

er to diagnose epithelial tumors at a time when they are still confined to
pelvis and highly curable.
In patients with advanced cancer, treatment approaches are also changing.
New combination chemotherapy regimens are under evaluation in clinical trials
which address issues such as high-dose chemotherapy and dose intensity are being
studied. It is our hope that this issue will provide to the reader an overview of
current approach to the management of this disease. In addition, we have
tried to provide a framework to help understand the forthcoming new thera-
peutic developments that are based on an ever-increasing understanding of the
biology of this complex disease.

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THE EPIDEMIOLOGY OF OVARIAN CANCER

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Ovarian cancer is the leading cause of death from a gynecologic malignancy among women in the United States and accounts for approximately 12,500 deaths per year.⁴² Although the etiology and pathophysiology of ovarian carcinogenesis are unknown, there is a growing body of epidemiologic literature that has identified several potential contributing factors. This article explores these findings, with a particular emphasis on the genetic and reproductive features characteristic of the disease.

GENERAL FEATURES

Ovarian cancer is a disease of older age, with incidence rates increasing with each decade of life and peaking in the mid to late 70s⁷⁷ (Fig. 1). The rate of rise in incidence appears to accelerate coincident with the time of natural menopause.^{20, 64} Malignant ovarian tumors do occur in childhood and represent 1% to 3% of all pediatric malignancies, but approximately two thirds of these are of the germ cell type.^{3, 12, 19}

Age-adjusted rates for ovarian cancer have remained relatively stable over the last two decades (Fig. 2), although mortality rates have climbed as a result of the aging of the population, and possibly owing to changing reproductive patterns.³⁶ Significant geographic and ethnic variations in rates have been observed. Rates are highest for white women in the industrialized countries of northern and western Europe and North America, and lowest in India and Asia.⁵³ The wide geographic variation observed is roughly correlated with average family size.²⁴ Within the United States, incidence rates for black, Hispanic, and American Indian women are approximately 40% lower than those for white women,⁴³ and among whites, rates are highest for Jewish

From the Cancer Control Science Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania

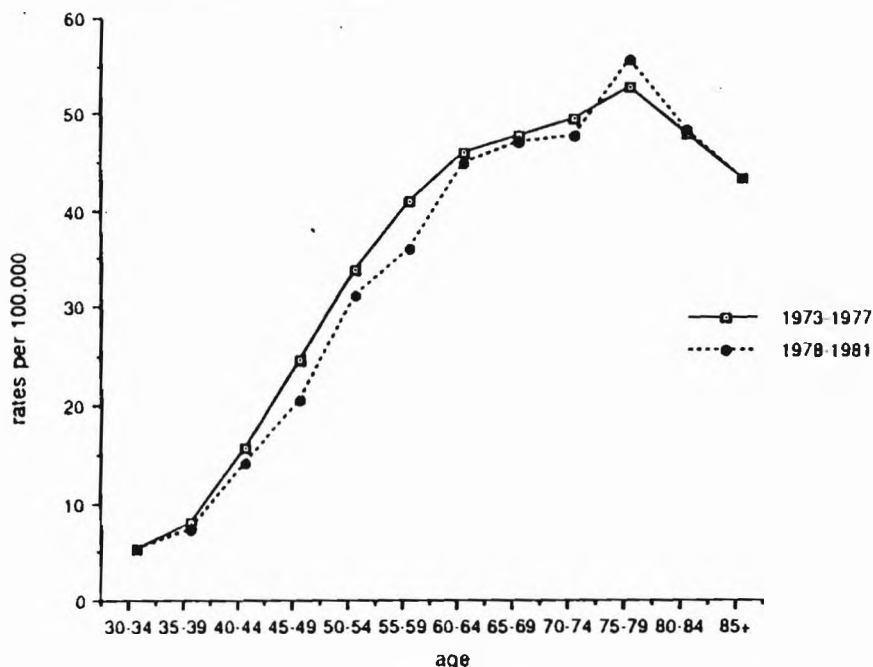


Figure 1. Ovarian cancer rates by age for 1973-1977 and 1978-1981. (Data from the SEER program: Cancer Incidence and Mortality in the United States, 1973-1981. US Department of Health and Human Services, 1984.)

women.²⁰⁻³¹ These ethnic differences in rates may be partially explained by variations in reproductive factors.

ENVIRONMENTAL FEATURES

Both the observed international and ethnic variations, and the shift in risk to that of the host country among migrant populations,²⁹ suggest a role for an environmental component such as diet in the etiology of ovarian cancer. Numerous case control studies, however, have failed to show an association between either weight or dietary fat intake and the risk for ovarian cancer.^{2-31, 46-49} A positive association between ovarian cancer risk and lactose consumption has, however, been found in a number of studies. Cramer¹⁵ correlated ovarian cancer incidence with both per capita milk consumption and lactase persistence worldwide and found both to be significantly positively associated. A case-control study conducted in 10 Boston-area hospitals demonstrated both higher levels of lactose consumption and lower levels of galactose converting enzymes in cases than in controls.¹⁶⁻²⁷ Although these data are very preliminary, there is theoretic support from the observation that women who suffer from galactosemia, an inborn error of metabolism resulting in elevated levels of serum galactose, often develop premature ovarian failure and hypergonadotropic hypogonadism.¹⁵ A series of animal studies has also documented ovarian failure in rodents fed a high galactose diet.⁴⁵

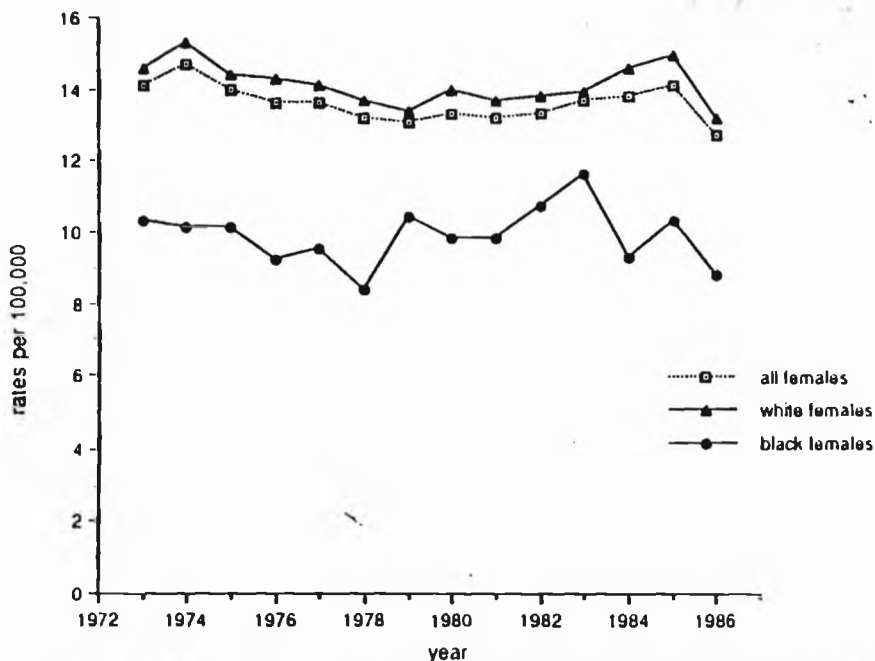


Figure 2. Age-adjusted ovarian cancer incidence by year and race for 1973–1986. (Data from the SEER program: Cancer Statistics Review, 1973–1986. US Department of Health and Human Services, 1989.)

Other environmental factors that appear to be unrelated to risk for ovarian cancer are occupation, alcohol, and smoking history.^{24–26} The noted increased incidence among women of higher socioeconomic status most likely reflects the combined effects of reproductive history and differential access to health care.²⁰

Of all the environmental factors implicated as causative agents in ovarian cancer, none has generated as much interest as talc and asbestos particles. Talc is a specific hydrous magnesium silicate that is chemically related to asbestos. Asbestos fibers are often found as contaminants of talc consumer products.²⁷ Interest in these compounds as causative agents for ovarian cancer stems from the recognition of asbestos as a cause of pleural and peritoneal mesothelioma, a disease that shares a common embryologic origin with ovarian cancer. Henderson et al²⁸ first reported finding talc particles in ovarian tissue obtained from nine women, three of whom had ovarian cancer. Cosmetic talc is thought to gain access to the ovaries by absorption through the vagina or cervix. In a case-control study in Boston, Cramer et al²⁹ reported a relative risk of 1.9 among individuals who used talc directly on the perineum or on sanitary pads. Whittemore et al³⁰ also showed a weak association between talc use and risk for ovarian cancer, but there was no dose-response relationship. Although these data are provocative, they offer only indirect evidence for an association between these products and ovarian cancer. With the removal of talc from most cosmetic preparations over the last decade, the issue may remain unresolved.

Likewise, with the introduction of widespread vaccination among children

against mumps, the causative role of this virus may never be clarified. First thought of as a potential etiologic agent for ovarian cancer because of its known association with clinical orchitis in males, mumps also belongs to the paramyxovirus family, which is associated with a number of rare but fatal chronic diseases.⁴⁷ Two case-control studies have found a lower self-reported history of mumps infection among cases^{48, 49}; however, when serum antibody levels were measured, they were similar for cases and controls, suggesting that ovarian cancer patients with mumps were more likely to have experienced subclinical disease, which may have led to subsequent chronic damage to the ovaries, depletion of oocytes, and alterations in gonadotropin levels.⁴⁹

Finally, there is evidence from the survivors of the atomic bomb experience that high doses of radiation increase the risk for ovarian cancer, particularly among those women who were less than 20 years of age at the time of exposure, with a latent period of approximately 20 years.⁵⁰

GENETIC FACTORS

Although the recognition of a hereditary component to ovarian cancer is very recent, heredity is emerging as one of the most important aspects of the disease. The number of known multicase ovarian cancer families has increased several-fold over the last 10 years. Extensive pedigree analysis by Lynch et al⁵¹ has led to the recognition of three patterns of familial ovarian cancer: (1) site-specific ovarian cancer only; (2) families in whom both breast and ovarian cancer predominate; and (3) the cancer family syndrome characterized by multiple cases of ovarian, breast, colon, and uterine cancer. Through careful analysis of these pedigrees, an autosomal dominant mode of inheritance with variable penetrance is postulated. A subset of approximately 5% to 10% of all ovarian cancers are thought to fit this genetic pattern.⁵² The most common relationship reported among cases is sibship, followed by mother-daughter.^{53, 54} Among these pedigrees, there is a significant reduction in risk for breast/ovarian cancer for relatives as their levels of coancestry decrease.⁵⁵ Other characteristics of familial ovarian cancer include an earlier age at onset and evidence of both paternal and maternal transmission^{56, 57}; however, there does not appear to be a significant difference in stage at presentation, histologic type or grade, or bilaterally between familial and sporadic cases.^{58, 59} The familial aggregation of ovarian cancer demonstrated by these pedigree analyses has been confirmed in numerous case-control studies. Data from the Alberta Cancer Registry have documented a statistically significant increase in affected family members among cases of ovarian cancer.⁶⁰ Tzonou et al⁶¹ found a familial aggregation of ovarian cancer among women with breast, ovarian, and uterine cancer in Greece. In Japan, Mori et al⁶² found a family history of breast, uterine, and ovarian cancer to be more common in cases than in controls. Using the multicenter data base assembled by the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute, Schildkraut and Thompson⁶³ demonstrated an odds ratio for ovarian cancer of 3.6 in first-degree relatives, and 2.9 in second-degree relatives, as compared with relatives of controls. The excess of affected relatives was seen in both maternal and paternal lines. Of interest is the fact that neither the SEER data nor a case-control study conducted by Harlow et al⁶⁴ in Washington state could demonstrate an increased risk of ovarian cancer for relatives of women with borderline tumors, suggesting a different carcinogenic pathway for this subtype of ovarian cancer.

The familial aggregation of breast and ovarian cancer is supported by data

from the Cancer and Steroid Hormone (CASH) Study,⁷ a population-based case-control study conducted by the Centers for Disease Control, in which Thompson and Schildkraut⁸ were able to demonstrate an elevated risk of developing breast cancer among first-degree relatives of women with ovarian cancer.

Finally, lending further support to a proposed genetic mechanism for ovarian cancer is the finding of clustering of both breast and ovarian cancer in the patients themselves. Data from the Birmingham Cancer Registry in England found a significant excess of second primaries in premenopausal women with either breast or ovarian cancer. Prior and Waterhouse⁹ and Gregg et al¹⁰ report a fivefold increase in rates of breast cancer occurring as a second primary among familial ovarian cancer cases, as compared with sporadic ovarian cases.

Although the precise genetic defect(s) associated with familial ovarian cancer has not yet been identified, a number of genetic alterations and associations have been described. Several investigators have noted decreased levels of α -L-fucosidase among women with ovarian cancer and their first-degree relatives,^{3, 40, 53} suggesting close linkage of the genetic loci that control α -L-fucosidase activity and ovarian carcinogenesis. Cytogenetic characterization of surgical specimens from ovarian tumors reveals extensive and complex structural chromosome abnormalities involving chromosomes 1, 3, 6,^{31, 72} chromosome 14,⁷⁰ and chromosome 17.^{31, 53} Marks et al⁴ reported overexpression of the p53 gene, a gene recently linked to familial breast cancer,⁴⁴ in 50% of 107 tumor samples. This growing body of evidence points to a multistep model of ovarian carcinogenesis in which a complex series of genetic events result in the initiation, development, progression, and metastatic potential of the disease.

REPRODUCTIVE FACTORS

Because the ovary is the major source of reproductive hormones in women, it is only natural that a great deal of attention has been paid to the role of reproductive factors in trying to understand the etiology of the disease. The one overwhelming observation that has emerged from almost every study in the literature is the relationship between pregnancy experience and risk for ovarian cancer. Women who develop ovarian cancer are significantly more likely to be nulliparous and to have had fewer pregnancies.^{11, 34, 48, 50, 64} The relative risk associated with nulliparity ranged in several case-control studies from 1.5 to 3.2.^{2, 6, 12, 18, 33, 46, 48, 76} Furthermore, there is a clear dose-response relationship associated with pregnancy history, with most studies reporting a steady decrease in risk with increased parity.^{6, 12, 31, 33, 48, 49, 76} When controlled for number of pregnancies, however, age at first pregnancy does not appear to affect risk.^{4, 6, 12, 38, 76} The role of oral contraceptive pills (OCPs), which mimic some of the physiologic changes induced by pregnancy, has also been carefully studied with regard to ovarian cancer. OCPs were first introduced in the United States in the 1960s. Most formulations include estrogen, progesterone, or a combination of the two, and they have been shown to suppress ovulation. Women with a history of OCP use have an overall reduction in risk of ovarian cancer of 40%.⁵⁴ The largest case-control study to document this effect was that conducted by the Centers for Disease Control, using the population-based cancer registry data of the eight SEER regions, and age-matched and area of residence-matched community controls. Ever-users of OCPs had a relative risk for ovarian cancer of 0.6. Risk reduction was apparent with as little as 3 months of use, increased in magnitude with duration of use, and persisted for as long

as 10 years after discontinuation of use.⁹ The observed reduction in risk applied to all histologic subtypes, including borderline tumors, and was not dependent on age at use, menopausal status, or measures of fertility or parity, although nulliparous women experienced a greater decrease in risk than did parous women.⁹ A summary of similar findings from a number of case-control studies is shown in Table 1. Note that the majority of these studies failed to show a relationship between replacement hormone therapy for menopausal symptoms and risk for ovarian cancer.^{2, 4, 31, 46} One exception is a case-control study in Washington state in which replacement hormone use was associated with a relative risk of 3.1 for the histologic subset of endometrioid tumors.⁷¹ A similar trend was noted in a study in Boston by Cramer et al,¹² and in a case-control study in Washington, DC.²⁸ Endometrioid tumors of the ovary are similar in appearance to tumors of the endometrium and may share common risk factors. No clear-cut relationship between age at menarche or age at menopause and risk for ovarian cancer has emerged from these studies.

Another series of studies investigated the role of sterilization procedures on subsequent risk of ovarian cancer. Irwin et al¹² examined the data from the Centers for Disease Control study to compare histories of tubal sterilization and hysterectomy with or without unilateral oophorectomy between cases and controls. A significantly reduced risk for ovarian cancer was seen in women who had undergone these procedures, particularly if they were performed at younger ages. Data from a case-control study in Washington, DC, demonstrated a 30% risk reduction in women undergoing hysterectomy with preservation of the ovaries.²⁸ Similarly, a case-control study performed in Japan, where incidence of ovarian cancer is low, found that a significantly lower proportion of ovarian cancer patients than controls had undergone permanent sterilization.⁴⁸ Of note is the observation by Cattanaich of decreased estrogen excretion in women who had undergone tubal ligation, a finding that he suggested may be related to localized procedure-induced vascular changes.⁷

Two alternative hypotheses have been advanced to explain the findings related to reproductive history that were described previously. One theory, first proposed by Fathalla,²⁰ contends that constant, uninterrupted ovulation leads to an increased risk for ovarian cancer by virtue of the repetitive trauma to the ovarian epithelium and resultant cellular proliferation associated with the ovulatory cycle, the potential for entrapment and transformation of epithelial inclusion cysts, or the repetitive exposure of the epithelium to steroid-rich follicular fluid or pituitary gonadotropins.^{14, 76} Thus, events such as pregnancy and OCP use, which suppress ovulation, would decrease this risk. A number of investigators have actually calculated measures of duration of ovulation by subtracting anovulatory time during pregnancy and lactation and while taking OCPs from time at risk for ovulation, and found a significant correlation

Table 1. RELATIVE RISK OF OVARIAN CANCER BY ORAL CONTRACEPTIVE USE

Reference (No.)	Cases/Controls		Relative Risk Estimate (95% Confidence Interval)
	Users	Nonusers	
CDC ⁸	197/439	2335/3867	0.6 (0.4-0.9)
Weiss ⁷¹	21/112	207/552	0.57 (0.05-1.92)
Rosenberg ³⁹	33/136	187/539	0.6 (0.4-0.9)
Wu ⁷⁶	188/299	619/1011	0.74 (0.52-1.06)
Cramer ¹¹	34/144	48/139	0.38 (0.15-0.96)
Harlow ²⁷	66/194	82/193	0.7 (0.4-1.2)
LaVecchia ³⁷	39/178	367/1104	0.68 (0.48-0.97)

between ovarian cancer risk and duration of ovulation.⁶⁻⁷⁶ Support for this theory also comes from a little-known experiment in which epithelial ovarian cancers were induced in hens by using prolonged fluorescent lighting to induce ovulation and maximize egg production.⁷⁵

The alternative hypothesis contends that nulliparity, rather than failing to offer protection against ovarian cancer, is actually a manifestation of an underlying reproductive disorder, which also predisposes to the development of the malignancy. At least four case-control studies have found a significantly increased prevalence of infertility among cases as compared with controls, whether infertility is measured by self-reporting of difficulty in conceiving,¹¹⁻¹⁴ physician-diagnosed infertility,⁷⁴ or number of years of unprotected intercourse without conception.¹⁶⁻¹⁹ Incidence of abortion, stillbirth, and congenital malformation do not appear, however, to be related to ovarian cancer risk.¹⁶⁻¹⁹ A role for altered pituitary gonadotropin levels, which may be the basis for subfertility in many women, is supported by the epidemiologic observation that incidence rates for ovarian cancer accelerate around the time of menopause, when gonadotropin levels also show a sharp rise.^{23,24} It is also consistent with the previously described apparent protection afforded by OCP use, which is associated with suppression of pituitary gonadotropins.⁹ In fact, a case-control study performed by Harlow et al²⁷ described an interactive effect between high lactose intake and the protection afforded by oral contraceptive use. They propose that it is in the setting of a hypergonadotropic state induced by high galactose levels that OCPs exert their protective, gonadotropin-lowering effect. The altered gonadotropin theory is also consistent with the observed reduction in risk following sterilization procedures, which have been shown to alter ovarian blood flow and hormonal activity.¹⁻³⁰ There are also some data from murine models in which elevated gonadotropin levels are associated with ovarian tumor promotion, but the applicability of this model to human carcinogenesis is questionable.⁴¹

SUMMARY

The epidemiologic data collected to date have provided some important and provocative clues as to the etiology of ovarian cancer. The recognition of familial clustering of this disease has led to exciting advances in understanding the genetics involved. The contribution of endocrine factors has been well documented in the epidemiologic literature, leading to important insights into the process of carcinogenesis. Clearly, additional information is needed to further explain the interplay of genetic, physiologic, and life style factors, to lead to a better understanding of the disease, and ultimately to a means of prevention and control.

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PATHOLOGY: EPITHELIAL TUMORS

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This article reviews the pathology of epithelial tumors of the ovary, with particular emphasis on newer information in the literature. Most of the discussion is devoted to the most common category of malignant epithelial tumors, which have usually been referred to in recent years as "coepithelial" or "surface epithelial" cancers.^{1,2} These are designated "epithelial-stromal tumors" in the revised classification of ovarian tumors of the World Health Organization (WHO) (Table 1) that is currently being finalized.³ Also discussed briefly is a recently described highly malignant epithelial tumor that does not appear to belong in this category, but that is important for oncologists to recognize, the small cell carcinoma.⁴ Although almost all types of surface epithelial-stromal tumor include those of borderline malignancy, those of endometrioid,^{5,6} clear cell,^{7,8} and transitional cell⁹ are rare and will not be discussed. Some particularly important problems in the differential diagnosis of epithelial cancers of which the clinician should be aware will be mentioned; these problems have been discussed in greater detail elsewhere, along with others.¹⁰

Surface epithelial-stromal tumors account for approximately two thirds of all ovarian neoplasms, and their malignant forms for about 90% of ovarian cancers.¹¹ In the designation given to a neoplasm in this category, three criteria are considered. The two most important are the cell type, i.e., mucinous, and so on—and whether the tumor is benign, of so-called borderline malignancy, or frankly malignant. The final two criteria are architectural features that pertain to whether the tumor is exophytic, endophytic, or both. If exophytic growth is present, the word "surface" is added to the designation, e.g., serous surface papillary carcinoma. The second relates to the proportions of the epithelial and stromal components of the neoplasm. In surface epithelial-stromal tumors are predominantly epithelial, with a minor portion derived from the ovarian stroma; when a tumor has a pre-

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VIII International Symposium on Endocrinology and Development: Multiple Endocrine Diseases: Growth Hormone Action: Intersexuality

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This publication presents selected contributions from three workshops relating to endocrinology and development. The first section aims to help clinicians and basic scientists understand the pathogenesis and clinical features of different polyendocrine syndromes. A comprehensive review of the anabolic and metabolic effects of growth hormone action is provided in the second section. Finally, the last section is devoted to the interesting mechanisms of sexual differentiation in man. Readers will find that the publication offers challenging perspectives and suggests exciting new directions for further research.

Contents

Introductory Lecture

LeRoith, D., Shemer, J., Roberts, C. T., Jr.: Evolutionary Origins of Intercellular Communication Systems: Implications for Mammalian Biology

Multiple Endocrine Diseases

Scherbaum, W.A.: Introduction · Riley, W.J.: Autoimmune Polyglandular Syndromes · Calender, A.: Schuffenecker, J.: Lenor, G.M.: The Genetics of Multiple Endocrine Neoplasia (MEN) · Padberg, B.C.: Holl, K.; Schröder, S.: Pathology of Multiple Endocrine Neoplasias 2A and 2B: A Review · Raus, F.; Zink, A.: Clinical Features of Multiple Endocrine Neoplasia Type 1 and Type 2

Anabolic and Metabolic Effects of Growth Hormone Action

Seeger, P.: Introduction · Clemmons, D.R.; Underwood, L.E.: Role of Insulin-Like Growth Factors and Growth Hormone in Reversing Catabolic States · Gerner, J.M.: Growth Hormone Actions on Fat Distribu-

tion and Metabolism · Haymond, M.W.; Horber, F.F.: The Effects of Human Growth Hormone and Prednisone on Whole Body Estimates of Protein Metabolism · Caprio, S.; Bouwmeester, D.; Tamborlane, V.: Growth Hormone and Insulin Interactions

Intersexuality

Sizonenko, P.C.: Introduction · Swaab, D.F.; Gooren, L.J.G.; Hofman, M.A.: Gender and Sexual Orientation in Relation to Hypothalamic Structures · Hawkins, J.R.: Genetic Determinants of Testis Development in Normal and Abnormal Individuals · Voutilainen, R.: Differentiation of the Fetal Gonad · Jossio, N.: Anti-Müllerian Hormone and Sertoli Cell Function · Hughes, I.A.; Williams, D.M.; Baich, J.A. et al.: Male Pseudohermaphroditism: Clinical Management, Diagnosis and Treatment · Aarskog, D.: Syndromes and Genital Dysmorphology

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Review

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Ovarian Surface Epithelium and Human Ovarian Cancer

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Abstract

Approximately 80-90% of adult ovarian cancers are assumed to originate from ovarian surface cells. The morphology of the ovarian surface epithelium changes constantly, exhibiting features such as crypts, inclusion cysts, villous processes and different forms of müllerian epithelium. The unique nature of ovarian surface features and their absence in the immediately adjacent peritoneal mesothelium suggest that local factors may play an important part in modifying the growth and morphology of the ovarian surface epithelium. Recent studies tend to emphasize oncogene activation, along with concomitant cytogenetic changes, in the development of ovarian cancer. This review focuses on the roles of exogenous and endogenous factors and ovulation in the pathogenesis of ovarian cancer.

Introduction

Ovarian cancer is a disease that is still largely unnameable to curative treatment. Although accounting for only 4% of all cancers, ovarian cancer causes an estimated 6% of deaths from all types of malignancies in women [1]. Every year, more women die in the industrialized nations from this cancer than from all types of uterine cancer, which is three times as frequent [2]. The main reason for this discouraging situation is the fact that over 70% of all ovarian cancers are first discovered at a stage that permits practically no curative therapy and no effective screening [3]. Less than 25% of women with ovarian cancer survive the initial diagnosis by more than 5 years [4]. Epithelial tumors account for 80-90% of all ovarian cancers and are presumed to originate from the surface epithelium [5-7]. They rarely appear before the age of 10; their frequency then increases exponentially until the age of 40, and the highest incidence is in women in their early 60s.

Dynamic Morphology of the Ovarian Surface Epithelium

The serosa-like cell layer of the ovary develops from the celomic epithelium. During the fetal period, the surface epithelium is subject to constant morphological changes. After approximately the 10th fetal week, a multi-layered epithelium with occasional papillary projections and a basement membrane separating it from the neighboring stroma develops out of a single-layered epithelium with no basement membrane [8]. The increase in proliferative activity is associated with a high nucleo-cytoplasmic ratio, nuclear polymorphism, prominence of nucleoli, and abundance of free and bound ribosomes [9, 10]. The separation of the superficial epithelium from the cortical stroma by a basement membrane is not yet complete, since islands and strands of surface epithelium are present in the cortex during the entire fetal and perinatal period [5, 10]. For this reason, cortical inclusion cysts, which by

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Fig. 1. Ovarian surface epithelium with cilia. $\times = 1,420$.

Fig. 2. Cytoplasmic vacuoles (*) and macropinocytosis (arrow) in an ovarian surface cell. $\times = 14,400$.



their nature have the same proliferative activity as the superficial epithelium, are also found in the fetal ovary [11].

The surface morphology of the ovary changes constantly during the whole course of life [9]. A single-layered ovarian surface epithelium with a regular cuboidal cell structure is usually found resting upon the tunica albuginea and the stroma below it. Since this epithelium possesses the characteristics of the müllerian duct, endometrioid and endocervical cell forms can also develop from it [5, 8]. At various points, the ovarian surface cells exhibit not only microvilli but also numerous cilia (fig. 1), whose surface distribution is regionally diverse in one and the same ovary and whose emergence is both cyclical and age related [12]. Frequent caveolae also indicate that the ovarian surface cells have high absorptive activity [13]. These caveolae can be regarded as morphological evidence of the endocytosis of material from the abdominal cavity [14]. It is possible that the uptake of substances by the ovarian surface cells is further aided by macropinocytosis, in which cytoplasmic evaginations entrap the material to be incorporated, forming small vacuoles (fig. 2). Numerous crypts are present on the surface of the ovary. The epithelium at the base of these crypts occasionally extends even deeper to form small cell nests, which can

give rise to inclusion cysts, probably due to sealing off at the surface epithelium. If there is proliferation in the opposite direction, papillary excrescences form on the surface. Also biochemically detectable in the superficial epithelium of the mature ovary is a characteristic pattern of four polypeptides of the intermediate filament cytokeratin, which is also present in Müller's tissue and in epithelial ovarian cancers [15-17]. It is noteworthy that Miettinen et al. [18] found the intermediate filament prekeratin in normal superficial epithelium, in inclusion cysts and in epithelial ovarian tumors. Ultrastructurally, scattered desmosome-tonofilament complexes (fig. 3) are found in the superficial epithelial cells of the mature ovary. According to studies by Ferczy and Roth [19], such complexes appear with predilection in endometrioid ovarian carcinomas and Brenner tumors; moreover, these tumor forms have very dense intermediate filaments.

