3. Studies of Cancer in Experimental Animals

In this section, only relevant studies on estrogens and progestogens alone and in combination that were published subsequent to or were not included in the previous evaluation (IARC, 1999) are reviewed in detail. Studies that were reviewed previously are summarized briefly.

3.1 Estrogen–progestogen combinations

The results of studies reviewed previously (IARC, 1979, 1999) on the carcinogenicity of combinations of estrogens and progestogens that are used in combined oral contraceptives are summarized below (see Tables 22 and 23).

The incidence of pituitary adenomas in female and male mice was increased by administration of mestranol plus chlormadinone acetate, mestranol plus ethynodiol diacetate, ethinylestradiol plus ethynodiol diacetate, mestranol plus norethisterone, ethinylestradiol plus norethisterone (females only) and mestranol plus norethynodrel. The latter combination also increased the incidence of pituitary adenomas in female rats.

The incidence of benign mammary tumours was increased in intact and castrated male mice by ethinylestradiol plus chlormadinone acetate and in castrated male mice by mestranol plus norethynodrel. In male rats, the incidence of benign mammary tumours was increased by administration of ethinylestradiol plus norethisterone acetate. This combination did not cause tumour formation in any tissue in one study in female monkeys.

The incidence of malignant mammary tumours was increased in female and male mice by ethinylestradiol plus megestrol acetate, in female and male rats by ethinylestradiol plus ethynodiol diacetate, and in female rats by mestranol plus norethisterone and mestranol plus norethynodrel.

Table 21. Association between oral contraceptive use and the risk for other cancers

Reference, location	Age (years)	Cancer type	Oral contraceptive use	Cases	Controls	Odds ratio (95% CI)	Comments
Nelson *et al.* (2001), Los Angeles, USA	18–75	Intermediate or high-grade B-cell non-Hodgkin lymphoma	Never	111	93	1.00	Matched, fitting education, place of birth
			Ever	66	84	0.47 (0.26–0.86)	
			< 5 years	43	53	0.50 (0.26–0.95)	
			≥ 5 years	21	29	0.35 (0.15–0.82)	
Glaser *et al.* (2003), San Francisco Bay Area, USA	19–79	Hodgkin lymphoma	Never	87	91	1.0	Multivariate parsimonious model that includes variables significant at $p < 0.10$ (reproductive history, socio-economic status)
			≤ 2.2 years	91	79	1.2 (0.8–1.9)	
			2.3–5.3 years	72	73	0.9 (0.6–1.5)	
			> 5.3 years	62	82	0.8 (0.5–1.3)	
Vessey *et al.* (2003), England and Scotland	25–39	Lymphoma/ haemopoietic cancer	Never	16	–	1.0	OFPA cohort of 17 032 Caucasian women; adjusted for parity, social class, smoking
			< 4 years	6	–	0.9 (0.3–2.5)	
			4–8 years	8	–	1.0 (0.4–2.5)	
			> 8 years	11	–	1.2 (0.5–2.7)	
Schiff *et al.* (1998), Rochester, MN, USA	≥ 20	Central nervous system lymphoma	Never	35	52	1.0	
			Ever	3	19	0.3	
Gago-Dominguez *et al*. (1999), Los Angeles, USA	25–74	Renal-cell cancer	Never	258	255	1.0	Adjusted for age, education, hysterectomy
			Ever	164	167	1.0 (0.7–1.4)	
Olshan *et al.* (1999), Canada and USA	< 20	Neuroblastoma	*Maternal use during first trimester*				Odds ratios also unity for oral contraceptive use in previous year or ever
			No	442	444	1.0	
			Yes	17	15	1.0 (0.5–2.1)	

Table 21 (contd)

Reference, location	Age (years)	Cancer type	Oral contraceptive use	Cases	Controls	Odds ratio (95% CI)	Comments
Schüz *et al.* (2001), Germany	≤ 7	Neuroblastoma	*Maternal use during pregnancy*				
			No	159	1671	1.0	
			Yes	4	26	5.7 (1.5–23)	
			Unspecified	16	70	NR	
Palmer *et al.* (1999), USA	≥ 18	GTD	Never	36	98	1.0	Matched analysis; $p = 0.03$ for trend with duration
			Ever	199	315	1.8 (1.2–2.8)	
Parazzini *et al.* (2002), Greater Milan area, Italy	13–56	GTD	Never	164	306	1.0	Risk increased with duration
			Ever	104	130	1.5 (1.1–2.1)	
Taioli & Wynder (1994), USA	20–89	Lung adeno-carcinoma	Never	134	229	1.0	
			Ever	46	74	0.8 (0.5–1.5)	
Beral *et al.* (1999), United Kingdom	16–79	Lung	Never	40	–	1.0	RCGP cohort of 46 000 women
			Ever	75	–	1.2 (0.8–1.8)	
Kreuzer *et al.* (2003), Germany	≤ 75	Lung	Never	528	557	1.00	Adjusted for age, region, education, smoking variables
			Ever	279	354	0.69 (0.51–0.92)	
			< 5 years	86	105	0.69 (0.46–1.03)	
			5–11 years	87	130	0.65 (0.44–0.95)	
			≥ 12 years	102	115	0.69 (0.47–1.02)	
			Nonsmokers				Adjusted for age, region, education, time since stopped smoking
			Never	–	–	1.00	
			Ever	–	–	1.18 (0.78–1.79)	
			Smokers				
			Never	–	–	1.00	
			Ever	–	–	0.50 (0.34-0.74)	

