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The hormone replacement therapy and incidence of gall stones in some selected post menopausal women using HRT

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Abstract
Accumulated evidence from human and animal studies showed that high levels of estrogen significantly stimulate the activity of 3-hydroxy-3- methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in hepatic cholesterol biosynthesis, even under high dietary cholesterol loads. These observations suggest that there may be an increased delivery of cholesterol to bile from de novo synthesis in the liver. Furthermore, studies in humans and animals suggested that estrogen could augment the capacity of dietary cholesterol to induce cholesterol supersaturation of bile. More recently, it is observed that high doses of estrogen significantly enhance intestinal cholesterol absorption, mostly due to an upregulated expression of intestinal sterol influx transporter Niemann-Pick C1 like 1 protein (NPC1L1) via the intestinal ERα pathway. Despite these observations, no information is available on the metabolic abnormalities underlying major sources of the excess cholesterol leading to the super saturation of bile and the formation of cholesterol gallstones induced by estrogen. Randomised controlled trials and observational studies have shown a clear increase in the risk of gallbladder disease (cholelithiasis, cholecystitis, or cholecystectomy as outcomes) with use of hormone replacement therapy by postmenopausal women. Thus, in this study analysis was done to observe the incidence of Gall Stones in HRT users, their biochemical profile related to serum levels of hormones, effect on hepatic efficiency was studied and a positive relation was found between the use of oral route of HRT and incidence of development of cholesterol containing gall stones.

Keywords: estrogen, supersaturation of bile, cholelithiasis, postmenupausal women, bilirubin, cholesterol

Introduction
In vertebrates, the gallbladder is a small hollow organ where bile is stored and concentrated before it is released into the small intestine. In humans, the pear-shaped gallbladder lies beneath the liver, although the structure and position of the gallbladder can vary significantly among animal species. It receives and stores bile, produced by the liver, via the common hepatic duct, and releases it via the common bile duct into the duodenum, where the bile helps in the digestion of fats. At any one time, 30 to 60 millilitres (1.0 to 2.0 US fl oz) of bile is stored within the gallbladder.

The gallbladder can be affected by gallstones, formed by material that cannot be dissolved – usually cholesterol or bilirubin, a product of hemoglobin breakdown. These may cause significant pain, particularly in the upper-right corner of the abdomen, and are often treated with removal of the gallbladder called a cholecystectomy. Cholecystitis, inflammation of the gallbladder, has a wide range of causes, including result from the impaction of gallstones, infection, and autoimmune disease. Gallstones form when the bile is saturated, usually with either cholesterol or bilirubin. Most gallstones do not cause symptoms, with stones either remaining in the gallbladder or passed along the biliary system. When symptoms occur, severe "colicky" pain in the upper right part of the abdomen is often felt. If the stone blocks the gallbladder, inflammation known as cholecystitis may result. If the stone lodges in the biliary system, jaundice may occur; and if the stone blocks the pancreatic duct, then pancreatitis may occur. Gallstones are diagnosed using ultrasound. When a symptomatic gallstone occurs, it is often managed by waiting it to be passed naturally. Given the likelihood of recurrent gallstones, surgery to remove the gallbladder is often considered. Some medication, such as ursodeoxycholic acid, may be used; and lithotripsy, a procedure used to break down the stones, may also be used.

In the developed world, 10–15% of adults have gallstones. Gallbladder and biliary related diseases occurred in about 104 million people (1.6%) in 2015 and they resulted in 106,000 deaths. Women more commonly have stones than men and they occur more commonly after the age of 40. Gallstone disease refers to the condition where gallstones are either in the gallbladder or common bile duct. Gallstones may be asymptomatic, even for years. These gallstones are called "silent stones" and do not require treatment. The size and number of gallstones present does not appear to influence whether people are symptomatic or asymptomatic. A characteristic symptom of gallstones is a gallstone attack, in which a person may experience colicky pain in the upper-right side of the abdomen, often accompanied by nausea and vomiting, that steadily increases for approximately 30 minutes to several hours. A person may also experience referred pain between the shoulder blades or below the right shoulder. These symptoms may resemble those of a "kidney stone attack". Often, attacks occur after a particularly fatty meal and almost always happen at night, and after drinking.

In addition to pain, nausea, and vomiting, a person may experience a fever. If the stones block the duct and cause bilirubin to leak into the bloodstream and surrounding tissue, there may also be jaundice and itching. This can also lead to confusion. If this is the case, the liver enzymes are likely to be raised. Pigment gallstones are most commonly seen in the developing world. Risk factors for pigment stones include hemolytic anemias (such as from sickle-cell disease and hereditary spherocytosis), cirrhosis, and biliary tract infections. People with erythropoietic protoporphyria (EPP) are at increased risk to develop gallstones. Additionally, prolonged use of proton pump inhibitors has been shown to decrease gallbladder function, potentially leading to gallstone formation. Cholesterol modifying medications can affect gallstone formation. Statins inhibit cholesterol synthesis and there is evidence that their use may decrease the risk of getting gallstones. Fibrates increase cholesterol concentration in bile and their use has been associated with an increased risk of gallstones.

