Hypertension is a very common and serious health problem affecting millions of people worldwide. Hypertensive individuals are at an increased risk for the development of heart disease, stroke, renal failure and associated complications. Excess sugar, salt or deficit of antioxidant vitamins in diet are contributing factors for hypertension. In addition to lifestyle factors such as diet, genetic factor may also play a vital role in the etiology of hypertension. There is a complex relationship existing between hypertension, antioxidants and oxidative stress. Oxidative stress is considered to have a crucial role in the pathophysiology of hypertension (Beg et al., 2011). Hypertension is symptomless until the development of serious complications, hence often known as "the silent killer" (Siyad, 2011).

Estrogen levels decline with menopause. Whether this decline is in accordance with estrogen receptor levels and associated co-morbidities need to be elucidated. Studies on estrogen receptor status among hypertensive postmenopausal women are scarce. Hence it is imperative to assess estrogen and estrogen receptor status in hypertensive postmenopausal women with clinical complications.

The experimental procedure pertaining to the study “Status of Estrogen and its Receptor Types as possible Diagnostic and Therapeutic Tool in Postmenopausal Women with Co-morbidities” was conducted in four phases:

**Phase I**

3.1 Anthropometric and Biochemical Profile of the Participants of the Study
3.1.1 Selection of the Participants for the Study
3.1.2 Sample Collection
3.1.3 Anthropometric Measurements
3.1.4 Hematologic Analysis
3.1.5 Assessment of Cardiac Biomarkers
3.1.6 Assessment of Atherogenic Indices
3.1.7 Assessment of Renal Biomarkers

Phase II

3.2 Antioxidant Status and DNA Damage in the Participants
3.2.1 Assessment of Enzymatic and Non-Enzymatic Antioxidant Activities
3.2.2 Assessment of Lipid Peroxidation and Protein Oxidation Indices

Phase I

3.1 Anthropometric and Biochemical Profile of the Participants of the Study
3.1.1 Selection of the Participants for the Study

During the study period 412 postmenopausal women who visited five hospitals namely KTVR Hospital, Kalpana Medical Centre, KG Hospital, SPT Hospital and Saaol Heart Centre in Coimbatore, Tamil Nadu were selected. A written informed consent was obtained from the participants (Appendix-1).
Status of Estrogen and its Receptor Types as possible Diagnostic and Therapeutic Tool in Postmenopausal Women with Co-morbidities

Figure 8
Grouping of the participants

Target Population
412 Postmenopausal women - selected

Questionnaire
Informed consent

Data Analysis

Inclusion Criteria:
Hypertensive blood pressure ≥140/90 or taking antihypertensive medications, Menopause >2yrs

Exclusion Criteria:
thyroid abnormalities, congenital heart disease, cancer, jaundice, infectious diseases, received/receiving hormone replacement therapy, undergone hysterectomy

398 subjects – further selected for the study

Experimental group
(n=301)

Hypertensive Postmenopausal women (n=88)
Hypertensive Postmenopausal women with Diabetes (n=55)
Hypertensive Postmenopausal women with Renal Insufficiency (n=118)

Control group
(n = 97)

Normotensive Postmenopausal women (n=87)
Questionnaire was distributed to elicit personal details, family history, medical history and personal habits of the participants (Appendix-2). Institutional Ethics Committee clearance (HEC.2011.25) was obtained for the study. Subjects with blood pressure ≥140/90 or those taking antihypertensive medications were categorised as hypertensive. Menopause was confirmed by the absence of menstruation for more than two years. Participants with hypertension, diabetes and renal insufficiency were alone included in the study. Those having thyroid abnormalities, congenital heart disease, cancer, jaundice, infectious diseases, who received/receiving hormone replacement therapy and undergone hysterectomy were excluded from the study.

Thus out of 412 subjects considered, 398 subjects of age group 47-92 years were selected and categorised into four groups namely normotensive postmenopausal women (group-1: n=97), hypertensive postmenopausal women (group-2: n=88), hypertensive postmenopausal women with diabetes (group-3: n=95) and hypertensive postmenopausal women with renal insufficiency (group-4: n=118). Normotensive postmenopausal group served as the control. Figure 8 is the flow chart showing the grouping of the participants.

