4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

The distribution of progestogens is described in the monograph on Combined estrogen–progestogen contraceptives. That of estrogens is described below.

4.1.1 *Humans*

Little more has been discovered about the absorption and distribution of estrone, estradiol and estriol products and conjugated equine estrogens in humans since the previous evaluation (IARC, 1999). Greater progress has been made in the identification and characterization of the enzymes that are involved in estrogen metabolism and excretion. The various metabolites and the responsible enzymes, including genotypic variations, are described below (see Figures 3 and 4). Sulfation and glucuronidation are the main metabolic reactions of estrogens in humans.

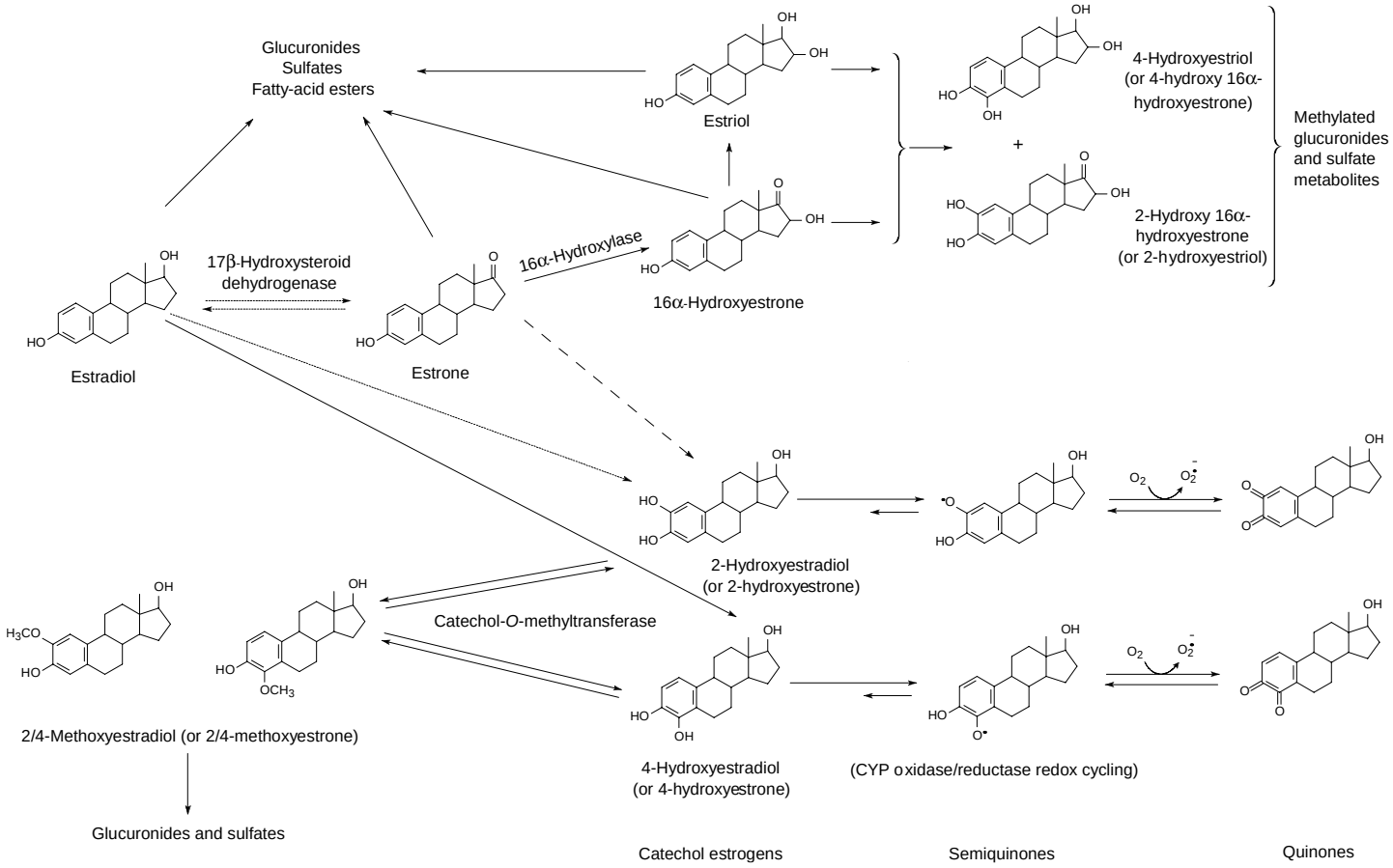
(*a*) *Metabolites*

(i) *Estrogen sulfates*

Several members of the sulfotransferase (SULT) gene family can sulfate hydroxysteroids, including estrogens. The importance of SULTs in estrogen conjugation is demonstrated by the observation that a major component of circulating estrogen is sulfated, i.e. estrone sulfate (reviewed by Pasqualini, 2004). In addition to the parent hormones, estrone and estradiol, SULTs can also conjugate their respective catechols and also methoxyestrogens (Spink *et al*., 2000; Adjei & Weinshilboum, 2002). The resulting sulfated metabolites are more hydrophilic and can be excreted.

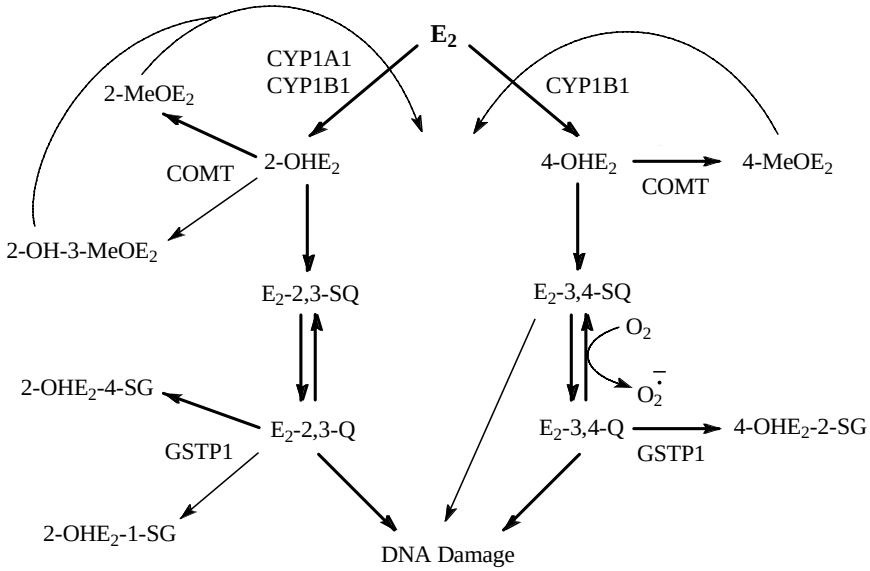
In postmenopausal breast cancers, levels of estrone sulfate can reach 3.3 ± 1.9 pmol/g tissue, which is five to nine times higher than the corresponding plasma concentration (equating gram of tissue with millilitre of plasma) (Pasqualini *et al*., 1996). In contrast, levels of estrone sulfate in premenopausal breast tumours are two to four times lower than those in plasma. Since inactive estrone sulfate can serve as a source for biologically active estradiol, it is of interest that various progestogens caused a significant decrease in the formation of estradiol when physiological concentrations of estrone sulfate were incubated with breast cancer cells MCF-7 and T47D (reviewed by Pasqualini, 2003).

Figure 3. Pathways of the metabolism and redox cycling of estradiol, estriol and estrone



Modified from Yager & Liehr (1996)

Figure 4. The estrogen metabolism pathway is regulated by oxidizing phase I and conjugating phase II enzymes



Adapted from Dawling *et al.* (2003)

COMT, catechol-*O*-methyltransferase; CYP, cytochrome P450; E_2, estradiol; GSH, glutathione; GST, glutathione *S*-transferase; $MeOE_2$, methoxyestradiol; OH, hydroxy; OHE_2, hydroxyestradiol; Q, quinone; SG, *S*-glutathione (oxidized); SQ, semiquinone

CYP1A1 and CYP1B1 catalyse the oxidation of E_2 to the catechol estrogens 2-OHE_2 and 4-OHE_2. The catechol estrogens are either methylated by COMT to methoxyestrogens (2-$MeOE_2$, 2-OH-3-$MeOE_2$, 4-$MeOE_2$) or further oxidized to semiquinones (E_2-2,3-SQ, E_2-3,4-SQ) and quinones (E_2-2,3-Q, E_2-3,4-Q). The methoxyestrogens exert feedback inhibition on CYP1A1 and CYP1B1, as indicated by the curved arrows, and reduce the formation of oxidative E_2 metabolites. The estrogen quinones are either conjugated by GSTP1 to GSH-conjugates (2-OHE_2-1-SG, 2-OHE_2-4-SG, 4-OHE_2-2-SG) or they form quinone–DNA adducts (e.g. 4-OHE_2-N7-guanine) or oxidative DNA adducts via quinone–semiquinone redox cycling (e.g. 8-OH-deoxyguanosine). The same pathway applies to estrone. The thicker arrows indicate preferential reactions.

(ii) *Estrogen glucuronides*

Estradiol and estrone and their respective catechols are recognized as substrates by various isoforms of the uridine-5′ diphosphate (UDP)-glucuronyltransferase (UGT) enzyme family. Several isoforms were more active towards catechol estrogens than towards the parent hormones (Albert *et al.*, 1999; Turgeon *et al.*, 2001). The resulting glucuronidated metabolites are more hydrophilic and can be excreted in bile and urine.

(iii) *Estrogen fatty acid esters*

Several steroids, including estradiol, have been shown to undergo esterification to long-chain fatty acids in a number of mammalian tissues (Hochberg, 1998). The responsible enzyme, fatty acyl-coenzyme A (CoA):estradiol-17β-acyltransferase, has a pH optimum of

5–5.5, which distinguishes it from the related enzyme, acyl-CoA:cholesterol acyltransferase (optimal pH ~7.0) (Xu *et al*., 2001a,b). The fatty acyl-CoA:estradiol-17β-acyltransferase shows specificity for the D-ring, especially the C-17β group of the estrogen molecule. The vicinity of a bulky 16α-hydroxy group appears to hamper the accessibility to the C-17β hydroxyl, which results in a reduced rate (28%) of esterification of estriol compared with estradiol (Pahuja *et al*., 1991). The D-ring esterification of estradiol has two effects: (i) the bulky fatty acid moiety prevents the binding of estradiol fatty acid to the estrogen receptor; and (ii) the fatty acid moiety shields the D-ring from oxidative metabolism to estrone. Thus, estradiol fatty acid may play a role in the action of estrogen by affecting the intracellular equilibrium between estrone and estradiol.

In the circulation, estradiol fatty acids are mainly bound by plasma lipoproteins; the majority (54%) are recovered in the high-density lipoprotein (HDL) and 28% in the low-density lipoprotein (LDL) fractions (Vihma *et al*., 2003a). They are present in very small amounts in the blood of premenopausal women, although their concentration increases 10-fold during pregnancy, from 40 pmol/L in early pregnancy to 400 pmol/L in late pregnancy (Vihma *et al*., 2001). Treatment of postmenopausal women with either oral or transdermal estradiol for 12 weeks resulted in a differential effect on serum estradiol fatty acids and non-esterified estradiol. Both types of application led to similar median concentrations of free (non-protein-bound) estradiol but only the oral therapy caused an increase (27%) in median serum estradiol fatty acid (Vihma *et al*., 2003b). The change during treatment in serum concentrations of estradiol fatty acid, but not those of non-esterified estradiol correlated positively with enhanced forearm blood flow responses *in vivo*. These data suggest that an increase in serum estradiol fatty acid may contribute to the effects of oral treatment with estradiol, compared with those of an equipotent transdermal dose.

(iv) *Oxidative metabolism*

Estradiol and estrone undergo extensive oxidative metabolism via the action of several cytochrome P450 (CYP) monooxygenases. Each CYP favours the hydroxylation of specific carbons, altogether, the CYP enzymes can hydroxylate virtually all carbons in the steroid molecule, with the exception of the inaccessible angular carbons 5, 8, 9, 10 and 13 (Badawi *et al*., 2001; Lee *et al*., 2001, 2002, 2003a,b; Kisselev *et al*., 2005). The generation of hydroxyl and keto functions at specific sites of the steroid nucleus markedly affects the biological properties of the respective estrogen metabolites, i.e. different hydroxylation reactions yield estrogenic, non-estrogenic or carcinogenic metabolites. Quantitatively and functionally, the most important reactions occur at carbons 2, 4 and 16.

Catechol estrogens

2- and 4-Hydroxyestrone, -estradiol and -estriol have been shown to serve a physiological function, to have some hormonal activity and to be substrates in the oxidative estrogen metabolism pathway. In their physiological function, they mediate the activation of dormant blastocysts for implantation into the receptive uterus. Specifically, 4-hydroxyestradiol produced in the uterus from estradiol mediates blastocyst activation for implanta-

tion in a paracrine manner. This effect is not mediated by the estrogen receptor but via prostaglandin synthesis (Paria *et al*., 1998, 2000). The oxidative metabolism of estrogens to catechol estrogens is generally thought to terminate the estrogenic signal, although catechol estrogens retain some binding affinity to the estrogen receptor. Treatment of MCF-7 cells with 2- and 4-hydroxyestradiol increased the rate of cell proliferation and the expression of estrogen-inducible genes such as the progesterone receptor (*PR*) gene and *pS2*. Relative to estradiol, 2- and 4-hydroxyestradiol increased proliferation rate, level of PR protein and *pS2* mRNA expression by 36 and 76%, 10 and 28% and 48 and 79%, respectively (Schütze *et al*., 1993, 1994).

Catechol estrogens occupy a key position in the oxidative pathway of estrogen metabolism (see Figures 3 and 4). They are products as well as substrates of CYP1A1 and CYP1B1 (Hachey *et al*., 2003; Dawling *et al*., 2004). Specifically, CYP1A1 converts estradiol firstly to 2-hydroxyestradiol and then to the estradiol-2,3-semiquinone and estradiol quinone. CYP1B1 converts estradiol firstly to 2- as well as to 4-hydroxyestradiol and then to the corresponding semiquinones and quinones. Estrone is metabolized in a similar manner by CYP1A1 and CYP1B1 (Lee *et al*., 2003a). The catechol estrogens also serve as substrates for catechol-*O*-methyltransferase (COMT), which catalyses *O*-methylation by forming monomethyl ethers at the 2-, 3- and 4-hydroxyl groups. Conjugated equine estrogens are also substrates for COMT (Yao *et al.*, 2003). COMT generated two products from 2-hydroxyestrogens, but only one product from 4-hydroxyestrogens (Dawling *et al*., 2001; Lautala *et al*., 2001; Goodman *et al*., 2002). With 2-hydroxyestradiol and 2-hydroxyestrone, COMT catalysed the methylation of the 2- and 3-hydroxy groups, which resulted in the formation of 2-methoxyestradiol and 2-hydroxy-3-methoxyestradiol and 2-methoxyestrone and 2-hydroxy-3-methoxyestrone, respectively. In contrast, for 4-hydroxyestradiol and 4-hydroxyestrone, methylation occurred only at the 4-hydroxyl group, which resulted in the formation of 4-methoxyestradiol and 4-methoxyestrone, respectively. 3-Methoxy-4-hydroxyestradiol and -estrone were not produced by COMT.

The observation that catechol estrogens are carcinogenic in animal experiments (IARC, 1999) has prompted studies in human tissues. Examination of microsomal estradiol hydroxylation in human breast cancer showed significantly higher 4-hydroxy-:2-hydroxyestradiol ratios in tumour tissue than in adjacent normal breast tissue (Liehr & Ricci, 1996), while the breast cancer tissue samples contained fourfold higher levels of 4-hydroxyestradiol than normal tissue from benign breast biopsies (Rogan *et al*., 2003). Comparison of intra-tissue concentrations of estrogens (estrone, estradiol, estriol), hydroxyestrogens (16α-hydroxyestrone, 2-hydroxyestrone, 2-hydroxyestradiol, 4-hydroxyestrone, 4-hydroxyestradiol) and methoxyestrogens (2-methoxyestrone, 2-methoxyestradiol, 4-methoxyestrone, 4-methoxyestradiol) in normal and malignant breast revealed the highest concentration of 4-hydroxyestradiol in malignant tissue (Castagnetta *et al*., 2002). The concentration (1.6 nmol/g tissue) determined by combined high performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) was more than twice as high as that of any other compound. [The Working Group noted that such high levels in neoplastic mammary

tissue suggests a mechanistic role of 4-hydroxyestradiol in tumour development; see also Section 4.4.]

16α-Hydroxyestrogens

An analysis of 15 CYP isozymes showed that CYP1A1, 3A4, 3A5 and 2C8 catalysed the 16α-hydroxylation of both estrone and estradiol (Badawi *et al*., 2001; Lee *et al*., 2003a,b). In contrast, CYP3A7 distinguished the two estrogen substrates with > 100 times higher maximum velocity of the enzyme:Michaelis-Menten constant (V_{max}:K_m) ratio for the 16α-hydroxylation of estrone than that of estradiol. The difference in reaction rates is most probably due to the difference in structure at the C-17 position of estrone and estradiol. The presence of the 17-ketogroup in estrone appears to be essential for recognition of the substrate and 16α-hydroxylation by CYP3A7 (Lee *et al*., 2003b).

Similarly to catechol estrogens, 16α-hydroxylated estrogens are hormonally active, chemically reactive and potentially mutagenic. 16α-Hydroxyestrone possesses the unique property of binding covalently to the estrogen receptor and other nuclear proteins, such as histones. Mechanistically, a Schiff base is formed from 16α-hydroxyestrone by a reaction with amino groups in proteins. The Schiff base, in turn, undergoes Heyns rearrangement to result in the formation of a stable 16-keto-17β-amino estrogen adduct (Miyairi *et al*., 1999).

Bradlow *et al.* (1996) proposed that increased formation of 16α-hydroxyestrone and estriol may be associated with an increased risk for developing breast cancer. They presented the hypothesis that the ratio of the two urinary metabolites 2-hydroxyestrone:16α-hydroxyestrone is inversely correlated with the risk for breast cancer. They chose the numerator 2-hydroxyestrone to reflect the 'good' C-2 hydroxylation and the denominator 16α-hydroxyestrone to reflect the 'bad' C-16α hydroxylation pathways of estrogen metabolism. Enzyme immunoassays for simultaneous quantitation of 2- and 16α-hydroxyestrone levels in urine have been developed and improved to correlate with results obtained by GC–MS (Falk *et al*., 2000). The enzyme immunoassay has been applied to the analysis of blood samples from premenopausal women. Current users of oral contraceptives had a significantly lower plasma 2-hydroxyestrone:16α-hydroxyestrone ratio than non-users ($p = 10^{-21}$) (Jernstrom *et al*., 2003b).

Results from epidemiological studies on the association between 2- and 16α-hydroxylation and breast cancer are inconsistent. Several case–control studies found an increased risk for breast cancer associated with a lower 2-hydroxyestrone:16α-hydroxyestrone ratio (Ho *et al*., 1998; Zheng *et al*., 1998), while other groups did not observe a difference in this ratio between controls and patients (Ursin *et al*., 1999). All of these studies measured metabolites after the diagnosis of breast cancer, which raises the possibility that the results may have been affected by the tumour. Two prospective studies addressed this issue but also yielded inconsistent results. The first was carried out in women on the island of Guernsey, United Kingdom. Urine samples were collected and stored in the 1970s when all women were healthy. Almost 20 years later, Meilahn *et al*. (1998) analysed the samples and

reported a median of 1.6 for the 2-hydroxyestrone:16α-hydroxyestrone ratio in 42 postmenopausal women who had developed breast cancer and 1.7 in 139 matched control subjects. Compared with women in the lowest tertile category of 2:16α-hydroxyestrone ratio, women in the highest tertile had an odds ratio for breast cancer of 0.71, but the 95% CI was wide and was not statistically significant (95% CI, 0.29–1.75). Analysis of premenopausal women in the Guernsey cohort showed no difference between cases and controls. The second prospective study of Italian women had a shorter average follow-up of 5.5 years (Muti *et al*., 2000). The odds ratio in postmenopausal women was 1.31 (95% CI, 0.53–3.18). In the premenopausal group, women in the highest quintile of the 2:16α-hydroxyestrone ratio had an adjusted odds ratio of 0.55 (95% CI, 0.23–1.32). A third type of epidemiological study examined urinary metabolites in women of different ethnic groups that are known to have different rates of breast cancer. One study examined healthy postmenopausal women randomly selected from the Singapore Chinese Health Study (67 subjects) and the Los Angeles Multiethnic Cohort Study (58 subjects). Although the incidence of breast cancer is substantially lower in Singaporean women than among American women, there were no significant differences between the groups in urinary 16α-hydroxyestrone levels or 2:16α-hydroxyestrone ratios (Ursin *et al*., 2001). Finally, no differences were found in premenopausal women with or without a family history of breast cancer (Ursin *et al*., 2002).

(v) *Methoxyestrogens*

Methoxyestrogens are methyl ether metabolites of catechol estrogens produced by COMT. In addition, 2-methoxyestradiol is not just a by-product of estrogen metabolism but is also endowed with antiproliferative activity. It has been shown to inhibit the proliferation of both hormone-dependent and hormone-independent breast cancer cells (LaVallee *et al*., 2003). The antiproliferative effect is not limited to breast cancer cells but extends to leukaemia, and pancreatic and lung cancer cells (Schumacher *et al*., 1999; Huang *et al*., 2000). Human xenograft studies in animal models have demonstrated the oral bioavailability and a high therapeutic index of methoxyestrogens with no sign of systemic toxicity. These features and their broad antitumour activity against a variety of tumour cells have led to the current testing of methoxyestrogens as potential therapeutic agents in clinical trials (Pribluda *et al*., 2000; Schumacher & Neuhaus, 2001). Several synthetic analogues were equally as effective as 2-methoxyestradiol or were even more potent than the endogenous compound (Wang *et al*., 2000; Brueggemeier *et al*., 2001; Tinley *et al*., 2003).

The antiproliferative effect of 2-methoxyestradiol appears to be concentration-dependent and to involve several mechanisms. At nano- and micromolar concentrations, 2-methoxyestradiol disrupted microtubule function, induced apoptosis and inhibited angiogenesis (Klauber *et al*., 1997; Yue *et al*., 1997; Huang *et al*., 2000; LaVallee *et al*., 2003). At concentrations ≥ 1 μM , it caused chromosome breaks and aneuploidy (Tsutsui *et al*., 2000).

