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

Fluorescent AGEs

Non-fluorescent 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 (Šebekova 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/macrophages and also exists in endothelial, mesangial, neuronal and other mesenchymal cells. AGE receptor 1 (AGE-R1) formerly known as oligosaccharyltransferase complex-48 (OST-48) is a main receptor involved in active turnover 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 TIDM patients, which was correlated with increased level of serum AGEs (He et al., 2001). Another receptor involved in 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 pathological elicitting 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

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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.
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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 antiseif 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 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 C1q, 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 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 T1DM 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 Genel, 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
Chapter 2: Analysis of CICs in clinical plasma

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 ethylenediaminetetraacetate (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 (HbAlc), 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 DIVI 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\(^5\) (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 \(\mu\)m and length 20 mm) (Waters Corporation) was used for preconcentrating and desalting of the injected sample with 0.1% mobile phase B at a flow rate of 5 \(\mu\)l/min. Nano-LC separation was performed using an Ethylene Bridged Hybrid (BEH)-C18 (1.7 \(\mu\)m x 75 \(\mu\)m x 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/\(\mu\)l), 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\(^5\) 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
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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 HIS 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 (Advanta, 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

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, croline, 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 Na2HPO4, 2 mM KH2PO4, 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.