Results
Chapter 1
Prediction of Metabolic Syndrome in Diabetic and Hypertensive Subjects
The metabolic syndrome is a combination of several factors which may share a common aetiology and each of which is a risk factor contributing to CVD. In the past few years the concept of metabolic syndrome has been a permanent topic of debate, particularly concerning its pathogenesis and clinical usefulness, and today still no consensus has been reached (Grundy, 2006). This is partially reflected by the use of several definitions, based on different diagnostic criteria, among them the definition proposed by the WHO, NCEP/ATP III, and that of EGIR are all the rage (Alberti and Zimmet, 1998). The various definitions have been shown to identify different individuals hindering comparisons between different studies and different populations. The NCEP/ATP III definition has however been found to be the most straightforward to implement because the five criteria (abdominal adiposity, hypertension, hypertriglyceridemia, low HDL, and hyperglycemia) were clearly defined. Although not included in the diagnostic criteria, an increase in the secretion of apoB-containing lipoproteins and pro-inflammatory cytokines may also contribute to the increased incidence of diabetes and CVD (Eckel, 2007). It has also been reported previously that the metabolic syndrome is well characterized by the presence of smaller, denser lipoprotein particles that increase their susceptibility to oxidative modification and a diminished serum paraoxonase (PON-1) activity that is a major determinant of the antioxidant capacity of HDL. These may be contributory factors to the increased presence and severity of coronary diseases in such patients (Garin et al., 2005). In general, the greater the number of metabolic syndrome components, the greater is the risk for these outcomes.

The newly detected diabetic and hypertensive subjects were consequently chosen for this study, to identify their risk of developing metabolic syndrome by estimating the parameters proposed by NCEP/ATP III along with the other risk markers.
1.1: Anthropometric observations

As earlier reports have proposed the involvement of elevated blood glucose and increased blood pressure in metabolic syndrome (Grundy et al., 2004), both diabetic and hypertensive subjects were included in the present work. Table 1.1 represents the anthropometric characteristics of control (C), diabetic (D) and hypertensive subjects (HT). It clearly depicts the elevated levels of fasting blood glucose (FBG) and blood pressure (BP) along with the records of age, family history and waist circumference of all subjects.

Several reports have suggested that prevalence of metabolic syndrome is associated with age. In the third National Health and Nutrition Examination Survey (NHANES III) performed in the USA, the prevalence of metabolic syndrome increased from 6.7% among participants of 20-29 years of age to 43.5% for 60-69 year-olds and was 42.0% for participants of 70 years or older (Alexander et al., 2003). Similar pattern was observed in this study as well. Our observations (table 1.1) illustrate that most of the diabetic and hypertensive subjects were from the age group of 50-59 years.

The effect of generalized obesity is also extremely important so much so that, in populations where obesity is more common in women than in men, the prevalence of metabolic syndrome is likely to be higher in women than in men. This trend can be observed in Indian, Iranian and Turkish populations (Onat et al., 2002; Azizi et al., 2003; Gupta et al., 2003; Ramachandran et al., 2003; Ozsahin et al., 2004). However, the pattern observed in the present study was in-a-way opposite as it showed higher waist circumference of men as compared to women. Besides, table 1.1 also suggests that the waist circumference of both hypertensive and diabetic subjects is always greater than that of control subjects.

The available data describing the role of genetic factors in determining the prevalence of metabolic syndrome are limited and many findings have yet to be replicated in other populations (Corella and Ordovas, 2004). The data from this study suggests, subjects having a family history of
either diabetes or hypertension are at a higher risk of developing the same disease and hence metabolic syndrome (table.1.1).

1.2: Lipid profile
The dyslipidemia typically found in subjects with the metabolic syndrome includes an elevated concentration of plasma TGs, a fraction of LDL in which the particles are smaller and denser than normal, and a low concentration of HDL cholesterol (Alberti and Zimmet, 1998). Fig.1.2 depicts the concentration of total cholesterol in all the participants of this study. It perceives that the average cholesterol of diabetic subjects (D) was maximum followed by hypertensive (HT) and control subjects (C). This result was consistent to the previous reports that have suggested an alteration in cholesterol metabolism during insulin deficiency (Young et al., 1988). It is known that in type 2 diabetic subjects, cholesterol absorption efficiency is low (Simonen et al., 2002), whereas the synthesis of cholesterol is high (Abrams et al., 1982). This could be a primary factor in modifying cholesterol metabolism (Young et al., 1988).

The figure also demonstrates the concentration of TGs in all study groups. Mean TGs were highest in hypertensive subjects (HT), followed by diabetic subjects (D) and lowest in control subjects (C). A meta-analysis of 17 prospective, population-based studies found that an increase in plasma triglyceride of 1 mM, or 89 mg/dl, was associated with an increased risk of CVD (Hokanson and Austin, 1996). The Physician's Health Study found that the relative risk of myocardial infarction increased with increasing postprandial triglyceride concentrations (Stampfer et al., 1996). A study by Jeppesen et al. (1998) has suggested that high plasma triglycerides were associated with an increased risk of ischemic heart disease. Evidence also exists that a combined measurement of plasma triglyceride and HDL, provides a strong index of risk of CVD (Gaziano et al., 1997). Eventually, measurement of HDL was also performed in this study and is demonstrated
in the same figure. No significant variation in HDL levels was observed among the three categories. Moreover, the values observed for diabetic (D) and hypertensive subjects (HT) were almost always similar. In addition, the LDL levels among control, diabetic, and hypertensive subjects are also illustrated and it is deduced that the concentration of LDL was highest in hypertensive subjects (HT), followed by diabetic (D) and control subjects (C).

1.3: sdLDL oxidation

A lipoprotein profile characterized by the predominance of small, dense LDL particles is one of the key elements of atherogenic dyslipidemia in metabolic syndrome (Krauss and Siri, 2004). *In vitro* studies have suggested that sdLDL particles confer increased atherosclerotic risk due to their increased susceptibility to oxidation (Chait et al., 1993). It was discovered that a combination of heparin and magnesium precipitate a part of LDL which remained in the supernatant. This fraction was identified as sdLDL and found very similar to the one isolated by ultracentrifugation. The supernatant obtained thus, was used as a source of sdLDL in the present study. It was oxidized in-vitro using cupric chloride (details given in 'methods') and the kinetics was observed in terms of peak time. The results presented in fig.1.3 indicate the earliest peak of oxidation of sdLDL among hypertensive subjects (HT) followed by diabetic (D) and control subjects (C). Thus, suggesting hypertension as a major risk to atherogenesis and metabolic syndrome. This result was in agreement with the previous reports that have found an inverse relationship between the lag time of in vitro LDL oxidation and the severity and progression of coronary atherosclerosis (Regnstrom et al., 1992) suggesting the enhanced susceptibility to oxidation may underlie the excess vascular disease observed in patients with diabetes. This is also consistent with the studies showing that the mean lag time of sdLDL oxidation is lower in patients with diabetes than in controls (Dimitriadis et al., 1995; Yoshida et al., 1997).
<table>
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<td>7</td>
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<tr>
<td>FBG (mg/dl)</td>
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<td>172 ± 1.8</td>
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<td>BP (mm Hg)</td>
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<td>50-59</td>
<td>50-59</td>
</tr>
<tr>
<td>WC (cm)</td>
<td></td>
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<td></td>
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<td>86 ± 1.5</td>
<td>105 ± 1.6</td>
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<tr>
<td>Females</td>
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<td>95 ± 1.6</td>
<td>93 ± 2.4</td>
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<td>Positive</td>
<td>12%</td>
<td>40 %</td>
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<td>Negative</td>
<td>88%</td>
<td>60 %</td>
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</table>

Table.1.1: Anthropometric observations in diabetic and hypertensive subjects.

The blood glucose level was determined by glucose oxidase method proposed by Trinder (1969). The diabetic state was confirmed when FBG concentration exceeded 120 mg/dl. The blood pressure (BP) and waist circumference (WC) for each subject was measured by the standard techniques (as described in 'methods'). Results are expressed as mean ± S.D. or percentage, wherever applicable.

C  Control group
D  Diabetic group
HT Hypertensive group
Fig.1.2: Lipid profile of diabetic and hypertensive subjects.

The concentration of serum TC, TGs, and HDL was measured by an enzymatic colorimetric test using commercial kits from Ranbaxy Diagnostic Div., India. LDL concentration was calculated using the Friedewald formula. Results are expressed as mean ± S.D.

C  Control group
D  Diabetic group
HT Hypertensive group
Fig.1.3: sdLDL oxidation time of diabetic and hypertensive subjects.

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al (1989). 10 µl of supernatant containing sdLDL, oxygenated PBS and 32 µl of 1 mM CuCl₂ was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored at 234 nm. Results are expressed as mean ± S.D.

C Control group
D Diabetic group
HT Hypertensive group
1.4: PON-1 activity

PON-1 is an HDL-associated enzyme that has been identified as an important determinant of the capacity of HDL to prevent oxidative modification to LDL. Aviram et al. (1998) showed that the esterolytic activity of PON-1 in serum HDL correlated inversely with the susceptibility of HDL to oxidation. The activity of this enzyme has also been reported to be decreased in cardiovascular diseases (Navab et al., 1997 and McElveen et al., 1986) and in diabetes mellitus (Mackness et al., 1998 and Sakai et al., 1998). A few scientists have observed reduced levels of serum PON-1 in the patients with metabolic syndrome (Garin et al., 2005). In light of these observations, serum PON-1 levels were measured in this study and the results obtained were quite similar. Fig.1.4 shows that the PON-1 activity was significantly reduced in case of hypertensive (HT) and diabetic subjects (D). However, no such diminution was seen in the control subjects (C) without diabetes or hypertension. This supports the notion that qualitative changes to the LDL and HDL profiles in metabolic syndrome is accompanied by a reduction in the capacity of one of the main antioxidant activities associated with HDL (Garin et al., 2005). This could be a contributory factor to the increased incidence and severity of CVD observed in metabolic syndrome affected populations.