Methoxyestrogens are also substrates for CYP1A1 and CYP1B1, which catalyse their *O*-demethylation to catechol estrogens, and thus effectively reverse the COMT reaction by

which they were formed (Dawling *et al*., 2003). Specifically, both CYP1A1 and CYP1B1 demethylated 2-methoxy- and 2-hydroxy-3-methoxyestradiol to 2-hydroxyestradiol, and CYP1B1 additionally demethylated 4-methoxyestradiol to 4-hydroxyestradiol. Thus, CYP1A1 and CYP1B1 recognize as substrates both the parent hormone estradiol and the methoxyestrogens, 2-methoxy-, 2-hydroxy-3-methoxy- and 4-methoxyestradiol. Kinetic analysis showed that estradiol and the methoxyestrogens are alternate substrates, each of which is catalysed by the same enzyme but by a different type of reaction (Dawling *et al*., 2003). Because they are converted to identical catechol estrogen products, each inhibits formation of 2- and 4-hydroxyestradiol from the other substrate in a non-competitive manner. It has been proposed that methoxyestrogens exert feedback inhibition on CYP1A1 and CYP1B1, which affects the entire oxidative metabolic pathway of estrogen in several ways. First, CYP1A1 and CYP1B1 generate catechol estrogen substrates for COMT and, at the same time, compete with COMT by converting the catechol estrogens to estrogen quinones. In turn, the methoxyestrogens generated by COMT are alternate substrates for CYP1A1 and CYP1B1 and inhibit oxidation of the parent hormone estradiol (and most probably also that of the catechol estrogens). Second, the inhibition occurs at a strategic point in the pathway where it branches into 2- and 4-hydroxycatechol estrogens. This may be important in view of the apparent difference in carcinogenicity of these two substances (Liehr & Ricci, 1996; Cavalieri *et al*., 2000). Third, all three products of the COMT-mediated reaction (i.e. 2-methoxy-, 2-hydroxy-3-methoxy- and 4-methoxyestradiol) act as inhibitors, and thereby maximize the feedback regulation (Dawling *et al*., 2003). Fourth, the feedback regulation occurs at the step in the pathway that precedes the conversion to estrogen semiquinones and quinones, and thereby reduces the formation of reactive oxygen species during semiquinone–quinone redox cycling and the potential for estrogen-induced DNA damage (Dawling *et al.*, 2003).

(vi) *Estrogen–glutathione conjugates*

The labile estrogen quinones react with a variety of physiological compounds, including amino acids such as lysine and cysteine and the tripeptide, glutathione (γ-glutamyl-cysteinyl-glycine, GSH) (Cao *et al*., 1998). MS analysis of the GSH–estrogen isomers revealed that the catechol estrogen attachment is at the cysteine moiety of GSH, and the cysteine sulfur binds to an A-ring carbon vicinal to the catechol carbons, i.e. C-1 or C-4 in 2-hydroxyestradiol and C-2 in 4-hydroxyestradiol (Ramanathan *et al*., 1998). Thus, the point of attachment of the -*S*-glutathione (-SG) moiety is always directly adjacent to an oxygen-bearing carbon, in line with all other known quinone–GSH conjugates (Bolton *et al*., 2000).

GSH is the most abundant intracellular non-protein thiol and is found at concentrations that range from 0.1 to 10 mM. In an in-vitro study, Hachey *et al*. (2003) used 0.1 mM GSH and recombinant, purified glutathione *S*-transferase P1 (GSTP1) and estradiol and observed a faster rate of estrogen quinone conjugation in the presence of GSTP1 than in the absence of the enzyme. 2-Hydroxyestradiol and 4-hydroxyestradiol did not form conjugates with GSH alone or in the presence of GSTP1. These data indicate that the enzy-

matic conversion of catechol estrogens to estrogen quinones by CYP1B1 is a necessary step for the subsequent GSH conjugation reaction. The enzymatic reaction with GSTP1 yielded only mono-conjugates, i.e. 2-hydroxyestradiol-1-SG, 2-hydroxyestradiol-4-SG and 4-hydroxyestradiol-2-SG. There was no evidence of bis-conjugates, such as 2-hydroxyestradiol-1,4-bisSG and 4-hydroxyestradiol-1,2-bisSG. GSTP1 is also a target for equine catechol estrogens (Yao *et al.*, 2002). Equine catechol significantly decreased GSH levels and the activity of GSTP1-1 in human breast cancer cells.

All GSH conjugates are catabolized via the mercapturic acid pathway. First, the glutamyl moiety is removed from the GSH conjugate by transpeptidation, which is catalysed by γ-glutamyl transpeptidase. The resulting cysteinylglycine conjugate is then hydrolysed by cysteinyl-glycine dipeptidase to yield the cysteine conjugate. The final step entails acetylation to the *N*-acetylcysteine conjugate, a mercapturic acid compound. Estrogen–GSH conjugates are excreted in the urine mostly as *N*-acetylcysteine conjugates but also as cysteine conjugates (Todorovic *et al.*, 2001). Thus, estrogen quinones are detoxified in tissues by GST-mediated GSH conjugation and the resultant GSH conjugates are catabolized to *N*-acetylcysteine conjugates that are readily excreted.

(*b*) *Enzymes*

(i) *CYP1A1*

Although other CYP enzymes, such as CYP1A2 and CYP3A4, are involved in hepatic and extrahepatic hydroxylation of estrogen, CYP1A1 and CYP1B1 display the highest level of expression in breast tissue (reviewed by Jefcoate *et al*., 2000; Lee *et al*., 2003a). The human gene for CYP1A1 is polymorphic. Apart from the wild-type (*CYP1A1*1*), 10 alleles have been described in different populations. However, several are very rare and of unknown functional significance (Karolinska Institutet, 2007). The most common alleles that result in amino acid substitutions are *CYP1A1*2* (462Ile → Val) and *CYP1A1*4* (461Thr → Asn). Kisselev *et al*. (2005) expressed and purified CYP1A1.1, CYP1A1.2 and CYP1A1.4 proteins and performed enzymatic assays of estrogen hydroxylation in reconstituted CYP1A1 systems. All three CYP1A1 isoforms catalysed the hydroxylation of estradiol and estrone to 2-, 15α-, 6α- and barely detectable 4-hydroxylated estrogen metabolites. The CYP1A1.2 variant had a significantly higher catalytic activity, especially for 2-hydroxylation. The catalytic efficiencies for 2-hydroxyestradiol and 2-hydroxyestrone were 5.7- and 12-fold higher, respectively, compared with the wild-type enzyme. Several studies found no overall association between the risk for breast cancer and the polymorphisms in codons 461 and 462 (Huang *et al*., 1999; reviewed by Mitrunen & Hirvonen, 2003).

In addition to genetic variation, there is a striking interindividual variation in *CYP1A1* expression. For example, Goth-Goldstein *et al*. (2000) measured *CYP1A1* mRNA expression in 58 non-tumour breast specimens from 26 breast cancer patients and 32 cancer-free individuals by reverse transcription-PCR. *CYP1A1* expression varied between specimens by ~400-fold and was independent of *CYP1A1* genotype and age of the patient.

A second study used quantitative immunoblotting of normal and malignant breast tissues and observed ~150-fold differences in CYP1A1 protein expression between individuals (El-Rayes *et al*., 2003). Attempts to explain such a high degree of interindividual variation in CYP1A1 expression have focused primarily on genetic polymorphisms within the *CYP1A1* gene with inconsistent results. Since the expression of CYP1A1 is induced via the aryl hydrocarbon receptor (AhR), Smart and Daly (2000) extended the investigation to the *AhR* gene, and observed that *AhR*-mediated induction of CYP1A1 appears to be influenced by the 1721G → A (554Arg → Lys) polymorphism in exon 10 of the *AhR* gene. The 554Arg residue lies close to the transactivation domain of the AhR protein. Individuals who had at least one copy of the variant 1721A allele showed significantly higher levels of CYP1A1 activity compared with individuals who were negative for the polymorphism ($p = 0.0001$). Levels of 3-methylcholanthrene-induced CYP1A1 activity in lymphocytes also varied by sex: women exhibited significantly lower activity than men (Smart & Daly, 2000). The authors suggested that interindividual variation in levels of CYP1A1 activity appears to be associated more with regulatory factors than with polymorphisms in the *CYP1A1* gene.

(ii) *CYP1B1*

CYP1B1 is the main enzyme that converts estradiol to 4-hydroxyestradiol (Jefcoate *et al*., 2000). Since animal studies have implicated 4-hydroxyestradiol in the development of cancer, the expression of CYP1B1 in hormone-responsive tissues such as the breast has attracted interest. Murray *et al.* (2001) performed several immunohistochemical studies of CYP1B1 expression in the breast. Breast cancer tissue but not normal breast tissue expressed CYP1B1. Forty-six of 60 (77%) invasive breast cancers showed cytoplasmic staining of tumour cells, which ranged from strong in 10 to moderate in 12 and weak in 24 cases. There was no relationship between the presence of CYP1B1 and the histological type or grade of the tumour, the presence of lymph node metastasis or estrogen receptor status (McFadyen *et al*., 1999). Immunohistochemical analysis of CYP1B1 expression also revealed cytoplasmic staining in a wide range of other cancers of different histogenetic types, including cancers of the colon, oesophagus, lung, brain and testis. Similar to the breast, no immunostaining occurred in corresponding normal tissues (Murray *et al.*, 1997). These findings contradict the observation that normal human mammary epithelial cells isolated and cultured from reduction mammoplasty tissue of seven individual donors expressed significant levels of CYP1B1 (< 0.01–1.4 pmol/mg microsomal protein) as determined by immunoblot analysis (Larsen *et al*., 1998). The discrepancy between the studies regarding the presence of CYP1B1 protein in normal mammary epithelium may be due to the use of different antibodies, to the induction of CYP1B1 as a result of the isolation of the mammary epithelial cells from mammoplasty tissue or to their in-vitro culture over 6 days (Murray *et al*., 2001).

Several polymorphisms have been identified in the *CYP1B1* gene, four of which are associated with amino acid substitutions: 48Arg → Gly, 119Ala → Ser, 432Val → Leu and 453Asn → Ser (Stoilov *et al*., 1998; McLellan *et al*., 2000). There is considerable ethnic variation in the frequency of these polymorphisms. For example, the 432Val allele

is present in approximately 70% of African-Americans, 40% of Caucasians and less than 20% of Chinese (Bailey *et al*., 1998; Tang *et al*., 2000).

Several investigators have examined the effect of *CYP1B1* polymorphisms on enzyme function (Shimada *et al*., 1999; Hanna *et al*., 2000; Li, D.N. *et al*., 2000; McLellan *et al*., 2000; Lewis *et al*., 2003). Although all studies analysed the 4- and 2-hydroxylation of estradiol by CYP1B1, a comparison of the results needs to take into account differences in expression systems (bacteria, yeast), assay conditions (microsomal membranes, purified proteins) and the type of analysis of estrogen metabolites (HPLC, GC–MS). Some studies also provided an incomplete definition of constructs, i.e. only two or three of the four amino acids were listed. For these reasons, the results are inconsistent, although it appears that there is at best a two- to threefold difference in catalytic activity between wild-type CYP1B1 and any variant isoform.

Several studies have examined the association of *CYP1B1* polymorphisms with the risk for breast and endometrial cancer. Two case–control studies that involved 1355 Caucasian and African-American women found no association with the risk for breast cancer (Bailey *et al*., 1998; De Vivo *et al*., 2002). Another case–control study of 186 Asian cases of breast cancer and 200 Asian controls found that women with the 432Leu/Leu genotype had a 2.3-fold (95% CI, 1.2–4.3) elevated risk for breast cancer compared with women with the 432Val/Val genotype (Zheng *et al*., 2000). Sasaki *et al*. (2003) examined 113 Japanese patients with endometrial cancer and 202 healthy controls. Women who had the homozygous 119Ser/Ser and 432Val/Val genotypes had relative risks for endometrial cancer of 3.32 (95% CI, 1.38–8.01) and 2.49 (95% CI, 1.10–5.66) compared with those who had wild-type *CYP1B1*. McGrath *et al*. (2004) examined codons 432 and 453 in women who had endometrial cancer within the Nurses' Health Study (222 cases, 666 controls). Carriers of the 453Ser allele had a significantly decreased risk for endometrial cancer (odds ratio, 0.62; 95% CI, 0.42–0.91), and there was no association with the 432Val $\rightarrow$ Leu polymorphism. A case–control study of postmenopausal Swedish women (689 cases, 1549 controls) examined polymorphisms at codons 119, 432 and 453 and found no evidence for an association between *CYP1B1* genotype and risk for endometrial cancer (Rylander-Rudqvist *et al*., 2004). However, two studies observed an association of the 432Val/Val genotype with expression of estrogen receptor in breast cancer patients (Bailey *et al*., 1998; De Vivo *et al*., 2002). Another study noted a significant association between the 119Ser/Ser genotype and expression of estrogen receptors α and β in endometrial cancer patients (Sasaki *et al*., 2003). One study of postmenopausal women found that carriers of the 432Leu and 453Ser alleles had modestly higher plasma levels of estradiol but similar levels of estrone and estrone sulfate (De Vivo *et al*., 2002), while another study found no such association (Tworoger *et al*., 2004). The 432Leu $\rightarrow$ Val polymorphism was also investigated in relation to other cancers and showed no association with lung cancer but increased risks for ovarian cancer associated with the 432Leu allele and for prostate cancer associated with the 432Val allele (Tang *et al*., 2000; Watanabe *et al*., 2000; Goodman, M.T. *et al*., 2001).

(iii) *Catechol*-O-*methyltransferase (COMT)*

The enzymatic activity of recombinant, purified COMT has been determined for methylation of the catechol estrogen substrates 2- and 4-hydroxyestradiol and 2- and 4-hydroxyestrone (Dawling *et al*., 2001; Lautala *et al*., 2001; Goodman *et al*., 2002). COMT catalysed the formation of monomethyl ethers at the 2-, 3- and 4-hydroxyl groups. Dimethyl ethers were not observed. The rates of methylation of 2-hydroxyestradiol and 2-hydroxyestrone yielded typical hyperbolic patterns, whereas those of 4-hydroxyestradiol and 4-hydroxyestrone exhibited a sigmoid curve pattern (Dawling *et al*., 2001). Thus, COMT interacts differently with the 2- and 4-hydroxyestrogen substrates. Methylation of 2-hydroxyestrogen substrates exhibits Michaelis-Menten saturation kinetics and yields two products, i.e. 2- and 3-methoxyestrogens. In contrast, the methylation of 4-hydroxyestrogen substrates displays sigmoid saturation kinetics that indicates cooperative binding and yields only a single product, i.e. 4-methoxyestrogen. The main structural difference between 2- and 4-hydroxy catechol estrogens is the proximity of the 4-hydroxyl group to the B-ring of the steroid. The 2- and 3-hydroxyl groups in 2-hydroxyestrogen appear to be similar in reactivity, whereas the 3- and 4-hydroxyl groups in 4-hydroxyestrogen differ in reactivity to the point that, in the latter, only the 4-hydroxyl group becomes methylated.

Dawling *et al*. (2001) compared the enzymatic activity of wild-type (108Val) COMT with that of the common variant (108Met). The 108Met variant, unlike wild-type COMT, was thermolabile, and led to two- to threefold lower levels of production of methoxyestrogen. These results differ from those of Goodman *et al*. (2002) but are in agreement with two other studies (Lachman *et al*., 1996; Syvänen *et al*., 1997). Dawling *et al*. (2001) developed an enzyme-linked immunosorbent assay to quantify COMT in breast cancer cell lines and determined that ZR-75 and MCF-7 cells contain similar amounts of COMT, but differ in genotype and enzymatic activity. The catalytic activity of variant COMT in MCF-7 cells was two- to threefold lower than that of wild-type COMT in ZR-75 cells. Since COMT is expressed ubiquitously, it appears that the *COMT* genotype significantly affects levels of catechol estrogens throughout the body. However, Goodman *et al*. (2002) found no difference between breast cancer cell lines of different *COMT* genotypes (MCF-10A and ZR-75-1 with high activity allele $COMT^{HH}$, and NCF-7 and T47D with low activity allele $COMT^{LL}$), except for the formation of 2-methoxyestradiol.

Immunohistochemical analysis of benign and malignant breast tissue revealed the presence of COMT in the cytoplasm of all epithelial cells. Immunoreactive COMT was also observed in the nucleus of some benign and malignant epithelial cells. There was no correlation between histopathology and the number of cells with nuclear COMT, size of foci that contained such cells or intensity of nuclear COMT immunostaining. Staining of both intra- and interlobular stromal cells was always of a much lower intensity than that of epithelial cells in the same tissue sections (Weisz *et al.*, 2000).

Several epidemiological studies have examined the association of *COMT* genotype with the risk for breast cancer. A meta-analysis of 13 studies published through to July 2004 did not support the hypothesis that the low-activity variant of COMT, as a single factor, leads to increased risk for breast cancer (Wen *et al*., 2005). However, Goodman, J.E. *et al*.

(2001) observed an association between the risk for breast cancer, *COMT* genotype and micronutrients in the folate metabolic pathway. These micronutrients (i.e. cysteine, homocysteine, folate, vitamin B12, pyridoxal 5′-phosphate) are known to influence levels of the methyl donor *S*-adenosylmethionine, and *S*-adenosylhomocysteine, a COMT inhibitor that is generated by the demethylation of *S*-adenosylmethionine. High-activity homozygous *COMT*1* cases of breast cancer had significantly lower levels of homocysteine ($p = 0.05$) and cysteine ($p = 0.04$) and higher levels of pyridoxal 5′-phosphate ($p = 0.02$) than homozygous *COMT*1* controls. In contrast, low-activity homozygous *COMT*2* cases had higher levels of homocysteine ($p = 0.05$) than low-activity homozygous *COMT*2* controls. An increase in the number of *COMT*2* alleles was significantly associated with an increased risk for breast cancer in women with levels of folate below the median (p for trend = 0.05) or levels of homocysteine above the median (p for trend = 0.02). No association was seen between vitamin B12, *COMT* genotype and risk for breast cancer (Goodman, J.E. *et al*., 2001). These findings are consistent with a role of certain folate pathway micronutrients in the mediation of the association between *COMT* genotype and the risk for breast cancer. At the same time, these results illustrate the complex interaction of genetic and nutritional factors in the development of breast cancer. Equally complex is the interaction of the C*OMT* genotype with other risk factors such as mammographic density (Hong *et al*., 2003).

Lavigne *et al*. (2001) examined the effect of estrogen metabolism on oxidative DNA damage (8-hydroxy-2′-deoxyguanosine [8-OH-dG]) in 2,3,7,8-tetrachlorodibenzo-*para*-dioxin-pretreated MCF-7 cells exposed to estradiol with and without Ro41-0960, a specific inhibitor of COMT. Administration of the COMT inhibitor blocked the formation of 2-methoxyestradiol and, at the same time, increased the levels of 2-hydroxyestradiol and 8-OH-dG. During inhibition of COMT, increased oxidative DNA damage was detected in MCF-7 cells exposed to concentrations of estradiol as low as 0.1 μM, whereas, when COMT was not inhibited, no increase in 8-OH-dG was detected at concentrations of estradiol ≤ 10 μM. These results demonstrate that COMT activity is protective against oxidative DNA damage associated with catechol estrogen metabolites. In the absence of COMT activity and methoxyestrogens, a linear relation was observed between levels of 2- plus 4-hydroxyestradiol and 8-OH-dG. However, this relationship did not remain under experimental conditions that allowed limited formation of methoxyestrogens (when cells were treated with a lower concentration of COMT inhibitor), i.e. 8-OH-dG levels were lower than those expected for a given concentration of 2- plus 4-hydroxyestradiol in the presence of 2-methoxyestradiol. The authors suggested that 2-methoxyestradiol may reduce the formation of 8-OH-dG.

(iv) *Glutathione* S-*transferases*

Hachey *et al*. (2003) determined that GSTP1 and CYP1B1 are coordinated in sequential reactions, i.e. 4- and 2-hydroxyestradiol did not form GSH conjugates in the presence of GSTP1 unless they were first oxidized by CYP1B1 to their corresponding quinones. CYP1B1 metabolized estradiol to two products, 4- and 2-hydroxyestradiol, and further to

estradiol-3,4-quinone and estradiol-2,3-quinone, while GSTP1 formed three products, 4-hydroxyestradiol-2-SG, 2-hydroxyestradiol-4-SG, and 2-hydroxyestradiol-1-SG, the last of which in smaller amounts. The rate of conjugation was in the order 4-hydroxyestradiol-2-SG > 2-hydroxyestradiol-4-SG >> 2-hydroxyestradiol-1-SG, which indicated a difference in the regiospecific reactivity of the two quinones. Estradiol-2,3- and estradiol-3,4-quinones are products of CYP1B1- and substrates of GSTP1-mediated reactions but also react non-enzymatically with other nucleophiles, as indicated by a 10-fold concentration gap between catechol estrogens and GSH–estrogen conjugates. It has been suggested that, although both reactions are coordinated qualitatively in terms of product formation and substrate utilization, the quantitative gap would enable the accumulation of estrogen quinones and their potential for DNA damage.

Based on protein levels, GSTP1 is the most important member of the GST family expressed in breast tissue (Kelley *et al.*, 1994; Alpert *et al*., 1997). However, two other GST isoforms, GSTM1 and GSTA1, are also expressed in mammary epithelium, although at lower levels. About 50% of Caucasian and 30% of African women possess the *GSTM1* null genotype and therefore completely lack GSTM1 expression in all tissues including the breast (Garte *et al*., 2001). GSTs are known to have selective as well as overlapping substrate specificities. It is unknown at present whether GSTM1 and GSTA1 are capable of conjugating estrogen quinones similarly to GSTP1 (Hachey *et al*., 2003).

The *GSTP1* gene also possesses two polymorphisms in codons 104 (Ile → Val) and 113 (Ala → Val) that are associated with altered catalytic activity towards polycyclic aromatic hydrocarbons (Hu *et al*., 1997; Ji *et al*., 1999). It is unknown at present whether the GSTP1-mediated conjugation of estrogen quinones varies between the *GSTP1* wild-type and its variants.

Several epidemiological studies found no overall association between polymorphism in *GSTP1* codon 104 and the risk for breast cancer (reviewed by Mitrunen & Hirvonen, 2003). The polymorphic allele in codon 113 showed a tendency for an increased risk in one study and a protective effect in another (Krajinovic *et al.*, 2001; Maugard *et al.*, 2001). A comprehensive review of 15 studies of *GSTM1* published through to 2002 found no overall evidence for an association of the *GSTM1* null genotype with risk for breast cancer (Mitrunen & Hirvonen, 2003).