Blaustein [5, 11] distinguishes two types of inclusion cysts: an immature type with a primitive celomic epithelium that emerges during fetal development, as mentioned above, and a mature type that possibly forms around the time of ovulation and in which the epithelium is quite variable. Cystadenomas and cystadenocarcinomas are believed to develop from inclusion cysts [20], and certain tumor markers have been found in both inclusion

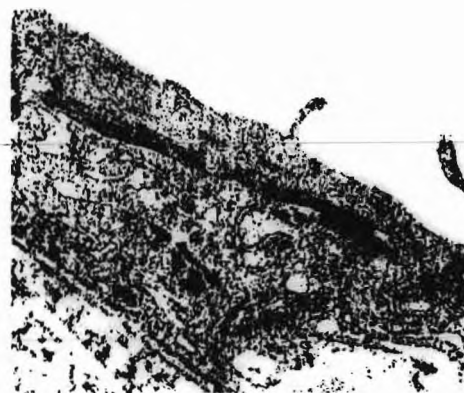


Fig. 3. Desmosome-tonofilament complex in a human ovarian surface cell. $\times = 16,400$.

cysts and serous ovarian tumors [21]. Blaustein et al. [21] found carcinoembryonic-antigen-positive cells in some inclusion cysts, which can be taken as a sign of the immaturity and undifferentiation of certain cortical inclusion cysts. Elevated levels of human chorionic gonadotropin and carcinoembryonic antigen have been noted in the fluid of some inclusion cysts, cystadenomas and cystadenocarcinomas [5]; this suggests that all three have the primitive embryonic celomic epithelium as their origin. It has also been reported that CA 125, an antigen associated with ovarian carcinoma and present in nearly all serous tumors of the ovary, is also to be found in inclusion cysts, but not in normal ovarian surface cells [22, 23]. Inclusion cysts form an actively proliferating cell compartment that is responsive to hormonal stimulation and is capable of neoplastic change under certain conditions. However, not only do inclusion cysts develop around the time of ovulation, but prominent papillae also develop - at least in animal experiments - at the site of the postovulatory defect [24]. These excrescences can result, under both physiological and pathological conditions, in the formation of superficial structures that resemble neoplastic lesions in most respects [25, 26]. Inclusion cysts derived from invagination and entrapment of ovarian surface cells in the cortex are more frequently observed in the later reproductive years and after the menopause [27]. Indirect evidence of increased proliferative activity in the senescent ovarian surface epithelium is also provided by the presence of cell

stratification and papillary projections [27]. It remains to be seen whether these morphological changes, which coincide with the peak age of ovarian carcinoma [28], are compensatory to cortical shrinkage or secondary to other stimuli [29].

Exogenous Factors

Ovarian carcinoma exhibits marked differences in frequency throughout the world. For example, it is three to seven times more common in western countries, such as the USA, Great Britain, and Scandinavia than in Japan [30]. The incidence of ovarian cancer is very low in Asia, particularly in Japan, which has the lowest age-adjusted incidence rate (2.8/100,000) [31]. This lack of uniformity in incidence is most marked among women over the age of 50. However, in women who emigrate from Japan to the USA, the risk of contracting ovarian cancer increases above that of their relatives who remain in Japan. In fact, the incidence among postmenopausal Japanese women living in the USA is practically the same as that of postmenopausal women born in the USA [32]. Woodruff [33] believes that the greater incidence of ovarian cancer noted in many industrialized countries [30, 34] is attributable to exogenous factors. Exogenous substances can ascend into the abdominal cavity via the genital tract in less than 30 min [35], and there have been a few reports that the risk of ovarian cancer is lower after hysterectomy [36, 37] and tubal ligation [38], but other studies have been unable to confirm this [39]. Talc granules have frequently been found in epithelial ovarian cancers. Cramer et al. [40] found that the risk of ovarian cancer was three times higher among women who regularly dusted their perineal or underclothing with talcum powder for cosmetic reasons than among women who did not do so. Keal [41] found a tenfold increase in incidence of ovarian cancer among women working in the asbestos industry, but Newhouse et al. [42] were unable to confirm this in a prospective study. Talc and asbestos are chemically similar magnesium silicate compounds, and talcum powder is often slightly contaminated with asbestos [43]. Pleural and peritoneal mesotheliomas have repeatedly been observed in persons exposed to asbestos or talcum powder [44, 45]. On the other hand, reports rejecting a connection between talc and epithelial ovarian tumors have also appeared [46, 47]. Ovarian tumors have been induced in mice by the application of carcinogenic polycyclic aromatics, e.g., 7,12-dimethylbenz[a]anthracene [48], which undergoes hormone-dependent metabolism to reactive

intermediates in the rat ovary [49]. In vivo, the activity of this carcinogen was found to increase fivefold during stimulated cycles in human granulosa tissue compared with human granulosa cells from natural cycles [50].

The possibility that carcinogenic substances enter the ovarian surface epithelium via the genital tract should be seriously considered [51]; however, exogenous factors are difficult to record in a cancer register, and investigations of this subject are often speculative in nature.

Although both ovarian and peritoneal surface cells are exposed to exogenous factors, peritoneal mesothelioma is much less common than epithelial ovarian carcinoma [52]. Also extremely rare are the so-called 'extraovarian serous carcinomas' [26, 53]. What is it, then, that makes the ovarian surface epithelium so much more susceptible to malignant transformation than the peritoneal mesothelium?

Endogenous Factors

The constantly changing external morphology of the ovarian surface epithelium contrasts markedly with the consistent structural uniformity of the bordering peritoneal mesothelium [54, 55]. This strongly suggests that local factors govern the morphogenesis of the ovarian surface epithelium. Some authors believe that the superficial epithelium of the ovary reacts like the endometrium to steroid hormones and may tend towards hyperplastic or even neoplastic reactions [8]. Proliferative lesions (papillary adenomas and adenocarcinomas) have been produced in animal experiments by the long-term application of diethylstilbestrol [56].

It has been shown that the ovarian surface cells possess 17- β -hydroxysteroid dehydrogenase activity [5, 57] and hormone receptors [58, 59]. In addition, estrogen and progesterone receptors have been found in certain ovarian superficial neoplasms [60, 61]. Hamilton et al. [59] have reported that the growth of ovarian surface cells in cell cultures is stimulated by androgens and inhibited by antiestrogens. However, treatment of advanced ovarian carcinoma with antiestrogens has rarely been successful [62]. The formation of so-called 'papillary excrescences' appears to be a hormone-dependent process: such papillary structures in man are more often found in polycystic ovaries and after the menopause, i.e. in circumstances where there are high gonadotropin levels [9, 63, 64]. The genesis of papillary lesions appears to be enhanced by diethylstilbestrol but suppressed by oral contraceptives, either directly or via negative gonadotropin feedback [56,

65]. Various findings argue in favor of the latter possibility: the increased incidence of ovarian neoplasms in the postreproductive years, the proliferative response of ovarian superficial epithelial cells to gonadotropins in culture [66], and the presence of gonadotropin binding sites in ovarian neoplasms [67]. Two studies published in 1942 showed an increased proliferation rate of the superficial epithelium of the mouse ovary in the periovulatory period and after estrogen application [68, 69]. The high proliferative potential of the ovarian surface cells has also been noted in *in vitro* cell cultures [70]. During proliferation, structures such as intracytoplasmic lumina and cysts appeared, which are frequently found in epithelial ovarian tumors [71].

Oncogenes, including those of the *ras* family, are highly regulated to maintain and control cellular proliferation [72]. Oncogene activation can occur through point mutations, gene rearrangements or amplification and can result in the production of increased or altered gene products with tumorigenic potential. A single case of serous cystadenocarcinoma with known *c-k-ras* amplification was followed over a period of several months [73]. *c-k-ras* amplification did not change with tumor progression over this period, and the authors concluded that *ras* p21 was most likely involved in the early stages of carcinogenesis. Kotylo et al. [74] found relatively higher levels of *ras* p21 in ovarian tumors of low malignant potential and serous cystadenocarcinomas, in contrast to normal ovaries and serous cystadenomas. No significant differences in *ras* p21 expression distinguished tumors of low malignant potential from serous cystadenocarcinomas. The overlap in p21 expression between these two groups is interesting and indicates that *ras* p21 might be involved at an early stage of tumor growth or carcinogenesis. Probably oncogene alteration or activation are not sole determinants of tumor progression.

Ovulation and the Ovarian Surface Epithelium

The fact that pregnancy and hormonal contraceptives [75] reduce the risk of developing ovarian carcinoma supports the theory that continuously repeated ovulation is an important factor in the pathogenesis of this neoplasm [76, 77].

It may be the repeated rupture of the superficial epithelium of the ovary during ovulation that increases the risk of neoplasia. The development of crypts and inclusion cysts in the ovarian stroma may be due to continuously repeated periovulatory traumatization of the surface epi-

Table 1. Follicular stimulation and development of ovarian cancer in 4 patients

Case No.	Age, years	Ovulation induction regime	Ovarian carcinoma	Reference*
1	26	clomiphene, HCG (1 cycle)	borderline papillary serous carcinoma (FIGO stage Ib)	86
2	32	FSH, LH (8 cycles) 1-year lapse FSH, LH (3 cycles)	endometrioid carcinoma (FIGO stage IIa)	87
3	25	clomiphene, FSH, LH (1 cycle)	papillary serous carcinoma (FIGO stage IIlb)	88
4	34	clomiphene, FSH, LH (1 cycle)	papillary serous carcinoma (FIGO stage IIlb)	89

thelium and repair of the surface defect [78, 79]. These ruptures are repaired by cell division, and growth cases when repair is complete. It is proposed that these repeated episodes of proliferation allow the promotion of cells already bearing allele loss and those carrying inactivated tumor suppressor genes. This may lead to uncontrolled cell division and malignant transformation [80, 81].

The suppression of ovulation and the inhibition of gonadotropin secretion have been interpreted as protective mechanisms, since oral contraceptives have a protective effect against the development of ovarian carcinoma [75]. It remains to be established whether ovulation also has an effect on the formation of germ cell tumors or sex cord stromal tumors [65, 82].

The technique of *in vitro* fertilization has steadily improved in recent years, with the goal of achieving a pregnancy rate as high as possible. The fact that the probability of pregnancy rises in proportion to the number of embryos implanted has led to the stimulation of follicle maturation in the hope of obtaining many follicles and many conceptions [83]. Due to improvements in freezing techniques, follicle stimulation is used in grand style in order to provide a certain 'supply' of embryos. Gonadotropins, either with or without antiestrogens (clomiphene), are used in the first line for follicle stimulation.

Excessive estrogen secretion has been found in association with ovarian, endometrial and breast carcinomas, and excessive gonadotropin secretion has been noted with ovarian cancer [84]. Persistent stimulation of the ovary with gonadotropins may have a direct carcinogenic effect or may act in conjunction with elevated estrogen concentrations [2, 85]. Since currently employed stimulation protocols have been in use for only a few years, it is obvious that nothing conclusive can yet be said about the possible later consequences of excessive ovarian stimulation. Three cases reported in the literature [86-88] and 1 case of our own [89], in whom there was a direct chronological relationship between hormonal stimulation of the ovaries and the development of ovarian carcinoma, are

described in table 1. Even though these individual reports are in no way proof of a direct connection between hyperstimulation and the induction of ovarian neoplasia, it is nevertheless quite conceivable that the growth of a preexisting tumor is substantially accelerated by such hormonal therapy. It is a matter of particular concern that the tumors described developed with remarkable rapidity in all 4 cases. On the other hand, it may be coincidental that ovarian carcinoma developed in this subgroup after induction of ovulation, and the natural history of the disease may have been interrupted with earlier intervention and diagnosis during the infertility treatment [90].

Conclusion

The ovarian surface epithelium is a dynamic tissue with considerable morphological variety. It can grow vertically downwards to form crypts and inclusion cysts and can also form superficial papillary excrescences. Moreover, metaplastic transformation to the various types of müllerian epithelium can occur. These growth processes appear to be affected by endogenous and/or exogenous factors, and it is conceivable that these factors also induce neoplastic lesions on the ovarian surface epithelium. What effect exogenous stimulation of the ovaries, e.g. as used in *in vitro* fertilization, may have, is unclear. It remains for future research to identify endogenous and/or exogenous factors responsible for triggering neoplastic processes in the ovarian surface cells.

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The Biology of Ovarian Cancer Development

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Background. In theory, all the cell types that comprise the human ovary have the potential for malignant transformation. The vast majority of malignant ovarian tumors in the human, however, arise from the ovarian surface epithelium. These cells have important functions during reproductive life; they contribute to follicular rupture and by cell division repair the wound that accompanies ovulation. There has been much speculation that the rapid cycles of cell division associated with wound repair contribute significantly to the development of ovarian cancer. Such speculation is based on the observation that ovarian cancer occurs most frequently at the end of a woman's reproductive life and is associated with nulliparity. It is of potential significance that, unlike most epithelia, these cells are not replaced through replenishment stem cells with the development of one end-stage cell and one cell with continued growth potential. Rather, the division of an ovarian surface epithelial cell yields two daughter cells with equal potential for subsequent growth. Thus, all potential mutations as they accumulate are passed on to near-exponentially expanding subsequent generations of cells that can acquire additional mutations that could confer the malignant phenotype.

Methods. We have developed a model to test the hypothesis that repeated cell division by ovarian surface epithelial cells contributes to development of malignancy. In this model, rat ovarian surface epithelial cells are isolated and subjected *in vitro* to repetitious cell division to mimic in a simple way growth of the surface epithelium *in vivo*.

Results. These cells develop a malignant phenotype based on loss of contact inhibition, the ability for sub-

strate independent growth, tumorigenicity in athymic mice, and cytogenetic changes.

Conclusions. Our data support the involvement of tumor suppressor genes in the development of ovarian cancer. *Cancer* 1993; 71:530-6.

Key words: ovarian cancer, malignant transformation, tumor suppressor genes, cytogenetics, surface epithelium.

The etiology of ovarian cancer is unknown,^{1,2} but there are some data that suggest that multiple factors may contribute to the initiation and progression of the disease. The vast majority of ovarian carcinomas arise as a result of malignant transformation of the ovarian surface epithelium.³⁻⁶ This simple epithelium, composed of a single layer of cells, often is described as modified peritoneal mesothelium.

This paper is divided into two parts. The first part summarizes some of the information available on the role of hormones and growth factors in regulation of normal and malignant ovarian surface epithelium in the context of how regulatory substances may affect disease etiology and progression. The second part contains experimental data supportive of the long-standing hypothesis that incessant or repetitious ovulation contributes to the initiation of ovarian cancer.

Features of the Normal and Malignant Ovarian Surface Epithelium

In adult life, the ovarian surface epithelial cells are a single layer of nondescript cells that vary from squamous to cuboidal and low columnar.^{5,7,8} The generally unremarkable features of these cells suggest that they might have little role in normal ovarian function, but this does not appear to be the case. The ovary has two major functions: (1) the production of steroid hormones, and (2) the timely release of ova. Based on data from several species, ovarian surface epithelial cells are believed to have a pivotal role in the latter process.^{2,9,10}

As the time of ovulation approaches, ovarian sur-

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face epithelial cells (in the area of the ovulation fossa) produce large lysosomal bodies that migrate to the basal region of the cells. These large bodies are excreted from the cells' basal surfaces before ovulation. It is believed the lysosomal bodies contain enzymes that participate in the breakdown of the tunica albuginea and thus aid in follicular rupture.⁹ The process of ovulation results in a wound at the ovarian surface that is repaired by rapid growth of ovarian surface epithelial cells.¹¹ These surface epithelial cells also may participate in the production of a new tunica albuginea.¹² The requirement for regulation of both processes (i.e., lysosome production and wound repair) suggests that the functions of ovarian surface epithelial cells are controlled in complex ways.

There is potential for a diverse range of regulatory molecules to influence function and growth of ovarian surface epithelial cells. These signals may reach their target by autocrine, endocrine, or paracrine routes. The latter of these mechanisms (i.e., paracrine) may have special significance to the surface epithelium in that the ovarian surface is bathed by follicular fluid at the time of ovulation. Direct experimental evidence on the roles of various hormones and growth factors in control of ovarian surface epithelial cells, however, is limited. The potential for hormones and growth factors to regulate growth and function of these cells is suggested by the presence of appropriate receptors for many of these regulatory substances in both the normal and malignant ovarian surface epithelial cells. Estrogen receptors, for example, have been detected in both normal and malignant ovarian surface epithelial cells.^{11,13-15} Independent laboratories have reported estrogen receptors in the normal rat ovarian surface epithelium, whereas there is less evidence for the presence of estrogen receptors in human ovarian surface epithelial cells.¹¹ There have been numerous studies, however, showing the presence of estrogen receptors in malignant tumors of ovarian surface epithelial cell origin.^{13,16-18} The presence of estrogen receptors in the malignant counterpart of a given cell type generally is considered to result from retention of a differentiated feature of the normal progenitor cell, rather than *de novo* synthesis as a result of malignant transformation. The complexity of ovarian surface epithelial cell regulation is indicated by the finding of estrogen production by ovarian cancer cell lines.^{19,20} Thus, estrogen, which generally is considered to mediate its effects through the endocrine route, may also have effects by both paracrine mechanisms (i.e., estrogen in follicular fluid) and autocrine mechanisms. The exact function of estrogen in ovarian surface epithelial cell regulation is not known. Limited evidence in the rat and mouse suggest estrogen is a weak mitogen.^{14,21} In some estrogen receptor-positive human ovarian

cancer cell lines it recently has been shown that estrogen is a strong mitogen^{17,22,23} and promotes the capacity for substrate-independent growth.²² It is possible that these effects are mediated indirectly by transforming growth factor α and β production.²⁴ In the PE04 ovarian cancer cell line, in which estrogen stimulated growth, it was observed that the hormone did not induce progesterone receptor synthesis,²² whereas in the OVCAR-3 ovarian cancer cell line estrogen induced progesterone receptor but not growth.^{22,25} Thus, it is possible that the divergent signals that mediate these two markers of estrogen action are uncoupled readily in ovarian cancer. This could explain why antiestrogen therapy is effective only in a small subset of patients with ovarian cancer.²⁶

The significance of progestagens and androgens to the regulation of normal and malignant ovarian surface epithelial cells is limited essentially to the findings of receptors for these two sex steroid hormone classes in ovarian tumors.²⁷ Only very limited data suggestive of a role for testosterone in stimulation^{23,28} and antiandrogens on inhibition of growth^{16,29} of ovarian cancer cells are available. It is noteworthy that the postmenopausal ovary is a net producer of androgens due to the continued formation of functional secondary interstitial tissue in the organ,^{30,31} and thus, if androgens stimulate growth of either normal or malignant ovarian surface epithelial cells, they may well contribute to disease etiology or progression (see below).

The roles of peptide hormones and growth factors in the regulation of the ovarian surface epithelium are not completely defined. It has been shown that the age-specific incidence of ovarian cancer increases abruptly at the time of the menopause.^{32,33} The age-related change in ovarian function results in loss of feedback inhibition on pituitary peptide hormone synthesis such that the pituitary produces constant increased amounts of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (15- and 5-fold normal levels, respectively) for a number of years after the climacteric.³⁴ This suggests that FSH and LH may somehow contribute to the development of ovarian cancer. The potential for direct effects of peptide hormones on the surface epithelium is shown by the existence of FSH and LH receptors in ovarian cancer specimens.^{35,36} In addition, peptide hormones have been shown to stimulate growth of normal rabbit ovarian surface epithelial cells *in vitro*³⁷ and cell lines derived from ovarian cancer specimens.³⁸

Receptors for epidermal growth factor (EGF; the best known of the peptide growth factors) have been found in both the normal and malignant human ovarian surface epithelium.^{38,39} Furthermore, EGF has been shown to be weakly to moderately mitogenic in rat⁴⁰ and human ovarian surface epithelial cells.⁴¹

It is of interest to consider how mitogens may come

into the proximity of the surface epithelium. This tissue is avascular and normally is separated from the ovarian cortex by a well defined tunica albuginea. Ovarian cancer is believed to arise most frequently in surface epithelial inclusion cysts. These cysts result from the proliferation of surface epithelial cells that either invade the ovarian cortex or are trapped within the cortex during ovulation or remodeling of the ovarian surface.^{2,42} Such cysts increase in number in relation to ovulatory activity, and thus are more prevalent in older aged women.^{41,43} It should be noted that the surface epithelial cells lining these inclusion cysts in the ovarian cortex are theoretically in close proximity to the mitogen producing components of the ovary, including the functional secondary interstitial tissue of the older aged women.

There is a reasonable amount of information suggestive that diverse regulatory molecules have the potential to stimulate growth of both normal and malignant ovarian surface epithelial cells. The question arises as to how growth-stimulatory effects might relate to disease etiology or manifestation. The potential for growth-stimulatory factors to influence disease manifestation appears straightforward. An alteration in the concentrations or patterns of production of growth-regulatory substances could stimulate slowly growing malignant cells such that tumor rapidly becomes manifest clinically. Alternatively, malignant transformation might include an altered responsiveness to such substances and thus more rapid growth.

The issue of how hormones and growth factors may directly influence the etiology of ovarian cancer is more complex. The simplest explanation is that any increase in DNA replication increases the likelihood of a mutation. Such mutations could be dominant, activating a protooncogene or producing an altered antioncogene product. Alternatively, recessive mutations could inactivate antioncogenes. Stimulation of mitotic activity may be of special significance in the ovarian surface epithelium. Ovarian surface epithelial cells, unlike most epithelia, behave as generative stem cells.⁴ Thus, the division of one of these cells is believed to yield two cells with equivalent potential for future growth. This is in contrast to replicative stem cells that simplistically yield two cells, one with loss of growth potential (i.e., destined for differentiation) and one stem cell with continued replicative potential. In the case of ovarian surface epithelial cells, a single mutation could be passed on to exponentially expanding progeny, and since transformation is essentially a game of numbers, the larger the number of cells available for additional mutations, the more likely it is that a mutation of significance to transformation will occur. It is straightforward to apply this pattern of replication to the simple model

of antioncogene inactivation, where both alleles of the antioncogene must be inactivated to achieve malignant transformation.⁴⁴ If replication increases the pool of cells with one inactivated allele (first hit) and also increases the likelihood of additional mutations (second hit), the process of replication in generative stem cells becomes doubly (or perhaps exponentially) dangerous. Thus, any growth-promoting stimuli could increase the possibility to accumulate combinations of potentially devastating mutational events that eventually result in malignancy.

Repetitious Ovulation and Ovarian Cancer Etiology

The observation that ovarian cancer is more prevalent in nulliparous women served as the basis for the development of a hypothesis that repetitious ovulatory activity is a factor in malignant transformation of the ovarian surface epithelium.⁴⁵ Alternatively, it has been suggested that an underlying hormonal imbalance might be responsible for infertility and that this, rather than ovulatory activity, may be responsible for the increased incidence of ovarian cancer observed in nulliparous women.⁴⁶ The fact that birth control pill use reduces ovarian cancer risk supports the former, more widely touted hypothesis.⁴⁷⁻⁵⁰ It also is noteworthy that tumors of the ovary derived from the surface epithelium are rare in animals. Germane to this, in nature animals ovulate seasonally and most frequently become pregnant shortly after ovulatory activity commences in the spring. Thus, the number of ovulations during life is quite low. The only exception to this is the incessantly ovulating domestic hen, which has a high incidence of ovarian cancer.^{51,52}

It is tempting to speculate how ovulatory activity could contribute to ovarian cancer etiology. If surface epithelial inclusion cysts are important in disease etiology, it is obvious that there would be a greater opportunity for such cysts to form in the more frequently ovulating person. In addition, the surface epithelial cell mitotic activity required to repair the wound created by the follicular rupture accompanying ovulation may be a factor in disease initiation. As noted above, mutations are more likely to occur in dividing as opposed to quiescent cells. Furthermore, because growth increases the pool of cells carrying a potential first mutation (first hit), the probability of an additional mutation occurring in a cell with a preexisting mutation is increased. It is of interest to speculate whether certain mechanisms to vigorously assure the fidelity of DNA replication, repair, and of chromosomal segregation might be inactive in surface epithelial cells because these cells would have been under little selective pressure from the standpoint

of evolution. In nature, such cells have a very limited requirement for replication, in contrast to the selective pressure placed on the skin or gastrointestinal mucosa, which during evolution might be expected to have been rigorously selected for the capacity for wound repair and luminal replenishment, respectively. In fact, proteins have been identified that are believed to have a role in the fidelity of DNA replication and chromosomal segregation and, thus, act to reduce the possibility of recombination errors and malignancy.⁵³⁻⁵⁶

Over the years, there has been a rather strong case made for the role of incessant ovulation in ovarian cancer etiology. Beside the recapitulation of the original hypothesis, however, previously there has been little more supportive epidemiologic data and even less biologic evidence. Recently, we have described a model in which ovarian surface epithelial cells from the rat undergo malignant transformation in association with prolonged exposure to a growth-promoting environment.^{2,40} Surface epithelial cells isolated from the rat ovary by selective trypsin treatment are placed in culture. These cells rapidly grow to cover the culture surface as if to cover the wound created by follicular rupture and, at confluence, mitotic activity ceases. These cells may then be repetitiously subcultured to mimic in vitro in a simple way the repeated requirement for growth produced by incessant ovulation in vivo. This

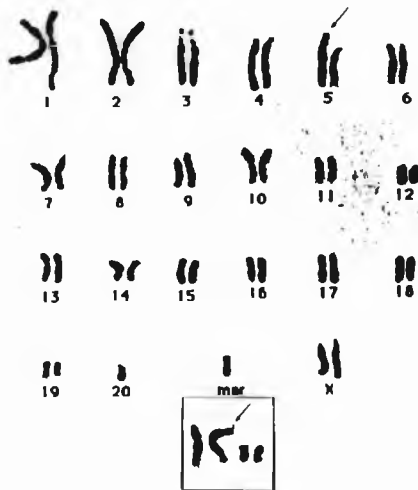


Figure 2. Karyotype of a Giemsa banded metaphase cell from late passage clone 7. Clonal changes include an unbalanced 5;12 derivative chromosome (arrow) and an unidentified marker (mar). Inset: chromosome pairs 5 and 12 from another cell; der(5;12) is arrowed.

prolonged growth results in the spontaneous acquisition of multiple features consistent with malignant transformation. The cells show loss of contact inhibition, acquisition of the capacity for substrate-independent growth, and, when injected intraperitoneally or subcutaneously into nude athymic mice, form tumors consistent with serous cystadenocarcinoma of ovarian origin.

We have examined the karyotypes of two spontaneously transformed clonal cell lines derived from ovarian surface epithelial cells and one spontaneously transformed mixed population surface epithelial cell line. In each cell line, the karyotypes revealed several cytogenetic abnormalities that could be consistent with oncogene activation or tumor suppressor gene inactivation. The most frequent changes involved chromosome 5 and chromosome 1.⁴⁰

One of the clonal rat ovarian surface epithelial cell lines, clone 7, compared for cytogenetic changes between early (normal phenotype) and late passage (transformed phenotype), showed cytogenetic changes of theoretical interest (Figs. 1 and 2). This cell line in early passage showed monosomy for chromosome 5 (Fig. 1), whereas late passage cells contained two copies

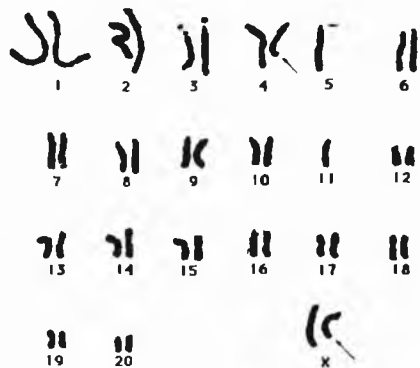


Figure 1. Karyotype of a Giemsa banded metaphase cell from early passage clone 7. Clonal changes include loss of one chromosome 5 and partial deletions of chromosomes 4 and X. Note that the remaining copy of chromosome 5 appears normal cytogenetically, but molecular analyses indicate that a small segment, including the interferon- α locus, is deleted from this chromosome (see Fig. 3). Loss of a chromosome 11 is a nonclonal change seen in this cell only. Arrows indicate structural alterations of 4 and X.

of chromosome 5, one apparently normal homologue and one defective copy consisting of an unbalanced derivative between chromosomes 5 and 12 (Fig. 2). We interpret this observation to indicate that there has been

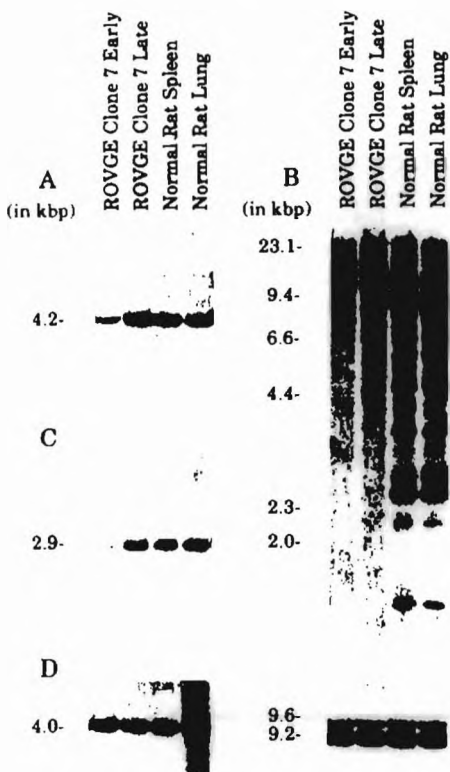


Figure 3. DNA blot analysis of early and late passage rat ovarian germinal epithelium (ROVGE) clone 7 cell lines for loss of specific loci of genes located on chromosome 5. High molecular DNA (10 μ g per lane) from the early and late passage clone 7 cells (indicated above) was digested with *NotI*, electrophoresed on a 1% agarose gel, and transferred to nylon membrane filters. Filters were hybridized sequentially with the following [^{32} P] labeled nick translated DNA probes: (A) interferon β , (B) interferon α , (C) *c-jun*, and (D) EGF receptor. The rat EGF receptor cDNA probe detects three bands (9.6, 9.2, and 4.0 kilobase pairs [kbp]), the 4.0 kbp band (left), which is closest in size to interferon- β and *c-jun*, and the 9.6 and 9.2 bands (right) are shown for comparison of DNA loading and transfer. Hind III digested λ phage DNA was used as molecular size markers. The fragment size is indicated in kilobase pairs to the left. DNA from normal rat spleen and lung is included as a control for normal gene dosage.

