GLYCATED PROTEINS AS AUTOANTIGENS: POTENTIAL BIOMARKER FOR GLYCATION MEDIATED DIABETIC COMPLICATIONS

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CERTIFICATE

This is to certify that the work presented in this thesis entitled, "**Glycated Proteins as autoantigens: Potential biomarker for glycation mediated diabetic complications**" by **Ms Shweta Bhat**, for the degree of **Doctor of Philosophy**, was carried out by the candidate under my supervision in the Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune-411008, India. This work is original and has not been submitted for any other degree or diploma to this or any other university. Any material that has been obtained from other sources has been duly acknowledged in the thesis.

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CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled **"Glycated Proteins as autoantigens: Potential biomarker for glycation mediated diabetic complications"** submitted for the award of the degree of **Doctor of Philosophy** in Biotechnology to the 'Savitribai Phule Pune University' has not been submitted by me to any other university or institution. This work was carried out by me at CSIR-National Chemical Laboratory, Pune, India. Such materials as obtained from other sources have been duly acknowledged in the thesis.

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Dedicated to my family

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– Shweta Bhat

List of Abbreviations

1D	: 1 Dimensional
2D	: 2 Dimensional
3-DG	: 3-Deoxyglucosone
ACN	: Acetonitrile
ADA	: American Diabetes Association
AFGP	: Alkyl Formyl Glucosyl Pyrrole
AGE-R1	: AGE receptor 1
AGE-R2	: AGE receptor 2
AGE-R3	: AGE receptor 3
AGEs	: Advanced Glycation End Products
ALEs	: Advanced Lipoperoxidation End Products
AMG	: Aminoguanidine
ANOVA	: One-way Analysis of Variance
APCs	: Antigen Presenting Cells
BCG	: Bromocresol Green
BEH	: Ethylene Bridged Hybrid
BSA	: Bovine Serum Albumin
CEL	: N-ε-Carboxy Ethyl Lysine
CICs	: Circulating Immune Complexes
CML	: N-ε-carboxy Methyl Lysine
CON	: Control subjects
CPCSEAI	: Control and Supervision of Experiments on Animals, India
DAMPs	: Danger-Associated Molecular Patterns
DM-MIC	: Diabetes with Microalbuminuria
DNA	: Deoxyribo Nucleic Acid
EDTA	: Ethylenediamminetetraacetate
ELISA	: Enzyme Linked Immune Sorbent Assay
FBG	: Fasting Blood Glucose
FL	: Fructosyl-Lysine
GD	: Gestational Diabetes
GLUT	: Glucose Transporter
GO	: Glyoxal
GOLD	: Glyoxal Lysine Dimer
HbA1c	: Glycated Haemoglobin
HDL	: High-Density Lipoprotein
HDL-CE	: High-Density Lipoprotein Cholesteryl Ester
HRMS	: High Resolution Mass Spectrometry

HSA	: Human Serum Albumin
ICs	: Immune Complexes
IDDM	: Insulin Dependent Diabetes Mellitus
IGT	: Impaired Glucose Tolerance
IP	: Immunoprecipitation
LC-MS^E	: Liquid Chromatography Mass Spectrometry At Elevated Energy
LDL	: Low Density Lipoprotein
MG-H1	: Methylglyoxal Derived Hydroimidazolone
MGO	: Methylglyoxal
MIC	: Microalbuminuria
MOLD	: Methyl Glyoxal Lysine Dimer
MS	: Mass Spectrometry
MSA	: Mouse Serum Albumin
NDD	: Newly Diagnosed Diabetes
NIDDM	: Non-Insulin Dependent Diabetes
OGTT	: Oral Glucose Tolerance Test
PAGE	: Polyacrylamide Gel Electrophoresis
PBG	: Postprandial Blood Glucose
PBS	: Phosphate Buffered Saline
PEG	: Polyethylene Glycol
PLGS	: Protein Lynx Global Server
PRPs	: Protease Pesistant Proteins
PRRs	: Pattern Recognition Receptors
PVDF	: Polyvinylidene Fluride
RA	: Rheumatoid Arthritis
RAGE	: Receptor For Advanced Glycation End Products
ROS	: Reactive Oxygen Species
RSA	: Rat Serum Albumin
SA-PE	: Streptavidin-Phycoerythrin
SDS	: Sodium Dodecyl Sulfate
SLE	: Systemic Lupus Erythematosus
SR	: Scavenger Receptor
STZ	: Streptozotocin
T1DM	: Type 1 Diabetes Mellitus
T2DM	: Type 2 Diabetes Mellitus
Th	: T-helper
TIC	: Total Ion Count
TMB/H_2O_2	: Tetramethyl Benzidine/Hydrogen Peroxide
VLDL	: Very Low Density Lipoprotein
WHO	: World Health Organization

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Thesis Abstract

All forms of diabetes are characterized by chronic hyperglycemia that is elevated levels of blood glucose. This imbalance in blood glucose level plays a central role in the development of diabetes specific microvascular pathologies such as nephropathy, retinopathy and neuropathy. The principal cause leading to diabetic complications is the non enzymatic modification of proteins known as glycation, which involves the series of reactions between a reducing sugar such as glucose, or a ketoaldehyde such as methylglyoxal (MG) and the amino groups of proteins known as glycation. Glycation eventually leads to formation of AGE modified proteins which exhibit altered structure and functionality from native proteins and even form aggregates. Such structurally altered glycated proteins present new immunological epitopes on their surface that are recognised by the host immune system as “no self” or “autoantigens” leading to generation of autoantibodies. The autoantibodies bind to modified antigens leading to formation of CICs. Autoimmune responses against modified self-antigens in the development of diabetic vascular complications represent a relatively unexplored concept. Comprehensive identification of novel antigens associated with CICs which are real time products of immune response, potentially could provide significant new mechanistic insight into the underlying disease process. Therefore, this thesis mainly focuses on identification, quantification and characterization of CIC associated glycated proteins acting as autoantigens using proteomic approaches. In addition the role of reactive immunization with CML AGE modified MSA were studied in normal mice.

Proteomic analysis of glycated proteins in CICs from clinical plasma

Elicitation of the immune response against AGE modified proteins generates autoantibodies directed against these modified proteins, eventually leading to formation of CICs. Hence with the objective of identification of glycated proteins acting as autoantigens we isolated CICs from clinical plasma samples from healthy control

(CON), prediabetes (IGT), newly diagnosed diabetes (NDD), diabetes (DM) and diabetes with microalbuminurea (DM-MIC). CIC associated proteins were identified quantified and characterized by using label free mass spectrometry. Serum albumin levels were found to be elevated in IGT, NDD and DM-MIC plasma in comparison to that of CON, which was also characterized to be AGE modified by western blotting and mass spectrometric analysis.

Evaluation of role of glycation in autoimmune response and formation of CICs using AGE inhibitor aminoguanidine (AMG)

To determine the role of glycation in the formation of immune complex, STZ induced diabetic mice were treated with or without prototype AGE inhibitor AMG. AMG decreased HbA1c and plasma AGEs in diabetic mice, as evidenced by fluorescence spectrometric, western blotting and mass spectrometric analysis .The annotated spectra of AGE modified peptides from mouse serum albumin and corresponding intensities were used to generate the heatmap. Mass spectrometric analysis of CICs showed elevated levels of serum albumin in diabetic mice plasma than non-diabetic mice plasma. AMG treatment reduced the albumin levels in the CICs of diabetic mice plasma. This observation was also evident by western blotting with anti-albumin antibodies. Elevated levels of albumin in CICs of diabetic mice were accompanied by a decline in plasma albumin levels. However, the decreased plasma albumin levels in diabetes were not restored by AMG treatment. In addition to serum albumin, the levels of apolipoprotein E (Apo E) (1.6), carboxylesterase 1C (1.6) and alpha 2 macroglobulin (1.4) were found to be increased in diabetic CICs. Alpha 1 antitrypsin and apolipoprotein A1 (Apo A1) were observed only in the CICs from diabetic mice plasma, but not in CICs from plasma of control mice or diabetic mice treated with AMG. These proteins were characterized to be AGE modified in mass spectrometric analysis.

Reactive immunization of mice with AGE-modified mouse serum albumin to understand immune response, its effect on glycation and albumin level.

Further we designed the experimental plan to study the effect of reactive immunization of normal mice with the CML AGE modified protein, to investigate if the immunization with self modified antigen has any effect on the level of blood glucose, glycation and AGE formation and level of serum albumin in the immune complex. The level of serum albumin in the immune complex from the control mice immunized with the CML-MSA increased significantly in comparison to the level of serum albumin in the immune complex from the control mice immunized with control-MSA. Initial results showed slight but significantly increased HbA1c in control mice immunized with AGE modified MSA accompanied by elevated serum albumin in CICs and also decrease in the total plasma albumin level.

CHAPTER 1

Introduction

1.1. Diabetes mellitus

Diabetes is a chronic metabolic disorder that occurs when there is an impaired secretion of insulin or defects in using the secreted insulin. Both the conditions eventually lead to persistent hyperglycemia that is elevated levels of blood sugar (Expert Committee 2003). This imbalance in blood glucose level in chronic conditions plays a determinant role in the development of diabetes specific microvascular pathology in the renal glomerulus, retina and peripheral nerve, the serious secondary complications known as nephropathy, retinopathy and neuropathy respectively (Brownlee et al., 1988).

1.1.1. Classification of Diabetes

Diabetes mellitus is classically classified into 3 types: (a) type 1 diabetes caused by the absolute insulin deficiency, (b) type 2 diabetes which is the result of insulin resistance and (c) gestational diabetes is a glucose intolerance condition or diabetes occurring in pregnant women.

1.1.1a. Type 1 diabetes mellitus

Type 1diabetes (T1DM) is historically known as insulin dependent diabetes mellitus (IDDM) or juvenile onset diabetes accounts for about 5-10% worldwide. This type of diabetes results from the autoimmune destruction of β-cells of islets of pancreas, eventually leading to absolute insulin deficiency. Genetic factors and environmental factors such as low temperature, virus, cow milk etc predispose one to develop autoimmunity against β-cells leading to T1DM. Elevated levels of blood glucose in T1DM are often associated with other symptoms like polyuria, polydipsia, and weight loss (Daneman, 2006; Rother, 2007).

1.1.1b. Type 2 diabetes

Type 2 diabetes (T2DM) or non-insulin dependent diabetes (NIDDM) caused by the insulin resistance, is often combined with reduced insulin secretion. Type 2 diabetic patients develop significantly reduced responsiveness to secreted insulin, which leads to diminished glucose uptake in peripheral tissues and potentiates the metabolic effects of glucose resultant of hyperglycemia (Ward et al., 1984). It is also characterized by the failure to inhibit hepatic glucose production by insulin along with dysregulated insulin secretion (DeFronzo, 1997). T2DM accounts for about 90-95 % of the diagnosed cases of diabetes all over the world.

1.1.1c. Gestational diabetes

Gestational diabetes (GD) affects about 3-5% pregnant women and mostly the condition is resolved within 48 h of delivery in 90% of the cases. The occurrence of gestational diabetes is also said to predispose 30-40 % women with the risk of developing T2DM within 5-10 years. However, it is treatable with efficient blood glucose level control and medical supervision throughout the pregnancy (Homko et al., 1996).

1.2. Global prevalence and economic impact

Epidemic of diabetes has grown to be a threatening global health crisis, a steadily growing challenge on the economies of developed as well as developing countries. It was estimated that diabetes affected at least 366 million people in 2011 and this number is expected to reach 552 million people by 2030 (Whiting et al., 2011). Diabetes related health expenditure was estimated to be 12% or $376 billion in 2010, a figure believed to go up to $490 billion in 2030 with steadily increasing rate of diabetes (Zhang et al., 2010). It is predicted that in developing countries during 2010 to 2030, there will be 69% increase in numbers of adults with diabetes and 20% increase in developed countries (Shaw et al., 2010). Top 10 countries with largest number of diabetic patients in 2010 and expected numbers in 2030 is shown in Table 1.1.

Table 1.1 List of top 10 countries with highest number of diabetic patients in 2010 and expected number in 2030

	2010		2030	
	Country	No. of adults with Diabetes (millions)	Country	No. of adults with Diabetes (millions)
1.	India	50.8	India	87.0
2.	China	43.2	China	62.6
3.	USA	26.8	USA	36.0
4.	Russian Federation	9.6	Pakistan	13.8
5.	Brazil	7.6	Brazil	12.7
6.	Germany	7.5	Indonesia	12.0
7.	Pakistan	7.1	Mexico	11.9
8.	Japan	7.1	Bangladesh	10.4
9.	Indonesia	7.0	Russian Federation	10.3
10.	Mexico	6.8	Egypt	8.6

It is alarming that by 2025 in India and China around 40% of the country's healthcare budget will be consumed by ~130 million diabetic patients, according to predictions of world health organization (WHO) (Farag et al., 2011). Approximately 180,000 people died in the United States, while 983,203 in India and 1,133,918 in China during 2011 due to diabetes (Figure 1.1). Perhaps, these numbers may not precisely represent the enormity of diabetes burden, since a large number of people are unaware of the fact that they have diabetes due to poor diagnosis (Scully, 2012).

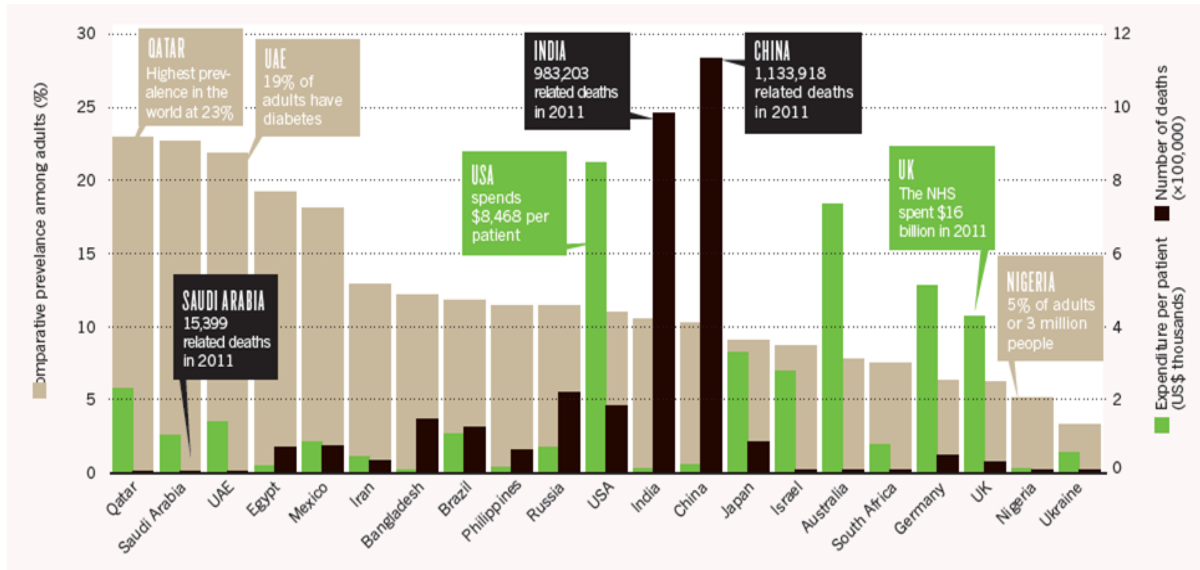


Figure 1.1 National prevalence of diabetes and number of diabetes related deaths during 2011. (Figure adapted from Scully, 2012).

1.3. Diabetes, glycation and advanced glycation end products

Irrespective of the type of diabetes, hallmark of the disease is persistent hyperglycemia and consequent abnormalities in carbohydrate, fat and protein metabolism (Brownlee, 1995). The inevitable consequence of hyperglycemia is enhanced glycation eventually forming advanced glycation end products (AGEs). Glycation is a non-enzymatic reaction between reducing sugars such as glucose and/or its auto-oxidation products with amino groups of nucleic acids, lipids , peptides and proteins This reaction was described for the first time by Louis Camille Maillard, who noticed the characteristic brown color when the mixture of amino acid and reducing sugars were heated together (Baynes et al., 1999), therefore it is also called Maillard's reaction or Browning.

1.3.1. Formation of AGEs

Glycation commences with the reversible reaction between reducing sugars and with ε-amino groups or N-terminal groups of the proteins (or free amine-containing

lipids or DNA) to form Schiff's base, which spontaneously rearranges to form a relatively stable entity called Amadori product (Brownlee, 1995). The Amadori products can undergo a series of reactions leading to the formation of highly reactive α-dicarbonyls and oxoaldehydes, such as 3-deoxyglucosone (3-DG), glyoxal (GO), and methylglyoxal (MGO), which are major AGE precursors (Baynes et al., 1999; Thornalley et al., 1999). AGEs can also be formed in nature by another process called autoxidative glycation or glycoxidation. Monosaccharides such as glucose exist in equilibrium with enediol. This enediol can undergo autoxidation in presence of metal ions forming an enediol radical, which in turn can undergo oxidation to form a dicarbonyl ketoaldehyde by reducing molecular oxygen to the superoxide radical (Wolff et al., 1987; Ahmed, 2005). These ketoimines and dicarbonyls which are products of Amadori rearrangements then indiscriminately react with lysine and arginine functional groups on bystander proteins, yielding a diverse range of irreversible and stable modifications. In addition to this, metal ion-catalyzed oxidation reactions of intermediates of lipid metabolism namely polyunsaturated fatty acids and arachidonic acid, can also lead to the modification of proteins. These protein adducts are known as advanced lipoperoxidation end products (ALEs). Both advanced glycation and lipoperoxidation end products are collectively termed as AGEs and display physiochemical characteristics such as fluorescence, brown colour, and intra or intermolecular cross-linking (Baynes et al., 1999; Horiuchi, 1996; Monnier et al., 1982; Vlassara, 1997; Bohlender et al., 2005). Most predominant and well characterized AGEs involved in development of diabetic complications include mainly, fructosyl-lysine (FL), N-ε-carboxymethyl lysine (CML), N-ε-carboxy ethyl lysine (CEL), argpyrimidine, pyralline, methylglyoxal derived hydroimidazolone (MG-H1), and imidazolones which are non fluorescent in nature, and pentosidine, crossline, vesperlysine, alkyl formyl glucosyl pyrrole (AFGP), methyl glyoxal lysine dimer(MOLD), glyoxal lysine dimer (GOLD) etc which are fluorescent in nature (Thornalley et al., 1999; Chen et al., 2015; Ghanem et al., 2011; Kalousova et al., 2004; Lieuw et al., 2004). The figure depicting formation of AGEs and representative fluorescent and non fluorescent AGEs are shown in Figure 1.2 and Figure 1.3.

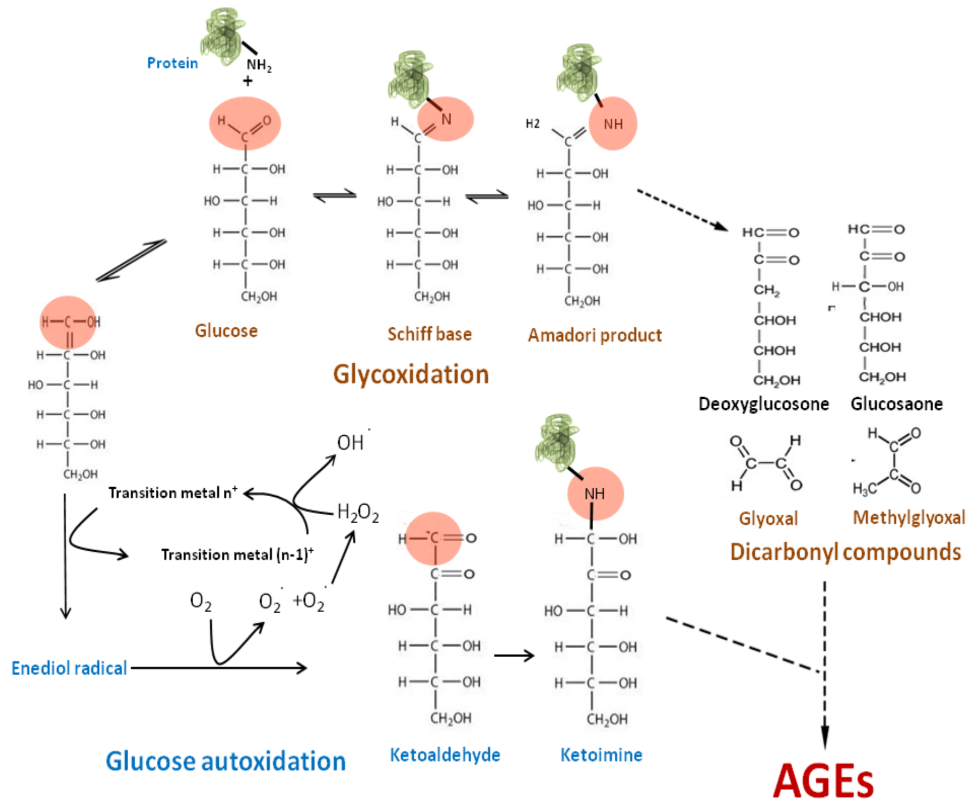
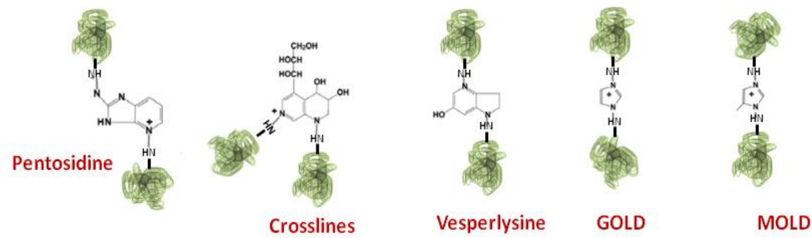


Figure 1.2 Formation of advanced glycation end products (AGEs).



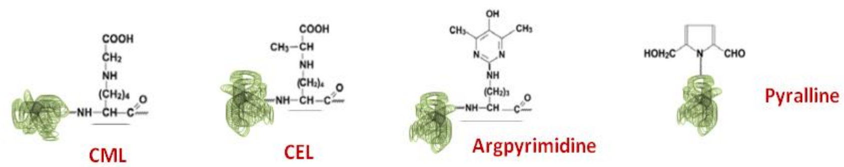


Figure 1.3 Various fluorescent and non fluorescent AGEs.

In addition to the accelerated formation of AGEs *in vivo* due to long standing hyperglycemia of diabetes, AGEs can also elevate in the body by consumption of dietary AGEs and can also aggravate the situation. Owing to their potential adverse effects these alimentary AGEs are also called glycotoxins (Šebeková et al., 2007). AGEs are reported to be present naturally in uncooked foods of animal origin. In addition, thermal processing associated with different cooking methods of modern diet such as roasting, grilling, broiling, searing, and frying induces and also accelerates the formation of newer AGEs (O'Brien et al., 1989; Vlassara et al., 2004; Goldberg et al., 2004; Uribarri et al., 2010). Oral AGEs have been implicated in mounting chronic risk for renal vascular lesion since excretion of orally absorbed glycotoxins is largely suppressed in diabetic nephropathy conditions (Koschinsky et al., 1997). The pronounced acute postprandial vascular dysfunction post consumption of meal with high AGE compared to that of low AGE diet in T2DM patients was also reported (Negrean et al., 2007).