3.1.2 Sample Collection

Five ml of blood was drawn from each of the subjects through venipuncture. Two ml blood collected in serum separator tubes was allowed to clot and centrifuged to obtain serum. Three ml of blood was collected in EDTA vacutainers and plasma was separated through centrifugation. Aliquots of serum and plasma were immediately stored at -20°C until analysis. Blood was stored at 4°C for further analysis.

3.1.3 Anthropometric Measurements

Obesity is a predisposing factor for hypertension, type 2 diabetes, menstrual disorders and other diseases. Body Mass Index (BMI) is a powerful predictor of type 2 diabetes and its co-morbidities, as it is a measure of general obesity (Gothankar, 2011). Obesity and overweight were found to have significant impact on health as they carry serious health burden (Guh et al., 2009).
Salvi (2012) opined Mean Arterial Pressure (MAP) as a vital parameter for evaluating blood pressure changes during cardiac cycle. The MAP remains unchanged from ascending aorta to peripheral arteries and this relative stability of MAP in the arterial tree is Pressure (DBP) at any value of MAP is known as elevated Pulse Pressure (PP). Central PP is regarded as an alternative measure of increased arterial stiffness of the central arteries whereas in elderly subjects, the brachial PP measurement is a widely recognized marker of arterial stiffness. In elderly, elevated pulse pressure is considered as a significant and independent predictor of cardiovascular outcomes and related mortality (Berbari and Jurjus, 2012). The measurement of PP provides further information beyond the systolic and diastolic blood pressures. Pulse pressure determination is a very simple process. A wide pulse pressure greater than 55-60 mmHg is attributable to decreased elasticity of large arteries and is an indicator of increased arterial stiffness and atherosclerotic risk (Lokaj et al., 2011). Vascular stiffness is a marker of vascular health and is considered to have additional independent prognostic value for adverse cardiovascular events (Steppan et al., 2011).

Height of the subjects was measured using height measuring tape and weight was measured using weighing scale. Blood pressure was recorded using mercury sphygmomanometer in sitting position. From systolic and diastolic blood pressure, Mean Arterial Pressure (MAP) and Pulse Pressure (PP) were calculated from the formula:

\[
\text{Mean Arterial Pressure} = \frac{(2 \times \text{Diastolic blood pressure}) + \text{Systolic blood pressure}}{3}
\]

\[
\text{Pulse Pressure} = \text{Systolic blood pressure} - \text{Diastolic blood pressure}
\]

BMI was calculated from the formula:

\[
\text{BMI} = \frac{\text{Weight in kg}}{(\text{Height in meter})^2}
\]

On the basis of the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, blood pressure less than 120/80 mmHg is categorised as normal, 120-139/80-89 mmHg
as prehypertension, 140-159/90-99 mmHg as stage I hypertension and ≥ 160/100 mmHg as stage II hypertension (Chobanian et al., 2003).

In 2009, India’s Health Ministry along with the Diabetes Foundation of India, the All-India Institute of Medical Sciences, Indian Council of Medical Research, the National Institute of Nutrition and 20 other health organisations jointly released guidelines for obesity. Based on this BMI was categorised as, Normal BMI: 18.0-22.9 kg/m², Overweight: 23.0-24.9 kg/m², Obesity: >25 kg/m² (Misra et al., 2009)

### 3.1.4 Hematologic Analysis

Mostly subclinical inflammatory reaction is accompanied by elevated hematological parameters and this contributes to the development of metabolic syndrome (Cucchiara et al., 2007). Impaired arterial stiffness is significantly associated with the markers of inflammation such as WBC count in prehypertensives (Celik et al., 2011)

Platelets of larger size were more active, both metabolically and enzymatically and had a higher thrombolic activity as compared with smaller platelets. They play an important role in the initiation of atherosclerotic lesions and coronary thrombus formation. Platelet volume indices and platelet count were suggested to help identifying patients at higher risk for acute coronary events (Ranjith et al., 2009).

