‘Diabetes’ is derived from Greek words. ‘Dia’ meaning ‘through’ and ‘bainein’ to go. The word diabetes was coined by Aretaeus (81-138AD) of Cappadocia to describe a condition where fluids not remain in the body but goes through as though the patient were a ‘siphon’. Indian scholars Charaka and Surruta in the 6th century BC named the disease “Madhumeha” meaning sweet tasting urine. Hence the credit of identifying the sweet nature of diabetic goes to Indian scholars. The mellitus which was added later on to ‘Diabetes’ refers to the sweet taste; ‘Mel’ in Greek means honey.

Susruta appears to have identified the cause of diabetes. He wrote that the disease was brought on by “gluttonous overindulgence in rice, flour and sugars” Susruta’s observations are relevant in view of the “gluttonous over indulgence of pure sugars” by the present day from developed countries or developing countries.

Among the four basic mechanisms underlying the diabetic complications, formation of advanced glycation end products is investigated in this study with an attempt to link diabetics and cardiovascular diseases.

Several large studies have shown the benefits of intensive control of blood glucose among diabetics. The Diabetes Control and Complications Trial (DCCT) was a major clinical study conducted from 1983 to 1993 on 1441 volunteers with type 1 diabetes. This study showed the benefits of keeping the HbA1c levels as close to the normal values of 6% as possible. This study was carried out in North America and Canada.

Many observational studies also support the correlation between glycemic control and diabetic complications in patients with type2 diabetes. However there have been only three randomized controlled trials attempting to test the benefits of glycemic control on the incidence of complications. The University Group Giabetes Program (UGDP) found no benefit of glycemic control (Cornfield 1971).

The second controlled trial in type 2 diabetes was conducted on 110 lean Japanese subjects. Glycemic control correlated with reduction in risk of microvascular complications in these subjects.
The third study was carried out on 153 men. This study showed no benefits of glycemic control on cardiovascular events.

The largest and the longest study on type 2 diabetics was conducted in UK, and is known as United Kingdom Prospective Diabetes Study (UKPDS). It recruited 5102 patients with newly diagnosed type 2 diabetes between 1977 and 1991. Patients were followed up for 10 years. This study confirmed related deaths.

Glucose and diabetes have not only a correlational relationship but also a causal relationship. Interestingly in the in vitro glycation of HDL, while glyoxal showed a dose dependent inactivation of HDL-PON, the glucose concentration up to 500mM was protective.

In an in vitro model used to characterize the protective effect of glucose on eye lenses subjected to oxidative stress glucose was able to protect the lenses from oxidative damage (Kletzky et al 1986). In an in vitro study high glucose levels were tested for this ability to protect against ischemia-induced synaptic transmission damage in hippocampal slices. Glucose at high concentrations of 10mM and 20mM significantly protected against ischemia-induced damage (Tian and Baker 2001). Interestingly at day 60, the high glucose was no longer protective suggesting that under prolonged exposure the Pro-oxidative effects may persist longer than the antioxidant effects.

Contrary to these effects, the AGE formation was seen with 10mM Glucose by fluorescence technique. If AGE is formed at even as low as concentration as 10mM glucose, how it could still protect PON from inactivation is not known.

It is possible that the amino groups modified by glycation are not essential for enzyme activity. PON has also shown to have histidine dyad in its active site. A catalytic mechanism involving histidine has been proposed by Harel et al. in 2004 (Fig.4.1).
PON active site has been shown to be a multiple tasking active site where the same active site has been used for both arylesterase as well as paraoxonase activity (Harel et al. 2004). In hydrolytic enzymes histidine imidazole is involved in a base catalysed rate determining step (Harel et al. 2004). Histidine often serves as a base, deprotonating a water molecule and generating a hydroxide ion that brings about hydrolysis.

The pH activity profile of PON suggested the presence of a basic amino near the active site (Harel et al. 2004). Another amino acid cys 284 is conserved in all PONs and has been attributed an antiatherogenic role (Aviram et al. 1998). Cys 284 is a part of a highly conserved sequence that includes active site His 285.