1.3.2. Clearance of AGEs

Catabolism of AGEs and turnover of biomolecules is brought about by cell surface bound AGE clearance receptor complexes via binding endocytosis and degradation. AGE detoxification system is predominantly found in mononuclear/machrophages and also exists in endothelial, mesangial, neuronal and other mesenchymal cells. AGE receptor 1 (AGE-R1) formarly known as oligosaccharyltransferase complex-48 (OST-48) is a main receptor involved in active turn over and negative regulation of inflammatory response mediated by AGEs (Li et al., 1996; Lu et al., 2004). Lowered expression of AGE-R1 was also reported in mononuclear cells in T1DM patients, which was correlated with increased level of serum AGEs (He et al., 2001). Another receptor involved n AGE degradation is AGE-R2, which was co-purified with AGE-R1 (Yang et al., 1991). Yet another member of this complex is AGE receptor R3 (AGE-R3) or galectin-3, which was found to be readily located to cytoplasm and to bind to AGEs with high affinity, on exposure to AGEs (Vlassara et al., 1995). The integral role of AGE-R3 in degradation of AGEs and maintenance of tissue integrity was

also demonstrated by enhanced AGE deposition and development of severe renal disease in mice deficient with AGE-R3 (Pugliese et al., 2001). AGE modification of the biomolecules acts as a signal for their turnover and impaired turnover of these modified entities is implicated in increasing the AGE burden and associated with molecular events of biological ageing and this is pronounced during conditions of diabetes (Vlassara et al., 1985; Radoff et al., 1988; Araki et al., 1992). Macrophage-associated receptors, which bind to AGEs are termed as scavenger receptors (SR) (Sano et al., 1999). There are two types SR-A and SR-B. SR-B1 has been reported to selectively mediate hepatic uptake of high-density lipoprotein cholesteryl ester (HDL-CE) without endocytic uptake of HDL apolipoproteins (Acton et al., 1996) and efflux of cholesterol from peripheral cells to HDL proteins (Chinetti et al., 2000; Ji et al., 1997). CD-36 is also a member of scavenger receptor family for AGEs involved in binding and intracellular AGEs (Ohgami et al., 2001). Even though these receptors are involved in AGE catabolism and turnover, their expression is regulated depending on the type of the tissue or cell and metabolic conditions. They can lead to reactive oxygen species (ROS) production, the release of proinflammatory molecules such as cytokines and growth factors causing cell activation and cell proliferation (Vlassara, 2001).

Yet another vastly studied receptor is are receptor for AGE or RAGE, which is known to be involved in pathways eliciting chronic cellular oxidant stress by binding not only to AGEs but the diverse spectrum of ligands including Ab-amyloid, amphoterin, components of the s100/calgranulin family to name a few. RAGE has been shown to be involved in intracellular signal transduction but not in endocytosis and turnover of the AGE-modified proteins (Mackic et al., 1998; Yan et al., 1999; Schmidt et al 1999). In addition to receptor mediated AGE removal antioxidant and enzymatic detoxification also exist in nature to ameliorate oxidant stress resulting from AGEs. These are namely molecules such as glutathione, enzymes such as aldose reductases, aldehyde dehydrogenases, glyoxalases and also metal ion chelation, since metal ions are involved in accelerating formation of AGEs (Shinohara et al., 1998; Thornalley et al., 1998). Also, it is important to note that part of the tissue derived or dietary AGE degradation

products are excreted in urine, which is hampered during diabetes and renal dysfunction causing retention of AGEs in tissues and circulation (Makita et al., 1994; Makita et al., 1991).

1.4. AGE modification of proteins in autoimmune response

The AGE modification of proteins alters their structure by structural distortion and loss of side chain charge which eventually leads to functional impairment (Ahmed et al., 2005). Glycated proteins show different functionality from native proteins, as well as may also react with other proteins, through crosslinking (Lapolla et al., 2000) and form of protease resistant aggregates (Bansode et al., 2013). Proteins being the functional units of the cell form an intrinsic part of its dynamic network. Their expression, activity and locations can be changed any time in response to the alteration in their structure and this alteration of the structure can elicit an autoimmune response as immune system tends to recognise them as “non self” or neo-self antigens.

1.5. Self tolerance of immune system

Immune system responses are highly remarkable due to high specificity and tight regulation, as it is involved in removal of foreign particles and maintains unresponsiveness to self components under homeostatic conditions (Van Parijs et al., 1998). During maturation of the immune system, immune cells that react against self-tissues are eliminated providing an immune system that is ‘tolerant’ to self. This self tolerance is achieved by the elaborate exposure of self peptides to the lymphoid system followed by removal of autoreactive thymus or bone marrow cells and hence developing B and T lymphocyte “anergy” in the peripheral circulation (Billingham et al., 1953; Kappler et al., 1987; Bretsche et al., 1970; Jenkins et al., 1987; Mueller et al., 1989; Schild et al., 1990). The antigen presenting cells (APCs) phagocytose the neo-self antigens and present them to autoreactive T cells which further leads to activation of cytotoxic T cells and B cells via production of cytokines (Doyle et al., 2012). Major histocompatibility complex (MHC) class I and II also play a key role in presentation of processed peptide antigens to CD8+ cytotoxic T cells and CD4+ T-helper (Th) cells,

respectively (Anderton, 2004; Germain et al., 1993). Cytotoxic T cells as name suggests, cause damage to the host tissue, whereas B cells express receptors on their surface and hence bind to the modified self antigen further differentiating into plasma cells which secrete more antibodies (Doyle et al., 2012; Janeway et al., 2001). In many cases antiself responses are usually generated in the process of mounting an immune response to foreign antigens, but result in autoimmune disease when poorly regulated. (Fairweather et al., 2007).

1.6. Circulating immune complexes (CICs)

The antibodies produced bind to the antigenic determinants which has evoked the immune response forming immune complexes (ICs) in circulation which are also known as circulating immune complexes (CICs). CICs are generally eliminated from the system by phagocytosis (Cornacoff et al., 1983) bringing about neutralization of the antigen. The biological impact and consequences of CICs depends on the nature of antigen and antibody and on the molar ratio of both (Weigle, 1961; Unanue et al., 1967; Cochrane et al., 1973; Haakenstad et al., 1977). The ICs of intermediate size with modest antigen antibody concentration are capable of complement activation by both classical and alternative pathways and further participate in of inflammatory responses (Muller-Eberhard, 1975; Gotze et al., 1976; Theofilopoulos et al., 1980). When they prevail in the system without being cleared off, localize in the vasculature and known to participate in the pathogenesis of complications. Deposition of the ICs along the vascular basement membranes increases the vasculature permeability by increased tissue injury which is caused by the accumulation of leukocytes as a result of complex reactions of complement pathways (Dixon, 1971; Cochrane et al., 1978).

1.7. AGE modification in elicitation of CICs and implications

It has been shown that CML is one of the important glycoxidation product in human and animal tissues, which is the major antigen recognised by the polyclonal anti-AGE antibodies (Reddy et al., 1995). Ikeda et al also showed that, CML contributes to be a major immunological epitope among AGEs (Ikeda et al., 1998). The evidence that AGEs

have antigenic properties lead to the hypothesis that AGE can act as antigen to elicit autoimmune response in diabetic condition. Further, Shibayama et al showed the presence of CML using monoclonal anti-CML antibody and in their subsequent studies demonstrated that CML structures in vivo serve as immunogens and elicit autoantibodies and t the activity of autoantibodies increased with the duration of diabetic status (Shibayama et al., 1999). Circulating autoantibodies binding specifically to reactive oxygen species (ROS) modified glycoxidative human serum albumin (HSA), were reported in serum from diabetic patients with retinopathy, nephropathy and atherosclerosis (Khan et al., 2010). This suggests the pivotal role ROS play in hyperglycemic conditions of diabetes in mediating immunopathogenesis. Another very important plasma protein reported to be glycated and known to elicit autoantibodies is low density lipoprotein (LDL) (Virella et al., 2003). These autoantibodies generated bind to the modified LDL leading to the formation of CICs. The elevated levels of CICs are shown to be associated with progression of retinopathy in T1DM patients (Virella et al., 2012). The CICs with modified LDL are in fact proinflammatory in nature are said to be diagnostic and prognostic biomarker for atherosclerosis in T1DM patients (Orekhov et al., 2014). CICs containing AGEs and antibodies are reported to get deposited in the glomerular basement membrane and hence participate in the pathogenesis of diabetic nephropathy (Velez, 2012). Thus the AGEs and CICs formed by the autoantibodies directed against AGE modified proteins may be involved in pathogenesis of various diabetic complications (Figure 1.4) (Bhat et al., 2014).

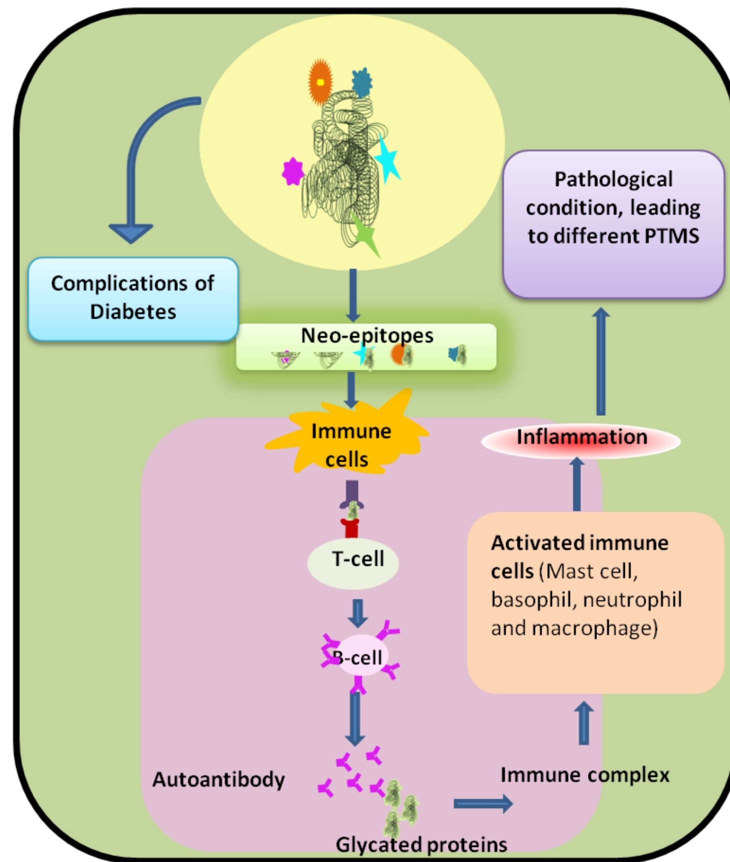


Figure 1.4 AGE modified proteins in CICs and their involvement in pathogenesis. (Adapted and modified from Bhat et al., 2014)

1.8.1. Isolation and characterization of CICs as an analytical approach for identification of new antigens

The significance and clinical use of CICs measurements in determining prognosis and monitoring patients with various autoimmune diseases is tremendous. For example, in patients with rheumatoid arthritis (RA), the presence of elevated levels of ICs are used as differentiating factor for RA from other inflammatory joint diseases, and also to find out severity of the disease (Luthra et al., 1975; Zubler et al., 1976; Nydegger et al., 1977). Classical methods of IC assays were based on the specific binding properties of Clq, Staphylococcus A, and cells with Fc and C receptors, which facilitate isolation of ICs (Svehag et al., 1976; Chenais et al., 1977; Heimer et al., 1978; Tucker et al., 1978; Casali

et al., 1979). Further based on these principles, biochemical assays and affinity chromatography techniques were developed to isolate and concentrate ICs and also for the separation of antigens and production of antiserum (Theofilopoulos et al., 1978; Natali et al., 1980; Zhao et al., 2008). Another method developed to analyse ICs, historically and widely used till date is polyethylene glycol precipitation (PEG) precipitation followed by enzyme linked immune sorbent assay (ELISA) (Ohlson et al., 1985; Tertov et al., 1990; Turk et al., 2002; Sobenin et al., 2013; Sabarinath et al., 2015; Hörl et al., 2016). The disadvantages of methods like C1q ELISA is that C1q must be present and accessible for isolation of ICs and in case of PEG purification, considerable amount of non IC-related proteins such as albumin, haptoglobin and α1-antitrypsin will also be precipitated (Robinson et al., 1989).

1.8.2. Proteomics and mass spectrometry for the analysis of CICs

With the advent of modern proteomic technologies, immunoprecipitation (IP) using protein G sepharose and/or PEG precipitation followed by 1 dimensional (1D) or 2 dimensional (2D) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and high resolution mass spectrometry (HRMS) approaches are being used for analysis of ICs and identification of IC associated new antigens in autoimmune diseases like RA and multiple sclerosis (Steendam et al., 2010; Srivastava et al., 2012). The drawback of isolation of CICs and associated antigens by IP using protein G is purification of free immunoglobulins along with ICs. However this can be overcome to certain extent by more sensitive detection approach like immunodetection and mass spectrometric characterization of antigens which will be of low abundance in isolated CICs. Proteomic technologies also offer a great opportunity not only for the identification of newer antigens but also to characterize post translationally modified antigens eliciting autoimmune response and formation of CICs (Cantin et al., 2004; Jensen et al., 2004; Wu et al., 2009; Steendam et al., 2010). The challenging task is identification and characterization of antigens with PTM that are evoking immune response and also not only single PTM but set of PTMs in the proteome can elicit

autoimmune response. Therefore it is of greater advantage to study the entire proteome with modifications. Quantitative label free liquid chromatography mass spectrometry (LC-MS) in combination with analytical methods to purify CICs, offers a great potential to identify as well as quantitate the novel autoantigens associated with CICs in the body fluids (Merl et al., 2013), which perhaps will provide better insights and aid in disease diagnosis as well as management.

1.9. Genesis and organization of thesis

Even though formation of AGE modification is an inevitable process during biological ageing in living organisms, the rate of formation is accelerated during the persistent hyperglycaemic conditions of all types of diabetes. Despite new therapeutic approaches hyperglycemia induced AGE modifications continues to play pivotal role in the pathogenesis of diabetic complications by causing elevated oxidant stress and also by interacting with RAGE. Yet another remarkable route through which AGE modification is involved in pathogenesis is, it renders the modified proteins immunogenic in nature, which are further recognised by the host immune system as “neoepitopes” or “non self” eventually generating autoantibodies. The antibodies generated against the AGE modified proteins bind to them forming CICs. The CICs containing AGE modified proteins have been implicated in pathogenesis of diabetic complications and comprehensive characterization of CICs in diabetic conditions can lead to identification of AGE modified proteins acting as autoantigens and involved in elicitation of immune response. Biomarkers can be measured in tissue or in body fluids like blood plasma, urine and are advantageous if protein profiles can be obtained from the body fluids that are collected using minimally invasive methodologies. The paradigm is that the disease state will change either the spectrum or the amount of modified protein. Hence identification and characterization of AGE modified proteins associated with CICs ideally can be disease-associated biomarkers and, consequently, proteomic analysis of plasma CICs should reveal diagnostic markers. Hence we decided to analyse CICs in plasma from clinical diabetes and healthy normal subjects using proteomic

approaches. Further to confirm the role of glycation in formation of CICs we studied the plasma CICs in streptozotocin (STZ) induced diabetic mice with or without treatment of prototype AGE inhibitor Aminoguanidine (AMG). Furthermore, immunization of antigens is reported in the development of autoimmune responses in some cases of autoimmune diseases and also immunization of animals with AGE modified proteins is implicated to protect against diabetic complications such as nephropathy. Here we evaluated the effects of immunization of CML modified mouse serum albumin on the level of glycation of haemoglobin, albumin glycation and level of albumin in plasma CICs and total plasma albumin levels in normal mice.

Major objectives of the thesis are

- Identification and characterization of AGE modified proteins in CICs from clinical diabetic plasma
- Evaluation of role of glycation in generation of autoantibodies and formation of CICs.
- To study the effects of immunization with AGE modified self protein.

Thesis is organized in the following manner

Chapter 1: Introduction

Chapter 2: Identification and characterization of glycated proteins acting as autoantigens in CICs of clinical plasma: A proteomic approach.

Chapter 3: Evaluation of role of glycation in autoimmune response and formation of CICs using AGE inhibitor AMG in mouse model.

Chapter 4: Reactive immunization of mice with AGE-modified mouse serum albumin to understand immune response, its effect on glycation and albumin level.

CHAPTER 2

Identification and characterization of glycated proteins acting as autoantigens in CICs of clinical plasma: A proteomic approach

2.1. Introduction

Formation and progressive accumulation of AGEs are the underlying factor for debilities associated with the normal ageing process. The hyperglycemic condition in diabetes promotes excessive accumulation of AGEs, which contribute to the development of diabetic complications. AGE modification of the proteins changes the chemical composition of proteins thereby by causing structural deformations. Glycation of proteins is implicated in decreased ligand binding and altered functions of proteins. For example glycation of albumin decreases its affinity for binding bilirubin and long chain fatty acids (Shaklai et al., 1984). Arginine directed modification of HSA resulted in loss of positive charge due to hydroimidazolone formation, which eventually leads to functional defects such as inhibition of binding of various ligands and albumin associated esterase activity (Ahmed et al., 2005). Further, it was also observed that AGE modification of the proteins renders them resistant to proteolytic activity and functional activity of these protease resistant proteins (PRPs) was also decreased in kidney proteins from diabetic rats (Bansode et al., 2013). In addition, these chemically modified structures in proteins can act as neo-self antigens or autoantigens, which can then lead to the generation of autoantibodies (Araki et al., 1992, Virella et al., 2003). The complex entities formed from the autoantigens and corresponding autoantibodies generally referred to as CICs. CICs were observed to negatively impact the *in vivo* determination and quantification of the AGE antibody titre (Turk et al., 2001). Further it is also reported that the immunoglobulin G type of antibodies constitute the major type of antibodies generated for AGE modified proteins (Shibayama et al., 1999). Sera from children with T1M were also shown to possess significantly increased levels of IgG in the

CICs and also increased levels correlated with the early diabetic nephropathy (Nicoloff et al., 2004).

Proteomic analysis of CICs aims to identify and characterize the proteins responsible for the elicitation of autoimmune response and hence associated with the disease pathology. Previously different groups have analyzed CICs in juvenile idiopathic arthritis by using proteomic approaches which mainly involved IP of IgG immune complexes using protein G sepharose followed by 2 DE for protein separation and identification of proteins using MS (Jason et al., 2009). Further a comprehensive profiling of CICs called as immune complexome analysis was performed, where immune complexome pull down was tryptically digested and proteins were identified by nano-LC-MS in the plasma from RA patients (Ohyama et al., 2011). A similar approach of immune complexome analysis was performed here in this study in the clinical plasma from different stages of diabetes. Isolation of CICs was done using protein G sepharose and then trypsin digestion of the CICs was followed by label free quantification of the associated proteins by using nano-LC MS/ MS. The proteins were also analyzed for AGE modification and characterization by MS and western blotting using anti-AGE antibodies.

2.2. Materials and methods

All the chemicals were procured from Sigma-Aldrich if not stated otherwise. MS Grade solvents and RapiGest (water, acetonitrile (ACN) and methanol) were purchased from J T. Baker (J T. Baker, PA). Primary antibodies used were procured from Abcam (Cambridge, UK) and secondary antibodies conjugated to HRP were from Bangalore Genei, India.

2.2.1. Clinical sample details

Clinical plasma samples were collected from the study subjects from Dr. Mohans' Diabetes Specialities Centre, Chennai, India with informed consent. The study was

performed according to the Helsinki Declaration and also approved by the institutional ethics committee of Madras Diabetes Research Foundation. Exclusion criteria followed before the collection of samples involved known cases of cancer, hematuria, hypothyroidism and a known history of inflammatory diseases or infection. All the clinical diagnostics were carried as per the American Diabetes Association (ADA) criteria. The study comprised of a total of 58 subjects in five clinical groups.

2.2.1.1 Types of clinical sample groups

2.2.1.1a Control subjects (CON, n = 12)

Subjects with normal glucose tolerance formed the control subjects group.

2.2.1.1b Prediabetes or impaired glucose tolerance (IGT, n=12)

Clinical prediabetes is characterized by the impaired glucose tolerance with glycated hemoglobin (HbA1c) in the range of 5.7% to 6.5%, fasting plasma glucose (FPG) of ≥ 100 mg/dl to <126 mg/dl and oral glucose tolerance test (OGTT) of ≥ 140 mg/dl to < 200mg/dl. Subjects fulfilling above criteria were considered in IGT group.

2.2.1.1c Newly diagnosed for T2DM (NDD, n=12)

Subjects who are diagnosed with diabetes with HbA1c ≥ 6.5% who are not on any medication were considered in NDD group.

2.2.1.1d T2DM (DM, n=12)

Patients known to be having T2DM with HbA1c ≥ 6.5%, FBG ≥ 126 mg/dl, and OGTT of ≥ 200 mg/dl, without any secondary complications were recruited for this study group.

2.2.1.1e Diabetes with microalbuminuria (DM-MIC, n=10)

Microalbuminuria (MIC) that is increased urinary albumin excretion through the glomerular filtration is considered to be initial stages of diabetic nephropathy. Subjects with known history of diabetes mellitus and having albumin excretion above 30mg/day were grouped in this category.

2.2.2. Preparation of blood plasma

Plasma was prepared from the peripheral blood collected in ethylenediamminetetraacetate (EDTA) vacutainers (BD Biosciences) followed by incubation at room temperature and centrifugation at 1500g for 15 min. Supernatant of plasma was collected. Biochemical parameters such as fasting blood glucose (FBG), postprandial blood glucose (PBG), glycated haemoglobin (HbA1c), oral glucose tolerance test (OGTT), HDL, very low density lipoprotein (VLDL) lipids, urea, and creatinine were analyzed immediately after the sample collection. Plasma was stored at -80 °C until used. Microalbuminuria complication was assessed by measuring urinary excretion of albumin of 24h. The clinical parameters of the study subjects are given in Table 2.1.

2.2.3. Isolation of CICs in plasma grouped based on Glycation level

Based on the similar HbA1c level of the subject an equal volume of plasma samples was pooled into 3 subgroups. Protein concentration was determined using Bio-Rad Bradford kit (Bio-Rad Laboratories, CA). 400 µg of plasma protein was further used for isolation of the CICs by using Protein G Sepharose. For the preparation of the resin and further experimental steps manufacturer's instructions were followed. Protein from clinical plasma (three subgroups made based on HbA1c, was incubated with 40 µl of protein G Sepharose. The final volume of the reaction mixture was adjusted to 600 µl with 1x IP buffer and incubated for 2 h at 4 °C on rotospin rotary mixer. The supernatant was separated after the incubation, and the beads were washed using 1x IP buffer for five times by centrifuging at 12000 g for 1 min. Complete removal of nonspecifically

interacting proteins was confirmed by measuring the absorption of wash fractions at 280 nm. The elution of column bound CICs fraction was achieved using 100 µl of 0.1% RapiGest (Waters, Milford, MA) in 50 mM ammonium bicarbonate buffer after intermittent vortexing for 20 min, followed by heating at 80 °C for 15 min and centrifugation at 12,000 g for 5 min. Protein estimation was performed using Bio-Rad Bradford kit (Bio-Rad Laboratories, CA).

2.2.4. Negative control experiments

The relevance of association of HSA with CICs and possibility of potential nonspecific binding to protein G sepharose or sepharose was evaluated (Ohyama et al., 2011). 400 µg of clinical CON and DM plasma and physiological concentration of HSA (50 µg/ µl) were incubated with either protein G sepharose or only sepharose column. Incubation and temperature conditions were maintained as used for the purification of plasma CICs. Unbound protein fraction was collected and non specifically bound proteins were washed. The bound fraction was eluted by incubating with 40 µl of Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiling for 5 min. Proteins were visualized after SDS-PAGE.

2.2.5. In-solution trypsin digestion

50 µl of isolated plasma CICs were reduced with 100 mM dithiothreitol at 60 °C for 15 min and alkylated using 200 mM iodoacetamide for 30 min at 25 °C under dark condition. After the denaturation CICs were digested with trypsin (1:20) for 18 h at 37 °C. The digestion reaction was stopped using 100% formic acid. Further the surfactant RapiGest removal was achieved by the addition of formic acid and incubation at 37 °C for 45 min followed by centrifugation at 14,000 g for 15 min. The peptides in the supernatant were collected leaving the precipitated surfactant.

