Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia resulting from defect in insulin secretion, insulin action or both. In 2014, estimated diabetic population was 387 million worldwide. Same year, about 67 million diabetic population diagnosed with disease in India, known as diabetic capital of world. Atherosclerosis is the major cause of premature death in diabetes. Dyslipidemia is a key feature of diabetic-atherosclerosis where LDL particles pose a major atherogenic threat. Atherogenicity of LDL may be influenced by damage to apoB100 by glycation. AGE-modified LDL, as well as other AGE-modified proteins, are also pro-inflammatory. The pro-inflammatory properties of modified LDL appear to be considerably enhanced as a consequence of their immunogenicity. The presence of antibodies to reductively glycated LDL leads to accelerated clearance but antibodies to non-reductively glycated LDL had no effect on its rate of clearance. It appears that the difference in antibody response and subsequent clearance rates may be in part the result of different degrees of apolipoprotein modification and in part the result of epitope differences. AGE-LDL may have pathophysiologic relevance, because it suggests the presence of circulating antigen-antibody complexes, which are believed to be potently atherogenic. Among all the reducing sugar, D-ribose is one of the most reactive sugar which plays an important role in the glycation of biomolecules. Moreover, the bioavailability of D-ribose makes this carbonyl species quite reactive and damaging, therefore having direct implication in diseases. Till now, no study stated the immunogenicity of D-ribose glycated LDL in patient’s sera. In view of the importance of glycation of DNA/LDL in diabetes & atherosclerosis, the present thesis entitled “Immunochemical studies on D-ribose glycated DNA/LDL: role in vascular and inflammatory diseases” incorporated the study of ribose induced glycation of DNA and LDL, in
generation of antibodies against these glycated conformers, and to examine the binding of induced antibodies against ribose modified LDL in diabetic atherosclerotic and diabetes-atherosclerotic subjects.

Phase I involved the study of physico-chemical characterization of D-ribose glycated calf thymus-DNA (CT-DNA) and LDL. In phase II, the specificity of induced antibodies against native and D-ribose glycated CT-DNA & LDL were detected in immunized rabbit’s sera by using ELISA. Furthermore, the histopathological study was performed on the rabbit’s kidney sections to detect immune complex deposition. In phase III of the thesis, induced antibodies against D-ribose-LDL were probed in T1 diabetes mellitus (T1DM), T2 diabetes mellitus (T2DM), atherosclerosis (ATH) and T2 diabetes mellitus-atherosclerosis (T2DM-ATH) patient’s sera with respect to healthy subjects by using ELISA.

The overall findings of the three different phases are appended below:

**Phase I**

In phase I the pilot experiments were performed to determine the time of incubation and optimum concentration of D-ribose, required for modification of CT-DNA. Generation of DNA Amadori products was confirmed from reduction of yellow NBT to purple monoformazan. UV-vis spectrophotometer characterized the sample showing increased hyperchromicity at 260 nm due to adduction-cum-free radical mediated damage to sugar phosphate backbone and exposure of DNA bases as a result of strand breaks, partial unfolding of double helix and unstacking of bases. Furthermore, the occurrence of an additional peak at 325 nm might be due to glycation of nitrogenous bases of DNA and the generation of DNA bound AGEs. In agarose gel electrophoresis pattern, glycated sample showed maximum mobility of DNA macromolecule as compared to
native analogue. This might be due to the generation of single strand breaks by glycation, formation of shortening or small size DNA showing faster mobility in comparison to the native form. Possible presence of fluorogenic AGEs in the glycated CT-DNA was verified with AGE-specific fluorescence at \( \lambda_{em} \) of 450 nm after excitation at \( \lambda_{ex} \) 370 nm. The increase in fluorescence intensity in glycated DNA was observed as compared to the native form. The increase in fluorescence intensity of glycated CT-DNA in comparison to native analogue suggests that glycation of DNA by D-ribose generates fluorescent DNA-AGEs, having heterocyclic structures which makes the glycation adducts to fluoresce. Structural changes in DNA were evaluated by ellipticity measurements. Glycated DNA showed negligible change in ellipticity. However, glycation corresponds to structural loss in the DNA molecule. Melting profile of modified and unmodified CT-DNA was analyzed in the temperature range of 30°C to 95°C. A decrease in Tm value of modified CT-DNA points toward helix destabilization as a result of modification. In thermal denaturation a decrease of 9°C in the Tm value of AGE-DNA points towards structural fluidity in the backbone of the DNA macromolecule as a result of glycation. This decrease in Tm value in case of glycated DNA point towards the susceptibility of the glycated DNA toward heat denaturation.