1.5: CRP levels

Recently several studies have drawn attention to the finding of elevated levels of CRP, a sensitive marker of inflammation, in subjects of metabolic syndrome or in association with its components (Haverkate et al., 1997; Koenig et al., 1999; Mendall et al., 1996). Elevated levels of CRP were also found in type 2 diabetic patients with features of metabolic syndrome (Pickup et al., 1997). Similar results were obtained following the estimation of serum CRP levels of the subjects involved in this study.
Fig. 1.5 represents the CRP levels in control, diabetic, and hypertensive subjects. It is clearly depicted that CRP levels of both diabetic (D) and hypertensive subjects (HT) were greater than that of control subjects (C). The importance of this finding stems from the evidence that elevation in CRP level is associated with an increased risk of metabolic syndrome and cardiovascular events (Florez et al., 2006). These results strongly suggest that CRP levels might provide an additional measure for identifying subjects for these co-morbidities and who therefore, would benefit from preventive interventions.

1.6: TNF-α levels

Metabolic syndrome accompanies nearly all patients who become obese, yet the relationship between adiposity and insulin sensitivity is not clear. A possible aetiology for insulin resistance in obesity is the production of the cytokine, TNF-α. Recent human studies have demonstrated elevated TNF-α expression in the adipose tissue of obese subjects, and its decreased expression following weight loss (Hotamisligil et al., 1995 and Kern et al., 1995). Nonetheless, the present study reveals higher levels of TNF-α at the onset of diabetes and hypertension (fig. 1.6). It is clearly depicted that TNF-α levels were always greater in case of hypertensive subjects (HT), followed by the diabetic (D) and control subjects (C). Previously reported elevation in the waist circumference of both diabetic and hypertensive subjects might provide a coherent explanation for such a change in TNF-α levels. These results are consistent to the earlier reports demonstrating a strong relationship between TNF-α and metabolic syndrome (Bertin et al., 2000). These findings strongly recommend the estimation of TNF-α in prediction of metabolic syndrome.
Fig. 1.4: PON-1 activity of diabetic and hypertensive subjects.

The assays were performed in a final volume of 250 µl containing 1 mM phenylacetate and 2 mM CaCl₂ in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 µl of serum and was read at 270 nm. Results are expressed as mean ± S.D.

C  Control group
D  Diabetic group
HT Hypertensive group
Fig. 1.5: CRP levels of diabetic and hypertensive subjects.

The concentration of serum CRP was measured by an enzymatic colorimetric test using a commercial kit from Calbiotech Inc., USA. Results are expressed as mean ± S.D.

C Control group  
D Diabetic group  
HT Hypertensive group
Fig. 1.6: TNF-α levels of diabetic and hypertensive subjects.

The concentration of serum TNF-α was measured by an enzymatic colorimetric test using a commercial kit from Immunotech SAS, France. Results are expressed as mean ± S.D.

C  Control group
D  Diabetic group
HT Hypertensive group
1.7: Apo-B levels
Apo-B is the primary apolipoprotein of low density lipoproteins (LDL), which is responsible for carrying cholesterol to tissues (Chumakova et al., 2005). It has recently been found to be associated with metabolic syndrome and a few studies have also revealed the association of Apo-B levels of diabetic persons with metabolic syndrome (Relimpio et al., 2002; Onat et al., 2007). In another study, including 77 non-diabetic postmenopausal overweight and obese women, apo-B was found to be the primary predictor when compared with various inflammatory markers (like IL-6, CRP, TNF-α and others) among a variety of risk parameters such as adiposity, blood pressure, insulin resistance, TGs, etc (Faraj et al., 2006). It was suggested that high apo-B would be associated with increased risk of developing coronary heart disease and diabetes. These findings stimulated the verification of apo-B levels in the diabetic and hypertensive subjects included in present study. This was done to predict the risk of metabolic syndrome. Results presented in fig.1.7 perpetuate that the apo-B levels were high in both diabetic (D) and hypertensive subjects (HT). The control subjects (C) having neither diabetes nor hypertension, showed a noticeably low level for apo-B. This result further adds to the hypothesis that apo-B probably has a potential role in the development of metabolic syndrome.

1.8: Correlation between NCEP/ATP III parameters and observed parameters of metabolic syndrome
The NCEP/ATP III guidelines to propose metabolic syndrome state that a person must have three of the following five abnormalities: abdominal adiposity, hypertension, hypertriglyceridemia, low high-density lipoprotein (HDL) cholesterol, and hyperglycemia. Eventually, the prevalence of metabolic syndrome in diabetic and hypertensive subjects included in this study was also estimated on the basis of same criteria. In addition, the role
of sdLDL oxidation and serum paraoxonase (PON-1) activity in prediction of metabolic syndrome has also been recognized recently (Nishtha et al., 2008a). Hence, it may be assumed that there exist some relationship between the parameters incorporated in NCEP/ATP III guidelines and the other parameters being evaluated in this study. Fig.1.8 represents a correlation between the NCEP/ATP III components and the other observed factors namely CRP, TNF-α and apo-B levels together with sdLDL oxidation time and PON-1 activity measured in each group. It was witnessed that a significantly positive correlation was found between the levels of CRP, TNF-α, apo-B and NCEP/ATP III parameters. This implies elevation in all these parameters as metabolic syndrome progresses. On the other hand, a significantly negative correlation was deduced between sdLDL oxidation time as well as PON-1 activity with respect to NCEP/ATP III parameters. This reveals the existence of an inverse relationship of these parameters to metabolic syndrome. Thus, explains their declination as metabolic syndrome progresses.
Fig. 1.7: Apo-B levels of diabetic and hypertensive subjects.

The concentration of serum Apo-B was measured by an enzymatic colorimetric test using a commercial kit from Giesse Diagnostics snc., Italy. Results are expressed as mean ± S.D.

C Control group  
D Diabetic group  
HT Hypertensive group
Fig. 1.8: Correlation between NCEP/ATP III parameters and observed parameters of metabolic syndrome in diabetic and hypertensive subjects.

Pearson's correlation analysis was used for determination of relationship between NCEP/ATP III parameters and observed parameters of diabetic and hypertensive subjects. The correlation coefficient (r) of indicated parameters was plotted against the following parameters to get a positive or negative relationship.

1. sdLDL oxidation time
2. PON-1 activity
3. CRP concentration
4. TNF-α concentration
5. Apo-B concentration
Chapter 2
Treatment of Metabolic Syndrome
(Human Studies)
Unit 2 (A)
Treatment of Metabolic Syndrome in Diabetic Subjects
Metformin \((1\text{-}(\text{diaminomethylidene})\text{-}3,3\text{-dimethylguanidine})\) is an inexpensive compound with documented glucose-lowering effect in both obese and non-obese subjects having type 2 diabetes (Consoli et al 2004; Donnelly et al 2006). The reduction of glycemic levels upon supplementation with metformin is done primarily by inhibiting hepatic glucose output (Leverve et al 2003). The beneficial effects of metformin on parameters of the metabolic syndrome in clinical diabetes were confirmed by the UK Prospective Study Group (UKPDS, 1998a) and it was the only drug in this mega-trial that significantly reduced cardiovascular events.

Insulin analogs have also been developed by modifying the amino acid sequence of the insulin molecule. Insulin is available in rapid, short, intermediate, and long-acting types that may be injected separately or mixed in the same syringe. Rapid-acting insulin analogs (insulin lispro and insulin aspart) are available, and other analogs are in development. Regular is short-acting insulin. Intermediate-acting insulins include lente and NPH. Ultralente and insulin glargine are long-acting insulins. Insulin preparations with a predetermined proportion of intermediate-acting insulin mixed with short or rapid-acting insulin (for example, 70% NPH/30% regular, 50% NPH/50% regular, and 75% NPH/25% insulin lispro) are also available (American Diabetes Association, 2002).

The present study investigates whether a monotherapy with metformin or insulin (mixture of intermediate and short acting insulin) has any beneficial role or not in metabolic syndrome and DNA damage. The parameters being analyzed herein follow the guidelines of NCEP/ATP III and include the determination of fasting blood glucose, HDL, and triglyceride levels in all the subjects involved. In addition to this, sdLDL oxidation time, serum paraoxonase (PON-1) activity, concentration of inflammatory markers (CRP and TNF-α) as well as apo-B and lipid peroxides levels were calculated as earlier reports from this laboratory have published their role in metabolic syndrome (Nishtha et al., 2008b). Previous studies have also demonstrated
an association between increased DNA damage and diabetes (Collins et al., 1998). In light of these observations, the extent of DNA damage in diabetic subjects as well as the effect of metformin or insulin monotherapy against this damage was also monitored.

2A.1: Anthropometric observations

Improved glycemic control in diabetes has, to date, not shown any significant reduction in CVD. In fact, cardiovascular events increased in the Veterans Affairs Cooperative Study on Glycemic Control and Complications in Type II Diabetes (VACSDM), and the decrease in cardiovascular events in the United Kingdom Prospective Diabetes Study (UKPDS) was not statistically significant. In contrast, insulin given acutely as an intravenous infusion has shown a favorable effect on several risk factors of CVD (Chaudhuri et al., 2004).

In an effort to identify the effect of glycemic control in reducing metabolic syndrome, a detailed analysis was performed on diabetic subjects receiving metformin monotherapy (500 mg, orally administered once daily), and diabetic subjects receiving insulin monotherapy (10 I.U. injected subcutaneously, twice a day). Diabetic subjects receiving either of the two monotherapies, as already prescribed by their physicians were on the regular treatment for past 5 months and not receiving any other medication for hyperglycemia. Measurement of all the components of metabolic syndrome, established in previous section was also performed in this study. Non-diabetic controls (C) as well as the diabetic subjects who were not receiving any hypoglycemic drug (D+ND) were also chosen in addition to the metformin or insulin supplemented subjects and their anthropometric observations are listed in table.2A.1. It is depicted that the diabetic subjects dependent on insulin (D+I) were from a comparatively elder age group. Waist circumference of all the categories remained almost same except the control
group (C), where it was found to be significantly low. However, a major difference was noticed in the blood glucose levels of all subjects. It is witnessed from the table that FBG levels reduced significantly in insulin treated subjects followed by metformin treated subjects among the diabetic population. This is coherent to the previous reports suggesting reduction in blood glucose in overweight diabetic subjects upon administration of metformin as well as insulin (Bailey and Turner, 1966; Inzucchi et al., 1998; Phillips et al., 2003).

2A.2: HDL levels

Previous studies of diabetes treatment were limited to the glycemic control only. Modern therapies however also imply the role played by blood lipids in the progression of diabetes (deFronzo and Goodman, 1995; Campbell, 2000). In the present study, HDL levels of all subjects were measured to judge their relevance to metabolic syndrome. Fig.2A.2 represents an insignificant variation in HDL levels of the four categories. Diabetic subjects receiving insulin or metformin monotherapy (D+I, D+M) however, reported slightly elevated amounts of HDL. Thus indicating a plausible role of insulin as well as oral hypoglycemics in improvement of lipid profile, which is a major decisive factor of metabolic syndrome. This result is consistent to the findings of Fonseca et al. (2006) highlighting increased HDL levels upon insulin and metformin administration.

