Chapter 1
Introduction
1.1. Menopause:

Menopause is the physiological cessation of menstrual cycles associated with advancing age in women. The menopause is sometimes referred to as change of life or climacteric. This process occurs in woman’s life due to normal aging process, as the ovaries stop producing estrogen, causing the reproductive system to gradually shut down. However, menopause can be surgically induced by bilateral oophorectomy, which is often done in conjunction with hysterectomy; the resulting cessation of menses as a result of reproductive organ removal is sometimes called “surgical menopause”.1,2

Menopause can thus be said to be a universal reproductive trend. Numerous physical and psychological symptoms have been recognized to the hormonal changes of menopause. This reproductive landmark is not always the same for all women in all cultures. The prevalence of menopausal symptoms varies widely not only among individuals of the same population but also between different ethnic populations. Even there is a great diversity in nature of symptom and frequencies across countries, even in the same cultures.1,2

Mean age at menopause ranges in Indian women from 40.32 to 48.84yrs, but some women enter menopause at younger age, especially if they have had cancer or another serious illness and undergone chemotherapy.3-10 Premature menopause (or premature ovarian failure) is defined as menopause occurring before the age of 40; it occurs in 1% of women. Other causes of premature menopause include autoimmune disorders, thyroid disease, and diabetes mellitus. Premature menopause is diagnosed by increasing levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH).11

The cessation of menses that is not due to surgical removal of the reproductive organs is the result of the eventual atresia of almost all oocytes in the ovaries. This causes an increase in circulating FSH and LH levels as there are a decreased number of oocytes responding to these hormones and producing estrogen. This decrease in the production of estrogen leads vasomotor symptoms such as hot flashes and palpitations, psychological symptoms such as depression, anxiety, irritability, mood swings and lack of concentration, and atrophic symptoms such as vaginal dryness and urgency of urination appear. Together with these symptoms, the woman may also have increasingly erratic menopausal periods.11
1.2. **Demographic assessment:**

It is well established that the clinical consequences of increased body fat mass and obesity include osteoarthritis, gallbladder disease, diabetes, cardiovascular and pulmonary insufficiency, hypertension and atherosclerotic cardiovascular disease (CVD), all of which are dependent on the severity and duration of obesity. Further, being overweight worsens all of the elements of the cardiovascular (CV) risk profile: hypertension, glucose intolerance, insulin-resistant glucose intolerance, dyslipemia, hyperuricemia, elevated fibrinogen and left-ventricular hypertrophy\(^{12}\).

According to the 2001 census, the total aged (60 years and above) population of India is approximately 110 million, or approximately 11% of the total population. This percentage will be increased to 14.8% by the year 2020. Many studies have had hinted that age is an important factor to which the CVD risk factors are related. The fact remains that the problems of high blood pressure (BP) and obesity, considered to be the major risk factors for CVD, are increasingly assuming global importance. Body weight is potentially an ideal modifiable risk factor, as it increases during the lifespan through late middle age. Age related differences in the regional body composition are also documented by higher waist diameters, higher waist to hip or waist to thigh ratios, and lower girths in the limbs in older than in younger subjects. Alterations in the lipid profile have also been associated with age. It is reported that the total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and atherogenic index are significantly high and high density lipoprotein cholesterol HDL-C is low in the women older than 45 years as compared to those women aged between 25 and 45 years\(^{13}\).

1.2.1. **Laboratory profile:**

The deleterious lipid profile and CV output has been ascribed to menopausal loss of estrogen. In the general population of post menopausal females (PMF), hormone replacement therapy (HRT) reduces CV risk by upto 50%. Approximately 25-50% of this reduction in risk has been attributed to changes in the levels of lipoproteins. Oral estrogen administration decreased LDL-C and increases HDL-C and triglycerides (TG) in normal menopausal females\(^{14-17}\).

C-Reactive protein (CRP) levels are higher in women taking HRT as compared with non HRT. However, whether or not the elevation in CRP that occurs with oral HRT use is
associated with increased risk in cardiovascular disease (CVD) are yet to determine. The Women's Health Initiative (WHI) observational study suggested that CRP is associated with future coronary risk irrespective of HRT status at baseline, although risk estimates for the prediction of cardiovascular events (CVEs) are nonsignificant in HRT users. Similarly, CRP has been shown to be a linear predictor of CVE in non HRT in one of the study, although a detailed analysis of HRT users has not yet been conducted in this population. Current American Heart Association (AHA)/Centers for Disease Control and Prevention (CDC) recommendations do not specify whether threshold cut-points for CRP are appropriate for HRT users, but suggest that additional prospective studies are needed to more precisely define risk at various strata and to assure consistency in subgroup populations\textsuperscript{18}.

Cross-sectional associations between HRT use and markers of inflammation in women aged 65 years and older from the Cardiovascular Health Study shows that the women treated with estrogen had 59% higher mean CRP levels as compared with nonusers, yet fibrinogen and plasminogen activator inhibitor-1 antigen levels are lower in HRT users\textsuperscript{19}. However these contradictory results may implicate a differential effect of HRT use on transcriptional control, clearance, or cytokine regulation of these proteins. Furthermore, a recent report from the Postmenopausal Estrogen/Progestin Interventions (PEPI) clinical trial indicated that levels of CRP increased with HRT use (estrogen alone and in combination with progestin) during a 3-year follow-up. A report of one small clinical study suggests that, the short-term (12-week) treatment with estradiol and sequentially combined HRT showed a significant increase level of CRP\textsuperscript{20}.