Red blood cell count, hemoglobin, hematocrit, red cell indices, white blood cell count and platelet count were performed as part of Complete Blood Count using Sysmex Automated Hematology Analyzer in all the participants. Differential leukocyte count was performed by staining and counting by the method of Raghuramulu et al. (1983) and is elaborated in Appendix- 3.

Glycated hemoglobin (HbA1c) reflects preceding 2 to 3 months average plasma glucose levels (Nathan et al., 2007). According to the International Expert Committee report, the main advantage is that it can be performed at any time and patient does not need to be in fasting condition and hence considered the gold
standard for monitoring glycemic control in diabetic patients. Now-a-days a considerable interest has been observed in using it as a screening test for persons at high risk of developing diabetes and also as a diagnostic test for diabetes (Nathan et al., 2009). The WHO Consultation recommended the acceptability of HbA1c, as an additional test to diagnose diabetes, a debilitating and deadly disease. The WHO recommended cut point for diagnosing diabetes is HbA1c value of 6.5%. But also suggested that a value less than 6.5% does not exclude diabetes diagnosed using glucose tests (World Health Organisation, 2011).

From each of the four groups, a sub-sample of thirty participants were selected for assessment of Glycated hemoglobin (HbA1c). HbA1c was assessed in the selected participants by Boronate Affinity Chromatography by the method of Rohlfing et al. (2000) as given in Appendix- 4.

3.1.5 Assessment of Cardiac Biomarkers

Dyslipidemia is characterised by total cholesterol > 200 mg/dl, HDL cholesterol < 35 mg/dl, LDL cholesterol > 130 mg/dl and triglycerides > 150 mg/dl and can be brought under control by using drugs to reduce low density lipoprotein cholesterol (LDL-C), decrease triglycerides and increase high density lipoprotein cholesterol (HDL-C) up to normal range thereby normalizing blood pressure as well cholesterol levels (Extremera, 2012). Serum total cholesterol (TC) was found to contribute significantly to both systolic blood pressure and diastolic blood pressure (Ejike et al., 2009).

Lipid profile was assessed in the participants of the study as given in Table 1.
Table 1
Details of analysis of serum lipid profile

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Reference</th>
<th>Appendix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
<td>Spectrophotometry</td>
<td>Kit method Allain et al., 1974</td>
<td>5</td>
</tr>
<tr>
<td>HDL Cholesterol</td>
<td>- do -</td>
<td>Kit method Gordon et al., 1977</td>
<td>6</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>- do -</td>
<td>Kit method Jacobs and VanDemark, 1960</td>
<td>7</td>
</tr>
</tbody>
</table>

The levels of low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) were calculated by using Friedewald equation:

VLDL cholesterol = Triglycerides/5
LDL cholesterol = Total cholesterol – (HDL cholesterol + VLDL cholesterol)

3.1.6 Assessment of Atherogenic Indices

Atherogenic indices are more appropriate markers of lipid profile than individual lipids concentrations, hence can be employed in cardiovascular risk prediction in patients with rheumatoid arthritis and other chronic inflammatory diseases (Popa et al., 2012). Yang et al. (2008) suggested atherogenic index as an important risk factor of atherosclerosis.

Atherogenic Index (LDL-C/HDL-C), Coronary Heart Disease Risk Ratio (HDL-C/TC), Cardiac Risk Ratio (TC/HDL-C), Atherogenic Coefficient ((TC-HDL) / HDL) and Atherogenic Index of Plasma (log (TG/HDL-C)) were assessed in the participants of the study.

3.1.7 Assessment of Renal Biomarkers

High blood pressure is the main culprit associated with increased progression of kidney disease, cardiovascular risk and mortality (Plantinga et al., 2009). Serum urea, creatinine and electrolytes are routine investigations for
essential hypertension. These tests are used to assess the overall level of cardiovascular risk and to look for any evidence of hypertensive end-organ damage and were suggested as sensitive markers for renal dysfunction (Thomas, 2007).

