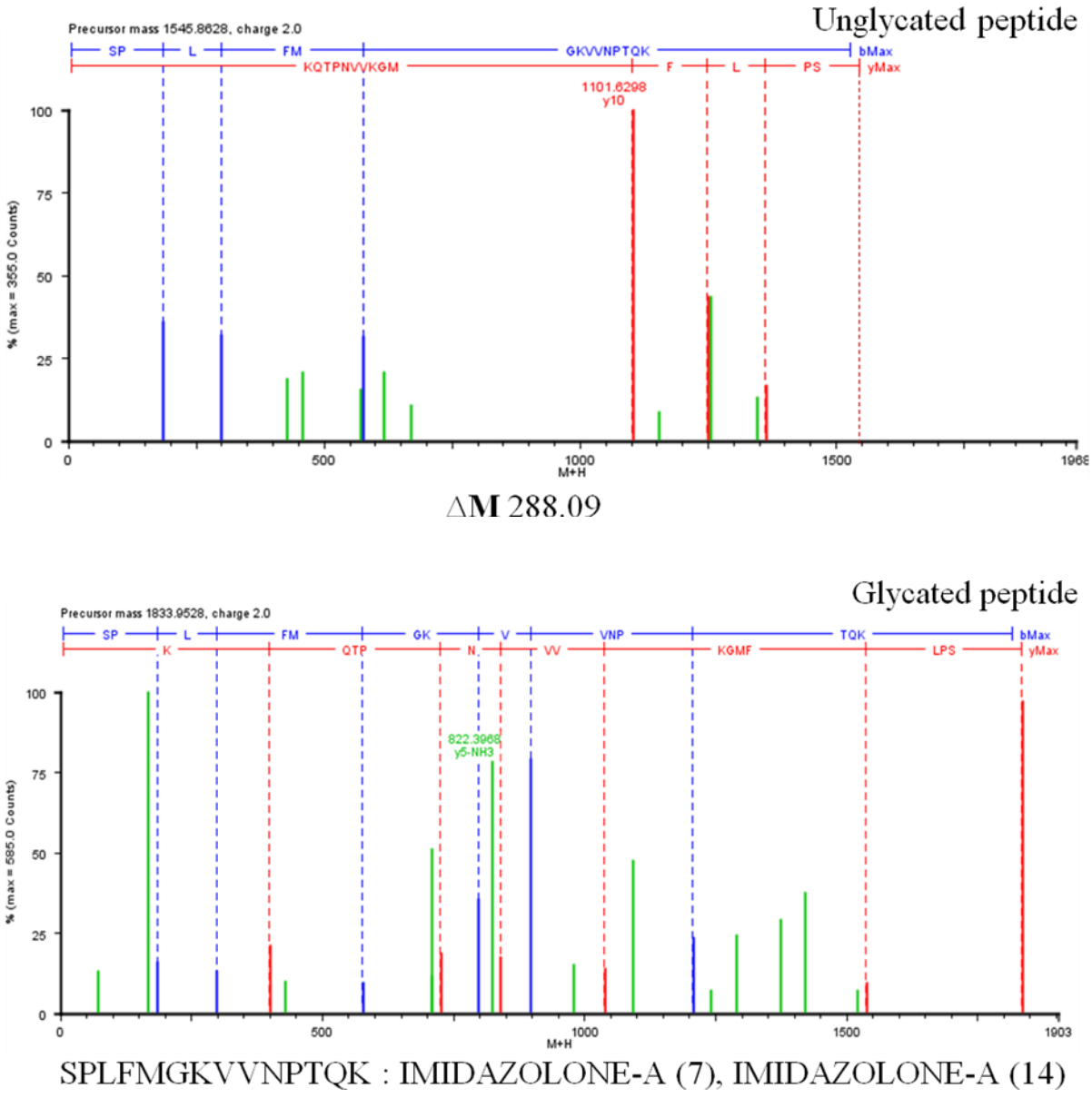


Figure A6. MS/MS annotation of Imidazolone-A modification

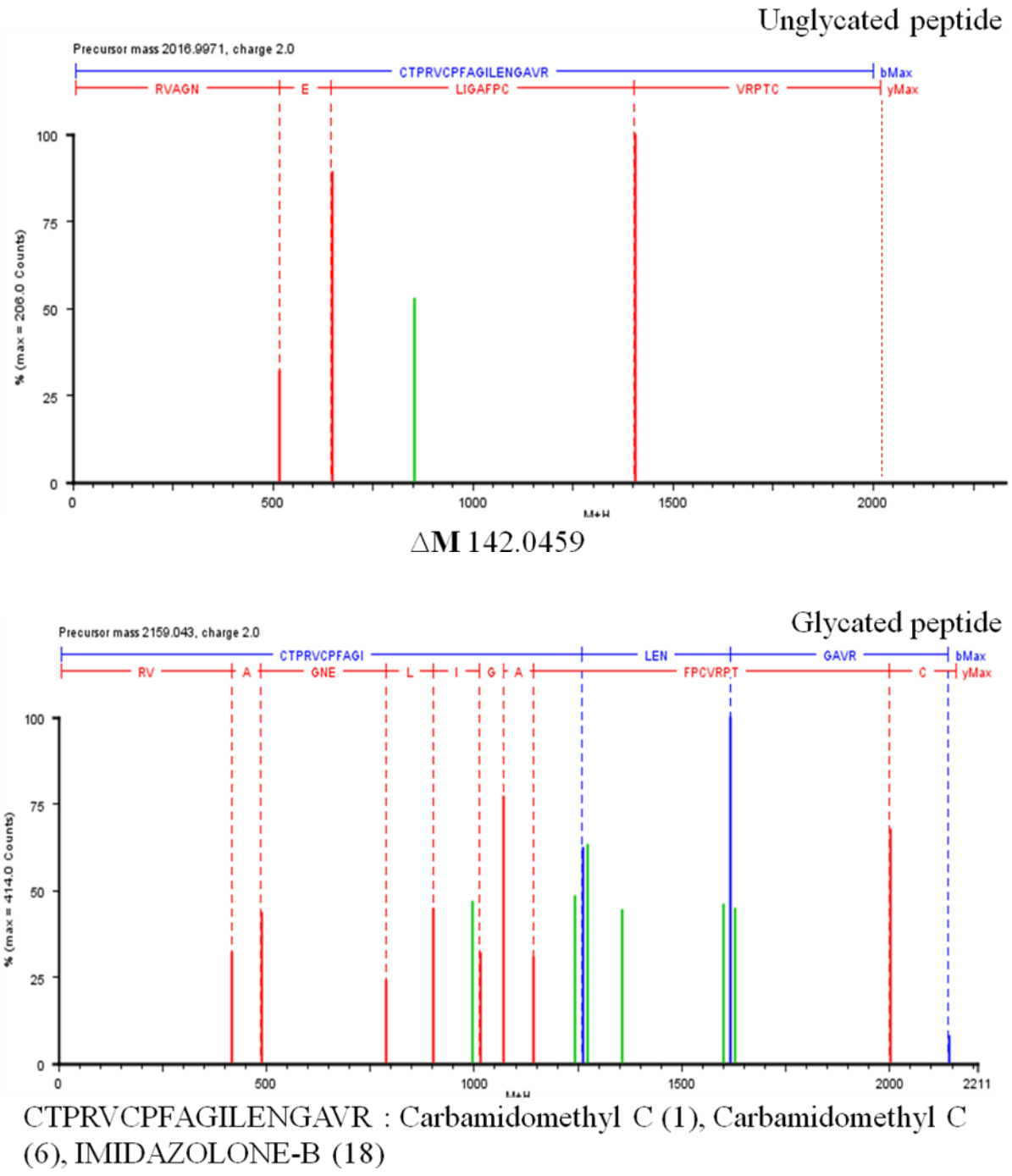