It is interesting to note that glycation brought about differential inactivation of phenyl acetate hydrolyzing activity and Paraoxon hydrolyzing activities.

Glycation may effect the residues in and around the active site whereby the binding of substrates may be affected, resulting in the observed differences in the catalysis.

**Fig.4.1 Catalytic Dyad of Histidine**
Most remarkable change after glycation was the 6 fold increase in thiolactonase activity. However this increase was seen only with glyoxal and not glucose.

Experiments to improve catalytic activity of paraoxonase have yielded many mutants with different levels of activities compared with the wild type. Site directed mutagenesis has shown against many fold changes in the enzyme activities against paraoxon or phenyl acetate.

Lactonase binding to the active site of PON contains a lys residue, Lys 192. Since lysine can be easily glycated, it can undergo glycative modification, why only glyoxal mediated glycation had increased the Thiolactonase activity activity but not glucose catalysed glycation is not understood.

Glycation with glyoxal or glucose showed a small but significant increase in Trp fluorscence. PON has atleat one Trp near the calcium and is part of the active site.

Amino group modification suggests that many amino groups are modified by glycation at higher concentration (1M) but not at 100 mM. Although there was no significant difference at 1 M concentration between glucose and glyoxal in the amino groups modified why only glyoxal modification increased thiolactonase activity is not known.

Purification of PON by cibacron Blue Sepharose affinity chromatography gave quantitative recovery of esterase activity was 62 % whereas paraoxonase activity was 10.8 fold greater. There are several possible explanations.

It is possible that glycation modified a subset of HDL which was poor in paraoxonase activity but rich in esterase activity. Consequently glycation would have reduced the activity thereby making the recovery about 62 %.

But why should the Paraooxonase activity increase 10.8 fold is not understood. PON activity does not have any physiologically relevant inhibitors. Hence removal of inhibitors from HDL cannot be offered as a mechanism.
Another possible mechanism is that glycation of proteins of HDL would cause a change in the structure of HDL-associated protein. These proteins could dissociate from the HDL particle. A loss of 15 KD protein from the HDL after glycation is suggestive of such a mechanism. Whether PON has any activator/inhibitor protein in the HDL particle is not known.

PON has been shown to be assembled in HDL particle containing Apo A1 has been demonstrated (Oda et al 2001). However activation or inhibition of PON 1 by Apo A1 or gylcated Apo A1 has not been shown. PON 1 has been shown to be affected by lipids, but modification of Lipids by glycation is not known.

Glycation had changed the mobility of HDL proteins on native PAGE. Still why it was quantitatively eluted from Ciacon bluse sepharose column is not known.

Interestingly 95% of the loaded paraoxonase activity came out in the wash and only 5% was bound to the column. It is possible that the HDL of Indian subjects is already modified and hence it does not bind to CBS column. But if it is was true then additional glycation should have ensured that 100% of the proteins is recovered in the flow through. But this was not the case.

The CD spectra of gylcated HDL showed significant changes when compared with native HDL. There was no difference in the glycation by gyloxal or by glucose as far as CD spectra are concerned. The CD spectra obtained by us is in agreement with the CD spectra reported in literature. CD of HDL was shown to have negative troughs at 208 nm and 222 nm which are characteristic of α helical structure (Gotto and Shore, 1969). The α helical nature of HDL proteins have been confirmed by other techniques like small angle neutron scattering (Wu et al 2009). Chetty et al 2012 have shown that the Apo A1 of HDL that is not in contact with the lipid assumes aperiodic structure.

In view of these findings it is interesting to note that glycation of HDL proteins decreased their α helical content and increases the β sheet content. B sheet structure can be considered as an extended α helical structure. This stretching of α helix is possible if the shape of the HDL particle changes due to glycation or the lipid
–protein interaction changes as a consequence of glycation. However these changes if any in HDL particle could not be determined.