In view of the structural perturbation of D-ribose glycated LDL (D-ribose-LDL) in comparison to native LDL, commercially available LDL was incubated with D-ribose for three weeks & characterized by various biophysical techniques and assays. Compared to 282 nm peak of native LDL, the increase in hyperchromicity reflects the exposure of chromophoric aromatic amino acid residues due to the unfolding and fragmentation of protein upon glycation. This could be attributed to the generation of LDL-AGEs. The formation of fluorescent
LDL-AGEs was confirmed by fluorescence spectroscopy by exciting AGE-LDL at 350 nm and recording the emission maxima at 450 nm. An increase of fluorescence intensity was observed in AGE-LDL, as compared to the native LDL. To study the effect of D-ribose induced glycation on the conformational characteristics of LDL, the CD spectral profile was recorded in the wavelength range of 200–250 nm. CD spectroscopy of native LDL showed characteristic spectrum of a primarily α-helical protein with negative minima at 210 nm indicating a significant loss of β-structure on glycation. Thermal denaturation of native and AGE-LDL was investigated between 30 to 95 °C. A decrease of 9 °C in the Tm value of AGE-LDL points towards the structural fluidity in the backbone of the LDL macromolecule as a result of glycation. This decrease in Tm value in the case of glycated LDL directs towards the susceptibility of the AGE-LDL for heat denaturation. The average carbonyl content (±SD) of three independent assays were corresponds to almost six fold increase in AGE-LDL as compared to native LDL. For the detection of early glycation products, native LDL alone showed almost negligible amount of ketoamine content in comparison to glycated LDL. Similarly, HMF that has been formed in glycated LDL was also maximum, whereas native LDL had almost negligible level of HMF.

Based on the combined in vitro results, it has been concluded that during the specified time of incubation of DNA and LDL with D-ribose, the structural perturbation caused in macromolecule as a result of glycation, points toward the formation of AGEs (DNA-AGEs, LDL-AGEs).

**Phase II**

In comparative analysis of D-ribose glycated CT-DNA and LDL case, antigenicity of D-ribose-modified CT-DNA and LDL were probed in female
rabbits. Results depicts that modified form is more immunogenic than its native form. D-Ribose-LDL antiserum showed very high titer antibodies in direct binding ELISA. Specificity of the D-ribose-modified CT-DNA and LDL antiserum were also investigated by competitive inhibition ELISA which was evident from inhibition in antibody activity using immunogen as inhibitor. The antiserum from obtained D-ribose-DNA and D-ribose-LDL immunized animals, exhibited wide range of heterogeneity in recognizing varied inhibitors that included DNA and LDL from different sources as well as free DNA bases and modified proteins. The antiserum showed considerably low recognition for native and OH modified conformers. The cross reactivity with various bases, DNA molecules and various protein, LDL, may be due to the recognition of epitopes. The cross reactivity shown with various bases may be due to the recognition of antigenic determinants which may be common with D-ribose glycated CT-DNA. Moreover, the binding of D-ribose glycated various amino acids and proteins revealed that their antigenic determinants or epitope might also common with D-ribose glycated LDL. The significant inhibition of glycated guanine and glycated Lysine residues might be due to identical or same epitope present on D-ribose glycated CT-DNA and D-ribose glycated LDL respectively. No significant inhibition in native and OH radical modified CT-DNA, bases, amino acids and proteins residues stated that antigenic determinants or epitopes present on these inhibitors were not similar to D-ribose-CT-DNA or D-ribose-LDL.