Figure A7. MS/MS annotation of Imidazolone-B modification

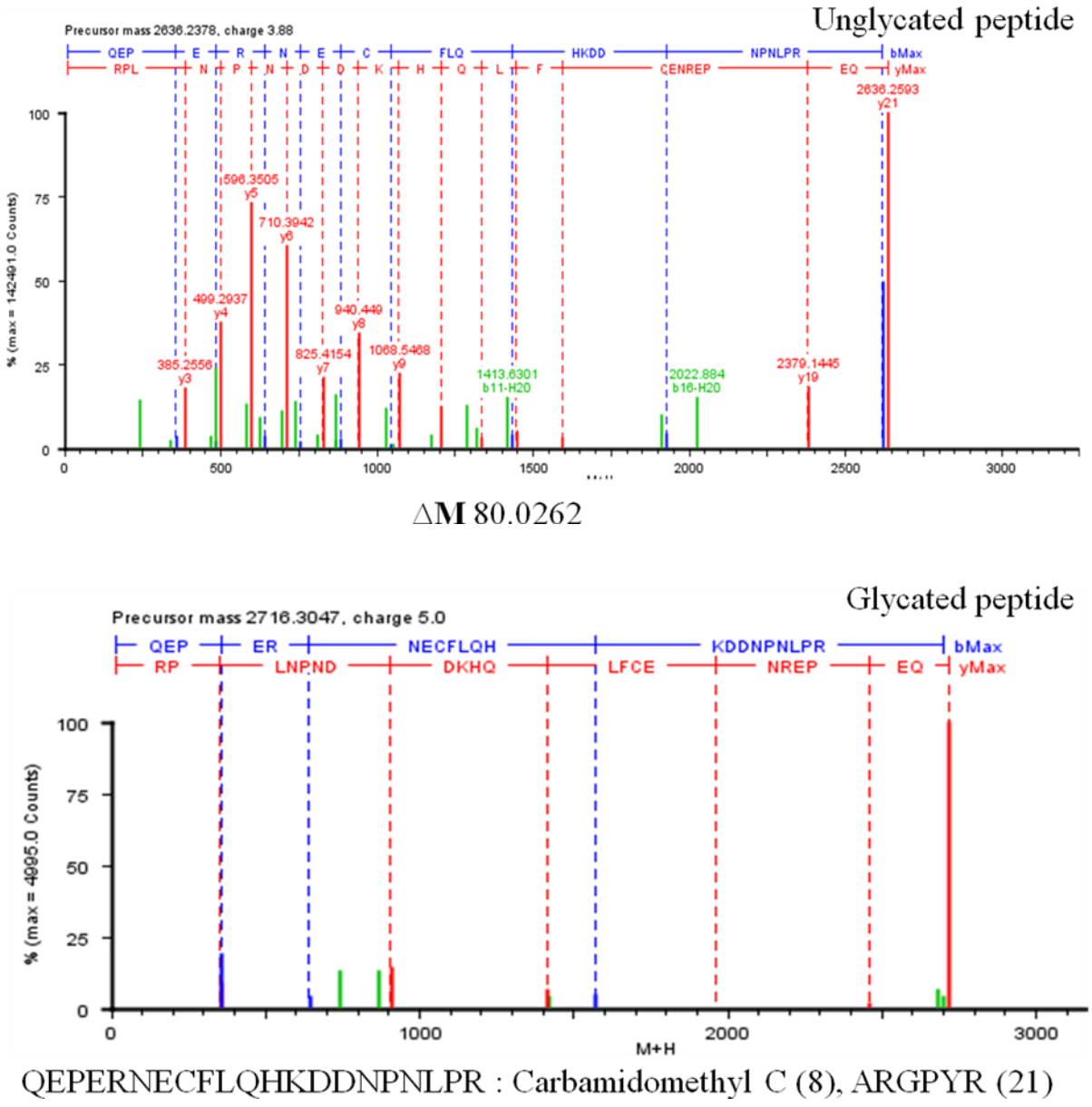


Figure A8. MS/MS annotation of Argpyrimidine modification