Etiopathology

As recently as 2005, women have had a positive attitude towards HRT, but based on the empirical data, these attitudes may be overly optimistic. Epidemiological investigations have found and clinical studies have confirmed that at all ages, women are twice as likely as men to form cholesterol gallstones, suggesting that estrogen may be an important risk factor for the formation of cholesterol gallstones in humans. Recently, we found that estrogen promotes cholesterol gallstone formation through increasing expression levels of the hepatic estrogen receptors (ER) α, but not β. Furthermore, the ERα-selective agonist propylpyrazole, but not the ERβ-selective agonist diarylpropionitrile, augments hepatic cholesterol output resulting in cholesterol supersaturated bile and gallstones. Similar to estrogen treatment, tamoxifen significantly promotes biliary cholesterol secretion and cholesterol gallstone formation in the gonadectomized increases gallstone prevalence in women. In addition, the lithogenic actions of estrogen can be totally abolished by its antagonist ICI 182,780. It is concluded, therefore, that the hepatic ERα plays a crucial role in the formation of gallstones in exposure to high levels of estrogen. It has already been established that biliary cholesterol hyper-secretion is the primary cause of cholesterol gallstones in humans and in several animal models of cholesterol gallstones. Accumulated evidence from human and animal studies showed that high levels of estrogen significantly stimulate the activity of 3-hydroxy-3- methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in hepatic cholesterol biosynthesis, even under high dietary cholesterol loads. These observations suggest that there may be an increased delivery of cholesterol to bile from de novo synthesis in the liver. Furthermore, studies in humans and animals suggested that estrogen could augment the capacity of dietary cholesterol to induce cholesterol supersaturation of bile. More recently, it is observed that high doses of estrogen significantly enhance intestinal cholesterol absorption, mostly due to an up-regulated expression of intestinal sterol influx transporter Niemann-Pick C1 like 1 protein (NPC1L1) via the intestinal ERα pathway. Despite these observations, no information is available on the metabolic abnormalities underlying major sources of the excess cholesterol leading to the super saturation of bile and the formation of cholesterol gallstones induced by estrogen. Randomised controlled trials and observational studies have shown a clear increase in the risk of gallbladder disease (cholelithiasis, cholecystitis, or cholecystectomy as outcomes) with use of hormone replacement therapy by postmenopausal women. Oestrogen administered orally is metabolised by the liver before entering the systemic circulation (“first pass metabolism”). Oestrogen administered transdermally avoids this first pass metabolism and it has been suggested that it might have a lesser effect on gallbladder disease than orally administered oestrogen. Hence it is examined the relation between method of administration and type of hormone replacement therapy and the incidence of gallbladder disease in a large cohort of postmenopausal women

Based on these previous work, we designed a hypothesis that HRT is strong etiological factor for the incidences of Gall stones in postmenopausal women.

Objectives

- To collect information regarding demographic data of HRT users (postmenopausal HRT users females)
- To select female subjects randomly from the society, who are age, sex and demographic data wise matched with HRT users, but not using HRT. I tried to take these subjects from the families of HRT users, so that other factors like family history, dietary habits can be controlled.
- Their details regarding receiving HRT was collected, especially regarding composition of HRT they are taking, whether taking only Estrogen or Estrogen and Progesterone both, because Estrogen affects the concentration of bile in Gall Bladder by making it supersaturated with Cholesterol and Progesterone reduces amplitude of contraction intensities, hence clearance of bile from the gall Bladder.
- The total lipid profile of both HRT users group and non users group is analyzed, as use of HRT increases serum lipids level, specially cholesterol, which precipitates Hyper-cholestrolemia, thickening of bile and filially cholesterol containing Gall stones.
- To analyze the serum Estrogen and Progesterone level in HRT users and Non users and correlated the hormonal levels with incidence of Gall Stones.
- The analysis of hepatic enzymatic profile was done as incidence of Gall stones affects hepatic profile and Liver

functioning status. This shows the severity of incidence of Gall Stones.