2.2.6. Liquid chromatography-mass spectrometry analysis

Peptide mixture after digestion was diluted in 3% ACN containing 0.1% FA in 1:3 ratio before subjecting to LC-MS^E (liquid chromatography mass spectrometry at elevated energy) analysis. Yeast alcohol dehydrogenase at the final concentration of 100 fmol was spiked along with peptides as an internal standard. Three technical replicates of mass spectra were acquired by using Nano Acquity UPLC system coupled to SYNAPT HDMS (Waters). The binary solvent system comprised 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). An online Symmetry C18 trapping column (internal diameter of 180 µm and length 20mm) (Waters Corporation) was used for preconcentrating and desalting of the injected sample with 0.1% mobile phase B at a flow rate of 5 µl/min. Nano-LC separation was performed using an Ethylene Bridged Hybrid (BEH)-C18 (1.7 µm × 75 µm × 250 mm) column (Waters Corporation) and peptides were eluted into the Nano-Lock Spray ion source using a gradient of 3 to 40% B for 95 min at a flow rate of 250 nl/min. The mass spectrometer was calibrated with MS/MS spectra of Glu-fibrinopeptide B (m/z 785.8426) (500 fmol/µl), and every 30s, the lock mass correction was done by the same peptide. All the MS runs were acquired at a resolution of about 9000 full width half maximum with a scan time of 0.75 s in a mass range of 50–2000 m/z with alternating low (4 eV) and high (15–40 eV) collision energy in a positive V-mode.

2.2.7. Data processing and database searching

Protein Lynx Global Server 2.5.1 (PLGS; Waters Corporation) software was used for analyzing the LC-MS^E data. The identification of the proteins and quantification was performed using reviewed human database (UniProt release 2013_09, 42, 897 entries) downloaded from UniProt). To the database alcohol dehydrogenase 1 (P00330) protein sequence of *Saccharomyces cerevisiae* was included to perform quantification analysis of the proteins. The initial search was carried out where in precursor and product ion tolerance was set to automatic, minimum three numbers of fragment ion matches per

peptide, minimum number of fragment ions matches per protein five, and minimum peptide matches per protein were set to one. Carbamidomethylated Cys (C) residues were set as fixed and Met (M) oxidation as variable modifications along with allowed missed cleavages site two. The false positive rate was 4%. The threshold for ion intensity was kept at 500 counts. The label free quantification was based on the product of femto mole of the internal standard (alcohol dehydrogenase 1) which was spiked and the ratio of the sum of signal intensities of three most abundant peptides of a protein to the sum of the signal intensities of three most abundant peptides of the internal standard. Further based on this estimation of molar amount of the protein injected on the column and the molecular weight information in the database, PLGS software determines the amount of protein in nanograms (Silva et al., 2006). Since the quantification is based on the three high abundant peptides of the proteins it's also called as HI3 label free quantification. Further, microgram of proteins present in CICs per millilitre of plasma was calculated by using dilution factor.

2.2.8. Western Blotting

10 µg of total plasma proteins or 10 µl of the isolated CICs were separated by running on 10% SDS-PAGE. Following the electrophoresis proteins were transferred onto PVDF membrane by using semi dry transfer technique. The free sites on the membranes were blocked by incubating with 5 % skimmed milk (HiMedia, India) in PBS buffer at 4 °C overnight. Blocked membranes were then probed either with anti AGE antibodies (1:1000) or anti serum albumin antibodies (1:5000) for 3h at 25 °C. Both the primary antibodies were procured from Abcam, Cambridge, UK. After the treatment with primary antibody membranes were washed with PBS-T (PBS with 0.05% Tween 20) and two washes with PBS and then incubated with secondary antibody conjugated to HRP (Bangalore Genei, India) at a dilution of 1:5000 for 1 h at 25 °C. The washes were repeated as above and bands were detected by using the WesternBright TM Quantum Western blotting detection kit (Advansta, Menlo Park, CA) by chemiluminescence as per the manufacturer's instructions. Analysis of the detected bands for the interpretation of

quantitative differences of protein or the extent of AGE modification between the experimental groups was performed by Licor Image Studio™ Lite software (Licor Biosciences). The signal from the control set was considered as 1 and the relative fold change in other groups with respect to control was calculated and bar graphs were plotted.

2.2.9. AGE modification analysis by LC-MS^E

HSA identified in clinical CICs was analyzed for identification of AGE modifications as described (Bhonsle et al., 2012). Variable glycation modifications namely Amadori (162.0528) at lysine or arginine, CML (58.0055) and CEL (72.0211) at lysine, MGH1 (54.0106) at arginine, and oxidation at Met and fixed carbamidomethylation of Cys residues were included. Targeted search was performed using ion accounting parameters wherein precursor ion tolerance was set to 300 ppm and product ion tolerance was set at 300 ppm. Ion intensity threshold for precursor was 500 and fragments were 10 counts. Minimum fragment match was set to 3 per peptide and missed cleavages allowed were 2. The false positive rate was set at 1%.

AGE modified peptides identified by the software PLGS were manually validated using following criteria: 1) Peptide should be confidently identified minimum in duplicate MS runs out of the triplicate runs acquired. 2) AGE modified peptides should have a missed cleavage at the modified residue, since the proteolytic enzyme trypsin does not cleave at the modified Arg or Lys residues (Huesgen et al., 2015). 3) There should be control or unmodified peptide with a similar fragment for each modified peptide. 4) The fragment ions should retain the modification and the accurate mass shift corresponding to the modification. 5) if modification is at the N terminus, then presence of b-ions retaining modification and unmodified y-ions; 6) if modification is at the middle position, then presence of b- or y-ions retaining modification; 7) presence of at least a few consecutive b- or y-ions; and 8) presence of complementary b- or y-ions. The

AGE modified peptides fulfilling the above criteria were then processed to remove unmatched noise peaks in the spectrum.

2.2.10. Relative quantification of fluorescent AGEs in plasma

Certain forms of AGEs possess fluorescent properties and exhibit characteristic excitation and emission wavelengths (Butko et al., 2014). Such fluorescent AGEs argpyrimidine, pentosidine, crosline, vesperlysine A or B, vesperlysine C, and imidazolone B, were quantified in the plasma samples (Table 2.2). 10µl of plasma was diluted to 100 µl in phosphate buffered saline (PBS) of composition 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, of pH 7.4 and the relative concentration of AGEs was measured by using fluorescence spectrofluorometer (Thermo, Varioskan Flash Multimode Reader).

2.2.11. Statistical analysis

The experiments were performed in triplicates. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to determine statistical significance. Data are expressed as mean± S.E. p-value <0.05 was considered as statistically significant.

2.3. Results and discussion

2.3.1. Elevated human serum albumin in CICs of plasma from IGT, NDD, and DM-MIC

Hyperglycemia associated with diabetes caused accelerated glycation of the proteins. AGEs are formed even during normal conditions, which is increased with biological ageing and during hyperglycemic conditions of diabetes the process of formation of AGEs is significantly accelerated. AGEs play a significant role in the development of secondary complications of diabetes.

2.3.1a. CICs in the plasma pooled based on glycation level

Hemoglobin is a protein found in erythrocytes of the blood and HbA1c has been used as a diagnostic marker for assessment of glycemic status over preceding three months (Goldstein et al., 1984). HbA1c is worldwide accepted diagnostic tool for diabetes and independent risk factor predicting T2DM (International Expert Committee 2009, David Edelman 2004). Considering these facts pooling of plasma was based on the level of HbA1c to evaluate the role of glycation in the elicitation of autoimmune response and formation of CICs. HSA was predominantly observed to be present in CICs from all the groups. The major finding was elevated HSA in the CICs from the plasma from IGT, NDD and DM-MIC. The bar graph depicting the levels of albumin in CICs from plasma is represented in Figure 2.1.

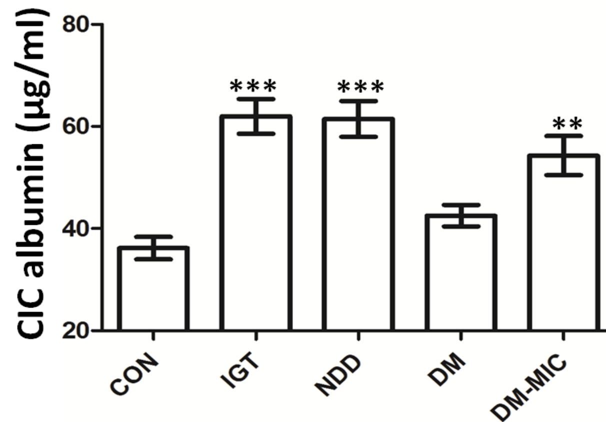


Figure 2.1 Bar graph depicts the quantity of serum albumin in CICs from clinical plasma samples. Label-free-based MS quantification of CICs from clinical CON (n=12), IGT (n=12), DM (n=12), DM-MIC (n=8). Significant difference was calculated by one-way ANOVA and represented at p<0.0001 (***), p<0.001 (**).

Complement system forms an integral part of the immune system (Janeway et al., 2001). Complement proteins are expressed in response to the formation of immune complexes and are known to be associated with immune complexes in the system

(Markiewski et al., 2007). Along with IgGs compliment factors such as C1, C3, and C4 were also identified in our study. The proteins associated with immunoglobulins were also quantified and the calculated fold change is given in Table 2.3.

Serum albumin is the most abundant protein in the blood plasma constituting about 50% of the total plasma proteins in the human blood (Evans et al., 2002). Serum albumin was also reported to get preferentially glycated (Farrugia, 2010; Bhonsle et al., 2008) compared to other plasma proteins. It consists of 59 Lys residues and known to get extensively glycated *in vitro* in plasma (Zhang et al., 2009). Glycated albumin was observed to get increased about two fold in T1DM patients and to increase further more in individuals with nephropathy (Schalkwijk et al., 1999). Glycated albumin was also reported to localize in the glomeruli of diabetic nephropathy patients, which increased with the severity of the damaged tissue (Sakai et al., 1996). Glycated albumin levels were also reported to be increased considerably in T2DM patients with coronary artery disease (Irshad et al., 2012). Conformational changes are introduced by glycation in albumin and increases the formation of amyloid beta cross sheets in it (Bouma et al., 2003), which may then lead to protein cross-linking and aggregation, and hence making it immunogenic. Autoantibodies for glycated albumin were also reported in T1DM (Arif et al., 2012). Circulating autoantibodies showed higher recognition of gluco-oxidatively modified HSA in patients with diabetic complications (Khan et al., 2010). Our findings were in line with previous studies showing an autoimmune response to AGE modified albumin in diabetic plasma and we identified AGE modified HSA associated with CICs which are real time products of the autoimmune response. Western blotting of CICs using anti-serum albumin antibody also showed a similar trend of elevated albumin in CICs from test samples in comparison to that of control (Figure 2.2a). Western blotting with anti-AGE (Figure 2.2b) and MS analysis for the characterization of AGEs also revealed AGE modification of HSA in the CICs. Representative AGE modified MS peptide is shown in Figure 2.3. List of all the AGE modified peptides identified in CICs are given in Supplementary data 2.1.

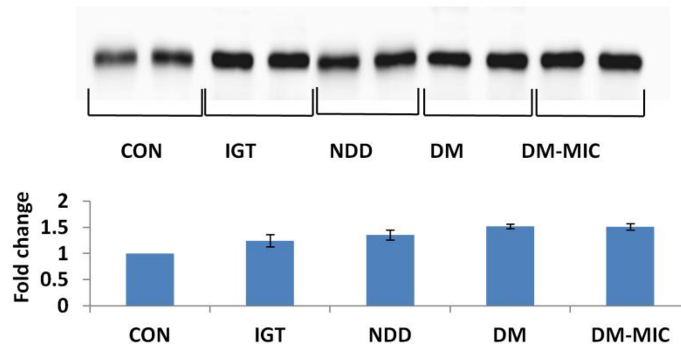


Figure 2.2a Western blotting analysis of CICs using anti-serum albumin antibody to validate mass spectrometric results. Representative blots of two groups from each condition are shown. Bar graph generated and fold change calculated by the antibody signal is represented with respect to control (considered as 1). Values are mean ± S.E.

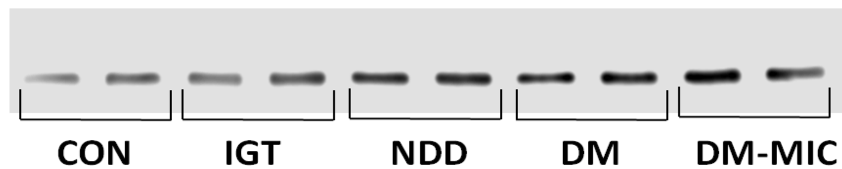


Figure 2.2b Western blotting analysis for AGE modification of serum albumin in CICs, using Anti-AGE antibodies.

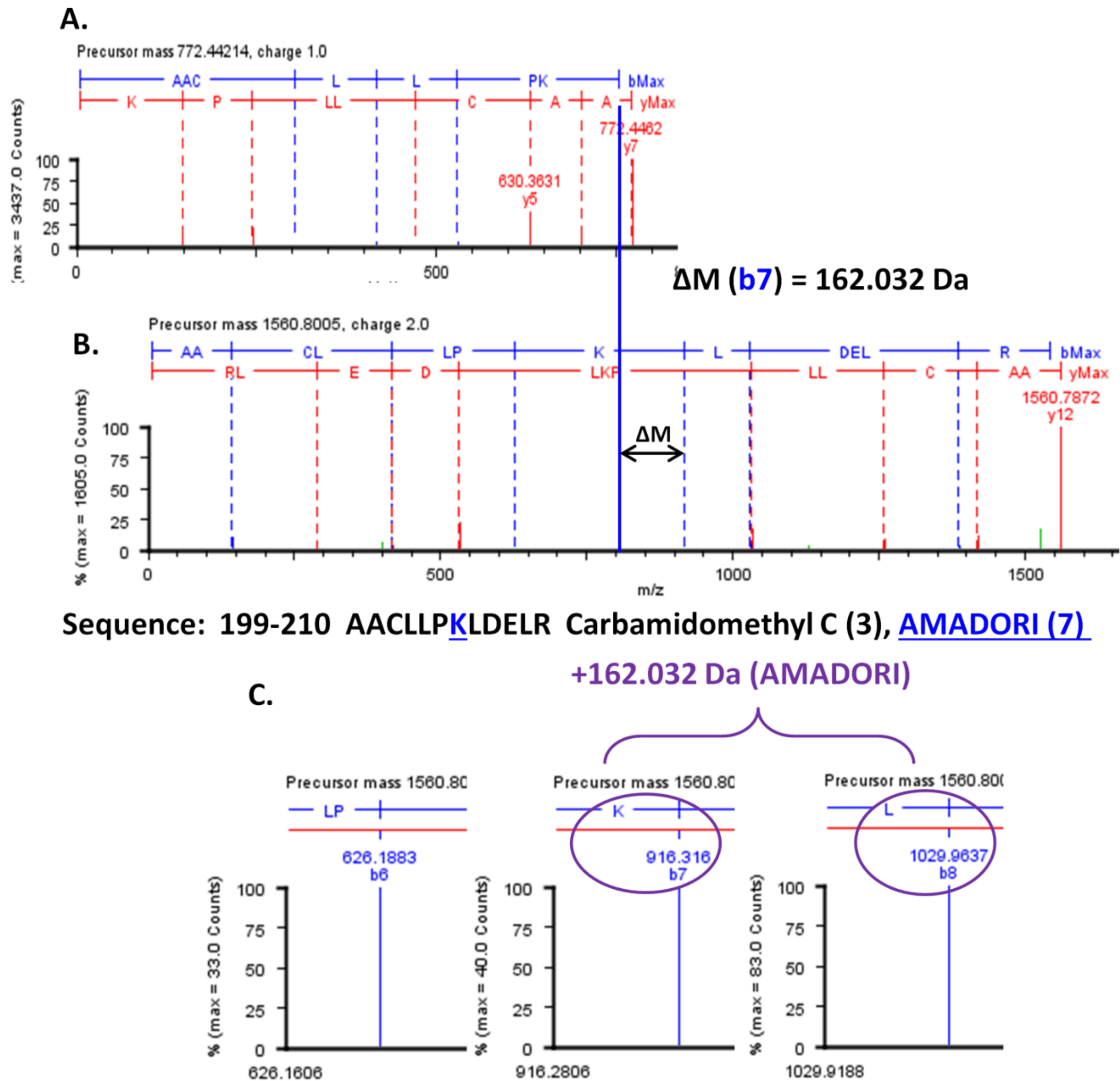


Figure 2.3 manually annotated AGE modified peptide of HSA identified in CICs. Where A is the modified peptide with AMADORI modification at K-205. B is corresponding unmodified peptide sequence and C shows zoomed spectra at modified residue with increase in mass corresponding to modification (AMADORI).

2.4. Specific interaction of HSA with IgGs

Negative control experiments showed that, the HSA was not non specifically interacting with neither sepharose nor protein G, and interaction was specific to antibodies and hence was present in CICs (Figure 2.4).

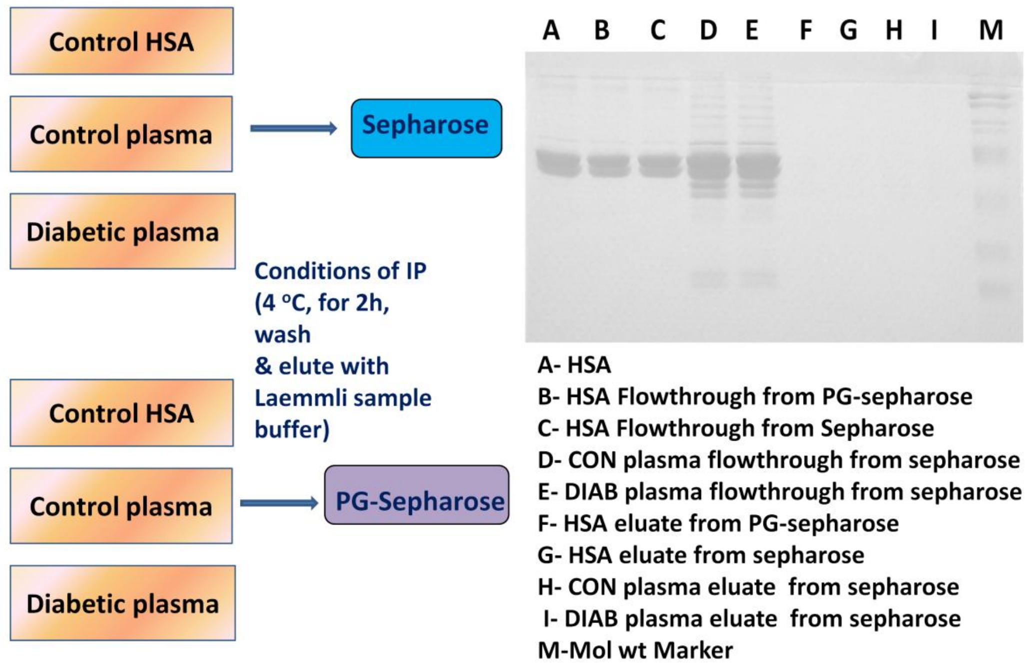


Figure 2.4 Evaluation of potential non specific binding of HSA to sepharose or Protein G-sepharose.

2.5. Plasma AGE analysis revealed increased AGE modification in plasma proteins from IGT, NDD, DM and DM-MIC

AGEs are increased in the plasma and tissue during chronic hyperglycemia and persistent oxidative stress associated with diabetes. Low molecular weight fluorophores which are degradation products of AGE modified proteins in the tissue are even considered as reliable markers of tissue AGE modification (Makita et al., 1991). LMW fluorophores were increased significantly in patients with diabetes, which was further enhanced in diabetic patients with nephropathy (Januszewski et al., 2005). Here we measured fluorescent AGEs and elevated fluorescent AGEs were observed in diabetic plasma and also the DM-MIC when compared to that of control plasma samples (Figure 2.5). Anti-AGE antibody was used in western blotting to detect AGE modified proteins in total plasma separated by 1D SDS PAGE (Figure 2.6). Increased plasma AGEs were detected in IGT, NDD, DM and DM-MIC samples compared to CON in plasma fluorescence as well as western blotting.

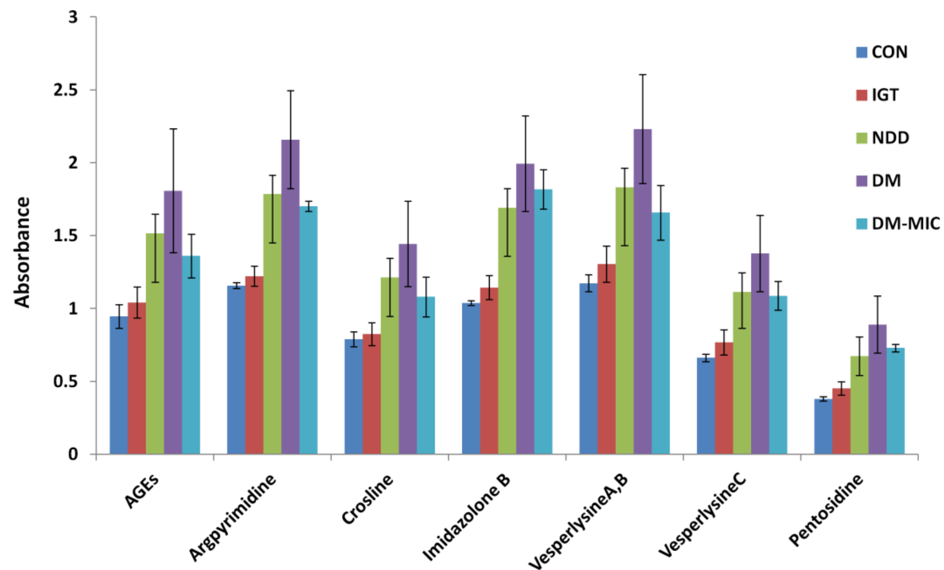


Figure 2.5 Bar graph depicts average intensity of fluorescent AGEs.

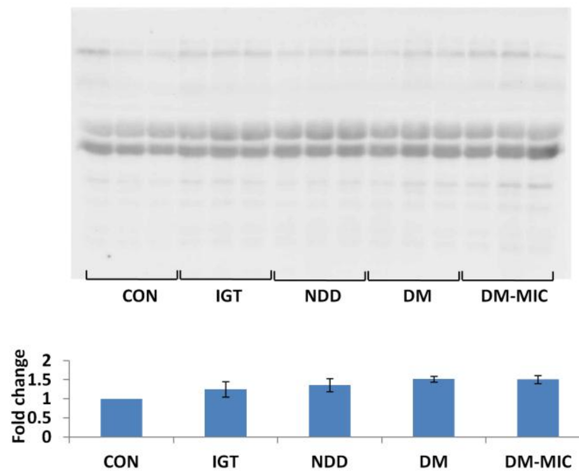


Figure 2.6 Western blotting analysis of total plasma proteins for AGEs in clinical samples. Bar graph represents fold change in intensity of AGE modification deduced from densitometry. The intensity of control is considered as 1. Values are mean ± S.E.