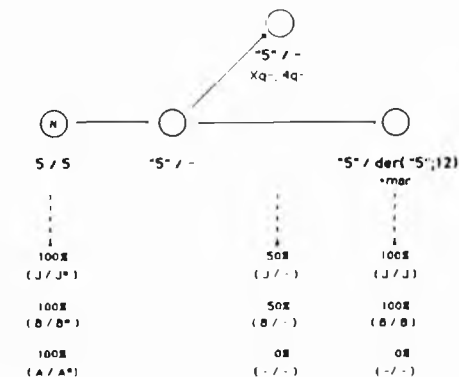


Figure 4. Schematic diagram illustrating proposed clonal evolution of ROVGE clone 7 cells. Normal surface epithelial cells (N) contain two normal copies of chromosome 5. We hypothesize that on in vitro culture, a single cell loses one copy of chromosome 5; the second copy (5^-) appears normal cytogenetically but has a microscopic deletion of a segment that includes the interferon α locus ($Xq-4q$). Late passage clone 7 cells have duplicated the defective chromosome 5 ($5^-/-$) and one of these copies participates in a rearrangement with a chromosome 12 derivative ($5^-/12$). The DNA signal intensity and allelic constitution of three different genes residing on chromosome 5 (i.e., *c-jun* alleles) and the interferon α gene locus [A/A^*] and [B/B^*]) are indicated. Both alleles of interferon α are lost in early and late passage clone 7 cells. Percent sign indicates the approximate gene dosage of the three genes as determined by Southern blot analysis. *mar* indicates the presence of an unknown marker chromosome. The existence of the hypothetical subpopulation of cells noted above is suggested by the presence of late passage clone 7 cells that do not contain cytogenetic deletions of chromosome X and chromosome 4. This would indicate that the hypothetical population ($5^-/-$) gave rise to the early passage clone 7 cells ($5^-/-$, $Xq-4q$) and to another subpopulation with the features of late passage clone 7. This latter subpopulation (at the time of early passage cytogenetic analysis) must have been a very small component of the culture, and thus was not observed in the chromosomal preparations examined and did not influence gene dosage. This latter subpopulation, however, apparently had a distinct growth advantage, and by late passage became dominant if not the entire population.

duplication of the single chromosome 5 of early passage cells. This view is supported by molecular data (see below). It has been hypothesized by others that one mechanism by which predisposing recessive somatic or germ line mutations could be unmasked is by the loss of a normal (wild type) chromosome (by mitotic nondisjunction) and reduplication of the remaining homo-

logue containing the aberrant allele.^{57,58} In our example, we examined the DNA of early and late passage clone 7 cells for three genes that reside on the long arm of chromosome 5: the interferon- α family, interferon- β , and *c-jun*.^{40,59} It would be anticipated in early passage clone 7 cells, which had one cytogenetically normal chromosome 5, that there would be one-half the diploid dosage for these genes. This was the case for *c-jun* and interferon- β (Fig. 3). Unexpectedly, all members of the interferon- α family were totally absent (Fig. 3). This indicates that there was a submicroscopic deletion in this apparently "normal" chromosome 5. A strong indication that the chromosome 5 portion of the unbalanced 5;12 derivative seen in late passage cells originated from the defective chromosome 5 of early passage cells is given by a doubling of the gene dosage for interferon- β and *c-jun* and the continued total absence of the interferon- α family in DNA of late passage cells (Fig. 3). This is perhaps the first experimental proof by a combination of cytogenetic and molecular methods, that the process of reduplication that returns a cell to disomy does indeed occur in transformed cells, and the process is summarized in Figure 4 along with the gene dosage evidence.

The cytogenetic and molecular changes in rat chromosome 5 we observe are in an area where a yet-to-be-cloned gene for the suppression of anchorage-dependent (SAI) growth is proposed to reside.⁶⁰ Furthermore, this portion of rat chromosome 5 has synteny with the short arm of human chromosome 9,⁵⁹ which reportedly shows frequent cytogenetic changes in human ovarian cancer⁴¹ and has been proposed as the site of a tumor suppressor gene.⁶²⁻⁶⁴

In conclusion, much work remains to be done to determine not only those genes involved in the development of the growth-dependent acquisition of the transformed phenotype in our rat model, but also to show the relationship of those findings to human ovarian neoplasms. Nonetheless, the development of this model has provided the first experimental evidence to support a role for repetitious ovulation in ovarian cancer etiology, and has proved a starting point to experimentally unravel potential genes involved in disease etiology.

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Age Contrast in Ovarian Pathology

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Background. Ovarian cancer accounts for approximately 23% of all gynecologic tumors and is the most common fatal gynecologic malignancy. These tumors can occur in women of all ages, but there are differences in the histologic types during various decades of life.

Conclusions. During infancy and childhood, the predominant type of neoplasms are those of germ cell origin, such as teratomas, dysgerminomas, and endodermal sinus tumors. In adults, epithelial neoplasms, or tumors that originate from the epithelium that covers the ovarian surface, are the most common, accounting for almost 85% of all neoplasms after the age of 50 years. The peak incidence of benign epithelial tumors occurs between the ages of 20 and 40 years. Young women (30 to 40 years of age) are frequently affected by the so-called "tumors of low malignant potential," which have excellent prognosis. Older women, on the other hand, usually have the most aggressive forms of ovarian cancer, present with advanced disease, and have a dismal prognosis. *Cancer* 1993; 71:537-44.

Key words: ovarian cancer, germ cell and epithelial tumors.

Ovarian cancer is the fifth leading cause of death in women in the United States, with an estimated incidence of 50 cases per 100,000 women and nearly 12,000 deaths annually.^{1,2} Approximately 25% of all gynecologic malignant tumors are of ovarian origin, but ovarian cancer is the most common fatal gynecologic malignancy.

Unfortunately, these neoplasms remain asymptomatic until massive ovarian enlargement causes compression of pelvic structures, ascites, abdominal distention, or distant metastasis. Only in a small percentage of patients will the tumors be found at an early stage, confined to the ovaries.³

Ovarian cancer may occur in infancy and childhood. The incidence increases with age, with the greatest increase being diagnosed in the fifth decade. Jaffe et al.⁴ investigated the distribution of neoplasms by decades of life and found that germ cell tumors were the most common in children, more than 40% of all ovarian tumors. The chance of a tumor being malignant was higher with an overall risk of malignancy of 15% in premenopausal women and 45% in elderly patients.

Predisposing Factors

Predisposing factors include:

- Tumors occur predominantly in young women, 15-40 years of age
- Family history
- Mumps parotitis
- Low parity
- Environmental factors such as exposure to radiation
- Persistent ovulation

The ovary is a complex structure that can give rise to neoplasms present from different germ cell lineages. Malignant neoplasms can develop from any of these lineages, and therefore ovarian tumors are classified according to the cell of origin. The classification is described in Table 1. There are differences in the type of tumors found in different age groups; for example, germ cell neoplasms predominate in children and young adults, while epithelial neoplasms are rare in this age group and predominate in postmenopausal women.

Tumors Occurring in Young Premenopausal Women

Germ Cell Neoplasms

These neoplasms account for 15-20% of all ovarian lesions and 15-20% of all gynecologic malignancies.

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A case-control study of epithelial ovarian cancer

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With data from a study of 296 patients with primary epithelial ovarian cancer and 343 patients hospitalized because of other conditions, we estimated ovarian cancer risk in accordance with reproductive and other factors. Risk was greatest among women of lower parity, especially among women who said they planned to have children but could not. The protective effect of oral contraceptives seen in other studies was observed only in subgroups of our study population. Women who had breastfed their children had decreased risk, but the number of months of breastfeeding was not related to risk. Incomplete pregnancies did not provide the protection seen for live births. A family history of ovarian cancer and a medical history of breast cancer were both strong risk factors. None of the nonreproductive factors that we examined, including childhood illnesses, tobacco and alcohol consumption, obesity, and selected adult diseases, was convincingly associated with risk. (Am J Obstet Gynecol 1989;161:10-6.)

Key words: Ovarian cancer, endogenous estrogens, infertility, oral contraceptives, epidemiology

The causes of ovarian cancer are mainly unknown.¹⁻³ Known risk factors are age (incidence rises until about age 60 years), race (white women are at higher risk than nonwhite women), low parity, infertility, a history of endometrial or breast cancer, a family history of ovarian cancer, and exposure to radiation. Probable protective factors are oral contraceptives and surgical menopause. Although the importance of reproductive and contraceptive history has been firmly established, questions remain. How do parity, pregnancy, and fetal loss jointly affect risk? Does the timing of the first birth affect risk? Is infertility an independent risk factor, separate from the effects of low parity? In addition, many nonreproductive factors have been suggested. With interview and medical records data from a case-control study, we have estimated ovarian cancer risk according to various reproductive and other suggested risk factors to add to our knowledge with regard to these persistent issues.

Methods

Study methods are described in detail elsewhere.⁴ All eligible cases involved women aged 20 to 79 years who resided in the Washington, D.C., metropolitan area and who were first diagnosed by operation with microscopically confirmed primary epithelial ovarian cancer during the period August 1978 to June 1981. Cases in-

cluded tumors of low malignant potential as well as frankly malignant tumors because risk factors for the two groups were similar.⁵ For all participants with ovarian cancer, we obtained the diagnostic microscopic slides and pertinent medical records. After review we excluded 30 women found not to have definite primary ovarian cancer of the epithelial type by microscopic (H.J.N.) or clinical (L.M.) evaluation. Several of these cases had been classified as mucinous ovarian cancer but actually were of colorectal origin.

We identified 400 cases and interviewed 296 women (74%). Losses were a result of death ($n = 44$), disability ($n = 12$), physician's refusal ($n = 8$), patient's refusal ($n = 33$), or other reasons ($n = 7$). As shown in Table I, serous and endometrioid types predominated.

Control participants were identified from hospital discharge lists and were matched with women with epithelial ovarian cancer according to hospital, age, and race (Table II). A woman was not eligible to be a control if her discharge diagnosis was psychiatric or was potentially related to the major exposures of interest. For all potential control participants, we confirmed in the physician and hospital records that the women had at least one ovary intact and excluded those who did not because they were not at risk of ovarian cancer. We identified 439 controls of whom we interviewed 343 (78%). Losses were a result of death ($n = 13$), disability ($n = 8$), physician's refusal ($n = 11$), patient's refusal ($n = 50$), or other reasons ($n = 14$).

Trained, experienced medical interviewers obtained informed consent and administered a standardized questionnaire in the study participant's home shortly after diagnosis. (Questionnaire is available on request.)

Effects of ovarian cancer risk were measured by the

Volume 161
Number 1

Table I. Ovarian cancer cases according to histologic type

Histologic type	Number	Percentage
Serous	84	28
Serous, low malignant potential	35	12
Mucinous	17	6
Mucinous, low malignant potential	9	3
Endometrioid	76	26
Endometrioid, low malignant potential	8	3
Clear cell	12	4
Mixed epithelial	25	8
Undifferentiated	30	10
TOTAL	296	100

estimated rate ratio—the ratio of ovarian cancer incidence in an exposed group to that in the corresponding unexposed group. A rate ratio of 2.0 for nulliparity, for example, indicates that the incidence rate is twice as great among the nulliparous women as among the parous women; conversely, a rate ratio of 0.5 indicates that the incidence is half as great. The rate ratio estimates were adjusted for the effects of confounding variables by stratified contingency table analysis (presented in the tables) and by logistic regression models, which gave similar results.⁶ All trend tests used scored categorized data and were two-tailed.

For each estimate presented we adjusted for age and race. We assessed whether there were confounding effects of parity, difficulty conceiving, recent administration of oral contraceptives, surgical menopause, menopausal estrogen use, or family history of ovarian cancer, and we adjusted the estimates accordingly. If the number of confounders held constant exceeded three, we used a logistic regression model to produce smoothed estimates.

To derive unbiased estimates of the effects of tobacco, alcohol, and adult medical conditions, it was necessary to include in the control group only women who were admitted for conditions not caused by (or protected by) the exposure under assessment. Without such exclusions, a hospital-based control series would produce spurious results because the population of hospitalized women generally includes, for example, more smokers than the whole population of women at risk for ovarian cancer.

Results

A total of 12% of the patients with ovarian cancer and 14% of the controls were black, and the remainder were white. The mean age at diagnosis was 54.4 years among women with cancer, and 54.7 years among controls. Because the control participants were chosen from the same hospitals as were the women with cancer, they were similar in social class and religion.

Greater parity was related to reduced risk in our study (Table III), and pregnancies that ended in spon-

Case-control study of epithelial ovarian cancer 11

Table II. Discharge diagnoses among control group

Disease category	Number	Percentage
Infectious disease	6	2
Neoplasm	22	5
Endocrine-metabolic diseases	19	5
Disease of blood or blood-forming organs	4	1
Disease of nervous system	20	8
Disease of eye, ear, or mastoid	26	8
Varicose veins, hemorrhoids	5	1
Respiratory diseases	27	8
Digestive system diseases	55	16
Urinary diseases	18	5
Skin disorders	6	2
Musculoskeletal diseases	75	22
Congenital anomalies	4	1
Ill-defined conditions	18	5
Fractures and other injuries	38	11
TOTAL	343	100

taneous or induced abortion or stillbirth did not, as a group, confer any protection. When the effects of the different pregnancy outcomes were further separated, stillbirths appeared to increase risk, induced abortions appeared to slightly decrease risk, and spontaneous abortions appeared not to affect risk, but the estimates for the specific outcomes were rather unstable. There was also no clear pattern of risk when women were categorized according to the number of months pregnant with the different outcomes, adjusted for the effects of parity. It was necessary to adjust for the effects of parity in the examination of fetal loss because the two were correlated: about one fifth of the nulliparous women had some fetal loss, whereas about one half of the women with five or more live births had fetal loss. The strong protective effect of parity was seen in almost all subgroups and was strongest among women aged 20 to 39 years and weakest among those aged 60 to 79 years.

The protection afforded by greater parity was not explained by an effect of earlier first birth. On the other hand, as shown in Table IV, the apparent protection associated with earlier first birth, when this variable was considered alone, was eliminated by adjusting for parity. Also shown in Table IV is reduced risk among women who had breastfed their children, but an erratic relationship is shown between lifetime months of breastfeeding and risk.

The total group of women who had taken oral contraceptives showed no altered risk (Table V), but women who had taken them ≥ 3 years were at slightly reduced risk, as were women who had taken them recently. Women who took compounds with high levels of progestogen showed no excess ovarian cancer risk (data not shown). Those who quit after only 1 or 2 months, usually because of side effects, were not at

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Table III. Estimated rate ratios according to pregnancies, births, and fetal losses

	Group with cancer	Control group	Estimated rate ratio*	95% Confidence interval
Total pregnancies				
0	70	71	1.0	—
1	57	45	1.4	0.8-2.4
2	49	71	0.7	0.4-1.2
3	49	65	0.8	0.5-1.4
4	35	37	1.0	0.4-1.2
5+	35	54	0.7	0.4-1.2
Trend test		p (trend) = 0.10		
Live births				
0	89	84	1.0	—
1	54	53	1.0	0.6-1.7
2	71	85	0.8	0.5-1.3
3	43	60	0.7	0.4-1.2
4+	39	61	0.6	0.4-1.1
Trend test		p (trend) = 0.03		
Losses, adjusted for live births				
0	206	238	1.0	—
1	53	62	1.0	0.6-1.6
2	24	28	1.0	0.5-1.8
3+	13	14	1.2	0.5-2.7
Trend test		p (trend) = 0.70		

*Adjusted for age and race.

Table IV. Estimated rate ratios among parous women according to age at first birth and months of breastfeeding

	Group with cancer	Control group	Estimated rate ratio*	95% Confidence interval
Age at first birth, adjusted for parity				
<20	42	52	1.0	—
20-24	72	108	0.7	0.4-1.2
25-29	58	62	1.0	0.5-1.7
≥30	34	33	1.0	0.5-2.0
Trend test		p (trend) = 0.22		
Months of breastfeeding, adjusted for parity				
0	112	121	1.0	—
1-9	62	84	0.8	0.5-1.2
10-18	16	37	0.5	0.2-0.9
19-110	13	15	1.1	0.5-2.6
Trend test		p (trend) = 0.14		

*Adjusted for age and race by logistic regression.

increased risk. The effect of oral contraceptives was similar for parous and nulliparous women, but it varied somewhat with age at diagnosis. Among women younger than 40 years old, oral contraceptives were weakly protective (rate ratio = 0.7), but among women aged 40 to 59 years, they were not (rate ratio = 1.1). Only one woman older than 59 years of age had taken oral contraceptives.

The effects of infertility were assessed in several ways. Married nulliparous women had a 70% higher risk than unmarried nulliparous women, an excess relative risk that was limited to those who reported difficulty conceiving (Table VI). Among the married nulliparous participants there was little difference in risk between those who had been pregnant and those who

had not. In a separate confirmatory question, we asked married women who had never conceived why they had never been pregnant. Women who planned not to have children showed no increased risk compared with non-married nulliparous women (rate ratio = 1.0, 95% confidence interval = 0.4 to 2.4) but women who were unable to become pregnant had a corresponding rate ratio of 2.8 (95% confidence interval = 1.1 to 7.3). So few women reported each specific cause of infertility with confirmation by a physician that cause-specific estimates were quite unstable. Among parous women, a history of trouble conceiving was unrelated to risk (Table VI).

Participants were asked about a variety of medical factors found in previous studies to be related to risk of ovarian cancer. Our data on menopausal factors,

Table V. Estimated rate ratios according to oral contraceptive use for women younger than age 60 years

	Group with cancer	Control group	Estimated rate ratio*	95% Confidence interval
Never took oral contraceptives	115	131	1.0	—
Ever took oral contraceptives	74	78	1.0	0.7-1.7
Duration (months)				
1-11	23	16	1.6	0.7-3.4
12-35	16	19	1.0	0.4-2.2
36-59	10	12	0.8	0.3-2.3
60+	25	31	0.8	0.4-1.5
Trend test		p (trend) = 0.76		
Latency (years since first taken)				
0-5	2	3	0.4	0.0-4.3
5-9	12	15	0.7	0.2-2.2
10-14	29	31	1.0	0.3-2.3
15+	30	29	1.1	0.6-2.1
Trend test		p (trend) = 0.72		
Recency (years since last taken)				
10+	28	24	1.4	0.7-2.6
1-9	38	41	0.9	0.5-1.6
<1	7	13	0.5	0.1-1.6
Trend test		p (trend) = 0.75		

*Adjusted for age and race; referent group for all comparisons is women who never took oral contraceptives.

Table VI. Estimated rate ratios according to reported difficulty conceiving*

	Group with cancer	Control group	Estimated rate ratio†	95% Confidence interval
Nulliparous women				
Never married	30	37	1.0	—
Ever married	58	47	1.7	0.9-3.4
No trouble conceiving or never tried	32	33	1.3	0.6-3.4
Had trouble conceiving	26	13	2.8	1.1-7.3
Parous women				
No trouble conceiving	164	209	1.0	—
Had trouble conceiving	42	50	1.0	0.6-1.7

*Women with incomplete responses excluded.

†Adjusted for age and race.

including protective effects of hysterectomy and menopausal estrogen, are detailed elsewhere. The results for selected childhood and adult diseases are given in Table VII. None of the childhood diseases influenced risk, regardless of whether it occurred before, after, or around the time of menarche. An extensive analysis of mumps revealed only that the protective effect of parity was absent among women who did not recall having had mumps.

Among the adult diseases, shingles was significantly protective but chickenpox was not. High blood pressure was weakly associated with increased risk. Thyroid disease was apparently protective, and hyperthyroidism was more protective than hypothyroidism (rate ratio = 0.5 and 0.9, respectively), but patients could not reliably make this distinction. Adrenal disease was reported by five women in the control group but no patients with cancer reported it. Diabetes was weakly protective. A history of asthma was also protective, but

the effects of the illness and the asthma medication could not be distinguished because nearly all women with asthma had taken multiple medicines for their condition. Previous breast cancer was strongly related to risk but nulliparity, a strong risk factor for breast cancer and ovarian cancer, was present in only one of the 17 cases with previous breast cancer. There were only two histories of endometrial cancer among patients with cancer compared with four in the control group, a difference that was not statistically significant.

A family history of ovarian cancer in a sister, daughter or mother was reported by 13 patients with cancer and by only five women in the control group, which equals an elevated rate ratio of 3.3 (95% confidence interval = 1.1 to 9.4). No excess risks were seen for first-degree family histories of breast or endometrial cancer.

Obesity was not related to risk. We considered usual adult weight adjusted for height using Quetelet's index (weight ÷ height²) divided into quartiles. The rate ra-

Table VII. Estimated rate ratios according to history of selected childhood and adult diseases

Disease	Number of participants who recalled disease*			
	Group with cancer	Control group	Estimated rate ratio†	95% Confidence interval
Mumps				
No	87	92	1.0	—
Yes	192	220	0.9	0.6-1.4
German measles				
No	99	124	1.0	—
Yes	112	136	1.0	0.7-1.5
Measles				
No	46	47	1.0	—
Yes	181	222	0.8	0.5-1.3
Chickenpox				
No	49	59	1.0	—
Yes	228	257	1.0	0.7-1.6
Shingles				
No	273	284	1.0	—
Yes	18	38	0.5	0.3-0.9
High blood pressure				
No	189	213	1.0	—
Yes	96	97	1.2	0.8-1.8
Thyroid disease				
No	237	236	1.0	—
Yes	56	78	0.7	0.5-1.1
Diabetes				
No	271	274	1.0	—
Yes	21	27	0.8	0.4-1.5
Asthma				
No	279	280	1.0	—
Yes	13	26	0.4	0.2-0.9
Breast cancer				
No	278	337	1.0	—
Yes	17	5	4.2	1.4-13.2

*Participants with uncertain responses excluded.

†Adjusted for age and race.

tios in increasing quartiles were 1.0, 1.4, 1.0, and 1.1, respectively.

As shown in Table VIII, ovarian cancer risk was related to drinking but was not related to cigarette smoking. Alcohol use was measured as the typical weekly number of shot glasses of alcohol, bottles of beer, and glasses of wine consumed over the past 10 years, with total alcohol consumption computed as the sum of the three subtotals. The rate ratio rose slightly with increasing total alcohol consumption, and with each of the three kinds of alcohol considered separately. The rate ratios were highest for whiskey (rate ratio = 1.6, 95% confidence interval = 1.1 to 2.3), followed by wine (rate ratio = 1.3, 95% confidence interval = 0.9 to 1.9) and beer (rate ratio = 1.1, 95% confidence interval = 0.7 to 1.6).

Comment

The results of this investigation confirm the importance of parity, infertility, family history, and previous breast cancer in the cause of epithelial ovarian cancer. The results also suggest the relative unimportance of

some other factors associated with risk in previous epidemiologic investigations.

Several explanations of the protective effect of childbearing have been proffered, among them that pregnancy prevents ovulation, that it inhibits pituitary secretion of gonadotropins, or that an underlying problem leads both to low parity and increased risk. Estimation of the effects of different pregnancy outcomes may help reveal how childbearing reduces risk of ovarian cancer. If parity protected against ovarian cancer simply by the inhibition of ovulation, then a pregnancy would reduce risk approximately in proportion to its length, regardless of outcome. Our data suggest that pregnancies that lead to live births offer protection but those that lead to losses may not. Inasmuch as women with more live births tend to have had more losses, adjustment for parity is critical. One previous estimate of the rate ratio adjusted for parity was 0.8 per miscarriage.⁸ Other investigations of fetal loss are not directly comparable, either because they were not adjusted for the effect of parity or because they measured the fraction of pregnancies that ended in

Table VIII. Estimated rate ratios according to cigarette and alcohol use*

	Group with cancer	Control group	Estimated rate ratio†	95% Confidence interval
Cigarette smoking				
Never smoked	128	130	1.0	—
Quit	91	89	1.5	0.9-2.0
Current	70	83	0.8	0.6-1.5
Cigarette smoking (duration)				
Never smoked	128	130	1.0	—
10 years or less	28	23	1.1	0.6-2.2
11-30 years	65	66	0.9	0.6-1.5
31+ years	66	64	1.1	0.7-1.8
Trend test		<i>p</i> (trend) = 0.87		
Alcohol (average weekly consumption)				
0	109	122	1.0	—
Occasional drink	49	49	1.1	0.7-1.9
1-6 drinks	63	54	1.4	0.8-2.3
7-13 drinks	36	32	1.2	0.7-2.2
14+ drinks	34	26	1.5	0.8-2.8
Trend test		<i>p</i> (trend) = 0.14		

*Three participants without data on smoking duration or currency were deleted.

†Adjusted for age and race.

miscarriage rather than the number or months of various pregnancy outcomes.

Apart from the reduced risk associated with higher parity, there clearly seems to be a separate link between infertility and risk evident among the nulliparous women in our investigation. Previous studies have shown that married nulliparous women have a higher risk than single nulliparous women.^{1,9} We clarified this observation by showing that married nulliparous women who chose not to become pregnant were not at increased risk, but women who tried and failed definitely were at increased risk. Case-control studies of ovarian cancer cannot easily assess infertility as a risk factor, because older women must recall often ill-defined diagnoses from decades earlier. One follow-up study of infertile women found increased ovarian cancer risk among subjects with nonhormonal infertility (rate ratio = 3.2, 95% confidence interval = 0.5 to 32.9),⁹ but another did not.⁸ Both studies observed very few cancers and both derived the expected rates of ovarian cancer from the general population of parous and nulliparous women, so the effects of parity and infertility could not be separated. A long-term follow-up study of a large cohort of infertile women could discriminate between the effects of different kinds of infertility and could provide useful etiologic clues. For example, anovulation that leads to infertility should protect against ovarian cancer if incessant ovulation is a key mechanism.

Several reproductive factors probably are not strongly related to risk. A majority of studies indicate that the number of live births and not their timing is the key factor. Our finding of reduced risk to women

who breastfed their children confirms a previous observation¹⁰ and supports the hypothesis that anovulation reduces risk. The absence of dose response may reflect the poor correlation between the number of months a woman breastfeeds and the months of ovulation suppression because of other factors such as body mass and variability in breastfeeding regimens. Menstrual factors such as age at menarche, menstrual symptoms, and age at menopause do not appear to be strongly related to risk of ovarian cancer in these or other data, although the plateau in age-specific incidence around menopause strongly suggests a change in risk broadly related to cessation of ovarian function. It is not clear whether menopausal estrogen use affects risk. Hysterectomy does appear to decrease risk, but the association may reflect increased clinical surveillance rather than decreased risk.¹¹

An anomalous finding in this study was that women who take oral contraceptives had only a very slight reduction in ovarian cancer risk. The estimated rate ratios from many studies have been in the protective range of 0.5 to 0.7. We know of no biases in the current study that would distort our estimate of the effects of oral contraceptives. Other known risk factors showed the same patterns in this study as in other studies, and women with diagnoses potentially related to oral contraceptives were not selected as control participants. From all of the available data we infer that oral contraceptives protect some women from ovarian cancer.

A history of mumps infection has been found to be a weak protective factor in several previous investigations.^{12,13} This effect, if real, is certainly not strong. The biologic meaning is in doubt because recall of mumps

history has been shown to be a poor indicator of antibody titer.¹⁵ A careful examination of mumps exposure, assessed serologically, would be worthwhile. Most of the other childhood and adult diseases that we considered probably do not influence the development of ovarian cancer. The apparent protection conferred by a history of shingles, in the absence of an effect of chickenpox, has no ready explanation. Nor does the effect of asthma. The lack of effect of hypertension, obesity, or diabetes, accords with the inconsistent findings of previous studies. Two medical conditions that warrant further study are thyroid disease and adrenal disease. The examination of adult diseases as risk factors in a hospital-based case-control study is difficult because the control group should include only patients whose admission diagnosis is not related (positively or negatively) to the adult disease risk factor. Data from population-based studies with well-documented medical histories may clarify whether these two associations are real.

Women with a previous history of breast cancer were at substantially increased risk of ovarian cancer. This observation is not new¹⁶ and appears to represent a true association. One goal of our painstaking pathologic and clinical review was to exclude metastatic breast and colon cancer; we believe misdiagnosis does not explain the finding. A family history of ovarian cancer seems to pose at least a three- to fourfold increased risk, as indicated by our results and the results of others.⁷

Smoking was not related to ovarian cancer in this investigation, as in most previous work, but the effects of alcohol remain unclear. Our study and others (e.g., Esoli and Franceschi)¹⁷ estimate slightly increased incidence of ovarian cancer among drinkers, but other studies of ovarian cancer found reduced incidence among drinkers (e.g., Gwinn et al. and Byers et al.).¹⁸⁻²¹ The available data suggest that if there is an association, it is weak.

During the past decade, case-control interview studies with 200 to 500 women with ovarian cancer have offered numerous leads. Comparisons among existing studies suggest that some of the leads warrant further pursuit. Despite their cost, larger studies are needed to

give the statistical power to disentangle the effects of specific causes of infertility, varying patterns of oral contraceptives, and specific pregnancy outcomes.

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prepared cytologic smears and residual tissue should be sent to the cytology department. Tissue may be discarded if trophoblast is identified cytologically. In the rare exception in which trophoblast is not identified cytologically, tissue can be processed for histologic study. In 100 cases of first-trimester abortion, the authors estimated that microscopic study (because no fetal parts were identified) was necessary for verification of placenta in 66% of the cases.