- The difference in incidence of Gall stones was analyzed among different routes of insertion of HRT used –Oral and Trans-dermal route as some previous studies suggested that the route of HRT administration also affects the incidence of Gall stones.
- The incidence of Gall stones was correlated with the type of Estrogen used as HRT, as some previous studies suggested that Estrogen in the form of Estron precipitates maximum risk for Gall stones and Estriol precipitates least risk for developing Gall stones.
- The duration of HRT use was also taken and correlated with the incidence of developing Gall stones.
- Few subjects who are undergone procedures of removing Gall Stones, their removed stones are looked for composition, as in HRT users It is expected to have Cholesterol mainly, but of Bilirubin in non HRT users.
- Study area-Bilaspur city and Raipur city, by contacting HRT users from clinics of endocrinologist –Dr Kalpana Dash (Bilaspur), Dr Bhattacharya (Raipur).
- Study Time April 2013-December 2017.
- Sample Size-43 HRT users and 43 HRT on users.
- The procedure of all medical ethics were followed as the study was done randomly, thus permission from the concerned subjects were taken in written in prescribed format.

Materials & Methodology

Estimation of serum lipid profile

The following chemicals and biochemicals were utilized.

1. Chema Diagnostica Qualigens fine chemicals A devision of Glaxo India Ltd.
2. Span diagnostic limited, Surat, India.

a. Cholesterol Estimation Kit (one step method of Wybenga and Plleggi) (Catalog No. – 25924)
b. HDL Estimation Kit (One step method of Wybenga and Plleggi) (Catalog No.–25924)
c. Triglyceride Estimation Kit (Enzymatic colorimetric method GPO–PAP liquid stable single regent) (Catalog No. 77034 (6×250 ml)).

Optical Measurements

All routine colorimetric estimations were performed on Spectro-colorimeter 103 and Spectro-photometer 106, and Colorimeter 114, (5-filters) (Systronics, India).

Blood Collection

Blood sample (2 ml) was collected from the control and experimental group. Blood samples were collected preferably before meals. All analytics were done on serum and not on plasma, as EDTA interferes with lipid estimation procedures particularly with high density lipoprotein (HDL).

Apart from blood collection and estimation done in the lab, the reports of lipid profile analysis were done in the collaboration with Aakash Patho-Lab, Bilaspur and also from Abhay Tamrakar, Pathologist, Bilaspur, from Sardar Patel City Hospital, Bilaspur; The Reports were also collected from the Bose Pathological Lab Bilaspur. Reports of 22 lipid profiles were collected in experimental group and 26 reports were collected in control group. Rests were analyzed. The Blood samples were stored in cold temperature (4° C) before estimation, if necessary, but were discarded after 8 hours. They were equilibrated to room temperature before estimation.

Data pertaining to lipid profiles of pre-menopausal controls (66 in no.), 26 data were served as collected data. Rests of them were analyzed, but as they were in general agreements, so the collected and analyzed data were taken as a whole.

Similarly out of 66 sample data of lipid profiles of post-menopaused experimentals-22 data were collected data. Rests of them were analyzed, but as they were in general agreements, so the collected and analyzed data were representative of whole.

Isolation of serum

The blood samples obtained were stored at room temperature and then centrifuged at 4° to 8° C for 6 to 8 min at 3500 rpm to remove serum from the blood.

Estimation of total cholesterol (mg/100 ml)

Cholesterol in the blood sample was determined by the one step procedure of Wybenga and Plleggi (Catalog no-25924).This procedure is based on the oxidation of Cholesterol to Cholesterol Oxidase (CHO).This is again oxidized to Cholest 4-en 3-one and Hydrogen Peroxide. Hydrogen peroxide formed reacts with 4-amino antipyrine and 4-chlorophenol in the presence of peroxide (POD) to produce pink colored quinonemine dye. The intensity of the color produced is proportional to the cholesterol concentration in the sample. Briefly the assay comprises of the following reactions-

$$\text{Cholesterol Esterase} \xrightarrow{CE} \text{Cholesterol + Fatty Acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CHOD} \text{Choleste-4-en-3 one} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{4- Chlorophenol} \xrightarrow{POD} \text{Quinoneimine} + H_2O$$

(Coloured Dye)

Protocol: Reagent 1: Cholesterol reagent.
Reagent 2: Standard Cholesterol.

Serum, 0.25 ml and cholesterol reagent 5.00 ml were mixed in a test tube thoroughly and then kept in boiling water bath for 90 seconds. The tube was subsequently cooled to room temperature under running tap water. The optical density (O.D) of the test sample was read using a Spectrophotometer at 560 nm. Standard cholesterol solution was prepared by using 0.25 ml of standard cholesterol solution and was mixed with 5.0 ml of cholesterol reagent and carried through the same steps as applied to serum samples.

A blank solution was prepared by using 5.00 ml cholesterol reagent in test tube and carrying the subsequent steps as above. Absorbance of the cholesterol standard and serum samples were then read at 560 nm against the blank. All the reagents of the kit are stable at 2-8°C. As the reagent 1–Cholesterol reagent is corrosive, so mouth pipetting was avoided.

Linearity- This assay was linear up to 600-mg/100 ml cholesterol value.