2A.3: TGs levels

The metabolic syndrome is frequently associated with low HDL and hypertriglyceridemia (Steiner, 2004). Poorly controlled diabetes causes hypertriglyceridemia and therefore, drug treatment should be effective not only for the lipid disorder but also, in the best case, to improve insulin resistance and glucose tolerance (Meyers and Kashyap, 2004). It was
observed that the metformin treated group (D+M) demonstrated remarkably decreased concentration of TGs (fig.2A.3) followed by the levels in insulin treated group (D+I), and no drug group (D+ND). Thus, suggesting the role of oral hypoglycemics in reducing TGs levels and bringing it near normal levels as shown in the control group (C). This effect of metformin adds up to the reason why oral hypoglycemics are the drugs of first choice in diabetic subjects who also suffer from metabolic syndrome.

2A.4: Lipid peroxidation

It has recently been reported from this laboratory that concentration of lipid peroxides in oxidized sdLDL is an important marker of metabolic syndrome and oxidative stress (Nishtha et al., 2008b). Thus, the sdLDL was isolated, oxidized by cupric chloride and lipid peroxides were measured in the present study groups. Fig.2A.4 shows the lowest concentration of lipid peroxides in metformin treated group (D+M). The diabetic group amid insulin administration (D+I) also witnessed a noticeable reduction when compared to the group receiving no drug at all (D+ND). This suggests that the maximum lipid peroxidation occurred in diabetic group receiving no treatment and the lowest in non-diabetic control (C) group. This result also indicates that the production of lipid peroxides is increased upon initiation of diabetes which is abridged by the supplementation of either oral hypoglycemics or insulin. Similar studies involving both metformin and insulin as therapeutic targets for diabetes have also reported decreased lipid peroxidation (Fonseca et al., 2006). Thus, the result generates a necessity for the provision of adequate basal insulin or in other words, proper glycemic control to lower the lipid peroxidation and presumably, oxidative stress leading to metabolic syndrome.
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<td>FBG (mg/dl)</td>
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<td>170 ± 2.3</td>
<td>120 ± 1.7</td>
<td>136 ± 1.5</td>
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Table 2A.1: Anthropometric observations in untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The blood glucose level was determined by glucose oxidase method proposed by Trinder (1969). The diabetic state was confirmed when FBG concentration exceeded 120 mg/dl. The waist circumference (WC) for each subject was measured by the usual procedures as described in ‘methods’. Results are expressed as mean ± S.D.

C Control
D + ND Diabetic + No Drug
D + I Diabetic + Insulin
D + M Diabetic + Metformin
Fig.2A.2: HDL levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum HDL was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

C Control
D + ND Diabetic + No Drug
D + I Diabetic + Insulin
D + M Diabetic + Metformin
Fig. 2A.3: TGs levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum TGs was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

C     Control
D + ND Diabetic + No Drug
D + I  Diabetic + Insulin
D + M  Diabetic + Metformin
2A.4: Lipid peroxidation in untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

Lipid peroxides in the oxidized lipoproteins were determined by mixing 0.1 ml of the medium containing the modified lipoproteins with 1.0 ml of iodine-color reagent, incubating the mixture for 30 min at room temperature in the dark, and reading the absorbance at 365 nm. Results are expressed as mean ± S.D.

C          Control  
D + ND     Diabetic + No Drug 
D + I      Diabetic + Insulin  
D + M      Diabetic + Metformin
2A.5: PON-1 activity

Previous results from the same study have revealed insignificantly elevated concentration of HDL in diabetic subjects after insulin or metformin monotherapy. As serum paraoxonase (PON-1) is an HDL-associated enzyme that protects both HDL and LDL from oxidation, a curiosity was raised to determine the content of this enzyme also. The results obtained after performing PON-1 assay in all the four categories came out to be analogous to what was expected. Fig.2A.5 reveals the rise in activity of this enzyme in case of diabetic subjects treated with metformin (D+M) followed by those receiving insulin (D+I). The PON-1 activity of diabetic subjects receiving no medication (D+ND) showed significantly low-levels whereas; the non-diabetic control group (C) showed the highest levels of enzyme activity. These results were comprehensible to the earlier proposed hypothesis stating that metformin possess antioxidant properties and significantly lowers insulin resistance (Faure et al., 1999).

2A.6: CRP and TNF-α levels

Both CRP and TNF-α are the markers of inflammation that have been associated with diabetes, metabolic syndrome and CVD (Tracy et al., 1997). There are different views among scientists, regarding the effect of oral hypoglycemics as well as insulin analogues on the CRP levels of diabetic patients. Yudkin et al. (1999) hypothesized the association of high CRP levels and oxidative stress. Further, Dandona et al. (2002) established the anti-inflammatory properties of intravenous insulin. The results explained in fig.2A.6(a) indicate high CRP levels in the diabetic subjects receiving no drug (D+ND) as compared to the non-diabetic control subjects (C). However, an insignificant reduction in CRP levels was also noticed in diabetic subjects after insulin or metformin monotherapy (D+I, D+M).

The implication of TNF-α in the pathogenesis of metabolic syndrome has been attributed to the over-expression of TNF-α gene in the adipose
tissue of obese humans (Hotamisligil et al., 1995; Kern et al., 1995) and rodents (Hotamisligil, 1993; Hotamisligil et al., 1996). Moreover, certain studies have also reported an association of increased TNF-α with insulin resistance (Hotamisligil, 1993; Ruan and Lodish, 2003). Thus, the increment in the levels of TNF-α in diabetic subjects (D+ND) as displayed in fig.2A.6(b), may account for the development of metabolic syndrome. Despite this risk, a slender, non-significant attenuation of TNF-α levels was also observed after administration of insulin or metformin alone (D+I, D+M).

2A.7: Oxidation of sdLDL and Apo-B levels
Circulating levels of apolipoproteins reflect the number of, rather than the cholesterol concentration of, lipoprotein particles. Specifically, the level of apo-B reflects the number of triglyceride-rich VLDL particles and the number of LDL particles. It thus gives more credence to the number of sdLDL particles than the more regular measurement of LDL cholesterol (Lind et al., 2006). Assessment of qualitative changes in sdLDL is therefore more significant in metabolic syndrome because of its ability to get oxidized easily (Scheffer et al., 2003). Such an important role of Apo-B and sdLDL in determination of metabolic syndrome has provided grounds for their estimation in the present study. Therefore, the time taken for oxidation of sdLDL, isolated from different groups was monitored. Surprisingly, it was found that oxidation of sdLDL from diabetic subjects (D+ND) completed in minimum time when compared to the one observed for non-diabetic control subjects (C) and diabetic subjects treated with metformin (D+M) or insulin (D+I) [fig.2A.7(a)]. Increase in the time required for oxidation of sdLDL in diabetic subjects receiving metformin depicts qualitative changes in sdLDL particles of these subjects, and thus reducing their chances of developing atherosclerosis and metabolic syndrome.

Similarly, fig.2A.7(b) reveals the effect of metformin or insulin supplementation on the apo-B levels of diabetic subjects. It is quite
remarkable that apo-B levels significantly decreased when diabetic subjects were treated with metformin (D+M). A lesser reduction was also observed in the insulin treated subjects (D+I) as compared to those receiving no drug (D+ND). Induction of these changes in sdLDL and apo-B by metformin administration may be one of the mechanisms of the anti-atherogenic effect of such oral hypoglycemics.

2 A.8: Oxidative stress marker MDA

There is growing evidence that excess generation of highly reactive free radicals, largely due to hyperglycemia, causes oxidative stress, which further exacerbates the development and progression of diabetes and its complications (Johansen et al., 2005). Also it has been reported that uncontrolled hyperglycemia was associated with elevated levels of malondialdehyde (MDA), an important marker of oxidative stress, which may get reduced with the supplementation of numerous antioxidant compounds (Vega-Lopez et al., 2004). Therefore it was of interest to measure MDA levels in serum of the diabetic subjects receiving either metformin or insulin monotherapy. Fig.2A.8 demonstrates elevated MDA levels in the diabetic group receiving no treatment (D+ND) denoting increased oxidative stress. On the other hand, metformin and insulin treated groups (D+M, D+I) showed remarkably decreased MDA levels; metformin revealing enhanced antioxidant effect as compared to that of insulin. Hence, it may be affirmed that in diabetic condition, free radicals are elevated and metformin or insulin treatment attenuated these changes.
Fig. 2A.5: PON-1 activity of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The assays were performed in a final volume of 250 µl containing 1 mM phenylacetate and 2 mM CaCl₂ in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 µl of serum and was read at 270 nm. Results are expressed as mean ± S.D.

C                  Control
D + ND             Diabetic + No Drug
D + I              Diabetic + Insulin
D + M              Diabetic + Metformin
Fig.2A.6(a): CRP levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum CRP was measured by an enzymatic colorimetric test using a commercial kit from Calbiotech Inc., USA. Results are expressed as mean ± S.D.

C: Control
D + ND: Diabetic + No Drug
D + I: Diabetic + Insulin
D + M: Diabetic + Metformin
Fig. 2A.6(b): TNF-α levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum TNF-α was measured by an enzymatic colorimetric test using a commercial kit from Immunotech SAS, France. Results are expressed as mean ± S.D.

- **C**: Control
- **D + ND**: Diabetic + No Drug
- **D + I**: Diabetic + Insulin
- **D + M**: Diabetic + Metformin
Fig. 2A.7(a): sdLDL oxidation time of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. (1989). 10 \mu l of supernatant containing sdLDL, oxygenated PBS and 32 \mu l of 1 mM CuCl\textsubscript{2} was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored at 234 nm. Results are expressed as mean ± S.D.

- C: Control
- D + ND: Diabetic + No Drug
- D + I: Diabetic + Insulin
- D + M: Diabetic + Metformin
Fig.2A.7(b): Apo-B levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The concentration of serum Apo-B was measured by an enzymatic colorimetric test using a commercial kit from Giesse Diagnostics snc., Italy. Results are expressed as mean ± S.D.