2.6. Conclusion

In conclusion, we could identify that serum albumin is significantly elevated in the CICs in the clinical plasma from IGT, NDD, and DM-MIC and that HSA associated with CICs is also AGE modified. Western blotting analysis was in accordance with the mass spectrometric findings revealing elevated HSA in CICs and AGE modification. Previous studies showed that AGE epitopes on the proteins can act as neo-epitopes eliciting an autoimmune response and this immune response is positively correlated with complications of diabetes and the severity of the disease using mainly techniques like ELISA. However, this is the study where in for the first time CICs in the plasma from different conditions of hyperglycemia were isolated and analyzed comprehensively using immunoaffinity pull down by protein G sepharose IP and proteomic technology for identification, label free quantification and characterization. HSA was characterized to be AGE modified in the CICs and it was slightly but significantly elevated in the plasma CICs from IGT, NDD and DM-MIC.

Table 2.2 Characteristics of fluorescent AGE modifications.

Sl. No.	Type of AGE modification	Ex/Em (nm)
1.	AGEs	370/440
2.	Argpyrimidine	320/382
3.	Crosline	379/463
4.	Imidazolone B	320/398
5.	Vesperlysine A or B	366/442
6.	Vesperlysine C	345/405
7.	Pentosidine	335/385

Table 2.3 Label free quantification data of proteins identified in CICs of clinical samples.

Sl. No.	Accession No	Protein Name	Fold change			
			Prediab Vs Cont	Newdiab Vs Cont	Diab Vs Cont	Diab-MIC Vs Cont
1	A0M8Q6	Ig lambda 7 chain C	0.7	1.2	0.9	0.9
2	B9A064	Immunoglobulin lambda like polypeptide 5 OS Homo sapiens GN IGLL5 PE 2 SV 2	1.23	1.1	1.2	0.9
4	P01024	Complement C3 OS Homo sapiens GN C3 PE 1 SV 2	2.6	1.9	1.4	0.8
5	P01593	Ig kappa chain V I region AG OS Homo sapiens PE 1 SV 1	1.3	1.5	1.1	0.9
6	P01609	Ig kappa chain V I region Scw OS Homo sapiens PE 1 SV 1	0.9	1.5	0.9	1.0
7.	P01611	Ig kappa chain V I region Wes OS Homo sapiens PE 1 SV 1	1.0	1.1	0.9	0.9
8.	P01614	Ig kappa chain V II region Cum OS Homo sapiens PE 1 SV 1	0.5	1.1	0.5	0.9
9	P01617	Ig kappa chain V II region TEW OS Homo sapiens PE 1 SV 1	1.2	1.1	1.0	0.9

10	P01620	Ig kappa chain V III region SIE OS Homo sapiens PE 1 SV 1	1.4	0.8	0.9	0.7
11	P01622	Ig kappa chain V III region Ti OS Homo sapiens PE 1 SV 1	0.9	1.7	1.5	1.3
12	P01714	Ig lambda chain V III region SH OS Homo sapiens PE 1 SV 1	1.3	1.1	1.2	1.0
13	P01717	Ig lambda chain V IV region Hil OS Homo sapiens PE 1 SV 1	1.3	1.2	1.5	0.9
14	P01763	Ig heavy chain V III region WEA OS Homo sapiens PE 1 SV 1	1.3	1.4	1.1	1.2
15	P01764	Ig heavy chain V III region VH26 OS Homo sapiens PE 1 SV 1	1.1	1.3	0.9	1.0
16	P01765	Ig heavy chain V III region TIL OS Homo sapiens PE 1 SV 1	5.0	6.4	8.0	4.0
17	P01766	Ig heavy chain V III region BRO OS Homo sapiens PE 1 SV 1	1.2	1.2	1.0	0.9
18	P01767	Ig heavy chain V III region BUT OS Homo sapiens PE 1 SV 1	1.0	1.1	1.1	0.8
19	P01774	Ig heavy chain V III region POM OS Homo sapiens PE	1.6	1.8	1.0	1.5

		1 SV 1				
20	P01777	Ig heavy chain V III region TEI OS Homo sapiens PE 1 SV 1	1.1	1.1	1.1	0.6
21	P01779	Ig heavy chain V III region TUR OS Homo sapiens PE 1 SV 1	0.7	0.5	0.6	0.6
22	P01781	Ig heavy chain V III region GAL OS Homo sapiens PE 1 SV 1	1.3	1.5	1.0	0.9
23	P01834	Ig kappa chain C region OS Homo sapiens GN IGKC PE 1 SV 1	1.0	1.2	1.0	1.0
24	P01857	Ig gamma 1 chain C region OS Homo sapiens GN IGHG1 PE 1 SV 1	1.1	1.0	1.1	0.9
25	P01859	Ig gamma 2 chain C region OS Homo sapiens GN IGHG2 PE 1 SV 2	0.9	1.4	1.4	1.0
26	P01860	Ig gamma 3 chain C region OS Homo sapiens GN IGHG3 PE 1 SV 2	1.1	1.4	1.0	1.0
27	P01861	Ig gamma 4 chain C region OS Homo sapiens GN IGHG4 PE 1 SV 1	1.2	1.6	1.4	0.5
28	P01871	Ig mu chain C region OS Homo sapiens GN IGHM PE 1 SV 3	1.5	0.8	0.8	0.5
29	P01876	Ig alpha 1 chain C region OS	0.8	1.1	1.0	1.0

		Homo sapiens GN IGHA1 PE 1 SV 2				
30	P02747	Complement C1q subcomponent subunit C OS Homo sapiens GN C1QC PE 1 SV 3	0.8	0.9	0.9	0.7
31	P02768	Serum albumin OS Homo sapiens GN ALB PE 1 SV 2	1.7	1.7	1.2	1.5
32	P04206	Ig kappa chain V III region GOL OS Homo sapiens PE 1 SV 1	0.9	0.6	1.0	1.2
33	P04208	Ig lambda chain V I region WAH OS Homo sapiens PE 1 SV 1	1.0	0.8	0.9	0.5
34	P04220	Ig mu heavy chain disease protein OS Homo sapiens PE 1 SV 1	1.8	1.3	1.6	--
35	P04430	Ig kappa chain V I region BAN OS Homo sapiens PE 1 SV 1	1.2	1.2	0.8	1.0
36	P04433	Ig kappa chain V III region VG Fragment OS Homo sapiens PE 1 SV 1	0.9	0.9	1.0	0.7
37	P06312	Ig kappa chain V IV region Fragment OS Homo sapiens GN IGKV4 1 PE 4 SV 1	1.9	1.2	1.2	2.3
38	P18135	Ig kappa chain V III region HAH OS Homo sapiens PE 2 SV 1	1.5	0.7	0.4	0.3

39	P23083	Ig heavy chain V I region V35 OS Homo sapiens PE 1 SV 1	1.6	1.2	1.2	1.0
40	P80748	Ig lambda chain V III region LOI OS Homo sapiens PE 1 SV 1	1.2	1.3	1.4	0.9

CHAPTER 3

Evaluation of role of glycation in autoimmune response and formation of CICs using AGE inhibitor AMG in mouse model

3.1. Introduction

AGE modification of the proteins impairs the structural integrity of the proteins, which leads to the formation of neo-epitopes in the proteins rendering them autoantigens. Such neo-epitopes have danger-associated molecular patterns (DAMPs), which are recognized by the various pattern recognition receptors (PRRs) (Bianchi, 2007). This interaction further activates proinflammatory pathways eliciting autoantibodies leading to microvascular complications of diabetes. For better understanding of the molecular mechanisms of a disease, appropriate experimental models are necessary. In biomedical research animal models have been extensively used, since the same research in human beings is impeded mainly by the ethical restrictions. Also pathogenesis of the disease and therapeutic intervention strategies can be conveniently studied in animal models. Especially among the animal models being used, rodent models have extensively been used since the inducible pathology resembles that of humans, as well as due to their short generation time, economic considerations and small size they are convenient for experimental use. Among rodents inbred strains such as BALB/c mice are widely used because of the genetic uniformity which leads to lesser phenotypic variation (Jensen et al., 2016). Further STZ is a toxic glucose analog extensively used in diabetes research owing to its diabetogenic property. It is taken up by the pancreatic beta cells by glucose transporter GLUT 2 (Wang et al., 1998) and inside the cells it alkylates deoxyribo nucleic acid (DNA) and destroys the beta cells causing decreased or absolute inhibition of insulin production (Lenzen, 2008) which leads to persistent hyperglycemia mimicking conditions of diabetes. Inhibition of the AGE formation is one molecular target in preventing AGE mediated pathogenesis. The nucleophilic hydrazine compound AMG is a prototype AGE inhibitor and most

extensively investigated molecule for its AGE inhibiting properties and its efficacy (Brownlee M et al., 1986; Liparota et al., 1991; Corbett et al., 1992; Li et al., 1996; Corman et al., 1998). AMG prevents the formation of AGEs by selectively blocking reactive carbonyls on ketoamine products and on their derivatives such as 3-DG and glycolaldehyde (Edelstein et al., 1992).

Following our initial observations in the clinical experiments, in this study we evaluated the role of glycation and AGE modification in the elicitation of autoimmune response and formation of CICs in STZ induced diabetic mice BALB/c in presence or absence of the treatment with AGE inhibitor AMG. CICs were isolated and quantified by label free quantification method using nano LC-MS/MS. Cytokines were measured by using a multiplex assay, in plasma of control and diabetic mice treated with or without AMG to understand overall proinflammatory status in the hyperglycemia induced AGE formation and autoimmune response.

3.2. Materials and methods

All the reagent materials were procured from Sigma-Aldrich if not mentioned otherwise.

3.2.1. Mice sample details

The animal experiments were performed at the Experimental Animal Facility, National Centre for Cell Sciences, India. The guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCSEAI), and approved by Institutional Animal Ethics committee were followed in all the experiments. Diabetes was induced in BALB/c mice following the protocol as described in the literature (Kesavan et al., 2013). Male BALB/c mice with an average weight of 20g were selected. Following overnight fasting mice were injected with 50 mg/kg body weight of STZ in 50 mM citrate buffer, pH 4.5. The area near the mid ventral region was cleaned with an alcohol swab and 1 ml insulin syringe (26 gauge) was used for intraperitoneal injections. A similar regime was followed for five consecutive days to induce

hyperglycemia. Control mice (CON) were injected with 50mM citrate buffer in a similar fashion. The blood glucose levels were monitored with a glucometer (Bayer, Germany) to confirm the induction of diabetes. The animals with a blood glucose of 6.12 ± 1.7 mmol/l were considered diabetic. The glycation was monitored by measuring HbA1c using Nycocard HbA1c analyzer. Further, the diabetic animals were treated either with or without glycation inhibitor AMG at a dose of 1 g/l of drinking water for about 60 days after induction of diabetes. Diabetic groups were labeled DIAB and treated with AMG as DIAB-AMG. The body weight, blood glucose, and HbA1c of the animals were recorded. At the termination of the study, blood samples were collected in sterile tubes containing EDTA and plasma was obtained by centrifugation for 10 min at 1500 g. Mice were sacrificed by cervical dislocation and the organs were collected in liquid nitrogen by snap freezing after a brief washing with PBS and then stored at -80 °C.

3.2.2. Isolation of CICs from mouse plasma

The protein concentration of the plasma was determined using Bio-Rad Bradford kit (Bio-Rad Laboratories, CA). 400 µg of plasma protein from CON, DIAB, DIAB-AMG groups was processed to isolate the CICs using protein G sepharose resin prepared according to manufacturer's instructions. Final reaction volume was made up to 600 µl using 1x IP buffer supplied and it was incubated at 4 °C for 2h on rotospin rotary mixer with constant rotation. After the incubation the supernatant was discarded and 5 washes were given with 1x IP buffer by centrifuging at 12,000 g for 1 min. Absorption of the wash fractions at 280nm was monitored for the complete removal of non specific interaction of unbound proteins. The bound CICs were eluted with 100 µl of 0.1% RapiGest (Waters, Milford, MA) in 50 mM ammonium bicarbonate buffer with brief intermittent vortexing for 20 min and heating at 80 °C for 15 min. The eluate was collected in fresh tubes by centrifugation at 12,000 g for 5 min. The isolated CICs were further processed for either MS analysis or western blotting.

3.2.3. Relative quantification of fluorescent AGEs in plasma

Fluorescent AGEs argpyrimidine, pentosidine, crosline, vesperlysine A or B, vesperlysine C, and imidazolone B, were quantified in the diluted mouse plasma samples using fluorescence spectrofluorometer (Thermo, Varioskan Flash Multimode Reader) as described in the previous section (**II.2.10.**).

3.2.4. Western blotting

10 µg of the plasma proteins or 10 µl of purified CICs were separated by 10% SDS-PAGE and transferred using semi dry blotting technique onto Polyvinylidene fluride (PVDF) membrane. The protein transferred membranes were incubated overnight at 4 °C in blocking buffer containing 5% skimmed milk in PBS. Following day the free sites blocked membranes were incubated with anti-AGE antibody (Abcam, Cambridge, UK) in 1:1000 dilution 3h at 25 °C or with anti-serum albumin antibodies (Abcam, Cambridge, UK) for 1h at 25 °C. After this step membranes were washed once with PBS-T (PBS with 0.05% Tween 20) and then twice with PBS. Further, incubated with secondary antibody conjugated to HRP procured from Bangalore Genei, India. Antibody dilution of 1:5000 was used for 1 h at 25 °C for incubation. The bands were detected by chemiluminescence phenomena with WesternBright TM Quantum Western blotting detection kit (Advansta, Menlo Park, CA) by as per the manufacturer's instructions after washing of the membrane as above. Quantification of the detected bands for AGE modification between the sample groups was carried out by using Licor Image Studio™ Lite software (Licor Biosciences). The relative fold change of the experimental groups with respect to control considered as 1 was calculated and the results are depicted in a bar graph.

3.2.5. AGE modification analysis and Label free quantification of CIC associated proteins by LC-MS^E

3.2.5a. In-solution trypsin digestion

10 µg of plasma proteins or 50 µl of CICs were subjected to in-solution trypsin digestion. 10 µg of plasma protein was solubilized in 10 µl of 0.1% RapiGest dissolved in 50mM NH_4HCO_3 buffer to facilitate effective proteolytic cleavage. The reaction mixture containing proteins was then subjected to reduction and alkylation with 100 mM dithiothreitol at 60 °C for 15 min and 200 mM iodoacetamide for 30 min at 25 °C under dark condition. Trypsin digestion was brought about by adding the enzyme in 1:20 ratio and incubating at 37 °C for 18h. The digestion reaction was stopped with 100% formic acid and the surfactant RapiGest was precipitated by incubating with acidic pH at 37 °C for 45 min followed by centrifugation at 14000 g for 15 min. The precipitated surfactant was discarded and the digested peptide supernatant was collected.

3.2.5b. Liquid chromatography-mass spectrometry analysis

Peptide mixture was diluted in 3% ACN with 0.1% FA in 1:3 ratio before subjecting to LC-MS^E analysis by using Nano Acquity UPLC system coupled to SYNAPT HDMS (Waters). Yeast alcohol dehydrogenase at the final concentration of 100 fmol was spiked along with peptides as an internal standard. Three technical replicates of mass spectra were acquired for each sample. The binary solvent system comprised 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). An online Symmetry C18 trapping column (internal diameter of 180 µm and length 20mm) (Waters Corporation) was used for pre concentrating and desalting of the injected sample with 0.1% mobile phase B at a flow rate of 5 µl/min. Nano-LC separation was performed using an Ethylene Bridged Hybrid (BEH)-C18 (1.7 µm × 75 µm × 250 mm) column (Waters Corporation) and peptides were eluted into the Nano-Lock Spray ion source using a gradient of 3 to 40% B for 95 min at a flow rate of 250 nl/min. The mass spectrometer was calibrated with MS/MS spectra of Glu-fibrinopeptide B (m/z 785.8426) (500 fmol/µl), and every 30s, the lock mass correction

was done by the same peptide. All the MS runs were acquired at a resolution of about 9000 full width half maximum with a scan time of 0.75 s in a mass range of 50–2000 m/z with alternating low (4 eV) and high (15–40 eV) collision energy in a positive V-mode.

3.2.5c. Data processing and database searching

The acquired LC-MS^E data was analyzed using PLGS (Waters Corporation) software. Mouse database (UniProt release 2014_08, 17,023 entries) downloaded from UniProt, to which alcohol dehydrogenase 1 (P00330) protein sequence of *Saccharomyces cerevisiae* appended was used for identification and quantification of the proteins. Alcohol dehydrogenase 1 sequence was added to databank since a known amount of the protein was spiked as an internal standard for quantification of the identified proteins. During the analysis precursor and product ion tolerance were set to automatic. Minimum fragment ion matches per peptide were set to three, a minimum of five fragment ion matches per protein and minimum one peptide match was set per protein. Fixed carbamidomethylation and variable oxidation modifications were set at Cys (C) and Met (M) respectively. The false positive rate was set to 4% with two allowed missed cleavages and ion intensity threshold was set to 500 counts. Labe free quantification was performed as described previously (Silva et al 2006). The quantification data of the individual proteins was further used to calculate the microgram of proteins in CICs per milliliter of the blood plasma.

3.2.5d Relative quantification of AGEs in mouse plasma albumin and identification of AGE modified peptides in plasma CIC albumin

Mouse serum albumin (MSA) from plasma CICs was analyzed for identification of AGE modified peptides and plasma albumin for the relative quantification of AGEs between the experimental groups. The analysis was carried out as described previously (Bhonsle et al., 2012). Variable glycation modifications namely amadori (162.0528) at lysine or arginine, CML (58.0055) and CEL (72.0211) at lysine, (MGH1) (54.0106) at arginine, and oxidation at Met and fixed carbamidomethylation of Cys residues were

included during the search. Precursor ion tolerance was set to 300 ppm and product ion tolerance was set at 300 ppm in the targeted search performed. Ion intensity threshold 500 for precursor and 10 counts fragments intensity threshold was set along with 2 missed cleavages. The false positive rate was set at 1%. AGE modified peptides identified by the software PLGS were manually validated with set criteria (section II.2.9). The intensity of the AGE modified peptides was considered for the relative quantification of AGEs. Highest total ion count (TIC) across all the mass spectral acquisitions was used to normalize the intensities to rule out the possibility of experimental variation. Peptides consistently observed in two replications out of triplicate acquisitions with matching RT were considered for the final calculation of average intensity and the heatmap generation.

3.2.6. Measurement of total plasma albumin

Total plasma albumin was measured using Bromocresol green (BCG) method by Innoline^{TM} Albumin, Merck, NJ, USA kit according to manufacturer's instructions. The absorption was measured at 610 nm. The color intensity formed is directly proportional to the albumin concentration in the sample.

3.2.7. Quantification of cytokines

We analyzed the panel of cytokines using a multiplex assay. The levels of eight cytokines (IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-gamma, and TNF-alpha) were measured in plasma from mice using the Bio-Plex Pro Mouse Cytokine Group I Panel 8-Plex (Bio-Rad). The assay principle is similar to that of sandwich ELISA wherein magnetic beads or the microspheres are fluorescently dyed, each with a distinct color code or spectral identity facilitating the simultaneous detection of different molecules (multiplex) in a suspension. The beads are coupled with capture antibodies specific for the molecules of interest. These antibodies interact and bind to the molecule of interest in the sample. After several washes to remove unbound molecules, the biotinylated detection antibody is added which creates a sandwich complex. The detection is

brought about by the addition of streptavidin-phycoerythrin (SA-PE) conjugate which binds specifically to biotin. Phycoerythrin acts as a fluorescent reporter molecule. The concentration of the analyte is directly proportional to the fluorescence signal of the reporter molecule. The assay was performed according to manufacturer's instructions. Briefly, 50 µl of the solution containing microbeads coupled with antibodies specific for each of the cytokines to be measured was added to each well. Three washes were given with 100 µl of Bio-Plex wash buffer (wash step). 50 µl of plasma aliquots were diluted to 1:4 in Bio-Plex sample diluents and was added to each well and incubated for 30 min at 25 °C with constant shaking at 800 rpm. After the wash step as above, 25 µl of the detection antibody premix were added and incubated for 30 min at 25 °C followed by incubation with 50 µl of the streptavidin–phycoerythrin solution for 10 min at 25 °C. After another wash step, the beads were resuspended with 125 µl of the assay buffer. The signal was recorded using a Bio-Plex MAGPIX (Bio-Rad) multiplex reader. A standard curve was generated simultaneously for each cytokine and was used to determine the concentration of the cytokines in the plasma samples using Bio-Plex Manager version 6.2 software.

3.2.7. Statistical analysis

All experiments were carried out in triplicates. Statistical significance was determined by ANOVA followed by Tukey's multiple comparison tests was used to determine statistical significance. Data are expressed as mean± S.E. A *p*- value <0.05 was considered as statistically significant.

3.3. Results and discussion

Chronic hyperglycemia is the hallmark of diabetes leading to the formation of AGEs and is known to be the primary causal factor for vascular deterioration in the micro and macrovascular complications associated with diabetes (Brownlee et al., 1988). The role of glycation and the AGEs in the elicitation of immune response and

formation of CICs was evaluated in the diabetic mice model treated with or without AGE inhibitor AMG.

3.3.1. Physiological data

Diabetic animals showed a marked increase in the level of blood glucose and HbA1c in comparison to control animals. In addition, polydipsia and polyuria developed in diabetic animals, which was evident by the observation of water cans and the visible cage wetting. Further, AMG treated diabetic animals displayed decreased HbA1c but hyperglycemia persisted compared to control animals. Diabetic animals also showed a decrease in body weight compared to the control animals. The physiological data at the end of the study are shown in Table 3.1.

3.3.2. Diabetic mouse plasma revealed elevated plasma AGEs compared to control, and AMG treatment decreased the formation AGEs in diabetic mice

The plasma AGEs were studied by using three different approaches to determine the variance of AGE abundance in plasma of diabetic mice compared to that of control and the AGE inhibiting effect of AMG treatment on diabetic mice.

1. Relative quantification of fluorescent AGEs.
2. Western blotting analysis using anti-AGE antibodies.
3. Mass spectrometric quantification of AGEs in mouse plasma albumin.

STZ diabetic mice showed increased plasma AGEs as evident by all the three experimental approaches. The fluorescent AGEs, argpyrimidine, crosline, imidazolone B, Vasperlysin A or B, vesperlysin C and pentosidine showed significant increase in diabetic mice plasma and were found to be decreased in plasma from diabetic animals treated with AMG (Figure 3.1).

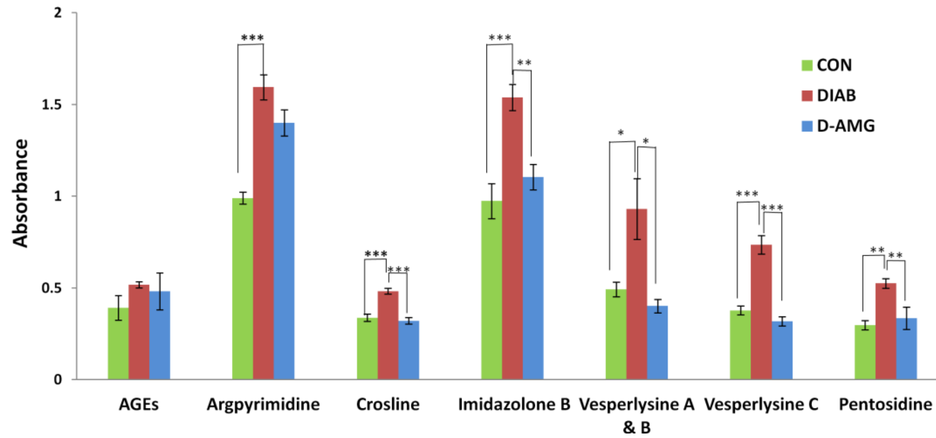


Figure 3.1 Bar graph depicting average intensity of fluorescent AGEs in mice plasma.