Estimation of High Density Lipoprotein (HDL) mg/100 ml

HDL in the blood/serum samples was determined by the procedure of Gorden *et al* (1977). The procedure is based on

the principle of production of Hydrogen Peroxide, which finally gives blue color. The optical Density of the developed color is measured at 600 nm, which is proportional to the HDL in the test sample. For the estimation of HDL mg (%) the diagnostic Kit of Span Diagnostics Ltd was used (Catalog No. 25924) based on the one step method of Wybenga and Plleggi. The principle behind the process is that Anti-human β Lipo-protein Ig in reagent A binds to lipoproteins (LDL, VLDL and Chylomicrons) other than HDL. This immuno-complex blocks cholesterol other than HDL. When reagent B is added, only HDL Cholesterol reacts with enzymatic chain (CHE-CO).Hydrogen Peroxide produced by enzymatic reaction yields a blue color complex upon oxidative condensation with F-DAOS and 4-APP in presence of peroxidase, whose absorbance is read at 600 nm, proportional to HDL Cholesterol concentration in the sample.

Protocol

For the estimation, 0.3 ml of fresh/stored serum was used. Firstly, the serum test samples were mixed with 0.3 ml precipitating reagent (Polyethylene Glycol 16%, Additives and Stabilizers). This is used to precipitate Lipo-proteins-LDL and VLDL. Both are mixed well and then kept at room temperature for about 10 minutes. After this, the solution was centrifuged at 2000 rpm for 15 minutes. From this 0.2 ml clear supernatant was taken and 5.0 ml Cholesterol Reagent was added to it. The contents were mixed well and then the tube was kept immediately in the boiling water bath for 90 seconds and cooled immediately to room temperature under running tap water. The optical density was read on a spectrophotometer at 600 nm. The same procedure was applied for preparing standard solution. The developed color was stable at least 10 minutes. It was kept away from strong light sources.

Linearity - This assay was linear up to 400 mg/100 ml levels.

Estimation of Triglyceride (TG) mg/100 ml

Triglycerides in the blood/serum samples were determined by the procedure of Bucolo David (1973). The procedure is based on the principal of production of red colored dye, Quinoneimine, which absorbs sharply at 510 nm. Briefly the assay comprises to the following reactions-

Lipase (serum/microbial)

1) Triglyceride----------->Glycerol + Fatty Acids

Glycerol Kinase

2) Glycerol +ATP----------->Glycerol 3- phosphate + ADP

Glycerol Phosphate Oxidase

3) Glycerol-3-phosphate+O_2---->Dihydroxy acetone phosphate+ H_2O_2

Peroxidase

4) H_2O_2+4-AAP+4-chlorophenol---------->Quinoneimine+H_2O

(Colored Dye)

For the estimation of Triglyceride (mg/100 ml) the diagnostic kit of Chema diagnostics Ltd Glaxo–was used which was azide free (catalog No. 77034) (6× 250 ml) was used. Triglycerides in the samples are hydrolyzed by microbial lipases to glycerol and free fatty acids (FFA). Glycerol is phosphorylated by Adenosine 5-triphosphate (ATP) to glycerol -3-phosphate (G-3-P) in a reaction catalyzed by glycerol-kinase (GK).G-3-P is oxidized to dihydroxy-acetone phosphate (DAP) in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GOP).In this reaction hydrogen peroxide (H_2O_2) is produced in equimolar concentration by the level of triglyceride present in the sample.H_2O_2 reacts with 4-Amino-antipyrine (4-AAP) and 4-chlorophenol, in a reaction catalyzed by peroxidase (POD).The result of this oxidative coupling is Quinoneimine, a red colored dye. The absorbance of this dye in solution is proportional to the concentration of triglycerides in the sample.

The reagents of the kit were supplied already in liquid, ready to use form. The kit for in vitro diagnosis was used.

Protocol

Serum, 0.02 ml was mixed with 2 ml reagent. Both were mixed well and incubate at 37° C for 5-8 min. The optical density was read at 510 nm in spectrophotometer. The same procedure was carried out for preparing standard solution. The absorbance of test and standard solutions were read at 510 nm against blank reagent.

Linearity – This assay was linear at least to 1000 mg/100 ml Triglyceride value.