The identification of trophoblastic epithelium in uterine contents removed by suction, curettage, or in spontaneously passed tissue does not certify interruption of pregnancy. Incomplete induced abortion, multiple or ectopic pregnancy, late intrauterine abortion of tubal pregnancy, and uterine anomalies are possible occurrences. In addition, partial moles, choriocarcinomas, and trophoblastic pseudotumors may be missed.

If material removed from the uterus does not correlate with the estimated gestational age or if there are other unusual findings, the clinician should take appropriate steps to explain the discrepancy.

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Review

EPIDEMIOLOGY AND ETIOLOGY OF OVARIAN CANCER: A REVIEW

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and Leo D. Lagasse, MD

Ovarian cancer is the most frequent cause of death from gynecologic malignancies in the western world. Much effort has been put into attempts to correlate differences in incidence rates with environmental, endocrinologic, and genetic factors. A review of the literature reveals that there is currently no evidence to incriminate any single etiologic factor for this group of tumors. There is growing evidence of familial predisposition in a small group of patients and of a relationship with reproductive history. If current knowledge of the epidemiology of ovarian cancer is to be translated into disease prevention, more attention should be paid to women at risk because of their family history, and more awareness should be made of the protective effect of oral contraceptives. (*Obstet Gynecol* 66:127, 1985)

The American Cancer Society indicates that cancer of the ovary is the sixth common malignancy in women in the United States (Table 1). Results of the third National Cancer Survey of the United States indicate that one of every 70 newborn females will develop the disease.¹ The highest incidence rates are reported in

Northwestern Europe and North America.²⁻⁶ (Table 2). The age-specific incidence rate increases from two per 100,000 for women in their 20s to 55 per 100,000 at the age of 70.¹ The greatest number of new patients occurs in the age group 50 to 59 years with a median age of 59 years. The mortality for ovarian cancer in Northwestern Europe and the United States is about 7.3 to 13 per 100,000, making ovarian cancer the most frequent cause of death from gynecologic malignancies, and the fifth leading cause of death from cancer in women (Table 1). The age-adjusted death rate shows an increase from 0.5 per 100,000 at age 20 to 45 per 100,000 at 75.¹ Since 1930 there has been a steady increase in both the incidence and mortality of ovarian cancer.¹ Epidemiologic investigations have not produced an adequate explanation for this phenomenon.

Environmental Factors

That an environmental factor might be of importance in ovarian cancer is suggested by the fact that the highest incidence of ovarian cancer occurs in industrialized countries, the only exception to this observation being Japan.^{1,3,6}

The hypothesis that environmental factors play an important role is strengthened by the observation that the incidence of ovarian cancer in Japanese women born in Japan and emigrating to the United States increases. In the postmenopausal age group it approximates the incidence of American women.^{1,3,6} A similar observation has been made for Jewish women in Israel. The incidence of ovarian cancer in Jewish women born in Europe or the United States is 14.3 per 100,000 and much higher than that reported for Jewish women born in Africa. In several studies, a higher incidence has been reported among women with higher education, which correlates with higher incomes and different dietary habits.^{1,3,6} This, in conjunction with the higher incidence in industrialized countries and the increased incidence among certain racial groups after migration to a country with a high incidence of the disease, is also strongly in support of an environmental influence.

It is clear that not everyone is equally susceptible to these environmental factors. This variation in susceptibility may be dependent on genetically controlled factors. Most of the investigations in this field have been focused on alimentation, products of industry, and extrinsic hormones. Alimentation research has mainly been focused on diets with a high intake of meat and animal fat, because these are the most significant areas in which diets of industrialized and nonindustrialized countries differ. The results of these investigations are contradictory.^{4,11-18} Byers et al.¹¹

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Table 1. Cancer Incidence and Death Rate in Women

Cancer	Incidence (%)	Death rate (%)
Breast	26	18
Cervix/rectum	15	15
Ovaries	13	5
Lung	9	17
Leukemia and lymphomas	7	9
Ovary	4	6
Uterus	4	3
Pancreas	3	5
Oral	2	1
Skin	2	1
Others	15	20

1983 estimated cancer incidence and cancer death by site in women

found no relationship between fat intake and ovarian cancer risk. Cramer et al.¹⁸ however, found that women above the highest quartile of animal fat consumption had a twofold increase in risk compared with those below the lowest quartile. The mechanisms by which animal fat could contribute to the genesis of ovarian cancer might be direct, via carcinogenic contaminants in the fat, or indirect via their ability to contribute to extragenital estrogen production from gut bacteria.¹⁹ A relationship between smoking and/or alcohol consumption and ovarian cancer has not been found.^{17,20} There are several reports concerning the relationship between ovarian cancer and caffeine consumption. This possible relationship was first reported by Stocks²¹ in 1970 and later confirmed by Trichopoulos et al.²² Byers et al.¹⁷ however, could not find any relationship between caffeine consumption and ovarian cancer risk. The possible relationship has been based on the fact that the highest incidence rates and mortality for ovarian cancer are found in Sweden,

which also has the highest rate of coffee consumption. A simple relationship between coffee consumption and ovarian cancer is confounded by the fact that coffee is a mixture of many different natural chemicals and many people add products such as artificial sweeteners and artificial cream to their coffee before consumption. The carcinogenicity of these individual chemicals has not been investigated. At this time there is no conclusive evidence for a causal relationship between caffeine consumption and ovarian cancer. An interesting finding is that of Byers et al.¹⁷ who reported an increased risk for ovarian cancer in association with decreased ingestion of vitamin A from fruit and vegetables sources. This finding occurred only in the 30 to 49 year age group.

Exogenous Chemicals

The highest incidence of ovarian cancer in industrialized countries suggests a relationship between this tumor and products of industry. Until now there has been little evidence to incriminate specific products. Currently, much attention is being focused on a possible relationship between ovarian cancer and materials of the talc-asbestos group (hydrous magnesium silicates).²³⁻²⁶ The clear relationship between asbestos exposure and pleural and peritoneal mesotheliomas and the similarity of ovarian cancer to mesotheliomas are in support of this theory. Graham and Graham²⁶ (1967) found particles similar to talc in ovaries with ovarian carcinoma. Newhouse et al.²⁷ found a slightly elevated frequency of ovarian carcinoma in female asbestos workers. Others^{24,25} have not confirmed this observation. Cramer et al.¹⁷ found that 42.8% of ovarian cancer patients regularly used talc powder in the perineal area, in contrast to 28.4% of a control group of women. He concluded that users of talc powder were at increased risk to develop ovarian cancer. It is of significance that in this study the majority of patients who subsequently developed ovarian cancer had no history of exposure to talc,¹⁷ suggesting at best a possible, but small, role as a promoting agent. Presumably the talc comes in contact with the ovaries after passage through the vagina, uterus, and fallopian tubes (pelvic contamination theory).²⁸ That this route of contamination is feasible was shown in 1961 by Egli and Newton²⁹ using carbon particles and in 1981 by Venter³¹ using labeled human albumin microspheres. Hargre et al.²⁸ however, could not demonstrate any effect of the use of talc on the relative risk of ovarian cancer. Even patients who used talc on their diaphragm did not show any increased risk compared with controls. This observation notwithstanding, talc has not been shown to induce tumors in other animals.³⁴

Table 2. International Incidence and Death Rate of Ovarian Cancer^{1, 8, 22, 30}

	Incidence per 100,000	Death rate per 100,000
Sweden	14.9	12.9
Norway	14.2	9.5
USA (white)	13.1	5.3
Israel	12.7	
German Democratic Republic	11.8	
West Germany	11.5	11.0
United Kingdom	11.1	9.1
Switzerland	10.6	
Finland	7.9	
Brazil	6.1	
India	4.6	
Japan	2.1	2.1
Netherlands		12.1

Talc plays any role in the cause of cancer, it still is not clear whether the active agent is the talc per se or the asbestos particles that are present in talc powder. This possible role becomes even less important because of the decreased inclusion of talc in cosmetic products after 1977.

Genetic and Familial Factors

Several studies report families in which more than one member developed a malignant tumor of the ovary.^{33,32-45} This phenomenon, however, is more the exception than the rule. Most women with ovarian carcinoma have no family history of the disease, although accurate data on family history frequently are not available.

A higher than expected incidence of ovarian tumors is found in women with Peutz-Jeghers syndrome,⁴⁶ basal naevus syndrome,⁴⁷ and gonadal dysgenesis.^{48,49} (Table 3). Most of these tumors are not of epithelial origin, however. One hundred forty families have been reported with familial ovarian cancer.^{33,32-45,50} Of these, 94 were published recently by the Familial Ovarian Cancer Registry.⁴⁵ The number of observed generations varies from two to four per family. In 131 of these 140 families, 321 women developed ovarian cancer.

There are several features that are characteristic of familial ovarian carcinomas. There is a tendency toward earlier onset, mean age being 47.7 years, compared with 59 years for epithelial ovarian cancer in general.¹¹ The serous type of tumor predominates (90 to 97%), and there is a trend toward more poorly differentiated adenocarcinomas.^{11,45} A high percentage of cases is bilateral (70 to 80%).¹¹ The hereditary pattern in these families is consistent with an autosomal dominant gene with variable penetration, transmitted through men or women.^{13,34,38,42} Lynch et al^{14,42} have performed the most comprehensive research in this field. After analysis of ten families in which 25 women developed ovarian cancer over three or four generations, they concluded that three different hereditary syndromes existed: 1) site-specific familial ovarian cancer syndrome—women in these families are at risk of developing ovarian cancer only. Hence, a

woman may be at risk if her mother or sister has ovarian cancer; 2) breast-ovarian cancer syndrome—the pedigree of these families is characterized by the presence of female members with breast and ovarian cancer, alone or in combination. Hence, a woman may have a 50% risk for ovarian cancer if her mother or sister had breast cancer and/or ovarian cancer; and 3) the cancer family syndrome—in the pedigree of these families, males and females are at risk of developing colon cancer and, to a lesser extent, other adenocarcinomas (ovary, endometrium). Consistent with an autosomal dominant inheritance factor females in these families should also have a 50% risk for ovarian cancer. Hence, a woman may be at risk for ovarian cancer if one of her relatives has one of the cancers of the cancer family syndrome.

The risk of female offspring developing ovarian cancer in these autosomal dominant familial syndromes may be as high as 50% in the first-degree female relatives. Although the exact pathogenesis of these syndromes is not understood, three theoretical explanations are possible⁴⁵: different genetic susceptibility of ovarian tissue to carcinogenic influences, a genetic defect that induces a hormonal imbalance which may be potentially carcinogenic, or a genetic defect causing an altered immunologic response resulting in ovarian cancer.

Of major significance for female offspring in these families is the question of prophylactic oophorectomy. All authors advise that consideration should be given to this procedure as soon as childbearing is completed. However, it is evident from the work of Tobachman et al⁵¹ that prophylactic oophorectomy does not give total protection against subsequent development of ovarian cancer. They describe 16 families with second-degree relatives who had documented ovarian cancer. Twenty-eight women in these 16 families underwent prophylactic oophorectomy and were observed for periods ranging from one to 20 years. The removed ovaries were histologically normal. During the follow-up period, three of the 28 women developed intraabdominal carcinomatosis. Histologic examination revealed lesions indistinguishable from ovarian cancer. These cases are consistent with the hypothesis that epithelial ovarian carcinoma may arise from any tissue originally derived from coelomic epithelium.²⁹

Graham et al⁵² were the first to describe hyperplastic epithelial foci in the ovaries of an asymptomatic woman, removed prophylactically because of a family history of ovarian cancer. Similar changes have been reported by Fraumeni et al¹⁴ who found these foci in three of eight asymptomatic women of cancer-prone families. Recently, Gusberg and Deligdisch⁵³ reported a similar finding in grossly normal ovaries removed

Table 3. Genetic Syndromes Associated With Ovarian Tumors

Syndrome	Tumor
Peutz-Jeghers syndrome ⁴⁶	Sex cord tumor
Multiple basal cell carcinoma ⁴⁷	Ovarian fibroma
Gonadal dysgenesis ^{48,49}	Dysgerminoma
	Gonadoblastoma in streak (xy) gonads

from three monozygous sets of twin sisters of three patients with invasive carcinoma of the ovary. They found hyperplastic and dysplastic foci that were not found in control ovaries from other women and suggested that these lesions may represent a precursor of ovarian cancer. This would support the concept of prophylactic removal of the ovaries. Lewis and Davison,¹¹ Lynch et al.,¹² Thor et al.,¹³ Skinner et al.,¹⁴ and Piver et al.¹⁵ reported a total of 18 asymptomatic women who underwent prophylactic oophorectomy. None of them reported histologic abnormalities in the removed ovaries. Occasionally, one can find benign mesothelial proliferation in the peritoneal cavity of healthy women. This makes it even more difficult to determine the significance of these lesions in a woman who is probably at high risk for ovarian cancer. Important, however, is the finding of Fraumeni et al.¹⁶ and Casberg and Deligdisch¹⁷ that the removed ovaries were macroscopically normal. If the lesion they describe proves to be characteristic of the dysplasia that precedes ovarian carcinoma, then the only way to diagnose the lesion is to remove both ovaries.

It is not easy to determine how many women will benefit from prophylactic oophorectomy. If the mother of a family has an autosomal dominant ovarian cancer, the chance of her daughters inheriting the gene for this tumor is 50%. In the absence of a genetic marker, there is no way to show if it is a genetic or random phenomenon if two members in one family are affected. If the chance of ovarian cancer during a lifetime is one in 70, the chance of this happening twice in one family is about (one in 70)², or only 0.02%. Given the low morbidity of prophylactic oophorectomy and the high mortality of ovarian cancer, it would seem prudent to consider this operation at the completion of childbearing in any woman who has two or more first-degree relatives affected with carcinoma of the ovary, or in whose family there is sufficient evidence of the breast-ovarian cancer syndrome or the cancer family syndrome. However, the woman concerned has to be aware of the incomplete data existing at this time.

Endocrinologic Factors

Many authors report differences in endocrine-related conditions in women with ovarian cancer compared with normal control subjects.^{18-21, 23, 24, 25, 29, 30, 31} Joly et al.²⁸ analyzed 399 women with ovarian cancer and found that women with the disease had an increased incidence of nulliparity, a lower mean number of pregnancies, and an increased incidence of miscarriage.

McCowan et al.³⁰ on the basis of a study of 197 women with ovarian cancer, estimated that nulligrav-

idas were 2.45 times more likely to develop malignant ovarian tumors and 2.9 times more likely to develop ovarian carcinoma of low potential malignancy than were women who had been pregnant three or more times. The risk of ovarian cancer in their series was reduced to 1/27 among women who had at least one pregnancy. This figure was not analyzed for full-term pregnancies, premature births, and abortions. In general in his series, women with ovarian cancer demonstrated a marked decrease in the number of live births. Similar experience has been reported by others.^{11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31} Unfortunately, in the study of Demopoulos et al.,¹¹ parity was calculated as the sum of all full-term gestations, premature deliveries, and abortions divided by the total number of patients. Whether or not the pregnancies in the study of Hildreth et al.²⁰ were full-term is not mentioned. Risch et al.²¹ analyzed the reproductive history of their patients in more detail and reported that patients with ovarian cancer, in addition to less full-term pregnancies, had fewer miscarriages and less total time breast-feeding. Casagrande et al.¹⁷ reported similar results for live births, but also reported a decreased risk in association with an increasing number of incomplete pregnancies. Cramer et al.²² found that the relative risk for ovarian cancer decreased significantly with increasing parity. They reported that compared with nulliparous women, women with one or two children had one-half the risk, women with three or four children one-third the risk, and women with five or more children had one-fourth the risk for ovarian cancer.

In contrast with these results, Wijnder et al.¹ and West¹⁶ found no differences in parity or marital status between ovarian cancer patients and control subjects. One explanation for these different findings may be the nature of the control groups. For example, West used patients with benign ovarian tumors as controls. Also, the risk of ovarian cancer is reported by Hildreth et al.²⁰ to increase with increasing age at first pregnancy. However, in their study it is not clear if this increased age is an independent risk factor or a result of the often associated lower parity. In all reported series the risk of ovarian cancer for women who had difficulties with conception was greater. Joly et al.²⁸ found that the risk also was increased in married but never pregnant women in comparison with women who never married and were never pregnant, and that this latter group in some instances had a smaller relative risk than women with one or two pregnancies. Joly's results suggest a relationship between the inability to get pregnant and ovarian cancer. One possible interpretation would be that the endocrinologic status in pregnancy protects against ovarian cancer, and that the lack of this protection makes infertile women a

higher risk for ovarian cancer. A second explanation could be that infertility and ovarian cancer are the result of the same abnormal gonadal status. This theory could explain why infertile women are more at risk than never married and never pregnant women. Because the patients studied were diagnosed between 1957 and 1966, oral contraceptives cannot have been of any importance in this group. However, these pregnancy-associated hormonal factors and abnormal gonadal status have not been identified. Cramer et al.¹⁹ could not demonstrate any difference in relative risk of ovarian cancer among infertile women and nulliparous women in general. They hypothesize that pregnancy may induce changes in the pituitary gland resulting in alterations of the secretion of tropic hormones and so in stimulation of the ovaries. The effect of nulligravida on the risk of ovarian cancer is summarized in Table 4.

Hildreth et al.⁴⁰ reported an increased risk of ovarian cancer in women with a late onset of menopause and decreased risk in women with a history of heavy menstrual flow. These results are in contrast with the findings of Wijnder et al.,⁵ who reported an increased risk of ovarian cancer associated with early menopause and a history of heavy menstrual flow. West,¹⁶ Newhouse et al.,¹⁹ Cramer et al.,¹⁹ and Annegers et al.¹¹ found no relationship between the age at menopause and the occurrence of ovarian cancer.

Exogenous Hormones

Most of the studies relating to exogenous hormones and ovarian cancer have found no significant etiologic relationship between the two.⁴ Hooover et al.¹⁶ observed 900 women who used conjugated estrogen for six months or more. They found four cases of ovarian cancer, whereas three would have been expected. There is no evidence of a relationship between diethylstilbestrol and ovarian cancer.^{46,47}

Several authors^{7,9,42,45} have reported a lower frequency of ovarian cancer after the use of oral contraceptives, although only four studies^{9,42,44} reached

Table 5. Effect of Nulligravida on the Risk of Ovarian Cancer

Author	Year	Relative risk (Nulligravida/gravid women)
Joly et al. ¹⁸	1974	1.8
Newhouse et al. ¹⁹	1977	2.5*
Annegers et al. ¹¹	1979	1.8
Casagrande et al. ¹	1979	1.8*
Demopoulos et al. ¹¹	1979	2.3
McGowan et al. ⁴⁸	1979	1.3-2.4
Hildreth et al. ⁴⁰	1981	1.7

* Nulliparity versus parity

statistical significance (Table 5). The most impressive study was reported by the Centers for Disease Control Cancer and Steroid Hormone Study.⁹ In their case control study they studied 179 women with ovarian cancer. Their major finding was that women who had ever used oral contraceptives had a significantly lower risk of ovarian cancer. The decrease in risk was directly related to the duration of oral contraceptive use: after a minimum of three months, the relative risk is declined from 1.0 to 0.6, and it declined further to 0.4 for women who used oral contraceptives for more than five years. This lower relative risk proved to be a long-term effect. This is in agreement with earlier reports.⁴⁴ They estimated that the incidence of ovarian cancer among women between 20 and 54 years of age in the United States might be 30% higher if oral contraceptives had not been used. Another interesting finding in the study was that the use of oral contraceptives by nulliparous women decreased their relative risk below that for parous women. A protective effect of oral contraceptives among nulliparous women also was found by Rosenberg et al.⁴⁴ and Cramer et al.,¹⁹ but only in very small samples. Contrary to the Centers for Disease Control study, Rosenberg et al.⁴⁴ and Cramer et al.¹⁹ found a further decrease of the relative risk in oral contraceptive users with increased parity. This protective effect could not be confirmed by Vessey et al.⁴⁶ and Romcharson et al.⁴⁷

Until now menopausal estrogen use has not been shown to have a significant influence in ovarian cancer. However, Weiss et al.⁴² found more menopausal estrogen users among women with endometrial cancers of the ovary. Cramer et al.¹⁹ found the same for endometrial cancers and clear-cell cancers. Cramer also observed a nearly statistically significant increased risk in women who had used menopausal estrogen in a cyclic rather than continuous fashion, and in women who had used these drugs for more than five years.

Because of the conflicting results on the carcinogenicity of long-acting progestones, Liang et al.⁴⁹ performed a follow-up investigation among 5000 women

Table 4. Familial Neoplasms of the Ovary⁴

Cystadenocarcinoma
Site-specific ovarian cancer
Breast-ovarian cancer syndrome
Cancer family syndrome
Dermoid tumor or teratoma
Sometimes associated with twinning
Dysgerminoma
Archeoblastoma
Sometimes associated with mucinous cystadenoma and thyroid adenoma

Table 6. Effect of Oral Contraceptive Use on Ovarian Cancer Risk

Author	Year	Relative risk (if ever used versus never used)
Newhouse et al. ¹⁷	1977	0.50 (0.31)
Castagnone et al. ¹⁸	1979	0.7 (0.6)
Wentz et al. ¹⁹	1981	0.52*
Hildreth et al. ²⁰	1981	0.5
Wullen et al. ²¹	1981	0.6
Rosenberg et al. ²²	1982	0.6*
Cramer et al. ²³	1982	0.38*
Risch et al. ²⁴	1983	0.89
CDK ²⁵	1984	0.6

* Significant

Only significant in women older than 40 years

who had received medroxyprogesterone for contraception. The women were studied for four to 13 years. No increased risk of ovarian, endometrial, or breast cancer was found.

Virus Infection

Ovarian cancer has been studied most in connection with mumps virus. West¹⁶ was the first to report that ovarian cancer patients had a decreased incidence of clinical mumps in their history, when compared with control subjects. He suggested a possible protective effect from mumps virus infection. Menzies et al.²⁶ confirmed that the incidence of clinical mumps among ovarian cancer patients was lower, but reported that the frequency of subclinical mumps was higher. Evidence for the latter is based on the presence of positive serologic evidence in patients with a negative mumps history. He also found that mumps complement-fixing antibody titers in these patients were lower. Menzies et al. hypothesized that these results may reflect an existing immunocompetence that facilitates the development of ovarian cancer probably through a direct etiologic role of mumps virus. Golan et al.²⁷ could not confirm these results. Lungenau²⁸ suggested that if mumps virus is involved in the cause of ovarian cancer, a decrease in the incidence of this disease after vaccination of the whole population can be expected. Data concerning mumps infection in countries with a low and high incidence of ovarian cancer are not known. Mumps virus has many affinities. Besides the parotid gland, it may affect other glands including the gonads. Mumps orchitis is known to occur in about 20% of infected males. The incidence of similar involvement of the ovaries is lower and estimated to be 5%.²⁹ The Royal College of General Practitioners and the Association for the Study of Infectious Diseases, however, published an incidence rate of mumps in Great Britain of 6.0 per 1000 and an incidence of mumps

ophoritis of only 0.1 to 0.2%.^{30,31} Unfortunately, the criteria for the diagnosis of oophoritis are not given in either study. This means that with an incidence of mumps of 6.0 per 1000 a year, the incidence of oophoritis must be about 1.2 per 100,000.

Cramer et al.²³ proposed that the mumps virus damages the ovaries and thereby causes premature depletion of oocytes, which can result in increased levels of gonadotropin. Oocyte loss in the animal model is associated with tumor growth.³² As the available data reveal an incidence of oophoritis that is much lower than the incidence of ovarian cancer, this theory remains highly speculative.

McGowan et al.³⁰ reported that rubella infection between the 12th and the 18th year increases the risk of ovarian carcinoma fourfold. He could not find this relationship after an infection in childhood. This observation has not been confirmed in publications of others.

Radiation

Although another possible reason for premature oocyte loss and ovarian failure is irradiation of pelvic organs, no increased incidence of ovarian carcinoma has been reported in women who underwent this therapy.³³

Etiology of Ovarian Cancer

Fathalla³⁴ was the first to propose that the etiology of ovarian cancer was related to incessant ovulation. The hypothesis is that the ovarian surface incurs minor trauma with each ovulation, which cumulatively could contribute to the development of an ovarian neoplasm. Cessation of ovulation by pregnancy or oral contraceptive use interrupts this process of repeated trauma and may thus protect against ovarian cancer. Pregnancy and the use of oral contraceptives also are associated with low gonadotropin levels. The high gonadotropin levels in the early postmenopausal years have been suggested to be of possible significance.^{35,36} In the experimental model, excess gonadotropin secretion seems to be related to the development of ovarian tumors.^{37,38} The incessant ovulation theory is also in accordance with those who have reported a higher risk of ovarian cancer in women with a late onset of menopause and a lower risk in those with an early onset. It is not in accordance with a higher risk of ovarian cancer in patients with excessive uterine bleeding insofar as this bleeding is a result of anovulation. The theories of incessant ovulation and excessive gonadotropin secretion are in agreement with the lower risk of ovarian cancer in parous women, and with the

lower risk in women who are nulliparous by desire and have used oral contraceptives for that reason. However, the theories are also in agreement with the higher risk observed in undesired infertile women who are nulliparous.

Based on these endocrinologic data, the authors must conclude that pregnancy and the use of oral contraceptives may protect against ovarian cancer, although undesired infertility in women who ovulate regularly increases the risk of ovarian cancer. Pregnancy and the use of oral contraceptives differ from undesired infertility to the cessation of ovulation and the suppression of gonadotropin release.

Pathogenesis of Ovarian Cancer

Cramer and Welch³⁹ have tried to unite the different etiologic factors in one theory. In this theory the first step toward malignant growth is the formation of inclusion cysts by entrapment of surface epithelium in the ovarian stroma. The second step is direct or indirect stimulation of the included epithelium by gonadotropins and extra- or intraglandular estrogen, resulting in differentiation, proliferation, and sometimes malignant transformation. However, the final stimulus to malignant transformation remains unknown (Figure 1). In this theory all factors that cause inclusion cyst formation, high extra- or intraglandular estrogen and factors that cause high gonadotropin levels, may make a woman at risk for ovarian cancer. Consequently, factors that prevent one or more of those stimuli may protect against this disease.

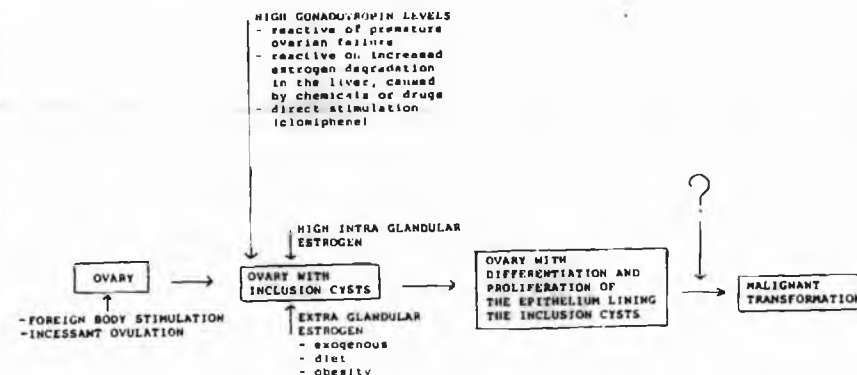


Figure 1. The etiology of ovarian cancer according to Cramer and Welch.³⁹

Prophylaxis for Ovarian Carcinoma

The group of patients who are at high risk for ovarian carcinoma can only be detected by a careful family history. The use of oral contraceptives for birth control may help to decrease their risk, and prophylactic oophorectomy after childbearing appears to be justified. However, the authors agree with Galsberg and Deligdisch⁴² that the women concerned have to be aware of the incomplete data existing at this time.

Dietary measures to prevent ovarian carcinoma could include a low intake of animal fat, and a high intake of vitamin A, although the evidence to support these recommendations is small.⁴³

For the general population, a prophylactic affect on the incidence of ovarian cancer might be expected should bilateral oophorectomy be performed in all patients 40 years of age or older receiving pelvic laparotomy for benign lesions.^{44,45} However, as Schweppe and Beller⁴⁶ calculated, to prevent one ovarian carcinoma 700 women would have to be castrated. So decision making about this subject becomes a matter of philosophy and personal attitude.⁴⁷ In the authors' opinion the decision should be weighed against the risks and the personal and general problems arising of the imposition of necessary long-term estrogen substitution.

Conclusions

Epidemiologic and etiologic research on ovarian cancer has hitherto included all cell types. As the large majority of the tumors are of epithelial origin, the

incidence of epithelial ovarian carcinoma is close to that of ovarian cancer in general. Epidemiologic and etiologic studies have identified a group of risk factors that appears to be directly or indirectly related to ovarian cancer. These factors can be grouped in four main categories: genetic, environmental, hormonal, and viral. In the present state of knowledge, it appears that the etiology of ovarian cancer is multifactorial. Efforts for early diagnosis and prophylaxis, which seem to be expensive for the whole population, are justified in the small group of women who may be at high risk for this lethal disease. From a clinical viewpoint, it is important to realize that a careful family history should be taken to identify daughters who are at high risk of developing ovarian cancer. For this group of patients the use of oral contraceptives for birth control may help to decrease the risk and prophylactic oophorectomy after completion of childbearing appears to be justified, although even with oophorectomy these patients remain at risk for carcinomas of extragonadal coelomic epithelium.