Our observations are in agreement with the previous studies which reported argpyrimidine, crossline, pentosidine, imidazolones in the diabetic animals (Baba et al., 2009; Bidasee et al., 2004). Western blotting analysis with anti-AGE antibodies and the mass spectrometric analysis of AGE modification in plasma albumin also revealed increased AGE modification of serum albumin from diabetic plasma, which was decreased in diabetic mice treated with AMG. Collectively these observations suggested that the AGEs were elevated considerably in the diabetic mice and the treatment of diabetic mice with AMG reduced the AGE levels as expected. The western blot and the heatmap generated from the intensities of AGE modified peptides from serum albumin is represented in the Figure 3.2, and Figure 3.3.

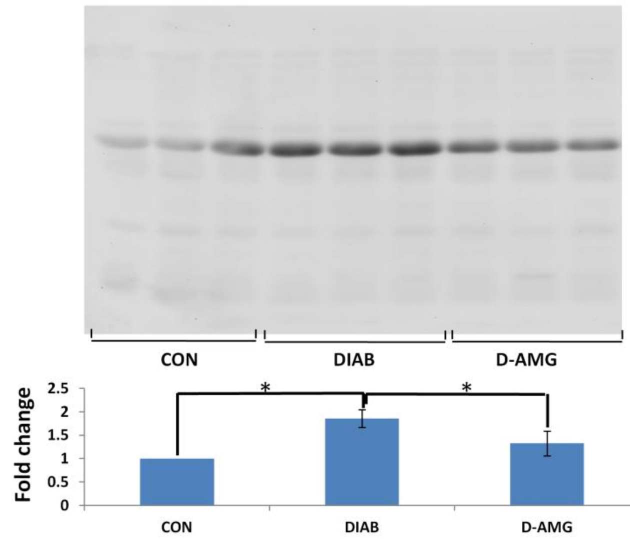


Figure 3.2 Western blotting analysis of total plasma proteins using anti-AGE antibodies; the extent of modification is represented by the fold change of the signal calculated densitometrically in comparison to control (considered 1). Values are mean ± S.E. Statistical significance of p 0.01 is represented by * as calculated by one-way ANOVA.

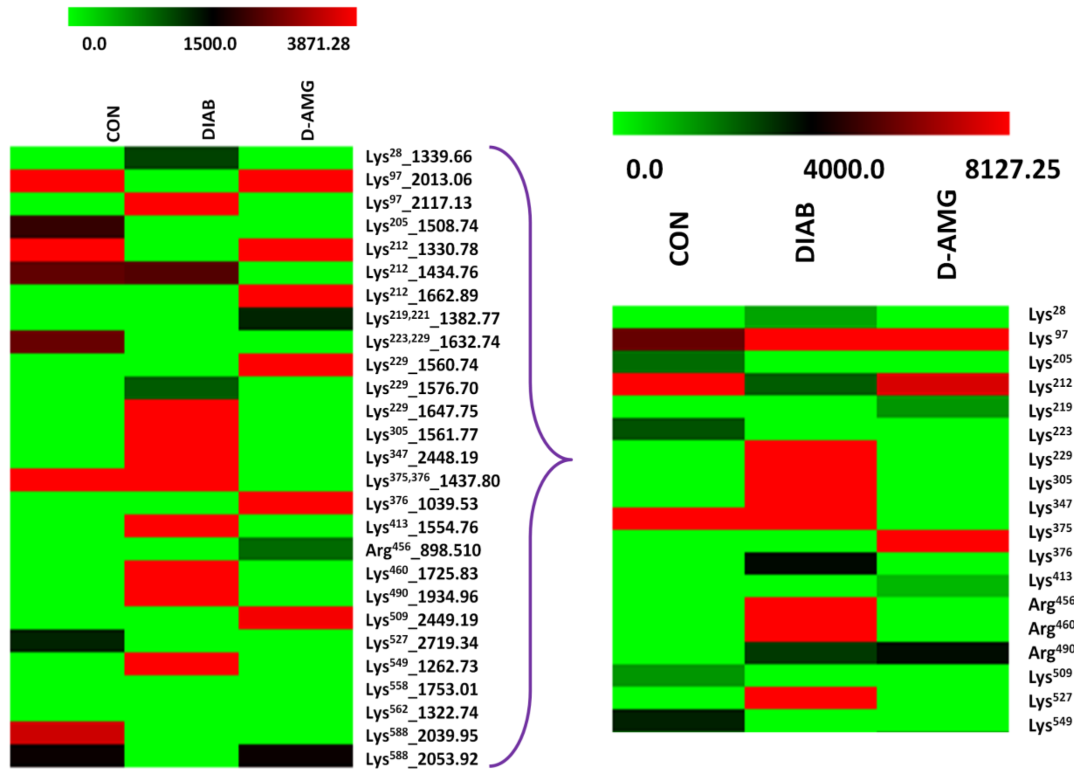
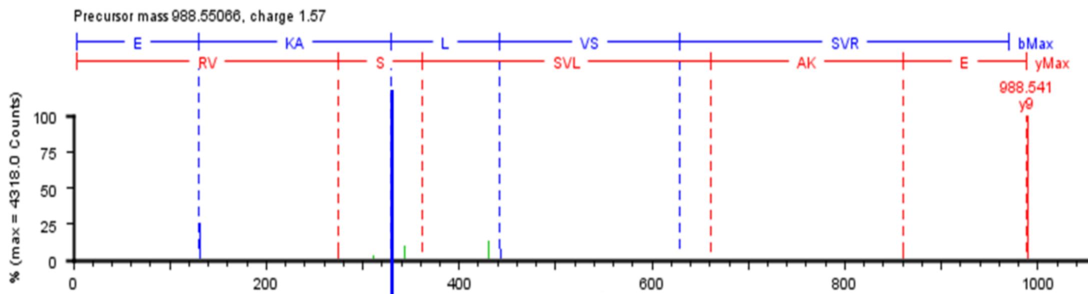


Figure 3.3 Heatmap showing relative quantification of AGE-modified peptides in serum albumin from mice plasma at PLGS identified modified residues. PLGS calculated peptide intensities were normalized with that of highest TIC. Average intensity of peptides containing modified residues was used for the generation of heatmap (n=2 biological replicates and three technical replications).

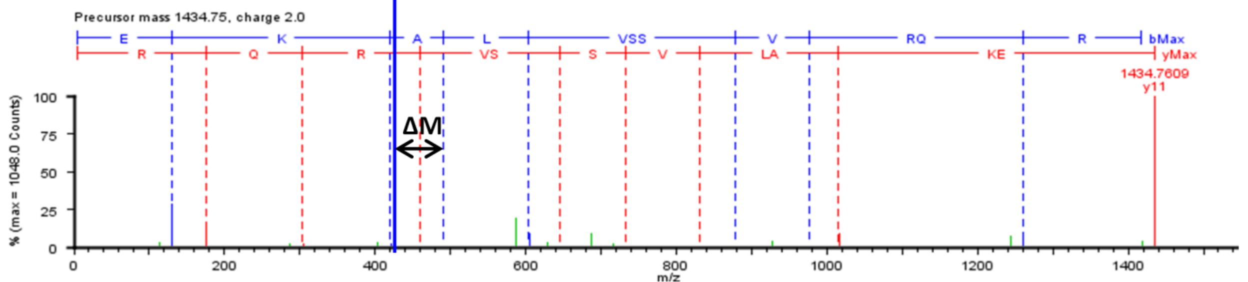
The details of the identified AGE modified peptides, the intensities used for the generation of the heatmap and manually annotated spectra are provided in supplementary data 3.1. Representative annotated AGE modified peptide spectral image is shown in Fig 3.4.

A.



ΔM (b3) = 162.036 Da

B.



Sequence: 211-221 EKALVSSVRQR AMADORI (2)

+162.036 Da (AMADORI)

C.

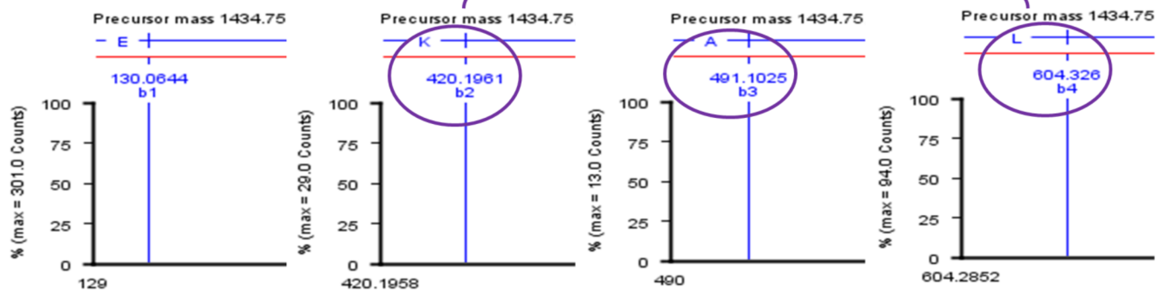


Figure 3.4 manually annotated AGE modified peptide of MSA, identified from mice plasma. Where A is the modified peptide with AMADORI modification at K-212. B is corresponding unmodified peptide sequence and C shows zoomed spectra at modified residue with increase in mass corresponding to modification (AMADORI).

3.3.3. Aminoguanidine treatment decreased the level of serum albumin in plasma CICs

After the findings of elevated and AGE modified human serum albumin in the CICs from diabetic plasma of clinical subjects, to establish the role of AGE modified proteins in the elicitation of autoimmune response and formation of CICs, STZ induced diabetic mice model was used with or without treatment of prototypic AGE inhibitor, AMG. Label free nano LC-MS quantification of CICs showed elevated levels of serum albumin in the CICs from diabetic mice. Whereas, treatment of diabetic mice with AMG reduced the levels of serum albumin in the CICs (Fig 3.5).

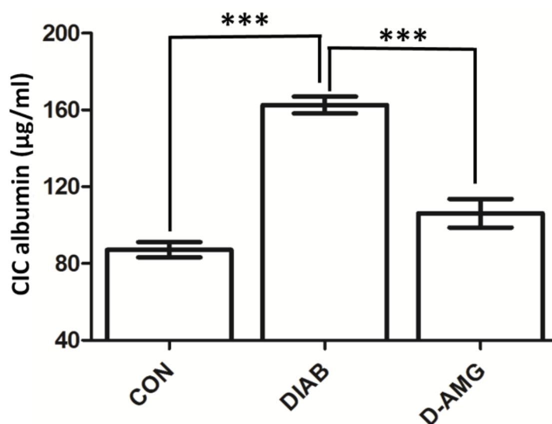


Figure 3.5 Bar graph depicts quantity of serum albumin in CICs from mice plasma samples. Label-free-based MS quantification revealed increased CIC albumin in DIAB mice compared with that of CON, which was reduced in the diabetic mice treated with AMG (D-AMG) (n=4) biological replicates and technical triplicates). Significant difference indicated by *** (at $p < 0.0001$) was calculated by one-way ANOVA analysis.

The concentration of serum albumin in the CICs was also determined by western blotting technique using anti-serum albumin antibodies and the trend remained same as observed by mass spectrometric quantification and is represented in Figure 3.6a. The AGE modification of serum albumin was evaluated using anti-AGE albumin western blotting (Figure 3.6b) and by mass spectrometric analysis. Representative, manually annotated spectra are shown in Fig 3.7.

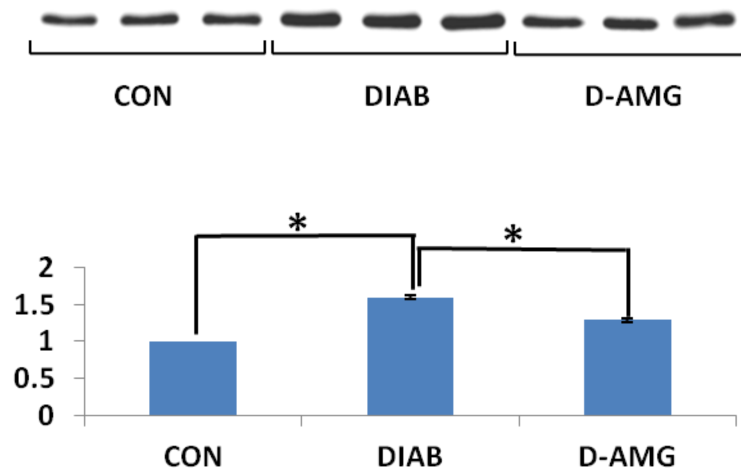


Figure 3.6a Western blotting analysis of CICs using anti-serum albumin antibodies. (n=3). Bar graph was plotted and fold change was calculated by the antibody signal is represented with respect to control (considered as 1). Values are mean ± S.E. Statistical significance of $p < 0.01$ is represented by * as calculated by one-way ANOVA.

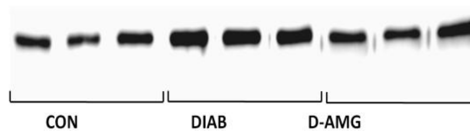
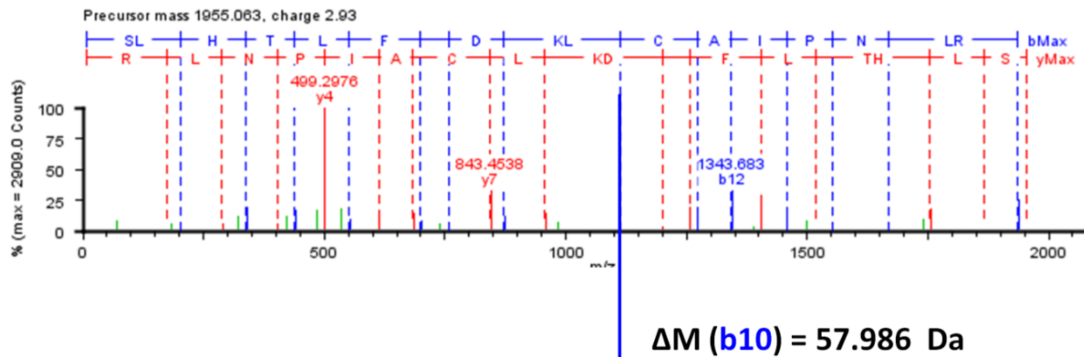
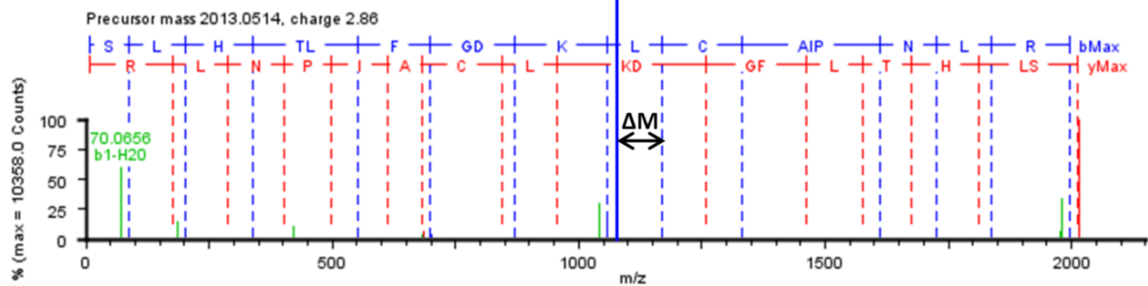


Figure 3.6b Anti-AGE Western blot of CICs from mice plasma (n=3).

A.



B.



Sequence: 89-105 SLHTLFGDKLCAIPNLR CML (9), Carbamidomethyl C (11)

C.

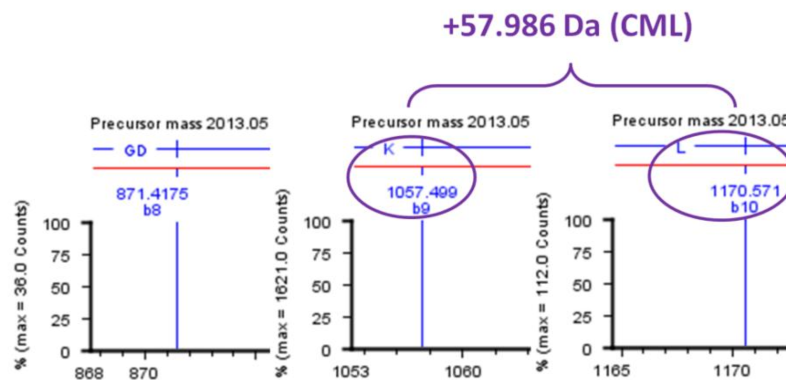


Figure 3.7 manually annotated AGE modified peptide of MSA identified in CICs. Where A is the modified peptide with CML modification at K-97. B is corresponding unmodified peptide sequence and C shows zoomed spectra at modified residue with increase in mass corresponding to modification (CML).

The characteristic peptide information and all the annotated peptides data are given in (Supplementary data No 3.2). The AGE inhibition property of AMG is the main protective factor in diabetic complications (Brownlee et al., 1986; Nicholls et al., 1989). Several lines of evidences from the previous studies have confirmed that AMG treatment prevents the complications of diabetes by reducing the *in vivo* accumulation of AGEs (Liparota et al., 1991; Cameron et al., 1992). AMG prevents the symptoms of diabetic nephropathy by inhibiting the formation of AGEs (Sugimoto et al., 1999). We exploited this property of AMG to evaluate whether the inhibition of AGE formation will have an effect on the CIC albumin level in diabetic plasma. The treatment with AMG decreased the AGE levels in plasma albumin and it also reflected in the decreased level of albumin in the CICs .

Furthermore, along with serum albumin, alpha 2 macroglobulin (1.4), carboxylesterase 1C (1.6), apolipoprotein E (Apo E) (1.6) were found to be elevated in CICs from diabetic mice plasma compared to that of control plasma or plasma of diabetic animals treated with AMG. Apolipoprotein A1 and Alpha 1 antitrypsin were observed exclusively in the CICs from diabetic plasma, but were not observed in CICs from control or diabetic animals treated with AMG. The relative fold change as calculated after mass spectrometric analysis is represented in (Fig 3.8).

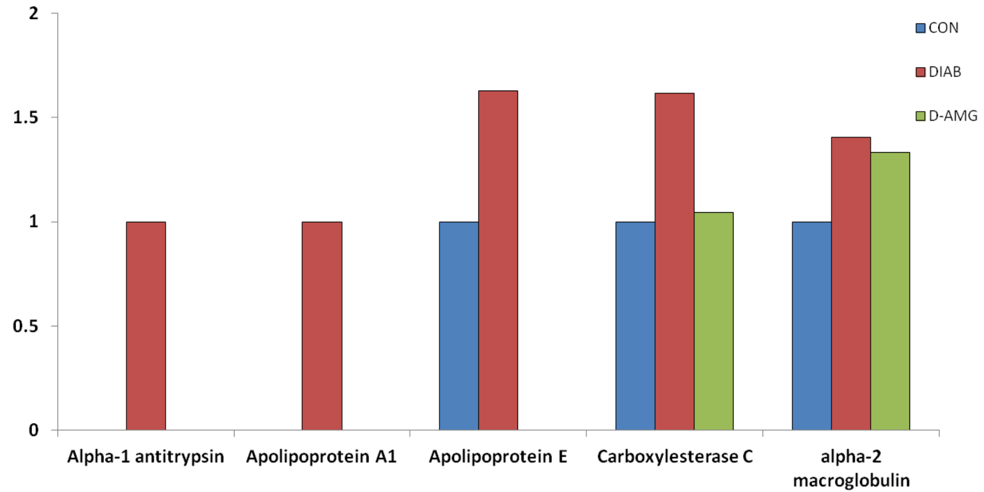


Figure 3.8 Relative fold change of CIC associated proteins in DIAB mice plasma treated with or without AMG, in comparison to CICs from CON mice plasma.

In addition to the relative fold change AGE modification analysis was also performed and the software identified AGE modified peptides of CIC associated proteins were manually validated and are represented in Figure 3.9.

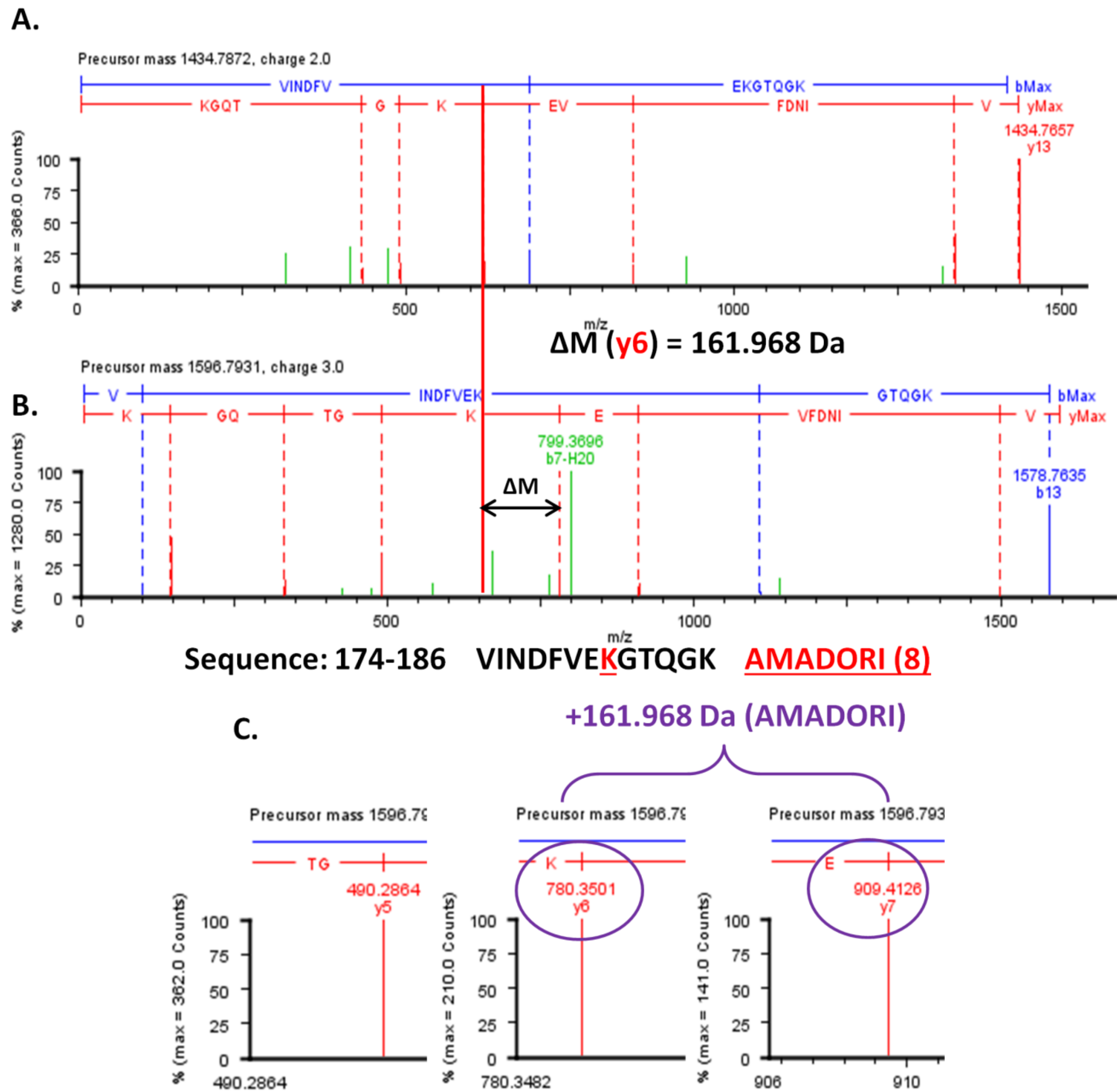


Figure 3.9a Manually annotated AGE modified peptide of Alpha 1 antitrypsin associated with CICs. B is the peptide with modification at K-181 with AMADORI. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (AMADORI).