(v) *Uridine-5′ diphosphate (UDP)-glucuronosyltransferases*

The UDP-glucuronosyltransferase (UGT) superfamily currently consists of 16 functional genes that are organized into two families of enzymes, UGT1 and UGT2 (King *et al*., 2000; Tukey & Strassburg, 2000). The study of UGTs was initiated by the hypothesis that UGT-mediated estrogen conjugation reduces catechol estrogen levels and thereby decreases the risk for breast cancer (Raftogianis *et al*., 2000). Similarly to the sulfotransferase (SULT) superfamily, several UGT isoforms are capable of estrogen conjugation, i.e. UGT1A1, -1A3, -1A7, -1A8, -1A9, -1A10, -2B4, -2B7, -2B11 and -2B15 (Lévesque *et al*., 1999; King *et al*., 2000; Turgeon *et al*., 2001). Although a comprehensive study of all known UGTs has not yet been performed, individual studies indicate that, of those tested,

UGT1A1, -1A3, -1A8, -1A9 and -2B7 have the highest activity toward estrogens (Albert *et al*., 1999; Tukey & Strassburg, 2000; Turgeon *et al*., 2001; Vallée *et al*., 2001). The parent hormones, estradiol and estrone, and their respective catechols are recognized as substrates, but individual isoforms display distinct differences in substrate specificity and conjugation efficiency. Comparison of UGT1A3 and -2B7 showed regioselective conjugation of estradiol, i.e. UGT1A3 only conjugated the C-3 hydroxyl group of the A-ring, whereas UGT2B7 conjugated the 17β-hydroxyl in the D-ring, to yield estradiol-3 and 17β-glucuronides, respectively (Gall *et al*., 1999). Several isoforms, including UGT1A1, -1A9 and -2B7, were more active towards the catechol estrogens than the parent hormones. In contrast, comparison of catechol estrogen substrates revealed that UGT1A1 and -1A3 were more active toward 2-hydroxyestradiol, while UGT1A9 and -2B7 conjugated 4-hydroxyestradiol more efficiently (Cheng *et al*., 1998; Albert *et al*., 1999). Although the catechols derived from estradiol and estrone are generally metabolized with similar efficiencies, UGT2B7 displayed seven- to 12-fold higher activity (1320 pmol/min/mg microsomal protein) towards 4-hydroxyestrone than 4-hydroxyestradiol, in spite of similar apparent K_m values (Cheng *et al*., 1998; Turgeon *et al*., 2001). The highest activity was recorded for the UGT1A9-mediated conjugation of 4-hydroxyestradiol (2500 pmol/min/mg) (Albert *et al*., 1999).

Few studies have examined UGT expression in breast tissue, and have usually been limited to the detection of the transcript. Of the isoforms with the highest activity toward estrogen conjugation, *UGT1A9* mRNA was detectable in breast tissue whereas *UGT1A1* mRNA was not detected (Albert *et al*., 1999; Vallée *et al*., 2001). UGT2B7 appears to be the only isoform that has been examined for both transcript and protein. *UGT2B7* transcript was present in normal mammary tissue, but not in T47D and ZR-75 breast cancer cells (Turgeon *et al*., 2001). A detailed immunohistochemical study (Gestl *et al*., 2002) demonstrated expression of UGT2B7 protein in normal mammary epithelium obtained from either reduction mammoplasties or tissue distant from invasive cancer in mastectomy specimens. In contrast, expression of UGT2B7 protein was significantly reduced in malignant cells. The observed difference in UGT2B7 expression between benign and malignant cells is consistent with the hypothesis that UGT-mediated conjugation of catechol estrogens prevents the formation of potentially carcinogenic estrogen quinones. Based on the efficiency of estrogen conjugation and expression in breast tissue, UGT1A9 and -2B7 may be considered to be the predominant isoforms in mammary metabolism of estrogen.

To date, polymorphisms have been described in seven of the 16 functional human UGT genes, namely UGT1A1, -1A6, -1A7, -1A8, -2B4, -2B7 and -2B15 (Lévesque *et al*., 1999; Huang *et al*., 2002; Miners *et al*., 2002). Altered catalytic activity has been shown for variants of UGT1A6, -1A7, -1A8 and -2B15, but the biological significance has yet to be proven (Huang *et al*., 2002; Miners *et al*., 2002). A polymorphism in UGT2B7 (268His → Tyr) exhibited similar efficiencies for the glucuronidation of a number of substrates for the wild-type and variant enzymes (Bhasker *et al*., 2000). Functional significance has only been convincingly demonstrated for a polymorphism in a TA repeat

element, $(TA)_{5-8}TAA$, of the UGT1A1 promoter. The length of the TA repeat appears to influence *UGT1A1* transcription, i.e. *UGT1A1* gene expression decreases with increasing number of repeats, and results in impaired glucuronidation of bilirubin in Gilbert syndrome. The *UGT1A1* polymorphism was associated with a marginal effect ($p = 0.06$) on the risk for breast cancer in premenopausal but not in postmenopausal African-American women (Guillemette *et al*., 2000). No association with risk was observed in a larger study of Caucasian women, and levels of circulating estradiol and estrone were not affected by the polymorphism (Guillemette *et al*., 2001).

(vi) *Sulfotransferases*

The SULT superfamily currently consists of 10 distinct enzymes that are classified into three families (SULT1, -2 and -4) based on the identity of amino acid sequence (Falany *et al*., 2000; Glatt *et al*., 2000; Adjei & Weinshilboum, 2002). Growing recognition of the carcinogenic potential of catechol estrogens has led to increased interest in the role of SULTs in the intracellular metabolism of estrogen (Raftogianis *et al*., 2000). These studies were initiated by the hypothesis that SULT-mediated estrogen conjugation reduces catechol estrogen levels and thereby decreases the risk for breast cancer.

The identification of new SULT isoforms during the past few years (Falany *et al*., 2000) has shown that earlier tissue studies frequently encompassed unrecognized isoforms, which obscured the issue of SULT specificity in estrogen conjugation. In a comprehensive study, Adjei and Weinshilboum (2002) prepared the known 10 recombinant SULT isoforms and determined that seven (1A1, 1A2, 1A3, 1E1, 2A1, 2B1a, 2B1b) catalysed the sulfate conjugation of catechol estrogens, whereas three (1B1, 1C1, 4A1) did not.

Although seven SULT isoforms were shown to conjugate estrogens, they differ significantly in their substrate affinity. There is consensus among investigators that only SULT1E1 can conjugate estradiol, and 2- and 4-hydroxyestradiol at nanomolar concentrations, in contrast to the micromolar concentrations observed for SULT1A1, -1A2, -1A3 and -2A1 (Faucher *et al*., 2001; Adjei & Weinshilboum, 2002). However, there is disagreement with respect to the sulfation of methoxyestrogens at nanomolar concentrations (Spink *et al*., 2000; Adjei *et al*., 2003).

Immunocytochemical studies have shown that SULT1E1 is the principal isoform in normal mammary epithelial cells derived from reduction mammoplasties, the non-tumour-derived cell line 184A1 and epithelial cells in normal breast tissues (Spink *et al*., 2000; Suzuki *et al*., 2003). Other isoforms, such as SULT1A1, were not detectable immunohistochemically in normal mammary epithelium, although reverse transcription-PCR revealed SULT1A1 and -1A3 mRNA in 184A1 cells (Spink *et al*., 2000). The expression pattern of SULT was almost converse in breast cancer cell lines and tissues. Virtually every malignant cell line expresses one or more members of the SULT1A subfamily. For example, SULT1A1 protein and mRNA levels were particularly high in BT-20, MCF-7, T47D and ZR-75 cells. In contrast, SULT1E1 was present in trace amounts or undetectable in most malignant cell lines (Spink *et al*., 2000; Falany *et al*., 2002). However, SULT1E1 was detected by immunohistochemistry in 50/113 (44.2%) invasive ductal carcinomas (Suzuki

et al., 2003). A subgroup analysis of 35 cases showed a significant correlation ($p < 0.01$) between the immunohistochemical SULT1E1 score and *SULT1E1* mRNA levels that was semiquantified by reverse transcriptase-PCR or with SULT1E1 enzymatic activity. Women who had SULT1E1-positive tumours had a better prognosis (longer disease-free interval [$p = 0.0044$] and overall survival [$p = 0.0026$]) than their SULT1E1-negative counterparts (Suzuki *et al*., 2003). Both the expression of SULT1E1 in normal mammary epithelium and the poor clinical outcome of SULT1E1-negative breast cancers support the view that SULT1E1-mediated conjugation is important in limiting long-term exposure of the mammary glands to carcinogenic catechol estrogens.

The *SULT1A1* and *-1E1* genes contain polymorphisms that are associated with decreased enzyme activity and thermal stability (Carlini *et al*., 2001; Adjei *et al*., 2003). Two *SULT1A1* polymorphisms have been described in codons 213Arg → His and 223Met → Val, which result in three alleles, *SULT1A1*1* (213Arg, 223Met), *SULT1A1*2* (213His, 223Met) and *SULT1A1*3* (213Arg, 223Val). Allele frequencies for *SULT1A1*1*, *-*2* and *-*3* were 65, 33 and 1% for Caucasians and 48, 29 and 23% for African-Americans, respectively (Carlini *et al*., 2001). A kinetic analysis of 2-methoxyestradiol showed similar K_m values for SULT1A1*1 and SULT1A1*2 (0.90 ± 0.12 and 0.81 ± 0.06 μM, respectively) (Spink *et al*., 2000). Three *SULT1E1* polymorphisms cause amino acid substitutions in codons 22Asp → Tyr, 32Ala → Val and 253Pro → His (Adjei *et al*., 2003). Kinetic studies with estradiol and the recombinant *SULT1E1* variant 22Tyr revealed an increase in apparent K_m, which resulted in a 40-fold lower activity compared with the wild-type enzyme (Adjei *et al*., 2003) and is consistent with the location of residue 22 at the entrance of the substrate-binding pocket (Pedersen *et al*., 2002). The striking decrease in enzyme activity and concentration observed for 32Ala → Val and 22Asp → Tyr are expected to have considerable impact on the mammary metabolism of estrogen. However, the allele frequency of these *SULT1E1* variants is < 1% (Adjei *et al*., 2003), which is much lower than the variant *SULT1A1* allele frequency, and raises the question whether they are indeed polymorphisms or mutations. One epidemiological study found an increased risk for breast cancer associated with the *SULT1A1*2* genotype (213Arg → His) (155 cases, 328 controls; odds ratio, 1.8; 95% CI, 1.0–3.2; $p = 0.04$) (Zheng *et al*., 2001). However, another study reported the lack of an association (444 cases, 227 controls; $p = 0.69$) (Seth *et al*., 2000).

(vii) *Steroid (estrone) sulfatase*

In contrast to the many SULTs, only one steroid sulfatase hydrolyses several sulfated steroids, including estrone sulfate, estradiol sulfate, dehydroepiandrosterone sulfate and cholesterol sulfate (Burns, 1983). Steroid sulfatase is not expressed in normal endometrium but was observed in 65/76 (86%) endometrial carcinomas (Utusunomiya *et al*., 2004). In contrast, the enzyme is expressed in both normal and malignant breast tissues (Chapman *et al*., 1995; Utsumi *et al*., 1999; Miyoshi *et al*., 2001). Utsunomiya *et al*. (2004) found a positive correlation ($p < 0.05$) between the steroid sulfatase:estrogen SULT ratio and shorter survival in patients with endometrial carcinomas, and suggested

that increased steroid sulfatase and decreased estrogen SULT expression may result in increased availability of biologically active estrogens.

Several studies have shown that progestogens can act as 'selective estrogen enzyme modulators' in hormone-responsive breast cancer cells (reviewed by Pasqualini, 2004). Specifically, several progestogens exert an inhibitory effect on estrone sulfatase, which produces estradiol, in conjunction with a stimulatory effect on SULT, which forms the inactive estrogen sulfate. These data help to explain the antiproliferative effect of progestogens in breast tissue. It was also shown in MCF-7 and T47D cells that estradiol inhibited estrone sulfatase in a dose-dependent manner (IC_{50}: concentration of estradiol that inhibits the activity of the enzyme by 50%, 8.8×10^{-10} M and 1.8×10^{-9} M, respectively) and thereby decreased its own formation by blocking the conversion of estrone sulfate to estradiol (Pasqualini & Chetrite, 2001).

(viii) *17β-Hydroxysteroid dehydrogenase*

17β-Hydroxysteroid dehydrogenase (17β-HSD) enzyme is responsible for the interconversion of 17-ketosteroids and their active 17β-hydroxysteroid counterparts, such as estrone, estradiol, androstenedione and testosterone. Six human genes that encode isozymes of 17β-HSD have been cloned (Peltoketo *et al.*, 1999). These isozymes are designated types 1–6 or HSD1–HSD6.

17β-HSD1 is a key enzyme in estrogen metabolism because it catalyses the conversion of estrone into the biologically more active estradiol. It is abundantly expressed in ovarian granulosa cells and placental syncytiotrophoblasts (Peltoketo *et al.*, 1999). 17β-HSD1 is detected in certain peripheral tissues, such as breast and endometrium, in addition to steroidogenic cells in the ovary and placenta. However, the degree of expression reported is quite variable. In breast cancers, for example, the detection of *17β-HSD1* mRNA varies from 16 to 100% (Gunnarsson *et al.*, 2001; Oduwole *et al.*, 2004) and the immunohistochemical staining of 17β-HSD1 ranges from 20 to 61% of cases (Poutanen *et al.*, 1992a,b; Sasano *et al.*, 1996; Suzuki *et al.*, 2000; Oduwole *et al.*, 2004). A positive, inverse or no correlation was observed between 17β-HSD1 expression and estrogen receptor-positive status in breast cancers (Sasano *et al.*, 1996; Suzuki *et al.*, 2000; Oduwole *et al.*, 2004). One study observed significantly higher expression of 17β-HSD1 in postmenopausal cancers while another study found no correlation with menopausal status (Suzuki *et al.*, 2000; Miyoshi *et al.*, 2001). Correlation between 17β-HSD1 expression and prognosis has been inconsistent, and either no association or a shorter overall and disease-free survival have been found in breast cancer patients (Suzuki *et al.*, 2000; Oduwole *et al.*, 2004). One reason for the discrepant data on 17β-HSD1 expression could be the amplification of the *17β-HSD1* gene, which was observed in 14.5% of postmenopausal breast cancers (Gunnarsson *et al.*, 2003). However, 17β-HSD1 is not expressed in normal or malignant endometrium (Utsunomiya *et al.*, 2001, 2003).

The *17β-HSD1* gene contains several polymorphisms, including a common one in exon 6 that results in the amino acid substitution 312Ser → Gly (Normand *et al.*, 1993). Several studies found no association of this polymorphism with either breast or endo-

metrial cancers (Feigelson *et al*., 2001; Wu *et al*., 2003; Setiawan *et al*., 2004). This is consistent with experimental data that show no difference in the catalytic activity of recombinant wild-type and variant 312 alleles (Puranen *et al*., 1994). Nevertheless, one study observed higher plasma levels of estradiol in lean women with the homozygous 312Gly/Gly genotype ($p = 0.01$) (Setiawan *et al*., 2004).

17β-HSD2 catalyses the conversion of estradiol into less potent estrone. In contrast to 17β-HSD1, expression studies of 17β-HSD2 yielded more consistent results. 17β-HSD2 is expressed in normal mammary epithelium but is frequently absent in breast cancer cells (Miettinen *et al*., 1999; Ariga *et al*., 2000; Suzuki *et al*., 2000; Gunnarsson *et al*., 2001; Oduwole *et al*., 2004). *17β-HSD2* mRNA was found in 10–31% of tumours and 17β-HSD2 protein was absent in all breast cancers (Suzuki *et al*., 2000; Gunnarsson *et al*., 2001). In contrast, 17β-HSD2 was regularly expressed in normal endometrium and was detected in 75% of endometrial hyperplasias and 37–50% of endometrial carcinomas (Utsunomiya *et al*., 2001, 2003). Since 17β-HSD2 preferentially catalyses the oxidation of estradiol to less active estrone, it has been suggested that the expression of 17β-HSD2 in proliferative glandular cells of endometrial disorders may represent an in-situ defence mechanism that modulates unopposed estrogenic effects (Utsunomiya *et al*., 2003).

17β-HSD5 (also known as aldo-keto reductase, AKR1C3) is expressed in normal breast and prostate (Penning *et al*., 2000). The level of 17β-HSD5 expression in breast cancer specimens was higher than that in normal breast tissue and 65% of 794 tumours labelled 17β-HSD5-positive (Oduwole *et al*., 2004). Since 17β-HSD5 recognizes a wide range of substrates, including estrogens, androgens, progestogens and prostaglandins, its role in breast tissue is uncertain.

Progestogens have a complex effect on 17β-HSD activity and can direct the interconversion of estrone to estradiol in both directions (reviewed by Pasqualini, 2004). Studies with the hormone-dependent breast cancer cells MCF-7 and T47D have shown that some progestogens stimulate the reductive activity of estrone to estradiol and thereby enhance cell proliferation (Coldham & James, 1990; Poutanen *et al*., 1990, 1992b; Peltoketo *et al*., 1996). Other progestogens favour the oxidation of estradiol to estrone and may thereby inhibit cell growth (Chetrite *et al*., 1999a,b).

(*c*) *Tobacco smoke*

Several compounds in tobacco smoke might affect estrogen metabolism by the induction of CYPs (Zeller & Berger, 1989). An epidemiological study of 27 premenopausal women (14 smokers, 13 nonsmokers) showed a significant increase in urinary excretion of 2-hydroxyestrone that is a result of 2-hydroxylation of reversibly oxidized estradiol (Michnovicz *et al*., 1986). [The concentration of 4-hydroxylated estrogen metabolites was not assessed by this assay.]

Berstein *et al.* (2000) used GC–MS to measure urinary excretion of catechol estrogens in six smoking and 10 nonsmoking postmenopausal women who received 2 mg/day estradiol valerate for 1 month. Before administration of estradiol valerate, smokers had significantly lower excretion of 16-epiestriol and 4-hydroxyestrone than nonsmokers. After

administration of estradiol valerate, much higher excretion of 2-hydroxyestrone and 4-hydroxyestradiol was observed in smokers compared with nonsmokers. These data indicate that only the combination of estradiol valerate and smoking (and not smoking itself) leads to an increase in potentially genotoxic catechol estrogens.

4.1.2 *Experimental systems*

(*a*) *Estrogen fatty acid esters*

Chronic treatment of ovariectomized rats with 0.5 or 5 nmol/day estradiol stearate for 10 or 23 days had a stronger stimulatory effect on mammary gland cell proliferation than treatment with equimolar doses of estradiol (Mills *et al*., 2001). Two commonly prescribed hypolipidaemic drugs, clofibrate and gemfibrozil, increase the size and number of hepatic peroxisomes upon administration to rodents. Treatment of rats with clofibrate caused a multifold increase in the hepatic microsomal formation of estradiol fatty acids (Xu *et al*., 2001a). The stimulatory effect of clofibrate on hepatic fatty acid esterification of estradiol was paralleled by enhanced estradiol-induced increases in the formation of lobules in the mammary gland and by increased incorporation of bromodeoxyuridine, a marker of cell proliferation, into these lobules (Xu *et al*., 2001b).

(*b*) *Catechol estrogens*

Catechol estrogens are carcinogenic in animal experiments. The experimental evidence was reviewed by Cavalieri *et al*. (2000) and showed that 4-catechol estrogens are more carcinogenic than the isomers 2-hydroxyestrogens. In addition to the induction of renal cancer in hamsters (Liehr *et al*., 1986), 4-hydroxyestradiol induces uterine adenocarcinoma, a hormonally related cancer, in mice. Administration of estradiol, 2-hydroxyestradiol and 4-hydroxyestradiol induced endometrial carcinomas in 7, 12 and 66%, respectively, of neonatally treated CD-1 mice (Newbold & Liehr, 2000). However, in adult ACI rats, administration of estradiol but not that of 2- or 4-hydroxyestradiol or 4-hydroxyestrone induced mammary tumours (Turan *et al*., 2004).

4.2 Receptor-mediated effects

As indicated in the monograph on Combined estrogen–progestogen contraceptives, there is evidence that not all of the effects of estrogens and progestogens used in hormonal therapy for the menopause are mediated through nuclear or other receptors. In addition, the effects of these steroids probably involve several molecular pathways and cross-talk between receptor- and/or non-receptor-mediated pathways. During the past decade, extensive growth in research on the mechanisms of action of hormones and on hormones and cancer has taken place, and several steroid hormone receptor subtypes and non-genomic mechanisms of action have been determined.

Hormonal therapy with ‘estrogens only’ is effective in the treatment of many aspects of the menopause, but the increased risk for endometrial cancer renders the prescription

of combined estrogen–progestogen products for women with a uterus essential. In this context, an ideal progestogen would prevent endometrial cancer and maintain the protective benefits of estrogens, which means that it should have no significant anti-estrogenicity, except in the endometrium.

The various components of hormonal therapy for the menopause have received increased attention in recent years. Information has become available on the progestogens used and on their hormonal activities and their binding affinities to various receptors and proteins. This information is summarized in Tables 17 and 18, which were compiled on the basis of information gathered by Sitruk-Ware (2002), Schindler *et al*. (2003), Shields-Botella *et al.* (2003), Sitruk-Ware (2004a,b) and Wiegratz and Kuhl (2004).

There has also been tremendous growth in research on the effects of postmenopausal hormonal therapy on a variety of non-cancer end-points related to endometrial function (vaginal bleeding), postmenopausal vasomotor symptoms, treatment of problems with the menstrual cycle, skin, bone and related calcium metabolism, the cardiovascular system and lipid metabolism. Many of these effects are probably at least in part mediated by mechanisms of steroid receptors. This topic is reviewed in Section 4.3.

4.2.1 *Combined estrogen–progestogen therapy*

(*a*) *Humans*

(i) *Breast*

No data were available on the effects of exposure to combined estrogen–progestogen therapy on the human breast in the previous evaluation (IARC, 1999). During the past 6 years, several reports have been published that are pertinent to this issue.

Hargreaves *et al*. (1998) obtained archival paraffin-embedded breast tissue samples from women who underwent surgery for benign (n = 61) or malignant (n = 124) breast disease and stained sections from these for the proliferation marker Ki-67 and the progesterone receptor. The median percentage of normal epithelial cells that stained for Ki-67 [using an unspecified antibody] was 0.19% (range, 0–3.66%) in breast samples of 111 women who did not receive hormonal therapy. This was not significantly different from the percentages in normal epithelial cells from 35 women who took estrogen only (0.22%; range, 0–1.44%) or 39 women who took combined estrogen plus progesterone therapy (0.25%; range, 0–2.80%). However, the median percentage of normal epithelial cells that stained for the nuclear progesterone receptor significantly increased from 4.8% (range, 0–39%) in 100 untreated women to 10.2% (range, 0.2–40%) in 31 women who took estrogen only and 6.7% (range, 0–44%) in 36 women who took combined estrogen plus progesterone therapy. This increased expression of the progesterone receptor is consistent with an effect of estrogen on breast cells.