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AN *IN VITRO* MODEL OF OVARIAN EPITHELIAL CARCINOGENESIS: CHANGES IN CELL-CELL COMMUNICATION AND ADHESION OCCURRING DURING NEOPLASTIC PROGRESSION

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To investigate the cellular mechanisms of ovarian epithelial carcinogenesis, a series of progressively transformed rat ovarian surface epithelial (ROSE) cell lines were developed and studied. Transfection of primary ROSE cells and an immortalized ROSE line (ROSE 199) with the pSV3neo plasmid (SV40 T-antigen) yielded transformed lines which retained epithelial morphology. *In vivo* selection of these pSV3neo cell populations resulted in further phenotypic transformation. Transfection of ROSE 199 with pSV3neo/c-H-ras^H (ras^H p21) resulted in a malignant line which appeared fibroblast-like and formed invasive sarcomas both in athymic mice and in immunocompetent rats. Gap junctional intercellular communication (GJIC) and cell-cell adhesion were studied in this series of ROSE lines. Both c-H-ras^H transformation and *in vivo* selection resulted in a significant reduction of GJIC between adjoining cells and a transition of *in vitro* migration as continuous epithelial sheets to the dissociation of individual cells. This apparent shift in cell adhesiveness was associated with reduced expression of the E-cadherin adhesion molecule. Our data suggest that neoplastic progression of the ovarian surface epithelium may be associated with concomitant reductions in GJIC, E-cadherin expression and functional adhesiveness.

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Adhesion mechanisms between tumor cells may permit detachment and dissemination during malignant progression towards metastasis (Larsen *et al.*, 1987; Takeichi, 1991). Gap junctions, which consist of membrane channels which connect the cytoplasmic compartments of adjoining cells, are thought to function primarily in the maintenance of tissue homeostasis and the exchange of regulatory molecules necessary for the control of normal cellular growth (Loewenstein, 1979). It has thus been hypothesized that loss of gap junctional intercellular communication (GJIC) may interfere with normal cellular growth regulation (Loewenstein, 1979). Indeed this theory has been supported by observations that many, but not all, tumor cells display reduced GJIC (Loewenstein, 1979; Klamt *et al.*, 1989). However, studies which demonstrate reduced GJIC in cells that are already neoplastic may not be sufficient for precise understanding of how communication is altered during the natural carcinogenic progression from normal to neoplastic cells. Analysis of GJIC characteristics among progressively transformed cell lines may provide useful information regarding how, and at what stage, communication is altered during neoplastic progression.

Cell-cell adhesion plays an important part in morphogenesis, maintenance of tissue architecture and immunologic function (Shimoyama *et al.*, 1989; Takeichi, 1991). As neoplastic populations progress towards malignancy, individual cells acquire the ability to detach from the primary tumor and metastasize. Disturbances in adhesion-molecule activity may play a significant role in the release of tumor cells (Takeichi, 1991). One major class of calcium-dependent homophilic cell adhesion molecules, the cadherins, have recently received considerable attention regarding their role in tumor progression. E-cadherin (uvomorulin), a trans-membrane 120-kDa protein expressed primarily in epithelial tissues, mediates early events in cellular adhesion necessary for the formation of junctional complexes, including gap junctions (Takeichi, 1991; Jonen *et al.*, 1991). Reduced E-cadherin expression may be associated with enhanced tumorigenicity (Navarro *et al.*, 1991) and invasiveness (Hehrens *et al.*, 1989).

The detection of multiple genetic defects within the cells of ovarian tumors supports the hypothesis that ovarian cancer may develop in a multistep manner (Lee *et al.*, 1990). The development of *in vitro* multistep models has been a recent approach used by investigators to study the biology of human epithelial cell carcinogenesis (Rhim *et al.*, 1990). Such models are generally based upon the *in vitro* immortalization and transformation of normal epithelial cell cultures by combinations of oncogenic transfection procedures and exposure to carcinogens (Rhim, 1992). In this study, a series of progressively transformed rat cell lines was developed to study ovarian

Ovarian cancer is the leading cause of death due to malignancy of the genital tract and the fifth most common cause of cancer death overall in American women (Borning *et al.*, 1992). A significant factor contributing to the high mortality rate is the lack of specific early symptoms manifested by the disease. Consequently, most patients are diagnosed with stage-III or stage-IV disease when metastases beyond the pelvic cavity are common and 80 to 95% of these women will die within 5 years (Peterson *et al.*, 1988). Unfortunately, this late presentation has limited our understanding of the cellular mechanisms of ovarian carcinogenesis and metastasis *in vivo*. Of the diverse cell types which comprise the ovary, all have the capacity to undergo neoplastic transformation; however, the common epithelial tumors are the most prevalent of the histologic types of ovarian tumors which have been described, accounting for 80 to 90% of all ovarian malignancies (Scully, 1979). These epithelial tumors are thought to arise from the neoplastic transformation of the ovarian surface epithelium (OSE) (Scully, 1979), a derivative of the coelomic epithelium which covers the embryonic gonadal ridge during development. Despite its importance, the number of studies investigating the *in vitro* culture of the mammalian OSE is limited (Hamilton *et al.*, 1980; Adams and Auersperg, 1981, 1985; Godwin *et al.*, 1992). While these studies have provided valuable insights into our evolving knowledge of the biology of the OSE, few have investigated the neoplastic transformation of the OSE *in vitro* (Adams and Auersperg, 1981; Godwin *et al.*, 1992; Hornby *et al.*, 1992).

Most, if not all, human and animal cancers develop in a stepwise manner from the accumulation of multiple genetic lesions within the cancerous cells, thus conferring several new phenotypic characteristics which distinguish them from normal cells (Klein and Klein, 1985). Perhaps the most serious of these is the ability of individual cells to detach from a primary tumor and spread to distant sites: the process of metastasis. It has been suggested that loss of gap junctions and other cell-

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carcinogenesis *in vivo* utilizing oncogene-mediated transformation. SV40 T-antigen (T-Ag) and *c-Ha-ras*¹ oncogenes were employed, based on the findings that T-Ag is a complete transforming protein in rodent cells, that *Ha-ras* has been implicated in the pathogenesis of ovarian cancer [Lee *et al.*, 1990], and that both have recently been employed in human epithelial cell systems to model carcinogenesis [Rhim, 1992]. Cell lines, which range from normal to malignant, were all derived from the natural target tissue for most ovarian cancers, the OSE. To address the role of cell-cell interactions in neoplastic progression of the OSE, functional GJC, cell adhesion and E-cadherin expression were analyzed. The model may prove useful in studies focusing on the mechanisms of metastatic progression during ovarian carcinogenesis.

MATERIAL AND METHODS

Cell lines and culture conditions

Tissue culture flasks were purchased from Corning (Corning, NY); culture dishes were obtained from Becton Dickinson (Houston, TX) and Lab-Tek slides were obtained from Nunc (Naperville, IL). Culture media, serum, general chemical reagents and other media additives were purchased from Sigma (St. Louis, MO), unless otherwise specified.

Primary rat ovarian surface epithelium (ROSE) cultures were isolated from normal Fisher 344 rats (28 to 42 d) utilizing the enzymatic procedure described by Hamilton *et al.* (1980). A spontaneously immortalized cell line derived from the continuous passage of a primary ROSE culture, ROSE 199, has been described [Adams and Andersperg, 1985]. COS-1, an SV40-transformed simian kidney cell line, BAlB/3T3, a continuous mouse fibroblast cell line, and T24, a human transition cell carcinoma line containing the activated *c-Ha-ras*¹ [Tabu *et al.*, 1982] oncogene were obtained from the ATCC (Rockville, MD). COS-1 and T24 were utilized as positive control cell lines for T-Ag and *ras*¹ p21 expression respectively. BAlB/3T3 was used as a negative control cell line for expression of both oncogenes.

Primary ROSE cultures were grown in Dulbecco's modified Eagle's medium nutrient mixture 1:12 (DMEM-F12) containing 15% fetal bovine serum (FBS) and supplemented with 100 IU ml⁻¹ penicillin G, 100 µg ml⁻¹ streptomycin and 0.25 µg ml⁻¹ amphotericin B (1% PSA). COS-1, BAlB/3T3, and T24 were all maintained in DMEM-F12 with 10% FBS and 1% PSA. All other cell cultures were grown in DMEM-F12 with 5% newborn calf serum (NCS) and 1% PSA. Cultures were incubated at 37°C in 5% CO₂/95% air at 100% humidity and routinely passaged 1:10 prior to reaching confluency. Living cultures were examined by phase contrast microscopy on an inverted microscope (Carl Zeiss, New York, NY) and photographed on technical pan film (Eastman Kodak, Rochester, NY).

Generation of transfected cell lines

The pSV2neo plasmid (ATCC) was generously provided by Dr. D. Bushce (Texas A&M University, College Station, TX). This plasmid is a derivative of the pSV2neo plasmid, which contains the neomycin antibiotic resistance (*neo*) gene as well as several restriction enzyme sites, into which the SV40 early-region genes have been incorporated [Southern and Berg, 1982]. The pSV2neo plasmid was transfected (via electroporation) into ROSE 199 and early passage ROSE cultures. Briefly, 10⁶ cells were routinely collected from monolayer cultures by trypsinization, transferred to a sterile cuvette, and chilled on ice for 10 min in 1 ml ice-cold Ca²⁺/Mg²⁺-free Dulbecco's phosphate buffered saline (DPBS). Twenty micrograms of plasmid were added to the cells and transfected by electroporation performed with a Transfector 300 system (Biotechnology & Experimental Research, San Diego, CA) at 300V, 200 µF. Cells expressing the *neo* and SV40 genes were

selected in media supplemented with 400 µg ml⁻¹ of the aminoglycoside antibiotic G418 (G418) (GIBCO, Grand Island, NY). Following 21 days of selection, cultures were maintained in 100 µg ml⁻¹ G418 and cloned with cloning rings.

The activated *ras* oncogene, *c-Ha-ras*¹, has been derived from the L1(T24) human bladder carcinoma cell line [Tabu *et al.*, 1982]. pSV2neo-*c-Ha-ras*¹, a pSV2neo plasmid into which this activated *c-Ha-ras*¹ oncogene has been inserted, was provided by Dr. G. Nicolson (M.D. Anderson Cancer Center, Houston, TX). The plasmid was transfected into ROSE 199 cells as described above. As a control, ROSE 199 cells were also transfected with the pSV2neo plasmid (ATCC, donated by Dr. D. Bushce) which contains no oncogenes.

Growth parameters

To assess the rate of cellular proliferation, population doubling times were determined for all cell lines. Cultures were seeded at low density (10³ cells/well) in each well (16 mm dia) of a 24-well plate. Triplicate cell counts were then determined daily for 8 days with a Coulter counter apparatus (Coulter, Hialeah, FL).

Assay for anchorage-independent growth

The potential of cells to form colonies in semi-solid medium was measured by standard procedures. Briefly, exponentially growing cells were routinely harvested with trypsin and suspended (10⁵ cells ml⁻¹) in a top agar consisting of 0.35% Bacto-agar (Lakewood Biochemicals, Irving, TX) in DMEM-F12 with 10% FBS. Top agar (2 ml) was plated onto 5 ml of solidified bottom agar, consisting of 0.75% Bacto-agar in DMEM-F12. Triplicate dishes were set up for each cell line examined. After 10 days, the dishes were examined and colonies >50 µm in diameter were counted. Cloning efficiencies were determined by the ratio: (colonies >50 µm dia/number of cells seeded) × 100%.

Tumorigenicity assays and development of tumor-derived cell lines

Congenitally athymic (*nude*) mice (Harlan, Indianapolis, IN) were injected s.c. with 10⁶ cells harvested from subconfluent monolayers suspended in DMEM-F12 without phenol red. The mice were monitored daily for evidence of palpable tumors. Cell lines were considered non-tumorigenic if they failed to form palpable tumors within 4 months after injection. Tumors which formed were allowed to grow to 1 cm diameter, at which time the animals were humanely killed; the tumors were excised, rinsed several times in DPBS and cut into small pieces. Several pieces from each tumor were fixed in 3% glutaraldehyde, 2% paraformaldehyde in 100 mM sodium cacodylate (Electron Microscopy Sciences, Fort Washington, PA), sectioned at 1 µm and stained with toluidine blue for evaluation of tumor histology. Tumor-derived cell lines were established by explant culture of the remaining tumor pieces, followed by selection with G418 (400 µg ml⁻¹). All cell lines that formed tumors in athymic mice, and the resultant tumor-derived cell lines, were similarly injected into Fisher 344 rats to assess neoplastic potential in immunocompetent hosts.

Immunocytochemistry

Primary antibody used for the indirect immunofluorescent detection of keratin intermediate filaments was purchased from Sigma (clone K8.13). Primary antibody for the detection of T-Ag was derived from a monoclonal cell line, Pab 405, a gift from Dr. V. Wilson (Texas A&M University, College Station, TX). Cell cultures were grown at low density on Lab-Tek chamber slides prior to fixation. Cultures examined for T-Ag were fixed for 10 min with 2% paraformaldehyde in 20 mM PBS, pH 7.3, washed in PBS alone, and then permeabilized with 1% Triton X-100 in PBS. Cultures examined for keratin intermediate filaments were similarly treated, except that they were fixed in 100% methanol (-20°C) and that the permeabil-

zation step was omitted. Washing and incubation steps were performed as described by Stein *et al.* (1991). Slides were mounted in glycerol with p-phenylenediamine (9.25 mM p-phenylenediamine, 90% glycerol in PBS, pH 8.0), and the samples were visualized with a Zeiss Photomicroscope III equipped for epifluorescence illumination.

SDS-PAGE and immunoblotting

Cell lines examined for the presence of ras p21 species and E-cadherin were extracted as described by Gimmlock *et al.* (1989) and Navarro *et al.* (1991) respectively. After washing the cultures with DPBS, the cells were scraped from the dish surface with a rubber policeman and centrifuged (1,800 g). The cell pellets were lysed in lysis buffer for 15 min on ice with occasional vortexing. Extracts were then centrifuged (6,000 g) for 15 min at 4°C to pellet insoluble material. The soluble protein extract (supernatant) was removed and quantitated with a modified Bradford analysis (Pierce, Rockford, IL). Extracts were mixed with an equal volume of sample buffer, boiled for 2 min and frozen at -80°C until analyzed.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein samples was conducted with a minigel apparatus (Bio-Rad, San Francisco, CA). E-cadherin and ras p21 proteins were separated on 8% and 15% gels respectively. They were then electroblotted with a Transphor electrophoresis unit (Bio-Rad) onto nitrocellulose membranes (Bio-Rad) at a constant 30 V overnight. Membranes were blocked overnight at room temperature in "blotto" (50 mM Tris-HCl, 2 mM CaCl₂, 8 mM NaCl, pH 8.0 with 5% Carnation non-fat dry milk, 0.2% NP-40 and 0.03% Na azide). The membranes were then probed for ras p21 species with membranes incubated with a broadly reactive anti-ras antibody (pan-ras (Ab-2), Oncogene Science, Uniondale, NY). Membranes which were probed for E-cadherin were first cut horizontally approximately at the level of 75 kDa, yielding high- and low-molecular-weight sections of the blots. The high-molecular-weight sections were incubated with a monoclonal antibody (DE-CMA-1, Sigma) which selectively binds E-cadherin (monomulin). The low-molecular-weight sections were probed with an anti-tubulin antibody (Ab-1, Oncogene Science) to ensure equal protein loading. After incubation with alkaline phosphatase-conjugated secondary antibody (Bio-Rad, Richmond, CA) the proteins were visualized by the use of the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Resulting bands were quantified by reflective scanning densitometry.

Assessment of functional gap junctional intercellular communication (GJIC)

GJIC was measured by fluorescence recovery after photobleaching (gap FRAP) technique (Wade *et al.*, 1986) with a Meridian ACAS 570 work station (Okemos, MI). Approximately 1×10^5 cells were plated into 35-mm dishes and incubated for 48 hr prior to analysis. The cells were washed several times with DPBS prior to labeling with $10 \mu\text{g ml}^{-1}$ 5(6)-carboxyfluorescein diacetate (CFDA) (Molecular Probes, Eugene, OR) in serum free, phenol red-free DMEM-F12 for 10 min at 37°C. The cells were then washed 3 times with the same media without CFDA, prior to gap FRAP analysis. Microscopic fields containing several adjacent cells were chosen for analysis of GJIC. An argon laser beam (488 nm) was focused through the objective lens of the inverted epifluorescent microscope onto a $1\text{-}\mu\text{m}^2$ area. Scanning parameters were optimized for maximal fluorescence detection and minimal cellular photobleaching. Selected cells were marked and photobleached with a series of laser point bleaches at sufficient intensity to reduce cellular fluorescence to 20 to 50% of pre-bleach, but without causing visible cell damage at the microscopic level. Isolated cells were bleached as negative controls; isolated cells and cells contacting others were marked

and left unbleached as positive controls. After bleaching, fluorescence recovery in the photobleached cells was monitored for 5 sequential scans beginning immediately postbleach, and then at 1 min intervals. Results of gap FRAP experiments were expressed as the average (percent) recovery of fluorescence $\pm$ standard error of the mean (SE-M). Statistical significance ($p < 0.05$) between the mean recovery rates of the ROSE cell lines was assessed with Duncan's multiple range test.

In vitro explant and migration assays

The *in vitro* migration and cellular detachment patterns of the ROSE cell lines was characterized with a bead explant assay (Stein *et al.*, 1992) and a wounding assay (Boyer *et al.*, 1989), according to previous descriptions. In the bead explant assay, Cytodex 3 microcarrier beads (Pharmacia, Piscataway, NJ), covered with cells, were suspended in culture medium before addition to 35 mm culture dishes at low density (50 to 100 beads to each of 10 dishes per cell line). Dishes were returned to the incubator and migration of cells off the beads onto the surface of the culture dish was monitored at 6, 12, 24 and 48 hr by phase-contrast microscopy. In the wounding assay, cells were plated on 35 mm culture dishes and allowed to reach confluence prior to "wounding" with a sterile 100- μl Eppendorf pipette tip to create a uniform linear cell-free area. Migration of the cells onto the acellular substrate from the wounded edge was then monitored at 12, 24 and 48 hr.

RESULTS

Growth properties of SV40 and c-H-ras^{val} transformed ROSE cell lines

Primary ROSE cultures which were enzymatically generated from juvenile Fisher 344 rat ovaries formed small colonies of relatively flat polygonal cells with defined borders after 2 or 3 days incubation, typical of primary ROSE cultures (Adams and Auerberg, 1981). Continued incubation resulted in "cobblestone-like" monolayers with few contaminating stromal cells, which were eliminated from ROSE cells at first passage as described (Adams and Auerberg, 1981). Early-passage ROSE cultures grew relatively slowly, with an approximate population doubling time of 29 hr during log-phase growth (Table I), reaching a saturation density of approximately 3×10^4 cells/cm². Indirect immunofluorescence with a broadly reactive anti-keratin antibody revealed strong perinuclear and cytoplasmic filament networks in the majority (>90%) of primary ROSE cells (data not shown), substantiating the surface-epithelial origin of the primary cultures (Hornby *et al.*, 1992).

Cells in primary culture are considered normal; however, as they develop into neoplastically transformed cells they acquire several additional properties of which immortalization is considered most important. ROSE 199 is a spontaneously immortalized cell line developed from the continuous passage of ROSE cultures which exhibits epithelial-like morphology in culture (Adams and Auerberg, 1985). In developing a multistep transformation model of the OSE, transformed cell lines were generated by incorporating viral (pSV3neo) and activated cellular (pSV2neo/c-H-ras^{val}) oncogenes into the immortalized ROSE 199 cell line.

Additionally, an early passage (p2) ROSE culture was transfected with pSV3neo. The cell lines resulting from pSV3neo transfection of ROSE 199 and ROSE were designated sv-ROSE 199 and sv-ROSE, respectively. A control cell line, neo-ROSE 199, was developed from pSV2neo transfection of ROSE 199. Sv-ROSE 199, sv-ROSE, and neo-ROSE 199 all exhibited epithelial morphology in culture (Fig. 1), closely resembling the parental cell lines from which they were derived. Incorporation of SV40 early-region genes and subsequent expression of T-Ag in the pSV3neo cell lines was

TABLE I. *IN VITRO* ANALYSIS OF LOG-PHASE GROWTH CHARACTERISTICS OF ROSE CELL LINES

Cell line ^a	Population doubling time	Cloning efficiency ^b (%)	Tumors sites in athymic mice	Fisher rats
ROSE	20.0 ± 2.8 ^c	0.0 ^d	ND ^e	ND
ROSE 199	17.1 ± 0.4 ^c	0.0 ^d	0/4	ND
neo-ROSE	17.9 ± 1.7 ^c	0.0 ^d	0/4	ND
sw-ROSE	18.0 ± 2.3 ^c	0.7 ± 0.1 ^f	4/4 (10) ^g	0/4
T-ras-ROSE	12.4 ± 0.4 ^c	9.5 ± 0.3 ^f	NA ^h	0/4
sw-ROSE 199	18.5 ± 1.2 ^c	4.9 ± 0.2 ^f	4/4 (6)	0/4
T-ras-ROSE 199	16.0 ± 1.2 ^c	10.0 ± 0.2 ^f	NA	0/4
ras-ROSE 199	8.6 ± 0.1 ^c	47.9 ± 0.2 ^f	4/4 (1)	4/4 (1)
T-ras-ROSE 199	9.0 ± 0.8 ^c	48.5 ± 0.3 ^f	NA	4/4 (1)

^aAll cell lines were analyzed at comparable passages except ROSE (first passage). Population doubling times were determined during log-phase growth and are expressed as mean hr ± SEM for triplicate cultures. Cell lines with the same letter were not significantly different ($p > 0.05$, Duncan's multiple range test). ^bCloning efficiency in soft agar = (number of clones $> 50 \mu\text{m}$ /number of cells seeded) $\times 100\%$, expressed as mean percentage ± SEM for triplicate assays. Cell lines with the same letter were not significantly different ($p > 0.05$, Duncan's multiple range test). ^cND, not done. Cell lines which failed to form tumors within 90 days in athymic mice were not tested for tumorigenicity in Fisher 344 rats. ^dNumbers in parentheses = latent period (weeks) until tumors were palpable. ^eNA, not applicable.

confirmed by immunofluorescent detection of the viral protein in 100% of the cells (data not shown). This expression appeared stable, since there were no changes in labeling uniformity or intensity in later passages (15 to 20 passages). T-Ag was not detected in neo-ROSE 199 or ROSE 199, or in ROSE lines subjected to similar immunocytochemistry (data not shown). The cell line developed from pSV2neo-c-H-ras¹² transfection of ROSE 199 was designated ras-ROSE 199. Unlike ROSE 199, this population grew in culture as randomly oriented fibroblast-like cells lacking extensive cell-cell associations (Fig. 1) and exhibited loss of contact inhibition (*i.e.*, cell piling) prior to reaching confluence. Immunoblot analysis of c-H-ras¹² expression in ras-ROSE 199 demonstrated an abundant level of the slower migrating ras¹² p21 species (Gimmerek *et al.*, 1989) in ras-ROSE 199 total cellular extracts (Fig. 2).

Population doubling times during log-phase growth of the transfected cell lines [sw-ROSE 199, ras-ROSE 199, neo-ROSE 199, and sw-ROSE], as well as the normal (ROSE) and immortalized (ROSE 199) lines, were determined (Table I). No significant difference in growth rates was detected between ROSE 199 and sw-ROSE 199, implying no effect on *in vitro* growth rate due to T-Ag expression in immortalized ROSE cells. On the other hand, stable expression of c-H-ras¹² in ROSE 199 resulted in a significant increase in growth rate. The doubling time of sw-ROSE was significantly shortened as compared with ROSE cultures. To date, sw-ROSE has undergone over 80 population doublings without exhibiting crisis or a decrease in growth rate, and requires much lower serum concentrations than ROSE cultures (5% NCS vs. 15% FHS respectively), indicating that pSV2neo is capable of immortalizing early-passage ROSE cultures.

The ROSE cell lines were evaluated for their ability to form colonies in soft-agar suspension cultures, an *in vitro* indicator of transformation (Table I). ROSE and ROSE 199 failed to form colonies after 10 days, remaining as single-cell suspensions. Stable expression of T-Ag in ROSE conferred an anchorage-independent phenotype as sw-ROSE formed colonies in soft agar; however the cloning efficiency was not significantly different from the non-transfected ROSE lines. Sw-ROSE 199 exhibited a significantly higher cloning efficiency than its parental line, thus demonstrating the ability of T-Ag

expression to confer anchorage independence on immortalized ROSE cells. After 10 days, ras-ROSE 199 exhibited a significantly greater cloning efficiency than all other lines. Neo-ROSE 199 failed to form colonies in soft agar, ruling out the possibility that the non-oncogenic components of the pSV2neo and pSV2neo-c-H-ras¹² plasmids (*i.e.*, pSV2neo) were responsible for the acquired characteristics of sw-ROSE, sw-ROSE 199 and ras-ROSE 199.

The hallmark of *in vitro* neoplastic transformation is the ability of cells to form tumors when injected into animals. The capacity of the continuous ROSE cell lines (ROSE 199, neo-ROSE 199, sw-ROSE 199, ras-ROSE 199 and sw-ROSE) to form tumors in athymic mice was investigated (Table I). While ROSE 199 and neo-ROSE 199 failed to form tumors at any of the injection sites, sw-ROSE and ras-ROSE 199 both formed palpable tumors at all sites after latent periods of 10 weeks and 6 weeks respectively. Grossly, the tumors were solid, rounded and smooth on the surface, with no obvious attachments to the skin or underlying body wall. Ras-ROSE 199 formed rapidly growing tumors which were irregular, highly vascularized and firmly adherent to the overlying skin and underlying thoracic musculature. Post mortem examination revealed no visible metastases to draining lymph nodes or internal organs secondary to any tumors. Histologically, the neoplasms formed by sw-ROSE 199 were composed of cords and large nests of tightly packed cuboidal to oval cells, with scant amounts of cytoplasm interspersed with numerous capillaries (Fig. 3). These poorly differentiated carcinomas histologically resembled ovarian serous papillary adenocarcinoma, as well as the undifferentiated ovarian carcinomas (Scully, 1979). Ras-ROSE 199 formed undifferentiated sarcomas composed of tightly packed fusiform cells with moderate amounts of cytoplasm and exhibiting abundant mitotic figures (Fig. 3). This neoplasm was capable of invading host tissue, as evidenced by numerous areas of skeletal muscle within the tumor sections. Sw-ROSE also formed poorly differentiated sarcomas, made up of whorls of fusiform cells with scant to moderate amounts of cytoplasm (Fig. 3).

Tumor-derived explant cultures were generated from each of the tumors described above, following selection in G418 (400 $\mu\text{g}/\text{ml}$) for 21 days, in an attempt to develop ROSE cell lines which were further transformed. Tumor-derived cell lines were subsequently cloned and designated T-sw-ROSE 199, T-ras-ROSE 199 and T-sw-ROSE (i.e., "tumor-derived"). Retention of T-Ag expression in the T-sw-ROSE 199 and T-sw-ROSE lines was confirmed by indirect immunofluorescence (data not shown); ras¹² p21 expression in T-ras-ROSE 199 was confirmed by immunoblotting (Fig. 2). Unlike their parental cell lines, both T-sw-ROSE 199 and T-sw-ROSE exhibited a distinct fibroblast like morphology in culture, growing in a disorganized fashion with less extensive cell-cell associations (Fig. 1). Neither cell line acquired a significantly higher growth rate as a result of tumorigenic selection; however, formation of more frequent and larger colonies in soft agar was observed (Table I). T-ras-ROSE 199 was phenotypically indistinguishable from the parental ras-ROSE 199 population, with virtually the same morphology (data not shown), growth rate and cloning efficiency in soft agar (Table I).

While tumor formation in immune-deficient rodents is a valid indicator of *in vitro* transformation, the ability of a cell line to form tumors in immunocompetent hosts is a more stringent indicator of true malignant potential. All of the cell lines which formed tumors in athymic mice, and the lines derived therefrom, were assayed for their tumorigenic capacity in syngeneic Fisher 344 rats (Table I). Ras-ROSE 199 and T-ras-ROSE 199 were the only cell lines which formed tumors, with a latent period similar to that seen in athymic mice. These tumors were grossly and histologically indistinguishable from those formed in athymic mice (data not shown).

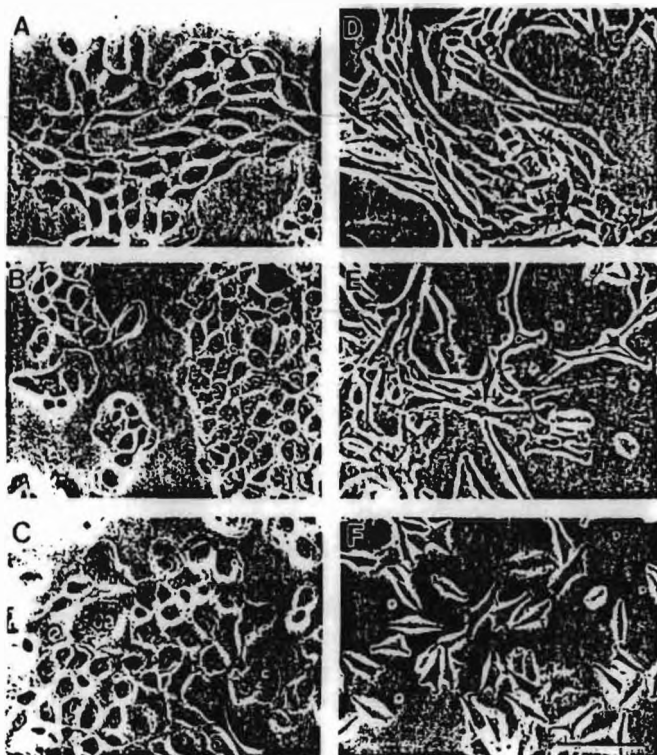


FIGURE 1 - Morphologic changes associated with progressive transformation of ROSE cell lines. Phase microscopic images of sv-ROSE 199 (a), sv-ROSE 199 (b), neo-ROSE 199 (c), Tsv-ROSE 199 (d), Tsv-ROSE 199 (e), and ras-ROSE 199 (f) cultures. c-Ha^{ras} transfection of ROSE 199 and tumorigenic selection of the SV40-transformed lines resulted in a shift in morphology from epithelial to fibroblast-like. Concomitant with this shift in morphology was an apparent reduction in cell-cell associations. Scale bar, 100 μ m.