Table 21 (contd)

Reference, location	Age (years)	Cancer type	Oral contraceptive use	Cases	Controls	Odds ratio (95% CI)	Comments
Vessey *et al.* (2003), England and Scotland	25–39	Lung	Never < 4 years 4–8 years > 8 years	15 9 12 18	– – – –	1.0 1.4 (0.6–3.5) 1.2 (0.5–2.6) 1.3 (0.6–2.8)	OFPA cohort of 17 032 Caucasian women; adjusted for parity, social class, smoking
Kreiger *et al.* (2001), Ontario, Canada	20–74	Pancreas	< 6 months ≥ 6 months	41 9	160 64	1.00 0.36 (0.13–0.96)	Multivariate, fitting age, oral contraceptives, hormonal menopausal therapy, obstetric history, body mass index, diet, smoking
Skinner *et al.* (2003), USA	30–55	Pancreas	Never Ever < 1 year 1–2.9 years 3–7.9 years ≥ 8 years	159 83 26 13 27 17	– – – – – –	1.00 1.21 (0.91–1.61) 1.45 (0.94–2.21) 0.78 (0.44–1.39) 1.38 (0.85–1.99) 1.26 (0.76–2.10)	Nurses Health Study; multivariate, fitting age, period, smoking, diabetes, body mass index, parity; age at baseline, 30–55 and followed up to 1998
Duell & Holly (2005), San Francisco Bay Area, USA	21–85	Pancreas	Never Ever < 1 year 1–2 years 3–7 years ≥ 8 years	135 102 18 25 18 41	402 394 70 140 73 103	1.00 0.95 (0.65–1.4) 0.85 (0.47–1.5) 0.67 (0.39–1.2) 0.92 (0.50–1.7) 1.4 (0.89–2.3)	Adjusted for age, education, smoking
Yen *et al.* (1987), Rhode Island State, USA	< 60	Gallbladder	Never Ever	6 4	70 6	1.0 7.8 (2.0–30)	

Table 21 (contd)

Reference, location	Age (years)	Cancer type	Oral contraceptive use	Cases	Controls	Odds ratio (95% CI)	Comments
WHO Collaborative Study (1989c), Chile, China, Columbia, Israel, Kenya, Mexico	NR	Gallbladder	Never Ever Current	49 9 1	269 86 8	1.0 0.6 (0.3–1.3) 0.9 (0.1–7.4)	
Moerman *et al.* (1994), Netherlands	35–79	Gallbladder and biliary tract	Never Ever	61 14	203 49	1.0 1.1 (0.5–2.4)	
Zatonski *et al.* (1997), Australia, Canada, Netherlands, Poland	64.9 (mean)	Gallbladder	Never Ever	132 20	558 142	1.0 1.0 (0.5–2.0)	
Gallus *et al.* (2001), Italy and Switzerland	< 79	Squamous-cell oesophageal cancer	Never Ever	110 4	392 33	1.0 0.24 (0.06–0.96)	Three hospital-based case–control studies pooled; adjusted for age, education, body mass index, energy intake, tobacco, alcoholic beverages

CI, confidence interval; GTD, gestational trophoblastic diseases; OFPA, Oxford Family Planning Association; RCGP, Royal College of General Practitioners

Table 22. Effects of combinations of various progestogens and estrogens on tumour incidence in mice

Combination	Pituitary adenomas		Mammary tumours			Uterine tumours	Cervical/ vaginal tumours
	Male	Female	Benign (males)	Malignant			
				Male	Female		
Chlormadinone acetate + mestranol	+	+					
Chlormadinone acetate + ethinylestradiol			+/c				
Ethynodiol diacetate + mestranol	+	+					
Ethynodiol diacetate + ethinylestradiol	+	+				+	
Lynestranol + mestranol					+/–		
Lynestranol + ethinylestradiol + 3-methylcholanthrene							+[a]
Megestrol acetate + ethinylestradiol				+	+		
Norethisterone acetate + ethinylestradiol	+/?	+/?					
Norethisterone + ethinylestradiol		+					
Norethisterone + mestranol	+	+					
Norethynodrel + mestranol	+	+	c		+/?		+
Norethynodrel + mestranol + 3-methylcholanthrene						+	–
Norgestrel + ethinylestradiol + 3-methylcholanthrene							+[a]

From IARC (1979, 1999)

+, increased tumour incidence; +/–, slighly increased tumour incidence; +/c, increased tumour incidence in intact and castrated animals; c, increased tumour incidence in castrated animals; +/?, increased tumour incidence, but not greater than that with the estrogen or progestogen alone

[a] Protection at doses 1/2000th and 1/200th that of a contraceptive pill for women; enhancement at a dose of 1/20th that of a contraceptive pill for women