Hofseth *et al*. (1999) obtained breast biopsies from women who were taking oral estrogen–progestogen therapy that contained conjugated equine estrogens (0.3–2.5 mg/day) or micronized 17β-estradiol (0.5–1.0 mg/day) plus medroxyprogesterone acetate (2.5–5.0 mg/day), from women who were taking these estrogens only or from women who did

Table 17. Overview of the spectrum of hormonal activities of progestogens used in hormonal menopausal therapy

Progestogen	Progesto-genic	Anti-estrogenic	Estrogenic	Androgenic	Anti-androgenic	Glucocorticoid	Antimineralo-corticoid
Chlormadinone acetate	+	+	–	–	+	+	–
Cyproterone acetate	+	+	–	–	+, +	+	–
Desogestrel	+	+	–	+	–	±, –	–
Dienogest	+	+, ±	–, ±	–	+	–	–
Drospirenone	+, +	+	–	–	+	?, –	+
Dydrogesterone	+	+	–	–	–, ±	?	±
Etonogestrel [3-keto-desogestrel]	+	+	–	+	–	±, –	–
Gestodene	+	+	–	+	–	±, +	+
Levonorgestrel/norgestrel	+	+	–	+	–	–	–
Medroxyprogesterone (acetate)	+	+	–	±	–	+	–
Norethisterone (acetate)	+, +	+	+	+	–	–	–
Progesterone	+, +	+	–	–	±	+	+
Trimegestone	+	+	–	–	±	–	±

Adapted from Wiegratz and Kuhl (2004); second value, for progestogenic activity only from Sitruk-Ware (2002); second value, except for progestogenic activity from Schindler *et al*. (2003)

+, effective; ±, weakly effective; –, ineffective; ?, unknown

Data are based mainly on animal experiments. The clinical effects of the progestogens are dependent on their tissue concentrations.

No comparable data were available for ethynodiol diacetate.

Note: This information should be viewed as only an indication of the hormonal activity and its order of magnitude of the various progestogens.

Table 18. Relative binding affinities of progestogens used in hormonal therapy for the menopause to steroid receptors and serum binding globulins[a]

Progestogen	PR	AR	ER	GR	MR	SHBG	CBG
Chlormadinone acetate	134	5	0	8	0	0	0
Cyproterone acetate	180	6	0	6	8	0	0
Desogestrel (as 3-keto-desogestrel)	300	20	0	14	0	15	0
Dienogest	10	10	0	1	0	0	0
Drospirenone	70, 19	65, 2	0, < 0.5	6, 3	230, 500	0	0
Dydrogesterone	150	0	?	?	?	?	?
Etonogestrel (3-keto-desogestrel)	300	20	0	14	0	15	0
Gestodene	180, 864	85, 71	0, < 0.02	27, 28	290, 97	40	0
Levonorgestrel/norgestrel	300, 323	45, 58	0	1, 7.5	75, 17	50	0
Medroxyprogesterone acetate	130, 298	5, 36	0, < 0.02	29, 58	160, 3.1	0	0
Norethisterone acetate	150, 134	15, 55	0, 0.15	0, 1.4	0, 2.7	16	0
Norgestimate/nomegestrol acetate	30	0	0	1	0	0	0
Progesterone	100	0	0	10	100	0	36
Trimegestone	660, 588	1, 2.4	0, < 0.02	9, 13	120, 42	?	?
Reference compounds (100%)	Progesterone	Metribolone (R1881)	17β-Estradiol	Dexamethasone (or cortisol)	Aldosterone	5α-Dihydro-testosterone	Cortisol

Adapted from Wiegrazt and Kuhl (2004a,b); second value from Sitruk-Ware (2004)
?, unknown; AR, androgen receptor; CBG, corticoid-binding globulin; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; SHBG, sex hormone-binding globulin
[a] Values were compiled by these authors by cross-comparison of the literature. Because the results of the various in-vitro experiments depend largely on the incubation conditions and biological materials used, the published values are inconsistent. These values do not reflect the biological effectiveness, but should be viewed as only an indication of the order of magnitude of the binding affinities of the various progestogens.
No comparable data were available for ethynodiol diacetate.

not take hormonal treatment. Compared with untreated women (n = 16–19), the percentage of epithelial cells in the inter- and intralobular ducts and the duct-lobular units that stained positive for proliferating cell nuclear antigen were significantly ($p < 0.01$) increased by approximately twofold in women who took estrogen alone (n = 21) and those who took estrogen plus progestogen (n = 15; ducts only); staining was increased by almost threefold in the duct-lobular unit cells of women who took estrogen plus progestogen (n = 19; $p < 0.01$). When another marker of proliferation (Ki-67) was examined, similar differences were found, but only the difference (approximately sixfold) for the duct-lobular unit cells of women who took estrogen plus progestogen was statistically significant ($p < 0.05$). There was a positive correlation between the percentage of epithelial breast cells that stained for markers of proliferation with the duration of both types of hormonal treatment, but this was only statistically significant for the treatment with estrogen plus progestogen when the Ki-67 marker was considered ($p = 0.03$). There was also a significant increase ($p \leq 0.01$) in the percentage of breast tissue occupied by epithelium, i.e. twofold for women who took estrogen only and threefold for women who took estrogen plus progestogen. The percentage of epithelial cells that were positive for the nuclear staining for the progesterone receptor was increased three- to fourfold ($p < 0.01$) in women who took estrogen only and approximately twofold in the lobular units of women who took estrogen plus progestogen ($p < 0.05$); again, the observed increase in the expression of the progesterone receptor is consistent with an effect of estrogen on these cells. No differences were observed in nuclear staining for the estrogen receptor.

Conner *et al*. (2001) studied 12 women who were treated continuously with 17β-estradiol (50 μg per day by skin patch) and either oral (5 mg per day) medroxyprogesterone acetate or vaginal (8 mg every 2 days) progesterone on days 15–26 of each cycle. They obtained fine needle aspiration biopsies during the last 2 days of the estrogen part of the cycle and during day 25 or 26 at the end of the estrogen plus progestogen part of the cycle after 6–8 weeks and after 14–16 weeks of treatment. The percentage of epithelial cells that stained for Ki-67 (using the MIB-1 antibody) was 1.4% at the end of the estrogen phase and 2.1% at the end of the estrogen plus progestogen phase of the cycle, but this was not statistically significant. There was no difference in Ki-67 staining between the two progestogen treatments. In follow-up studies, Conner *et al.* (2003, 2004a) examined women who received continuous oral 17β-estradiol (2 mg per day) plus norethisterone acetate (1 mg per day) or 17β-estradiol valerate (2 mg per day) plus dienogest (2 mg per day) for 6 months. In the first study, two groups of 13–17 women received estrogen plus either norethisterone acetate or estrogen plus dienogest. For both treatments combined, the mean percentage of epithelial cells that stained for Ki-67 in fine needle aspiration was statistically significantly increased ($p < 0.001$) from 2.2% (n = 28; median, 1.4%; range, 0–11.7%) at baseline to 9.1% (n = 30; median, 7.6%; range, 0–27.1%) after 3 months and 8.0% (n = 31; median, 5.7%; range, 0–25.9%) after 6 months of treatment. One woman who had a high baseline proliferation index showed a decrease in proliferation after treatment. The increases in cell proliferation index were similar for both hormonal treatments (Conner *et al.*, 2003). In the second study of 83 women who were treated with 17β-estradiol (2 mg per day) plus

norethisterone acetate (1 mg per day), the mean percentage of epithelial cells that stained for Ki-67 in fine needle aspiration biopsies was significantly ($p < 0.01$) increased from 2.2% (median, 1.9%; range, 0–11.9%) at baseline to 6.4% (median, 5.0%; range, 0–20.4%) after 6 months of treatment. There was a negative correlation between the rate of epithelial proliferation in this study and total and free serum testosterone levels (Conner *et al*., 2004a).

Valdivia *et al*. (2004) obtained breast core biopsies from 19 women at baseline and after treatment for 12 months with continuous conjugated equine estrogen (0.625 mg per day) and medroxyprogesterone acetate (5 mg per day). Of these women, 15 responded with an increase in percentage of epithelial cells that stained for Ki-67, one exhibited a decrease and three women had no change in this parameter; the increase from baseline was probably statistically significant, but this was not clear. Expression of the apoptosis marker Bcl-2 was increased in nine women, decreased in five and unchanged in five.

The results of all but one of these studies indicate that combined estrogen–progestogen menopausal therapy increases the rate of cell proliferation in the breast. The addition of progestogens appears to enhance significantly the modest increase in the rate of breast cell proliferation caused by estrogen-only therapy. This is consistent with the notion of an increase in risk for breast cancer associated with combined estrogen–progestogen menopausal therapy over that associated with estrogen-only menopausal therapy (see also IARC, 1999). Only the study of archival surgical specimens of women with breast disease by Hargreaves *et al*. (1998) did not show an increase in breast cell proliferation associated with combined estrogen–progestogen menopausal therapy. The other studies used either fine needle aspirates or core biopsies from women without breast disease, which may explain the discrepancy.

Mammographic density is a strong identifier of risk for sporadic breast cancer that exceeds the risk associated with elevated circulating levels of 17β-estradiol (Santen, 2003). Risk for breast cancer was increased in a number of case–control studies in which mammographic density was not only subjectively evaluated by radiologists but was also assessed by observer bias-free, automated, computer-assisted techniques. The relative risks were in the order of 4–6 for subjective evaluations and 3–4 for computer-assisted evaluations (Byng *et al*., 1997; Yaffe *et al*., 1998; Boyd *et al*., 1999; Li *et al*., 2005). A number of recent studies have reported on the effects of estrogen–progestogen therapy on mammographic density. Valdivia *et al*. (2004) (see study details above) observed an increase in mammographic density (BI-RADS method) in 11/19 women (58%) who took conjugated equine estrogen plus medroxyprogesterone acetate for 12 months, while density was decreased in only one woman and was unchanged in seven.

Conner *et al*. (2004b) randomized women to continuous oral treatment for 6 months with either 17β-estradiol (2 mg per day) plus norethisterone acetate (1 mg per day) (22 women) or 17β-estradiol valerate (2 mg per day) plus dienogest (2 mg per day) (23 women). Both treatments resulted in an increase in mammographic density (Wolfe method) over baseline values in 50–60% of these women; this change was statistically significant.

Christodoulakos *et al*. (2003) randomized 94 women to continuous oral treatment for 12 months with conjugated equine estrogen alone (0.625 mg per day) (25 women), equine estrogen (0.625 mg per day) plus medroxyprogesterone acetate (5 mg per day) (34 women) or 17β-estradiol (2 mg per day) plus norethisterone acetate (1 mg per day) (35 women); 27 untreated control women were also included. Mammographic density (Wolfe classification method) increased in 12% of women who took equine estrogen plus medroxyprogesterone acetate, 31% of women who took 17β-estradiol plus norethisterone acetate and in 8% of women who took estrogen only, whereas density did not increase in any of the control women. Density decreased in 26% of control women but in none of the women who took hormonal treatment. The difference from controls was statistically significant for all three treatment groups.

Georgiev and Manassiev (2002) found that breast density increased in 16% of 19 women who were treated with continuous oral 17β-estradiol (2 mg per day) plus dienogest (2 mg per day) or 17β-estradiol (2 mg per day) plus norethisterone acetate (1 mg per day) and were followed annually by mammography for 4 years using the Wolfe method.

Sendag *et al*. (2001) compared women who received continuous oral treatment with 17β-estradiol (2 mg per day) plus norethisterone acetate (1 mg per day) (44 women), conjugated equine estrogen (0.625 mg per day) plus medroxyprogesterone acetate (5 mg per day) (17 women), equine estrogen only (0.625 mg per day) (20 women) or transdermal 17β-estradiol (3.9 mg per week) (56 women) and 44 women who received a variety of sequential treatments with estrogen and estrogen plus progestogen. The mean follow-up was 20 months (range, 12–96 months) and the Wolfe method was used to assess breast density. Density was increased in 31% of women who took continuous estrogen plus progestogen and in 4% of women who took estrogen only, but did not change in women who received the sequential treatments with estrogen plus progestogen. More women (34%) who took the continuous treatment with 17β-estradiol plus norethisterone acetate had increased breast density than those who took continuous estrogen plus medroxyprogesterone acetate (24%).

Colacurci *et al*. (2001) randomized women to continuous treatment with transdermal 17β-estradiol (0.05 mg per day) plus nomegestrol acetate at one of two doses (5 mg per day, 26 women; or 2.5 mg per day, 25 women), 17β-estradiol only (23 women) or no treatment (controls; 23 women). Mammographic density (Wolfe method) after 12 months of treatment was increased in 35% and 43% of the women who took estrogen plus the high and low dose of nomegestrol acetate, respectively, in 21% of women who took estrogen only and in none of the control women. The differences from the control group were statistically significant.

Erel *et al*. (2001) assigned women to continuous oral treatment with conjugated equine estrogen (0.625 mg per day) plus medroxyprogesterone acetate (2.5 mg per day) (26 women), continuous treatment with estrogen (0.625 mg per day) plus medroxyprogesterone acetate (10 mg/day) for the last 10 days of the 28-day cycle (21 women) or continuous treatment with estrogen only (0.625 mg per day) (23 women). Women were followed by mammography for 4 years using the Wolfe method to assess breast density. Density was increased in 35% of women who took continuous estrogen plus progestogen,

in 19% of women who took estrogen plus cyclic progestogen and in 22% of women who took estrogen only. Although the differences between these three groups were not statistically significant, the results suggest that treatment with continuous estrogen plus progestogen is more likely to increase breast density than treatment with continuous estrogen only or estrogen plus sequential progestogen.

Lundström *et al.* (2001) studied women who took continuous oral conjugated equine estrogen (0.625 mg per day) plus medroxyprogesterone acetate (5 mg per day) (52 women) or estriol (2 mg per day) (51 women) or used a transdermal patch of 17β-estradiol (0.05 mg per day) (55 women) and were followed every 2 years by mammography using the Wolfe method to assess breast density. Density increased over baseline at the first 2-year visit in 40% of women who took continuous estrogen plus progestogen, in 6% of women who took oral estrogen only and in 2% of women who used a transdermal patch of estrogen only.

Collectively, these studies consistently show that approximately one third of women treated with continuous estrogen (by any route) plus oral progestogen respond with increased mammographic breast density. Treatment with continuous estrogen plus sequential progestogen resulted in fewer women developing increased breast density than treatment with continuous estrogen plus progestogen. Estrogen-only treatment appeared to result in increased breast density in fewer women. These findings correspond to the supposition that continuous estrogen plus progestogen therapy results in an increased risk for breast cancer.

(ii) *Uterus*

In the previous evaluation (IARC, 1999), it was concluded that the addition of progestogens reduces the increased rate of cell proliferation in the endometrium that is seen with estrogen-only therapy. The effects of estrogen only and their reduction by progestogens were dose-related. Two previous studies from the 1980s on cell proliferation concerned combined treatment with conjugated equine estrogens (Premarin®) and norethisterone. At least nine additional studies have been conducted with norethisterone, all but one of which combined the treatment with 17β-estradiol. In addition, studies have been carried out on six other progestogens combined with estrogen therapy. Many of these studies include histopathological analysis of endometrial biopsies. Although many studies have taken care to standardize this analysis, it should be noted that there is considerable potential for significant inter-observer and inter-study variation (Wright *et al.*, 2002)

Norethisterone (acetate) plus estrogen

Cameron *et al.* (1997) followed 14 postmenopausal women for 3 months during which they were treated with a dermal patch that released 0.05 mg per day 17β-estradiol for 7 days alternated with a patch that released 0.05 mg per day 17β-estradiol plus 0.25 mg per day norethisterone acetate for 3 days. End-of-study endometrial biopsies were obtained at the end of an estrogen-only period and at the end of an estrogen plus norethisterone acetate period. Staining for the proliferation marker Ki-67 was reduced at the end

of the estrogen plus norethisterone acetate period compared with the estrogen-only period, but staining for estrogen (α) and progesterone receptors and histological endometrial thickness did not differ. No endometrial hyperplasia was found.

Johannisson *et al*. (1997) randomized postmenopausal women in an open-label dermal patch study to continuous 0.05 mg per day 17β-estradiol plus doses of norethisterone acetate of 0.17 or 0.35 mg per day either continuously or sequentially on days 14–28. A reference group (not randomized) was treated with a continuous 0.05-mg per day 17β-estradiol patch and orally with either 1 mg per day norethisterone acetate or 20 mg per day dydrogesterone during the last 14 days of each cycle. End-of-study endometrial biopsies were obtained from 107–124 women per group after 13 cycles of 28 days. No significant differences were observed in the percentage of women with atrophic or proliferative endometrial histology and no malignancies occurred; only one case of endometrial hyperplasia developed in the group that received 17β-estradiol plus sequential norethisterone acetate at 0.35 mg per day.

Habiba *et al*. (1998) studied 103 postmenopausal women who were treated orally with 2 mg per day 17β-estradiol valerate continuously and 1 mg per day norethisterone on days 16–28 of the cycle. The women received a baseline and end-of-study endometrial biopsy after 6 months of therapy. Most women had inactive or non-secretory endometrial histology at baseline whereas over 90% had secretory morphology after 6 months of treatment. No cases of endometrial hyperplasia or carcinoma occurred.

Dahmoun *et al*. (2004) assigned postmenopausal women to continuous treatment with either 2 mg per day 17β-estradiol plus 1 mg per day norethisterone acetate or 0.625 mg per day conjugated equine estrogens plus 5 mg per day medroxyprogesterone acetate. The two treatment groups were analysed in combination after 1 year of treatment. Staining for the proliferation marker Ki-67 was increased in stromal cells, but was not affected in epithelial cells. Staining for estrogen (α) receptor was reduced in the epithelium but was only slightly reduced in stromal cells. Staining for a marker of apoptosis (TUNEL) and the progesterone receptor in stroma and epithelium were not affected by the treatments, nor was endometrial thickness as assessed by ultrasound.

Kurman *et al*. (2000) conducted a double-blind clinical trial in which postmenopausal women were randomized to continuous oral treatment with 1 mg per day 17β-estradiol only or 1 mg per day 17β-estradiol plus 0.10, 0.25 or 0.50 mg per day norethisterone acetate. End-of-study endometrial biopsies were obtained from 241–251 women per group after 12 months of treatment. In the estrogen-only group, 14.6% of women had endometrial hyperplasia, whereas only 0.8% of women who took estrogen plus 0.10 mg norethisterone acetate and 0.4% of women who took the two higher doses of norethisterone acetate had such lesions. In women over 65 years of age, 4/21 (19%) who took estrogen only had endometrial hyperplasia, while none of the women who received estrogen plus either of the doses of norethisterone acetate had this lesion (0/18, 0/18 and 0/19 women).

Iatrakis *et al*. (2004) conducted an open-label prospective study of continuous oral treatment with 1 mg per day 17β-estradiol and 0.5 mg per day norethisterone acetate of 124 postmenopausal women for up to 3 years. A concurrent control group (not randomized) of

130 untreated women was available. Endometrial thickness, as assessed by ultrasound, was virtually unaffected. In end-of-study endometrial biopsies, no differences in the percentage of women with atrophic, secretory or proliferative histology were observed between the treated and control women. No cases of endometrial hyperplasia or carcinoma occurred.

Wells *et al*. (2002) conducted an open-label prospective study of postmenopausal women who were given continuous oral treatment with 2 mg per day 17β-estradiol plus 1 mg per day norethisterone acetate for up to 5 years; the mean follow-up was 4.4 years. Endometrial biopsies were obtained at baseline, between 24 and 36 months and at the end of the study. Inactive or atrophic endometrium was found in 68/164 (41%) women who had had no hormonal treatment at baseline, in 157/465 (34%) women after 24–36 months of treatment and in 185/398 (46%) women at the end of the study. (The entire cohort consisted of a mixture of women who had had no hormonal treatment and women who had already been taking either sequential or estrogen-only hormonal therapy.) At baseline, 14/164 women had a secretory endometrial histology (9%); this increased to 162/465 (35%) and 102/398 (26%) women after 24–36 months of treatment and at the end of the study, respectively. No cases of endometrial hyperplasia or carcinoma occurred. Sturdee *et al*. (2000) reported on 9 months of follow-up in this study. At baseline the prevalence of complex hyperplasia was 5.3% and that of atypical hyperplasia was 0.7% in the entire group of 1196 women who completed 9 months of treatment, many of whom had previously taken hormonal therapy that may have induced these hyperplastic lesions. None of these women had endometrial hyperplasia after 9 months of treatment and no new cases arose.

Neven *et al*. (2004) reported results of the EURALOX (European double-blind clinical trial on raloxifene) in which postmenopausal women were randomized to continuous oral treatment with 2 mg per day 17β-estradiol plus 1 mg per day norethisterone acetate or 60 mg per day raloxifene alone. End-of-study endometrial biopsies were obtained after 12 months of treatment. The conclusion of a detailed histopathological analysis was that more endometrial pathology occurred in 261 women on estrogen plus norethisterone acetate than in 73 women on raloxifene (polyps, 4.3% versus 2.0%; $p < 0.05$; endometrial proliferation/hyperplasia, 8.8% versus 1.2%; $p < 0.001$; cystic atrophy, 5.5% versus 1.2%; $p < 0.001$). Very few cases of malignant or premalignant endometrial histology occurred in either group.

Portman *et al*. (2003) conducted a double-blind placebo-controlled clinical trial in which postmenopausal women were randomized to continuous oral treatment with placebo, ethinylestradiol at 0.005 mg per day without or with 0.25 or 1.0 mg per day norethisterone acetate, or ethinylestradiol at 0.01 mg per day without or with 0.5 or 1.0 mg per day norethisterone acetate. In addition, an open-label comparison group was given 0.625 mg per day conjugated equine estrogens plus 2.5 mg per day medroxyprogesterone acetate. After 12 months of therapy, 114–121 women in each group received an end-of-study endometrial biopsy. Endometrial hyperplasia was found in 23/118 (19%) of the women who took 0.01 mg per day ethinylestradiol only, but only in a maximum of one woman in each of the other groups. In the estrogen-only groups, 80–90% of women had

proliferative endometrial morphology, including the 19% of women with hyperplasia in the group who took 0.01 mg per day ethinylestradiol only. The occurrence of this morphology was reduced to 30–45% of women in all groups who were given co-treatment with norethisterone acetate, but was found in 70% of women who took estrogens plus medroxyprogesterone acetate. The differences between the co-treatment with norethisterone acetate and that with estrogen only or estrogen plus medroxyprogesterone acetate were statistically significant.