Menopausal symptoms appear at the time when a woman stops having menstrual periods, is not a disease or an illness. It is a transition between two phases of a woman’s life. Many women experience a variety of symptoms as a result of the hormonal changes associated with the transition through menopause. Around the time of menopause, women often loose bone density and their blood cholesterol levels may worsen, which increases their risk of heart disease. This list of common symptoms that occur during perimenopause and menopause was developed from the real-life experiences of hundreds of women. All symptoms were experienced by numerous women and were either cyclical in nature or responded to treatments (both traditional and alternative) known to address hormonal imbalances. The most common clinical symptoms associated with menopause were hot
flushes and night sweats, insomnia, headache and body-aches, fatigue, irritability, perspiration, palpitation, short breath, nervous tension and depression\textsuperscript{21-23}.

1.3. Method development and validation.

1.3.1. Method development and validation for estrogen estimation\textsuperscript{24}:

In order to quantify plasma concentrations of estrogens in clinical pathophysiology diagnosis and assessments, it is necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy and precision. Hence in many of the clinical studies the measurement of serum estradiol forms an integral part of the assessment of female reproductive function, including studies of infertility, oligo-menorrhea, and menopausal status. In addition, it is widely used for monitoring of ovulation induction as well as during preparation for in vitro fertilization\textsuperscript{25,26}. These applications account for the bulk of clinical estrogen analyses; estradiol is measured frequently and estrone rarely. The estradiol assays used are optimized for these clinical scenarios, which place only modest demands on assay sensitivity but require fast assay times and high throughput. However, a more sensitive estradiol assay, simultaneous measurement of estrone, or both are needed in many other clinical situations. These include inborn errors of sex-steroid metabolism, disorders of puberty, estrogen deficiency in men, and increasingly, therapeutic drug monitoring, either in the context of low-dose female hormone replacement therapy or antiestrogen treatment\textsuperscript{27-33}. There is also an increasing research-driven demand for high-sensitivity estrone and estradiol assays, e.g., to study breast cancer, male osteoporosis, Alzheimer disease, and cardiovascular disorders\textsuperscript{28,34-38}.

High-sensitivity estradiol immunoassays are challenging because physiologic serum concentrations of estradiol are typically <140 pmol/L (40 ng/L) in adult men and postmenopausal female and in both sexes during infancy and childhood. None of the commercially available automated direct estradiol assays appears to have sufficient sensitivity for the evaluation of estradiol in the sera of children and men. Assays with higher sensitivity are available, but they have traditionally been manual RIAs. Although some of these estradiol and estrone RIAs provide better sensitivity, they have several important drawbacks: They require handling of radioactive materials, organic extraction, chromatography, and prolonged incubation. Their quality control is also difficult in a routine laboratory and they are very susceptible to artifacts caused by nonspecific binding of
radioactivity, cross reactivity and other analytical interferences may occur. Most importantly, there is often very poor agreement among the results obtained by different RIAs, sometimes even assays from the same manufacturer, making patient follow-up over time or between laboratories, as well as longitudinal studies, extremely difficult. Similar issues are also relevant with regard to automated, chemiluminescence-based, direct estradiol immunoassays. College of American Pathologists (CAP) survey results for the past few years confirm that the performance of direct estradiol immunoassays needs to improve with respect to analytical accuracy and detection limit\(^{39}\).

Estrone and estradiol assays based on gas chromatography–mass spectrometry (GC-MS) address many of the shortcomings of automated immunoassays and RIAs, and they are considered to be the most accurate methodology\(^{40}\). However, sensitivity is often less than what can be achieved by sensitive RIAs, and run times may be longer than 30 min/sample, limiting throughput\(^{40-43}\). Liquid chromatography with tandem mass spectrometry detection (LC-MS-MS) has been shown to be superior to GC-MS in many scenarios, in terms of both sensitivity and sample throughput, and, in combination with isotope dilution, is also considered as a reference methodology\(^{44-46}\).

Hence it is essential to establish an assay capable of quantifying estrogens at lower concentrations. At the same, it is expected that this method would be efficient in analyzing large number of clinical samples obtained for pathophysiologically assessment, pharmacokinetic studies after therapeutic doses of estrogens.

1.3.2. Method development and validation for progesterone\(^{24}\):

Generally, the concentrations of progesterone and norgestrel in biological fluids are around the ng/mL level or even lower. The radioimmunoassay (RIA) method has been extensively employed in clinical examination owing to its sensitivity. Although sensitive, RIA is considered to be time consuming and labor-intensive. Moreover, it is not suitable for high throughput analysis\(^{47}\).