Table 23. Effects of combinations of various progestogens and estrogens on tumour incidence in rats

Combination	Pituitary adenomas		Mammary tumours			Liver				
	Male	Female	Benign (males)	Malignant		Adenoma		Carcinoma		Foci (females)
				Male	Female	Male	Female	Male	Female	
Ethynodiol diacetate + ethinylestradiol				+	+					
Ethynodiol diacetate + mestranol				?	?					
Megestrol acetate + ethinylestradiol			+/–	+/–	+/–	+/?	+/?			
Norethisterone acetate + ethinylestradiol			+			+		–	+	
Norethisterone + mestranol					+	+	–			
Norethynodrel + mestranol	+/?	+	+/?	+/?	+	+/?	–	–	–	+
Norethynodrel + mestranol + *N*-nitroso-diethylamine							–		–	+
Norgestrel + ethinylestradiol			+/–							

From IARC (1979, 1999)

+, increased tumour incidence; +/–, slighly increased tumour incidence; +/?, increased tumour incidence, but not greater than that with the estrogen or progestogen alone; ? conflicting result; –, no effect

In female mice, the incidence of malignant non-epithelial uterine tumours was increased by ethinylestradiol plus ethynodiol diacetate and that of vaginal or cervical tumours by norethynodrel plus mestranol. In mice treated with 3-methylcholanthrene to induce genital tumours, ethinylestradiol plus lynestrenol, ethinylestradiol plus norgestrel and mestranol plus norethynodrel increased the incidence of uterine tumours; however, this occurred only at the highest doses of ethinylestradiol plus lynestrenol and ethinylestradiol plus norgestrel that were tested. Lower doses inhibited the tumorigenesis induced by 3-methylcholanthrene alone.

In male rats, the incidence of liver adenomas was increased by mestranol plus norethisterone and by ethinylestradiol plus norethisterone acetate; the latter combination also increased the incidence of hepatocellular carcinomas in female rats. Liver foci, which are putative preneoplastic lesions, were induced in female rats by mestranol plus norethynodrel. In rats initiated for hepatocarcinogenesis with *N*-nitrosodiethylamine, mestranol plus norethynodrel increased the formation of altered hepatic foci.

Subcutaneous implantation and oral administration in rabbits

Virgin female New Zealand white rabbits, about 6 months of age and weighing 3.2–4.7 kg, were randomized into groups of five. Steroids (estradiol plus levonorgestrel or ethinylestradiol plus levonorgestrel) were delivered by subdermal implants in the neck or, in one case, by oral administration. The estimated dose of levonorgestrel was based on its loss from implants of the same type in women. Estimates of the doses of estradiol or ethinylestradiol delivered were based on measurements of in-vitro release from the implants used. Because of the high release and early exhaustion of steroid supply, the progestogen implants were replaced at 20-day intervals. The steroids administered orally were the commercially available combination pill Lo Femenal®; each tablet contained 30 μg ethinylestradiol and 300 μg norgestrel. Norgestrel contained equal amounts of levonogestrel and its inactive isomer. The pills were dispersed in 2 mL water and were administered intragastrically, but evaluation of the effects was confounded by the very low bioavailability of ethinylestradiol in this species when given orally. Hence, a new experiment was conducted in which levonorgestrel was delivered by oral pill and 30 μg of ethinylestradiol per day was delivered by implant for 8 weeks. At necropsy, lesions were noted and the weights of the uterus, liver and spleen were determined. Samples for tissue block preparation were taken from all lesions identified in organs or tissues, and samples were routinely taken from the uterus, spleen, lung, liver and bone marrow. Tissues were stained with haematoxylin and eosin and were studied microscopically. The animals were killed after 2 or 4.5 months. The incidences of tumours among the groups treated subcutaneously with a combination of estradiol and levonorgestrel are shown in Table 24. Estradiol alone resulted in decidualization, but did not induce deciduosarcoma, nor did tumours develop when estradiol was supplemented with lower doses of levonorgestrel, with the exception of an early atypical deciduosarcoma in one group. All animals in the groups treated with 66 or 233 μg/day levonorgestrel for 2 or 4.5 months had multiple deciduosarcomas. Neoplastic decidual

cells were found in the uterine blood vessels of many animals of these groups. In the other experiments by subcutaneous administration, ethinylestradiol was substituted for estradiol. The implants were in place for 6 months before the animals were killed. The experimental design and histological findings are summarized in Table 25. Ethinylestradiol alone induced deciduosarcomas in the spleen and ovary of one animal. In combination with a high dose of levonorgestrel (150 μg/day), even 10 μg/day ethinylestradiol produced many

Table 24. Incidence of malignant tumours in female New Zealand white rabbits treated subcutaneously with estradiol (E_2) and levonorgestrel (LNG)

Treatment time	E_2 (μg/day)	LNG (μg/day)	% of animals with deciduosarcomas (n = 4–5)				
			Uterus	Spleen	Liver	Ovary	Lung
2 months	0	0	0	0	0	0	0
	60	0	0	0	0	0	0
	60	8	0	25[a]	0	0	0
	60	25	0	0	0	0	0
	60	66	80	100	60	40	40[b]
	60	233	100	100	40	20	0
4.5 months	0	0	0	0	0	0	0
	6	233	20	0	0	0	0
	20	233	100	40	20	0	0
	60	233	100	75	25	0	25
	200	233	100	100	40	40	20