Other progestogens plus estrogens

Ferenczy and Gelfand (1997) conducted an open-label prospective study of postmenopausal women who were given continuous oral treatment with 2 mg per day 17β-estradiol and 10 mg dydrogesterone on days 15–28. Baseline biopsies from 146 women who completed the 12-month course of treatment showed predominantly atrophic endometrium, whereas biopsies taken after 12 months of treatment showed that endometrial histology was predominantly secretory. One endometrial hyperplasia was found, but no endometrial carcinomas.

Hänggi *et al*. (1997) randomized 35 postmenopausal women per group to oral treatment with either placebo, 2 mg per day of continuous 17β-estradiol plus 10 mg dydrogesterone on days 15–28 or 0.05 mg per day continuous 17β-estradiol by dermal patch plus 10 mg oral dydrogesterone on days 15–28. Ultrasound assessment revealed a 2.5- to threefold increase over baseline of endometrial thickness after 12 and 24 months of hormonal treatment. Biopsies taken at the same time-points showed a shift from a predominantly inactive or atrophic endometrial histology to a predominantly secretory morphology.

Ross *et al*. (1997) conducted a double-blind clinical trial in which postmenopausal women were randomized to continuous oral treatment with 2 mg per day 17β-estradiol plus 0.1, 0.25 or 0.5 mg per day trimegestone on days 15–28. In each of the three groups, 10–11 women were available for evaluation. After three cycles, biopsies were taken and 90–100% of the women in all groups had a secretory endometrial morphology; no hyperplasia was found in any of the groups.

Suvanto-Luukkonen *et al*. (1998) conducted an open-label clinical trial in which postmenopausal women were randomized to continuous treatment with a skin gel that released 0.15 mg per day 17β-estradiol into the circulation plus either an intrauterine device that released 0.02 mg per day levonorgestrel for up to 5 years, 100 mg per day oral micronized progesterone on days 1–25 or 100–200 mg per day vaginal progesterone on days 1–25. After 12 months of treatment, endometrial thickness (assessed by ultrasound) was not significantly changed from baseline. No change in endometrial histology was observed in end-of-study biopsies in the group that received 17β-estradiol plus intrauterine levonorgestrel but, in the groups that received 17β-estradiol plus oral or vaginal progesterone, morphology changed from predominantly atrophic at baseline (46/50 cases; 92%) to predominantly proliferative (13/18 cases [72%] in the oral progesterone-treated group and 8/14 cases [57%] in the vaginal progesterone-treated group).

Byrjalsen *et al*. (1999) conducted a double-blind clinical trial in which 55–56 postmenopausal women per group were randomized to placebo or continuous oral treatment with 2 mg per day 17β-estradiol plus 0.025 or 0.05 mg per day gestodene sequentially (days 17–28), 1 mg per day 17β-estradiol plus 0.025 mg per day gestodene sequentially (days 17–28) or 1 mg per day 17β-estradiol plus 0.025 mg per day gestodene continuously. After 2 years of follow-up, end-of-study biopsies were obtained and examined histologically. Treatment with continuous estrogen plus gestodene did not change the high percentage of women with atrophic endometrium (83%) compared with placebo (81%) but, in women who received sequential treatments, the majority (54–79%) had a secretory type of endometrial histology. In the latter groups, endometrial thickness, the histochemical expression of secretory markers and staining for estrogen and progesterone receptors in the endometrium were increased. One endometrial carcinoma developed in the group who took 1 mg 17β-estradiol and 0.025 mg gestodene sequentially. No cases of endometrial hyperplasia occurred.

van de Weijer *et al.* (1999) randomized 151 women to continuous oral treatment with 1 mg 17β-estradiol plus 5 or 10 mg dydrogesterone on days 15–28. Biopsies at baseline and after 13 cycles revealed that 98% of these women had no endometrial lesions; only one woman in each group developed either proliferative changes or hyperplasia.

Wahab *et al*. (1999) conducted a double-blind clinical trial in which postmenopausal women were randomized to continuous oral treatment with 2 mg per day 17β-estradiol plus 0.05, 0.1, 0.25 or 0.5 mg per day trimegestone on days 15–28. After 6 months of follow-up, biopsies were taken and compared with those of untreated control women who were not randomized. Extensive morphometric analysis of the endometrium was carried out. In the endometrium of treated women compared with that of untreated women, there was evidence of somewhat smaller glands and a clearly significantly reduced area occupied by glands, but no change in the number of glands per unit area. Glands with evidence of secretion were less frequent in the high-dose group only. Discriminant analysis revealed a significant relation with dose for the all histomorphometric parameters combined.

Jondet *et al*. (2002) randomized postmenopausal women to treatment with a skin gel that released 1.5 mg per day 17β-estradiol on days 1–24 plus oral administration of either 10 mg per day chlormadinone acetate (42 women) or 200 mg per day progesterone (63 women) on days 10–24. Endometrial biopsies were taken at baseline and at the end of the study (18 months). There was a shift in atrophic morphology from 92% of women who were affected at baseline to 20–27% who were affected after 18 months of treatment; at the same point in time, 63–77% of women had a secretory endometrial morphology versus 3% at baseline.

Drospirenone (1, 2 or 3 mg) in combination with 1 mg 17β-estradiol is a continuous combined product used in hormonal therapy. Phase II/III trials of these combinations have demonstrated that, at all three doses of drospirenone, the combination is associated with a highly favourable safety profile, with excellent endometrial protection after 1 and 2 years (no cases of hyperplasia or cancer) (Rubig, 2003).

The combination of 2 mg estradiol valerate with 2 mg dienogest is the first continuous combined hormonal menopausal therapy preparation to contain a progestogen with substantial anti-androgenic activity. This combination was compared with a continuous combination of 2 mg estradiol plus 1 mg norethisterone acetate. In a large-scale study (1501 women) (Von Schoultz, 2003), biopsy and ultrasound assessment demonstrated that estradiol valerate plus dienogest quickly and effectively achieved endometrial atrophy in the vast majority of subjects, which indicates a protective effect on the endometrium.

Fugère *et al.* (2000) conducted a double-blind clinical trial in which postmenopausal women were randomized to continuous treatment with 0.625 mg per day conjugated equine estrogens plus 2.5 mg per day medroxyprogesterone acetate (69 women) or 150 mg per day raloxifene (67 women). Endometrial thickness, as assessed by ultrasound, increased slightly but significantly after 1 and 2 years of follow-up in the group given estrogen plus progestogen but no change was observed in the raloxifene-treated group. In the former group, more women developed benign proliferative endometrial changes (19–24%) when biopsies were taken 1 and 2 years after the start of treatment, whereas in the raloxifene-treated group only 6% of women developed such changes, which did not differ from baseline (4–6%).

Chang *et al*. (2003) conducted a double-blind clinical trial in which postmenopausal women were randomized to 0.625 mg per day conjugated equine estrogens on days 1–25 plus 5 (102 women) or 10 (66 women) mg per day medroxyprogesterone acetate or 20 mg per day dydrogesterone (73 women) sequentially on days 12–25. After 10–12 cycles, no statistically significant changes in endometrial thickness were observed by ultrasound assessment. End-of-study biopsies were taken and flow cytometric analysis was performed on endometrial tissue. No differences in cell-cycle distribution were observed among the three treatment groups, in all of which 61–81% of women had secretory or proliferative endometrial morphology. Endometrial hyperplasia was found in two cases in the group that took 0.625 mg equine estrogens plus 5 mg medroxyprogesterone acetate. No cases of endometrial carcinoma occurred.

Overall, these studies confirm that addition of progestogens to estrogen therapy for the menopause prevents the development of endometrial hyperplasia and reduces the increased rate of endometrial cell proliferation caused by estrogen only. This beneficial effect was found for all progestogens studied, regardless of the route of administration and dose. Norethisterone was the most frequently studied progestogen and, even at the lowest dose examined in randomized studies (in the range of 0.1 mg per day), there was a maximal protective effect for both estrogen-induced hyperplasia and cell proliferation. Treatment with some, but not all, progestogens given sequentially in combination with continuous estrogen treatment resulted in increased endometrial thickness, but this has not been studied in a sufficiently rigorous fashion to draw any conclusion. Most women treated with estrogen only and, to a lesser extent, women who took the combined therapy had a proliferative or secretory type endometrial histology, whereas most untreated postmenopausal women have atrophic or inactive endometrial morphology.

(iii) *Other effects of hormonal therapy*

Conner *et al*. (2004b) (see above for study details) found statistically significant reductions in free and total serum testosterone and increases in sex hormone-binding globulin (SHBG) caused by the combination of 17β-estradiol and norethisterone, but no change in insulin-like growth factor (IGF)-I levels. However, the combination of 17β-estradiol and norethisterone (acetate) or dienogest did not alter total serum testosterone levels in another study (Conner *et al*., 2003; see above for details), although it also increased SHBG but did not affect IGF-I. Dören *et al*. (2001) and Hofling *et al*. (2005) reported essentially the same findings in postmenopausal women treated with 17β-estradiol (2 mg per day) and norethisterone acetate (1 mg per day) for 6 or 12 months but found a decrease in the circulating levels of IGF-binding protein-1 and -3, no effects on dehydroepiandrosterone or its sulfate and only minor effects on androstenedione. However, Chatterton *et al.* (2005) found a reduction in the level of dehydroepiandrosterone sulfate in women who took Prempro® (conjugated estrogens plus medroxyprogesterone acetate).

Other studies have investigated the effects of treatment with progestogen plus estrogen on the IGF axis in more detail and have found that a variety of progestogens in combination with 17β-estradiol result in decreases in total and free IGF-I and IGF binding protein-3, increases in IGF-binding protein-1 and no effect on IGF-II (Heald *et al*., 2000; Campagnoli *et al*., 2002). The magnitude of these effects appears to depend on the type of progestogens used (Biglia *et al*., 2003; Campagnoli *et al*., 2003), but apparently not on the route of administration of the estrogen and progestogens (Raudaskoski *et al*., 1998). However, in another study, significant differences were found between the effects of transdermal and oral treatment on IGF-I, SHBG and growth hormone-binding protein (Nugent *et al.*, 2003).

Another issue is the possibility that the various regimens used in hormonal therapy for the menopause may affect the metabolism of the hormonal agents used, as suggested by studies of estrogen metabolism (Seeger *et al*., 2000; Mueck *et al*., 2001, 2002) (see also Section 4.1).

These studies may suggest reduced androgenic stimulation, e.g. of the breast, and changes in the IGF axis. However, several of the progestogens used, such as norethisterone, have androgenic activity themselves and antigonadotropic effects reported for progestogens such as norethisterone may not be mediated by androgen receptor mechanisms (Couzinet *et al*., 1996). Furthermore, there is a lack of consistency in many observations, such as the inconsistent effects reported on the IGF axis. Nevertheless, these studies raise the possibility of complex interactions of the agents used in hormonal therapy for the menopause with various hormonal systems.

(*b*) *Experimental systems*

(i) *Animal studies*

Three studies of the effects of hormonal therapy regimens in cynomolgus monkeys that had been surgically rendered postmenopausal are summarized in Section 3.1.2 (Cline *et al*.,

1996, 1998; 2002a,b). In these studies, continuous treatment with conjugated equine estrogens slightly increased the rate of cell proliferation in the mammary gland after 2–3 years of exposure, but this increase was not statistically significant. Addition of medroxyprogesterone acetate to the continuous treatment with estrogen increased the rate of cell proliferation in the lobuloalveolar mammary tissue by 50–100% over control values. Staining of mammary tissue for progesterone receptor, an indicator of estrogenic activity, was markedly increased by treatment with estrogen only and this effect was reduced by the addition of progestogen to the treatment. A preliminary study (Isaksson *et al*., 2003) explored the effect of the same regimens on the immunohistochemical mammary expression of progesterone receptor-A and -B subtypes in cynomolgus monkeys. Treatment with progestogen alone did not significantly affect cell proliferation or expression of the progesterone receptor-A and -B. However, when ethinylestradiol plus norethisterone acetate was used as a regimen for only 1 year, cell proliferation was not increased (Suparto *et al*., 2003). One study of short duration in mice injected with 17β-estradiol and progesterone also found increased mammary cell proliferation in the combined estrogen plus progestogen group compared with the control groups (Raafat *et al*., 2001).

[These results are consistent with the observations in breast tissue of women who took conjugated equine estrogen plus medroxyprogesterone acetate as hormonal menopausal therapy but not those who took ethinylestradiol plus norethisterone.]

(ii) *Cell culture and other studies*

Studies of the effects of estrogen–progestogen combinations on breast cell proliferation *in vitro* were carried out with 17β-estradiol and a variety of progestogens, doses and treatment regimens. Lippert *et al*. (2000, 2001, 2002), Mueck *et al*. (2003) and Seeger *et al*. (2003a,b) determined the in-vitro effects of a range of progestogens on the proliferation of MCF-7 breast cancer cells induced by 10 nM 17β-estradiol, either combined for 5–7 days or sequentially using estrogen only for 4–5 days followed by combined exposure for 3–5 days. Although the results of these studies are not completely identical, they generally showed that norethisterone, medroxyprogesterone acetate, progesterone, chlormadinone acetate, dienogest, 3-keto-desogestrel, gestodene and levonorgestrel counteracted the cell proliferation induced by 17β-estradiol in these estrogen receptor-positive cells. The effects were stronger when exposure to the estrogen and progestogens occurred simultaneously for 5–10 days than when the progestogens were added 4–5 days after the start of estrogen treatment for 3–5 days in some, but not in all studies (Lippert *et al*., 2000, 2001). Although the consistency across these studies was not perfect, the continuous regimen with progesterone generally produced the strongest counteraction to the 17β-estradiol-induced stimulation of cell proliferation; medroxyprogesterone acetate gave an intermediate and norethisterone gave the weakest counteraction (Seeger *et al*., 2003a). The inhibitory effect required concentrations of progestogens greater than 1 nM. The equine estrogens, equilin and 17α-dihydroequilin, induced cell proliferation to a lesser extent than 17β-estradiol and progestogens inhibited their activity to a lesser extent than that of 17β-estradiol (Mueck *et al*.,

2003). [These findings suggest a protective effect on breast cancer of combined exposure to estrogen–progestogen but this does not correlate with the epidemiological data.]

Franke and Vermes (2002, 2003) and Franke *et al*. (2003) observed similar effects, but the potencies of progestogens to inhibit 17β-estradiol-induced cell proliferation differed from those found previously (Lippert *et al.*, 2001; Mueck *et al.*, 2003; Seeger *et al.*, 2003a,b), which may be related to the fact that they measured an indicator of cell proliferation, cyclin D, and not proliferation *per se*. They also found that apoptosis (measured by flow cytometry) was induced by 17β-estradiol and enhanced by progestogens, but the actual data were not presented in their reports. However, they used single high doses of both hormones (1 μM) and one time-point — 6 days of continuous treatment. Treatment of T47D human breast cancer cells with 17β-estradiol and medroxyprogesterone acetate resulted in a variety of changes in gene expression patterns (Mrusek *et al*., 2005). [Although the biological significance of these findings is not clear at present, the changes in gene expression patterns differed between treatments with estrogen only and those with estrogen plus progestogen.]

The endometrial effects of 17β-estradiol (10 nM) with or without medroxyprogesterone acetate (100 nM) were studied by Bläuer *et al*. (2005) in an organotypic culture system of primary human endometrial cells. 17β-Estradiol significantly doubled the percentage of cells that stained for Ki-67 over control values, whereas addition of the progestogen significantly reduced the percentage of Ki-67-positive cells to 50–70% of control values. The apparent effect of 17β-estradiol on cell proliferation required the presence of stromal cells and raised the possibility that the effect is indirect and stroma-mediated. These findings correlate with the supposition that the addition of progestogens to estrogen therapy confers a protective effect for the endometrium. Treatment of primary human endometrial cells with 17β-estradiol in the presence or absence of norethisterone acetate resulted in a variety of changes in gene expression patterns that differed depending on the presence of progestogen (Oehler *et al*., 2002). [Although the biological significance of these findings is not clear at present, the differences in gene expression patterns between treatment with estrogen only and estrogen plus progestogen may, once confirmed and extended, provide a mechanistic basis for differences in the known biological effects of the two treatments.]

4.2.2 *Individual estrogens and progestogens*

(*a*) *Humans*

No new data were available to the Working Group.

(*b*) *Experimental systems*

(i) *Estrogens*

Only one new study of estrogenic compounds that are used in hormonal therapy for the menopause (conjugated equine estrogens, ethinylestradiol or mestranol) that is rele-

vant to the evaluation of the carcinogenic risk of such therapy via the oral or other routes has been carried out since the previous evaluation (IARC, 1999).

17β-Estradiol has been shown to increase the generation of reactive oxygen species through anchorage- and integrin-dependent signalling to mitochondria. The 17β-estradiol-induced reactive oxygen species increased the phosphorylation of c-Jun and cyclic adenosine monophosphate-response element-binding protein and increased the transcriptional activity of redox-sensitive transcription factors, activator protein 1 and the phosphorylated element-binding protein; these are involved in growth of estrogen-dependent cancer cells (Felty *et al.*, 2005a). Inhibitors of protein synthesis, transcription and replication and function of mitochondria, as well as antioxidants, effectively reduced the estrogen-induced growth of breast cancer cells by blocking the estrogen-induced G_1/S transition of G_0-arrested MCF-7 cells (Felty *et al*., 2005b). These authors suggested that, in addition to the receptor activity of estrogens, other factors such as reactive oxygen species may be involved in the early growth of cancer cells (Felty *et al*., 2005b).

(ii) *Progestogens*

New studies of progestogens, including those most recently introduced, have been conducted that may be relevant to an evaluation of the carcinogenic risk of combined hormonal therapy via the oral or other routes, but no new studies were available on chlormadinone acetate, ethynodiol diacetate or norethynodrel.

Many studies described the influence of substituting active groups on the basic molecule of several progestogens — desogestrel, (3-keto-)desogestrel (etonogestrel), gestodene, levonorgestrel, norethisterone and drospirenone — on receptor binding, receptor transactivation and in-vivo hormonal activities (Deckers *et al*., 2000; Schoonen *et al*., 2000a; Garciá-Becerra *et al*., 2004); these are summarized in Section 4.2.3(*b*) of the monograph on Combined estrogen–progestrogen contraceptives in this volume.

A few studies have examined the role of estrogen and progesterone receptor subtypes on the activities of progestogens and their divergent tissue-specific effects. Estrogen receptor α but not estrogen receptor β appears to be activated by the A-ring 5α-reduced metabolites of both norethisterone and gestodene which have weak estrogenic activity (Larrea *et al*., 2001). However, Pasapera *et al.* (2002) found that the same metabolites of norethisterone activated both estrogen receptors α and β, and Rabe *et al.* (2000) obtained similar results for norethisterone but not for gestodene. These divergent findings may be related to the fact that the former study used HeLa and Chinese hamster ovary cells, whereas the latter studies used CV-1 monkey kidney cells and T-47D breast cancer cells or COS7 cells. Progesterone, norethisterone, levonorgestrel, desogestrol and gestodene are progestogens that are used in hormonal therapy and contraception. They bind with approximately equal affinity to the progesterone receptor subtypes A and B in MCF-7 cells, in Chinese hamster ovary cells stably transfected with these receptor subtypes and in in-vivo assays (Schoonen *et al*., 1998). However, after supertransfection of these receptor subtypes in different Chinese hamster ovary cell subclones, differences among the progestogens tested were found in the stimulation of reporter genes for the two receptor subtypes in diffe-

rent clones (Dijkema *et al*., 1998). These studies illustrate the critical roles of both metabolism and receptor-subtype specificity in the various hormonal effects of progestogens, while tissue or cell specificity appears to be another critical determinant of the activities of progestogens on receptor subtypes.

Progestogens may potentially affect not only factors that are related to tumour development and tumour cell growth. Some new evidence suggests that they may also affect factors that are related to tumour progression, such as angiogenesis. However, this is an emerging field of research that does not allow any conclusions to be drawn at present. For example, medroxyprogesterone acetate, progesterone, norethisterone, norgestrel and norethynodrel are mediated by the progesterone receptor B and have been shown to induce vascular endothelial growth factor in human breast cancer cells (Wu *et al*., 2004). Dienogest, on the contrary, was shown to inhibit tumour cell-induced angiogenesis (Nakamura *et al*., 1999) (See also Section 4.2.3(*b*) of the monograph on Combined estrogen–progestogen contraceptives).

Dydrogesterone inhibits the activity of estrogen sulfatase and 17β-HSD in the human breast cancer cell lines MCF-7 and T-47D and inhibits the conversion of estrone to 17β-estradiol in these cells (Chetrite *et al*., 2004).

Medroxyprogesterone acetate stimulated proliferation of the progesterone receptor-positive breast cancer cell line T-47D in a time-dependent manner with a biphasic dose–response (Thuneke *et al*., 2000). Induction of cyclin D1 was found to parallel the stimulation of cell proliferation at the same (fairly high) dose of 250 nM. In addition, medroxyprogesterone acetate appears to inhibit the induction of apoptosis by serum depletion of several human breast cancer cell lines at a non-cytotoxic dose of 10 nM. However, this effect was only found in progesterone receptor-positive cell lines and not in the progesterone receptor-negative cell line MDA-MB-231, which suggests that this is a progesterone receptor-mediated effect (Ory *et al*., 2001).

A study of norethisterone by Schoonen *et al*. (2000b) indicates that some of its various 3β- and 5α-reduced metabolites are much stronger estrogens or androgens *in vivo* than the parent compound. Their respective receptor-binding affinities and receptor-transactivation activities correlate with this observation. Rabe *et al.* (2000) found that norethisterone moderately transactivated estrogen receptor α in COS7 cells in a manner that was inversely related to dose. It transactivated estrogen receptor β somewhat more strongly; a concentration of 0.1 nM was strongly estrogenic (85% that of ethinylestradiol, which is 100% estrogenic) but higher (1 nM) and lower (0.01 nM) concentrations were far less estrogenic.

Nomegestrol acetate is a strong progestogen that is relatively devoid of other hormonal activities. Its properties have been reviewed by Shields-Botella *et al*. (2003).

Nestorone and trimegestone are newly synthesised progestogens that are less progestogenic than nomegestrol acetate but have activities that are in the same range as those of progesterone itself and are also relatively devoid of other hormonal activities. The properties and activities of nestorone have been reviewed and described by Kumar *et al*. (2000), Tuba *et al*. (2000) and Sitruk-Ware *et al*. (2003), and those of trimegestone by Zhang *et al*. (2000), Lundeen *et al*. (2001) and Winneker *et al*. (2003).