Calculations

The following formula was used to determine the mg/100 ml value of the following

(A) Total Cholesterol

(Normal level -130-250 mg/100 ml in adults)

$$\text{Serum Cholesterol (mg/100 ml)} = \frac{\text{Optical density of Test (Ax)}}{\text{Optical density of Standard (As)}} \text{ x } 200$$

(B) High Density Lipo-Protein (Hdl)

(Normal level - 35-75-mg/100 ml for adult female)

$$\text{HDL (mg/100 ml)} = \frac{\text{Optical density of Test}}{\text{Optical density of standard}} \text{ x } 50$$

(C) Estimation of Triglyceride (Tg)

(Normal level = 10-190 mg /100 ml for adult woman)

$$\text{TG (mg/100 ml)} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \text{ x } 200$$

(D) Calculation of Low-Density Lipoprotein (Ldl mg /100 ml)

For this the **fw** formula was adopted =

(Normal Range = 30-60 mg/100 ml)

a. Triglyceride mg/100 ml = x
b. X + HDL mg/100 ml = y
c. Total cholesterol –y = LDL (mg /100 ml)

Statistical Methods Used In The Analysis Of Data

For statistical analysis of data the package SPSS-STAT was used. Mean, Standard Deviation, Degree of Correlation-all are calculated by using this package, butt Value –Level Of Significance was manually calculated on the basis of two sample means by using the formula.

$$S = \frac{(m_1)^2 + (m_2)^2}{\sqrt{n_1 + n_2 - 2}}$$

$$t = \frac{(m_1)^2 - (m_2)^2}{S} \times \frac{\sqrt{n_1 \times n_2}}{n_1 + n_2}$$

- The demographic data of all the subjects and controls were collected.
- Serum Estrogens and Progesterone level was estimated by using Mini Vidas -500.
- The details of HRT regime was collected-The HRT users take 0.625 mg/day via Oral route (Premarin) and those HRT users who opt trans dermal route are taking 0.05 mg / day (Estraderm) (Women with hysterectomy) and Progesterone in the form of Progestin (Women with intact Uterus).
- As the damage of Gall bladder due to Gall stones affects the Hepatic Physiological Status, thus the following parameters are also analysed- The blood sugar of all the related persons were analyzed by using NYCOCARD.
- Analysing serum C- Peptide levels. Measuring C-peptide can help to determine how much of their own natural insulin a person is producing as C-peptide is secreted in equimolar amounts to insulin, because the liver metabolizes a large and variable amount of insulin secreted into the portal vein, thus to test affected Hepatic efficiency in subjects this biochemical parameters was taken.
- The Blood tests were done to assess the serum level of the following Hepatic enzymes to assess Hepatic status-
- Aspartate aminotransferase (AST or SGOT)
- Alanine aminotransferase (ALT or SGPT)
- Alkaline phosphatase, 5' nucleotidase,
- Gamma-glutamyl transpeptidase (GGT)
- LDH (Lactate dehydrogenase)

The AST and ALT readings in such cases are usually between twice the upper limits of normal and several hundred units/liter. One of the most common causes of mild to moderate elevations of these liver tests is a condition referred to as fatty liver (steatohepatitis or hepatic steatosis due to Gall Stones).

- Estimation of WBCs to diagnose the condition of any pancreatic infections due to Gall stones by using Neubauer's chamber, as incidence of Gall Stones increases WBC count by precipitating infective condition.
- Estimation of Coagulation panel (prothrombin time or PT), because in liver malfunctioning, the Prothrombin time is prolonged, because of lesser production of Fibrinigen, Prothrombin and other clotting factors.
- Estimation of Albumin level by using Autoanalyser –Star 21 model. In Hepatic stress due to Gall stones, the production and hence the blood level of Albumin is reduced significantly.
- Estimation of serum Bilirubin level was done by using Autoanalyser –Star 21 model. In Gall stones and Liver stress the conversion and clearance of Mono and Di Bilirubin Glucuronoid hampers significantly, thus serum level of free and conjugated Bilirubin is elevated.

Results & Discussion

Table 1: Statistics of serum lipid profile

Lipid Constituents	(Mean ± SD)		Change in percent-age value	(df =84) t value
	Non HRT Taking subjects	HRT Receivers		
Cholesterol (mg/ml)	1.57 (±0.19)	1.69 (±0.24)	↑ 18%	2.04 *,**
Triglyceride (mg/ml)	1.59 (±0.17)	1.88 (±0.18)	↑ 17%	1.81*,**
HDL (mg/ml)	0.61 (±0.04)	0.31 (±0.04)	↓ 20%	2.49*,**
LDL (mg/ml)	0.69 (±0.21)	0.98 (±0.25)	↑ 40%	4.04*,**

NS = Not significant.