From Jänne *et al.* (2001)
[a] Atypical deciduosarcoma
[b] Metastasis

Table 25. Incidence of malignant tumours in female New Zeland white rabbits treated subcutaneously with ethinylestradiol (EE) and levonorgestrel (LNG)

Treatment time	EE (μg/day)	LNG (μg/day)	Proliferation of hepatic bile ductules (%)	% of animals with deciduosarcomas (n = 5)				
				Uterus	Spleen	Liver	Ovary	Lung
6 months	0	0	0	0	0	0	0	0
	30	0	80	0	20	0	20	0
	10	150	20	100	100	20	0	0
	30	150	20	100	100	100	20	20[a]

From Jänne *et al.* (2001)
[a] Metastasis

deciduosarcomas. As in the experiments cited earlier, hyperplasia of splenic mesenchymal cells was seen in most animals in all groups that received estrogen. Proliferation of hepatic bile ductules was observed in 40% of rabbits that received ethinylestradiol. This lesion was not seen in any other test or control rabbits. In the experiment in which levonorgestrel was delivered by an oral pill and 30 µg/day ethinylestradiol were delivered by implant, one of five animals developed a spleen deciduosarcoma (Jänne *et al*., 2001). [This study clearly shows the carcinogenic effects of combinations of levonorgestrel with ethinylestradiol as well as with estradiol. It is interesting to note that, although the study is well designed, there is no human counterpart for deciduosarcomas. Hepatic bile duct proliferation is a more relevant lesion in this context.]

3.2 Estrogens used in combined oral contraceptives

The results of studies reviewed previously (IARC, 1979, 1999) on the carcinogenicity of estrogens used in combined oral contraceptives are summarized below (see Tables 26 and 27).

The incidence of pituitary adenomas was increased by ethinylestradiol and mestranol in female and male mice and by ethinylestradiol in female rats.

The incidence of malignant mammary tumours was increased by ethinylestradiol and mestranol in female and male mice and female rats; however, mestranol did not increase the incidence of mammary tumours in female dogs in a single study.

Ethinylestradiol increased the incidence of cervical tumours in female mice.

In female and male mice, ethinylestradiol increased the incidence of hepatocellular adenomas. In female rats, ethinylestradiol and mestranol increased the numbers of altered hepatic foci. In rats, ethinylestradiol increased the incidence of hepatocellular adenomas in females and males and of hepatocellular carcinomas in females, whereas mestranol increased the incidence of hepatic nodules and carcinomas combined in females.

The incidence of microscopic malignant kidney tumours was increased in male hamsters exposed to ethinylestradiol.

In female mice initiated for liver carcinogenesis and exposed to unleaded gasoline, ethinylestradiol increased the number of altered hepatic foci; however, when given alone after the liver carcinogen, it reduced the number of altered hepatic foci.

In female rats initiated for liver carcinogenesis, ethinylestradiol and mestranol increased the number of altered hepatic foci and the incidence of adenomas and carcinomas. Ethinylestradiol also increased the incidence of kidney adenomas, renal-cell carcinomas and liver carcinomas in male rats initiated with *N*-nitrosoethyl-*N*-hydroxyethylamine. In female hamsters initiated with *N*-nitrosobis(2-oxopropyl)amine, ethinylestradiol increased the incidence of renal tumours and the multiplicity of dysplasias.

Table 26. Effects of ethinylestradiol and mestranol alone and with known carcinogens on tumour incidence in mice

Estrogen	Pituitary adenoma		Malignant mammary tumours		Uterine tumours	Vaginal/ cervical tumours	Liver		
	Male	Female					Adenoma		Foci (females)
			Male	Female			Male	Female	
Ethinylestradiol	+	+	+	+	+	+	+	+	
Mestranol	+	+	+	+			–	–	
Ethinylestradiol + *N*-nitrosodiethylamine									Protective
Ethinylestradiol + *N*-nitrosodiethylamine + unleaded gasoline									+

From IARC (1979, 1999)
+, increased tumour incidence; –, no effect

Table 27. Effects of ethinylestradiol and mestranol alone and with known carcinogens on tumour incidence in rats

Estrogen	Pituitary adenoma (females)	Malignant mammary tumours (females)	Liver					Kidney	
			Adenoma		Carcinoma		Foci (females)	Adenoma (males)	Carcinoma (females)
			Male	Female	Male	Female			
Ethinylestradiol	+	+	+	+		+	+		
Mestranol		+				+/–	+		
Ethinylestradiol + *N*-nitrosoethyl-*N*-hydroxyethylamine					+			+	+
Ethinylestradiol + *N*-nitroso-diethylamine			+	+	+	+	+[a]		
Mestranol + *N*-nitrosodiethylamine			+	+	+	+	+		

From IARC (1979, 1999)
+, increased tumour incidence; –, no effect; +/–, slightly increased tumour incidence
[a] In one of three studies, ethinylestradiol initiated hepatocarcinogenesis.