Sodium is the most abundant electrolyte with a concentration ranging from 135 to 145mEq/L (135 to 145mmol/L) in the extracellular fluid compartment. In contrary only 10 to 14mEq/L of sodium is located in the intracellular fluid compartment. Na⁺ and its associate anions (Cl⁻ and HCO₃⁻) are responsible for approximately 90% to 95% of osmotic activity in the extracellular fluid. Excess sodium retention increases the extracellular fluid volume. Functions of sodium are found to be interrelated with potassium and are involved in nutrient transport, muscle contraction and nerve impulse transmission. Potassium is the major cation in intracellular fluid and the second most abundant cation in the body. The concentration of potassium in extracellular fluid is 3.5 to 5.0mEq/L and is considerably low compared to the levels in the intracellular fluid. The electrical membrane potential is regulated by the distribution of potassium between intracellular and extracellular compartments, which turn control the neuromuscular excitability as well as contractility of skeletal, cardiac and smooth muscle tissues. Serum potassium levels can be strikingly elevated if there is a very small addition of potassium to extra cellular fluid compartment (Porth, 2010). High serum sodium and chloride concentrations and increased urinary potassium excretion were found to adversely affect electrolyte and water imbalance leading to the pathogenesis of essential hypertension (Iyalomhe et al., 2008).

As opined by Johnson et al. (2003) and Beg et al. (2011) uric acid is a proficient scavenger of singlet oxygen species, superoxides and hydroxyl radicals and can chelate transition metals. It is distributed in comparatively high concentrations in the body. Besides its protective function owing to its antioxidant properties, it may also have a pathogenic role in the development of hypertension and cardiovascular disease. The harmful effects of uric acid are attributed to its stimulation of afferent arteriolaropathy and tubulointerstitial nephritis, thereby contributing to hypertension. It is also a causative factor for vascular smooth
muscle proliferation, endothelial dysfunction and impaired nitric oxide production, resulting in cardiovascular and renovascular diseases.

The serum markers for renal function were estimated by the procedures as given in Table 2

### Table 2

**Details of analysis of renal function parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Reference</th>
<th>Appendix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>Flame photometry</td>
<td>Wooton, 1964</td>
<td>8</td>
</tr>
<tr>
<td>Potassium</td>
<td>- do -</td>
<td>- do -</td>
<td>8</td>
</tr>
<tr>
<td>Chloride</td>
<td>Titrimetry</td>
<td>Van Slyke and Hiller, 1947</td>
<td>9</td>
</tr>
<tr>
<td>Total Protein, Albumin and Globulin</td>
<td>Spectrophotometry</td>
<td>Kingsley, 1939</td>
<td>10</td>
</tr>
<tr>
<td>Albumin/Globulin ratio</td>
<td>By calculation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>Spectrophotometry</td>
<td>Wybenga et al., 1971</td>
<td>11</td>
</tr>
<tr>
<td>Creatinine</td>
<td>- do -</td>
<td>Bonsnes and Taussky, 1945</td>
<td>12</td>
</tr>
<tr>
<td>Uric Acid</td>
<td>Spectrophotometry</td>
<td>Caraway, 1955</td>
<td>13</td>
</tr>
</tbody>
</table>

#### 3.1.7.1 Estimated Glomerular Filtration Rate (eGFR)

A crude measure of renal function is serum creatinine (Scr), which is a readily available parameter and can be easily entered into equations that estimate glomerular filtration rate at the bedside and is known as estimated glomerular filtration rate (eGFR) (Rule et al., 2004).

The most commonly advocated estimating equation for calculating the glomerular filtration rate (GFR) is the one that was developed by the Modification of Diet in Renal Disease (MDRD) Study Group. The MDRD study equation was developed in 1999, utilizing the data from 1628 patients with chronic kidney disease with a mean measured GFR of 40 ml/min/1.73 m². This formula estimates GFR adjusted for body-surface area. The equation is:
GFR = 186 × (SCr)^{-1.154} × (age)^{-0.203} × 0.742 (if the subject is female)
GFR is expressed in ml/min/1.73 m^2, SCr is serum creatinine expressed in mg/ml, and age is expressed in years (Levey et al., 1999).