Assessment of functional gap junctional intercellular communication (GJIC)

Gap FRAP, the ability of cells to recover carboxyfluorescein (CF) fluorescence after photobleaching via gap junctions (Wade *et al.*, 1986), was employed to study the relationship between progressive transformation of ROSE cells and perturbations in functional GJIC. GJIC in ROSE cultures was first examined to appraise recovery characteristics in the most normal cell type of those constituting the group of progressively transformed ROSE cell lines. All photobleached ROSE cells in contact with neighboring cells exhibited CF fluorescence recovery; this recovery was rapid, with most occurring in the first 1 to 2 min after bleaching (Fig. 4). The mean recovery rate at 1 min was $21.72 \pm 1.09\%$ (SEM).

The continuous ROSE cell lines were similarly subjected to gap FRAP analysis (Fig. 5). PSV3neo transformation of ROSE did not result in any appreciable reduction of GJIC, as sv-ROSE recovered at rates indistinguishable from normal ROSE cultures. The immortalized ROSE 199 line exhibited recovery rates significantly lower than ROSE cultures, averag-



FIGURE 2 - Immunoblot analysis of ras p21 expression in c-Ha^{ras} transfectant and control cell lines. Equal amounts of cellular extract from the 124 bladder carcinoma (lane 1), BALB/3T3 (lane 2), ras-ROSE 199 (lane 3), ROSE 199 (lane 4), and Tsv-ROSE 199 (lane 5) cell lines were separated (15% SDS-PAGE), electroblotted and probed with a pan-reactive anti-ras antibody.

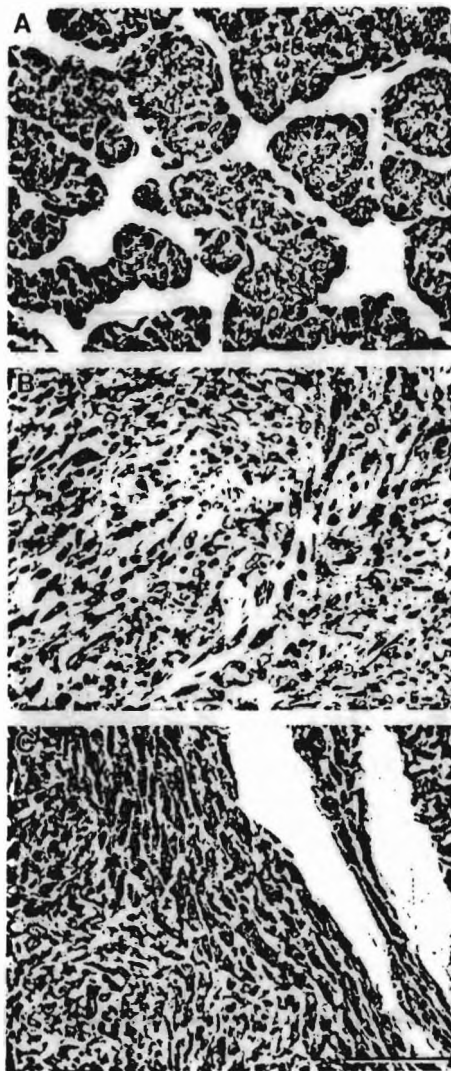


FIGURE 3. Histologic appearance of the tumors formed by sv-ROSE:199, sv-ROSE:199, and ras-ROSE:199 in athymic mice. Tumors resulting from sv-ROSE:199 (*a*) were poorly differentiated carcinomas. Both sv-ROSE:199 (*b*) and ras-ROSE:199 (*c*) formed poorly differentiated sarcomas. Note the invasion of skeletal muscle tissue exhibited by ras-ROSE:199. Scale bar, 50 μ m.

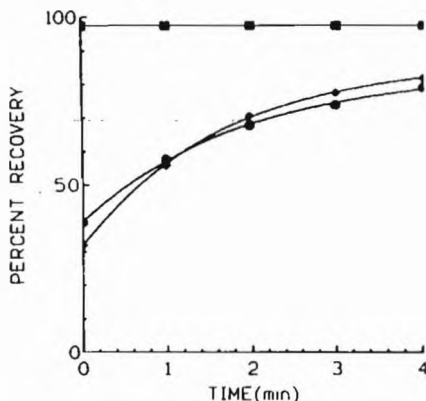


FIGURE 4. Graphic display of gap FRAP in primary ROSE cells. Primary ROSE cultures were fluorescently labeled with CFDA and subjected to gap FRAP. The recovery characteristics displayed in this figure are typical for normal ROSE cells in primary culture. Two ROSE cells (diamond and circle) were photobleached to a level of 20 to 50% of pre bleach levels. A control ROSE cell (square) was also marked and monitored over the 4-min scanning period.

ing $6.55 \pm 0.92\%$ (SEM) per minute. Similarly, pSV3neo transformation of the ROSE:199 line did not appreciably affect GJC, as sv-ROSE:199 and ROSE:199 recovered at similar rates. However, the highly transformed ras-ROSE:199 line exhibited a significantly lower rate of CF dye transfer. To determine the effect of further neoplastic progression upon GJC, gap FRAP was performed on the tumor-derived populations. Passage of the pSV3neo-transformed cell lines through athymic mice (sv-ROSE:199 $\rightarrow$ T-sv-ROSE:199 and sv-ROSE:199 $\rightarrow$ T-sv-ROSE:199) led to a significant reduction in functional communication. Similar passage of ras-ROSE:199 through athymic mice (ras-ROSE:199 $\rightarrow$ T-ras-ROSE:199) resulted in a population with lower gap FRAP rates; however, the decrease was not statistically significant ($p = 0.06$).

Effects of progressive transformation on cellular adhesion and migration characteristics *in vitro*

Necessary steps in the metastatic cascade are detachment and dissemination of individual cells from the primary tumor. Two migration assays were used to evaluate the ability of progressively transformed ROSE cells to disseminate from a primary population. In the first assay, cells were grown on spherical Cytodex beads and transferred to 35-mm culture dishes where they attached to the surface and migrated away from the primary population (Fig. 6). This assay has been used to investigate cell detachment and migration patterns in transformed granulosa cell lines (Stein *et al.*, 1992). Sv-ROSE cultures grew from the Cytodex beads as continuous epithelial sheets in a fashion identical to that exhibited by normal ROSE cultures. Similarly, both the ROSE:199 and sv-ROSE:199 cell lines migrated as continuous sheets from the cell-coated beads. In contrast, ras-ROSE:199 detached and migrated as individual cells away from the primary bead cell population. As a result of *in vitro* passage, T-sv-ROSE:199 acquired the ability to dissociate and migrate as individual cells. Much like its parental sv-ROSE line, T-sv-ROSE continued to migrate as an epithelial sheet; however, growth was more disorganized and

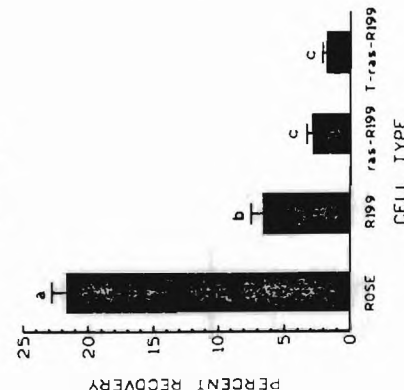
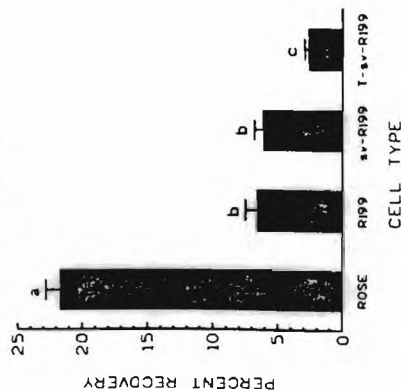
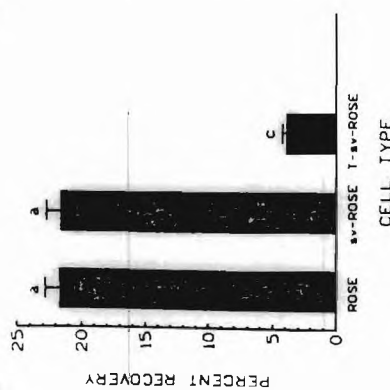


FIGURE 5—Reduction of GJC associated with progressive neoplastic transformation of ROSE. This graphic comparison of the ROSE cell lines shows that tumorigenic selection of the pSV3neo-transformed lines was associated with a reduction in GJC. Additionally, c-Ha-ras^{V600L} transformation of ROSE 199 resulted in a lower mean recovery rate. Each point and vertical bar represents the mean percent fluorescence recovery over the first minute after photobleaching $\pm$ SEM. The values are based on 36 to 51 determinations. Cell lines with the same letter were not significantly different ($p > 0.05$; Duncan's multiple range test).

individual cells were frequently noted at the periphery. T-ras-ROSE 199 displayed a pattern identical to ras-ROSE 199 (data not shown).

In a second migration assay, confluent monolayers were physically scraped with a pipette tip, creating a linear acellular area ("wound") into which cells then migrated (Fig. 6). The patterns observed in this assay were strongly supportive of the observations from the bead assay. ROSE, sv-ROSE, ROSE 199, and sv-ROSE 199 all grew onto the acellular substrate as continuous epithelial sheets. Both ras-ROSE 199 and its tumor-derived descendant T-ras-ROSE 199 grew rapidly into the wounded areas as individual cells. As with the bead assay, T-sv-ROSE 199 detached and migrated as single cells. T-sv-ROSE also exhibited dissociation of individual cells into the area of new growth.

Immunoblot analysis of E-cadherin expression

The precise mechanisms which allow cells destined for metastasis to detach from the primary tumor are not clear; however, there is growing evidence that cell-adhesion molecules may be involved. To further investigate the cell-cell adhesion changes observed in the migration assays described above, immunoblot analysis of E-cadherin expression in the cell lines of the ROSE model system was undertaken (Fig. 7).

The anti-E-cadherin antibody (DECMA-1) recognized a major protein species in both the ROSE and the ROSE 199 cell lines with an approximate molecular weight of 120 kDa, consistent with that reported for E-cadherin [Bhargava *et al.*, 1989]. This 120 kDa species was similarly expressed in both of the pSV3neo-transformed cell lines (sv-ROSE and sv-ROSE 199). Expression of E-cadherin in ras-ROSE 199 was diminished by 58% when compared with its parental ROSE 199 line. Similarly, selection for tumorigenicity altered E-cadherin expression in the transformed cell lines, since the 120 kDa species was diminished in all of the tumor-derived lines (by 56% in T-sv-ROSE, 64% in T-sv-ROSE 199 and 54% in T-ras-ROSE).

DISCUSSION

Multistep carcinogenesis is thought to proceed as a clonal phenomenon whereby a single defective cell clonally expands until some point where new sub-clones emerge and replace their predecessors due to a selective growth advantage [Klein and Klein, 1985]. Evidence is accumulating that ovarian cancer develops as a multistep process, since examination of ovarian tumor samples has revealed that several oncogenes and putative tumor-suppressor genes may act in concert to fully transform ovarian epithelial cells *in vivo* [Lee *et al.*, 1990]. To study the multistep development of cancer *in vitro*, a stepwise approach to neoplastic transformation *in vitro* is considered a reasonable strategy [Rhim *et al.*, 1990]. By means of *in vitro* oncogene-mediated transfection and *in vitro* selection procedures, a series of progressively transformed ROSE cell lines was generated to model ovarian carcinogenesis. The rationale for basing the system upon the OSE is obvious, since the majority of ovarian malignancies are thought to develop from the OSE [Scully, 1979]. Only one previous case of oncogene-

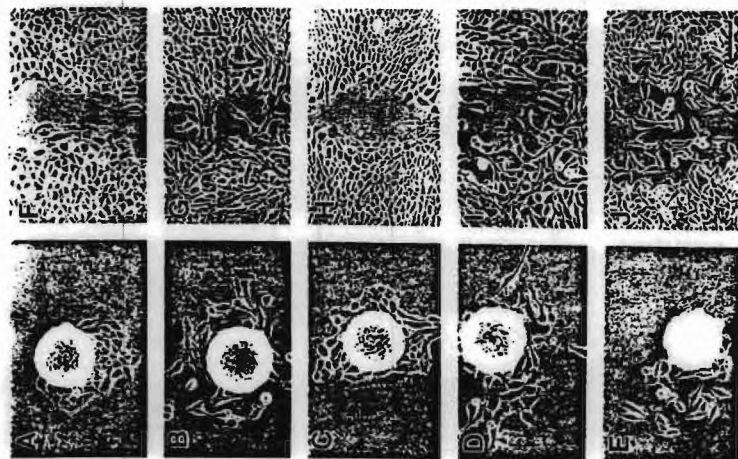


FIGURE 6—Effect of progressive neoplastic transformation on the appearance of cells migrating from cell-coated cytochalasin phase-contrast images of outgrowths from cell-coated beads 48 hr (a-d) and 24 hr (e) after plating. The right column similarly shows the appearance of cellular migration from wounded edges after 24 hr. The cell lines are: (a) ROSE, (b) T-sv-ROSE, (c) ROSE 199, (d) T-sv-ROSE 199, (e) ras-ROSE 199, and (f) T-ras-ROSE 199. It can be seen that both of the pSV3neo-transformed cell lines grow and spread as continuous epithelial sheets; however, their tumor-derived counterparts dissociated as individual cells. This pattern was also seen with ras-ROSE 199. Scale bar, 100 μ m.

mediated transformation of the OSE *in vitro* has been reported, that being via infection with an oncogenic virus (Kirsten murine sarcoma virus, Ki-MSV) [Adams and Auer-Sperg, 1981].

ROSE 199 is a spontaneously immortalized cell line derived from the continuous passage of ROSE cultures [Adams and Auer-Sperg, 1985]. This line has been characterized, and while it is epithelial in origin and morphology, it also possesses characteristics of mesenchymal cell types [Adams and Auer-Sperg, 1985]. In agreement with this earlier report, ROSE 199 cells exhibited an indefinite life span, reduced contact-inhibition and a shortened population doubling time when compared with ROSE cultures. To date, ROSE 199 has not shown any indication of spontaneous transformation *in vitro*. After 50 passages (over 110 population doublings), it is

FIGURE 7—Immunoblot analysis of E-cadherin expression in progressively transformed ROSE cell lines. The upper half of the blots were probed with an antibody against E-cadherin (1:20 kDa). The lower portions of the blots were probed for tubulin to check for equal protein loading (1:30 kDa). The lane assignments (both upper and lower blots) are: ROSE (lane 1), ROSE 199 (lane 2), sv-ROSE 199 (lane 3), ras-ROSE 199 (lane 4), sv-ROSE (lane 5), T-sv-ROSE 199 (lane 6), T-ras-ROSE 199 (lane 7), and T-sv-ROSE (lane 8). All of the bands were quantified by reflective scanning densitometry and the values are as follows: ROSE, 100; ROSE 199, 100; sv-ROSE, 100; ras-ROSE, 100; T-sv-ROSE, 100; T-ras-ROSE, 100; T-sv-ROSE 199, 0.015; sv-ROSE, 0.076; T-ras-ROSE, 0.09; T-sv-ROSE 199, 0.016; and T-sv-ROSE 199, 0.037.

morphologically unchanged and fails to form colonies in soft agar or tumors in athymic mice. The current data on ROSE 199 suggests that while it is not fully for neoplastically transformed, it has undergone pre-neoplastic changes in addition to immortalization [Adams and Auer-Sperg, 1985].

In the present study, normal ROSE cultures transfected with pSV3neo stably expressed T-Ag and exhibited several characteristics of *in vitro* transformation, including tumorigenic potential in athymic mice. Similar transformation of the immortalized ROSE 199 cell line resulted in a distinct transformed line (sv-ROSE 199) which, when compared with sv-ROSE, exhibited higher cloning efficiency in soft agar and a shortened tumor-forming latent period in athymic mice. Thus, the pSV3neo plasmid is capable of neoplastically transforming both ROSE and ROSE 199, but apparently to different degrees based upon their pre-transformation level of transformation. *In vitro* passage of the pSV3neo-transformed ROSE lines through athymic mice resulted in tumor-derived cell lines which exhibited enhanced capacity for anchorage-independent growth, yet did not form tumors in syngeneic hosts. The appearance of the tumor-derived pSV3neo lines suggested that progressive transformation of ROSE cells is accompanied by an epithelial-to-mesenchymal transition (EMT) which is thought to result from a significant change in the cellular program of differentiation [Dwyer *et al.*, 1989]. Examination of various human carcinoma cell lines has revealed an association between a shift in differentiation to the fibroblast-like phenotype and malignant progression [Frisen *et al.*, 1991]. Thus by placing additional selection pressure (growth *in vivo*) on the pSV3neo-transformed cells, new populations emerged which were further transformed.

c-Ha-ras^{V600L} transformation of ROSE 199 resulted in a dramatic shift towards malignancy. In addition to rapid growth in soft agar and in athymic mice, ras-ROSE 199 also formed tumors in syngeneic rats which readily invaded host tissues. c-Ha-ras^{V600L} transformation of ROSE 199 also resulted in a significant EMT. ROSE cultures infected with Ki-MSV (v-Ki-ras oncogene) exhibit a similar shift in morphology and are highly tumorigenic *in vitro* [Adams and Auer-Sperg, 1981]. v-Ha-ras infection of other rat epithelial cell types has yielded similar observations [De Vrijer *et al.*, 1990]. Thus, rodent epithelial cells may be particularly susceptible to transformation by the ras family of oncogenic proteins. Passage of ras-ROSE 199 through athymic mice did not confer any obvious growth advantage upon the tumor-derived population, as ras-ROSE 199 and T-ras-ROSE 199 were phenotypically indistinguishable. These results strongly suggest that the c-Ha-

ras⁺ transformed ROSE cell lines represent late stages of neoplastic progression of the OSE.

The tumors formed by sv-ROSE 199 were histologically consistent with serous adenocarcinoma of the ovary. Tumors of similar histology have been reported to develop in athymic mice inoculated with spontaneously transformed ROSE cells (Friedman *et al.*, 1992)—the ability of transformed ROSE cells to form tumors resembling human ovarian malignancies of clinical significance supports the usefulness of the *in vitro* ROSE model system. The ras-ROSE 199 population induced highly malignant sarcomas in athymic mice and normal rats. Ki-MSV-transformed ROSE cells have also been shown to form sarcomas in immunosuppressed rodents (Adams and Auerperg, 1981). The mesenchymal nature of the tumors formed by ras-transformed ROSE cells is consistent with the EMT observed in culture. As ras-ROSE 199 exhibits a mesenchymal phenotype *in vitro*, it is not surprising that it forms sarcomas *in vivo*. The tumor histology of the sv-ROSE tumors was not consistent with the assumption that transformed epithelial cells beget epithelial tumors. Sv-ROSE, which appears epithelial in culture, failed to form epithelial tumors in athymic mice. This ability of transformed ROSE cells to form tumors of mesenchymal character may be related to the embryonic mesodermal origin of the OSE. Although the OSE differentiates into epithelial tissue, it may retain the ability to revert to a more primitive mesenchymal differentiation program when progressively transformed. The plastic nature exhibited by the OSE may account for the histogenesis of the mesenchymal components often observed in ovarian tumors (Scully, 1979).

The results of the characterization experiments of the ROSE model system suggest that neoplastic transformation of the cell type responsible for the majority of ovarian malignancies may develop in a stepwise manner. Progressive *in vitro* transformation of the OSE was accomplished through oncogene-mediated transformation and *in vivo* selection procedures. When the entire series of ROSE cell lines is viewed as a progression from normality to malignancy, some degree of order becomes apparent. Based upon their growth characteristics, both *in vitro* and *in vivo*, the ROSE cell lines can be ordered as follows: normal (ROSE) → immortalization (ROSE 199) → early transformation (sv-ROSE and sv-ROSE 199) → intermediate transformation (T-sv-ROSE and T-sv-ROSE 199) → late transformation (ras-ROSE 199 and T-ras-ROSE 199). This ordering is based upon the phenotype of the ROSE cell lines and is employed to illustrate the levels of progressive transformation which was observed *in vitro*. The specific cell lines cited parenthetically are examples of the particular levels of transformation.

Although studies comparing GJIC of normal and fully transformed cells suggest that alterations in GJIC are associated with neoplastic transformation (Yamasaki, 1990; Luciwstein, 1979), information available on GJIC during the progressive stages of neoplastic development is limited (Klann *et al.*, 1989). Studies focusing on GJIC during mouse-skin carcinogenesis have demonstrated an association between neoplastic progression *in vivo* and the progressive loss of homologous GJIC (Klann *et al.*, 1989). Reduced GJIC has also been reported during progressive transformation *in vitro* (Stein *et al.*, 1991).

Primary ROSE cells were junctionally coupled in culture, as demonstrated by rapid dye transfer between contacting cells within the first few minutes after photobleaching. The rate of dye transfer between coupled ROSE cells was comparable to rates reported for other highly coupled cells (Stein *et al.*, 1991). The rate of dye coupling in sv-ROSE was not significantly different from that observed in ROSE cultures, indicating retention of a high level of GJIC. Similarly, dye coupling in

sv-ROSE 199 was not significantly different from that measured in ROSE 199 cells. In a recent report, pSV3neo transformation of primary rat granulosa cell cultures was not associated with any significant reductions in GJIC in the transformed populations (Stein *et al.*, 1992), consistent with the observations noted with the primary ROSE populations. *In vivo* passage of both pSV3neo-transformed-ROSE cell lines through athymic mice (sv-ROSE → T-sv-ROSE and sv-ROSE 199 → T-sv-ROSE 199) resulted in cell lines which were further transformed and exhibited reduced GJIC. These results suggested that pSV3neo transformation of ROSE cells had no immediate effect on GJIC, but that further neoplastic progression was associated with a significant reduction in GJIC. Similar observations of a reduction in GJIC upon *in vivo* passage of pSV3neo-transformed cell lines have been reported (Stein *et al.*, 1991, 1992).

c-H-ras⁺ transformation of ROSE 199 induced a significant decrease in the rate of dye transfer between adjoining cells in culture. The correlation between H-ras expression in transformed cells and decreased GJIC has been reported by other investigators (De Feijter *et al.*, 1990). Passage of ras-ROSE 199 *in vivo* (ras-ROSE 199 → T-ras-ROSE 199) appeared to somewhat reduce GJIC; however, this drop was not statistically significant. Considering the low rate of dye transfer displayed by the ras-ROSE 199 population, any further reductions may be rather subtle. Passage of ras-ROSE 199 *in vivo* did not appear to further transform the resultant population appreciably, since ras-ROSE 199 and T-ras-ROSE 199 were phenotypically indistinguishable. A similar observation has been reported for c-H-ras-transformed rat liver epithelial cells: passage *in vivo*, tumor-derived populations displayed growth characteristics and GJIC similar to that before *in vivo* passage (De Feijter *et al.*, 1990). Thus, the observation that *in vivo* passage of ras-ROSE 199 did statistically decrease GJIC is not in conflict with the observations noted with sv-ROSE and sv-ROSE 199 that neoplastic progression *in vivo* was accompanied by a reduction in GJIC. Taken together, the results of the gap FRAP studies performed on ROSE cell lines strongly suggest that progressive transformation of the OSE is accompanied by a progressive decrease in GJIC and that this reduction may occur very early, before the appearance of anchorage-independence, tumorigenicity and EMT, as evidenced by the difference in GJIC between ROSE and ROSE 199.

Results of *in vitro* cell-adhesiveness assays demonstrated that, whereas sv-ROSE and sv-ROSE 199 migrated as continuous epithelial sheets, the *in vivo* selected T-sv-ROSE and T-sv-ROSE 199 lines dissociated and migrated much more as individual cells. A similar transition in growth pattern was observed with c-H-ras⁺ transformation of ROSE 199. This transition in cell adhesiveness may represent progression towards a metastatic phenotype, as a reduction in tumor-cell adhesiveness *in vivo* is thought to contribute to cellular detachment from the primary tumor, a necessary step in metastasis (Takeichi, 1991). Similar transitions of *in vitro* growth patterns from adherent epithelial morphology to dissociated fibroblast-like morphology have been observed during transformation and neoplastic progression of other epithelial cell types (Belchens *et al.*, 1989; De Feijter *et al.*, 1990; Stein *et al.*, 1992) and are thought to represent a modulation in differentiation (Boyer *et al.*, 1989; Frixen *et al.*, 1991; Takeichi, 1991).

Considerable evidence has accumulated that cellular adhesion of epithelial cells, both *in vitro* and *in vivo*, is mediated by the E-cadherin cell-adhesion molecule (Takeichi, 1991; Shimoyama *et al.*, 1989). Modulation of cadherin-mediated intercellular adhesion is hypothesized to occur during the neoplastic progression of epithelial cells *in vivo*, resulting in malignant

cells capable of detachment and metastasis [Behrens *et al.*, 1989; Takeichi, 1991]. Several studies have indeed demonstrated that reduced E-cadherin expression may play a significant role in the progression of carcinoma cells towards a malignant phenotype [Frisen *et al.*, 1991; Navarro *et al.*, 1991]. In the present study, reduction in cell adhesiveness observed during neoplastic progression of ROSE cell lines was accompanied by a reduction in immunologically detectable E-cadherin expression on blots. Taken together with the results of previous studies, the results of our study suggest that neoplastic progression of the OSE may involve modulation of E-cadherin-mediated cell adhesion.

The observation of a concomitant reduction in E-cadherin-mediated cell adhesiveness and GJIC in the ROSE system is of particular interest. Recent studies have suggested a link between cadherin-mediated cell adhesion and the formation of junctional complexes, including gap junctions, reporting that transfection of cadherin cDNA into communication-incompetent cell lines results in the formation of functional gap junctions between transfected cells [Musil *et al.*, 1990; Jongen *et al.*, 1991]. Furthermore, the formation of gap junctions induced by E-cadherin expression appeared to involve post-translational modification (phosphorylation) of the gap-junction protein to a form capable of assembling into functional junctions [Musil *et al.*, 1990]. The observation that reduced E-cadherin expression in ROSE cells is associated

with a reduction in GJIC supports the results of previous studies suggesting that specific cell-cell adhesion may modulate gap-junction formation and thus functional coupling of cells. However, GJIC may not always correlate with E-cadherin expression, since E-cadherin appeared similar both in ROSE and in ROSE 199, but GJIC was dissimilar in the 2 populations.

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DIFFERENTIAL METABOLISM AND TRAFFICKING OF SPHINGOLIPIDS IN DIFFERENTIATED VERSUS UNDIFFERENTIATED HT29 CELLS

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Trafficking and metabolism of sphingolipids were examined in undifferentiated (G⁺) and differentiated (G⁻ reversed) HT29 human colon adenocarcinoma cell lines. Metabolic experiments employing a fluorescently labeled sphingolipid precursor, 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoylceramide (C₆-NBD-ceramide) revealed that both qualitative and quantitative differences exist in sphingolipid synthesis between the 2 cell lines. One of the C₆-NBD-sphingolipids synthesized in G⁺ cells is not found in the G⁻ reversed cells. Furthermore, the ratio of the 2 main products, C₆-NBD-glucosylceramide and C₆-NBD-sphingomyelin, differs: in G⁺ cells glucosylceramide is by far the main product, whereas G⁻ reversed cells synthesize C₆-NBD-sphingomyelin in slight excess. Once established, these ratios of sphingolipids are quickly restored metabolically when distortion of the ratio is caused by experimental manipulation. This indicates that they represent a true metabolic equilibrium situation of the 2 sphingolipids in these cells, while the distinct ratios are mainly determined by the NBD-lipid pool in the plasma membrane. Preferential synthesis and transfer of glucosylceramide from its site of synthesis to the cell surface do not occur when the plasma membrane pool of glucosylceramide is selectively removed. This suggests that instantaneous replenishment via specific signalling is probably not involved as a mechanism in re-establishing perturbed lipid pools. In conjunction with observations on distinct lipid trafficking pathways of glucosylceramide in G⁺ and G⁻ reversed cells, the present metabolic studies emphasize a relation between the expression of this glycolipid and the state of differentiation of HT29 cells.
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Glycosphingolipids are involved in certain processes such as cell differentiation and transformation (Hakomori, 1981; Hannun and Bell, 1989). These processes may be accompanied by changes in glycolipid composition. Quantitatively, changes in the relative amounts of various glycolipids may occur. In a qualitative sense, a certain type of glycolipid may disappear, which can then be used as a differentiation marker. It remains to be established, however, whether these changes are to be considered as a cause or a consequence of the differentiation process.

In the present study, several aspects of glycosphingolipid trafficking and metabolism were examined in the human colon adenocarcinoma cell line HT29. From the HT29 G⁺ cell population, a well-differentiated enterocyte-like cell population defined as HT29 G⁺ can be selected by completely withholding sugars from the growth medium (Zweibaum *et al.*, 1985). When this HT29 G⁺ cell line is again supplied with glucose, another differentiated cell line, G⁻ reversed, can be obtained (Zweibaum; personal communication). As such, this cell line has been used to study various processes in relation to differentiation (Gamet *et al.*, 1991; Viallard *et al.*, 1991). We have employed the latter system to study trafficking and metabolism of (glyco)sphingolipids, and addressed the question whether differentiation of the cells could affect these processes.