In recent years electrospray ionization (ESI) has become the most widely applied interface for liquid introduction into the mass spectrometer. Also, the advent of the electrospray ionization interface has greatly stimulated the use of another technique, atmospheric-pressure chemical ionization (APCI). The improvements in mass spectrometric methodology, such as electrospray ionization and atmospheric pressure chemical ionization,
have meant that assay methods with enhanced sensitivity, improved selectivity, robustness and high sample throughput have become possible. Therefore, high performance liquid chromatography with mass spectrometry (HPLC-MS), especially HPLC-MS-MS, is currently recognized as a powerful tool to characterize complex samples and to analyse biological samples.

Primary methods of measurement play an important role in metrology because they provide the essential first link in the chain of traceability. In order to be considered primary, two conditions for a method are required. First, it must be a method that is specific to a defined substance. Second, the values of all variables, or corrections that depend on other species or the matrix, must be known or calculable within appropriate uncertainty limits.

However, other methods are also developed for the same purpose like GC–MS method which has complexity such as derivatization of a sample, hence in contrast LC–MS-MS, which has the advantages of simplicity, accuracy, and speed of measurement, has replaced it. High performance liquid chromatography (HPLC) and enzyme immunoassay (EIA) have also been used as commercial methods in many clinical laboratories.

### 1.4. Estrogen and progesterone (comparison and pharmacokinetic (PK) study)

Estradiol & estrone are the primary circulating estrogens in women; the relative amounts depend on the menopausal status of the women. Estradiol and estrone are inter-convertible. The conversion rate of estradiol to estrone is higher than the reverse reaction. Both estrone and estradiol are metabolized in the liver and gastrointestinal tract to form glucuronide and sulfate conjugates and are excreted in the urine or feces. Estrone sulfate is the primarily circulating conjugate and provides a large stable pool of estrogen in the body. After administration of an intravenous dose of radio-labeled estradiol, less than 1% is excreted in the urine as unconjugated estrogen, and the elimination half-life is approximately 1 hr.

It is reported in USA premenopausal female, that estradiol concentrations range over the menstrual cycle from 40 to 60 pg/mL to 200 to 400 pg/mL, and estrone concentrations range from 40 to 60 pg/mL to 170 to 200 pg/mL. The ratio of estradiol to estrone ranges from 0.5 to 2.0. In postmenopausal women, estradiol and estrone concentrations are 5 to 20 pg/mL and 30 to 70 pg/mL, respectively, with the estradiol-to-estrone ratio significantly less than one.
Estradiol is highly protein bound (90%). The free, or unbound, steroid is generally considered the active component in blood and is available to bind to the estrogen receptors. The route of administration of estradiol significantly affects its elimination rate and metabolite profile. On oral administration, the bioavailability of micronized estradiol is only 0.1% to 12%. Because of significant first-pass metabolism, most of the estradiol is converted to estrone and estrone sulfate, resulting in plasma estrone concentrations 3 to 6 times higher than estradiol. Transdermal, vaginal, sublingual, and subcutaneous routes circumvent the first-pass metabolism and result in an estrone to estradiol ratio less than 1.7. Therefore, small doses can be used to achieve estradiol plasma concentrations similar to those resulting from oral therapy.

Since the discovery of progesterone in 1950s, synthetic oral progesterone has been used for a variety of gynaecological conditions. However, androgenic activity inherent in the synthetic compound precludes its liberal use in assisted reproductive technology because of the threat of teratogenic effects. Furthermore, synthetic progesterone used in hormonal replacement therapy (HRT) may partially reverse the estrogenic benefits on the cardiovascular system and lipoprotein metabolism.

Natural progesterone is devoid of any androgenic activity, extensively used in assisted reproduction, sometimes for long periods of time. The major problem with natural progesterone is its route of administration. Oral intake is the most convenient. However, rapid and extensive intestinal metabolism prevents adequate absorption. Injection (i.m.) assures reliable absorption, but is related to low compliance. It is painful, can cause local irritation and cold abscesses, and must be administered by trained medical personnel. Thus the vaginal route has become the most established way to deliver the natural progesterone as it is easy to administer and avoids liver first-pass metabolism. Other studies suggest that, drugs administered through the vaginal route are transmitted primarily to the uterus, where they achieve higher tissue concentrations than if administered orally or by i.m. injections. Many vaginal formulations have been assayed, mostly as suppositories, gelatin capsules and recently as bio-adhesive. Although the suppositories are easily inserted, they melt at body temperature and lead to disturbing vaginal discharge of estrone.

As menopausal health demand priority in Indian scenario due to increase in life expectancy and growing population of menopausal women, large efforts are required to
educate and make these women aware of menopausal symptoms. This will help in early recognition of symptoms, reduction of discomfort and fears and enable to seek appropriate medical care if necessary.

All the above discussed studies are performed in countries like USA, UK, Australia, UAE, New Zealand etc. These studies are necessary to understand the pattern of changes in the hormonal levels during pre menopause and post menopause period. The HRT is very well known therapy used for elevating the post menopausal syndrome of women. But many studies suggests that there is a great diversity in nature of symptoms and frequencies across countries even in the same culture.

For the use of estrogen and progesterone formulations in Indian PMF, there is a lack of well established pharmacokinetic data, so the data available from other countries are utilized. But its effectiveness should be established.