3.1.7.2 C-Reactive Protein (CRP)

C-Reactive Protein (CRP) serves as one of the most important inflammatory markers in chronic kidney disease (CKD), diabetes and cardiovascular diseases (Hansson, 2005). Elevated levels of CRP are associated with increased risk of chronic kidney disease. CRP not only serves as a biomarker, but might also function as a mediator in the early development of renal inflammation (Li et al., 2011).

From each of the four groups, a sub-sample of thirty participants were selected for assessment of CRP levels. CRP levels were assessed in the selected participants by agglutination method by Wadsworth and Wadsworth (1984) as explained in Appendix - 14.

Phase II

3.2 Antioxidant Status and DNA Damage in the Participants

3.2.1 Assessment of Enzymatic and Non-Enzymatic Antioxidant Activities

Activities of enzymatic and non-enzymatic antioxidants were assessed in the plasma of the participants.

3.2.1.1 Estimation of Catalase activity

Catalase (CAT) is a hemoprotein, largely located in peroxisomes. Catalase is an essential enzyme involved in the reduction of hydrogen peroxide and thereby involved in the detoxification of H_2O_2 (Punitha et al., 2005).

Catalase activity was estimated by the method of Luck (1974) as provided in Appendix – 15.

3.2.1.2 Estimation of Superoxide dismutase activity

A decline in superoxide dismutase (SOD) and catalase (CAT) activities can lead to an excess availability of superoxide radical (O_2•−) and hydrogen peroxide
(H₂O₂), which, in turn facilitates the generation of highly reactive hydroxyl radical (•OH), leading to the initiation and propagation of lipid peroxidation (Jain et al., 1990). SOD scavenges superoxide radical (O₂•−) to form O₂ and the less reactive H₂O₂. It is an important defense enzyme involved in reducing the toxic effects of O₂•− or other free radicals derived from secondary reactions (Manonmani et al., 2005).

The Superoxide dismutase activity was assessed by Beauchamp and Fridovich method (1971) and is given in Appendix – 16.

### 3.2.1.3 Estimation of Glutathione peroxidase activity

Glutathione peroxidase (GPx) has a key role in enzymatic defense system and is involved in the decomposition of hydrogen peroxide and other organic peroxides into their corresponding alcohols, at the expense of reduced glutathione. Glutathione peroxidase is a GSH-metabolizing enzyme. A drop in glutathione peroxidase activities may be due to the decreased availability of its substrate, reduced glutathione (GSH) as stated by Jain et al. (1990).

Glutathione peroxidase activity was estimated spectrophotometrically by the method of Rotruck et al. (1973), as elaborated in Appendix – 17.

### 3.2.1.4 Estimation of Glutathione-S-transferase activity

Glutathione-S-transferases (GST) catalyse the conjugation of reactive metabolites such as reactive electrophiles, oxidative stress products and carcinogenic compounds with GSH. Hence they play an important role in the detoxification process (Aw et al., 1991).

Glutathione-S-transferase activity was estimated in plasma photometrically by the method of Habig et al. (1974) as explained in Appendix – 18.

### 3.2.1.5 Estimation of Vitamin A

Preformed vitamin A and provitamin A carotenoids are the two forms of vitamin A ingested by humans. Preformed vitamin A consists of retinol and retinyl ester. Retinyl ester is predominantly present in foods from animal sources including dairy products, fish, meat (especially liver) and other organ meats, which
is converted to retinol in the small intestine. In tissues retinol is mostly esterified into retinyl ester with a long-chain fatty acid, such as palmitate, stearate or oleate, since it is unstable. The second principal form of vitamin A in diet is provitamin A, consisting of several types of carotenes namely α-carotene, β-carotene and β-cryptoxanthin. β-carotene constitutes the major form of dietary carotenoids having provitamin A activity (Ross, 2010).