In studies concerning the trafficking of lipids in relation to their metabolism, the use of fluorescently tagged lipids has proved to be of great value (Pagano and Sleight, 1985; Hockstra and Kok, 1992). We have employed C₆-NBD lipids in a number of lipid transport and metabolism studies (Kok *et al.*, 1989, 1990, 1991, 1992).

In a previous study (Kok *et al.*, 1991) we have shown that differences exist in endocytic trafficking of (C₆-NBD)-glucosylceramide between G⁺ and G⁻ reversed cells, as this glycolipid is specifically salvaged and recycled via the Golgi apparatus to the cell surface in G⁺ cells, but not in G⁻ reversed cells. The present study shows that there is also a pronounced difference in the synthesis of glucosylceramide in G⁺ versus G⁻ reversed cells, as revealed by monitoring sphingolipid metabolism in cells incubated with a fluorescent ceramide derivative (C₆-NBD-ceramide), a common sphingolipid precursor. It appears that long-term metabolic differences are reflected in short-term differences in trafficking of (NBD)-sphingolipids in the endocytic pathway.

Taken together, the results indicate that the expression and trafficking of glucosylceramide may be related to the state of cell differentiation, as, in contrast to its fate in the G⁻ reversed cells, the lipid is not only specifically sorted but also specifically metabolized in the HT29 G⁺ cell line.

MATERIAL AND METHODS

Material

1-β-D-glucosylsphingosine, cerebroside type II, N-palmitoyl-DL-dihydrocholesterol, sphingosylphosphorylcholine and D-sphingosine were purchased from Sigma (St. Louis, MO). 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoic acid was obtained from Molecular Probes (Eugene, OR). Uridine diphospho-D-[U-¹⁴C]glucose, ammonium salt (> 200 mCi/mmol) was supplied by Amersham (Aylesbury, UK).

Synthesis of C₆-NBD-labeled sphingolipids

C₆-NBD-glucosylceramide, C₆-NBD-galactosylceramide, C₆-NBD-lactosylceramide, C₆-NBD-sphingomyelin and C₆-NBD-ceramide were synthesized from C₆-NBD and 1-β-D-glucosylsphingosine, 1-β-D-galactosylsphingosine, 1-β-D-lactosylsphingosine, sphingosylphosphorylcholine and D-sphingosine, respectively, as described (Kishimoto, 1975). 1-β-D-galactosylsphingosine and 1-β-D-lactosylsphingosine were prepared (Goela *et al.*, 1987) from cerebroside (type II) and N-palmitoyl-DL-dihydrocholesterol, respectively. The C₆-NBD-lipids were quantified spectrophotometrically in a fluorometer (Perkin-Elmer-MPF-43) with an excitation wavelength of 465 nm and an emission wavelength of 530 nm, by reference to known amounts of C₆-NBD-phosphatidylcholine.

Cell culture

Monolayer cultures of HT29 cells were grown in DMEM containing 25 mM glucose, supplemented with 10% (vol/vol)

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Abbreviations: BSA, bovine serum albumin; C₆-NBD, 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl- or -hexanoic acid; DMEM, Dulbecco's modification of minimal essential medium; G⁺/G⁻, (HT29) cells growing in the presence of glucose; G⁺/G⁻ (Glc⁺/Glc⁻), (HT29) cells growing in the absence of glucose; (HPLC), (high-performance) thin-layer chromatography; FCS, fetal calf serum.

many individuals and organizations if we are to meet our ultimate goal—health for all our citizens.

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Epidemiology and Primary Prevention of Cancers of the Breast, Endometrium, and Ovary

A Brief Overview

JENNIFER L. KELSEY, PhD, AND ALICE S. WHITEMORE, PhD

ABSTRACT

Cancers of the breast, endometrium, and ovary account for 41% of incident cancers among women. Many risk factors for breast cancer have been identified, but most are associated with only modest elevations in risk. Also, of all the risk factors identified, few are likely to be affected by intervention programs. The pathogenesis of breast cancer is not well understood, but estrogen and possibly estrogen plus progesterone are likely to be etiologically involved. For endometrial cancer, a major etiologic pathway is exposure to estrogen without cyclic exposure to progesterone. Most of the established risk factors for endometrial cancer appear to affect risk at least in part through this pathway. Only a few risk factors for ovarian cancer have been identified. The two most commonly suggested etiologic mechanisms for ovarian cancer are (a) that suppression of ovulation reduces risk and (b) that suppression of pituitary gonadotropins reduces risk. Each hypothesis is consistent with some, but not all, of the data. Prospects for the primary prevention of these cancers are discouraging at present because few of the risk factors identified to date are readily subject to modification, especially for breast cancer, and the underlying etiologies of these cancers are not well understood, particularly for breast and ovarian cancers. *Ann Epidemiol* 1994;4:89-95.

KEY WORDS: Breast cancer, endometrial cancer, ovarian cancer, epidemiology, prevention.

INTRODUCTION

Cancers of the breast, corpus uteri, and ovary account for about 41% of newly diagnosed cancers and about 26% of cancer deaths among women (1). Thus, identification of risk factors in the hope that means of prevention can be suggested is of considerable societal importance. This review summarizes what currently is known of the epidemiology of cancers of these three sites, discusses the possible roles of hormones in their etiology, and briefly describes ongoing and planned randomized trials to evaluate possible preventive measures. Since far more epidemiologic research has focused on breast cancer than cancers of the other two sites, breast cancer receives the greatest emphasis. Most of the material on breast cancer epidemiology can be found in more detail in a recent issue of *Epidemiologic Reviews* (2), while much of the description of ovarian cancer epidemiology is derived from a recent series of articles by Whittemore and colleagues (3-7). With the exceptions of age and international variation, descriptive epidemiologic characteristics are not considered because of space limitations.

BREAST CANCER

Epidemiology

In the United States, breast cancer incidence rates increase steeply with age through the 40s, then continue to increase, but at a slower rate, through the rest of the life span (8). Generally accepted risk factors for breast cancer (8-16) are summarized in Table 1 according to the approximate magnitude of the relative risk associated with them (9). Most of the characteristics associated with relative risks higher than 2.0 are relatively uncommon. Also, of all these generally accepted risk factors, only obesity might be affected by intervention programs, and an effect from weight reduction is unlikely to be large.

Other attributes might be categorized as "probable" risk factors for breast cancer. Although they have been identified in several studies, either the supporting evidence is somewhat inconsistent or there is concern that unidentified confounding may account for the association. Tallness, for instance, has been associated with increased risk in many studies, but not all (16). Most studies, although there are exceptions, suggest that high parity protects against breast cancer diagnosed after the age of 50, even after taking into account the association between high parity and early age at first full-term pregnancy (15). However, multiparity may be associated with an increased risk for breast cancer diagnosed before the ages of 40 to 50 (15). A relatively short

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TABLE 1. Established risk factors for breast cancer*

Factor (reference)	High-risk group	Low-risk group
	Relative risk > 4.0	
Age (8)	≥ 50 y	< 30 y
Geographic area (8)	North America, northern Europe	Asia, Africa
Mother and sister with history of breast cancer, especially if diagnosed at an early age (9)	Yes	No
Biopsy confirmed benign proliferative breast disease with atypia (10)	Yes	No
Atypical epithelial cells in nipple aspirate fluid (11)	Yes	No fluid produced
	Relative risk of 2.1-4.0	
Nodular densities on mammogram (12)	Densities occupying < 75% of breast volume	Parenchyma composed entirely of fat
History of cancer in one breast (13)	Yes	No
Mother or sister with history of breast cancer (9)	Yes	No
Biopsy confirmed benign proliferative breast disease without atypia (10)	Yes	No
Hyperplastic epithelial cells without atypia in nipple aspirate fluid (11)	Yes	No fluid produced
Radiation to chest in moderate to high doses (14)	Yes	No
	Relative risk of 1.3-2.0	
Removal of ovaries before age 40 (15)	No	Yes
Nulliparity, breast cancer at age ≥ 40 y (15)	Yes	No
Age at first full-term pregnancy (15)	≥ 30 y	< 20 y
Age at menarche (15)	< 11 y	≥ 15 y
Age at menopause (15)	≥ 55 y	< 45 y
History of primary cancer in endometrium, ovary (13)	Yes	No
Body mass, breast cancer at age ≥ 50 y (16)	Obese	Thin
Body mass, breast cancer at age < 50 y (16)	Thin	Obese

* Adapted from Kelsey (9).

number of days between menstrual periods appears to be associated with an increased risk, although more study of this issue is needed before conclusions are reached (15). Most studies that included relatively large numbers of young women who used oral contraceptives for 10 to 15 years or longer suggest that the risk for breast cancer is slightly increased among these long-term users (17). Likewise, most of the relatively small number of studies that included sufficient numbers of women who used estrogen replacement therapy for 10 to 15 years or longer indicate that such long-term use is associated with a slightly elevated risk (18). The limited data presently available suggest that use of hormone replacement therapy with both estrogen and progestin increases risk at least as much as use of estrogen alone (19). Women who used diethylstilbestrol (DES) in the 1940s through the 1960s to prevent early termination of pregnancy appear to have an elevated risk, but the possibility that the reasons for its use were the important factor rather than the DES itself cannot be ruled out (20). Most studies indicate that alcohol consumption is associated with a modestly increased risk, but in the absence of a generally accepted causal mechanism, confounding by a hitherto unidentified risk factor cannot be excluded (21). Some evidence, but not all, suggests that lactation, particularly if it is practiced for many years as in certain Asian countries, protects

against breast cancer, independently of the association of duration of lactation with early age at first birth and multiparity (15). Most of these potential etiologic factors appear to confer only modest changes in risk, with relative risks in the high-risk group compared to the low-risk group generally less than 2.0.

Finally, exposures that might be categorized as "possible" etiologic factors, and that are probably at most weakly associated with breast cancer risk, include lack of physical activity (22), exposure to electromagnetic fields (22), and certain components of diet, such as a low intake of vitamin A and a high intake of calories during childhood and adolescence (16). Although an increased risk from a high-fat diet has been hypothesized on the basis of animal experiments and variation in incidence rates among countries, most evidence from epidemiologic studies does not support this hypothesis (16), at least in respect to a high-fat diet in adulthood. Relatively few studies have evaluated the etiologic role of these "possible" risk factors other than diet, and no firm conclusions can be reached at this time.

Possible Etiologic Pathways

The biology of breast cancer is not well understood and is undoubtedly complex, but evidence strongly indicates that

ovarian hormones are important in its pathogenesis (23). The increased risks associated with early menarche and late menopause and the protection afforded by early removal of the ovaries suggest that longer exposure to ovarian hormones increases risk. The elevation in risk from postmenopausal obesity is consistent with involvement of estrogen, since most estrogen in postmenopausal women is derived from conversion from androstenedione in adipose tissue, and the greater the amount of adipose tissue, the higher the concentration of circulating estrogen. Also, in obese individuals, less estrogen is bound to sex hormone-binding globulin and is more available to target tissue than in thinner women. Possible associations of breast cancer risk with shorter length of time between menarche and menopause, long-term use of hormone replacement therapy, long-term use of oral contraceptives, parity, and lactation also suggest hormonal involvement.

It is believed that ovarian hormones affect breast cancer risk by increasing mitotic activity, which in turn increases the likelihood that DNA damage will be converted into a stable genetic error (24). Estrogen alone induces some cell division, but estrogen and progesterone together (as during the luteal phase of the menstrual cycle) may induce more cell division. Thus, exposures associated with elevated estrogen levels, and possibly elevated levels of both estrogen and progesterone, are at present of considerable interest as potential risk factors for breast cancer. Evidence for a role of other endogenous hormones, such as prolactin and androgens, is less compelling, but further study is needed.

Heredity appears to be important in the etiology of some breast cancers, especially breast cancers diagnosed at early ages (9). As breast cancer susceptibility genes are identified with new technologies in molecular biology, the study of gene-environment interactions will be of great interest. Little is known about etiologic pathways for increased risk related to disease susceptibility genes.

ENDOMETRIAL CANCER

Epidemiology

Endometrial cancer is rare before the age of 40 years, after which incidence rates increase steeply with age until 60 to 64 years and then decrease somewhat (25). Several risk factors for endometrial cancer, shown in Table 2, are well established, and relative risks are generally higher than 2.0 (13, 25-32). Although estrogen replacement therapy without progestin increases the risk for endometrial cancer (see Table 2), risk appears not to be increased if progestin is added cyclically for 10 days or more per month to estrogen as a hormone replacement regimen (33). Use of sequential oral contraceptives (34), early age at menarche (35), and tallness (35) are probably also risk factors, while cigarette smoking is probably protective (35). Some evidence suggests

that a family history of endometrial cancer (29) and a history of diabetes increase risk (25), while late age at last pregnancy may be protective (36). However, studies of these factors have either been few (family history of cancers, late age at last pregnancy) or inconsistent (diabetes), and no conclusions can be reached at this time.

Etiologic Pathway of Several Risk Factors

Most of the risk factors identified for endometrial cancer involve exposure to estrogen with insufficient cyclic exposure to progesterone, and this explanation is generally accepted as a major etiologic pathway for the development of endometrial cancer. Unopposed estrogen increases the frequency of mitosis in the endometrial cells, while progestogens markedly reduce mitotic activity (37). All of the generally accepted factors associated with an increased risk (except pelvic irradiation and possibly pregnancy) are believed to contribute to the development of endometrial cancer at least in part through this pathway, while combination oral contraceptives and particularly exposure to depot-medroxyprogesterone acetate (DMPA) provide greater exposure to progesterone and are thereby protective. Cigarette smoking may be protective because of a possible association with lowered circulating estrogen levels (37). A late age at last pregnancy could be a marker for decreased risk; being able to become pregnant at a late age may indicate that a woman has more normal ovulatory function and thus sufficient exposure to progesterone compared to a woman who is unable to become pregnant relatively late in her reproductive life (36). Hereditary predisposition to endometrial cancer has not been well studied.

EPITHELIAL OVARIAN CANCER

Epidemiology

Cancer of the ovary has been less well studied than breast and endometrial cancers, and only a few risk factors have been identified with any degree of certainty (3). Epithelial ovarian cancer, the most common histopathologic type, is uncommon before the age of 40, after which incidence rates increase until the ages of 70 to 79 and then decrease somewhat (25). Established risk factors for epithelial ovarian cancer are listed in Table 3 (4, 13, 25, 26, 38). The familial aggregation is believed to be in part attributable to hereditary susceptibility (39, 40). A gene located to chromosome 17q21 has been found to predispose to breast and ovarian cancer in some families as well as to some early-onset breast cancers (41). Possible risk factors that have not been extensively studied or for which the evidence is inconsistent or difficult to interpret include perineal talc use (42), fertility drugs (4), galactose from lactose in milk (43), caffeine consumption (44), and a history of mumps (45), while rubal

TABLE 2. Established risk factors for endometrial cancer

Factor (reference)	High-risk group	Low-risk group
	Relative risk > 4.0	
Age (25)	60-64 y	< 40 y
Geographic area (26)	North America, northern Europe	Asia, Africa
Use of estrogen replacement therapy (27)	≥ 5 y	None
	Relative risk of 2.1-4.0	
Body mass (28)	Obese	Thin
No. of pregnancies (29)	≥ 4	0
Age at menopause (30)	≥ 55 y	< 45 y
Use of combination oral contraceptives (31)	No	≥ 4 y
Use of DES/PA (32)	No	Yes
	Relative risk of 1.3-2.0	
History of primary cancer in breast (33)	Yes	No
Associations that appear strong, from clinical observations, but are difficult to quantify exactly		
Endometrial hyperplasia (25)	Yes	No
Estrogen-producing ovarian tumors (25)	Yes	No
Stein-Leventhal syndrome (25)	Yes	No
Pelvic irradiation (25)	Yes	No

DES/PA = diethylstilbestrol/progesterone acetate

ligation and hysterectomy possibly are protective (4). Age at menarche, age at menopause, age at first birth, and estrogen replacement therapy appear to have little or no effect on risk (4).

Possible Etiologic Pathways of Some Risk Factors

Two main biologic explanations have been put forward to explain the associations between reproductive variables and risk of epithelial ovarian cancer. The first is that suppression of ovulation reduces risk. Fathalla (46) hypothesized that ovarian carcinogenesis involves some mechanical sequelae of ovulation, such as trauma or mitotic stimuli to the ovarian epithelium. This hypothesis suggests that pregnancy and oral contraceptive use protect against ovarian cancer by inhibiting ovulation. The second is that suppression of secretion of pituitary gonadotropins decreases risk. Gardner (47) and Stadel (48) hypothesized that exposure of the ovarian epithelium to persistently high circulating levels of pituitary gonadotropins increases the likelihood of malignancy. This hypothesis suggests that pregnancy and oral contraceptive use protect against ovarian cancer by inhibiting pituitary secretion of gonadotropins.

While a number of epidemiologic observations support both hypotheses, there are exceptions. Differential risk reduction associated with pregnancy and oral contraceptive use (pregnancy appearing to be the more effective, particularly in young women) (4) conflicts with the first hypothesis; this hypothesis predicts equal protection per year of pregnancy and per year of oral contraceptive use, since these conditions are equally effective in suppression of ovulation. This difference is more consistent with the second hypothesis, because the low potency oral contraceptives may be less

effective than pregnancy in inhibiting pituitary secretion of gonadotropins.

On the other hand, observations of reduced ovarian cancer risk associated with lactation conflict with the second hypothesis, because lactation increases secretion of follicle-stimulating hormone. This result supports the first hypothesis, since lactation induces partial inhibition of ovulation. Also in conflict with the gonadotropin suppression hypothesis is the absence of protection associated with estrogen replacement therapy, since conjugated estrogens suppress pituitary hormones in postmenopausal women, although not to premenopausal levels.

Some findings, including the observations that age at menarche and age at menopause are not at all or only weakly

TABLE 3. Established risk factors for epithelial ovarian cancer

Factor (reference)	High-risk group	Low-risk group
	Relative risk > 4.0	
Age (25)	70-79 y	< 40 y
Geographic area (26)	North America, northern Europe	Asia, Africa
	Relative risk of 2.1-4.0	
Family history of ovarian cancer (38)	Yes	No
No. of pregnancies (4)	0	≥ 4
Oral contraceptive use (4)	None	≥ 6 y
	Relative risk of 1.3-2.0	
Breast-feeding (4)	None	> 1 y
History of primary cancer in breast (13) or endometrium (25)	Yes	No

related to risk, are inconsistent with both hypotheses. Odds ratio attenuation resulting from errors in reporting personal characteristics may be responsible for some of these inconsistencies. Further research is needed to clarify the etiologic roles of ovulation and gonadotropin stimulation, both of which may enhance carcinogenesis in the ovarian epithelium. Identification of interactions between genetic and environmental factors will also be of great interest.

A COMPARISON OF SELECTED RISK FACTORS

The incidence rates of cancers of the breast, endometrium, and ovary in various countries around the world are relatively highly correlated (26), and the demographic characteristics of high-risk women are somewhat similar for the three cancers (white women after ages 40 to 50, never-married women, and probably Jewish women, and women of high social class) (25). In addition, multiple primary cancers involving these three sites occur somewhat more often than would be expected by chance (13). It therefore used to be thought that they might to some extent have similar etiologies. However, comparison of other risk factors indicates that there are in fact many differences. Whereas long-term use of oral contraceptives may slightly increase the risk for breast cancer, use of combination oral contraceptives decreases the risk of endometrial and ovarian cancers. While use of estrogen replacement therapy strongly increases the risk for endometrial cancer, long-term use is probably associated with only a slightly increased risk for breast cancer and no alteration in risk for ovarian cancer. Although data are limited at this time, hormone replacement therapy with both estrogen and progesterin is probably associated with an increased risk of breast cancer, but no alteration of risk for endometrial cancer. After the age of 50 years, obesity is associated with a modestly increased risk for breast cancer, a more marked increased risk for endometrial cancer, and no alteration in risk for ovarian cancer. Below the age of 50 obesity appears to be protective against breast cancer, increases the risk for endometrial cancer, and has no effect on ovarian cancer risk.

Thus, in formulating ideas for preventive measures, care must be taken that while attempting to reduce the frequency of one of these cancers, the incidence of another is not increased. Also, preventive measures for one of these cancers could, of course, have an adverse effect on other body organs.

PROSPECTS FOR PRIMARY PREVENTION

Prospects for primary prevention of these cancers are discouraging at present. Most attention has focused on the prevention of breast cancer. As noted above, of all the

established risk factors for breast cancer, only postmenopausal obesity might be amenable to intervention efforts, and it is associated with only a modest elevation in risk. If such exposures as long-term use of hormone replacement therapy, long-term use of oral contraceptives, and alcohol consumption are firmly established as risk factors, then some preventive efforts may be applied, although other adverse and beneficial effects of these agents, including those related to risk of endometrial and ovarian cancer, will need to be considered. Use of oral contraceptives and of hormone replacement therapy that contains progesterin as well as estrogen could reduce the incidence of endometrial cancer compared to the incidence when replacement estrogen alone is used, but use of such a combined regimen could adversely affect risk for other diseases, such as breast cancer and coronary heart disease. Sufficiently little is known of the epidemiology of ovarian cancer that it is difficult to suggest many preventive measures; use of oral contraceptives provides some protection and could be recommended for women at very high risk.

Despite the apparent lack of primary prevention measures suggested by analytic epidemiologic studies, three primary prevention trials in which breast cancer is a major end point are now underway or are about to start in the United States. The randomized trial component of the Women's Health Initiative will have as its primary objective to determine whether a low-fat diet, hormone replacement therapy, and a calcium/vitamin D regimen in adulthood affect the risk for coronary heart disease, breast cancer, colon cancer, and hip fracture. However, observational epidemiologic studies provide little evidence that a high-fat diet in adulthood increases the risk for breast cancer, so it is unlikely that a low-fat diet in adulthood will decrease risk. Pharmacologic interventions also are being tried, in part because of the lack of feasible methods of primary prevention by environmental or behavioral change. A large randomized trial, the Breast Cancer Prevention Trial, has already begun in the United States and Canada; in this trial the chemotherapeutic agent tamoxifen, which has been shown to reduce the frequency of contralateral breast cancer and to increase the length of disease-free survival in women with cancer in one breast, is being administered to apparently healthy women 35 years and older, exclusive of women at very low risk of breast cancer. There is some evidence that tamoxifen also may favorably affect mortality from coronary heart disease and loss of bone mass in the vertebrae (but not the forearm) of postmenopausal women with breast cancer. However, little is known of the effects of tamoxifen in healthy women; potential side effects of concern include such rare but serious complications as thromboembolism, endometrial cancer, liver toxicity, and ocular changes, and such common but less serious side effects as hot flashes, vaginal dryness, and irregular menstrual periods (49). Also, there is evidence that tamoxifen may be associated with a

loss of bone mass in premenopausal women, and there is concern that its effect on cardiovascular disease risk in premenopausal women, in whom endogenous estrogen levels are high, could be different from its effect in postmenopausal women, in whom endogenous estrogen levels are low.

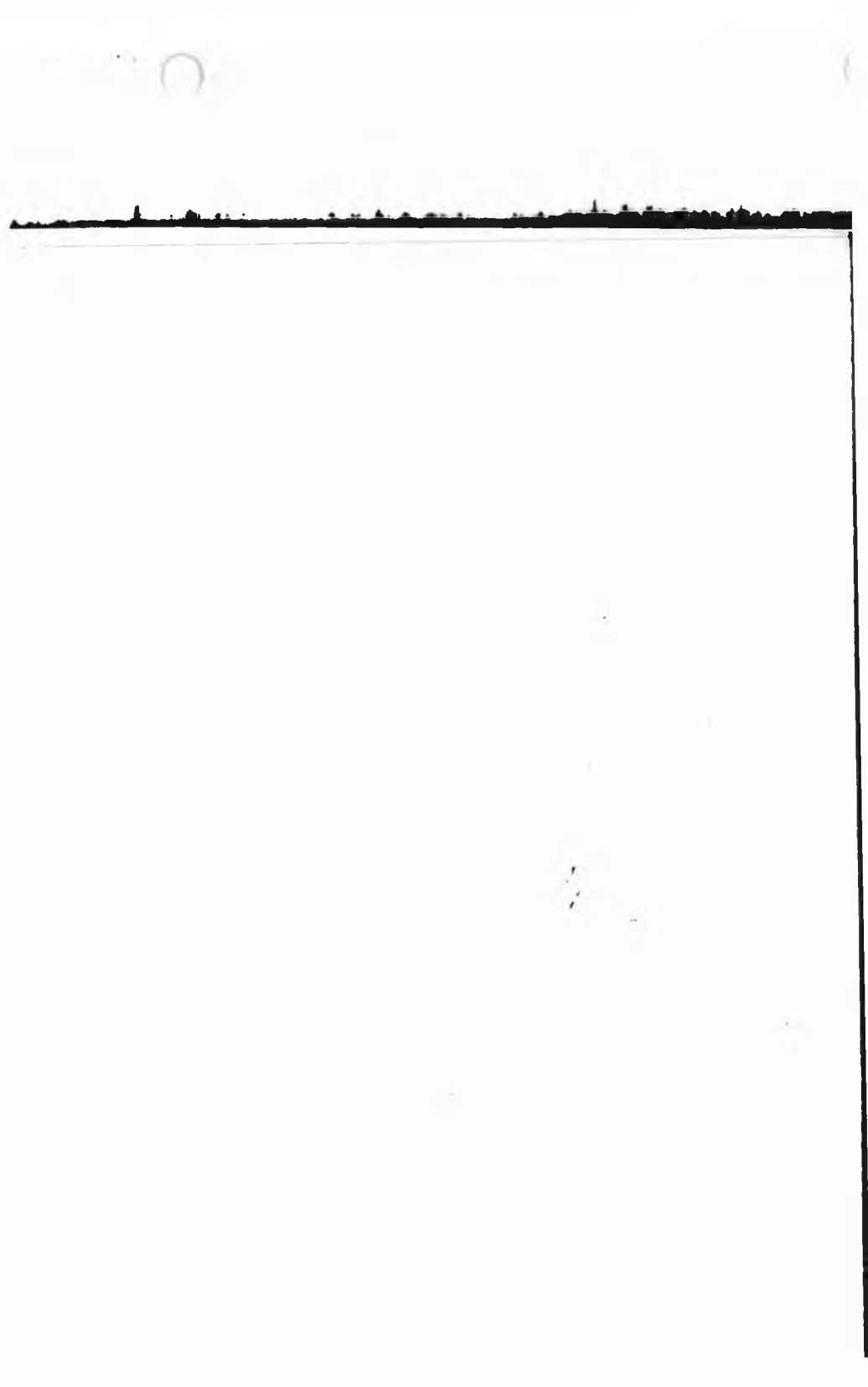
Finally, a small randomized trial in Los Angeles (50) is evaluating a prototype hormonal contraceptive regimen that contains (1) a gonadotropin-releasing agonist to suppress ovulation, (b) a low-dose estrogen, and (c) a short course of progestogen every fourth month, in the hope that this combination of agents will decrease the risk for breast, endometrial, and ovarian cancers. This formulation is based on the assumptions that the "unopposed estrogen hypothesis" accounts for endometrial cancer risk factors, the total frequency of ovulation is an important determinant of ovarian cancer risk, and an "estrogen-plus-progestogen hypothesis" explains breast cancer risk. However, with the exception of the "unopposed estrogen hypothesis," there is considerable uncertainty about the other etiologic pathways, and also about the effects of this regimen on other body systems, such as bone and the cardiovascular system.

In summary, for the majority of women there is not much cause for optimism at present for primary prevention of these cancers by modification of the environment or lifestyle, except for weight loss in postmenopausal women. Primary preventions by pharmacologic agents are being evaluated, but such interventions are risky in view of their largely unknown mechanisms of action and unknown effects on other body systems.

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Dietary Factors and the Risk of Epithelial Ovarian Cancer^{1,2}

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ABSTRACT—Dietary factors in the etiology of ovarian cancer were investigated with the use of data from a case-control study conducted in Northern Italy on 455 histologically confirmed epithelial carcinomas and 1,385 control subjects in the hospital for acute conditions unrelated to any of the known or potential risk factors for cancer of the ovary. Women with ovarian cancer reported significantly elevated frequency of consumption of meat (relative risk (RR) = 1.8 for ≥ 7 vs. < 4 portions/wk), ham (RR = 1.9 for ≥ 4 vs. < 2 portions/wk), and higher subjective scores of fat intake (RR = 2.1 for highest vs. lowest scores), particularly butter. In contrast, consumptions of fish, green vegetables, carrots, and wholemeal bread or pasta were less frequent in cases; the corresponding risk estimates for highest versus lowest frequencies ranged between 0.6 and 0.8. These results were not materially modified by adjustment for indicators of socioeconomic status, parity, and other identified determinants of ovarian cancer. No relation emerged between alcohol consumption and ovarian cancer risk. The present study provides interesting indications that help to explain the considerable variations in ovarian cancer rates in different populations and, if confirmed, could, in principle, have important public health implications. Due caution, however, is required in interpreting the present results because of the limitations of available information and of the uncertainties of other published material concerning diet and ovarian cancer.—*JNCI* 1987; 79:663-669

There are considerable variations in incidence and mortality rates from cancer of the ovary. On an international scale, the cumulative incidence was sixfold higher in Denmark than in Japan (1), and also within Italy mortality rates were about 2.5 times higher in Northern areas than in the South (2, 3). Therefore, the North-South gradient in Italy was greater for ovarian cancer than for any other neoplasm in females (2). These broad variations can be explained in part in terms of reproductive variables; for instance, ovarian cancer mortality rates in various Italian regions were strongly and negatively related with the average number of births per woman of reproductive age ($r = -0.76$) (4). Strong correlations, however, were evident also with economic indicators and dietary variables, on an international (5) and Italian scale (i.e., -0.81 for bread, -0.84 for pasta, $+0.62$ for meat, and $+0.79$ for milk consumption), which could not be completely accounted for by confounding for reproductive variables after computation of first-order partial correlation coefficients (4).