Progesterone is a natural progestogen that is used in a highly bioavailable, micronized form in hormonal therapy in combination with estrogens (de Lignières, 1999). Differential metabolism occurs in normal and malignant human breast tissue and results in 4-pregnene and 5α-pregnane metabolites that have opposite effects on MCF-7 breast cancer cell proliferation *in vitro* (Wiebe *et al*., 2000). The 5α-pregnane metabolites stimulate, whereas the 4-pregnene metabolites inhibit cell proliferation. Progesterone and 17β-estradiol affect IGF-I and IGF-binding protein -2 and -3 in a complex but non-synergistic manner, and not all of these effects can be blocked by the anti-estrogen tamoxifen or the anti-progestogen RU486 (mifepristone) (Milewicz *et al*., 2005a). Similarly, progesterone has been shown to stimulate local production of growth hormone in human breast cancer explants, which cannot be counteracted by RU486 (Milewicz *et al.*, 2005b). Progesterone inhibits the 17β-estradiol-stimulated proliferation of MCF-7 cells more strongly than either medroxyprogesterone acetate or norethisterone (Seeger *et al*., 2003a); this may have implications for the risk for breast cancer in women who are treated with either progesterone or a synthetic progestogen in combination with estrogen (Fournier *et al.*, 2005).

Medroxyprogesterone acetate or synthetic progestogen R5020 (as surrogate of progesterone) induced distinctly different changes in gene expression in progesterone receptor-negative Ishikawa endometrial cells that had been stably transfected with either progesterone receptor A or B (Smid-Koopman *et al*., 2005). In both cases, however, the cells responded to medroxyprogesterone acetate or synthetic progestogen R5020 by growth inhibition and induction of apoptosis, which suggests that there is no difference between the two subtypes in the molecular pathways involved in these responses. In a rat endometrial cell line that expresses progesterone receptor, however, progestogen R5020 prevented apoptosis; this was counteracted by RU486 (Pecci *et al*., 1997). [These results indicate that the progesterone receptor is required for these types of response of endometrial cells to progestogens, but suggest that the responses are highly cell type-specific.]

4.3 Side-effects other than genetic or cancer-related effects

Estrogen–progestogen therapy was designed to provide estrogen to women in order to relieve the vasomotor effects of the menopause and progestogen to modulate the adverse effects of estrogen on the uterus. The actual effects of the combination of these two types of hormone may differ from those of estrogen alone, depending on the target tissue considered. The addition of progestogen may ameliorate the adverse effects of estrogen at some sites, but counteract its possible beneficial effects at other sites. The complexity and interactions of estrogen–progestogen combinations should be borne in mind when considering these therapies.

4.3.1 *Cardiovascular effects*

It is generally believed that women are relatively protected from the development of coronary artery disease until the menopause: the incidence of cardiovascular disease in

women lags behinds that in men by approximately 20 years (Colditz *et al.*, 1987; Grundy *et al*., 1999). Ovarian hormones appear to be involved in the maintenance of these lower rates, since ovariectomized women who do not take hormonal therapy have an incidence of cardiovascular disease similar to that of men of the same age and, at any given age, postmenopausal women have a higher incidence of cardiovascular disease than women who menstruate normally (Wuest *et al.*, 1953; Gordon *et al.*, 1978).

A large body of evidence from observational studies has suggested that postmenopausal hormonal supplementation is associated with a 35–50% reduction in cardiovascular mortality and morbidity (Stampfer & Colditz, 1991). However, the majority of case–control and cohort studies on this subject have been conducted on women who used estrogen-only therapy. These hypothetical benefits have not been confirmed by several recent randomized trials of generally asymptomatic postmenopausal women (Hulley *et al.*, 1998; Herrington *et al.*, 2000; Manson *et al.*, 2003; Anderson *et al.*, 2004). Progestogens that are added to estrogens in combined hormonal therapy to reduce the risk for uterine malignancy have a number of potential adverse effects on the cardiovascular system, which may alter their efficacy in postmenopausal women. [Because combined hormonal therapy is so widely used, it is of pivotal importance to know whether or not it has an effect on cardiovascular disease.] Progestogens can have various effects on the vasomotor system, which are dependent on the agent and the dose regimen, and may also induce vasoconstriction of estrogenized vessels (Horwitz & Horwitz, 1982; Lin *et al.*, 1982).

It should be noted that hormonal therapy is designed to address symptoms that are related to reduced production of female hormones in the peri- and postmenopausal intervals. The menopause may involve changes other than cessation of estrogen and progestogen production, and the use of exogenous hormones may not mimic premenopausal physiology.

(*a*) *Biological effects of estrogens on the cardiovascular system*

The putative protective effect of estrogens on the cardiovascular system has for a long time been associated with their beneficial effect on the metabolism and deposition of cholesterol, which contributes to the inhibition of the formation of atherosclerotic plaque in the arterial walls (Bush & Barrett-Connor, 1985; Bush *et al.*, 1987). Although early reports suggested that up to 50% of the protective effect of estrogens on coronary artery disease was attributable to favourable changes in plasma lipids, it is now believed that the changes in lipids induced by estrogens are probably not relevant (Rossouw, 2000; Rossouw *et al.*, 2002).

Estrogen deprivation has been associated with an increased risk for coronary artery disease and poorer vascular functions in women (Wuest *et al.*, 1953; Gordon *et al.*, 1978; Colditz *et al.*, 1987). Acute and chronic administration of estrogens to estrogen-deficient individuals restores the endothelium-dependent vasodilatation of coronary arteries that is lost after the menopause (Herrington *et al.*, 1994; Collins *et al.*, 1995; Volterrani *et al.*, 1995).

In the past decade, it has become clear that ovarian hormones have significant effects on arterial blood flow (Gilligan *et al.*, 1994a; Reis *et al.*, 1994; Collins *et al.*, 1995). The vascular effects of ovarian hormones may differ according to their chemical structure, and it is important not only to differentiate the effects of estrogens from those of progestogens but also to distinguish between the effects of different estrogens. Estrogens induce vasodilation while estrogen depletion leads to vasomotor instability, diminished vasodilatory activity and enhanced sensitivity to vasoconstrictor stimuli (Kronenberg *et al.*, 1984; Penotti *et al.*, 1993; Gilligan *et al.*, 1994a; Herrington *et al.*, 1994; Reis *et al.*, 1994; Collins *et al.*, 1995). Ovarian hormones act at all levels of the arterial structure — the endothelium, the vascular smooth muscle and the nerve endings in the adventitia in almost all the arterial systems; they act very rapidly and at both non-genomic and genomic levels (Kronenberg *et al.*, 1984; Jiang *et al.*, 1991, 1992a; Penotti *et al.*, 1993; Gilligan *et al.*, 1994a; Herrington *et al.*, 1994; Reis *et al.*, 1994; Collins *et al.*, 1995).

(i) *Calcium-antagonistic action of estrogens*

Early in-vitro studies showed that 17β-estradiol has a relaxing effect on isolated rabbit and human coronary artery rings and cardiac myocytes contracted both by activation of receptor-operated and potential-operated calcium channels, due to a calcium-antagonistic effect (Jiang *et al.*, 1991, 1992a,b; Chester *et al.*, 1995). Subsequent in-vitro studies in animals and humans produced further evidence that estrogens have calcium-antagonistic properties, which account for a new non-endothelium-dependent mechanism of relaxation of coronary and peripheral arteries. The calcium-antagonistic property of estrogen was confirmed in coronary vascular myocytes by measuring cytosolic concentration, contraction and calcium current. Sudhir *et al.* (1995) demonstrated that estrogens cause dilation of coronary conductance and resistance arteries in dogs when administered acutely into the coronary circulation. This in-vivo effect was shown to be endothelium-independent and partially mediated by effects on calcium channels. Calcium-antagonistic properties of estrogen have also been demonstrated in uterine arteries, cardiac myocytes and vascular smooth muscle cells (Stice *et al.*, 1987; Jiang *et al.*, 1991, 1992a; Sudhir *et al.*, 1995). Since it has been proposed that calcium channel blockers may reduce the progression of atherosclerosis in animals, it has been suggested that estrogens may reduce the progression of coronary artery disease by a similar mechanism in humans (Collins *et al.*, 1993).

(ii) *Endothelial action of estrogens*

Another important component of the effect of estrogens on the vascular system is mediated through the endothelium. In-vivo studies have demonstrated that estrogens potentiate the endothelium-dependent vasodilator response to acetylcholine in the coronary arteries of animals and humans (Gilligan *et al.*, 1994a; Reis *et al.*, 1994; Collins *et al.*, 1995). The effect of estrogens on the restoration of altered endothelial function was demonstrated *in vitro*, and in animals and humans *in vivo* in different vascular beds. Williams *et al.* (1990) reported that a reversal of acetylcholine-induced vasoconstriction was produced by subcutaneous implants of 17β-estradiol in ovariectomized monkeys fed

an atherogenic diet for 30 months. Volterrani *et al.* (1995) showed that 17β-estradiol reduces peripheral vascular resistance and increases peripheral blood flow in menopausal women. Collins *et al.* (1995) showed that acute administration of 17β-estradiol reverses the coronary constrictor effect of acetylcholine in postmenopausal women with coronary artery disease and that the effect is gender-dependent. Reis *et al.* (1994) demonstrated an increase in coronary blood flow and epicardial cross-sectional area and decreased resistance in postmenopausal women 15 min after an intravenous infusion of ethinylestradiol. Abnormal coronary vasomotor responses to acetylcholine were also attenuated. Similar results were obtained by Gilligan *et al.* (1994a,b) in women who received continuous infusions of estrogens to achieve physiological concentrations of intracoronary 17β-estradiol. These studies indicated that estrogens influence vascular tone by enhancing the production of an endothelium-derived relaxant factor (nitric oxide). Estrogen receptors have been identified in endothelial cells from human aorta, and coronary and umbilical arteries (Kim-Schulze *et al*., 1996; Venkov *et al*., 1996). Caulin-Glaser *et al.* (1997) demonstrated that estradiol induced a rapid (within 30 min) increase in the production of nitric oxide in human umbilical vein endothelial cells grown in cell culture and that this action was inhibited when the estradiol receptor was blocked. Estrogen can induce calcium-dependent nitric oxide synthase activity, which results in an increased release of nitric oxide. Using inhibitors of nitric oxide synthase, Tagawa *et al.* (1997) demonstrated that estrogens considerably improve both nitric oxide-mediated and non nitric oxide-mediated vasodilation in the peripheral vasculature of the forearm in humans.

Since coronary artery tone plays a significant role in the pathogenesis of ischaemic cardiac syndromes, the effect of estrogens on the reactivity of coronary arteries may be important to the cardiovascular effects of these hormones. Rosano *et al.* (2006) showed that intracoronary administration of 17β-estradiol attenuates the vasoconstrictor effect of methylergometrine in women with coronary artery disease, which suggests that 17β-estradiol has a direct effect on the smooth muscle of coronary vessels in humans.

(iii) *Inflammatory effects of estrogens*

C-Reactive protein is a marker of inflammation that has been associated with the risk for coronary heart disease in menopausal women (Ridker *et al.*, 2000; Pradhan *et al.*, 2002). Estrogen administered with or without progestogen rapidly and substantially increases plasma concentrations of C-reactive protein in menopausal women (van Baal *et al*., 1999; Cushman *et al.*, 1999; Ridker *et al.*, 1999). The effects of hormonal therapy on other markers of inflammation are not consistent with those on C-reactive protein, which suggests that they may be related to metabolic hepatic activation (Silvestri *et al.*, 2003). [The extent to which hormonal therapy affects cardiovascular risk through inflammation is unknown.]

(iv) *Route of administration*

To establish differences in alteration of some risk factors for cardiovascular disease (such as lipoproteins, fibrinogen) according to the route of administration of hormonal

menopausal therapy, a study was carried out among women aged 50–65 years who received hormones orally, transdermally or by implantation. Initial therapy consisted of oral or transdermal estrogen alone for 3 months, followed by concomitant cyclical, continuous oral or transdermal administration of norethisterone, as appropriate, for a further 3 months. A separate group of women received an implant of estrogen only followed by an implant of estrogen and testosterone combined. All regimes lowered LDL cholesterol; the oral route was more potent than the parenteral route. Risk factors for cardiovascular disease were significantly reduced in women who used both oral and transdermal estrogen–progestogen therapy compared with untreated menopausal women (controls), although some of the benefit of estrogen alone on fibrinogen and HDL were attenuated (Seed *et al.*, 2002).

(*b*) *Biological effects of progestogens on the cardiovascular system*

Progesterone receptors are present in the arterial wall, and the effects of progestogens on arteries are therefore probably mediated by these receptors as well as by down-regulation of the estradiol receptor. Progestogen therapy has various effects on arterial function; it can stabilize arteries that are in a state of vasomotor instability, but may also induce vasoconstriction of estrogenized vessels (Mercuro *et al.*, 1999). Because progestogens have estrogenic effects in some systems and also have progestational mineralo-corticoid and androgenic effects, there has been some concern that combined estrogen–progestogen therapy may modify some of the effects of estrogens on the cardiovascular system (Williams *et al.*, 1994; Adams *et al.*, 1997).

(i) *Progestogens and lipid profile*

Estrogen-only therapy lowers total serum cholesterol and LDL cholesterol, increases HDL cholesterol and produces an effect upon plasma triglycerides, which is dependent upon the route of administration of the estrogens and the baseline plasma levels of lipids (Walsh *et al.*, 1991). It also stimulates the removal of cholesterol from the systemic circulation, which results in an increase in reverse cholesterol transport (Tikkanen *et al.*, 1982).

In contrast to estrogens, progestogens induce hepatic lipase activity, which increases the degradation of HDL-cholesterol. Accordingly, the addition of a progestogen to estrogens tends to attenuate the increase in serum HDL-cholesterol and the decrease in LDL-cholesterol that are obtained with estrogen only, an effect that may be related to the biochemical structure, dose, androgenic potency and regimen of the progestogen. Progestogens that have pure progestogenic activity do not alter lipid metabolism; 19-nortestosterone derivatives reduce HDL cholesterol, while 17α-hydroxyprogesterone derivatives and non-androgenic progestogens (19-norpregnane derivatives) seem to have little effect and progesterone has no detrimental effect on plasma lipids (Tikkanen *et al.*, 1986; Rijpkema *et al.*, 1990; Walsh *et al.*, 1991; The Writing Group for the PEPI Trial, 1995).

(ii) *Progestogens and coronary atherosclerosis*

The vasodilatory and anti-atherogenic effects of estrogens on normal and diseased arteries are well known. Estrogens may influence the progression of coronary atherosclerosis and, when administered either acutely or chronically, may reverse acetylcholine-induced vasoconstriction in both animals and humans. When administered in combination with estrogens, progestogens may interfere with these effects.

Adams *et al.* (1997) evaluated the effect of estrogen-only and estrogen–progestogen therapy in ovariectomized monkeys fed an atherogenic diet and found that estrogens alone or in association with progesterone or medroxyprogesterone acetate significantly reduced (by 50–70%) the degree of coronary atherosclerosis when therapy began soon after oophorectomy, but not when it began later (Williams *et al.*, 1994, 1995; Adams *et al.*, 1997; Clarkson *et al.*, 1998, 2001). Nevertheless, it should be noted that the effect of progestogens on the arteries of non-human primates may be mediated by different metabolic pathways than those present in human arteries. Therefore, the effects of progestogens on atherosclerosis and vascular function cannot be extrapolated fully from animals to humans.

In a randomized, placebo-controlled trial, Herrington *et al.* (2000) compared the effect of hormonal supplementation with conjugated equine estrogens alone or in combination with medroxyprogesterone acetate on the progression of coronary atherosclerosis in normocholesterolaemic postmenopausal women (aged ≥ 55 years) with proven coronary artery disease. After a mean follow-up of 3.2 years, no significant difference in mean coronary artery stenosis was found between women allocated to estrogen alone, estrogen plus progestogen or placebo.

The evaluation of intima-media thickening of arteries may help to identify initial stages of atherosclerosis. Several studies have shown that long-term estrogen therapy or combined hormonal therapy are effective in delaying the progression of early stages of atherosclerosis by reducing the intimal thickening in users of hormone compared with non-users (Espeland *et al.*, 1995a; Liang *et al.*, 1997; Hodis *et al.*, 2001).

At present, no role has been established for combined hormonal therapy in the prevention of the progression of atherosclerosis in postmenopausal women. From a preventive aspect, the inhibition of plaque formation and progression of small plaques is more important than a reduction in the size of pre-existing atherosclerotic plaques.

(iii) *Progestogens and vascular reactivity*

Progestogens have vasoactive properties that are partly mediated by non-nuclear receptors. Since the expression of these receptors on the cell surface is influenced by levels of circulating estrogen, exposure to estrogens may affect the response of the vascular tree to progestogens.

Several studies have evaluated the effect of progesterone and other progestogens on coronary arteries *in vitro* and have demonstrated an endothelium-independent mechanism of relaxation that differs minimally between a variety of substances (Jiang *et al.*, 1992c). Miller and Vanhoutte (1991) assessed relaxation in coronary artery strips from ovariecto-

mized dogs treated with estrogen, progesterone or estrogen plus progesterone. The relaxation response was similar in the coronary arteries of animals that received estrogen and in those that received progesterone, while it was minimally reduced in the group treated with the combined therapy. Therefore, it seems that there is little or no detrimental effect of progesterone on vasomotility, at least *in vitro* (Rosano *et al*., 2003). However, pure progesterone is not commonly used in combined hormonal therapy.

Studies carried out *in vivo* suggested that synthetic progestogens may antagonize the dilator effect of estrogens in experimental animals. Two studies evaluated the separate and combined effects of conjugated equine estrogens and medroxyprogesterone acetate on the coronary reactivity of atherosclerotic monkeys. Exposure to estrogen increased coronary dilator responses and blood flow reserve, while co-administration of the progestogen resulted in a 50% reduction in dilation (Williams *et al.*, 1994; Adams *et al*., 1997). Again, the effect of synthetic progestogens cannot be fully translated to women, due to the different metabolic pathways that operate in animals and humans.

Androgenic progestogens have been reported to reduce the beneficial effect of estrogens on vascular reactivity to a greater extent than progesterone and less androgenic progestogens; similar results were found in studies of carotid artery stiffness (Vitale *et al.*, 2001; Rosano et *al*., 2001; Gambacciani *et al*., 2002; Rosano *et al*., 2003).

(*c*) *Biological effects of hormones on risk for thrombosis*

Estrogens have many different effects on the coagulation system. These include increases in the levels of prothrombin fragments 1 + 2 and reductions in those of the anticoagulant factors, protein S and antithrombin, and may also occur after transdermal administration (Teede, *et al*., 2000; Post *et al.*, 2003). These modifications predict a change towards a more pro-coagulant state (which was confirmed in studies that examined activated protein C resistance or thrombin generation) that is not counterbalanced by an increase in fibrinolytic activity (Teede *et al*., 2000). It is currently unclear how these effects are induced at the molecular level of the estrogen receptor. At the cellular level, these effects are probably under genetic control, because the haemostatic system of some women appears to be more sensitive to the effect of estrogens than that of others. How estrogens and progestogens interact in their effect on thrombosis is also unclear. It appears that estrogens are pro-thrombotic rather than pro-atherogenic, which explains an increase in risk for thrombosis in former users (Bloemenkamp et *al*., 1998; Koh *et al.*, 1999); again genetic factors may play an important role (Bloemenkamp *et al.*, 2002).

Selective modulators of the estrogen receptor, such as tamoxifen and raloxifene, have anti-estrogenic effects on breast and endometrial tissue and are used in the treatment and prevention of breast cancer. However, these drugs have estrogenic effects on blood clotting (Peverill, 2003).

(*d*) *Effects of the use of estrogen and estrogen plus progestogen on the risk for cardiovascular disease*

(i) *Coronary heart disease*

The effects of hormonal therapy on coronary heart disease have been evaluated in two randomized trials. The HERS and the WHI have provided critical data on the effect of hormonal therapy on primary and secondary prevention in postmenopausal women with or without coronary artery disease (Hulley *et al.*, 1998; Rossouw *et al.*, 2002). Neither study found a protective effect of fixed-dose hormonal therapy. For continuous combined hormones, the WHI reported a hazard ratio for coronary heart disease of 1.24 (95% CI, 1.00–1.54) over an average of 5.2 years of follow-up; most of the apparent risk occurred during the first year (hazard ratio, 1.81; 95% CI, 1.09–3.01). These effects did not differ by age (Manson *et al.*, 2003). In the HERS secondary prevention trial, the hazard ratio for coronary heart disease for the same combined hormonal therapy over an average of 4.1 years of follow-up was 0.99 (95% CI, 0.80–1.22) with some evidence of an increased risk in the first year (Hulley *et al.*, 1998). The WHI found no significant effects on the risk for coronary heart disease of estrogen alone over an average of 6.8 years of follow-up (hazard ratio, 0.91; 95% CI, 0.75–1.12). The data suggested the possibility that younger women experience a reduction in risk (*p* value for interaction, 0.14; hazard ratio, 0.56; 95% CI, 0.30–1.03) (Anderson *et al.*, 2004).

Few epidemiological studies have investigated the effect of combined hormones, but all have suggested that estrogen plus progestogen therapy may be more effective than estrogen therapy alone in the reduction of cardiovascular events. The majority of the observational studies suggested that the risk for coronary artery disease was reduced in women who received both estrogen only and estrogen–progestogen therapy (Stampfer & Colditz, 1991).

All of the observational studies were conducted in healthy women who used hormonal therapy for reasons other than the menopause and who were generally at low risk for coronary heart disease. The effect of the adjunct of a progestogen to estrogens in women who are at increased risk for cardiovascular disease may differ. The discrepancies with the randomized trials require that the observational studies be viewed cautiously. Biases inherent in cohort and case–control studies and variability of dosing, duration and other time-dependent effects of hormonal therapy must be taken into account in order to present a balanced view of the results. Careful control for confounding and an allowance for an adverse effect during the first 2 years of exposure, with attenuation of this effect in subsequent years, has been shown to align the results from observational studies with those of randomized trials (Prentice *et al.*, 2005). Petitti (1994) argued that compliance bias could account for some of the observed benefits. It has been suggested that the women who were included in the hormonal therapy groups were of higher socioeconomic class, had healthier habits and exercised regularly. Barrett-Connor (1991) demonstrated that a healthy women bias exists at least for the women who were included in the Rancho Bernardo (CA, USA) study population (Barrett-Connor *et al.*, 1989). Not all study populations were restricted to upper class

retirement areas, however, and no socioeconomic differences were noted in the Nurses' Health Study (Grodstein *et al.*, 2000) or other studies. Selection bias probably exists in nearly all studies, since women who take hormonal therapy tend to exercise more and have healthier habits. Therefore, estimates of risk reduction in women who take ovarian hormones may be biased towards finding a protective effect that may be due in part to a healthier lifestyle (Nelson *et al.*, 2002a).