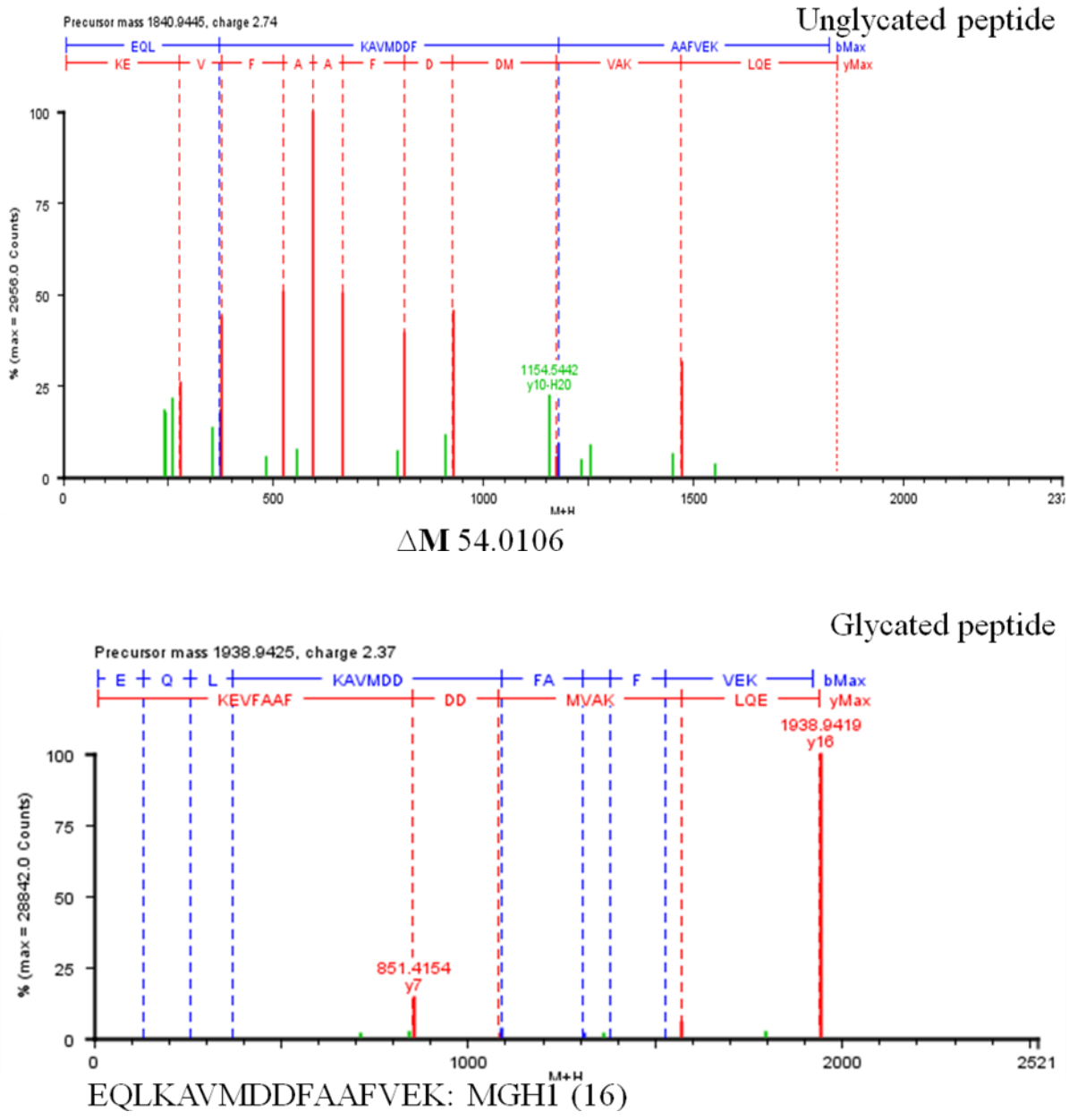


Figure A9. MS/MS annotation of MG-H1 modification

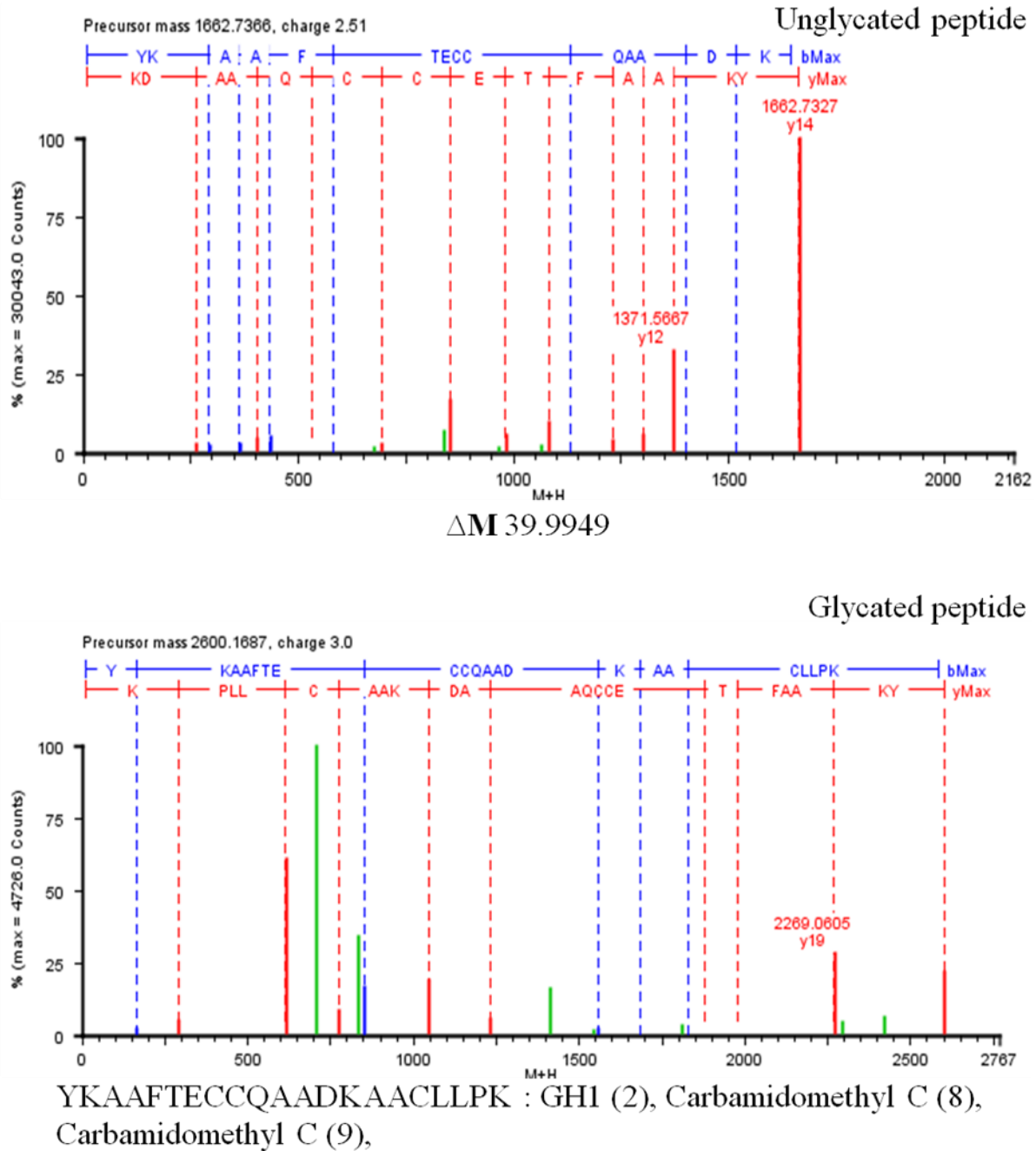