3.2.1 *Subcutaneous implantation*

(*a*) *Mouse*

A total of 272 female CD-1 (ICR) mice, 9 weeks of age, were divided equally into 17 groups and received subcutaneous implants into the back of cholesterol pellets (31.84 mg) that contained 0 or 0.16 mg estrone, estradiol, estriol, 2-hydroxyestrone, 2-hydroxyestradiol, 2-hydroxyestriol, 2-methoxyestrone, 2-methoxyestradiol, 2-methoxyestriol, 4-hydroxyestrone, 4-hydroxyestradiol, 16β-hydroxyestrone diacetate, 16-epiestriol, 16,17-epiestriol, 16α-hydroxyestrone or 17-epiestriol. The pellets were renewed every 7 weeks throughout the experiment. At 10 weeks of age, each mouse received a single injection of 12.5 mg/kg bw *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG) dissolved at a concentration of 1.5% (w/v) in polyethylene glycol into one of the uterine cavities via the vagina. The experiment was terminated at 30 weeks of age, when all surviving animals were autopsied to obtain reproductive organs, and the uterus and ovaries were weighed and processed for histological examination. Endometrial proliferative lesions were classified histologically into two categories — hyperplasia and adenocarcinoma — and the incidence is given in Table 28. The results indicated that estrogens and their metabolites affect endometrial carcinogenesis in mice initiated with ENNG in a manner that is dependent on their metabolic attributes. Estrogens (estrone, estradiol and estriol) and their metabolites that belong to the 16α-hydroxylation pathway (16α-hydroxyestrone and 17-epiestriol) and the upstream of the 16β-hydroxylation pathway (16β-hydroxyestrone diacetate) exerted both promoting and additive co-carcinogenic effects on endometrial carcinogenesis in ENNG-initiated mice as shown by the development of invasive adenocarcinomas. Estrogen metabolites that belong to the downstream of the 16β-hydroxylation pathway exerted mainly promoting effects in this experimental model, as shown by the enhanced development of endometrial hyperplasia (16-epiestrol), or exerted no effect (16,17-epiestriol) (Takahashi *et al.*, 2004).

(*b*) *Hamster*

Groups of 10 male Syrian hamsters, 4–6 weeks of age, were implanted subcutaneously with 25-mg pellets of estradiol, 17α-estradiol, ethinylestradiol, menadione, a combination of 17α-estradiol and ethinylestradiol or a combination of ethinylestradiol and menadione [no effective dose provided]. The hamsters received a second identical pellet 3 months after the initial treatment. A control group of 10 animals was sham-operated and left untreated. The animals were killed after 7 months and inspected macroscopically for tumour nodules on the surface of each kidney (see Table 29). No tumour nodules were detected in untreated hamsters or in groups of hamsters that were treated with 17α-estradiol, ethinylestradiol, menadione or a combination of 17α-estradiol and ethinylestradiol for 7 months. In contrast, hamsters treated with either estradiol or a combination of ethinylestradiol and menadione for 7 months developed renal tumours. The tumour incidence was 90% in the group treated with estradiol (versus 0% in untreated controls; $p < 0.05$, Fisher's exact test) and 30% in the group treated with a combination of ethinylestradiol and menadione. The mean number of tumour nodules per hamster was higher in the group treated with estradiol compared

Table 28. Incidence of endometrial proliferative lesions in female CD-1 mice treated with a single dose of ENNG and various estrogens

Treatment	Effective no. of animals	Endometrial hyperplasias[a,b]				Adeno-carcinomas[b]	Total[b]
		+	++	+++	Total		
Control	16	2 (13)	6 (38)	1 (6)	9 (56)	0	9 (56)
Estrone	16	1 (6)	1 (6)	2 (13)	4 (25)	12 (75)*	16 (100)*
Estradiol	16	0	2 (13)	1 (6)	3 (19)	13 (81)*	16 (100)*
Estriol	16	1 (6)	7 (44)	2 (13)	10 (63)	6 (38)*	16 (100)*
2-Hydroxyestrone	16	5 (31)	6 (38)	2 (13)	13 (81)	0	13 (81)
2-Hydroxyestradiol	16	7 (44)	4 (25)	1 (6)	12 (75)	0	12 (75)
2-Hydroxyestriol	16	6 (38)	8 (50)	1 (6)	15 (93)*	0	15 (93)*
2-Methoxyestrone	16	7 (44)	3 (19)	0	10 (62)	0	10 (62)
2-Methoxyestradiol	16	7 (44)	7 (44)	1 (6)	15 (93)*	0	15 (93)*
2-Methoxyestriol	16	8 (50)	8 (50)	0	16 (100)*	0	16 (100)*
4-Hydroxyestrone	15	4 (27)	5 (33)	1 (7)	10 (67)	0	10 (67)
4-Hydroxyestradiol	16	6 (38)	4 (25)	2 (13)	12 (75)	0	12 (75)
16β-Hydroxyestrone diacetate	15	2 (13)	7 (47)	2 (13)	11 (73)	4 (27)*	15 (100)*
16-Epiestriol	16	9 (56)*	5 (31)	2 (13)	16 (100)*	0	16 (100)*
16,17-Epiestriol	16	5 (31)	6 (38)	1 (6)	12 (75)	0	12 (75)
16α-Hydroxyestrone	15	1 (7)	6 (40)	4 (27)	11 (73)	4 (27)*	15 (100)*
17-Epiestriol	15	1 (7)	2 (13)	1 (7)	4 (27)	11 (73)*	15 (100)*