C Control  
D + ND Diabetic + No Drug  
D + I Diabetic + Insulin  
D + M Diabetic + Metformin
Fig. 2A.8: MDA levels of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

MDA in serum was determined fluorimetrically by thiobarbituric acid reaction (TBA) as described in 'methods'. TBA reacting substance was expressed in terms of MDA as nmol/ml of serum and was measured at 553 nm emission and 515 nm excitation wavelength. Results are expressed as mean ± S.D.

C = Control
D + ND = Diabetic + No Drug
D + I = Diabetic + Insulin
D + M = Diabetic + Metformin
2A.9: SOD and Catalase activity
Maritim and colleagues recently reviewed in detail that diabetes has multiple effects on the protein levels and activity of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase, which further augment oxidative stress by causing a suppressed defense response (Maritim et al., 2003). SOD immediately converts superoxide radical to hydrogen peroxide, which is then detoxified to water either by catalase in the lysosomes or by glutathione peroxidase in the mitochondria. A plethora of studies have been performed to determine the activity of these enzymes upon induction of diabetes and after supplementation with metformin or insulin (Ramanathan et al., 1999; Pavlovic et al., 2000; Kocic et al., 2007). All these studies have emphasized the role of these hypoglycemics in ameliorating oxidative stress and implicate that such a treatment significantly increased the level of antioxidant enzymes and reduced the amount of lipid peroxidation. The results indicated in fig.2A.9(a,b) also witnessed similar outcomes and elucidate that the enzyme activity of SOD as well as catalase was highest in the non-diabetic control group (C) and decreased significantly in diabetic subjects receiving no treatment (D+ND). It is interesting to note that though both metformin and insulin monotherapy succeeded in augmentation of SOD and catalase activity, the results of treatment with metformin (D+M) showed much significant values as compared to that of treatment with insulin (D+I). This investigation suggests that the application of metformin monotherapy in diabetic subjects improves the antioxidative enzyme status considerably and thus adds to an important preventive measure for oxidative stress.

2A.10: DNA damage
Insulin dependent diabetes mellitus is associated with increased oxidative stress in vivo and under conditions of oxidative stress; damage to cellular biomolecules (lipids, proteins, carbohydrates, and DNA) can occur (Hannon-Fletcher et al., 2000). Such damage in the DNA of diabetic subjects can be
measured by a technique known as comet assay. The comet assay, can potentially measure DNA lesions in any organ or tissue even in the absence of mitotic activity. Cells are examined using fluorescent microscope. Cells look like comets with a bright fluorescent head and tail, the length and intensity of which are related to the number of strand breaks (Dobrzynska, 2005).

Traditionally, peripheral blood lymphocytes have been preferred because they are regarded as sentinel cells being early warning signals for adverse health effects (Tice et al., 2000). The assay involves treatment of small number of cells layered on glass slides and sandwiched between layers of agarose. Furthermore, agarose embedded cells are lysed to generate a nucleoid body composed of nuclear DNA stripped of some attached protein and its histones. These nucleoid bodies are then subjected to limited electrophoresis that cause the damaged DNA to migrate away from the undamaged DNA forming a “comet”, in which the ‘head’ of the comet is the undamaged DNA and the ‘tail’ the damaged DNA. Therefore, the more damaged a cell’s DNA is, the greater the amount of DNA in the tail (often measured as olive tail movement, OTM or tail length) (Almeida et al., 2006). The size of the comet and the distribution of fluorescence within it are correlated quantitatively with the frequency of DNA breaks (Fairbairn et al., 1995).

Several studies have shown increases in the level of DNA damage in subjects with poor glycemic control (Collins et al., 1998 and Lorenzi et al., 1987), and no significant changes in subjects with good glycemic control (Anderson et al., 1998). To verify the extent of DNA damage in untreated diabetic subjects and those receiving a hypoglycemic treatment, fresh lymphocytes were isolated from the blood samples of each subject and the procedures of comet assay were followed. Interestingly, this study also presented similar results when DNA damage was assessed for non-diabetic control subjects (C), diabetic subjects receiving no medication (D+ND), and
diabetic subjects treated with oral metformin (D+M) or sub-cutaneously supplied insulin (D+I). Fig.2A.10 represents the extent of DNA damage in all the above stated categories and witnesses no significant alteration in tail-length of subjects from any of the three diabetic groups. DNA damage in the non-diabetic control subjects (C) was however lesser than any other category stated. This result implies that supplementation of oral hypoglycemics or even insulin does not provide any significant protection to the DNA damage.

2A.11: Correlation between NCEP/ATP III parameters and observed parameters

Present study engrosses the determination of glycemic control and its effect on metabolic syndrome. The NCEP/ATP III components (abdominal adiposity, hypertension, hypertriglyceridemia, low HDL, and hyperglycemia) as well as the parameters observed in present study (CRP, TNF-α and apo-B levels together with sdLDL oxidation time, lipid peroxidation, and PON-1 activity) have been shown to be involved in metabolic syndrome in the earlier chapter. Several reports have also revealed an increased DNA damage in diabetes (Collins et al., 1998; Lorenzi et al., 1987; Anderson et al., 1998). In an effort to establish a relationship between these factors and NCEP/ATP III parameters in diabetic subjects with the administration of insulin or metformin, Pearson’s correlation analysis was performed. The plotted values of correlation coefficient (r) in fig.2A.11 reveal a negative correlation between (i) the values of PON-1 activity and the levels of TGs and FBG, (ii) lipid peroxidation and HDL, (iii) CRP and HDL, (iv) sdLDL oxidation time and levels of TGs and FBG, (v) TNF-α and HDL, (vi) apo-B and HDL, (vii) DNA damage and HDL. Thus it adds to an important observation depicting the reduction in lipid peroxidation, and levels of CRP, apo-B and TNF-α together with the DNA damage upon elevation in HDL concentration followed by insulin or metformin administration. Similarly, the decrease in TGs and FBG
concentration as a result of the treatment with these hypoglycemics was found to be responsible for increased PON-1 activity and the time required for oxidation of sdLDL. This implies that regulation of FBG, TGs and HDL levels after treatment with hypoglycemics, may play a crucial role in preventing the development of metabolic syndrome and other risk factors associated with it.
Fig. 2A.9(a): SOD activity of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

The assay medium in a final volume of 3 ml consisted of 50 μl sample (serum) and 0.05 M tris-succinate buffer, pH 8.2 (2.85 ml). After incubation at 25°C for 20 min, the reaction was initiated by the addition of 8 nM pyrogallol. The change in absorbance was recorded spectrophotometrically at 420 nm for 3 min. One enzyme unit (E.U.) is defined as the amount of enzyme required to cause 50% inhibition of the rate of pyrogallol auto-oxidation. Results are expressed as mean ± S.D.

C Control
D + ND Diabetic + No Drug
D + I Diabetic + Insulin
D + M Diabetic + Metformin
Fig.2A.9(b): Catalase activity of untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

Catalase activity was measured by following the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide (H₂O₂). The reaction was allowed to proceed in 0.05 M phosphate buffer (pH 7.0) containing 1 ml H₂O₂ (30 mM) and 50 µl sample. One enzyme unit (E.U.) is defined as the amount of enzyme decomposing 1 µM H₂O₂ per min at 25°C. Results are expressed as mean ± S.D.

C        Control
D + ND   Diabetic + No Drug
D + I    Diabetic + Insulin
D + M    Diabetic + Metformin
Fig. 2A.10: DNA damage in untreated diabetic subjects, and diabetic subjects treated with insulin or metformin monotherapy.

DNA breakage in the lymphocytes isolated from each subject was measured by comet assay proposed by Singh et al. (1988) as described in 'methods'. Comet tail length (μmeters) was observed and plotted against each group. Results are expressed as mean ± S.D.

- C: Control
- D + ND: Diabetic + No Drug
- D + I: Diabetic + Insulin
- D + M: Diabetic + Metformin
Fig.2A.11: Correlation between NCEP/ATP III parameters and observed parameters with insulin or metformin monotherapy.

Pearson's correlation analysis was used for determination of relationship between the components of NCEP/ATP III and the parameters observed in diabetic subjects with the administration of insulin or metformin. The correlation coefficient (r) of indicated parameters was plotted against the following parameters to obtain a positive or negative relationship.

1. PON-1 activity
2. Lipid peroxidation
3. CRP concentration
4. sdLDL oxidation time
5. TNF-α concentration
6. Apo-B concentration
7. DNA damage
Unit 2 (B)
Treatment of Metabolic Syndrome in Hypertensive Subjects
Angiotensin II [formed from angiotensin I in a reaction catalyzed by angiotensin converting enzyme (ACE, kininase II)] is a potent vasoconstrictor, the primary vasoactive hormone of the rennin-angiotensin system and an important component in the pathophysiology of hypertension. It also stimulates aldosterone secretion by the adrenal cortex. Losartan and its principal active metabolite block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the angiotensin I receptor found in many tissues (vascular smooth muscle, adrenal gland) and thus called as Angiotensin receptor blocker (ARB) or Angiotensin receptor antagonist (ARA) (Vitale et al., 2005).

Ramipril, on the other hand inhibit ACE in human subjects and animals. Inhibition of ACE results in decreased plasma angiotensin II which leads to decreased vasopressor activity and a decreased aldosterone secretion. Although ACE inhibitors are clinically effective, their use has been hampered by adverse effects such as temporary and reversible decline in glomerular filtration rate (GFR), angioedema and cough; which are thought to be caused due to inhibition of aldosterone synthesis and release (Pitt et al., 1997 and Garvas, 1999). Moreover, with prolonged use, the effectiveness of ACE inhibitors may be attenuated by generation of angiotensin II through non-ACE pathways or by up regulation of angiotensin I because of stimulation of rennin secretion. Several clinical trials (Anderson et al., 2000; Stigant et al., 2000; Brenner et al., 2001; Lewis et al., 2001) have however, established that these limitations of ACE inhibitors may be circumvented by use of the newer ARA.