Figure A10. MS/MS annotation of G-H1 modification

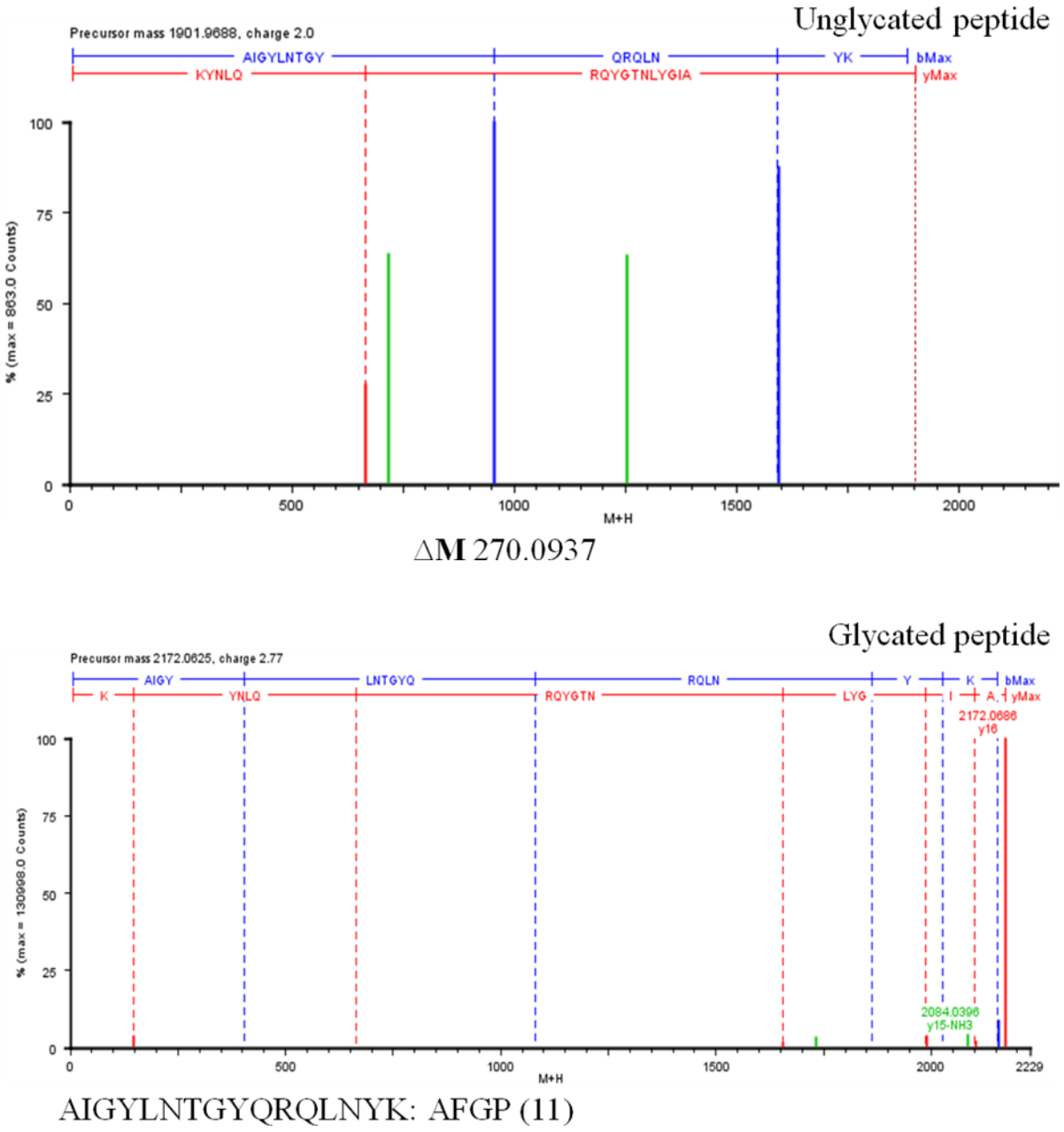


Figure A11. MS/MS annotation of AFGP modification

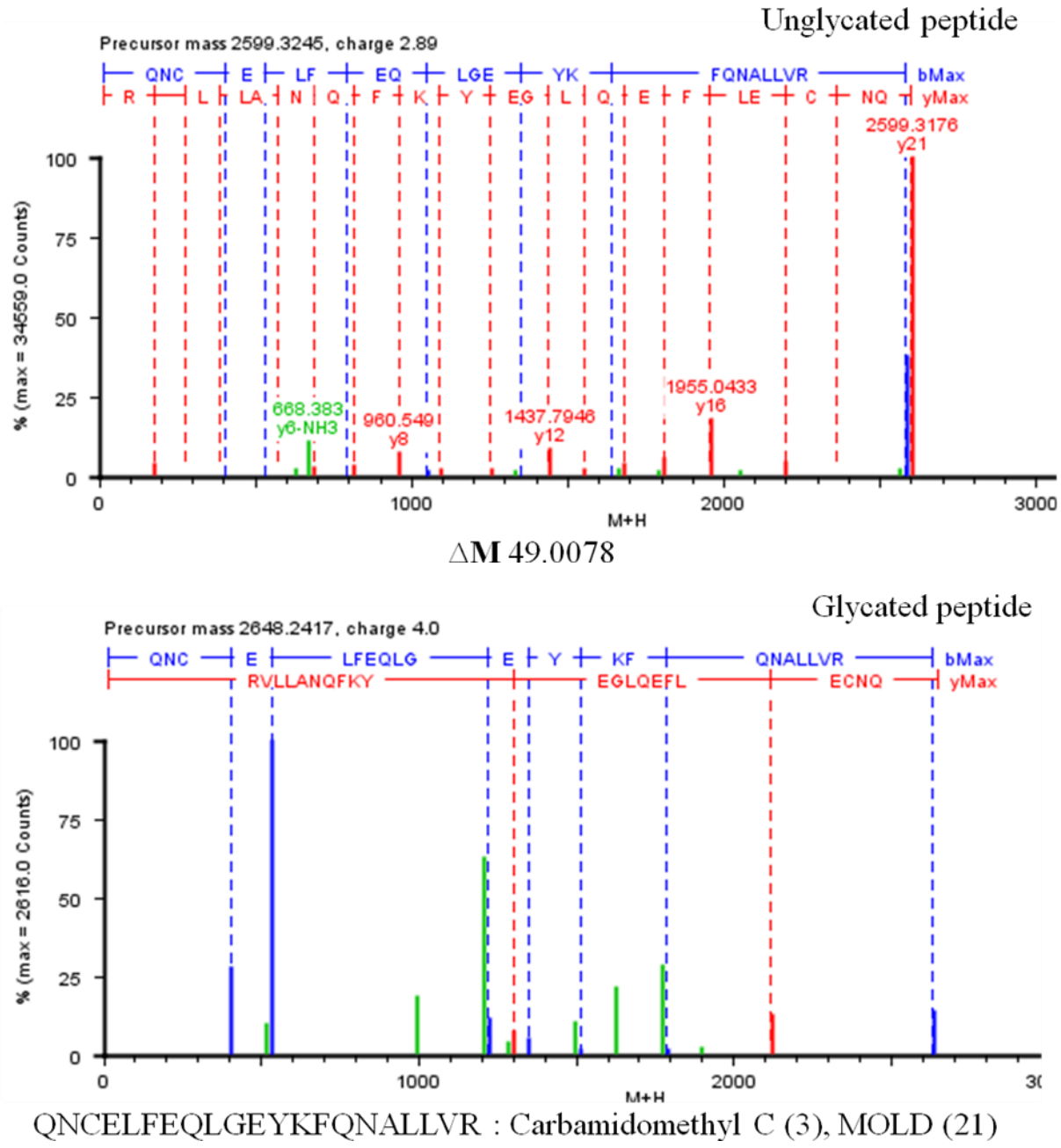