In histological examination of kidney sections of study of NZW female rabbits native LDL showed a normal kidney morphology having normal glomerular, tubular, and vascular structure. Whereas D-ribose-LDL immunized rabbits revealed hyper cellular and congested glomeruli with thickened basement
membrane indicative of immune complex deposition. In the fluorescence microscopic examination, low fluorescence signal was detected in FITC-labeled kidney sections of rabbits immunized with native LDL. However, kidney sections of D-ribose-LDL-immunized rabbits showed GBM having a series of intense fluorescence points. The deposition of immune complexes in the kidney points toward the possible involvement of D-ribose in glomerulonephritis.

In conclusion, the phase II clearly demonstrated that D-ribose glycated LDL is more antigenic than D-ribose glycated CT-DNA, which prompted us to evaluate the binding specificity of induced antibodies against glycated LDL in diabetic, atherosclerotic and diabetic-atherosclerotic subjects.

**Phase III**

Based on the high antigenic activity, antibodies against glycated LDL were probed in patient’s sera with respect to healthy subjects. Oxidation of lipoproteins logically resulted in an increase in protein carbonyl contents, a recognized biomarker of oxidative stress. The average carbonyl contents (±SD) of three independent assays of patient’s sera (T1DM, T2DM, ATH and T2DM-ATH) were high whereas HC sera had almost negligible level of carbonyl content. This corresponds to almost four to six fold increases in carbonyl contents in patient’s sera as compared to HC. For the detection of early glycation products, the ketoamine moieties formed in patients sera were measured calorimetrically by NBT assay. HC alone showed almost negligible amount of ketoamine content, whereas patient’s sera had maximum ketoamine content. Similarly, HMF that has been formed in the early glycation of LDL was determined as thiobarbituric acid reactive substance after hydrolysis. This shows that early and intermediate glycation product concentrations were higher in patient’s sera in comparison to HC. The higher yield of HMF in patient’s serum
sample is in agreement with the NBT assay result. The pilot study was performed to screen out the positive sera samples (sera showing higher binding with immunogen) from T1DM, T2DM, ATH and T2DM-ATH subjects. Out of total 74.42% sera samples of T1DM, 43.42% of T2DM, 50.94% of ATH and 76.39% sera samples of T2DM-ATH showed enhanced binding with the glycated LDL as compared to the native form. Competitive ELISA was carried out to analyze the binding specificity of circulating antibodies in patients to native and glycated LDL. In T1DM, T2DM, ATH and T2DM-ATH group, it has been observed that maximum inhibition has been observed with glycated LDL in comparison to native LDL. These results indicate substantial recognition of the glycated LDL by serum antibodies in patients. It might be possible that duration of the disease may be having an effect on the quantity/titre of anti-LDL antibodies. Immunogenicity of glycated LDL has been increased two folds upon glycation with D-ribose which may lead to the formation of antibodies, immune complexes and development of other complications in T1DM, T2DM, ATH and T2DM-ATH patients.

Based on the above evidences it has been concluded that in vitro treatment of LDL with D-ribose causes biophysical and biochemical alterations in LDL resulting in the formation of LDL-AGEs. Vigorous humoral response in animals immunized with D-ribose-LDL suggests an alteration in the LDL structure, resulting in the generation of neo-epitopes, which are recognized as alien by the immune system, leading to the robust production of antibodies. These induced antibodies against D-ribose-LDL were probed in patient’s sera in comparison to HC. From clinical data it is conclude that anti LDL antibodies were found in the sera of these diseases & there presence was also related to the disease and its complications. So a large prospective and multicentric study will be fruitful in
ascertaining the role of anti-glycated LDL antibodies in the secondary complications associated with different diseases.