Plasma vitamin A level was estimated photometrically as elaborated by Bayfield and Cole (1980) and is given in Appendix – 19.

3.2.1.6 Estimation of Vitamin E

Vitamin E, a fat-soluble antioxidant is generally considered to be the most important inhibitor of lipid peroxidation. Intake of vitamin E prevents the generation of free radicals (Yoshihara et al., 2010).

Contribution of labile hydrogen to a lipid or lipid peroxyl radical by α-tocopherol yields α-tocopheroxyl radical. The α-tocopheroxyl radicals formed is reduced to the original α-tocopherol form by ascorbic acid (Kojo, 2004). Vitamin E and vitamin C are interconnected by this recycling process (Packer et al., 1997).

The plasma vitamin E level was estimated by Quaife et al. method (1949). The procedure is given in Appendix – 20.

3.2.1.7 Estimation of Vitamin C

Vitamin C protects lipids from peroxidative damage, by working together with vitamin E to regenerate α-tocopherol from α-tocopheroxyl radicals in membranes and lipoproteins. Vitamin C participates in protecting protein thiol group against oxidation by elevating the levels intracellular glutathione. Vitamin C and vitamin E, together limit oxidative damage in humans and thereby reduce the risk of certain chronic diseases. They are commonly known as ‘antioxidant vitamins’ (Naziroglu and Butterworth, 2005). Vitamin C is a hydrophilic antioxidant and disappears faster than any other antioxidant when plasma gets exposed to reactive oxygen species (May and Qu, 2011).
Vitamin C estimation was performed by the method of Omaye et al. (1979) and the procedure is given in Appendix – 21.

### 3.2.1.8 Estimation of Reduced Glutathione

Reduced glutathione (GSH) is an intracellular thiol rich tripeptide. GSH execute excellent role in protecting cells and tissue structures from oxidative damage (Yu, 1994). Reduced glutathione is necessary for the recycling of vitamin C. GSH inturn act as a substrate for glutathione peroxidase enzymes engaged in protecting and preventing the detrimental effect of free radicals (Mohamadin et al., 2011).

Reduced glutathione was estimated as explained by Moron et al. (1979) in Appendix – 22.

### 3.2.2 Assessment of Lipid Peroxidation and Protein Oxidation Indices

A rise in lipid peroxidation level is considered to be the main consequence of an increased oxidative stress (Al-Rawi, 2011). An increase in thiobarbituric acid reactive substances (TBARS) is an indirect evidence of increased production of free radicals (Maritim et al., 2003). The lipid peroxide product, TBARS is evaluated as an index of lipid peroxidation (Mohamadin et al., 2011).

TBARS was assessed by the procedure of Niehaus and Samuelsson (1968) and is elaborated in Appendix- 23.

Protein carbonyls are the most prominent irreversible oxidized protein products produced in the body as a result of unrelenting exposure to oxidants. The levels of protein carbonyls indicate the extent of damage to proteins (Benzie and Strain, 1999). Griffiths had demonstrated the chemical stability of protein carbonyls as three months when stored at -80°C. This chemical stability makes them suitable parameter for laboratory measurement (Griffiths, 2000).

The classical and highly sensitive assay for the detection of protein carbonyl groups involves their reaction with 2,4-dinitrophenylhydrazine (DNPH), leading to the formation of a stable product, 2,4-dinitrophenylhydrazone, followed by the
spectrophotometric quantification of these acid hydrazones at 370 nm (Levine et al., 1990 and Levine et al., 1994). Spectrophotometric DNPH assay is very sensitive for the determination of carbonyl content in purified proteins (Dalle-Donne et al., 2001), in mixture of proteins such as cellular extracts, tissue homogenates, plasma and in isolated proteins. This method has become the most extensively employed measure of protein oxidation in several human diseases (Dalle-Donne et al., 2003).

Plasma protein carbonyl was assessed by the procedure of Levine et al. (1990) and is provided in Appendix-24.