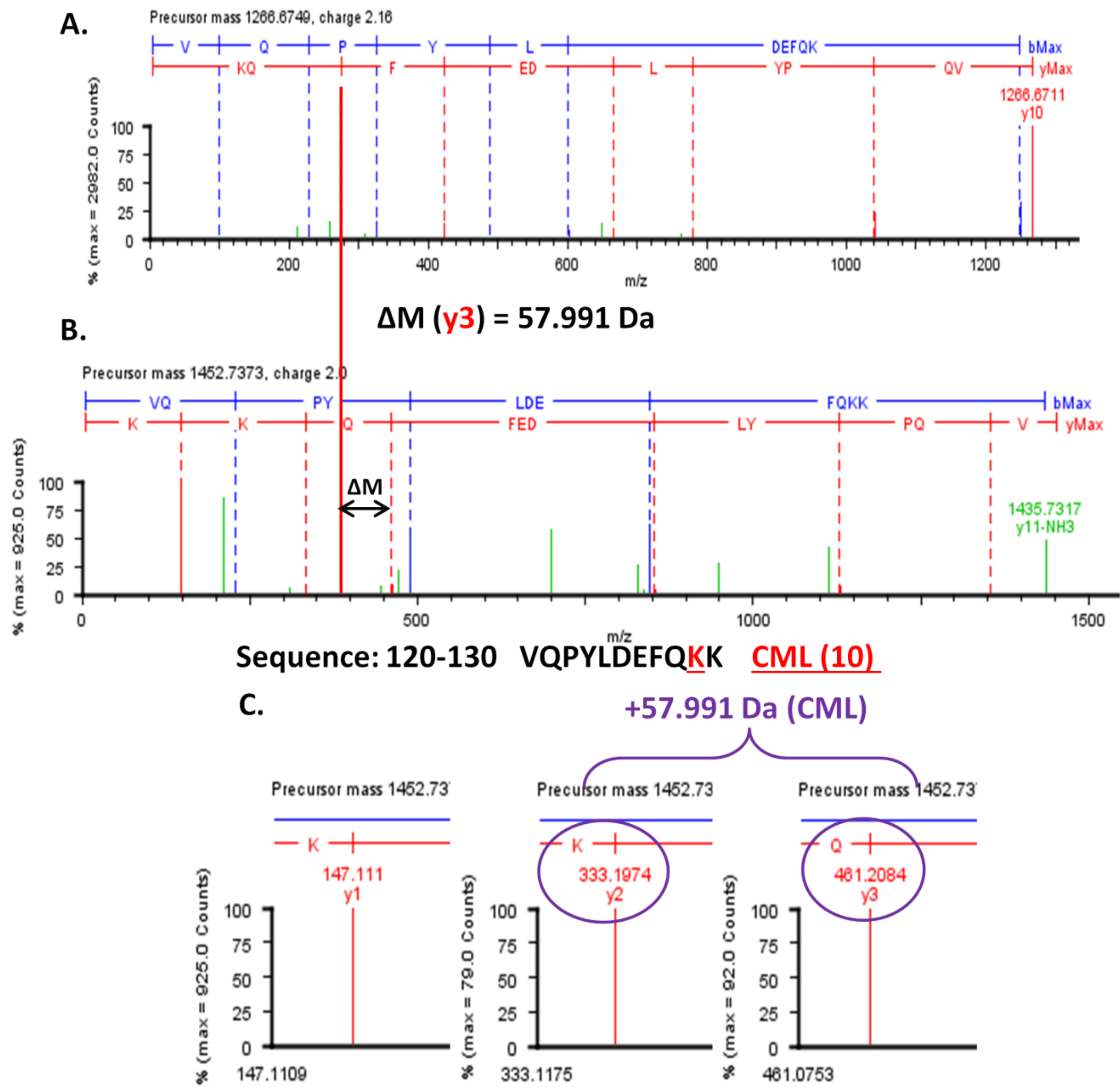


Figure 3.9b Manually annotated AGE modified peptide of Apolipoprotein A1 associated with CICs. B is the peptide with modification at K-129 with CML. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (CML).

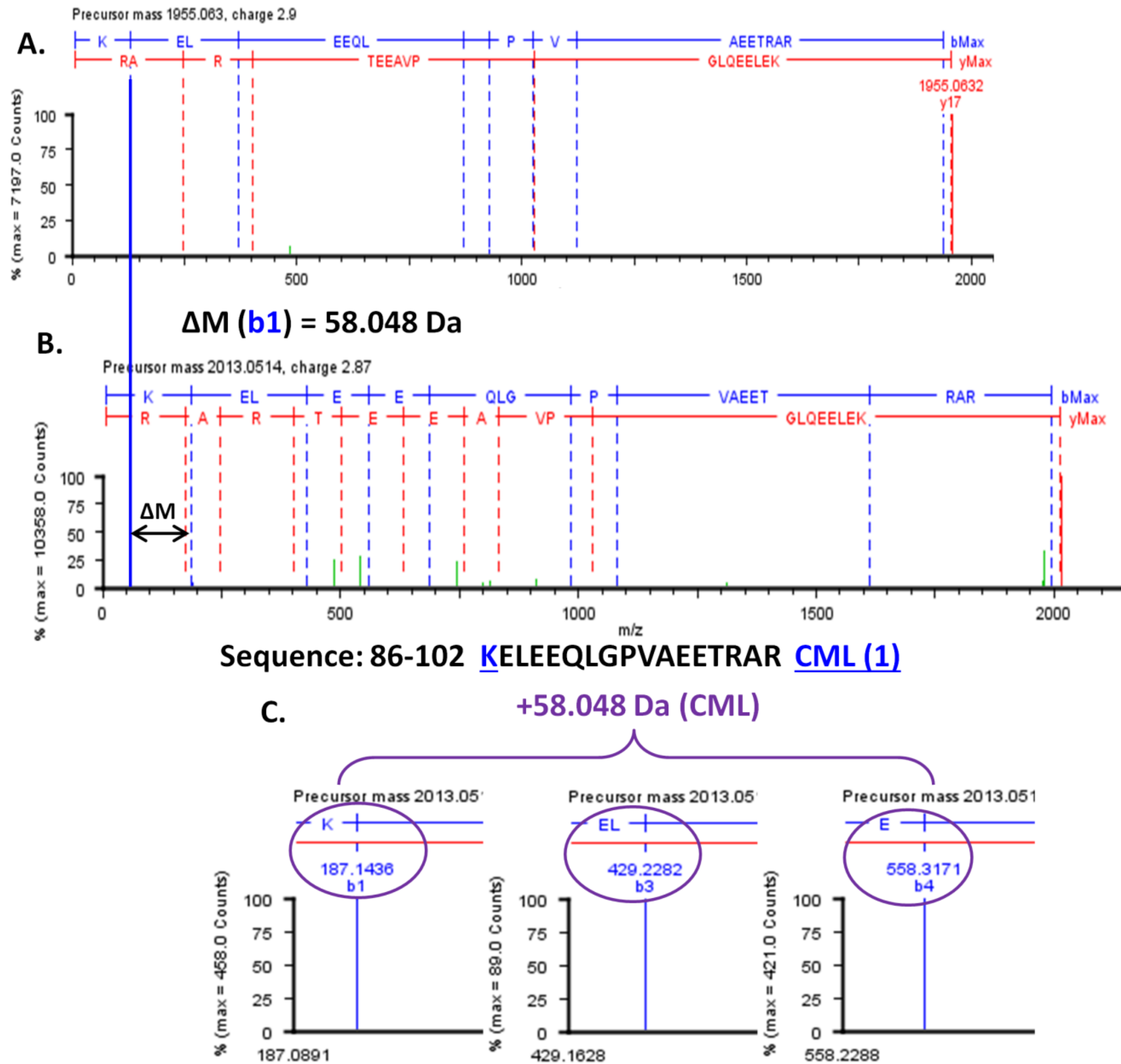


Figure 3.8c Manually annotated AGE modified peptide of Apolipoprotein E associated with CICs. B is the peptide with modification at K-86 with CML. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (CML).

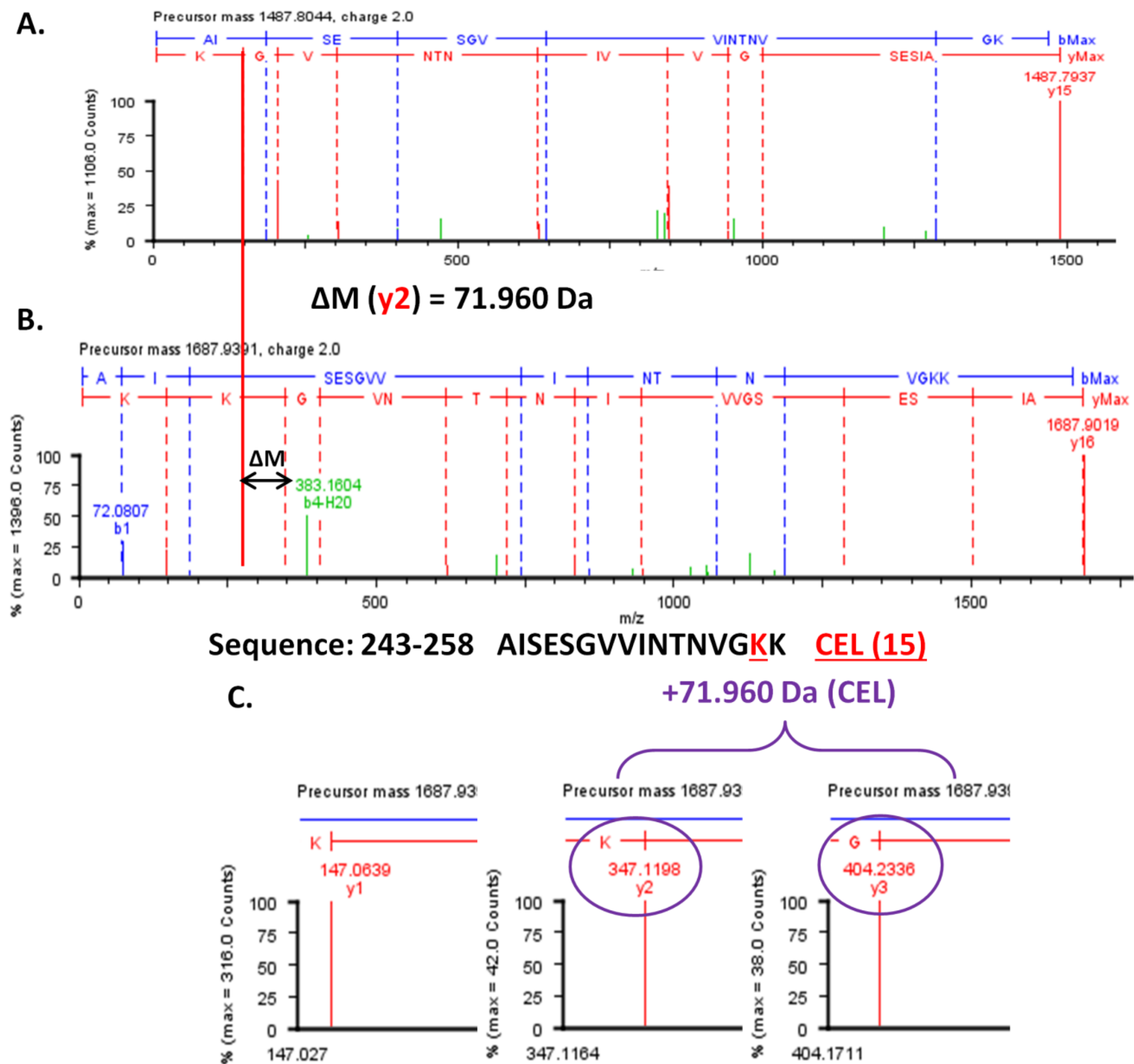


Figure 3.8d Manually annotated AGE modified peptide of Carboxylesterase C associated with CICs. B is the peptide with modification at K-257 with CEL. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (CEL).

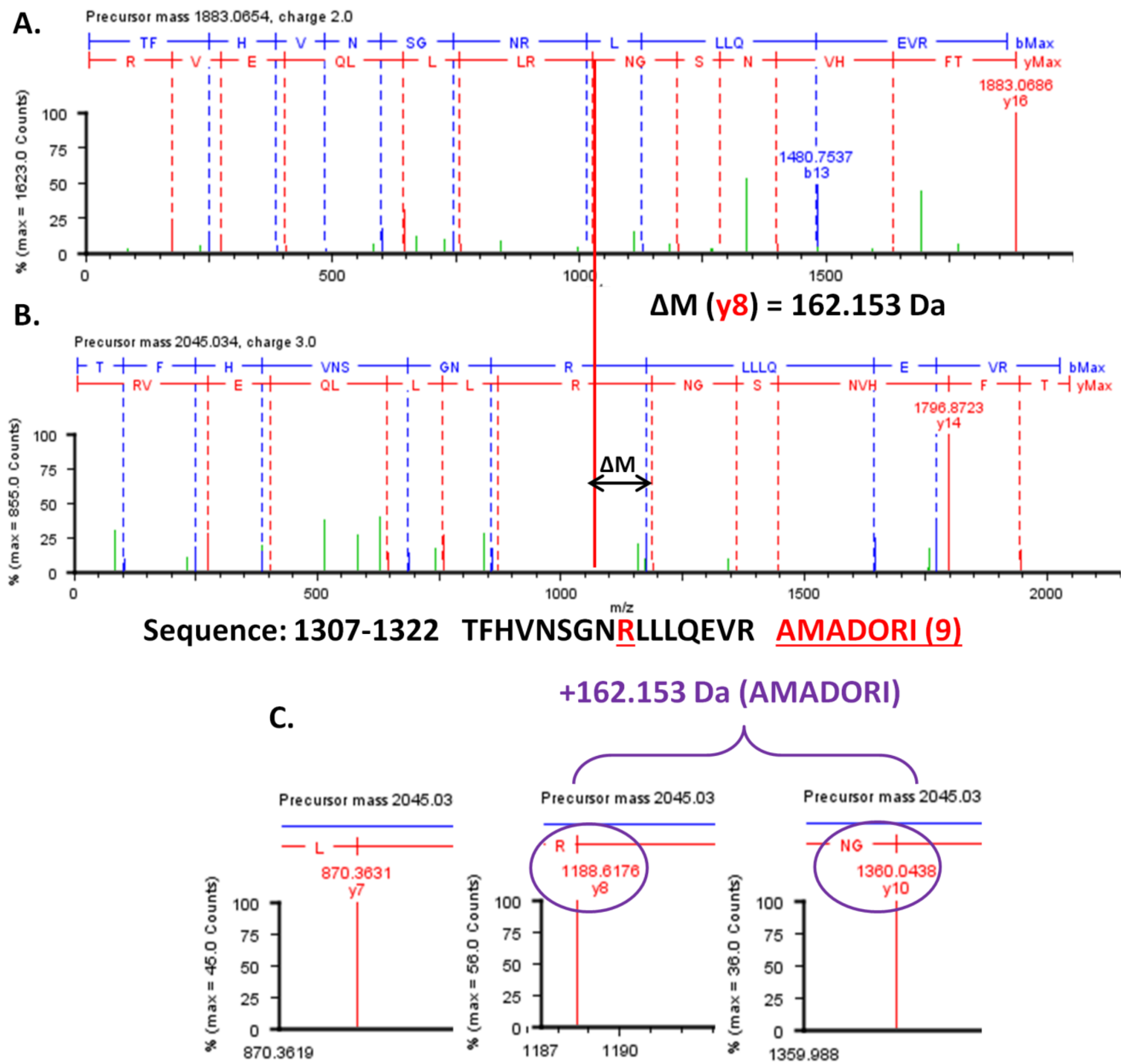


Figure 3.8e Manually annotated AGE modified peptide of Alpha 2 macroglobulin associated with CICs. B is the peptide with modification at R-1315 with AMADORI. A is corresponding unmodified peptide. C is the zoomed portion showing mass shift corresponding to modification (AMADORI).

The entire list of identified AGE peptides and their annotated spectra are given in supplementary data 3.3. Earlier researchers have reported the glycation of Apo E, Apo A1, alpha 2 macroglobulin, and alpha 1 antitrypsin in diabetic conditions (Schalkwijk et al., 2012). In this study we identified these proteins to be associated with CICs in the plasma and have been found to be AGE modified. Apo A1 is a major constituent of high density lipoproteins, which is reported to be proatherogenic when glycated, leading to coronary artery disease in diabetic patients (Hedrick et al., 2000). Even though LDL are very well known to get glycooxidatively modified and to be associated with immune complexes leading to diabetic complications (Virella et al., 2003; Virella et al., 2012), in our present study we did not identify LDL in the CICs. This may be owing to the fact that short duration of diabetes and the different experimental approach used in the current study. Further, alpha 1 antitrypsin is a well known circulating serine protease inhibitor, inhibiting proteases like trypsin, elastase, thrombin and proteinase-3 (Korkmaz et al., 2010). These serine proteases activate receptors known as protease activated receptors (PARs) on the immune cells such as neutrophils, eosinophils and macrophages, which is an essential step in inflammatory responses (Shpacovitc et al., 2008). Hence inhibition of protease inhibitors, in turn, contributes to decrease in the inflammatory proangiogenic processes. During diabetes glycation of alpha 1 antitrypsin is reported to impair its function and also the plasma level is said to be decreased in nonobese diabetic mice (Ortiz et al., 2014). Yet another protein identified in CICs and found to be AGE modified was Carboxylesterase 1C. Carboxylesterases are mainly involved in detoxification (Potter et al., 2006) and drug metabolism (Laizure, et al 2013). The esterase is linked to diabetes since the activity of the lens esterase is decreased with normal ageing associated senile cataract (Kamei, 1996) and in diabetic patients (Solerte et al 1986; Aoyagi, et al 1985). Carnosine, an endogenous dipeptide containing histidine and beta alanine was shown to prevent the inactivity of esterase caused due to glycation *in vitro* (Yan et al., 2005).

3.3.4. Plasma albumin was decreased in diabetic plasma

BCG method for the quantification of albumin is a classical method being used (Doumas et al., 1971). Plasma prepared with EDTA can be used where as heparin interferes with the assay (Bonvicini et al., 1979). The interferences like hemolysis do not affect the estimation since the complex formed has the distinct absorption maxima. The estimation showed the significantly decreased level of total plasma albumin in diabetic mice plasma condition to that of control mice. However, regardless of the AMG treatment we did not observe any increased albumin level in the AMG treated diabetic mice group (Figure 3.9).

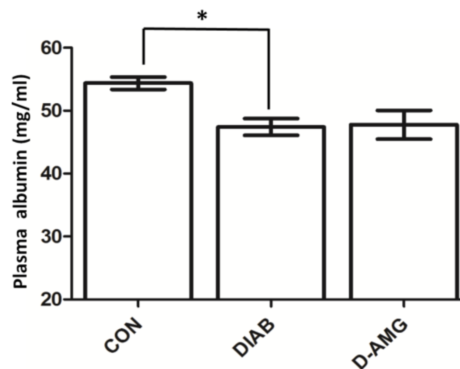


Figure 3.9 Graphical representation of total plasma albumin estimated by BCG method (n = 4 biological replicates in three technical replications). Values are mean ± S.E. Statistical significance of $p < 0.05$ is represented by * as calculated by one-way ANOVA.

In a previous study, AMG prevented the development of albuminuria, mesangial expansion and kidney tissue AGE fluorescence in STZ induced diabetic rats when treated for 32 weeks (Liparota et al., 1991).

3.3.5. Proinflammatory cytokines were increased in diabetic mice plasma and which were decreased with AMG treatment

The proinflammatory cytokines TNF-alpha, IL-1b, and IL-2 were increased markedly in diabetic mice compared to that of control mice, as analyzed by Bio-plex assay. The plasma from diabetic mice treated with AMG showed decreased level of these proinflammatory cytokines. Cytokines are short polypeptides with low molecular weight involved in regulation of the immune response. Previous reports suggest that the elevation in the level of inflammatory cytokines such as IL-1b and IL-6 together cause subclinical inflammation and precede the development of T2DM (Spranger et al., 2003) and also proinflammatory cytokines induced by the AGE interaction with the receptors play a determinant role in development of microvascular complications of diabetes (Navarro-Gonza´lez et al., 2008). GM-CSF is an important proinflammatory cytokine (Ikuta et al., 2011), however, in this study, we did not observe any significant change in its level. The levels of IL-10 were observed to be decreased in diabetic mice compared to control and the AMG treatment increased the levels of IL-10 in our study. The low production of IL-10, a strong operating anti-inflammatory cytokine is shown to be associated with metabolic syndrome and type 2 diabetes (Van Exel et al., 2002). IL-5 is one of the type 2 cytokines, which can be host protective or can drive pathogenicity when dysregulated (Wynn, 2015). In the present study IL-5 levels were decreased in diabetic mice to that of control mice or diabetic mice treated with AMG. The bar graph showing different cytokines in the analysis is depicted in Figure 3.10.

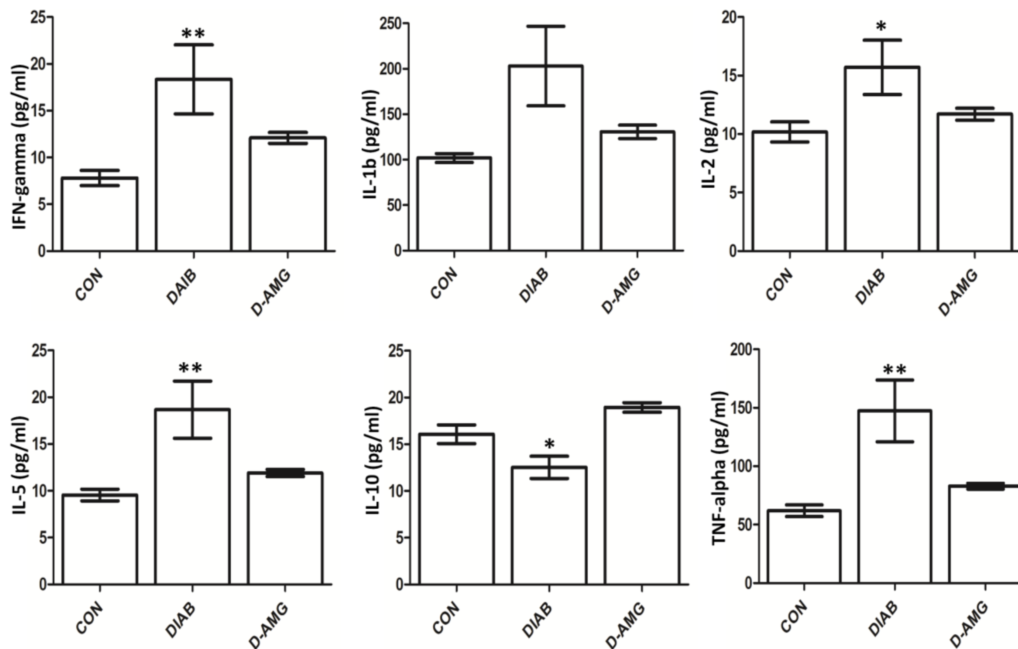


Figure 3.10 Measurement of cytokines in mice plasma. Bar graph depicting cytokines in plasma samples from CON, DIAB, and D-AMG mice (n = 3 biological replicates in technical triplicates. Values are mean ± S.E. Significant difference at $p < 0.01$ is indicated by ** and $p < 0.05$ is indicated by *.