From Takahashi *et al*. (2004)
ENNG, *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine
[a] Hyperplasias are classified based on the degree of cellular atypism into three subcategories: +, slight; ++, moderate; +++, severe
[b] Numbers of animals (percentage incidence)
* Significantly different from control value ($p < 0.05$, Fisher's exact test)

Table 29. Influence of different estrogens and menadione on renal carcinogenesis in male Syrian hamsters

Treatment	No. of animals with tumours/ no. of animals	Mean no. of tumour nodules per hamster
Estradiol	9/10[a]	7.0 ± 3.16[b]
17α-Estradiol	0/10	0
Ethinylestradiol	0/10	0
17α-Estradiol + ethinylestradiol	0/10	0
Menadione	0/10	0
Menadione + ethinylestradiol	3/10[c]	1.6 ± 2.67
Untreated	0/10	0

From Bhat *et al*. (2003)
[a] $p < 0.05$ compared with untreated controls and the menadione + ethinylestradiol-treated group by Fisher's exact test
[b] $p < 0.05$ compared with untreated controls by the $\div^2$ test; $p < 0.05$ compared with the menadione + ethinylestradiol-treated group by using the unpaired *t* test
[c] $p < 0.10$ compared with untreated controls by Fisher's exact test

with the group treated with a combination of ethinylestradiol and menadione or the untreated control group (Bhat *et al*., 2003).

3.2.2 *Subcutaneous injection*

Mouse

Groups of outbred female CD-1 mice [initial number of animals per group not specified] were given daily subcutaneous injections of 2 μg estrogen (2- or 4-hydroxyestradiol, estradiol or ethinylestradiol) dissolved in corn oil or corn oil alone (as a control) on days 1–5 of life. Mice were killed at 12 or 18 months of age. Tissue sections were stained and evaluated by light microscopy. The number of mice with reproductive tract tumours was determined. The incidence of uterine adenocarcinomas was compared by Fisher's exact tests (see Table 30). Both of the catechol estrogens examined (2- and 4-hydroxyestradiol) induced tumours in CD-1 mice. 2-Hydroxyestradiol was more carcinogenic than estradiol: 14 and 11% of the rodents developed uterine adenocarcinomas at 12 and 18 months, respectively, after neonatal administration of catechol estrogen. In contrast, 4-hydroxyestradiol was considerably more carcinogenic than 2-hydroxyestradiol and produced a 74% (12 months) and 56% (18 months) tumour incidence after treatment during the neonatal period. Ethinylestradiol also induced more tumours (38% at 12 months and 50% at 18 months) than estradiol. This study, which was designed to demonstrate the carcinogenic properties of catechol estrogens, also included a group that was treated with ethinylestradiol in which uterine adenocarcinomas were induced at a high incidence (Newbold & Liehr, 2000). [It should be

Table 30. Uterine adenocarcinomas in groups of female CD-1 mice injected subcutaneously with various estrogens during the neonatal period

Compound	Incidence of uterine adenocarcinoma No. of animals with tumour/total no. of animals (%)		
	12 months	18 months	Total
Control (corn oil)	0/12 (0)	0/22 (0)	0/34 (0)
2-Hydroxyestradiol	3/21 (14)	2/19 (11)	5/40 (12)*
4-Hydroxyestradiol	14/19 (74)	9/16 (56)	23/35 (66)**
Estradiol	0/5[a]	1/10 (10)	1/15 (7)
Ethinylestradiol	9/24 (38)	9/18 (50)	18/42 (43)**

From Newbold & Liehr (2000)
* $p < 0.05$ versus corn oil controls (Fisher's exact test)
** $p < 0.01$ versus corn oil controls (Fisher's exact test)
[a] These data were published previously (Newbold *et al*., 1990).

noted that studies in neonatal mice could have additional limitations for the extrapolation of the effects of hormones in adult women who take oral contraceptives.]