Figure A12. MS/MS annotation of MOLD modification

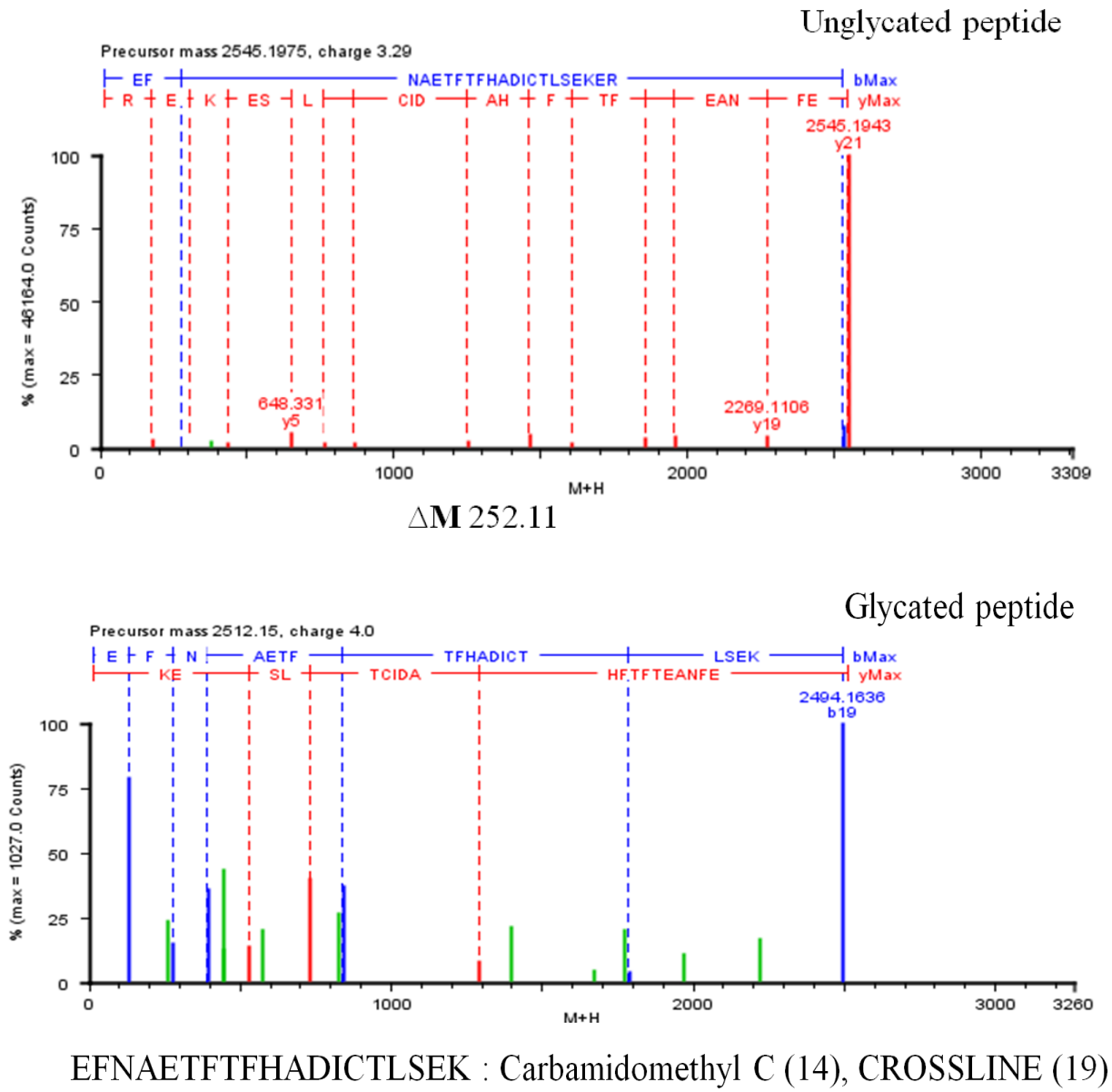


Figure A13. MS/MS annotation of Crossline modification

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Career Objective:

To excel research career in proteomics, cell biology and disease biology related areas.

Education:

2005-2007: Master of Science in Medical Biotechnology. Center for Biotechnology, Pravara Rural University, Loni, India.