* P<0.05 level **P<0.01 level [SD values showed in parenthesis]

Table 2: Relevant biochemical profile of HRT users

Parameters	HRT Users	n	HRT Non Users	n	Significant Difference "t"
Serum Glucose	206 mg %	30	96 mg %	30	1.98*,**
C-Peptide Level	3.044 ng/mL	30	0.9 ng/mL	30	0.957*
Estrogens	167 picograms per millileter	30	309 picograms per millileter	30	7.132*,**
Progesterone	0.13 ng/mL.	30	0.27 ng/ml	30	1.152*,*
Aspartate aminotransferase (AST or SGOT)	126 Units /L	30	15 units /L	30	1.322*,**
Alanine aminotransferase (ALT or SGPT)	243 Units/L	30	29 /L	30	8.066*,**
Alkaline phosphatase	209 U/L	30	56 U/L.	30	1.81*
gamma-glutamyl transpeptidase (GGT)	81 U/L	30	29 U/L.	30	4.41*,**
LDH (Lactate dehydrogenase)	218 U/L	30	122 U/L	30	1.005*
Prothrombin time	31 Seconds	30	9.8 seconds.	30	6.93*,**
Total Albumin	2.1 g / dL	30	3.9 g/dL	30	4.56*,**
serum Bilirubin	3.4 mg/dL	30	0.18 mg/dL	30	1.90*
Total WBC Count	32,800 /dL	30	11,7,00 /µL	30	2.09*,**
Ultra sound	Grade -3 to 4 of Gall stones	11	Clear Gall Bladder	9	--
MRI	Gall Stones seen	4	Normal MRI	2	--

Table 3: Estimated serum estrogen level pg/ml

Age Range	Serum Estradiol In HRT Users	Incidence of Gall Stones	Serum Estradiol In HRT Non Users	Incidence of Gall Stones	Level of correlation with occurrence of Gall Stones (r)
45-50	233pg/ml	High	87 pg/ml	No	0.977*,**
50-55	187pg/ml	Low	63pg/ml	No	
60-65	308pg/ml	High	23pg/ml	No	
65+	293pg/ml	Medium	36pg/ml	No	

Table 4: Estimated serum progesterone level pg/ml (n=07) (Intramuscular Progesterone -10 mg/ ml with Estrogen)

Age Range	Serum Progesterone In HRT Users	Incidence of Gall Stones	Serum Progesterone In HRT Non Users	Incidence of Gall Stones	Level of correlation with occurrence of Gall Stones (r)
45-50	9 ng /ml	High	3ng /ml	No	0.052*
50-55	11 ng/ml	Low	5 ng /ml	No	
60-65	7ng /ml	High	9 ng ml	No	
65+	8ng /ml	Medium	4 ng /ml	No	

Table 5: Duration of HRT Use and Incidence of gall stones

Age Range	Duration of use	Incidence of Gall Stones	Level of correlation with occurrence of Gall Stones (r)
45-50	2-4 years	High	0.0445*
50-55	5-7 years	medium	
60-65	8-9 years	High	
65+	Above 10 years	Medium	

Table 6: Route of Estrogen administration and Effect on Serum Estrogen level.

Age Range	Serum Estradiol In HRT Users Oral Route	Serum Estradiol In HRT Non Users Trans dermal Route	Level of correlation with incidence of Gall Stones (r)	
			With Oral Route	With Trans Dermal Use
45-50	273pg/ml	187 pg/ml	0.552	0.029
50-55	387pg/ml	203pg/ml		
60-65	229pg/ml	213pg/ml		
65+	244pg/ml	136pg/ml		

Table 7: Route of Estrogen administration and Incidence of Gall Stones

Age Range	Serum Estradiol In HRT Users Oral Route	Incidence of Gall Stones	Serum Estradiol In HRT Non Users Trans dermal Route	Incidence of Gall Stones
45-50	273pg/ml	High	187 pg/ml	No
50-55	387pg/ml	Low	243pg/ml	Low
60-65	229pg/ml	High	213pg/ml	No
65+	244pg/ml	Medium	136pg/ml	No

Table 8: Types of Estrogens used and Effect on Serum Estrogens Level

Age Range	Serum Estradiol In Estrone Users	Incidence of Gall Stones	Serum Estradiol In Estradiol Users	Incidence of Gall Stones	Serum Estradiol In Estriol Users	Incidence of Gall Stones
45-50	314pg/ml	High	287 pg/ml	No	185 pg/ml	No
50-55	227pg/ml	Low	243pg/ml	mild	211pg/ml	no
60-65	317pg/ml	High	199pg/ml	No	247pg/ml	mild
65+	298pg/ml	Medium	266pg/ml	moderate	193pg/ml	no

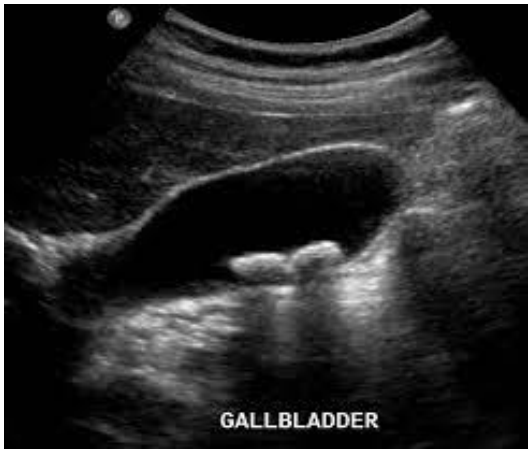
Table 9: Composition of Gall stones in HRT Users and Non Users as found after surgical removal, (n-08)