(ii) *Stroke*

The WHI trial reported an increase in risk for stroke from estrogen plus progestogen therapy (hazard ratio, 1.31; 95% CI, 1.02–1.68) that was restricted to ischaemic stroke (hazard ratio, 1.44; 95% CI, 1.09–1.90) (Wassertheil-Smoller *et al.*, 2003). The WHI trial of estrogen alone was interrupted because of the observed increase in risk for stroke (hazard ratio, 1.39; 95% CI, 1.10–1.77) (Anderson *et al.*, 2004). The HERS trial reported a hazard ratio for estrogen plus progestogen of 1.18 (95% CI, 0.83–1.66) for non-fatal stroke and 1.61 (95% CI, 0.73–3.55) for fatal stroke during an average follow-up of 4.1 years (Simon *et al.*, 2001a). The Women's Estrogen for Stroke Trial conducted on 664 postmenopausal women who had had a recent ischaemic stroke or a transient ischaemic attack found no beneficial effect of 17β-estradiol (1 mg per day) for stroke (hazard ratio, 1.1; 95% CI, 0.8–1.6) or mortality (hazard ratio, 1.2; 95% CI, 0.8–1.8) after a mean follow-up of 2.8 years (Viscoli *et al.*, 2001). A meta-analysis of randomized trials found a significant increase in risk for stroke (relative risk, 1.44; 95% CI, 1.10–1.89) with no substantial variation between studies (Gabriel-Sanchéz *et al.*, 2005).

A meta-analysis of nine observational studies suggested that hormonal therapy is associated with a small increase in risk for stroke (relative risk, 1.12; 95% CI, 1.01–1.23) that is primarily confined to thromboembolic stroke (relative risk, 1.20; 95% CI, 1.01–1.40) (Nelson *et al.*, 2002b).

(iii) *Thrombosis*

The risk for venous thrombotic disease was increased twofold (hazard ratio, 2.06; 95% CI, 1.57–2.70) for estrogen plus progestogen in the WHI trial (Cushman *et al.*, 2004) and almost threefold in the HERS trial (hazard ratio, 2.89; 95% CI, 1.50–5.58) (Hulley *et al.*, 1998). Pulmonary embolism and deep vein thrombosis were similarly affected. The WHI trial reported a smaller increase with estrogen alone (hazard ratio, 1.33; 95% CI, 0.99–1.79) (Anderson *et al.*, 2004).

(*e*) *Estrogen–progestogen therapy in postmenopausal women*

The divergent results of observational and randomized studies on cardiovascular endpoints have led many authors to stress the superiority of randomized clinical trials over observational studies, but have not solved the dilemma of the effect of hormonal therapy on the cardiovascular system. The discrepancies in the results of observational and randomized studies are related to methodological issues and to differences between study populations, hormonal regimens and time- and age-dependent biological effects of hormones

during different periods of the lives of women. Observational studies typically did not distinguish between the hormonal regimens used, whereas the randomized studies examined a fixed dose of continuous combined conjugated equine estrogens plus medroxyprogesterone acetate. Although the treatment regimens in the two types of study differed, the dose of estrogen and progestogen used probably played an important role. More recent progestogens that have fewer androgenic and mineralo-corticoid effects may influence the risks for cardiovascular disease differently. Several varied opinions on the potential cardiovascular effects — beneficial and adverse — of hormonal therapy for postmenopausal women are still emerging, and the selection of patients, dose regimen and timing of treatment are probably critical (Rosano *et al.*, 2004).

It is possible that factors other than methodological differences may explain the divergent effects of hormonal therapy noted in observational and randomized studies. The randomized clinical studies were conducted exceptionally well and methodological design is not an issue. A key difference between the observational and randomized studies is the women under study: in the observational studies, the exposed women chose to take hormonal therapy for menopausal symptoms and represented long-term compliers while, in the randomized studies, the absence of severe menopausal symptoms was a prerequisite for inclusion in the study. This seemingly small difference may have important implications, since symptomatic women are younger and have clinical symptoms that suggest an effect of a lack of estrogen on several organs or systems. The low prevalence of symptoms may indicate a physiological adaptation to lower levels of ovarian hormone in these women, due to a slow decline in estrogen levels or the long time lapse since menopause, and therefore a new homeostasis. These and other biological explanations for the divergent results of observational and randomized studies should be considered in detail (Rosano *et al*., 2004).

(i) *Ageing and cardiovascular response to estrogen–progestogen therapy*

Several studies that evaluated the effect of hormonal therapy on intermediate markers of coronary heart disease in women and in non-human primates indicated substantial benefits, although they also suggested some adverse effects (Scarabin *et al.*, 1999; Walsh *et al.*, 2000; Silvestri *et al.*, 2003). Clinical and experimental evidence suggests that the putative cardio-protective and anti-atherogenic effects of ovarian hormones are receptor-mediated and endothelium-dependent (Caulin-Glaser *et al.*, 1997; Mikkola *et al.*, 1998). Both estrogen receptors and endothelial function are markedly influenced by the time at which estrogen deprivation and progression of the atherosclerotic injury occur. Evidence indicates that expression of the estrogen receptor in the arterial wall is sharply diminished with increasing age, which might be related to an age-related increase in methylation of the promoter region of the estrogen receptor gene in vascular areas with atherosclerosis (Post *et al.*, 1999).

Time since menopause and the presence of atherosclerosis are associated with a reduced cardio-protective effect of estrogens, while the unfavourable effects of hormones

on coagulation remain unaltered. Therefore, in early postmenopausal women such as those included in observational studies, hormonal therapy may be cardio-protective because of the responsiveness of the endothelium to estrogens while, in late postmenopausal women, hormonal therapy has either no effect or even a detrimental effect because of the predominance of the pro-coagulant or plaque-destabilizing effects over the vasculo-protective effects. It is possible that hormonal therapy inhibits atherosclerosis in younger women but may not be able to inhibit progression of atherosclerosis and complicated plaques that lead to coronary events in older women. This hypothesis has also been suggested by randomized studies of postmenopausal cynomolgus monkeys in which estrogens had no effect on the extent of coronary artery plaque in those assigned to estrogen alone or to estrogen combined with medroxyprogesterone acetate beginning 2 years (approximately 6 human years) after oophorectomy (Williams *et al*., 1995). When given to younger monkeys soon after oophorectomy, hormonal treatment resulted in a 50% reduction in the extent of plaques (Clarkson *et al*., 2001; Mikkola & Clarkson, 2002).

The effect of atherosclerosis and ageing on the vascular responsiveness to hormonal menopausal therapy has been also analysed in several clinical studies (Herrington *et al.*, 2001). In the Cardiovascular Health Study, women (over 65 years of age) who had established cardiovascular disease had a flow-mediated vasodilator response that was equal among women who used hormones (estrogen alone or estrogen combined with progestogen) and those who did not; among women (over 65 years of age) who had no cardiovascular disease, users of hormones had a 40% better response than non-users (Herrington *et al.*, 2001). In the Estrogen Replacement and Atherosclerosis Trial, a randomized trial that involved women (aged ≥ 55 years) who had documented coronary disease, no effect of estrogen alone or of estrogen combined with progestogen was found on the diameter of coronary arteries (Herrington *et al*., 2000). In contrast, in the Estrogen in the Prevention of Atherosclerosis Trial, in which younger women (aged ≥ 45 years) who had no cardiovascular disease were randomly assigned to 17β-estradiol or placebo, the average rate of progression of carotid atherosclerosis was lower in women assigned to estrogen (Hodis *et al.*, 2001).

(ii) *Characteristics of the study populations*

Women recruited in the randomized studies comprised a broader age range and were representative of individuals for whom prevention interventions would be contemplated. Observational studies recruited women who were exposed to hormonal therapy primarily for short-term relief of menopausal symptoms and longer-term use for the prevention or treatment of osteoporosis. Recently, a new class of progestational agents with anti-aldosteronic properties has been developed: the prototype of this class is drospirenone, a progestational agent that can reduce water retention, body weight and blood pressure (Keam & Wagstaff, 2003). The addition of this newer progestogen to estrogens in hormonal therapy schemes may help to minimize the side-effects of estrogen therapy that are related to water and salt retention and may represent a new strategy for the treatment of postmenopausal women (Pollow *et al.*, 1992; Krattenmacher, 2000).

In a randomized trial of 230 hypertensive postmenopausal women, treatment with drospirenone plus 17β-estradiol was not associated with a higher incidence of hyperkalaemia than treatment with placebo in patients with and without type-2 diabetes mellitus and concomitant use of angiotensin-converting enzyme inhibitors, angiotensin receptor antagonists or ibuprofen. Drospirenone plus 17β-estradiol was found to reduce both systolic and diastolic blood pressure compared with the placebo group (Preston *et al.*, 2005).

Body mass index is an important marker of endogenous estrogen in postmenopausal women and has been associated with risk for cardiovascular disease especially when it is ≥ 25 kg/m^2 (Wilson *et al.*, 2002). In a very large cohort of 290 827 postmenopausal women, the coronary benefits of hormonal therapy were found exclusively in women with a lower body mass index (< 22 kg/m^2) (Rodriguez *et al.*, 2001). In the WHI, there was no significant interaction with body mass index, which mean was 28.5 (standard deviation [SD], 5.9) (Manson *et al.*, 2003).

Randomized studies of hormones in younger women who seek treatment for menopausal symptoms are not informative with regard to hormonal effects on rates of cardiovascular disease, because of the very low incidence rates in this population.

4.3.2 *Other effects*

(*a*) *Established benefits*

(i) *Control of vasomotor symptoms*

The primary indication for use of hormonal therapy during the menopause is vasomotor symptoms. Numerous studies have documented the beneficial effects of estrogen, either alone or combined with progestogen, for the relief of hot flushes/flashes and night sweats. MacLennan *et al.* (2004b) recently reviewed the randomized, double-blind placebo controlled trials of hormonal therapy and reported a summary measure of the reduction in the frequency of hot flushes of 75% (95% CI, 64.3–82.3%) relative to placebo, accompanied by similarly important reductions in the severity of symptoms.

(ii) *Prevention of osteoporosis and fractures*

Hormonal therapy has also been shown to be effective for the prevention or treatment of osteoporosis and bone fractures. In the WHI trial, the risk for hip fractures was reduced by both estrogen alone (hazard ratio, 0.61; 95% CI, 0.41–0.91) and by estrogen plus progestogen (hazard ratio, 0.67; 95% CI, 0.47–0.96) (Cauley *et al.*, 2003; Anderson *et al.*, 2004). Beneficial effects on bone mineral density have been shown for other hormonal preparations (Recker *et al.*, 1999; Lees & Stevenson, 2001; Arrenbrecht & Boermans, 2002; Civitelli *et al.*, 2002).

(*b*) *Overview of evidence for other effects*

The efficacy of hormonal therapy for the above indications has typically been established in randomized trials of up to a few hundred women and a follow-up of a few months for vasomotor symptoms and 3 years for osteoporosis. These trials lack sufficient

power to establish rates of disease or rarer side-effects with adequate precision. Further, because of the strong evidence for beneficial effects on vasomotor symptoms, such trials often lack a placebo group, which obscures any inference of hormonal effects on other outcomes. Two large randomized trials, the WHI (Rossouw *et al*., 2002) and the HERS (Grady *et al.*, 1998) are the primary exceptions. Both of these were randomized, double-blinded, placebo-controlled trials of hormonal therapies that tested chronic disease prevention strategies in the USA. The WHI trial involved separate placebo-controlled comparisons of estrogen plus progestogen (conjugated equine estrogens plus medroxyprogesterone acetate) in 16 608 postmenopausal women aged 50–79 years with an uterus and estrogen alone in 10 739 postmenopausal women in the same age range with prior hysterectomy (Stefanick *et al*., 2003). The HERS trial tested the same combined hormonal regimen in 2764 postmenopausal women under 80 years of age who had a uterus and documented coronary artery disease. Additional details for both trials are provided in Section 2. Because of the strength of the evidence derived from these two studies, most of the information summarized here on other effects of hormonal therapy relies on data from these trials. For many outcomes, important additional information derives from the Postmenopausal Estrogen/Progestin Interventions (PEPI) study, another randomized, double-blinded, placebo-controlled trial conducted in the USA to evaluate the effects of three combined hormonal regimens and one estrogen-alone therapy on biomarkers of cardiovascular disease in 840 postmenopausal women aged 45–65 years (Espeland *et al.*, 1995b). Data from other randomized trials are described only when they contradict these findings or introduce different inferences. There is also an immense body of data from observational studies that include many features of health that cannot be summarized adequately here. Mention is made of these data only when significant controversy exists between the findings from trials and observational studies.

(i) *Quality of life and symptoms associated with menopause or ageing*

Quality of life

Improvement in other symptoms that are commonly associated with ageing or menopause have been reported, occasionally in conjunction with overall quality of life. The reported benefits vary across studies; the differences are probably attributable to the populations studied and the dimensions of symptoms and quality of life used. No clinically significant effects on measures of general quality of life were observed with estrogen plus progestogen therapy in the WHI trial (Hays *et al.*, 2003a) despite significant improvements in vasomotor symptoms and vaginal or genital dryness (Barnabei *et al.*, 2005). In the HERS trial, estrogen plus progestogen decreased hot flushes, vaginal dryness and sleep troubles (Barnebei *et al.*, 2002) and improved depression scores, but was associated with worsening of some health measures (i.e. more rapid decline in physical function scores and in energy/fatigue) (Hlatky *et al.*, 2002). Trials designed specifically to test the effect of hormonal therapy on vasomotor symptoms as their primary objective generally reported an

improvement in quality of life, derived primarily from the relief of hot flushes, night sweats and related conditions (e.g. Wiklund *et al.*, 1993).

Vaginal bleeding

Common side-effects of hormonal therapy are vaginal bleeding, breast tenderness, urinary incontinence and headaches. Among women with a uterus, bleeding rates vary by type of hormonal therapy, schedule of progestogen, age and time since initiation of therapy. The prevalence of bleeding with both sequential and continuous formulations has led to numerous attempts to identify hormonal therapy regimens that provide good relief of vasomotor symptoms while minimizing bleeding (e.g. Saure *et al.*, 1996; Al-Azzawi *et al.*, 1999; Cano *et al.*, 1999; Saure *et al.*, 2000; Mendoza *et al.*, 2002). Intermittent and continuous treatment with progestogen has been used to reduce bleeding, especially in older women. In the WHI estrogen plus progestogen trial, 51% of women randomized to continuous combined therapy but less than 5% of women who took placebo reported vaginal bleeding within the first 6 months. The proportion who reported bleeding in the active hormonal therapy group declined thereafter but remained above 11% throughout the study (Barnabei *et al.*, 2005). These estimates do not take into account the non-compliance to the therapy (approximately 42% by year 5) (Rossouw *et al.*, 2002), some of which was a result of bleeding. Most of the bleeding in the active hormonal therapy group was classified as spotting (Barnabei *et al.*, 2005). Bleeding, especially after the first few months of use, causes concern and may lead to significantly increased rates of endometrial biopsy (Anderson *et al.*, 2003). Intermittent treatment provides only a marginal improvement in bleeding rates (Cano *et al.*, 1999), but some studies have found reduced bleeding with higher doses of progestogen (Al-Azzawi *et al.*, 1999). Many smaller randomized trials lacked either a placebo control or a common active treatment control group against which to judge the relative effects. Nevertheless, few significant differences in symptom control or vaginal bleeding were reported.

Breast symptoms

The frequency of breast symptoms, variously reported as breast tenderness, discomfort, pain or mastalgia, is also significantly increased by hormonal therapy. In the WHI trial, 9.3% of asymptomatic women randomized to combined hormones reported breast tenderness at year 1 compared with 2.4% who took placebo, and this proportion remained elevated at year 3 (odds ratio, 2.55; 95% CI, 0.98–6.64) (Barnabei *et al.*, 2005). In the PEPI trial, the risk for greater breast discomfort was doubled by all three combined hormonal regimens relative to placebo (range of odds ratios, 1.92–2.33). Corresponding risks for estrogen alone were not increased over those for placebo (odds ratio, 1.16; 95% CI, 0.70–1.93) (Greendale *et al.*, 1998), which suggests that these breast symptoms may be an effect of protestogen.

Mammographic screening

Use of exogenous hormones interferes with mammographic screening. In the WHI trial, Chlebowski *et al.* (2003) reported that 9.4% of women assigned to combined hormones had an abnormal mammogram during the first year of use compared with 5.4% of placebo-treated women ($p < 0.001$), a period during which there was no excess incidence of breast cancer. This pattern of increased incidence of mammographic abnormalities persisted throughout the follow-up. In a study ancillary to the PEPI trial, Greendale *et al.* (2003) reported increased breast density with all three combined hormonal regimens (change from baseline in adjusted mean mammographic per cent density ranged from 3.1 to 4.8%), which was significantly different from the rate of change in the placebo group (–0.07%; 95% CI, –1.50–1.38%). Use of estrogen alone resulted in a non-significant 1.17% (95% CI, –0.28–2.62%) adjusted mean change, which again suggests that progestogen is the active agent in these breast changes. However, in a randomized, double-blinded, placebo-controlled multi-arm trial of raloxifene and estrogen, the mean breast density in women who took estrogen was significantly greater than that in the other arms (Freedman *et al.*, 2001).

Urinary incontinence

Urinary incontinence is adversely affected by hormonal therapy. Asymptomatic women randomized to estrogen alone in the WHI Trial experienced an increased risk for self-reported urinary incontinence at 1 year, including all subtypes: stress (hazard ratio, 2.15; 95% CI, 1.77–2.62), urge (hazard ratio, 1.32; 95% CI, 1.10–1.58) and mixed urinary incontinence (hazard ratio, 1.79; 95% CI, 1.26–2.53). The addition of medroxyprogesterone acetate did not alter these effects substantially. Among women who reported urinary incontinence at baseline, the risk for worsening the self-reported frequency of incontinence, amount of leakage, limitation in activities and bother associated with the symptoms was significantly elevated by both hormonal regimens at 1 year (Hendrix *et al.*, 2005). In a 3-year randomized, double-blind, placebo-controlled osteoporosis prevention trial (Goldstein *et al.*, 2005), 7% of 158 women randomized to conjugated equine estrogens reported new or worsening urinary incontinence compared with 1.3% of the 152 women randomized to placebo ($p \leq 0.02$). In the HERS trial, Grady *et al.* (2001) found that 39% of women who took estrogen plus progestogen reported worsening symptoms compared with 27% of women who took placebo ($p = 0.001$). Several smaller, short-term, randomized, double-blind, placebo-controlled trials of hormonal therapy have been carried out in incontinent women (Wilson *et al.*, 1987; Fantl *et al.*, 1996; Jackson *et al.*, 1999); some of these used objective measurements of response, but none identified any significant therapeutic response.

Headaches

Frequency and duration of headaches may be affected by hormonal therapy. In the WHI trial, the incidence of headaches or migraines in the placebo-treated group was 4.7% and was modestly increased by estrogen plus progestogen at 1 year to 5.8% (odds ratio,

1.26; 95% CI, 1.08–1.46) (Barnabei *et al.*, 2005). Vestergaard *et al.* (2003) did not find an effect on the occurrence of headache after 5 years in the Danish Osteoporosis Prevention Study. In women who are known to suffer from migraine headaches, however, there is evidence of an increase in the frequency of attacks, the number of days with headache and analgesic consumption over 6 months of observation during continuous combined, continuous sequential or cyclical sequential hormonal therapy (Facchinetti *et al.*, 2002).

(ii) *Incidence of disease*

In addition to the more common symptoms associated with hormonal therapy, there is increasing evidence of hormonal effects on other clinical outcomes.

Gallbladder disease

Randomized trials have shown a significant increase in the rates of gallbladder disease and biliary tract surgical procedures following hormonal therapy. In the WHI trial, Cirillo *et al.* (2005) reported a hazard ratio for estrogen alone of 1.67 (95% CI, 1.35–2.06) for the incidence of hospitalized gallbladder disease or related surgical procedures over an average of 7.1 years of follow-up and a hazard ratio for estrogen plus progestogen of 1.59 (95% CI, 1.28–1.97) over a mean 5.6 years of follow-up (attributable risk of 31 and 20 cases per 10 000 person–years, respectively). The HERS results on estrogen plus progestogen for the same combined outcome over the initial mean 4.1 years of follow-up were similar (hazard ratio, 1.38; 95% CI, 1.00–1.92) (Simon *et al.*, 2001b) and became significant (hazard ratio, 1.48; 95% CI, 1.12–1.95) during the 6.8-year average open-label extended follow-up period (Hulley *et al.*, 2002). [The Working Group noted that these results suggest that the effect is primarily a function of estrogen alone and may be dependent on duration.] Several observational studies have also found evidence of an adverse effect of hormonal therapy on gallbladder disease (e.g. Mamdani *et al.*, 2000; Boland *et al.*, 2002).

Dementia and cognitive function

Despite preliminary evidence of improved cognitive function following hormonal therapy, randomized trials have not provided evidence of a benefit. On the contrary, data from these trials suggest an increased risk for dementia and a negative impact on cognitive function in women randomized to hormones. The strongest evidence derives from the WHI Memory Study, which is an ancillary study of women over 65 years of age at randomization into one of the WHI hormone trials. In this subset of older participants, the combined hormonal therapy group experienced a significantly increased risk for probable dementia relative to the placebo-treated group (hazard ratio, 2.05; 95% CI, 1.21–3.48) over an average of 4 years of follow-up (Shumaker *et al.*, 2003). A somewhat smaller increase was observed with estrogen alone relative to placebo (hazard ratio, 1.49; 95% CI, 0.83–2.66) over an average of 5 years (Shumaker *et al.*, 2004). The increases in the incidence of probable dementia were not explained by those observed in the incidence of stroke. Rapp *et al.* (2003) and Espeland *et al.* (2004) found an adverse effect of hormonal therapy on global cognitive function for both combined hormones and estrogen alone.