To find evidence regarding the involvement of ACE inhibitors and ARAs in prevention of hypertension and thereby metabolic syndrome, all the parameters of metabolic syndrome were analyzed once again; though in context with antihypertensive monotherapy this time.
2B.1: Anthropometric observations

Table 2B.1 demonstrates the anthropometric characteristics of all the study subjects chosen for this section. A total of 99 subjects were selected, out of which 79 were the newly diagnosed hypertensive subjects, and the remaining 20 were normotensive controls (C). The hypertensive population was further sub-divided into three categories: those receiving no medication (HT+ND) at all, those receiving ramipril (HT+R) (5 mg orally administered, once in a day) as a monotherapy, and finally those receiving losartan (HT+L) (50 mg orally administered, once in a day) as a monotherapy prescribed by their physicians, for past 5 months. The groups receiving losartan (HT+L) and ramipril (HT+R) as a monotherapy comprised of elder subjects, compared to those belonging to the no drug (HT+ND) as well as control group (C). No significant difference was observed in waist circumference of the three hypertensive groups. Thus it can safely be said that neither ramipril, nor losartan play any remarkable role in the improvement of waist circumference or bringing it to near normal levels as displayed by control group (C). Observed reduction in systolic and diastolic blood pressure as a result of ramipril and losartan monotherapy, are also indicative of the role of these anti-hypertensive drugs in ameliorating metabolic syndrome.

2B.2: HDL levels

Besides the lowering of blood pressure, certain anti-hypertensives may have different pleiotropic effects on the pathophysiology of metabolic syndrome (Brenner et al., 2001). Accordingly, serum HDL concentration was measured for all the subjects involved. Fig.2B.2 put across no major variation in HDL levels of any of the group and thus suggests no significant role of ARA and ACE inhibitors in altering the lipid profile of hypertensive subjects. This result is in concordance with the previous reports suggesting only marginal effects of these drugs on lipid profile (Sica and Bakris, 2002).
2B.3: TGs levels

Akin to the alteration in HDL, increment of TGs levels is also an important indication of metabolic syndrome. As conceived in the former result, that showed no remarkable change in HDL even after ramipril or losartan monotherapy, the present result (fig.2B.3) also depicts that none of the anti-hypertensives have any beneficial effect over dyslipidemia. It is clearly evident that the TGs levels of non-treated (HT+ND), ramipril treated (HT+R), as well as the losartan treated group (HT+L) was almost the same but higher than that observed for the control group (C). Thus, it greatly emphasizes the need for some combination therapy, where drugs specifically capable of reducing lipid disorder should be used in addition to these ARA or ACE inhibitors in order to obtain a preventive measure for metabolic syndrome.

2B.4: Lipid peroxidation

Hypertension is a risk factor for atherogenesis (Kannel and Sorlie, 1975). The increased risk of CVD in hypertensive subjects correlates with blood pressure and may be linked to other factors including oxidative modification and accumulation of lipids in the vascular wall (Alleman and Weidmann, 1995). The effect of both ramipril and losartan was thereby observed against the concentration of lipid peroxides in the oxidized sdLDL of each subject involved in this study. Fig.2B.4 demonstrates the lowest lipid peroxidation in normotensive control subjects (C) and highest in the hypertensive subjects with no medication (HT+ND). However, it is clearly depicted that both ramipril (HT+R) and losartan (HT+L) monotherapy succeeded in reducing the amount of lipid peroxidation in hypertensive category. Thus, suggesting an important contribution of these anti-hypertensives towards suppression of oxidative stress, a major culprit of metabolic syndrome.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>C (n=20)</th>
<th>HT + ND (n=19)</th>
<th>HT + R (n=28)</th>
<th>HT + L (n=32)</th>
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<tr>
<td>Males (n)</td>
<td>9</td>
<td>10</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>Females (n)</td>
<td>11</td>
<td>9</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Age (Yrs)</td>
<td>33 ± 2.1</td>
<td>39 ± 3.6</td>
<td>42 ± 2.8</td>
<td>44 ± 3.2</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>86 ± 1.3</td>
<td>98 ± 2.3</td>
<td>96 ± 3.1</td>
<td>95 ± 3.2</td>
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<tr>
<td>BP (mm Hg)</td>
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<tr>
<td>(Sys)</td>
<td>126 ± 1.3</td>
<td>131 ± 2.3</td>
<td>115 ± 2.1</td>
<td>117 ± 2.6</td>
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<tr>
<td>(Dia)</td>
<td>75 ± 1.2</td>
<td>78.8 ±1.5</td>
<td>68.3 ± 2.0</td>
<td>71 ± 1.6</td>
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Table.2B.1: Anthropometric observations in untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The waist circumference (WC) and blood pressure (BP) for each subject was measured by the usual procedures as described in 'methods'. Results are expressed as mean ± S.D.

C Control
HT + ND Hypertensive + No Drug
HT + R Hypertensive + Ramipril
HT + L Hypertensive + Losartan
Fig. 2B.2: HDL levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum HDL was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

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<td>C</td>
<td>Control</td>
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<tr>
<td>HT + ND</td>
<td>Hypertensive + No Drug</td>
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<tr>
<td>HT + R</td>
<td>Hypertensive + Ramipril</td>
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<tr>
<td>HT + L</td>
<td>Hypertensive + Losartan</td>
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Fig. 2B.3: TGs levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum TGs was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

- **C** Control
- **HT + ND** Hypertensive + No Drug
- **HT + R** Hypertensive + Ramipril
- **HT + L** Hypertensive + Losartan
Fig. 2B.4: Lipid peroxidation in untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

Lipid peroxides in the oxidized lipoproteins were determined by mixing 0.1 ml of the medium containing the modified lipoproteins with 1.0 ml of iodine-color reagent, incubating the mixture for 30 min at room temperature in the dark, and reading the absorbance at 365 nm. Results are expressed as mean ± S.D.

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<td>C</td>
<td>Control</td>
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<tr>
<td>HT + ND</td>
<td>Hypertensive + No Drug</td>
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<td>HT + L</td>
<td>Hypertensive + Losartan</td>
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2B.5: PON-1 activity

The activity as well as concentration of PON-1 has been reported to be decreased in CVD (Navab et al., 1997) and diabetes (Sakai et al., 1998). The results published from this laboratory have also proposed a diminished PON-1 activity in hypertension (Nishtha et al., 2008a). Following the same guideline, PON-1 activity of hypertensive subjects dependent upon ramipril or losartan was also measured. Surprisingly, the enzyme activity was found to be low in hypertensive subjects receiving no treatment (HT+ND) as compared to those receiving ramipril (HT+R) or losartan (HT+L) monotherapy (Fig.2B.5). Control group (C), however displayed the highest enzyme activity. This result is indicative of a plausible role of ARA and ACE inhibitors in maintaining the antioxidant status by upregulating the levels of PON-1. Thus, suggesting reduction in oxidative stress and the risk of metabolic syndrome.

2B.6: CRP and TNF-α levels

Rapidly evolving work now demonstrates that CRP has several direct effects at the level of the vessel wall (Pasceri et al., 2001). These observations along with basic research into the inflammatory mechanism of both diabetes and vascular dysfunction provide strong evidence that insulin resistance and atherosclerosis share a common inflammatory basis. Based on these evidences, CRP levels were evaluated in the normotensive as well as hypertensive subjects receiving either ramipril or losartan. This was performed in an attempt to verify the role of these drugs against inflammation, critically developing into metabolic syndrome. Fig.2B.6(a) represents a rise in CRP levels of hypertensive subjects receiving no treatment (HT+ND) while a significant drop in hypertensive subjects when treated with losartan (HT+L) and ramipril (HT+R). Nonetheless, the normotensive control group (C) revealed the lowest concentration of CRP.
Based on the evidences that elevated levels of another pro-inflammatory cytokine, TNF-α, also contribute substantially to metabolic syndrome (Devaraj et al., 2004), this research was further extended for the estimation of this component too. Fig.2B.6(b) witnesses a significant reduction in TNF-α levels of the losartan treated group (HT+L) as compared to that of no drug (HT+ND) and ramipril treated group (HT+R). The later ones, however showed almost similar values. The control group (C) showed an obvious lowest concentration of TNF-α. These results throw light on the pleiotropic effects of these drugs on the pathophysiology of metabolic syndrome.

2B.7: Apo-B levels and oxidation of sdLDL

Clinical assessment of the apo-B containing lipoproteins has fostered the investigations for measurement of standard lipids and their use as indices of atherogenic lipoprotein abnormalities of the metabolic syndrome (Austin et al., 1990). Consequently, apo-B levels were evaluated in all the subjects involved in this study and illustrated in fig.2B.7(a). The group containing normotensive control subjects (C) showed lowest concentration of apo-B; while no significant variation was depicted in the apo-B content of hypertensive subjects receiving no drug (HT+ND) and those receiving ramipril or losartan (HT+R, HT+L) as a monotherapy.

Similarly, fig.2B.7(b) represents the time taken for oxidation of sdLDL isolated from all the four categories. It is clearly indicated that the sdLDL isolated from all hypertensive subjects got oxidized in lesser duration of time as compared to that of normotensive control subjects (C). An almost equal amount of time was however noticed when rest of the three hypertensive groups (HT+ND, HT+R, HT+L) were compared to each other. Thus suggesting that ramipril and losartan are unable to reduce apo-B levels or more specifically, to create any qualitative changes in the lipoprotein profile of hypertensive subjects.
Fig. 2B.5: PON-1 activity of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The assays were performed in a final volume of 250 µl containing 1 mM phenylacetate and 2 mM CaCl$_2$ in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 µl of serum and was read at 270 nm. Results are expressed as mean ± S.D.

- **C**  Control
- **HT + ND**  Hypertensive + No Drug
- **HT + R**  Hypertensive + Ramipril
- **HT + L**  Hypertensive + Losartan
Fig. 2B.6(a): CRP levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum CRP was measured by an enzymatic colorimetric test using a commercial kit from Calbiotech Inc., USA. Results are expressed as mean ± S.D.

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<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hypertensive + No Drug</th>
<th>Hypertensive + Ramipril</th>
<th>Hypertensive + Losartan</th>
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<tr>
<td>C</td>
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<td>HT + ND</td>
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Fig. 2B.6(b): TNF-α levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum TNF-α was measured by an enzymatic colorimetric test using a commercial kit from Immunotech SAS, France. Results are expressed as mean ± S.D.

C       Control
HT + ND  Hypertensive + No Drug
HT + R   Hypertensive + Ramipril
HT + L   Hypertensive + Losartan
Fig. 2B.7(a): Apo-B levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The concentration of serum Apo-B was measured by an enzymatic colorimetric test using a commercial kit from Giesse Diagnostics snc., Italy. Results are expressed as mean ± S.D.

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<td>HT + R</td>
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<td>HT + L</td>
<td>Hypertensive + Losartan</td>
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Fig. 2B.7(b): sdLDL oxidation time of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. (1989). 10 μl of supernatant containing sdLDL, oxygenated PBS and 32 μl of 1 mM CuCl$_2$ was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored at 234 nm. Results are expressed as mean ± S.D.