Age Range	Serum Estradiol In HRT Users	Composition of Gall Stones	Serum Estradiol In HRT Non Users	Incidence of Gall Stones
45-50	233pg/ml	Cholesterol Monohydrate	87 pg/ml	calcium bilirubinate
50-55	187pg/ml	Cholesterol –Bilirubin	63pg/ml	calcium bilirubinate
60-65	308pg/ml	Cholesterol Monohydrate	23pg/ml	calcium bilirubinate
65+	293pg/ml	Cholesterol Monohydrate	36pg/ml	calcium bilirubinate

Oestrogens administered orally are absorbed in the gut and travel through the portal vein to the liver where they are extensively metabolised through conjugation before entering the systemic circulation. The hepatic profile is also found affected due to the higher cholesterol level. Metabolites are excreted through the bile and urine. Transdermal oestrogens are administered in lower doses than oral oestrogens and are absorbed through the skin and directly into the circulation. By avoiding first pass metabolism there should be lower concentrations of oestrogens and their metabolites in the bile and this may well explain the lower risk of gallbladder disease that we observed with transdermal therapy. Among users of oral hormone therapy we found a dose effect, with increased relative risks of gallbladder disease among users of higher doses compared with lower doses. These findings suggest that the concentration of oestrogen and its metabolites in the bile affect the risk of gallbladder disease. Use of equine oestrogen was associated with a slightly higher risk of gallbladder disease than estradiol and this may be because the equine preparations consist largely of conjugated oestrogens, which are metabolised somewhat differently from estradiol; however, it is unknown what effects this may have on biliary concentrations of hormone. Oestrogen implants, which can result in high blood concentrations of oestrogen but avoid the first pass metabolism, were also found to increase the relative risk of gallbladder disease to a level somewhere between oral users and transdermal users.Our results suggest that the addition of progestogens to oestrogen therapy does not have a large additional effect on the risk of gallbladder disease. This is in agreement with findings from the women's health initiative randomised controlled trial.1 Furthermore, among users of transdermal oestrogens, the risks did not vary appreciably if the progestogens were administered orally or transdermally, although owing to the number of cases

statistical power was limited.

In past users of hormone replacement therapy the risk of gallbladder disease was lower than in current users and decreased gradually with increasing time since therapy stopped although some excess risk still remained 10 years after stopping. This finding suggests that oestrogen induces some persistent change in the gallbladder. Similar findings have been described in other studies and one possible explanation is the formation of asymptomatic gallstones, which continue to increase a woman's risk of gallbladder disease long after stopping hormone replacement therapy. Results from one study suggest that the risk of gallbladder disease may increase with increasing duration of use; however we found no significant trends. Finally, we did not compare the risks associated with past use of oral and transdermal formulations because a large proportion of past users provided no information on the type of hormone replacement therapy used and evidence suggests that recall of brand names by past users is poor. Among users of oral hormone replacement therapy the relative risk, but not the absolute risk, of gallbladder disease associated with hormone replacement therapy was found to decrease with increasing body mass index. A similar attenuation of the hormone replacement therapy associated relative risks in overweight and obese women has been observed with breast cancer and endometrial cancer. The absolute risk of gallbladder disease increases with increasing body mass index probably because circulating levels of endogenous oestradiol are higher in overweight and obese women and the addition of exogenous oestrogens may alter total oestrogen levels to a lesser extent than in thinner women. It has been suggested that the attenuated relative risks with increasing body mass index might be because we could not exclude every woman who had a cholecystectomy before recruitment, although additional analyses suggest this is unlikely.



Strengths and Limitations

Our results are based on hormone replacement therapy use reported at recruitment and although some changes in use occurred during follow-up this would not substantially alter our findings. Admissions for gallbladder disease occurred a mean of 3.3 years after recruitment and, on the basis of resurvey data, a small proportion of never users, only 1% annually, started use after recruitment. The proportion of current users stopping therapy was similar for women using oral and transdermal preparations and few women switched between oral and transdermal therapies. Sensitivity analyses showed that our estimates of relative risk were essentially unchanged after censoring women at major events that may alter the use of hormone replacement therapy. Also, after censoring follow-up at July 2002 (to coincide with publication of the first results from the women's health initiative trials), significant differences between the effect of transdermal therapy and oral therapy persisted.

In this cohort, the findings relating to the method of administration of hormone replacement therapy are specific for gallbladder disease, as we found no differences between transdermal therapy and oral therapy for increased risk of breast cancer and ovarian cancer, or the reduced risk of fracture. Prescribing of transdermal oestrogen rather than oral oestrogen differs according to some factors such as history of hysterectomy and bilateral oophorectomy (see table 1⇑) but we were able to adjust for these as well as other potential confounders that could influence the choice of hormone replacement therapy such as socioeconomic status, body mass index, and other medical history.