3.2.3 *Oral administration to transgenic mice*

Sixty female *p53* (+/–) mice (heterozygous female *p53*-deficient CBA mice, in which exon 2 of the lateral *p53* allele was inactivated, were the F_1 offspring of heterozygous *p53*-deficient male C57 BL/6J mice that had been back-crossed with female CBA mice (Tsukada *et al*., 1993)) and 60 female wild-type *p53* (+/+) litter mates, 6 weeks of age, were each divided into four groups of 15 animals. [The Working Group noted the small number of animals.] All mice received an intraperitoneal injection of 120 mg/kg bw *N*-ethyl-*N*-nitrosourea (ENU) in physiological saline, followed by no further treatment (Group 1) or were fed *ad libitum* diets that contained 1 ppm ethinylestradiol (Group 2), 2.5 ppm ethinylestradiol (5 ppm for the first 4 weeks reduced to 2.5 ppm thereafter because of marked body weight depression; Group 3) or 2000 ppm methoxychlor [a weakly estrogenic pesticide] (Group 4) for 26 weeks. Individual body weights in each group were measured every week. One of the 15 *p53* (+/–) mice in Group 2 died during the early period of the experiment. After the end of the 26-week experiment, surviving animals were killed and autopsied. The uterine tissues were sectioned and stained for microscopic examination. The differences in the incidence of uterine proliferative lesions were assessed by the Fisher's exact test. Multiple nodules (5–20 mm in diameter) of the uterine horn suggestive of uterine tumours were observed in seven, nine, 12 and seven *p53* (+/–) mice in Groups 1, 2, 3 and 4, respectively. The absolute uterine weights and uterine weight/body weight ratios of *p53* (+/–) and *p53* (+/+) mice in Groups 2, 3 and 4 were significantly higher than those in the Group 1 mice. The uterine weights of *p53* (+/–) mice, especially in Group 3, were considerably

increased because of marked growth of uterine tumours. Uterine proliferative lesions were classified into endometrial stromal polyps, endometrial stromal sarcomas, adenocarcinomas, atypical hyperplasias and endometrial glandular hyperplasias. Atypical hyperplasias were classified into two cell types — clear and basophilic — characterized by small proliferative foci of endometrial glandular epithelia with atypia. Non-atypical endometrial glandular hyperplasias were composed of increased numbers of endometrial glands with occasional cysts. The incidence of uterine stromal tumours in *p53* (+/–) mice was 87% (47% stromal sarcomas, 40% polyps), 85% (64% stromal sarcomas, 21% polyps), 87% (stromal sarcomas) and 53% (stromal sarcomas) in Groups 1, 2, 3 and 4, respectively; there was a significant difference in the incidence of stromal sarcomas between Groups 1 and 3 (see Table 31). In *p53* (+/+) mice, only stromal polyps were seen at an incidence of 20, 13, 0 and 0% in Groups 1, 2, 3 and 4, respectively; these values displayed a clear decrease compared with the incidence of stromal tumours in the groups of *p53* (+/–) mice. The incidence of clear-cell type atypical hyperplasias in *p53* (+/–) mice was 0, 14, 60 and 0% in Groups 1, 2, 3 and 4, respectively; that in *p53* (+/+) mice was 0, 7, 53, and 0%; the difference between Groups 1 and 3 was significant in both cases ($p < 0.05$, Fisher's exact test). For atypical hyperplasias of the basophilic cell type, there were no significant differences among the groups [incidence not specified]. One *p53* (+/–) mouse in Group 3 developed a clear-cell adenocarcinoma. The incidence of glandular hyperplasias in *p53* (+/–) mice was 60, 79, 60 and 27% in Groups 1, 2, 3 and 4, respectively, whereas that in *p53* (+/+) mice was 60, 80, 100 and 100%; the incidence in Group 3 and Group 4 *p53* (+/+) mice showed significant differences from values in Group 1 (Mitsumori *et al.*, 2000). [This study suggests that ethinylestradiol possibly exerts tumour-promoting (co-carcinogenic) effects on stromal and epithelial proliferative lesions of the uterus in *p53*-deficient mice initiated with ENU.]

3.3 Progestogens used in combined oral contraceptives

The results of studies that were reviewed previously (IARC, 1979, 1999) on the carcinogenicity of progestogens used in combined oral contraceptives are summarized below (see Tables 32, 33 and 34).

The incidence of pituitary adenomas was increased by norethisterone in female mice and by norethynodrel in female and male mice and male rats.

The incidence of malignant mammary tumours was increased in female mice by lynestrenol, megestrol acetate and norethynodrel. In female rats, lynestrenol and norethisterone slightly increased the incidence of malignant mammary tumours. In male rats, norethisterone also slightly increased the incidence of malignant mammary tumours, while norethynodrel increased the incidence of both benign and malignant mammary tumours. In female dogs, chlormadinone acetate, lynestrenol and megestrol acetate increased the incidence of benign and malignant mammary tumours; however, lynestrenol had a protective effect at a low dose but enhanced tumour incidence at two higher doses. Levonorgestrel did not increase the incidence of mammary tumours in one study in dogs.

Table 31. Incidence of lesions (percentage) in female *p53* (+/+) and (+/–) CBA mice injected intraperitoneally with ENU and given EE or MXC in the diet

Treatment	*p53* (+/–) Mice (n = 15)[a]					*p53* (+/+) Mice (n = 15)				
	Uterine stromal tumours		Atypical hyperplasias		Glandular hyperplasias	Uterine stromal tumours		Atypical hyperplasias		Glandular hyperplasias
	Polyps	Stromal sarcomas	Clear-cell type	Basophilic cell type[b]		Polyps	Stromal sarcomas	Clear-cell type	Basophilic cell type[b]	
ENU	40	47	0	NR	60	20	0	0	NR	60
ENU + 1 ppm EE	21	64	14	NR	79	13	0	7	NR	80
ENU + 2.5 ppm EE	0	87*	60*[c]	NR	60	0	0	53*	NR	100*
ENU + MXC	0	53	0	NR	27	0	0	0	NR	100*

From Mitsumori *et al*. (2000)
ENU, *N*-ethyl-*N*-nitrosourea; EE, ethinylestradiol; MXC, methoxychlor; NR, not reported
* Significantly different from the ENU group ($p < 0.05$, Fisher's exact test)
[a] ENU + 1 ppm EE group (n = 14)
[b] Crude number not specified in the paper, but the authors state that there were no significant differences among treatment groups.
[c] One animal in this group developed a clear-cell adenocarcinoma.