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<td><strong>HT + R</strong></td>
<td>Hypertensive + Ramipril</td>
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<td><strong>HT + L</strong></td>
<td>Hypertensive + Losartan</td>
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2B.8: Oxidative stress marker MDA
Increased vascular oxidative stress could be involved in the pathogenesis of hypertension (Miyajima et al., 2007), a major risk factor for cardiovascular disease mortality. Also it has been reported that in essential hypertension ROS may increase due to a diminution of the activity of antioxidant enzymes (Pedro-Botet et al., 2000). To explore any protective role offered by ACE inhibitors or ARAs against oxidative stress, this study evaluated the levels of MDA, the oxidative stress damage index in hypertensive subjects. Fig.2B.8 demonstrates highly elevated MDA levels in the group of hypertensive subjects receiving no drug at all (HT+ND) as compared to the normotensive subjects (C). However, the treatment with ramipril alone (HT+R), significantly reduced the MDA levels. A relatively lesser amount of decrease was also noticed in the losartan treated group (HT+L). These findings are consistent to the previous reports suggesting reduction in oxidative stress and improvement in the endothelial function in hypertensive patients following ramipril and losartan monotherapy (Beatriz et al., 2007; Barath et al., 2006).

2B.9: SOD and Catalase activity
The occurrence of oxidative stress may arise from a primary decrease in the antioxidant defense system or from an elevation of ROS concentration. This derangement leads to oxidative damage to the structure of biomolecules, likely involving the antioxidant enzymes, thus contributing to the oxidative stress found in hypertensive subjects, but not in normotensive subjects (Rodrigo et al., 2007). As a consequence of decreased activity of antioxidant enzymes SOD and catalase, a reduction in the endothelium dependent vasodilation of the vascular smooth muscle cells of hypertensive subjects could be expected (Lassègue and Griendling, 2004). The treatment with antihypertensives such as ramipril or losartan may however provide a protection from these damaging consequences. Therefore the assays were performed for
determination of SOD as well as catalase activity in the hypertensive subjects receiving no drug and those receiving ramipril or losartan as a monotherapy. Fig.2B.9(a,b) reveal reduced activity of both SOD and catalase in the hypertensive group receiving no treatment (HT+ND) suggesting high oxidative stress. The treatment with ramipril (HT+R) and losartan (HT+L) however successfully replenished the antioxidant enzyme activities in the hypertensive subjects. Interestingly, the effect of ramipril therapy was more pronounced than that of losartan therapy, suggesting its greater antioxidant capacity. Similar protective effects of ramipril and losartan against hypertension have been reported earlier (Khattab et al., 2004; Barath et al., 2006) and this study also put forward an important role of ramipril in ameliorating oxidative stress.

2B.10: DNA damage

Earlier studies have shown an association between hypertension and the oxidation of LDL, and particularly the fact that its susceptibility to oxidation is greater in patients with essential hypertension than in normotensive subjects (Pierdomenico et al., 1998). It is suggested that oxidative modification of LDL could promote and accelerate the development of atherosclerosis (Witztum, 1994). Some studies have demonstrated the presence of DNA damage and DNA adducts in the atherosclerotic lesions and tissues (Binkova et al., 2002 and Martinet et al., 2002). The result presented in fig.2B.10 is consistent to the above reports, as a significant DNA damage was observed in hypertensive patients of all the three categories (HT+ND, HT+R, HT+L) as compared to the control group (C). However, the three groups of hypertensive subjects showed an almost equal extent of DNA damage; suggesting no beneficial role of ramipril or losartan against DNA damage, a major factor of metabolic syndrome.
2B.11: Correlation between NCEP/ATP III parameters and observed parameters

Present work focuses the effect of anti-hypertensive drugs on metabolic syndrome. The NCEP/ATP III components (HDL, TGs, and BP) as well as the parameters observed (CRP, TNF-α and apo-B levels together with sdLDL oxidation time, lipid peroxidation, PON-1 activity, and extent of DNA damage), have been employed to ascertain the potential of metabolic syndrome in hypertensive and normotensive subjects chosen for this study. However, to find a relationship between these factors and NCEP/ATP III parameters in hypertensive subjects with the administration of ramipril or losartan therapy, Pearson’s correlation analysis was performed. The plotted values of correlation coefficient (r) in fig.2B.11 reveal a negative correlation between (i) the values of PON-1 activity and the levels of TGs together with diastolic BP, (ii) lipid peroxidation and HDL, (iii) CRP and HDL, (iv) sdLDL oxidation time and HDL (v) TNF-α and TGs, (vi) apo-B and HDL (vii) DNA damage and HDL. Thus it may be suggested that reduction in BP and TGs levels upon ramipril or losartan monotherapy instigate the elevation of PON-1 activity in hypertensive subjects. Also the increased HDL concentration may facilitate the decrease in lipid peroxidation, levels of CRP and apo-B along with the amendment of DNA damage as well as the time taken by sdLDL for oxidation. Changes in the TNF-α level however, was shown to be related with the reduction of TGs levels. This implies that improvement in BP, TGs as well as HDL levels on account of ramipril or losartan monotherapy play a pivotal role in regulation of several other metabolic syndrome parameters in hypertensive subjects.
Fig. 2B.8: MDA levels of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

MDA in serum was determined fluorimetrically by thiobarbituric acid reaction (TBA) as described in ‘methods’. TBA reacting substance was expressed in terms of MDA as nmol/ml of serum and was measured at 553 nm emission and 515 nm excitation wavelength. Results are expressed as mean ± S.D.

C          Control
HT + ND    Hypertensive + No Drug
HT + R     Hypertensive + Ramipril
HT + L     Hypertensive + Losartan
Fig. 2B.9(a): SOD activity of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

The assay medium in a final volume of 3 ml consisted of 50 µl sample (serum) and 0.05 M tris-succinate buffer, pH 8.2 (2.85 ml). After incubation at 25°C for 20 min, the reaction was initiated by the addition of 8 nM pyrogallol. The change in absorbance was recorded spectrophotometrically at 420 nm for 3 min. One enzyme unit is defined as the amount of enzyme required to cause 50% inhibition of the rate of pyrogallol auto-oxidation. Results are expressed as mean ± S.D.

<table>
<thead>
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<th>Treatment</th>
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<td>C</td>
<td>Control</td>
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<tr>
<td>HT + ND</td>
<td>Hypertensive + No Drug</td>
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<tr>
<td>HT + R</td>
<td>Hypertensive + Ramipril</td>
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<tr>
<td>HT + L</td>
<td>Hypertensive + Losartan</td>
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Fig.2B.9(b): Catalase activity of untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

Catalase activity was measured by following the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide ($\text{H}_2\text{O}_2$). The reaction was allowed to proceed in 0.05 M phosphate buffer (pH 7.0) containing 1 ml $\text{H}_2\text{O}_2$ (30 mM) and 50 µl sample. One enzyme unit is defined as the amount of enzyme decomposing 1 µM $\text{H}_2\text{O}_2$ per min at 25°C. Results are expressed as mean ± S.D.

C Control
HT + ND Hypertensive + No Drug
HT + R Hypertensive + Ramipril
HT + L Hypertensive + Losartan
Fig.2B.10: DNA damage in untreated hypertensive subjects, and hypertensive subjects treated with ramipril or losartan monotherapy.

DNA breakage in the lymphocytes isolated from each subject was measured by comet assay proposed by Singh et al. (1988) as described in ‘methods’. Comet tail length (μmeters) was observed and plotted against each group. Results are expressed as mean ± S.D.

C Control
HT + ND Hypertensive + No Drug
HT + R Hypertensive + Ramipril
HT + L Hypertensive + Losartan
Fig.2B.11: Correlation between NCEP/ATP III parameters and observed parameters with ramipril or losartan monotherapy.

Pearson's correlation analysis was used for determination of relationship between the components of NCEP/ATP III and the parameters observed in hypertensive subjects with the administration of ramipril or losartan therapy. The correlation coefficient (r) of indicated parameters was plotted against the following parameters to obtain a positive or negative relationship.

1. PON-1 activity
2. Lipid peroxidation
3. CRP concentration
4. sdLDL oxidation time
5. TNF-a concentration
6. Apo-B concentration
7. DNA damage
Chapter 3
Treatment of Metabolic Syndrome
(Animal Studies)
Hitherto, it has been recognized that diabetic subjects exhibit high oxidative stress due to persistent and chronic hyperglycemia, thereby depleting the activity of antioxidative defense system and promoting the generation of free radicals. To this end, it has become clear that ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing diabetic complications that may also lead to metabolic syndrome. There are evidences suggesting that antioxidants, such as α-tocopherol (vitamin E), have potential benefits with respect to CVD. Alpha-tocopherol has been shown to decrease lipid peroxidation, inhibit platelet adhesion, aggregation and smooth muscle cell proliferation, to exert anti-inflammatory effect on monocytes and to improve endothelial function (Harris et al., 2002). Animal studies have shown that vitamin E protects development of cholesterol-induced atherosclerosis by inhibiting protein kinase C activity in smooth muscle cells \textit{in vivo} (Kartal et al., 2003).

Ascorbic acid (vitamin C) is the main water-soluble antioxidant in human plasma (Frei et al., 1990). Earlier it was reported that long-range, heavy dose ascorbic acid therapy tends to normalize considerably, but not completely, the disturbances in certain blood fat metabolism factors, such as total cholesterol, triglycerides and lipoprotein lipase (LPL) activity (Sokoloff et al., 1967). Several studies have demonstrated that ascorbic acid improves endothelial-dependent vasodilatation in hypertension and diabetes. It has been proposed that such effects may be due to scavenging of superoxide anions and its reactive oxygen intermediates (Ting et al., 1996).

Earlier the role of metformin and insulin was verified against diabetes and metabolic syndrome, no significant improvement was observed though. This study therefore, is an attempt to investigate whether the supplementation of vitamin C or E in diabetic animals could be an alternative to prevent oxidative stress and metabolic syndrome or not. For this purpose, a total of 12 rabbits were distributed into four experimental groups of three rabbits per group: non-diabetic (control, G1); alloxan-induced
diabetic (G2); diabetic supplemented with 150 mg/kg body weight of vitamin C (G3) and diabetic supplemented with 1000 I.U./kg chow of vitamin E (G4), administered daily as an oral supplementation via gavage for a period of two weeks. Thereafter, 5 ml fasting blood was collected from each animal and serum was isolated to determine all the parameters of oxidative stress and metabolic syndrome.