Grady *et al.* (2002b) reported no significant differences between the effects of estrogen plus progestogen and placebo among women at 10 HERS centres from six end-of-study measurements of cognitive function (mean age at time of testing, 71 ± 6 years). One measurement, verbal fluency, was significantly worse ($p = 0.02$) in women who were randomized to estrogen plus progestogen. In the PEPI trial, in which the average age of participants was 56 years (SD, 4.3 years; somewhat younger than those in the WHI or HERS), no significant differences were found between the placebo-treated group and the four hormonal therapy groups at 12 or 36 months for self-reported forgetfulness, concentration or distraction (Reboussin *et al.*, 1998). No randomized trial has been conducted to examine the risk for dementia in younger women. Several very small, short-term randomized trials of hormonal therapy in women with dementia have been conducted (Henderson *et al.*, 2000; Mulnard *et al.*, 2000; Asthana *et al.*, 2001), each of which reported some modest improvements in a subset of the cognitive measurements examined but no consistent pattern of effects.

In contrast, observational studies have mostly reported substantially lower rates of dementia associated with hormonal therapy but with variable relationships between duration and recency of use. In a Cache County Study report, women who had ever used hormonal therapy had a reduced risk for Alzheimer disease compared with non-users (odds ratio, 0.59; 95% CI, 0.35–0.96); the reduction was concentrated in former users or those who had more than 10 years of exposure. The risk for Alzheimer disease in current users of hormonal therapy was not affected (odds ratio, 1.08; 95% CI, 0.59–1.91). According to the authors, this pattern of effects suggests a limited window of time in which a beneficial effect of hormonal therapy on the risk for Alzheimer disease exists (Zandi *et al.*, 2002). Baldereschi *et al.* (1998) reported a reduced risk for Alzheimer disease with ever use of estrogen (odds ratio, 0.28; 95% CI, 0.08–0.98) in the Italian Longitudinal Study on Aging, which was an analysis of data from 1582 postmenopausal women in eight Italian cities. A similar reduction in risk for Alzheimer disease was reported in the Baltimore Longitudinal Study of Aging cohort of 472 peri- or postmenopausal women (mean age at enrollment, 61.5 years). After 16 years of follow-up, the hazard ratio for Alzheimer disease for ever use of oral or transdermal estrogen was 0.46 (95% CI, 0.21–1.00), but no effect of duration was observed (Kawas *et al.*, 1997). Paganini-Hill and Henderson (1994) reported a reduced risk for dementia in users of estrogen (odds ratio, 0.69; 95% CI, 0.46–1.03) and evidence of a stronger effect with higher dose and longer duration of use in a nested case–control study within the Leisure World cohort of southern California.

[Whether hormonal therapy used early in the menopausal period has a beneficial effect on dementia rates or whether these observed reductions arise from subtle patterns of prescription, adherence, survival or other biases remains unanswered.]

Diabetes

The risk for non-insulin-dependent diabetes has been found to be reduced by use of estrogen plus progestogen. In the WHI trial, rates of self-reported diabetes were reduced by 21% (95% CI, 7–33%) (Margolis *et al.*, 2004), and the observed reduction in the HERS

trial was 35% (95% CI, 11–52%) (Kanaya *et al.*, 2003), each of which was accompanied by corresponding changes in fasting glucose and insulin levels.

Other diseases or conditions

The potential impact of hormonal therapy on other age-related health conditions (e.g. osteoarthritis, rheumatoid arthritis, lupus, macular degeneration, cataract, Parkinson disease) has been examined in observational studies and in some randomized trials. The evidence for substantial effects on these disease processes is limited at this time, but additional studies would provide much needed clarification (Cooper *et al.*, 2002; d'Elia *et al.*, 2003; Abramov *et al.*, 2004; Currie *et al.*, 2004; Freeman *et al.*, 2004).

Mortality

Randomized trials have not shown a statistically significant effect on mortality during average intervention periods that ranged from 5.2 to 6.8 years (Hulley *et al.*, 2002; Roussouw *et al.*, 2002; Anderson *et al.*, 2004). Additional follow-up of the WHI cohorts will be of particular interest. A recent meta-analysis of 30 trials also found no effect of hormonal therapy on mortality (odds ratio, 0.98; 95% CI, 0.87–1.18). Further analyses suggested a possible reduction in mortality due to factors other than cancer or cardiovascular disease (Salpeter *et al.*, 2004).

4.4 Genetic and related effects

Estrogen may contribute to the promotion of uterine, breast, ovarian and cervix tumours. It is thought to sustain the growth of preneoplastic and malignant cells by acting through estrogen receptor-mediated signalling pathways that regulate the production of growth factors that maintain clonal growth of both cell types. Epidemiological observations and mathematical models derived therefrom have suggested that most malignant cells accumulate several genetic changes in the genes or chromosomes as they evolve into cancer. Since the previous evaluation (IARC, 1999), many studies in experimental systems on the possible direct genetic and genotoxic effects of steroid sex hormones as a factor in carcinogenesis have been published, and these are summarized in Tables 19 and 20. The data that are now available indicate more strongly that some of these hormones and their metabolites can cause DNA damage, which can potentially induce genetic alterations in cells.

As described in detail in Section 4.1 of this monograph, estrogens, i.e. estrone, 17β-estradiol, estriol and 17α-ethinylestradiol, are activated by aromatic hydroxylation at the C-2 and C-4 positions that is catalysed by CYPs. Subsequently, peroxidase enzymes convert them into catechol estrogens, i.e. 2-hydroxyestrogens and 4-hydroxyestrogens (Roy & Liehr, 1999; Cavalieri *et al.*, 2000; Roy & Singh, 2004). Catalytic oxidation of these two catechol estrogens gives rise to the corresponding estrogen-2,3-quinone and estrogen-3,4-quinone, which react with DNA to form adducts (Roy & Liehr, 1999;

Table 19. Genetic and related effects of estrogens in experimental systems

Test system	Results[a]		Dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Estradiol				
Gene mutation, Chinese hamster V79 cells, *Hprt* locus, *in vitro*	+	NT	0.01 nM [0.0027 ng/mL]	Kong *et al.* (2000)
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *Hprt* locus, *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	1 µg/mL	Tsutsui *et al.* (2000a)
DNA single-strand breaks, comet assay, human peripheral blood lymphocytes *in vitro*	+	NT	50 µM [13.6 µg/mL]	Anderson *et al.* (1997)
DNA single-strand breaks, comet assay, human sperm *in vitro*	+	NT	10 µM [2.7 µg/mL]	Anderson *et al.* (1997)
DNA single-strand breaks, human MCF-7 cells *in vitro*	+	NT	1 nM [0.27 ng/mL]	Yared *et al.* (2002)
DNA single-strand breaks, human MCF-7 cells *in vitro*	+	NT	10 nM [2.7 ng/mL]	Rajapakse *et al.* (2005)
Sister chromosome exchange, human peripheral blood lymphocytes *in vitro*	+	+	25 µg	Ahmad *et al.* (2000)
Micronucleus formation, human MCF-7 cells *in vitro*	+	NT	1 nM [0.27 ng/mL]	Yared *et al.* (2002)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	+	+	25 µg	Ahmad *et al.* (2000)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	–	+	10 µg	Ahmad *et al.* (2000)
Aneuploidy, human MCF-7 cells *in vitro*	–	NT	3.6 µM [1 µg/mL]	Fernandez *et al.* (2005)
Micronucleus formation, mouse bone-marrow cells *in vivo*	–		150 mg/kg bw ip	Ashby *et al.* (1997)
Micronucleus formation, rat and mouse bone-marrow cells *in vivo*	–		1250 mg/kg bw ip	Shelby *et al.* (1997)
2-Hydroxyestradiol				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *Hprt* locus, *in vitro*	–	NT	3 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	NT	3 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	1 µg/mL	Tsutsui *et al.* (2000a)

Table 19 (contd)

Test system	Results[a]		Dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA single-strand breaks, human MCF-7 cells *in vitro*	+	NT	100 nM [30 ng/mL]	Rajapakse *et al.* (2005)
Aneuploidy, human MCF-7 cells *in vitro*	–	NT	3.6 µM [1 µg/mL]	Fernandez *et al.* (2005)
4-Hydroxyestradiol				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *Hprt* locus, *in vitro*	+	NT	1 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	NT	3 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	1 µg/mL	Tsutsui *et al.* (2000a)
DNA single-strand breaks, human MCF-7 cells *in vitro*	+	NT	100 nM [30 ng/mL]	Rajapakse *et al.* (2005)
Aneuploidy, human MCF-7 cells *in vitro*	–	NT	3.6 µM [1 µg/mL]	Fernandez *et al.* (2005)
2-Methoxyestradiol				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *in vitro*	+	NT	0.1 µg/mL	Tsutsui *et al.* (2000b)
Gene mutation, Syrian hamster embryo cells, *Hprt* locus, *in vitro*	+	NT	0.3 µg/mL	Tsutsui *et al.* (2000b)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	NT	0.3 µg/mL	Tsutsui *et al.* (2000b)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	0.3 µg/mL	Tsutsui *et al.* (2000b)
Estrone				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *Hprt* locus, *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	30 µg/mL	Tsutsui *et al.* (2000a)
DNA single-strand breaks, comet assay human MCF-7 cells *in vitro*	+	NT	0.1 nM [0.03 ng/mL]	Yared *et al.* (2002)

Table 19 (contd)

Test system	Results[a]		Dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Micronucleus formation, human MCF-7 cells *in vitro*	+	NT	0.1 nM [0.03 ng/mL]	Yared *et al.* (2002)
2-Hydroxyestrone				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *Hprt* locus, *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	NT	3 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	0.3 µg/mL	Tsutsui *et al.* (2000a)
4-Hydroxyestrone				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *in vitro*	+	NT	3 µg/mL	Tsutsui *et al.* (2000a)
Gene mutation, Syrian hamster embryo cells, *Hprt* locus, *in vitro*	+	NT	1 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	NT	3 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	3 µg/mL	Tsutsui *et al.* (2000a)
2-Methoxyestrone				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *Hprt* locus, *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	10 µg/mL	Tsutsui *et al.* (2000a)
16α-Hydroxyestrone				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *Hprt* locus, *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+	NT	10 µg/mL	Tsutsui *et al.* (2000a)

Table 19 (contd)

Test system	Results[a]		Dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Estriol				
Gene mutation, Syrian hamster embryo cells, Na^+/K^+ ATPase, *Hprt* locus, *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
Aneuploidy, Syrian hamster embryo cells *in vitro*	–	NT	10 µg/mL	Tsutsui *et al.* (2000a)
DNA single-strand breaks, human MCF-7 cells *in vitro*	+	NT	10 nM [3 ng/mL]	Yared *et al.* (2002)
Micronucleus formation, human MCF-7 cells *in vitro*	–	NT	0.1 mM [28.9 µg/mL]	Yared *et al.* (2002)
Ethinylestradiol				
Salmonella typhimurium TA100, TA1535, TA98, TA97a, reverse mutation	–	–	10 mg/plate	Hundel *et al.* (1997)
Unscheduled DNA synthesis, rat hepatocytes *in vitro*	+	NT	1 µM [0.3 µg/mL]	Martelli *et al.* (2003)
Unscheduled DNA synthesis, human hepatocytes *in vitro*	–	NT	50 µM [15 µg/mL]	Martelli *et al.* (2003)
Sister chromosome exchange, human peripheral blood lymphocytes *in vitro*	+	+	1 µg/mL	Hundal *et al.* (1997)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	+	NT	1 µg/mL[c] 48 h	Hundal *et al.* (1997)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	–	+	10 µg/mL[d] 6 h	Hundal *et al.* (1997)
Sister chromosome exchange, mouse bone-marrow cells *in vivo*	+		1 mg/kg bw ip	Hundal *et al.* (1997)
Micronucleus formation, mouse bone-marrow cells *in vivo*	+		1 mg/kg bw ip	Hundal *et al.* (1997)

[a] +, positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; ip, intraperitoneal
[c] Tested for 48 h without exogenous metabolic system only
[d] Tested with exogenous metabolic system only for 6 h. The result was negative without exogenous metabolic system after an exposure of 6 h.

Table 20. Genetic and related effects of progestogens in experimental animals

Test system	Results[a]		Dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Progesterone				
Unscheduled DNA synthesis, rat hepatocytes *in vitro*	–	NT	50 µM [15.7 µg/mL]	Martelli *et al.* (2003)
Unscheduled DNA synthesis, human hepatocytes *in vitro*	–	NT	50 µM [15.7 µg/mL]	Martelli *et al.* (2003)
Micronucleus formation, rat liver *in vivo*	+		100 mg/kg bw po	Martelli *et al.* (1998)
Medroxyprogesterone				
Unscheduled DNA synthesis, rat hepatocytes *in vitro*	–	NT	50 µM [17.2 µg/mL]	Martelli *et al.* (2003)
Unscheduled DNA synthesis, human hepatocytes *in vitro*	–	NT	50 µM [17.2 µg/mL]	Martelli *et al.* (2003)
Norethisterone				
Unscheduled DNA synthesis, rat hepatocytes *in vitro*	±	NT	10 µM [3 µg/mL]	Martelli *et al.* (2003)
Unscheduled DNA synthesis, human hepatocytes *in vitro*	–	NT	50 µM [15 µg/mL]	Martelli *et al.* (2003)
Sister chromosome exchange, human peripheral blood lymphocytes *in vitro*	–	–	75 µg/mL	Ahmad *et al.* (2001)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	–	–	75 µg /mL	Ahmad *et al.* (2001)
Micronucleus formation, rat liver *in vivo*	–		100 mg/kg bw po	Martelli *et al.* (1998)
Norgestrel				
Sister chromosome exchange, human peripheral blood lymphocytes *in vitro*	+	+	25 µg/mL	Ahmad *et al.* (2001)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	+	+	25 µg/mL	Ahmad *et al.* (2001)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	–	+	10 µg/mL	Ahmad *et al.* (2001)

Table 20 (contd)

Test system	Results[a]		Dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cyproterone acetate				
DNA single-strand breaks, comet assay, rat hepatocytes *in vitro*	+	NT	10 µM [4 µg/mL]	Mattioli *et al.* (2004)
Unscheduled DNA synthesis, male rat hepatocytes *in vitro*	–	NT	10 µM [4 µg/mL]	Mattioli *et al.* (2004)
Unscheduled DNA synthesis, female rat hepatocytes *in vitro*	+	NT	10 µM [4 µg/mL]	Mattioli *et al.* (2004)
Gene mutation, Big Blue™ transgenic Fischer 344 rats, *LacI* gene, *in vivo*	+		75 mg/kg bw × 1 po	Krebs *et al.* (1998)
Levonorgestrel				
Gene mutation, mouse lymphoma L5178Y cells *in vitro*	–	NT	NG	Jordan (2002)
Chromosomal aberrations, Chinese hamster ovary fibroblasts *in vitro*	–	NT	NG	Jordan (2002)
Desogestrel				
Salmonella typhimurium [strains NG], reverse mutation	–	–	NG	Jordan (2002)
Micronucleus formation, female rat liver *in vivo*	–		NG	Jordan (2002)
Potassium canrenoate				
DNA single-strand breaks, rat hepatocytes *in vitro*	+	NT	10 µM [3.7 µg/mL]	Martelli *et al.* (1999)
Unscheduled DNA synthesis, rat liver *in vitro*	+	NT	30 µM [11 µg/mL]	Martelli *et al.* (1999)
Micronucleus formation, rat hepatocytes *in vitro*	+	NT	30 µM [11 µg/mL]	Martelli *et al.* (1999)
DNA single-strand breaks, human hepatocytes *in vitro*	+	NT	30 µM [11 µg/mL]	Martelli *et al.* (1999)
DNA single-strand breaks, human peripheral blood lymphocytes *in vitro*	–	NT	90 µM [33 µg/mL]	Martelli *et al.* (1999)
Unscheduled DNA synthesis, human liver *in vitro*	–	NT	90 µM [33 µg/mL]	Martelli *et al.* (1999)
Micronucleus formation, human hepatocytes *in vitro*	–	NT	90 µM [33 µg/mL]	Martelli *et al.* (1999)
Micronucleus formation, human peripheral blood lymphocytes *in vitro*	–	NT	90 µM [33 µg/mL]	Martelli *et al.* (1999)

Table 20 (contd)

Test system	Results[a]		Dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA single-strand breaks, male rat liver *in vivo*	+		325 mg/kg bw po	Martelli *et al.* (2002)
DNA single-strand breaks, female rat thyroid and bone marrow *in vivo*	+		325 mg/kg bw po	Martelli *et al.* (2002)
DNA single-strand breaks, rat testes and ovary *in vivo*	+		81 mg/kg bw po	Martelli *et al.* (2002)
Micronucleus formation, rat liver *in vivo*	–		325 mg/kg bw po	Martelli *et al.* (2002)
Micronucleus formation, rat bone marrow polychromatic erythtocytes *in vivo*	–		325 mg/kg bw po	Martelli *et al.* (2002)
Drospirenone				
Unscheduled DNA synthesis, rat hepatocytes *in vitro*	+	NT	1 µM [0.36 µg/mL]	Martelli *et al.* (2003)
Unscheduled DNA synthesis, human hepatocytes *in vitro*	–	NT	50 µM [18.3 µg/mL]	Martelli *et al.* (2003)

[a] +, positive; –, negative; ±, equivocal; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; NG, not given; po, oral

Cavalieri *et al.*, 2000). These adducts can form stable modifications that remain in the DNA unless they are removed by repair. Alternatively, the modified bases can be released from DNA by destabilization of the glycosydic bond and result in the formation of depurinated or depyrimidinated sites (Cavalieri *et al.*, 2000).

4.4.1 *Humans*

At present, only two studies have reported the presence of catechol estrogen adducts in human breast tissue. Embrechts *et al.* (2003) analysed estrogen adducts in the DNA from 18 human samples: five malignant breast tumour samples, five samples of tissues adjacent to the tumour and eight alcohol-fixed and paraffin-embedded malignant breast tumour samples. Almost every DNA sample showed the presence of deoxyguanosine adducts of 4-hydroxyestradiol and 4-hydroxyestrone. In four patients who had used conjugated equine estrogens, 4-hydroxyequilenin–DNA adducts, derived from conjugated equine estrogen metabolites, were detected. In seven patients, deoxyadenosine adducts of 4-hydroxy-17α-ethinylestradiol were observed. The formation of catechol estrogen quinone-derived DNA adducts has also been reported in two breast samples that were collected from one woman with and one woman without breast cancer (Markushin *et al.*, 2003). The catechol quinone-derived adducts identified were 4-hydroxyestradiol-1-N3-adenosine, 4-hydroxyestrone-1-N3-adenosine and 4-hydroxyestradiol-1-N7-guanine.

4.4.2 *Experimental systems* (Tables 19 and 20)

Liquid chromatographic–tandem MS analysis of mammary fat pads of rats injected with 4-hydroxyequilenin showed a dose-dependent increase in DNA single-strand breaks and formation of alkylated guanine adducts that are prone to depurination; stable cyclic deoxyguanosine and deoxyadenosine adducts and other oxidized bases were also observed (Zhang *et al.*, 2001). Injection of 4-hydroxyestradiol or estradiol-3,4-quinone into the mammary glands of female ACI rats resulted in formation of the depurinating adducts 4-hydroxyestradiol-1-N3-adenosine and 4-hydroxyestradiol-1-N7-guanosine (Li *et al.*, 2004). 4-Hydroxyestradiol-GSH conjugates were also detected. Recently, 4-hydroxy catechol estrogen conjugates with GSH or its hydrolytic products (cysteine and *N*-acetylcysteine) were detected in picomole amounts both in tumours and hyperplastic mammary tissues from ERKO/Wnt-1 mice and demonstrated the formation of estrogen-3,4-quinones (Devanesan *et al.*, 2001). DNA adducts derived from 2-hydroxyestrogen-quinone have been shown to be mutagenic, and primarily produced G→T and A→T mutations in simian kidney (COS-7) cells (Terashima *et al.*, 2001). Estradiol-3,4-quinone reacted rapidly to form 4-hydroxyestradiol-1-N3-adenosine adducts that are depurinating adducts. Numerous A→G mutations in H-*ras* DNA were observed in SENCAR mouse skin treated with estradiol-3,4-quinone (Chakravarti *et al.*, 2001). [These studies indicate that certain estrogen metabolites can react with DNA to form adducts. Such adducts or the apurinic sites they generate in DNA can give rise to mutations and these, in turn, could contribute to the development of tumours.]

Recently, it was reported that estrogen-induced mammary gland tumours in female ACI rats show losses and gains in chromosomes. Cells with an increased copy number of the *c-myc* gene (7q33), one on each of the three homologues of a trisomy of chromosome 7, were observed. A frequency of aneuploidy of 61% in sporadic invasive human ductal breast cancers and 71% in ductal carcinoma *in situ* was also observed. The authors asserted that the estrogen-induced mammary tumours in female ACI rats resembled human ductal carcinoma *in situ* and invasive ductal breast cancer because they are aneuploid and exhibit a high frequency of *c-myc* amplification (Li *et al.*, 2002).

Mitochondria are significant targets of estrogen (reviewed by Roy *et al.*, 2004; Felty & Roy, 2005a,b). Recently Felty *et al.* (2005a) reported that physiological concentrations of 17β-estradiol stimulate a rapid production of intracellular reactive oxygen species which, in epithelial cells, depends on cell adhesion, the cytoskeleton and integrins. Induction of the production of reactive oxygen species by estradiol occurs much more rapidly than the estrogen receptor-mediated interaction with the genome. Furthermore, estradiol-stimulated production of reactive oxygen species does not depend on the presence of the estrogen receptor in breast cancer cells because it was equal in both estrogen receptor-positive cell lines MCF7, T47D and ZR75.1 and the estrogen receptor-negative cell line MDA-MB 468. Exposure of human mammary epithelial cells to 2- or 4-hydroxyestradiol has been shown to produce reactive oxygen species and a subsequent increase in the formation of 8-OHdG (Hurh *et al.*, 2004; Chen *et al.*, 2005). This finding shows that formation of reactive oxygen species following exposure to estradiol could explain oxidative damage in hormone-dependent tumours and subsequent genetic alterations reported earlier (Malins & Haimanot, 1991; Musarrat *et al.*, 1996; Yamamoto *et al.*, 1996; Malins *et al.*, 2001). Mutations have recently been reported to occur following exposures to physiological and pharmacological concentrations of estrogens (Kong *et al.*, 2000; Singh *et al.*, 2005).

It has been shown that catechol estrogens can induce aldehydic DNA lesions in calf thymus DNA (Lin *et al.*, 2003). Equilin and equilenin are major constituents of Premarin®, a widely prescribed drug used in estrogen therapy for the menopause. These equine estrogens are metabolized, respectively, to 4-hydroxyequilin and 4-hydroxyequilenin, which, in turn, are oxidized to products that react with DNA. Mutations induced by 4-hydroxyequilin have been identified in supF plasmid shuttle vectors that were transfected in human fibroblast cells (Yasui *et al.*, 2003).