Table 32. Effects of various progestogens alone or with a known carcinogen on tumour incidence in mice

Progestogen	Pituitary adenoma		Mammary tumours		Uterine tumours	Vaginal/ cervical tumours	Liver			
	Male	Female	Benign (males)	Malignant (females)			Adenoma		Carcinoma	
							Male	Female	Male	Female
Chlormadinone acetate							+/–			
Cyproterone acetate							+[a]	+/–[a]	+[a]	+[a]
Ethynodiol diacetate			c				+/–			
Lynestrenol				+			+			
Megestrol acetate				+				+		
Norethisterone acetate							+/–			
Norethisterone		+					+/–			
Norethynodrel	+	+	c	+				+/–		
Norethynodrel + 3-methyl-cholanthrene					+	–				

From IARC (1979, 1999)

+, increased tumour incidence; +/–, slightly increased tumour incidence; –, no effect; c, increased incidence in castrated males

[a] Dose exceeded the maximum tolerated daily dose

Table 33. Effects of various progestogens alone or with a known carcinogen on tumour incidence in rats

Progestogen	Pituitary adenoma (males)	Mammary tumours			Liver				
		Benign (males)	Malignant		Adenoma		Carcinoma (males)	Foci	
			Male	Female	Male	Female		Male	Female
Cyproterone acetate					+[a]	+[a]			+[b]
Ethynodiol diacetate		+							
Lynestrenol				+/–					
Norethisterone acetate					+	+		+	+ or –[c]
Norethisterone		+/–	+/–	+/–	+				
Norethynodrel	+	+	+		+	+	+		–[c]
Norethynodrel + *N*-nitrosodiethylamine									+

From IARC (1979, 1999)
+, increased tumour incidence; +/–, slightly increased tumour incidence; –, no effect
[a] Liver adenomas detected only at high doses
[b] Tested for initiating activity; the results were positive in one study in which it was administered for 5 days and negative when it was administered as a single dose.
[c] Tested as a single dose for initiating activity

Table 34. Effects of various progestogens on mammary tumour incidence in female dogs

Progestogen	Benign	Malignant
Chlormadinone acetate	+	+
Lynestrenol	+[a]	+[a]
Megestrol acetate	+	+

From IARC (1979, 1989)
+, increased tumour incidence
[a] In this study, lynestrenol had a biphasic effect, with protection at the low dose (10 times the human contraceptive dose) and enhancement at 50 and 125 times the human contraceptive dose.

In female mice treated with 3-methylcholanthrene to induce uterine tumours, norethynodrel further increased the tumour incidence.

In male mice treated with chlormadinone acetate, ethynodiol diacetate, lynestrenol, norethisterone or norethisterone acetate, the incidence of liver adenomas was increased. Megestrol acetate increased the incidence of adenomas in female mice. Cyproterone acetate increased the incidence of liver adenomas and that of hepatocellular carcinomas in male and female mice, but at levels that exceeded the maximum tolerated dose. In rats, the incidence of liver adenomas was increased by norethisterone acetate (males and females), norethisterone (males), norethynodrel and cyproterone acetate (males and females). The numbers of altered hepatic foci in female rats were also increased by norethisterone acetate and cyproterone acetate. In female rats treated with *N*-nitrosodiethylamine to initiate hepatocarcinogenesis, norethynodrel increased the number of altered hepatic foci. Norethynodrel alone was shown to increase the incidence of hepatocarcinomas in male rats.

Levonorgestrel in combination with *N*-nitrosobis(2-oxopropyl)amine did not increase the incidence of renal dysplastic lesions or tumours in female hamsters.

Oral administration to dogs

Groups of three female beagle dogs, 9–10 months old, were treated orally for 91 days with 0.03, 0.3 or 3 mg/kg bw per day dienogest. Three control animals were given the vehicle (0.5% carboxymethylcellulose) alone. On the day after the last treatment, the animals were killed and their mammary glands and pituitary glands were removed. The mammary glands from dogs treated with dienogest for 91 days showed dose-dependent proliferation. Dogs given 3 mg/kg bw per day showed severe alveoli hyperplasia and a large number of vacuoles in the alveolar cells. The pituitary glands from dienogest-treated dogs showed slight hypertrophy compared with those from control dogs (Ishikawa *et al.*, 2000). [Dienogest has progestational activity and caused proliferation of mammary gland epithelial cells in dogs, although no conclusive evidence of a carcinogenic effect was provided. As part of this same study, and with a similar design, dienogest showed no effects in female rats or monkeys.]