Although some non-NHS funded admissions would not be included in our follow-up, privately funded treatment in the United Kingdom is limited, and we have previously shown that in this cohort such admissions are uncommon, as most self reported cholecystectomies were included in our linked NHS hospital admission data. We excluded from the analyses women with a record of admission for gallbladder disease before recruitment as most of these women will have had a cholecystectomy and would no longer be at risk of an event. For women recruited in Scotland, hospital records were available from 1981 (when participants were a mean age of 36). In these women, had we not excluded those with an admission before recruitment, the relative risks for gallbladder disease in current users of hormone therapy compared with never users would have been 1.70 instead of 1.71, and the relative risks for transdermal therapy versus oral therapy would have been 1.13 versus 1.83 instead of 1.13 versus 1.85. For women recruited in England, hospital records were available only from 1997; however, the small difference in relative risks in Scotland suggests that not excluding comparable women in England would have a minimal effect on our estimates.

Despite these limitations, the study has several strengths. All women were registered with the NHS, and by linking to NHS hospital records we had virtually complete follow-up, with objective recording of gallbladder disease. The large sample size allowed us to compare reliably a variety of types of hormone replacement therapy. The prospective design ensured that use of hormone replacement therapy was ascertained before outcomes thereby reducing biases resulting from recall or differential prescribing, and we were able to adjust for many potential confounders and found our results to be consistent. Reporting of hormone replacement therapy use in this population compares well with prescription data, 31 and outcomes were coded by the NHS hospitals, independent of the study investigators and participants. Additionally, both the accuracy of the linkage process and the coding of gallbladder disease has been shown to be good and as misclassification of hormone replacement therapy use at recruitment or of gallbladder disease from the hospital records should be non-differential, this would result in an underestimate of the effects found.

Implications for practice

Gallbladder disease is common in middle aged women. In the UK over five years an estimated 1.1% of middle aged women

who have never used hormone replacement therapy are admitted to hospital for a cholecystectomy. Use of transdermal oestrogen increases the risk to 1.3% and use of oral oestrogens increases this to 2.0%. Transdermal hormone replacement therapy is, however, generally more costly than oral therapy and can cause local skin reactions. Whereas the number of women using hormone replacement therapy halved between 2002 and 2005, about 1 million UK women were taking it in 2005 and most used oral preparations. For women who choose to use hormone replacement therapy, one cholecystectomy could be avoided for every 140 users of transdermal therapy rather than oral therapy over a five year period.

Conclusion

A significant difference in serum Estrogen level among HRT users and Non users was observed (t Value - 7.132) in the case of Estrogen and T value 1,152 in the case of Progesterone. Also significant difference in serum Bilirubin level (t value-1.90) was observed amonh HRT users and non users, Ahigh positive correlation was observed among high serum level of Estrogen and incidence of Gall stones (r = 0.477).The longer duration use of HRT is also found to be related with higher possibility of developing Gall stones (0.445). The oral route of hormone administration is found significantly associated with development of Gall stones than trans dermal route (r = 0.552). A biochemical profile of affected hepatic status was observed in all Liver Function tests. WBC count was also found significantly increased in affected persons. The gall stones were found maximally cholesterol containing as expected in HRT users. These data suggest an increase in risk of biliary tract disease among postmenopausal women using HRT therapy. In this study the Gallstone formation is thought to rely on 3 factors: super saturation of biliary cholesterol due to hepatic hyper secretion, nucleation of cholesterol monohydrate crystals, and gallbladder hypomotility. The liver has estrogen receptors, and the presence of endogenous estrogens causes cholesterol saturation in the bile, inhibition of chenodeoxycholic acid secretion, and increased cholic acid content. Progestins inhibit gallbladder contraction, encourage bile stasis, and have been shown to decrease the gallbladder's response to cholecystokinin. So, the subjects who are using both hormones are found affected by the very disease. Here the observational evidence suggests that estrogen therapy, including the use of oral and trans dermal postmenopausal estrogen therapy, is an important risk factor for gallbladder disease and specially those patients who are using oral route. The duration of HRT use is observed to be related with incidence of Gall stones production, the type of Estrogen use is also affecting the incidence significantly as Estron form of hormone is most potent in comparison to other forms of this hormone. As the HRT not only affects the incidence of Gall stones, but also affects the composition of it, in HRT users are found as expected the stones are having cholesterol part maximally. Thus, this report provides new evidence on the effect of estrogen therapy, with or without progestin, on gallbladder stone disease incidence and gallbladder surgical outcomes. Use of hormone replacement therapy by postmenopausal women increases the risk of gallbladder disease. This finding agrees with previous reports.

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