2002-2005: Bachelor of Science in Biotechnology, University of Mumbai, India.

Awards and Achievements:

1. Best Oral Presentation Award. 'Role of albumin in regulation of plasma protein glycation in diabetes' at the Graduate Students Meet 2011, held at ACTREC, Navi Mumbai.

2. Best Poster Award. "Regulation of Glycation by Serum Albumin" presented on the National Science Day, 2011 at the CSIR-National Chemical Laboratory, Pune.

3. Junior and Senior Research Scholarship Award. 2008 to 2012 from the Lady Tata Memorial Trust, Mumbai, India.

4. Best Publication Award. Publication in highest impact factor journal (Biological Sciences) during the year 2011-2012 at CSIR-National Chemical Laboratory, Pune.

Research Experience:

August 2008 to till date: Ph.D student at CSIR- National Chemical Laboratory (NCL), Pune, India. Thesis title: "Regulation of Glycation in Diabetes: Identification and Characterization of Glycated Proteins". (*Research supervisor: Dr. M J. Kulkarni*).

Diabetes is characterized by hyperglycemia. Glucose modifies proteins by a nonenzymatic reaction called glycation. Protein glycation leads to the formation of heterogeneous fluorescent molecules, "advanced glycation end

products" (AGEs). AGEs are associated with pathogenesis of diabetic complications. In view of this, AGE modified proteins were identified and characterized in diabetic clinical and mice model plasma samples. Further, regulation of glycation by high abundant albumin was comprehensively studied. Additionally, differential protein expression in non diabetic, controlled diabetic and poorly controlled diabetic clinical samples was studied.

October 2007 to July 2008: Project Assistant-II at CSIR-NCL, Pune, India. Project: "Proteomics of diabetic complications, with special references to AGEs". (*Research supervisor: Dr. M J. Kulkarni*).

Association of AGEs and AGE modified protein in development of diabetic complications. AGE modified proteins were identified in plasma from streptozotocin induced diabetic mice models.

January 2007 to July 2007: Post Graduation dissertation at Center for Biotechnology, Pravara Rural University, India. Research title: "DNA fingerprinting of beta thalassemia patients". (*Research Supervisor: Dr. R J. Raynade*).

Publications:

1. 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 ***J Proteome Res*** 2012 Feb 3; 11 (2): 1391-6.

2. Bhonsle HS, Singh SK, Srivastava G, Boppana R, Kulkarni MJ. Albumin competitively inhibits glycation of less abundant proteins. ***Prot Pep Lett*** 2008; 15: 663-667.

3. Golegaonkar SB, **Bhonsle HS**, Boppana R, Kulkarni MJ. Discovery of rifampicin as a new antiglycating compound by MALDI-TOF-MS based insulin glycation assay. ***EurJ Mass Spectrom*** 16: 221-226, 2010.

4. Korwar AM, **Bhonsle HS**, Chougale AD, Kote SS, Gawai KR, Ghole VS, Koppikar CB, Kulkarni MJ. Analysis of AGE modified proteins and RAGE

expression in Invasive ductal carcinoma. ***Biochem Biophys Res Commun*** 419: 490–494.

5. Korwar AM, **Bhonsle HS**, Ghole VS, Gawai KR, Koppikar CB and Kulkarni MJ. Proteomic profiling and interactome analysis of ER positive / HER2/neu negative invasive ductal carcinoma. (Under review. ***OMICS: A Journal of Integrative Biology***)

6. Bhonsle HS, Korwar AM, Chougale AD, Dhande NL, Giri AP, Shelgikar KM, Kulkarni MJ. Proteomics studies revealed that Apoliprotein AI is downregulated in poorly controlled diabetes. (Communicated to ***Clinical Biochemistry*** March 6th, 2012).

Conferences Attended:

1. Presented poster on Albumin competitively inhibits glycation of less abundant proteins during 5th Asia Oceania Human Proteome Organization (AOHUPO), 14th Association for the Promotion of DNA Finger Printing and Associated DNA Technologies (ADNAT) and 1st Proteomics Society India (PSI) on 21-25 February 2010 held at CCMB, Hyderabad, India.

2. Presented poster on Albumin competitively inhibits glycation of less abundant proteins at International Symposium on Mass Spectrometry in Life Sciences on 27- 29 September 2009 held at the National Centre for Biological Sciences (NCBS), Bangalore, India.

3. Attended scientific meet on 'Current trends in proteomics and Brainstorm discussion on plasma proteomics' on February 17-18 2008 held at Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India.

References:

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Declaration:

All information above is true and to the best of my knowledge.

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