3.4. Conclusion

In this chapter we discussed the role of glycation in the elicitation of autoantibodies and the formation of CICs in STZ induced diabetic mice model treated with or without AGE inhibitor AMG. AMG reduced the plasma AGEs and specifically AGE modification of serum albumin. Serum albumin levels were significantly increased in diabetic mice plasma CICs and also it was characterized to be AGE modified, which corroborated our initial findings of elevated HSA in the clinical diabetic plasma CICs. Additionally treatment with AGE inhibitor AMG decreased the serum albumin levels in the CICs in the plasma. We have also observed decreased serum albumin levels in the plasma of diabetic mice. Previous studies have reported the decreased transcription of

albumin gene during DM (Barrera-Hernandez et al., 1996) and also there are reports showing the decreased levels of serum albumin during conditions of heavy proteinuria of diabetic nephropathy (Viswanathan et al., 2004). Considering the previous reports and the observations of the current study, one can hypothesize that immune response against the AGE modified albumin may partly contribute to the reduced levels of plasma levels of albumin along with the other contributing factors such as decreased albumin transcription and proteinuria of chronic hyperglycemic conditions. In conclusion, this study suggests that AMG mediated AGE inhibition regulates the serum albumin levels in CICs of diabetic mice confirming the role of glycation and subsequent AGE modification in the elicitation of autoimmune response and the formation of CICs. The entire clinical study and animal model study design for the CICs analysis is depicted in a combined way in Figure 3.11.

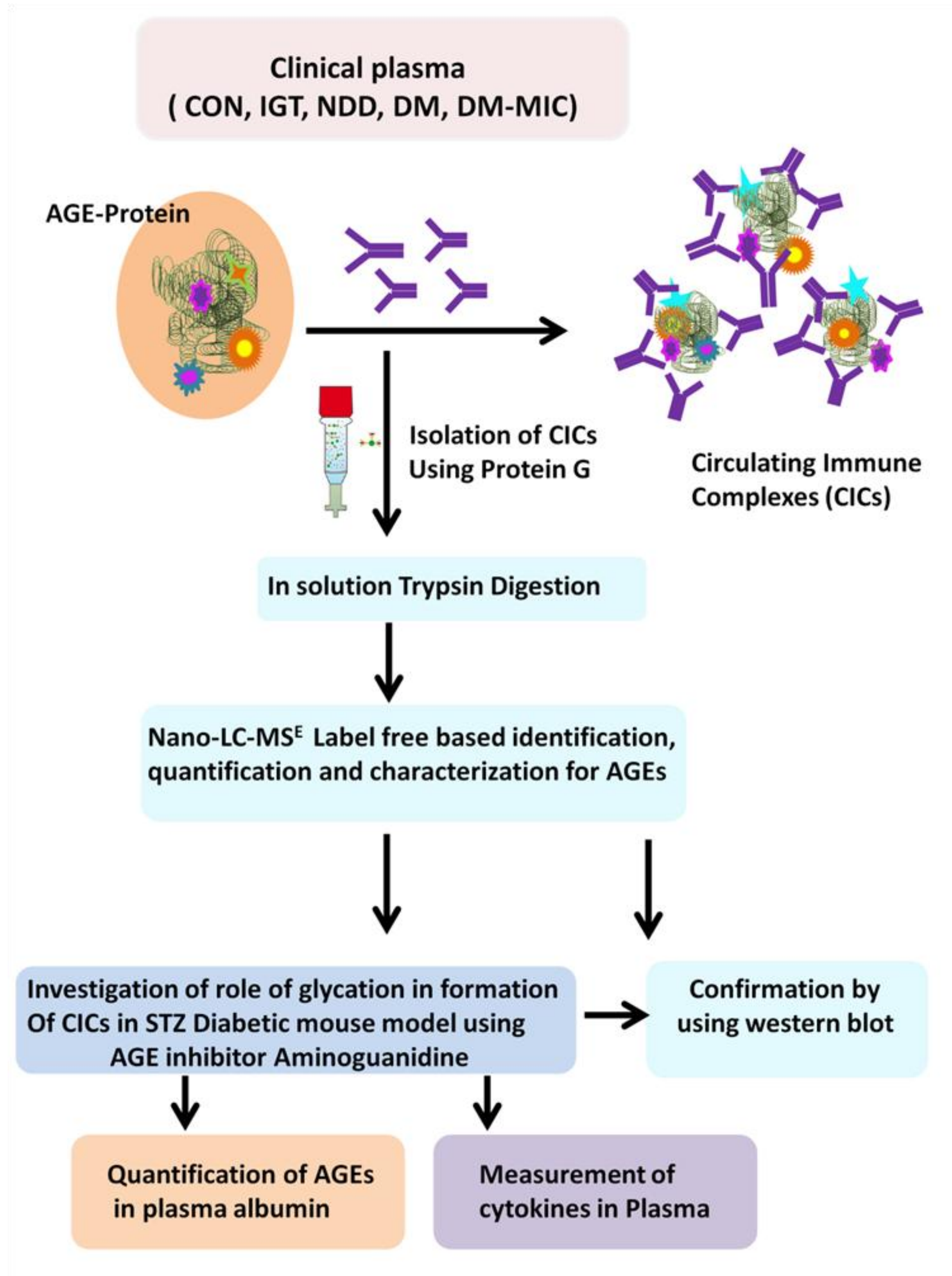


Figure 3.11 Study overview.

Table 3.1 Physiological data.

Sl. No.	Treatment	Body Weight (gms)	Blood Glucose (mg/dL)	HbA1c (%)
1.	CON (n=6)	26.28 ±2.27	102.5± 7.5	4.2±0.17
2.	DIAB (n=6)	20.4 ±1.74	550 ± 44	8.15 ± 0.24
3.	D-AMG (n=6)	22.0 ± 0.89	284.8 ± 56	6.8 ± 0.47

CHAPTER 4

Reactive immunization of mice with AGE-modified mouse serum albumin to understand immune response, its effect on glycation and albumin level

4.1. Introduction

Advanced glycation end product modification of proteins elicits immunogenicity leading to the production of autoantibodies. The autoantibodies bind to the AGE modified antigens and form CICs. AGE modified and oxidized LDL (Ox-LDL) have been shown to elicit autoimmune response, which further leads to modified LDL-IC. It has been shown both in experimental animals and clinical subjects that, the ICs containing modified LDL and corresponding antibodies are proinflammatory and proatherogenic in nature (Lopes-Virella et al., 1991, Lopes-Virella et al., 1998). Ox-LDL-ICs have also been reported to contribute to atherosclerosis and also act as diagnostic and prognostic biomarker for atherosclerosis and macrovascular diseases in T2DM (Klimov et al., 1988; Tertov et al., 1990; Orekhov et al., 2014). In addition, high concentrations of AGE-LDL and Ox-LDL in CICs have been reported to be associated with progression of retinopathy in the patients with T1DM (Lopes-Virella et al., 2012). Further, immunization of AGE-LDL has been shown to inhibit the progression of atherosclerosis in diabetic apoE and LDL receptor (LDLR) null mice model (Zhu et al., 2014). Recently, there was a report which demonstrated that pre immunization of mice with AGE-modified bovine serum albumin inhibits progression of nephropathy post diabetes induction (Mashitah et al., 2015).

In our previous studies we observed elevated serum albumin in the plasma from diabetic CICs in clinical as well as mice model and it was decreased in the diabetic mice treated with AGE inhibitor Aminoguanidine. Based on this experimental evidences, further we evaluated the effect of immunization of normal mice with AGE modified self protein that is MSA. Specifically CML AGE modified MSA (CML-MSA) was used for the immunization of mice, since CML is known to be major immunogenic epitope in vivo (Ikeda et al., 1996). The level of glycation was monitored by measuring HbA1c and

plasma albumin glycation was quantified using MS. CICs albumin in the plasma from mice immunized with CML-MSA and mice immunized with unmodified MSA was determined using nano LC-MS^E and western blot using anti serum albumin antibody. Also the level of plasma albumin and cytokines were measured.

4.2. Materials and methods

The entire reagents used were brought from Sigma-Aldrich if not mentioned otherwise.

4.2.1. Preparation of CML-AGE modified MSA

CML-MSA was prepared as described previously in the literature (Ikeda et al., 1996). MSA (50mg/ml) was incubated with 0.15M sodium cyanoborohydride and 0.05M glyoxylic acid in 0.2M sodium phosphate buffer pH 7.4. The final volume was adjusted to 10 ml with phosphate buffer. This reaction mixture was incubated at 37 °C for 24h followed by washes with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, of pH 7.4). Control MSA was processed similarly without modifying agents. All the pipette tips, collection tubes and glass wares were sterilized prior to use. Sterilized water was used for the preparation of buffers and aseptic conditions were maintained throughout. Modification was confirmed with western blotting using anti-CML antibody.

4.2.2. Mice sample and experimental details

The mice experiments were performed at the Experimental Animal Facility, National Centre for Cell Sciences, India. The protocol followed was in accordance with guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCSEAI). The experimental procedures followed were also approved by Institutional Animal Ethics committee.

4.2.2a. Reactive immunization of mice with CML-MSA

Control mice were separated into two groups each and immunized with either CML modified MSA or unmodified control MSA. Control mice immunized with unmodified protein were named as C-ICP, control mice immunized with CML-MSA as C-IMP. Pre-immune plasma was collected from the animals before starting the immunization schedule. CML-modified MSA was given emulsified with Freund's complete adjuvant and sterile PBS (0.1M ph7.4) in equal volumes, by intraperitoneal injections for the first time (0.1 ml, 30ug/mouse). Titer of the antibody in the sera was evaluated by ELISA, from the blood (50-100ul) collected from the tail vein after 14 days. This was followed by subsequent 3 doses of immunizations emulsified with Freund's incomplete adjuvant. Corresponding control serum albumin was also administered similarly. Plasma and organ samples were collected at the termination of the experiment.

4.2.3. Autoantibody titer ELISA

Indirect ELISA was performed for the determination of the autoantibody titer. Nunc "MaxiSorp (Nunc-Immuno™ 96 MicroWell™ maxisorp #442404) plates were coated with either 500 ng CML-MSA or C-MSA in 100 µl 50 mM carbonate buffer (pH 9.6) and incubated overnight at 4 °C. Excess solution was carefully discarded and unbound sites were blocked by incubating with 300 µl of 5 % skimmed milk at 37 °C. 100 µl of mouse plasma in diluted 1:100 in blocking buffer was used as primary antibody and incubated for 2h at 37 °C. After the incubation plate was washed once with 300 µl of PBS-T (PBS with 0.05% tween 20) and twice with 300 µl of PBS. Then the wells were incubated with 100 µl of rabbit anti-mouse antibody conjugated with HRP (Bangalore Genei, India) in 1:5000 for 1h at 37 °C. After the washes, plates were incubated with 100 µl of tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) (Bangalore Genei, India) under dark condition at room temperature for 20 min. H_2O_2 is substrate for enzyme HRP and TMB is the chromogen used. HRP releases nascent oxygen from H_2O_2, which oxidizes

TMB to TMB oxide a blue colored complex. Reaction was stopped using 100 µl 1M sulphuric acid. The absorbance of the colored complex (yellow) was read at 450 nm.

4.2.4. Isolation of CICs from mouse plasma

The protein concentration of the plasma was determined using Bio-Rad Bradford kit (Bio-Rad Laboratories, CA). 400 µg of plasma protein from C-ICP, C-IMP, groups was processed to isolate the CICs using protein G sepharose resin. The isolation protocol was followed according to manufacturer's instructions. The resin was washed thrice with 1 ml of 1x IP buffer provided along with the kit. Final reaction volume of the plasma was made up to 600 µl using 1x IP buffer and it was incubated at 4 °C for 2h on rotospin rotary mixer with constant rotation. After the incubation the supernatant was discarded and 5 washes were given with 1x IP buffer by centrifuging at 12,000 g for 1 min. Absorption at 280 nm was monitored for the wash fractions to confirm the complete removal of unbound non specifically interacting proteins. The bound CICs fraction was eluted with 100 µl of 0.1% RapiGest (Waters, Milford, MA) in 50 mM ammonium bicarbonate buffer with brief intermittent vortexing for 20 min and heating at 80 °C for 15 min. The eluate was collected in fresh tubes by centrifugation at 12,000 g for 5 min. The isolated CICs were further analyzed with MS or western blotting.

4.2.5. Western blotting

10 µg of the plasma proteins or 10 µl of purified CICs were subjected to western blotting for AGE analysis or quantification of albumin in CICs. Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membrane. The membranes were then incubated overnight at 4 °C in blocking buffer containing 5% skimmed milk in PBS. The following day blocked membranes were incubated with anti-AGE antibody (Abcam, Cambridge, UK) in 1:1000 dilution 3h at 25 °C or with anti-serum albumin antibodies (Abcam, Cambridge, UK) for 1h at 25 °C. After this step membranes were washed once with PBS-T (PBS with 0.05% Tween 20) and then twice with PBS. Further, incubated with secondary antibody conjugated to HRP (Bangalore Genei, India), at 1:5000 for 1 h at 25

°C. The bands were detected by Western Bright TM Quantum Western blotting detection kit (Advansta, Menlo Park, CA) as per the manufacturer's instructions after washing of the membrane as above. Licor Image Studio™ Lite software (Licor Biosciences) was used for quantification of the detected bands between the sample groups. The relative fold change of the experimental groups with respect to control considered as 1 was calculated and the bar graph was plotted.

4.2.6. Label free quantification of CIC associated proteins and AGE modification analysis by LC-MS^E

4.2.6a. In-solution trypsin digestion

In-solution trypsin digestion 10 µg of plasma proteins or 50 µl of CICs was carried out. 10 µg of plasma protein was solubilized in 10 µl of 0.1% RapiGest in 50mM NH_4HCO_3. The reaction mixture containing proteins was then subjected to reduction and alkylation with 100 mM dithiothreitol at 60 °C for 15 min and 200 mM iodoacetamide for 30 min at 25 °C under dark condition. Trypsin digestion was brought about by adding the enzyme in 1:20 ratio and incubating at 37 °C for 18h. The digestion reaction was stopped with 100% formic acid and the surfactant RapiGest was precipitated by incubating with acidic pH at 37 °C for 45 min followed by centrifugation at 14000 g for 15 min. The precipitated surfactant was discarded and the digested peptide supernatant was collected.

4.2.6b. Liquid chromatography-mass spectrometry analysis

Peptide mixture was diluted in 3% ACN with 0.1% FA in 1:3 ratio before subjecting to LC-MS^E analysis by using Nano Acquity UPLC system coupled to SYNAPT HDMS (Waters). Yeast alcohol dehydrogenase at the final concentration of 100 f mol was spiked along with peptides as an internal standard. Three technical replicates of mass spectra were acquired for each sample. The binary solvent system comprised 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). An online Symmetry C18 trapping column (internal

diameter of 180 µm and length 20mm) (Waters Corporation) was used for pre concentrating and desalting of the injected sample with 0.1% mobile phase B at a flow rate of 5 µl/min. Nano-LC separation was performed using an BEH-C18 (1.7 µm × 75 µm × 250 mm) column (Waters Corporation) and peptides were eluted into the Nano-Lock Spray ion source using a gradient of 3 to 40% B for 95 min at a flow rate of 250 nl/min. The mass spectrometer was calibrated with MS/MS spectra of Glu-fibrinopeptide B (m/z 785.8426) (500 fmol/µl), and every 30s, the lock mass correction was done by the same peptide. All the MS runs were acquired at a resolution of about 9000 full width half maximum with a scan time of 0.75 s in a mass range of 50–2000 m/z with alternating low (4 eV) and high (15–40 eV) collision energy in a positive V-mode.

4.2.6c. Data processing and database searching

The acquired LC-MS^E data was analyzed using PLGS 2.5.1 (Waters Corporation) software. Mouse database (UniProt release 2014_08, 17,023 entries) downloaded from UniProt, to which alcohol dehydrogenase 1 (P00330) protein sequence of *Saccharomyces cerevisiae* appended was used for identification and quantification of the proteins. Alcohol dehydrogenase 1 sequence was added to databank since a known amount of the protein was spiked as an internal standard for quantification of the identified proteins. During the analysis precursor and product ion tolerance were set to automatic. Minimum fragment ion matches per peptide was set to three, minimum of five fragment ion matches per protein and minimum one peptide match was set per protein. Fixed carbamidomethylation and variable oxidation modifications were set at Cys (C) and Met (M) respectively. The false positive rate was set to 4% with two allowed missed cleavages and ion intensity threshold was set to 500 counts. Label free quantification was performed as described previously (Silva et al., 2006). The quantification data of the individual proteins was further used to calculate the microgram of proteins in CICs per milliliter of the blood plasma.

4.2.6d. Relative quantification of AGEs in mouse plasma albumin and identification of AGE modified peptides in plasma CIC albumin

MSA from plasma CICs was analyzed for identification of AGE modified peptides and plasma albumin for the relative quantification of AGEs between the experimental groups. The analysis was carried out as described previously (Bhonsle et al., 2012). Variable glycation modifications namely Amadori (162.0528) at lysine or arginine, CML (58.0055) and CEL (72.0211) at lysine, MGH1 (54.0106) at arginine, and oxidation at Met and fixed carbamidomethylation of Cys residues were included during the search. Precursor ion tolerance was set to 300 ppm and product ion tolerance was set at 300 ppm in the targeted search performed. Ion intensity threshold 500 for precursor and 10 counts fragments intensity threshold was set along with 2 missed cleavages. The false positive rate was set at 1%. AGE modified peptides identified by the software PLGS were manually validated with set criteria (section II.2.8). The intensity of the AGE modified peptides was considered for the relative quantification of AGEs. Highest TIC across all the mass spectral acquisitions was used to normalize the intensities to rule out the possibility of experimental variation. Peptides consistently observed in two replications out of triplicate acquisitions with matching RT were considered for the final calculation of average intensity and the heatmap generation.

4.2.7. Measurement of total plasma albumin

Total plasma albumin was measured using BCG method by Innoline^{TM} Albumin, Merck, NJ, USA kit according to manufacturer’s instructions. The absorption was measured at 610 nm. The intensity of the color formed is directly proportional to the albumin concentration in the sample.

4.2.8. Quantification of cytokines

We analyzed the panel of cytokines using a multiplex assay. The levels of eight cytokines (IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-gamma, and TNF-alpha) were measured in plasma from mice using the Bio-Plex Pro Mouse Cytokine Group I Panel 8-Plex (Bio-Rad). The assay principle is similar to that of sandwich ELISA wherein magnetic beads or the microspheres are fluorescently dyed, each with a distinct color code or spectral identity facilitating the simultaneous detection of different molecules (multiplex) in a suspension. The beads are coupled with capture antibodies specific for the molecules of interest. These antibodies interact and bind to the molecule of interest in the sample. After several washes to remove unbound molecules, the biotinylated detection antibody is added which creates a sandwich complex. The detection is brought about by the addition of SA-PE conjugate which binds specifically to biotin. Phycoerythrin acts as a fluorescent reporter molecule. The concentration of the analyte is directly proportional to the fluorescence signal of the reporter molecule. The assay was performed according to manufacturer’s instructions. Briefly, 50 µl of the solution containing microbeads coupled with antibodies specific for each of the cytokines to be measured was added to each well. Three washes were given with 100 µl of Bio-Plex wash buffer (wash step). 50 µl of plasma aliquots were diluted to 1:4 in Bio-Plex sample diluents and was added to each well and incubated for 30 min at 25 °C with constant shaking at 800 rpm. After the wash step as above, 25 µl of the detection antibody premix were added and incubated for 30 min at 25 °C followed by incubation with 50 µl of the streptavidin–phycoerythrin solution for 10 min at 25 °C. After another wash step, the beads were resuspended in 125 µl of the assay buffer. The signal was recorded using a Bio-Plex MAGPIX (Bio-Rad) multiplex reader. A standard curve was generated simultaneously for each cytokine and was used to determine the concentration of the cytokines in the plasma samples using Bio-Plex Manager version 6.2 software.

4.2.9. Statistical analysis

All experiments were carried out in triplicates. Statistical significance was determined by student's t-test. Data are expressed as mean± S.E. A *p* value <0.05 was considered as statistically significant.

4.3. Results and discussion

Elevated levels of AGEs play a determinant role in the development of diabetic micro and macro vascular complications. AGE modification of the proteins also renders them immunogenic resulting in autoantibody formation. A previous study has reported that administration of AGE modified rat serum albumin (AGE-RSA) in normal rats resulted in vascular dysfunction (Vlassara et al., 1992). In our present study we studied the effects of reactive immunization with AGE-CML modified mouse serum albumin (CML-MSA) in mice.

4.3.1. Physiological data

Glycation was monitored by measurement of blood glucose and HbA1c. The blood glucose levels between mice injected with C-IMP and C-ICP did not vary significantly, although a slight increase in glucose was observed in C-IMP. However, the glycated HbA1c was increased in the C-IMP. The data are represented in table 4.1.

4.3.2. Autoantibody titer was increased against CML-MSA in C-IMP

ELISA showed increased antibody titer for CML-MSA in the plasma from C-IMP at 14th day of immunization compared to that of pre-immunization collected plasma. This was considered before subsequent immunizations were performed. Results of ELISA are depicted in Figure 4.1.

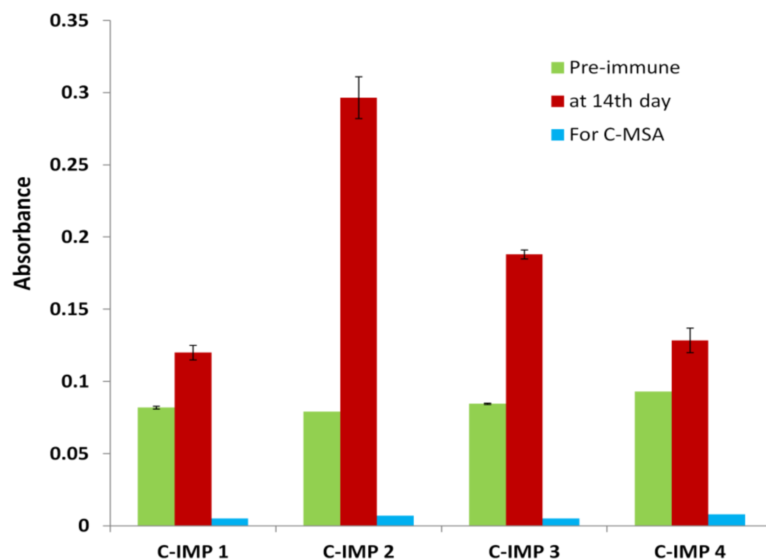


Figure 4.1 Graphical representation of antibody titre against CML-MSA and unmodified MSA (C-MSA) in mice plasma at 14th day of immunization. Data represents values obtained in plasma from 4 individual mice.

4.3.2. C-IMP mouse plasma albumin revealed increased AGE modification to that of C-ICP albumin

AGE modification in the plasma albumin was quantified by mass spectrometric analysis. PLGS software identified AGE modified peptides were manually validated. TIC normalized AGE modified peptide intensity was used to plot heatmap. The data revealed increased AGE modification in the albumin from C-IMP mouse plasma. The heatmap generated is represented in Figure 4.2. The annotated peptides, MS/MS characteristics and intensity normalization details are provided in supplementary data 4.1. Representative annotated spectra is given in Figure 4.3.