3.1: Elevated parameters of metabolic syndrome

3.1(a): FBG levels

It has been recognized previously that an increase in plasma glucose to above normal levels typically develops late in the course of metabolic syndrome (Genuth et al., 2003). This study therefore, employs the measurement of FBG in all the four animal groups involved. Fig.3.1(a) demonstrates, that the FBG level was highest for the diabetic group (G2) and lowest for the non-diabetic, control group (G1). The groups supplemented with antioxidants, vitamin C and vitamin E (G3 and G4), however attained an intermediate level, with the lowest FBG level in G3, among treated groups. This is consistent with the previous findings suggesting that antioxidants have potential benefits with respect to CVD (Vieira and Vianna, 2005).

3.1(b): TGs levels

Altered metabolism of triglyceride-rich lipoproteins and their elevated levels in serum can contribute substantially to the risk of ischemic heart disease in patients with metabolic syndrome (Krauss and Siri, 2004). In light of these observations, serum TGs levels were also estimated in the present study. Fig.3.1(b) demonstrates that the concentration of TGs was highest in diabetic group (G2), signifying atherogenic dyslipidemia and a possibility for the development of metabolic syndrome. Further, the supplementations of vitamin C and Vitamin E (G3 and G4) to the diabetic rabbits lead to the reduction in their TGs levels. This finding is in agreement to the proposal
drawn by Vieira and Vianna (2005) that vitamin supplementation may be used as an alternate therapy to treat lipid disorder for diabetic subjects.

3.1(c): Apo-B levels
Following the elevation in TGs levels, a somewhat greater portion of apo-B is observed in VLDL. There is growing evidence that all apo-B containing lipoproteins are atherogenic and elevated apo-B levels cause atherogenic dyslipidemia (Grundy, 2004). Consequently, apo-B levels were determined for all groups involved in this study and illustrated in fig.3.1(c). It was observed that diabetic group (G2) contained highest apo-B levels followed by vitamin E (G4) and vitamin C (G3) supplemented groups. The lowest apo-B levels attained by G3 suggest that vitamin C may play an important role in regulating metabolic syndrome and associated abnormalities.

3.1(d): Lipid peroxidation
Diabetes is known to be associated with increased oxidative stress in vivo (Zozulinska et al., 1996). The increased oxidative stress together with poor metabolic control enhances lipid peroxidation in diabetic subjects, which has been proposed to be related with aetiology of diabetic complications or metabolic syndrome (Armstrong and Al-Awadi, 1991). In the present study, concentration of lipid peroxides was determined in the oxidized sdLDL isolated from the serum of each animal, by the method proposed by el-Saadani et al. (1989). Results obtained in fig.3.1(d) conceive that the animals belonging to diabetic group (G2) had the greatest amount of lipid peroxides; whereas those supplemented with antioxidants such as vitamin C or vitamin E (G3, G4) had lesser amounts of lipid peroxides in their serum. This result rationally explains that lipid peroxidation may occur due to depletion of endogenous antioxidants in sdLDL. Hence, the inverse relationship between plasma levels of dietary antioxidants and the risk of metabolic syndrome observed in several epidemiological studies can be clearly understood.
Fig.3.1(a): FBG levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The FBG level was determined by glucose oxidase method proposed by Trinder (1969). The diabetic state was confirmed when FBG concentration exceeded 200 mg/dl. Results are expressed as mean ± S.D.

- **G1**: Control
- **G2**: Diabetic
- **G3**: Diabetic + Vitamin C
- **G4**: Diabetic + Vitamin E
Fig.3.1(b): TGs levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum TGs was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

G1 Control
G2 Diabetic
G3 Diabetic + Vitamin C
G4 Diabetic + Vitamin E
Fig. 3.1(c): Apo-B levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum Apo-B was measured by an enzymatic colorimetric test using a commercial kit from Giesse Diagnostics snc., Italy. Results are expressed as mean ± S.D.

G1  Control
G2  Diabetic
G3  Diabetic + Vitamin C
G4  Diabetic + Vitamin E
Fig.3.1(d): Lipid peroxidation in untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

Lipid peroxides in the oxidized lipoproteins were determined by mixing 0.1 ml of the medium containing the modified lipoproteins with 1.0 ml of iodine-color reagent, incubating the mixture for 30 min at room temperature in the dark, and reading the absorbance at 365 nm. Results are expressed as mean ± S.D.

G1  Control
G2  Diabetic
G3  Diabetic + Vitamin C
G4  Diabetic + Vitamin E
3.1(e): CRP levels

Persons with metabolic syndrome commonly have high levels of CRP (Ridker et al., 2003). Evidence is growing that an elevation of serum CRP levels predicts the development of type 2 diabetes and occurrence of major CVD events (Pradhan et al., 2001). In order to validate the necessity of estimating this inflammatory marker as a component of metabolic syndrome, the serum CRP levels were measured. Fig. 3.1(e) shows that maximum concentration of CRP was found in the diabetic group (G2), followed by vitamin E and C supplemented groups (G4, G3) and finally the non-diabetic, control group (G1). The surprisingly reduced CRP concentration in the antioxidant supplemented groups indicates the role of these vitamins in warding off the complications associated with metabolic syndrome. Numerous studies have confirmed that CRP levels are elevated in patients with metabolic syndrome (Tracy, 1999; Rifai and Ridker, 2001; Jialal and Devaraj, 2003) but none, best to our knowledge, have yet reported the suppression of CRP levels after antioxidant supplementation. Nonetheless, Frohlich et al. (2000) have demonstrated the association between CRP and the features of metabolic syndrome in a population-based study. It was concluded that mean CRP levels increased steadily as the number of metabolic abnormalities increased.

3.1(f): TNF-α levels

TNF-α is another proinflammatory cytokine secreted by monocytes-macrophages, endothelial cells, and to a large extent, by adipocytes. Several studies have shown that levels of TNF-α are an important regulator of insulin sensitivity (Hotamisligil, 1993) and that neutralization of TNF-α improves insulin sensitivity in fa/fa rats but not in obese humans with diabetes (Arner, 2003 and Kern et al., 1995). The results avowed in fig. 3.1(f) coherently states that TNF-α levels were raised in the group of diabetic rabbits (G2). The levels were reduced significantly, in the groups supplemented with vitamin C (G3) and vitamin E (G4). It may be thus assumed that treatment with vitamins or
antioxidants suppress the exacerbation in insulin resistance and other metabolic abnormalities that are probably caused by elevation of proinflammatory cytokines and other factors of metabolic syndrome.

3.2: Declined parameters of metabolic syndrome

3.2(a): HDL levels
An inverse relationship between the levels of HDL and the risk of developing premature CVD has been a consistent finding in many prospective population studies. In several of these studies, the level of HDL has been the single most powerful lipid predictor of future CVD events (Assmann et al., 1998; Manninen et al., 1992; Stamler et al., 1986). The NCEP/ATP III criterion also puts forth the importance of decreased HDL concentration in diagnosing metabolic syndrome. Consequently, HDL concentration was determined in all the four groups participating in this study. Fig.3.2(a) shows that the diabetic group (G2) has lowest concentration of HDL followed by the vitamin E group (G4), vitamin C group (G3), and finally the control group (G1). Thus, above result indicates the efficacy of vitamins in improving HDL profile; thereby supporting the hypothesis of Gordon et al (1989) which suggests that, for every 1 mg/dl (0.025 mM) increase in HDL-C, the CVD risk is reduced by 2% to 5%.

3.2(b): PON-1 activity
Paraoxonase-1 (PON-1) is an HDL-associated enzyme that possesses antioxidative properties. In addition, diabetes is characterized by increased oxidative stress and decreased PON-1 activity. The mean serum PON-1 activity for G1, G2, G3 and G4 is demonstrated in fig.3.2(b). The highest and the lowest enzyme activity corresponding to the control group G1 and diabetic group G2, strongly suggest the reduction of arylesterase activity of PON-1 during oxidative stress. However, a noticeable elevation in the enzyme activity of the groups supplemented with vitamin C and E (G3, G4)
indicates the protection offered by antioxidants, possibly through maintaining the balance of excess free radicals and antioxidant defenses. This result also highlights the significance of decreased oxidative stress and increased PON-1 activity in recuperating diabetes as well as metabolic syndrome. Rozenberg et al. (2008) have reported that increasing PON-1 expression in mice could attenuate the development of diabetes, a phenomenon which could be attributed to the antioxidative properties of PON-1 and decreased oxidative stress.

3.2(c): sdLDL oxidation

Previous reports have established that not only quantitative but also qualitative changes to lipoprotein profiles contribute to the increased incidence of coronary diseases (Gardner et al., 1996). Another important contributor to increased coronary risk is oxidative stress (Chisolm and Steinberg, 2000). In addition, there may be a pathological link between these parameters as modified forms of low density lipoproteins such as sdLDL are more susceptible to oxidative stress (de Graff et al., 1991). Similarly, fig.3.2(c) demonstrates the average time required for the oxidation of sdLDL. It is clearly depicted that sdLDL of diabetic group (G2) got oxidized earliest as compared to other experimental groups. One means by which sdLDL could increase the risk of vascular diseases is their greater susceptibility to oxidation (de Graff et al., 1991). Diabetes is associated with increased oxidative stress (Ceriello, 2003b) and anti-oxidant protective mechanisms are suggested to be sub-optimal in diabetes and metabolic syndrome (Ford et al., 2003). Eventually, the time required for oxidation of sdLDL in vitamin supplemented groups (G3 and G4) was more than that required by the diabetic group receiving no such supplementation. The sdLDL isolated from vitamin C supplemented group however, procured maximum time to get oxidized, conferring a better preventive measure for diabetes as well as metabolic syndrome.
Fig. 3.1(e): CRP levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum CRP was measured by an enzymatic colorimetric test using a commercial kit from Calbiotech Inc., USA. Results are expressed as mean ± S.D.

G1 Control
G2 Diabetic
G3 Diabetic + Vitamin C
G4 Diabetic + Vitamin E
Fig. 3.1(f): TNF-α levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum TNF-α was measured by an enzymatic colorimetric test using a commercial kit from Immunotech SAS, France. Results are expressed as mean ± S.D.