In order to mimic the effects of glycation invivo, human umbilical cord was used. Umbelical cord has vein and two arteries. The vein was filled with gyloxal in HBSS and allowed to stand for two days at $4^\circ$C. There were no detectable changes in the umbilical vein when the sections were stained by Hematoxyin-eosine, but the formation of AGE culd be detected by fluorescence. This finding suggests that Nonenzymatic glycation is capable of forming AGEs in the arteries and veins of diabetis. Formation of AGE would certainly alter rhe physiological properties of the arteries and veins. The auto fluorecence of AGEs have been used to detect AGEs in skin and cornea (MEirwaldt et al 2004, Tseng et al 2010).

Injury to arteries described as the initiating cause for atherosclerosis could very well be that the “injury” was a result of advanced glycation end product formation. Injury to endothelial cells has been experimentally demonstrated in diabetic, sucrose-fed aged rats. (Arbogast et al 1984).

We wanted to investigate whether this glycative modification by gyloxal and glucose could be inhibited by endogenous/natural molecules. Our results show that vitamin C could prevent glycation by gyloxal but not glucose. Where as vitamin E could prevent the glycative loss of PON activity of HDL caused by both gyloxal and glucoase. The phytochemical quercitin was also able to prevent the loss of PON activity of HDL by gyloxal but not glucose. In this its action was similar to that of vitamin C Since Vitamin C and quercitin are both water soluble molecules, it is possible that gyloxal exerts an aqueous phase stress which these chemicals are able to neutralize. On the other hand vitamin E being a lipid soluble molecule was a better protective agent than ascorbic acid or quercetin. It is possible that vitamin E partitions into the lipid phase where it can effectively prevent the glycative modification of HDL.

Studies with Antibodies

In our study we used Apo A1 as a molecule to glycate and raise antibodies. Apo A1 is tightly associated with HDL and can be an integral part of the HDL
molecule. To our knowledge we did not find any HDL particle not containing Apo A1. Moreover in our electrophoresis we found the Apo A1 band distinct from other protein bands. Hence we choose to electro-elute this band and use it to raise antibodies.

HDL particle as such was not used since there are some common proteins between HDL and LDL Collins and Oliver (2010) have found 26 peptides /proteins common to both HDL and LDL though at different concentrations.

In the silver staining of the electrophoresis of Apo A1, pure electro-eluted Apo A1 gave three bands at equal distance. This suggests that the protein could associate with itself and form oligomers which may not get dissociated under conditions of SDS- PAGE.

Relatively low antibody titre fund in the blood of the rabbits. Apo A1 being an animal protein, its antigenicity may be low in the rabbits. Gylcation may not be changing the protein structure drastically for it to become highly antigenic. When two different samples of Apo A1 were glycated separately, their ability to react with the antibody was different. This suggests that the glycated Apo A1 may only be slightly be modified.

In our study to determine the level of glycated HDL in Diabetics, we did not find any significant difference between control subjects and diabetics. The mean glycated Apo A1 was 2.34±1.0 ng/ml. Even though there were some high values, there were equally low values also. It is surprising that the glycated Apo A1 is not significantly different from control subjects who were all young people with the mean age of 25.3 years. In an earlier study we had showed that the PON values of 288 young people was low and that even smoking did not affect it further (Mahadesh Prasad et al 2010). These results are consistent with our observation. We had suggested that HDL of Indians is already modified at an early age. This study confirms the suggestion we had made.

Since non enzymatic glycation is well established in sugars like fructose, increased sugar consumption may be the reason for the non enzymatic glycation of serum proteins even at ayound age. In 2008-09 Indians consume 233 lakh tons of
sugar. This was increased from 218 lakh tons from the previous year. According to the statistics of sugarcane breeding institute Coimbatore, in India, the consumption of sugars has sturdily increased from about 5 kg per annum per person in 1960 to about 17 kg per annum per person now (Fig.4.2).