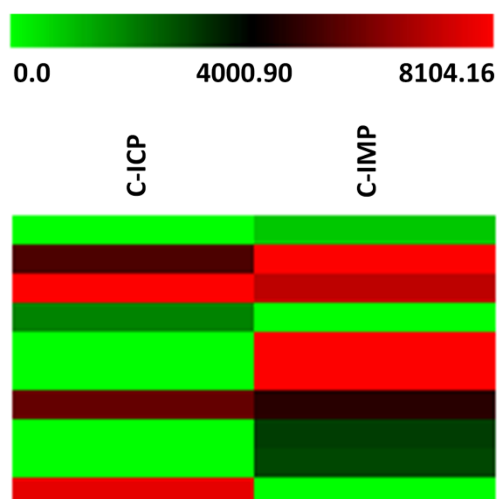
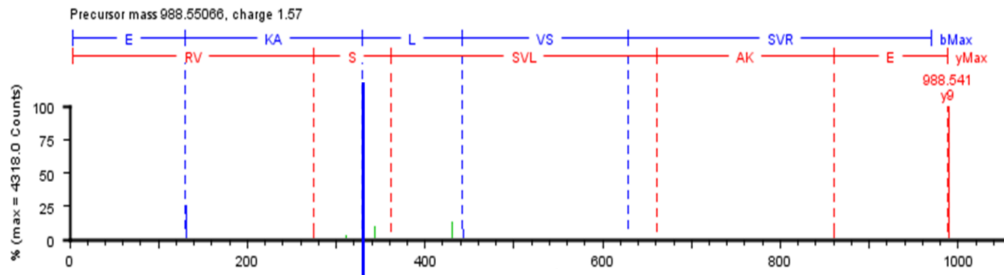
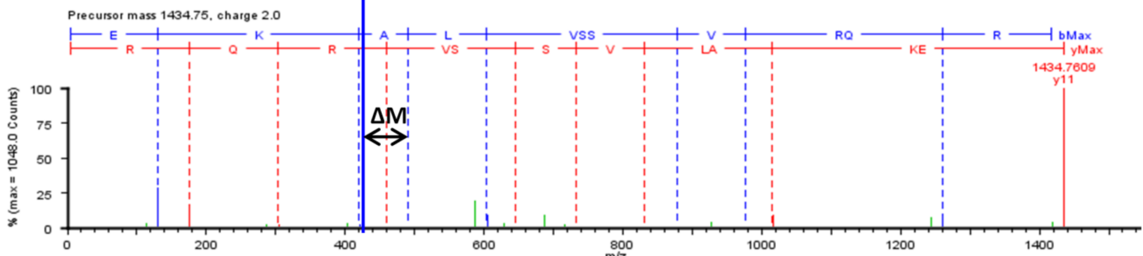


Figure 4.2 Heatmap showing relative quantification of AGE-modified peptides in albumin from mice plasma at PLGS identified modified residues. PLGS calculated peptide intensities were normalized with that of highest TIC. The average intensity of peptides containing modified residues was used for the generation of heatmap (n = 2 biological replicates and three technical replications).

A.



B. ΔM (b3) = 162.036 Da



Sequence: 211-221 EKALVSSVRQR AMADORI (2)

C.

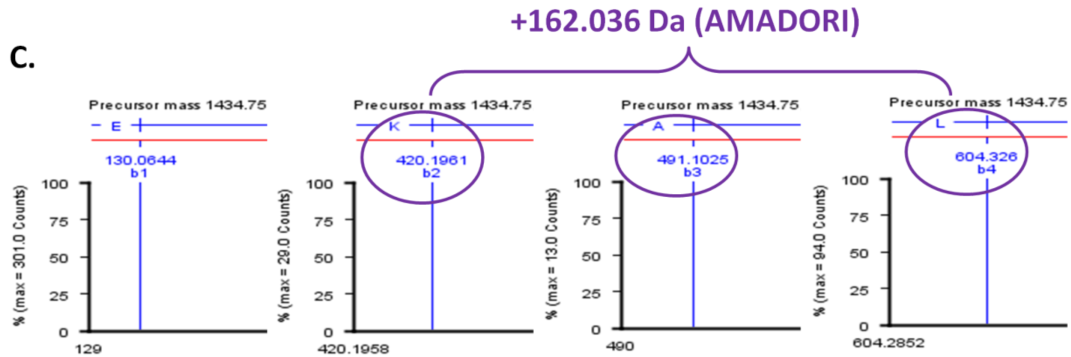


Figure 4.3 manually annotated AGE modified peptide of MSA in mice plasma. B represents modified peptide with AMADORI at Lys K-212. B is corresponding unmodified peptide. C is zoomed portion showing mass shift corresponding to AMADORI.

4.3.3. Immunization with CML-MSA increased albumin in the plasma CICs

Following our previous observation of elevated plasma albumin in the CICs of diabetic mice, the effect of reactive immunization with CML-MSA on the plasma CICs was studied. Label free nano LC-MS^E quantification of CICs showed elevated levels of serum albumin in the C-IMP mice plasma compared to that of C-ICP mice group. The mass spectrometric quantification data was used to plot the bar graph and is represented in Figure 4.4.

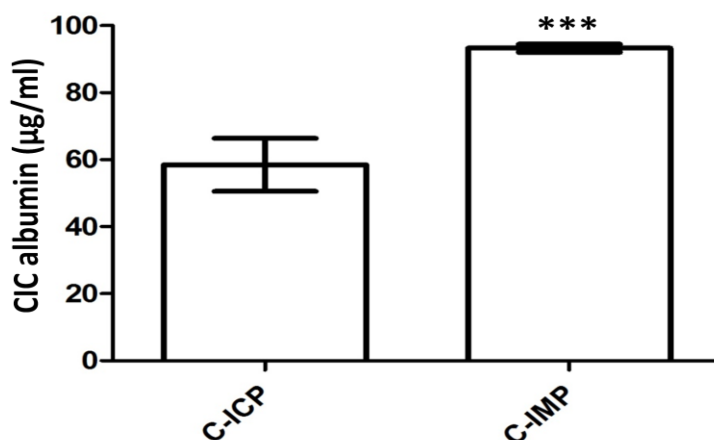


Figure 4.4 Label-free-based MS quantification revealed increased CIC albumin in C-IMP mice compared with that of C-ICP (n = 3) (biological replicates and technical triplicates). Significant difference indicated by *** (at $p < 0.0005$) was calculated by Student's t-test.

Further the western blot analysis using anti-serum albumin antibody for the determination of the concentration of serum albumin in the CICs also revealed elevated albumin in CICs from C-IMP (Figure 4.5).

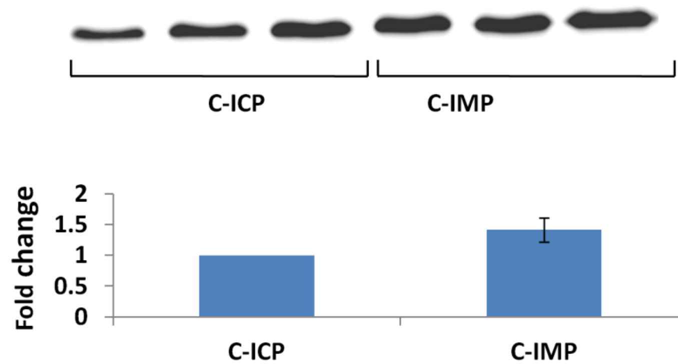


Figure 4.5 Western blotting analysis of CICs using anti-serum albumin antibodies (n =3). Bar graph was plotted and fold change was calculated by the antibody signal is represented with respect to control (considered as 1). Values are mean ± S.E.

In previous chapters we have discussed the observation of elevated serum albumin levels in the CICs from clinical IGT patients, NDD who are not on medicine and DM-MIC. Diabetic mice also showed increased albumin in CICs compared to that of normal control mice. Furthermore, our investigation confirmed that the AGE modification of proteins results in elicitation of immune response and CIC formation in diabetes, since diabetic mice treated with AGE inhibitor AMG exhibited decreased albumin in the CICs.

4.3.4. Serum albumin in CICs was also AGE modified

The albumin in CICs was also analyzed for AGE modification by western blotting analysis using anti-AGE antibody. Albumin was found to be AGE modified in the CICs and is represented in Fig 4.6.

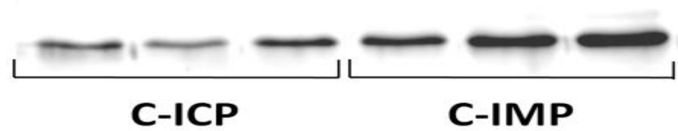


Figure 4.6 Anti-AGE Western blot of CICs from mice plasma (n = 3).

Previous reports suggest that AGE modification of proteins leads to the generation of autoantibodies which leads to the formation of CICs (Turk et al., 2003).We observed AGE-modified serum albumin in real time CICs.

4.3.5. Proinflammatory cytokines were elevated with immunization of CML-MSA

Two most important proinflammatory cytokines TNF-alpha and INF-gamma were found to be increased in the plasma from mice immunized with CML-MSA compared to that of C-ICP group of mice. TNF-alpha and INF-gamma are considered to be prototypic proinflammatory cytokines involved in inflammation and are associated with autoimmune response (Moudgil et al., 2011). Elevated levels of TNF-alpha have been reported in obesity related insulin resistance and impaired glucose tolerance (Hotamisligil et al., 1995; Zinman et al., 1999; Moller et al., 2000). INF-gamma is known to be associated with chronic inflammation and autoimmune response. INF-gamma is produced with the commencement of inflammation and subsequently it participates in augmentation of the inflammatory process (Zhang, 2007). Further chronic inflammation is linked to impairment in metabolic homeostasis and the development of type 2 diabetes (Hotamisligil et al., 2008; Li et al., 2011). Data is represented in Figure 4.7. We did not observe any significant changes in other cytokines analysed (Figure 4.8).

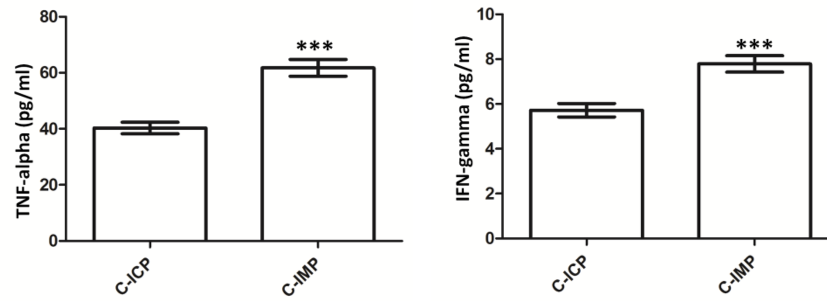


Figure 4.7 Measurement of cytokines in mice plasma. Bar graph depicting cytokines in plasma samples from C-ICP and C-IMP mice (n = 3 biological replicates in technical triplicates). Values are mean ± S.E. Significant difference at is indicated by *** at $p < 0.0005$ as calculated by Student's t-test.

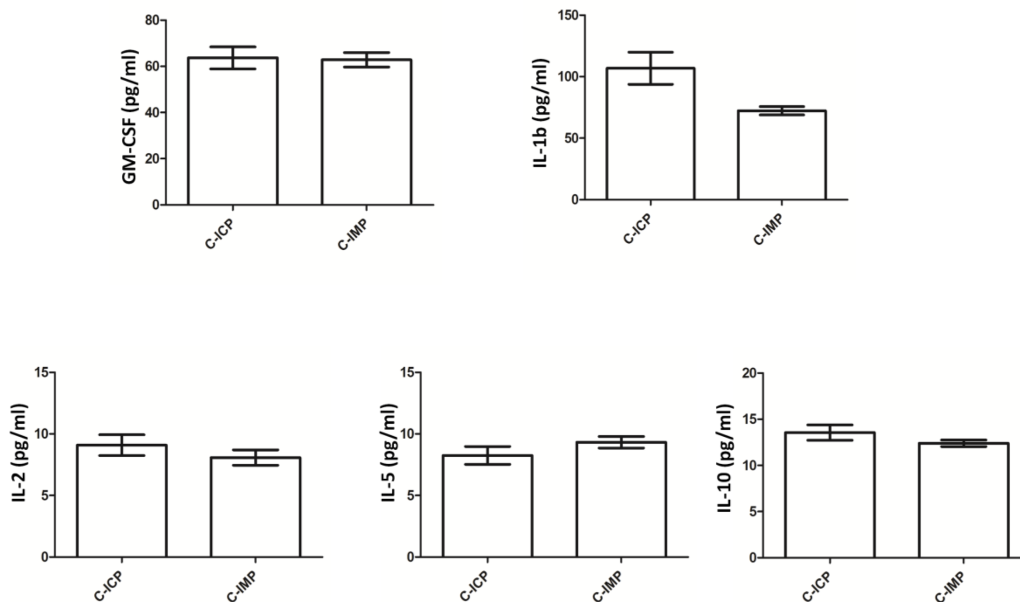


Figure 4.8 Measurement of cytokines in mice plasma. Bar graph depicting cytokines in plasma samples from C-ICP and C-IMP mice (n = 3 biological replicates in technical triplicates). Values are mean ± S.E.

4.3.6. Total plasma albumin level was decreased in C-IMP group

Total plasma albumin estimation using BCG method showed a considerable decrease in their levels in C-IMP as compared to that of C-ICP. This observation along with increased AGE modification in the MSA in immunized mice suggests that the autoimmune response with immunization of CML-MSA might eventually lead to autoantibody generation against albumin in the plasma and hence leading to decreased level of plasma albumin. Bar graph representing the data is shown in Figure 4.9.

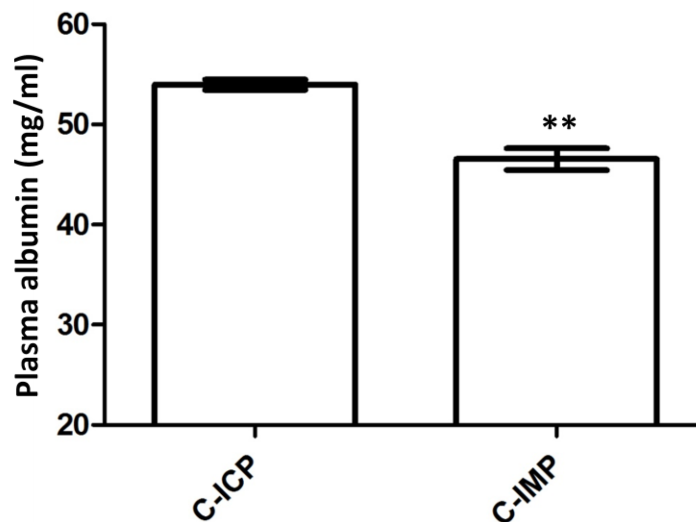


Figure 4.9 Graphical representation of total plasma albumin estimated by BCG method (n = 3 biological replicates in three technical replications). Values are mean ± S.E. Statistical significance of $p < 0.005$ is represented by ** as calculated by Student's t-test.

There are previous reports that show immunization with an antigenic peptide of a protein can mount autoimmunity against the entire protein. Systemic lupus erythematosus (SLE) is an autoimmune disorder which is characterized by the presence of autoantibodies against many different components of the cell including double stranded DNA and ribonucleoproteins Ro (or SS-A) and La (or SS-B) (Tan, 1989; Hahn et al., 1997; Harley et al., 1997; Craft, 1997). It was shown that immunization with short

peptides of Ro autoantigen protein lead to the development of autoimmunity against entire ribonucleoprotein (Scofield et al., 1996, Deshmukh et al., 2000), this is explained by inter and intra molecular epitope spreading in the heterogeneous autoimmune response (Topfer et al., 1995; Kinoshita et al., 1998; Tseng et al., 1997; Craft et al., 1997). Sjogren's syndrome is another chronic and progressive systemic autoimmune disease which occurs along with another autoimmune disease such as SLE or rheumatoid arthritis (RA) primary biliary cirrhosis, polymyositis, or scleroderma (Manoussakis 2001). The autoantigens recognized in Sjogren's syndrome are Ro/La (or SSA/SSB) ribonucleoprotein particle and these are present in all the cell types. Hence, eventually the manifestations of this disease involve the lungs, kidneys, skin, muscles, bone marrow, joints, and/or vasculature (Harley et al., 1992). Immunization of Balb/c mice with short peptides from the 60-kDa Ro antigen recapitulated the illness similar to Sjogren's syndrome and model exhibited high-titer anti-Ro and anti-La as found in humans with Sjo¨gren's syndrome (Scofield et al., 2005).

4.3.7. Conclusion

In our present study we observed increased HbA1c and increased AGE modification of serum albumin in the plasma from mice immunized with CML-MSA. This was accompanied by the increased level of proinflammatory cytokines, elevated levels of serum albumin in the CICs and an overall decrease in the level of plasma albumin. We also have observed previously that low levels of plasma albumin are associated with increased HbA1c and plasma protein glycation (Bhonsle et al., 2012). In conclusion it appears that immunization of CML modified self protein ie MSA induces autoimmune response leading to enhanced formation of CICs and subsequent decrease in the plasma albumin level, which in turn might cause the increased AGE modification in serum albumin and increased HbA1c.

Table 4.1 Data showing levels of blood glucose and HbA1c.

Sl. No.	Parameter	C-ICP (n=4)	C-IMP (n=4)	*p*-value
1.	Blood sugar (mg/dl)	81.7 ± 24.9	112.5 ± 27.3	0.148
2.	% HbA1C	4.22 ± 0.3	5.32 ± 0.2	0.0007

Summary and future perspectives

This thesis mainly deals with study of glycated proteins acting as autoantigens in elicitation of autoimmune response and formation of CICs in diabetes induced chronic hyperglycemia. We investigated IgG immune complexome using protein G sepharose affinity chromatography followed by label free based mass spectrometric quantification of CIC associated proteins in different stages of clinical diabetes. The major finding with the clinical studies was elevated levels of HSA in the CICs of IGT, NDD and DM-MIC compared to healthy CON. However, there was no significant difference in the CIC HSA level in DM group, which could possibly be due to ongoing treatment and management of diabetes. Western blotting with anti-AGE antibodies and MS analysis also confirmed that the HSA was AGE modified in CICs, and also HSA in the plasma from diabetic conditions such as NDD, DM and DM-MIC was modified to a greater extent compared to healthy control as measured by plasma fluorescence for AGEs and western blotting.

To corroborate our observations and results of the CIC analysis of clinical plasma, STZ induced diabetic mice with or without treatment with AGE inhibitor AMG was used as study model to study CICs. AMG treatment has been shown to ameliorate the development of complications in diabetes in vivo by inhibiting the AGE formation. Diabetic mice plasma analysis revealed pronounced elevation of serum albumin in the CICs compared to that of CON mice plasma. Which was further decreased in the plasma CICs from mice treated with AMG which was also accompanied by overall decreased AGE modification in plasma proteins. MS analysis of MSA for AGE modification also showed decreased AGE modified peptide intensities in diabetic mice treated with AMG. In addition quantification of cytokines which are integral part of immune response, that participate in pathogenesis of inflammation was performed to understand autoimmune response as a consequence of hyperglycemia mediated AGE modification. This analysis showed marked increase in the levels of proinflammatory cytokines TNF-alpha, IL-1 beta, IL-2, in diabetic mice plasma compared to that of control and treatment of AMG had counter effects on levels of increased proinflammatory cytokines in diabetes.

The findings of clinical work on CICs was corroborated with mice model study, wherein we found increased albumin levels in CICs from diabetic mice, which in turn was reduced with treatment of diabetic mice using AMG. It is also reported in earlier studies that transcription of albumin gene itself is reduced in conditions of diabetes mellitus via reduced binding activity of hepatocyte nuclear factor 1 and also reduced serum albumin levels were observed in T2DM patients with nephropathy who suffer from heavy proteinuria. In light of previous findings and our current study one can prudently conclude the involvement of autoimmune response directed against AGE modified serum albumin, as an important contributing factor for the reduction of serum albumin levels in diabetic patients along with proteinuria and decreased albumin gene expression.

Further, there are previous reports that reactive immunization with small portion of an antigenic protein can bring about autoimmune response against entire protein, which is demonstrated in case of antigens of systemic autoimmune disorders such as SLE and Sjogren's syndrome. To understand the effect of reactive immunization with AGE modified self protein, we studied CICs from mice immunized with CML-MSA. Interestingly, our data suggested increased levels of MSA in CICs from mice plasma immunized with CML-MSA compared to that of mice immunized with unmodified MSA. This was accompanied by slight but significant increase in AGE modification in plasma albumin, decreased albumin level in plasma and increased HbA1c in group of mice with reactive immunization of CML-MSA to that of unmodified MSA immunization. However there are also studies which have reported inhibition in development of nephropathy in diabetic mice immunized with AGE modified BSA and the protective role of autoantibodies generated against AGE proteins is also implicated. It can also be because the peptide epitopes exposed with immunization with AGE modified non self protein such as bovine serum albumin (BSA) and epitopes of a self protein MSA are differently recognized by the immune system involving intricate complex pathways. The area which needs still to be explored is the mechanism of autoimmune response to AGE modified self proteins and non self proteins.

In conclusion we propose that longitudinal and large prospective cohort studies using advanced high throughput technologies of proteomics and other interdisciplinary approaches of immunological techniques in diabetic conditions should be performed, which might unravel deeper insights of the mechanisms of AGE modification induced autoimmune response and formation of CICs. This might also help in understanding effect of glycated protein serum albumin which is the most abundant plasma protein and also the combined effect of other proteins prone to glycation and autoimmune response in pathogenesis of inflammation which is believed to be the major underlying factor not only in the development of diabetic complications but also development of insulin resistance and diabetes.

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Curriculum Vitae

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Publications:

1. **Bhat S**, Jagadeeshaprasad MG, Patil YR, Shaikh ML, Regin BS, Mohan V, Giri AP, Balasubramanyam M, Boppana R, Kulkarni MJ. Proteomic insight reveals elevated levels of albumin in circulating immune complexes in diabetic plasma.
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2. Vannuruswamy G, Jagadeeshaprasad MG, Kashinath K, Keshavan SK; **Bhat S,** Korwar AM, Chougale AD, Boppanna R, Reddy SD, Kulkarni MJ. Molecules with O-acetyl, not N-acetyl group, protect protein glycation by acetylating lysine residues. RSC Adv., 2016, 6, 65572-65578, DOI: 10.1039/C6RA11313C (**Impact Factor: 3.840**).

3. Korwar AM, Vannuruswamy G, Jagadeeshaprasad MG, Jayaramaiah RH, **Bhat S,** Regin BS, Ramaswamy S, Giri AP, Mohan V, Balasubramanyam M, Kulkarni MJ. Development of Diagnostic Fragment Ion Library for Glycated Peptides of Human Serum Albumin: Targeted Quantification in Prediabetic, Diabetic, and Microalbuminuria Plasma by Parallel Reaction Monitoring, SWATH, and MS^E. Mol Cell Proteomics. 2015 Aug; 14(8):2150-9. doi: 10.1074/mcp.M115.050518. Epub 2015 May 28. (**Impact Factor: 5.912**)

4. **Bhat S**, Mary S, Banarjee R, Giri AP, Kulkarni MJ. Proteomics Clin Appl. 2014 Feb; 8(1-2):19-34. doi: 10.1002/prca.201300068. Review. (**Impact Factor: 2.956**)

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Book Chapter:

1**. Bhat S,** Mary S, Giri AP, Kulkarni MJ. *Advanced glycation end products and diabetic complications.* In Mechanisms of vascular defects in diabetes, (Ed: Kartha CC), Springer (2016).

Manuscripts under preparation:

1. Bhat S, Jagadeeshaprasad MG, Shaikh ML, Giri AP, Boppana R, Kulkarni MJ. Reactive immunization with CML-MSA of normal mice increases glycation in haemoglobin and plasma albumin accompanied by decreased total albumin level.

Education and Honors

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- Trypsin digestion and data independent acquisition on NanoLC-synapt G1 HDMS, MALDI-TOF/TOF 5800, Micro LC-Triple TOF 5600, Q Exactive(HR-MS/MS) Benchtop Quadrupole-Orbitrap Mass Spectrometer.
- Expression analysis and PTM analysis (Glycation) of mass spectrometry data.
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Conferences and presentations

1. International symposium on "**Proteomics Beyond IDs**" and Fourth Annual Meeting of Proteomics Society, India. **CSIR-National Chemical Laboratory**, **Pune** during November 22-24, 2012
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