G1    Control
G2    Diabetic
G3    Diabetic + Vitamin C
G4    Diabetic + Vitamin E
Fig. 3.2(a): HDL levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The concentration of serum HDL was measured by an enzymatic colorimetric test using a commercial kit from Ranbaxy Diagnostic Div., India. Results are expressed as mean ± S.D.

G1  Control
G2  Diabetic
G3  Diabetic + Vitamin C
G4  Diabetic + Vitamin E
Fig. 3.2(b): PON-1 activity of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The assays were performed in a final volume of 250 μl containing 1 mM phenylacetate and 2 mM CaCl₂ in 20 mM Tris-HCl buffer, pH 8.0 in the presence of 0.1 μl of serum and was read at 270 nm. Results are expressed as mean ± S.D.

- **G1** Control
- **G2** Diabetic
- **G3** Diabetic + Vitamin C
- **G4** Diabetic + Vitamin E
Fig. 3.2(c): sdLDL oxidation time of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. (1989). 10 µl of supernatant containing sdLDL, oxygenated PBS and 32 µl of 1 mM CuCl₂ was added to a 2 ml quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored at 234 nm. Results are expressed as mean ± S.D.

- G1: Control
- G2: Diabetic
- G3: Diabetic + Vitamin C
- G4: Diabetic + Vitamin E
3.3: Oxidative stress marker MDA

In recent years it has been known that, the most important factor that increases the free radicals production in diabetes is the hyperglycemic status, which can induce damage such as lipid peroxidation. Present approaches to diabetic therapy involve mainly drugs enhancing insulin secretion or signaling as well as inhibitors of endogenous glucose production (Aguirre et al., 1998), while the role of antioxidants as agents important for restoring the redox balance of the organism is still underestimated. This study therefore focuses the role of vitamin C or E alone in suppressing oxidative stress and related anomalies in alloxan-induced diabetic rabbits. Fig.3.3 illustrates the highest MDA levels, in diabetic animals (G2). Besides, it was also shown that supplementation with vitamin C (G3) as well as vitamin E (G4) successfully reduced the MDA levels. Present results hence, signify the involvement of antioxidant therapy in ameliorating oxidative stress.

3.4: SOD and Catalase activity

Under normal circumstances, free radicals that are produced through biological processes and in response to exogenous stimuli are controlled by various enzymes and antioxidants in the body. SOD and catalase are paradigm of such enzymes and generate the antioxidant defense system (Cheryl et al., 1996). A group of researchers have previously reported that a single dose of alloxan produced a decrease in the liver and pancreatic SOD and catalase activities during the development of alloxan-induced diabetes (Hamden et al., 2008). Akin to these findings, our results represented in fig.3.4(a) and (b) also demonstrate changes in the activity of antioxidant enzymes SOD and catalase, when verified in the serum of diabetic rabbits after the supplementation of vitamin C or E. It is elucidated that the specific activity of both SOD and catalase drop off significantly with diabetes and raised concomitantly after vitamin therapy as seen in G2, G3 and G4.
Nonetheless, the extent of recovery was slightly greater in G3, the vitamin C supplemented group, as compared to G4, the vitamin E supplemented group; suggesting it to be more effectual against oxidative stress.

3.5: DNA damage

Increased blood glucose levels stimulate the production of ROS, which can cause damage to biological macromolecules such as proteins and DNA (Bonnefont-Rousselot, 2002). In the present study, DNA damage was observed in the lymphocytes of alloxan-induced diabetic rabbits with or without the supplementation of antioxidants. The damage (expressed as tail length, μmeters) was measured in all the four groups G1, G2, G3 and G4 via comet assay (as described in ‘methods’). The representative photographs displaying the images of comets obtained after DNA damage before and after the supplementation of vitamin C or E is shown in fig.3.5(a). The corresponding tail length was also determined and represented in fig.3.5(b). Maximum DNA damage was observed in diabetic rabbits (G2) depicting the association of diabetes with increased oxidative stress. The antioxidants supplemented groups however experienced a lesser extent of DNA damage and it was observed that the reduction was more pronounced in vitamin C supplemented group (G3) as compared to the vitamin E supplemented group of diabetic rabbits. This result justifies the scavenging of free radicals by these vitamins and reduction of ROS, thereby ameliorating DNA damage in diabetes and metabolic syndrome.

3.6: Correlation between DNA damage and metabolic syndrome

A number of studies have reported the relation between DNA damage and diabetes (Hannon-Fletcher et al., 2000; Lima et al., 2007; Demirbag et al., 2005). To determine the association of DNA damage with metabolic
syndrome, Pearson's correlation analysis was performed. The correlation coefficient (r) obtained is an easy indicator of the role of elevated as well as declined parameters of metabolic syndrome in carrying out DNA damage. Fig.3.6 represents the result obtained after the correlation analysis and indicates positive correlation coefficient for the elevated factors and negative correlation coefficient for the declined factors of metabolic syndrome. This is an indication of enhanced DNA damage with increase in CRP, TNF-α, lipid peroxides, TGs and apo-B levels together with an increment in blood glucose. However, the negatively correlated factors propose an inverse relationship and explain the association of DNA damage with a decline in PON-1 activity, HDL concentration and the time required for oxidation of sdLDL. It can thus, safely be said that DNA damage do also occur in metabolic syndrome.

3.7: Correlation between NCEP/ATP III parameters and observed parameters with antioxidants

Present study contemplates the prediction as well as prevention of metabolic syndrome and DNA damage in diabetic animals after antioxidant supplementation. Though NCEP/ATP III criteria were involved herein to determine metabolic syndrome, other parameters analyzed throughout the study, were also taken into consideration. These parameters incorporated the analysis of serum PON-1 activity, time required for oxidation of sdLDL, concentration of lipid peroxides together with CRP, apo-B and TNF-α levels in serum, along with the estimation of DNA damage. In an effort to reveal a relationship between these factors and NCEP/ATP III parameters (FBG, TGs and HDL) in the diabetic rabbits with supplementation of vitamin C or E, Pearson's correlation analysis was performed. The plotted values of correlation coefficient (r) in fig.3.7 reveal a negative correlation between (i) the values of PON-1 activity and the levels of TGs and FBG, (ii) lipid peroxidation and HDL, (iii) sdLDL oxidation time and levels of TGs and FBG,
(iv) CRP and HDL, (v) apo-B and HDL, (vi) TNF-α and HDL, (vii) DNA damage and HDL. Thus it adds to an important observation depicting that the reduction in lipid peroxidation, and levels of CRP, apo-B and TNF-α together with the DNA damage occur due to an elevation in HDL concentration; an outcome of antioxidant supplementation. Similarly, the decrease in TGs and FBG concentration as a result of vitamin C or E supplementation was found to be responsible for increased PON-1 activity and the time required for oxidation of sdLDL. This implies that FBG, TGs and HDL levels play a fundamental role in regulation of several other metabolic syndrome parameters.
Fig. 3.3: MDA levels of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

MDA in serum was determined fluorimetrically by thiobarbituric acid reaction (TBA) as described in 'methods'. TBA reacting substance was expressed in terms of MDA as nmol/ml of serum and was measured at 553 nm emission and 515 nm excitation wavelength. Results are expressed as mean ± S.D.

- G1: Control
- G2: Diabetic
- G3: Diabetic + Vitamin C
- G4: Diabetic + Vitamin E
Fig. 3.4(a): SOD activity of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

The assay medium in a final volume of 3 ml consisted of 50 μl sample (serum) and 0.05 M tris-succinate buffer, pH 8.2 (2.85 ml). After incubation at 25°C for 20 min, the reaction was initiated by the addition of 8 nM pyrogallol. The change in absorbance was recorded spectrophotometrically at 420 nm for 3 min. One enzyme unit (E.U.) is defined as the amount of enzyme required to cause 50% inhibition of the rate of pyrogallol auto-oxidation. Results are expressed as mean ± S.D.

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<tr>
<td>G2</td>
<td>Diabetic</td>
</tr>
<tr>
<td>G3</td>
<td>Diabetic + Vitamin C</td>
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<tr>
<td>G4</td>
<td>Diabetic + Vitamin E</td>
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Fig. 3.4(b): Catalase activity of untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

Catalase activity was measured by following the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide (H$_2$O$_2$). The reaction was allowed to proceed in 0.05 M phosphate buffer (pH 7.0) containing 1 ml H$_2$O$_2$ (30 mM) and 50 µl sample. One enzyme unit (E.U.) is defined as the amount of enzyme decomposing 1 µM H$_2$O$_2$ per min at 25°C. Results are expressed as mean ± S.D.

G1    Control
G2    Diabetic
G3    Diabetic + Vitamin C
G4    Diabetic + Vitamin E
Fig. 3.5(a): DNA damage in untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants (representative photographs).

DNA breakage in the lymphocytes isolated from each rabbit, as measured by comet assay proposed by Singh et al. (1988) (details of the method are described in 'methods').

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<td>Diabetic + Vitamin C</td>
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<td>G4</td>
<td>Diabetic + Vitamin E</td>
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Fig.3.5(b): Extent of DNA damage in untreated diabetic rabbits, and diabetic rabbits supplemented with antioxidants.

DNA breakage in the lymphocytes isolated from each rabbit was measured by comet assay proposed by Singh et al. (details of the method are described in 'methods'). Comet tail length (µmeters) was observed and plotted against each group. Results are expressed as mean ± S.D.

G1  Control
G2  Diabetic
G3  Diabetic + Vitamin C
G4  Diabetic + Vitamin E
Fig.3.6: Correlation between DNA damage and parameters of metabolic syndrome as measured in diabetic rabbits.

Pearson's correlation analysis was performed to determine the relationship between DNA damage and the observed parameters of metabolic syndrome. The plotted values of correlation coefficient (r) indicate the positive or negative relationship between DNA damage and following parameters:

1. PON-1 activity
2. Lipid peroxidation
3. CRP concentration
4. sdLDL oxidation time
5. HDL concentration
6. TGs concentration
7. Fasting blood glucose levels
8. TNF-α concentration
9. Apo-B concentration
Fig. 3.7: Correlation between NCEP/ATP III parameters and observed parameters with antioxidants.

Pearson's correlation analysis was used for determination of relationship between the components of NCEP/ATP III and the parameters observed with vitamin supplementation. The correlation coefficient (r) of indicated parameters was plotted against the following parameters to obtain a positive or negative relationship.

1. PON-1 activity
2. Lipid peroxidation
3. sdLDL oxidation time
4. CRP concentration
5. Apo-B concentration
6. TNF-α concentration
7. DNA damage