**Fig.4. 2 Trends in per capita consumption of white sugar and Gur-Khandsari in India**

Hence it is possible that the blood proteins of even the young people are glycated. In fact the mean glycated Apo A1 in the control group was higher than that of the diabetics. Our results are consistent with the values reported by Caines et al. who found that in nondiabetic subjects the glycated Apo A1 was 2.1±0.8 %  (Caines et al.1989 ).

The amount of glycated HDL present in the blood showed a complex relationship with HDL-C. At HDL-C levels < 45 mg/dl their appears to be no correlation between HDL-C and glycated HDL. However at HDL-C levels >45 mg/dl it showed a negative correlation. At higher concentrations of HDL, it may act as anti-inflammatory and antioxidant to a greater extent there by reducing the harmful effects
of glycation. HDL did not have the ability to break the AGEs once formed. Hence it is reasonable to assume that HDL may prevent the formation of AGEs.

HbA1c linearly correlated with glycated HDL suggesting that the factors which can glycate hemoglobin can also glycate HDL and other proteins.

When a sub group of the diabetics having high HbA1c (> 8.0 %) were compared with those who could control the HbA1c well within ranges (< 6%), though the glycate HDL was higher in the high HbA1c group, the differences were not significant.

When glyated HDL was treated with the antibody a precipitate was found. This precipitate has PON1 activity. It was surprising that glycation with glyoxal which actually inactivates PON activity had given more PON activity in the precipitate than the glucose catalyzed glycated HDL.

Since we had used antibody to glycated Apo A1 it is possible that the PON and Apo A1 are getting differentially glycated and the highly glycated ApoA1 containing HDL it does not glycate PON equally thereby higher amount of PON activity in the supernatant of glyoxal treated HDL. Thus, these results suggests that glyoxal being highly reactive, would react with the most accessible substrate. In this case it would be apoA1 rather than paraoxonase.

When HDL or serum were treated with the antiglycated HDL-antibody, a small precipitate was found suggesting that presence of glycated HDL in blood. When this glycated HDL was removed by precipitation the activity of PON increased. This suggests that glycated HDL may act as a competitive inhibitors of native HDL.

If the glycated HDL was removed from serum, it should be anti-inflammatory. However this was not seen in DCFH fluorescence study.

**HbA1c profile of South Indians.**

In order to study about the glycemic control excreted by diabetics in Mysore, this study was taken up. We found that as the duration of diabetes increased the HbA1c levels also increased. However when the duration increased beyond 14 years, the HbA1c actually decreased. This seemingly paradoxical observation has a simple
answer. With increasing duration of diabetes the subjects with high HbA1c would have died leaving only those with good glycemic control to survive.

When distribution of samples based on this HbA1c was carried, there were more females than males with higher HbA1c. There could be many explanations to this observation. It is well known that women live longer than men. Thus diabetic women would also live longer than diabetic men.

Hemoglobin levels had no effect on the HbA1c levels. It was only the blood glucose that decided the HbA1c and not the hemoglobin levels. In order to demonstrate in vitro glycation of hemoglobin, erythrocytes were washed and used for glycation with glucose or glyoxal. While glucose increased the levels of HbA1c in a dose dependent manner, glyoxal actually decreased HbA1c at the highest dose (100mM). The reason for this could be that at 100mM glyoxal could be toxic to erythrocytes causing their lysis. Only intact cells were used for the determination of HbA1c.

**Blood glucose estimation: plasma or serum**

In large scale studies where blood samples are collected a field station and analysed at a central laboratory, there is a critical factor which may affect many of the blood parameters. In order to evaluate the effect of time of storage of blood and the type of sample plasma or serum were investigated.

We found that serum can equally will be used to assay blood glucose level since the serum values were only 1.15% lower than the corresponding plasma values. Moreover, if the analysis is carried out with a time gap of over 10 min, there is going to be a decrease in the total blood glucose estimated.

These factors need to be seriously considered particularly when automated analysis of large number of samples is carried out.