Analysis of geographical variations in cancer rates or correlational studies can only provide suggestive hypotheses on underlying causes, which should be thoroughly tested in ad hoc studies. A few case-control studies conducted in North America reported that animal fat consumption increased the risk (6), whereas beta-carotene (7) or elevated alcohol (8) consumption

might provide some protection against ovarian carcinogenesis. However, available evidence is rather controversial, and the results from the dataset analyzed more in detail (from the Roswell Park Memorial Institute) were largely inconclusive (7).

Using data from a case-control study conducted in Northern Italy, we decided, therefore, to reconsider the potential role of a few selected dietary items or indicators in the etiology of epithelial ovarian cancer.

SUBJECTS AND METHODS

Since 1979, we have been conducting a case-control study of ovarian cancer. The general design and methodology of the study have been previously described (9).

Trained interviewers identified and questioned women admitted for ovarian cancer and for a wide spectrum of other conditions to university and general hospitals in the areas under surveillance. On the average, less than 2% of the eligible women (cases and controls) refused to be interviewed.

A standard questionnaire was used to obtain information on sociodemographic characteristics and habits; medical histories with special emphasis on hormonal and gynecologic features; obstetric data, including age at menarche and at menopause and menstrual pattern; number of births, miscarriages, and induced abortions, age at first pregnancy, at first and last birth; history of

ABBREVIATIONS USED: CI = confidence interval; RR = relative risk.

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TABLE 1.—Distribution of 455 cases of epithelial ovarian cancer and 1,385 controls according to age, Milan, Italy, 1983-86

Age group, yr	Ovarian cancer		Controls	
	No.	Percent	No.	Percent
< 30	14	3.1	63	4.5
30-39	25	5.5	182	13.1
40-49	104	22.9	271	19.6
50-59	154	33.8	347	25.1
60-69	111	24.4	353	25.5
70-74	47	10.3	169	12.2

lifetime use of oral contraceptives and female hormones for other indications.

From January 1983, the subjects were also asked about their frequency of consumption per week of ten selected food items, including the major sources in the Italian diet of retinoids (milk and dairy products, meat, fish, liver, and eggs), and carotenoids (carrots, green vegetables, and fruit). Subjective scores (low, intermediate, and high) were used to obtain information on fat intake (butter, margarine, and oil) and on consumption of wholemeal bread or pasta.

Thus the present report is based on data collected between January 1983 and June 1986.

Cases.—The cases studied were women below the age of 75 with histologically confirmed epithelial ovarian cancer diagnosed within the year preceding the interview, who had been admitted to the Obstetrics and Gynecology Clinics of the University, the National Cancer Institute, and the Ospedale Maggiore of Milan (which includes the four largest teaching and general hospitals in the metropolitan area). A total of 455 cases, aged 22-74 (median age = 55 yr) were included. Among them, 84% had serous carcinomas; 3%, endometrioid; 5%, mucinous; 3%, clear cell; and 5%, poorly differentiated epithelial carcinomas.

Controls.—The controls were women admitted for acute conditions to several specialized University Clinics and to the Ospedale Maggiore of Milan for diseases other than malignant, hormonal, or gynecologic, diag-

nosed within the year before the interview. The catchment area of cases and controls was well comparable, since over 85% of the subjects interviewed resided in the same region—Lombardy. After exclusion of women who had undergone oophorectomy, a total of 1,385 controls, aged 21-74, were considered in the present analyses. Their median age was 54 years, and their age distribution was well comparable with that of the cases (table 1). Among the control subjects, 31% were admitted for traumatic conditions (mostly fractures and sprains), 25% had non-traumatic orthopedic disorders (mostly low back pain and disk disorders), 16% were admitted for acute surgical conditions (mostly abdominal, such as acute appendicitis or strangulated hernia), and 28% had other illnesses, such as ear, nose, and throat or dental disorders.

Data analysis.—We computed the mean frequencies of consumption of available dietary items among cases and controls, together with their standard errors. For the food items showing significant differences between cases and controls, we calculated the RRs of ovarian cancer, together with their 95% approximate CIs, according to three levels of consumption including, as far as possible, comparable numbers of cases and controls combined (i.e., approximate tertiles).

An index of fat intake was simply derived by adding the scores (low = 1; intermediate = 2; high = 3) of self-reported intake of the three major types of dietary fat (butter, margarine, and oil).

Indices of retinol and beta-carotene (pro-vitamin A) intake were computed with the use of tables of nutrient values of the Italian Department of Agriculture (10) or, in the absence of Italian data, standard American tables (11). These indices should clearly be interpreted as relative estimates of vitamin A intake, since the limited dietary information available did not cover all the possible sources of vitamin A.

In the computation of RRs, decade of age was controlled for by stratification and the Mantel-Haenszel procedure (12). For multiple levels of exposure, significance was assessed by means of the linear trend test given by Mantel (13). To allow simultaneously for the effects of several potential confounding factors, we used

TABLE 2.—Average frequencies of intake of selected food items among 455 cases of epithelial ovarian cancer and 1,385 controls, Milan, Italy, 1983-86

Food item	Average frequency of intake, portions per week		Difference		P value*
	Cases	Controls	Cases - controls	SE	
Milk	6.0	6.2	-0.2	0.32	NS
Meat	5.4	5.0	+0.4	0.13	<.001
Liver	0.5	0.6	-0.1	0.06	NS
Carrots	1.2	1.5	-0.3	0.10	<.001
Green vegetables	7.8	8.5	-0.7	0.22	<.001
Fresh fruit	12.1	11.9	+0.2	0.78	NS
Eggs	1.8	2.0	-0.2	0.13	NS
Ham	2.5	2.3	+0.2	0.10	<.05
Fish	0.9	1.1	-0.2	0.09	<.05
Cheese	4.8	5.1	-0.3	0.16	NS

*NS = not significant.

multiple logistic regression, with maximum likelihood fitting (14). Included in the regression equations, besides the dietary factors considered, were terms for age (in cardinal form), interviewer, marital status, social class, education, parity, age at first birth, age at menarche, menopausal status, age at menopause, body mass index, and oral contraceptive and other female hormone use.

RESULTS

The average weekly frequencies of consumption of available dietary items are reported in table 2. Consumption of milk, cheese, liver, eggs, and fresh fruit did not significantly differ for cases and controls but although differences in absolute terms were small (e.g., 5.4

TABLE 3.—Distribution of 655 cases of epithelial ovarian cancer and 1,386 controls according to frequencies of consumption of selected food items, Milan, Italy, 1983-86

Food item and portions per week	Ovarian cancer		Controls ^a		RR estimates (95% CI)	
	No.	(%)	No.	(%)	M-H ^b	Multivariate ^c
Meat						
<4	114	(25.1)	455	(32.9)	1 ^d	1 ^d
4-6	131	(28.8)	409	(29.5)	1.30 (0.98-1.73)	1.30 (0.96-1.85)
≥7	210	(46.2)	521	(37.6)	1.63 (1.25-2.12)	1.60 (1.21-2.12)
χ^2 (trend)					12.93 (<i>P</i> < .001)	15.75 (<i>P</i> < .001)
Ham						
<2	136	(29.9)	487	(35.2)	1 ^d	1 ^d
2-3	227	(49.9)	706	(51.0)	1.17 (0.91-1.49)	1.13 (0.87-1.46)
≥4	92	(20.2)	192	(13.9)	1.85 (1.35-2.53)	1.55 (1.11-2.16)
χ^2 (trend)					12.00 (<i>P</i> < .001)	4.67 (<i>P</i> = .03)
Fish						
<1	220	(48.4)	591	(42.7)	1 ^d	1 ^d
1	162	(35.6)	496	(35.8)	0.87 (0.69-1.10)	0.94 (0.73-1.21)
≥2	73	(16.0)	298	(21.5)	0.66 (0.43-0.90)	0.84 (0.61-1.16)
χ^2 (trend)					6.68 (<i>P</i> = .01)	0.69 (NS)
Carrots						
<1	197	(43.3)	557	(40.2)	1 ^d	1 ^d
1	129	(28.4)	319	(23.0)	1.11 (0.86-1.45)	1.19 (0.90-1.58)
≥2	129	(28.4)	508	(36.7)	0.70 (0.55-0.91)	0.81 (0.62-1.06)
χ^2 (trend)					6.41 (<i>P</i> = .01)	2.29 (NS)
Green vegetables						
<7	125	(27.5)	342	(24.7)	1 ^d	1 ^d
7	185	(40.7)	419	(30.3)	1.18 (0.90-1.55)	1.10 (0.83-1.47)
≥8	145	(31.9)	624	(45.1)	0.69 (0.45-0.78)	0.61 (0.46-0.82)
χ^2 (trend)					17.01 (<i>P</i> < .001)	18.99 (<i>P</i> < .001)
Wholegrain bread or pasta						
Never	886	(78.8)	954	(69.1)	1 ^d	1 ^d
Occasionally	82	(18.0)	258	(18.7)	0.88 (0.66-1.16)	0.95 (0.71-1.27)
Frequently	37	(8.1)	169	(12.2)	0.60 (0.41-0.88)	0.63 (0.42-0.93)
χ^2 (trend)					6.62 (<i>P</i> = .01)	4.45 (<i>P</i> = .03)

^aIn some items, the sum of the strata does not add up to the total because of missing values.

^bMantel-Haenszel estimates adjusted for age in decades.

^cEstimates from multiple logistic regression equations including terms for age, interviewer, marital status, social class, education, parity, age at first birth, age at menarche, menopausal status, age at menopause, body mass index, oral contraceptive and other female hormone use, retinol and carotene indices, added score of fat consumption and alcohol intake, besides the food items considered in this table. NS = not significant.

^dReference category.

vs. 5 times per week for meat consumption), women with ovarian cancer reported more frequent consumption of meat and ham and less frequent consumption of fish, carrots, and green vegetables.

The corresponding RR estimates are shown in table 3. The point estimates for highest versus least frequent consumption levels were 1.63 for meat, 1.85 for ham, 0.66 for fish, 0.70 for carrots, and 0.59 for green vegetables, and all the age-adjusted tests for linear trend in risk were statistically significant. Moreover, frequent consumption of wholegrain bread or pasta was associated with significantly reduced ovarian cancer risk (RR = 0.60, 95% CI = 0.41-0.88, table 2).

Allowance for identified potential distorting factors, as well as reciprocal allowance for various dietary items included in table 3, did not materially modify the estimated RRs for meat, green vegetables, and wholegrain foods. The association with ham was less evident, and associations with fish and carrots were no longer significant after multivariate analysis.

Indices of monthly intake of retinol (preformed vitamin A) and beta-carotene were computed on the basis of the frequencies of consumption of food items listed in table 2 (milk, meat, liver, eggs, fish, and cheese for retinol; carrots, green vegetables, and fresh fruit for carotene). In the univariate analysis, no relation emerged between indices of retinol intake and ovarian cancer risk, but a significant inverse association was evident with beta-carotene intake. Compared with women whose carotene index was under 150,000 IU per month, the risk estimate was 0.62 for those whose index was over 200,000 IU, and the trend in risk was highly significant (table 4). However, after multivariate analysis with inclusion of dietary factors as well as other major identified covar-

iates, the inverse relation of ovarian cancer risk with beta-carotene was no longer evident.

In table 5, subjective scores of fat intake in cases and controls are reported. Elevated butter consumption was associated with about twice the ovarian cancer risk (RR = 2.3 univariate, 1.9 multivariate). The large majority of the subjects interviewed reported low scores of margarine consumption; consequently, little relation was evident between margarine intake and ovarian cancer risk. No association was observed with oil consumption either, even though oil was the fat item with highest intake scores. Consequently, the positive association between added fat consumption score was chiefly attributable to the influence of butter on the overall index. The multivariate RRs presented in table 5 were derived from two different models, one including the three single fat items and the other including the added index only.

The distribution of ovarian cancer cases and the comparison group according to total alcoholic beverage consumption (wine, beer, and spirits) is given in table 6. Compared to women who did not drink alcohol, the RRs were slightly above unity for drinkers, but there was no tendency for the risk to increase with higher alcohol consumption.

DISCUSSION

The findings of the present study indicate that the diet of Italian women with epithelial ovarian cancer differs in several aspects from that of a hospital-based comparison group of subjects with acute nonneoplastic conditions. In particular, more frequent meat and ham consumption and higher fat intake (in particular, butter) in

TABLE 4. Distribution of 455 cases of epithelial ovarian cancer and 1,385 controls according to estimated monthly intake of retinol and beta-carotene, Milan, Italy, 1985-86

Index	Ovarian cancer		Controls		RR estimates (95% CI)	
	No.	(%)	No.	(%)	M-H ^a	Multivariate ^b
Retinol ^c						
<100	149	(32.7)	470	(33.9)	1 ^d	1 ^d
100-150	134	(29.5)	341	(24.6)	1.23	1.18
					(0.94-1.61)	(0.89-1.57)
>150	172	(37.8)	574	(41.4)	0.96	0.94
					(0.75-1.24)	(0.72-1.22)
χ^2 (trend)					0.18	0.14
					(NS)	(NS)
Carotene ^c						
<150	215	(47.3)	508	(36.7)	1 ^d	1 ^d
150-200	109	(24.0)	377	(27.2)	0.68	0.76
					(0.52-0.88)	(0.53-1.10)
>200	131	(28.8)	500	(36.1)	0.62	0.94
					(0.48-0.80)	(0.66-1.61)
χ^2 (trend)					14.73	0.17
					(P<.001)	(NS)

^aMantel-Haenszel estimates adjusted for age in decades.

^bEstimates from multiple logistic regression. Allowance was made for identified potential distorting factors (see "Subjects and Methods" and footnote c, table 3).

^cIn thousand international units.

^dReference category.

TABLE 5.—Distribution of 455 cases of epithelial ovarian cancer and 1,385 controls according to subjective scores of fat intake, Milan, Italy, 1983-86

Type of fat and consumption score	Ovarian cancer		Controls		RR estimates (95% CI)	
	No.	(%)	No.	(%)	M-H ^a	Multivariate ^b
Butter						
Low	241	(53.0)	879	(63.5)	1 ^c	1 ^c
Intermediate	179	(39.3)	449	(32.4)	1.47 (1.17-1.84)	1.41 (1.11-1.80)
High	35	(7.7)	57	(4.1)	2.27 (1.47-3.51)	1.93 (1.20-3.11)
χ^2 (trend)					20.52 (<i>P</i> <.001)	12.61 (<i>P</i> <.001)
Margarine						
Low	370	(81.3)	1,157	(83.5)	1 ^c	1 ^c
Intermediate-high	85	(18.7)	228	(16.5)	1.16 (0.88-1.52)	1.11 (0.83-1.49)
Oil						
Low	90	(19.8)	221	(16.0)	1 ^c	1 ^c
Intermediate	291	(64.0)	1,002	(72.8)	0.73 (0.56-0.96)	0.71 (0.55-0.96)
High	74	(16.3)	182	(11.7)	1.17 (0.80-1.69)	1.29 (0.87-1.92)
χ^2 (trend)					0.21 (NS)	0.64 (NS)
Combined ^d						
Low	198	(43.5)	738	(53.3)	1 ^c	1 ^c
Intermediate	150	(33.0)	453	(33.1)	1.22 (0.96-1.55)	1.15 (0.90-1.48)
High	107	(23.5)	188	(13.6)	2.12 (1.60-2.82)	2.14 (1.59-2.88)
χ^2 (trend)					23.78 (<i>P</i> <.001)	18.63 (<i>P</i> <.001)

^aMantel-Haenszel estimates adjusted for age in decades.^bEstimates for multiple logistic regression. Allowance was made for major identified potential distorting factors. (See "Subjects and Methods" and footnote c, table 3; two equations were fitted, one including various types of fat and the other the combined score only.)^cReference category.^dComputed as the simple sum of scores (1=low; 2=intermediate; 3=high) of the various fat items and subdivided as follows: 3-4=low; 5=intermediate; 6-9=high.

seasoning were associated with elevated ovarian cancer risk, whereas more frequent vegetable consumption showed a protective effect.

We were unable to explain these findings in terms of selection bias, since cases and controls were drawn from similar catchment areas, and the participation rate was

practically complete. With regard to confounding, allowance for several identified potential distorting factors, including measures of body weight and indicators of socioeconomic status, parity, oral contraceptive use, and other known determinants of ovarian cancer risk failed to explain the observed associations with various dietary

TABLE 6.—Distribution of 455 cases of epithelial ovarian cancer and 1,385 controls according to total alcoholic beverage consumption, Milan, Italy, 1983-86

Alcoholic beverage consumption, drinks per day	Ovarian cancer		Controls		RR estimate (95% CI)	
	No.	(%)	No.	(%)	M-H ^a	Multivariate ^b
0	134	(29.5)	506	(36.5)	1 ^c	1 ^c
<2	162	(33.4)	391	(28.2)	1.45 (1.11-1.89)	1.24 (0.94-1.65)
2-3	138	(30.3)	397	(28.7)	1.31 (1.00-1.72)	1.08 (0.80-1.40)
>3	31	(6.8)	91	(6.6)	1.30 (0.82-2.04)	1.32 (0.78-2.37)
χ^2 (trend)					2.44 (NS)	0.40 (NS)

^aMantel-Haenszel estimates adjusted for age in decades.^bEstimates from multiple logistic regression. Allowance was made for identified potential distorting factors (see "Subjects and Methods" and footnote c, table 3).^cReference category.

factors, namely meat, green vegetables, wholegrain foods, and butter. Likewise, a major impact of information bias is unlikely, since the possible relation of diet with ovarian cancer risk was probably unknown to the majority of the subjects interviewed.

The results of this study are in agreement with evidence from descriptive epidemiology showing, on both an international (5) and an Italian national (4) scale, strong positive correlations between measures of fat intake and ovarian cancer mortality. In this sense, they provide useful clues to explain regional and international differences in ovarian cancer incidence and mortality (1-3).

They also confirm the findings of a study conducted in North America and utilizing population controls (6), which showed significantly higher ovarian cancer risk in women reporting more frequent consumption of butter, and an approximately twofold elevated risk estimate in the highest quartile of animal fat consumption.

The negative relation with vegetables and wholegrain bread or pasta is also of potential interest in relation to defining a low-risk diet for ovarian cancer in contrast with a high-risk one characterized by elevated meat and animal fat intake.

In this regard, data collected in an Italian population may be particularly helpful in studies of diet and cancer, on account of the considerable heterogeneity of these components in the Italian diet.

A plausible biologic explanation for the present findings can be related to the lower urinary levels of estrogens and higher plasma sex-hormone binding globulin levels in vegetarian women (15), since female hormones and their bioavailability are probably related to ovarian carcinogenesis, although at present it is difficult to define clearly the role of various hormones. Further, studies in rodents showed positive relations between high-fat diets and promotion for another female hormone-related neoplasm, i.e., breast cancer, although the relation was not homogeneous for various types of fats, olive oil (which is the most commonly used oil in Italy), and other monounsaturated fats in particular showing little promoting effect (16). This evidence from animal studies should be carefully considered in light of the several similarities in the epidemiology of breast and ovarian cancer (9, 17).

The present epidemiologic findings, if accurate, would have important public health implications, since the dietary factors analyzed are, at least in principle, modifiable, and the magnitude of the RRs in this study is similar to that of the best identified reproductive correlates of ovarian carcinogenesis (9), which are, obviously, scarcely amenable to intervention.

The results of this study, however, should be considered with caution, since available information was limited to merely subjective scores of fats or wholegrain food intake and to the number of portions per week of a restricted list of dietary items, with no assessment of their size. Consequently, major uncertainties are obviously inherent in any evaluation of specific micronutrients (18, 19), and, particularly, in the negative rela-

tion between indices of beta-carotene and ovarian cancer risk. Further, the observation that inclusion of specific food items (i.e., green vegetables, carrots, and fruits) substantially reduced the relation between beta-carotene and ovarian cancer risk suggests that little residual effect for this index was evident besides that attributable to its various components. It is thus possible that other components of a vegetable-rich diet are indeed protective in the absence of any specific protection conveyed by beta-carotene on ovarian carcinogenesis.

Further caution in drawing inferences from the findings of the present investigation is indicated by a study of dietary factors and ovarian cancer based on the Roswell Park Memorial Institute dataset. The results of that study, in fact, were largely inconclusive in relation to fats, vegetables, fibers, and also vitamin A, with the sole exception of a negative association with beta-carotene that, however, was restricted to the subgroup of women below age 50 (7).

In this study, there was no relation between alcohol consumption and ovarian cancer risk. Thus these findings do not confirm the suggestion of a lower ovarian cancer risk in heavy drinkers reported in the Centers for Disease Control's Cancer and Steroid Hormone Study and tentatively explained in terms of depressed levels of gonadotropins and hence anovulation induced by large doses of alcohol (8).

In conclusion, although the present study has substantial limitations, chiefly in terms of the restricted amount of information available, it offers interesting indications that help to explain peculiar features of the descriptive epidemiology of ovarian cancer, and, if confirmed, could have important practical public health implications.

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Frequent Loss of Heterozygosity on Chromosomes 6q, 11, and 17 in Human Ovarian Carcinomas¹

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ABSTRACT

Recently, tumor-specific allele loss has been shown to be an important characteristic of some tumors. When such loss includes one or more growth-regulatory genes, it may allow the expression of tumorigenicity. Using Southern blots, we analyzed normal and tumor DNA samples from 19 ovarian cancer patients, using a series of polymorphic DNA probes that map to a variety of chromosomal loci. Of 14 informative cases, tumor-specific allelic loss was observed in nine (64%) at the estrogen receptor (*ESR*) gene locus on chromosome 6q. On chromosome 17p at the *D17S28* and *D17S30* loci, allelic losses were also detected in 6 of 8 (75%) and 9 of 14 (64%) cases, respectively. Allelic loss at the *HRAS1* gene locus on chromosome 11p occurred in 5 of 11 (46%) informative cases. The relatively high incidence of these allelic losses observed on chromosome 6q represents the first implication by molecular genetic analysis of this chromosomal region in a human malignancy, and it thus appears to be a genetic change specific to ovarian carcinoma. DNA sequence losses on 11p and 17p, also reported for other cancers, may reflect the presence of tumor- or growth-suppressor genes on these chromosomes that are important in the genesis of many tumor types, including ovarian malignancies.

INTRODUCTION

Although ovarian carcinoma is the most frequent cause of death from gynecological malignancies in the Western World (1), the histogenesis and biological characteristics of these tumors are not well understood. Furthermore, little is known about potential molecular genetic alterations that may contribute to development of such tumors or to disease progression.

It is now well established that molecular genetic aberrations can alter the expression of genes and may play a role in malignant transformation (2). Recent studies indicate that loss or inactivation of genes at specific chromosomal loci may contribute to the development of certain childhood tumors such as retinoblastoma (3), Wilms' tumor (4), and rhabdomyosarcoma (4). It has been hypothesized that loss of heterozygosity at a specific locus may indicate loss of genetic material that suppresses the tumor phenotype. Such negative growth-regulating genes have been called "tumor-suppressor genes" or "anti-oncogenes" (5). Because these allelic losses occur not only in hereditary but also in nonhereditary tumors, it has been suggested that negative growth-regulating genes are commonly lost in adult malignancies (6-12). It has been further suggested that the inactivation of a gene is achieved more readily than its activation through specific mutations. Consequently, the loss of an important negative growth-regulating gene may occur even more frequently during tumorigenesis than activation of a positive growth-regulating gene (13).

Even though the identification of consistent chromosomal

aberrations in fresh ovarian tumors has been very limited, nonrandom structural or numerical aberrations have been reported for several different chromosomes (14-17). We, therefore, analyzed 19 fresh ovarian cancers for possible tumor-specific allelic loss at 18 different chromosomal loci. Because of cytogenetic reports of 6q deletions in this disease, we were particularly interested in the 6q region, as well as in other chromosomal regions that frequently show allelic loss in other tumors.

MATERIALS AND METHODS

Patients and Tissues. Primary and metastatic malignant ovarian tumors from a total of 19 patients at the M. D. Anderson Cancer Center (Houston, TX) were obtained from surgical pathology immediately after surgery. The fresh solid tissues were dissected free of fat and necrotic tissue. The specimens were stored at -70°C until DNA was extracted. The histological diagnosis of the tumors studied was established by the Department of Pathology, from paraffin-embedded sections. The histological grading was performed according to a system described by Mauch *et al.* (18). Peripheral blood samples were obtained from the same patients from whom the tumor tissues were procured. Stages of disease were assigned according to the classification scheme accepted by the general assembly of International Federation of Gynecology and Obstetrics (19).

Preparation of High Molecular Weight DNA. Tumor tissue, pulverized in liquid nitrogen as described (20), and lymphocytes from peripheral blood samples were lysed in EDTA/sodium dodecyl sulfate solution, proteinase K digested, phenol/chloroform extracted, ethanol precipitated, resuspended in sterile water, and spectrophotometrically quantified as previously described (20).

Allelic Loss Analysis. Fifteen μ g of high molecular weight DNA were digested to completion with the appropriate restriction endonuclease as recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, MD), size fractionated in a 0.8-1.2% agarose gel, denatured, neutralized, and transferred to a nylon filter (21). Hybridization to oligo-primed ³²P-labeled (22) probes was conducted at 42°C for 48-72 h. The filters were then washed at 60°C for 60 min in 0.1× saline sodium citrate (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate and autoradiographed at -70°C using intensifying screens. The following probes and restriction enzymes were used: chromosome 1, pAT3 (*Pst*I) (23); chromosome 2, pYNZ15 (*Taq*I) (24) and IMR-6 (*Eco*RI) (25); chromosome 5, pHGR (*Bcl*I) (26); chromosome 6, pHHH157 (*Bam*HI) (27), pHM2.6 (*Eco*RI) (28), and pOR3 (*Pvu*II) (29); chromosome 7, HER-A64-1 and HER-A64-3 (*Hind*III) (30); chromosome 11, c-Ha-ras1 (*Bam*HI) (31), pHINS300 (*Eco*RI) (32), and SS6 (*Bam*HI) (33); chromosome 12, pSW480-cDNA-5 (*Taq*I) (34); chromosome 17, pYNH37.3 (*Taq*I) (35), pYNZ22 (*Msp*I or *Bam*HI) (36), and cEFD52 (*Pvu*II) (37); chromosome 19, pHP450 (*Sac*I) (38); and chromosome 22, L335 (*Hind*III) (39).

Patients' constitutional DNA samples that were informative, i.e., heterozygous, for a given marker displayed restriction fragments corresponding to each of two alleles in the resultant autoradiograph. Allelic loss from the matched tumor DNA was indicated by the disappearance of restriction fragments specific for either allele.

RESULTS

Loss of heterozygosity occurred frequently at several chromosomal loci in ovarian carcinomas. We examined a total of

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²To whom requests for reprints should be addressed, at Molecular Analysis Incorporated, 8000 El Rio, Houston, TX 77054.

18 loci from 10 different chromosomes. Representative results are displayed in Figs. 1-3. Loss of heterozygosity was observed at loci on seven different chromosomes: chromosomes 2, 6, 7, 11, 17, 19, and 22. The frequency of allele loss at individual loci ranged from one of eight (13%) at the *MYB* locus on

chromosome 6q to six of eight (75%) at the *D17S28* locus on chromosome 17p (Table 1). Loss of heterozygosity at one or more loci was detected in 75% (14 of 19) of ovarian carcinomas. The incidence of loss of heterozygosity was remarkably high on chromosomes 6q (64% at the *ESR* gene locus), 17p (75 and 64% at the *D17S28* and *D17S30* loci, respectively), and 11p (46% at the *HRAS1* gene locus).

Allelic Losses on Chromosome 6. To characterize allelic loss on chromosome 6, we used three different polymorphic DNA probes: one for the short arm (*D6S29* locus) and two for the long arm (*MYB* and *ESR* loci). Loss of heterozygosity occurred at high frequency in the terminal region of the long arm of chromosome 6, the area to which the *ESR* gene maps (Table 1 and Fig. 3). The frequencies of loss of heterozygosity were similar in both primary tumors (63%) and metastatic lesions (67%) at the *ESR* locus. Of six individuals heterozygous at both the *ESR* and *MYB* loci, five (83%) showed an allelic loss at only the *ESR* locus. A polymorphic DNA probe (pHHH157) for the *D6S29* locus on the short arm detected nine informative individuals, none of whom revealed an allelic loss.

Allelic Losses on Chromosome 17. On chromosome 17p at the *D17S28* and *D17S30* loci, loss of an allele was observed in 6 of 8 (75%) informative individuals and in 9 of 14 (64%) informative patients, respectively (Table 1 and Fig. 2). Among 14 samples that showed loss of heterozygosity on the short arm of chromosome 17 (*D17S28* or *D17S30*) and that also were informative at the *D17S26* locus on the long arm, six (43%) showed concurrent allelic losses. Of eight samples from informative individuals who revealed *ESR* gene allelic loss and also were informative at a chromosome 17p locus (*D17S28* or *D17S30*), five (63%) showed simultaneous allelic losses on both chromosomes.

Allelic Losses on Chromosome 11p. Among the 19 normal/tumor DNA sample pairs we examined, 11 (58%) individuals were heterozygous and thus informative. In 5 of these 11 (46%) cases, loss of heterozygosity occurred. The fact that we did not observe any allelic loss at the *INT2* locus (on 11q) suggests that the *HRAS1* allelic loss was largely due to subchromosomal structural changes.

Allelic Losses on Other Chromosomes. To determine whether these restriction fragment length polymorphism allelic losses were specific for chromosomes 6q, 11p, and 17p in ovarian

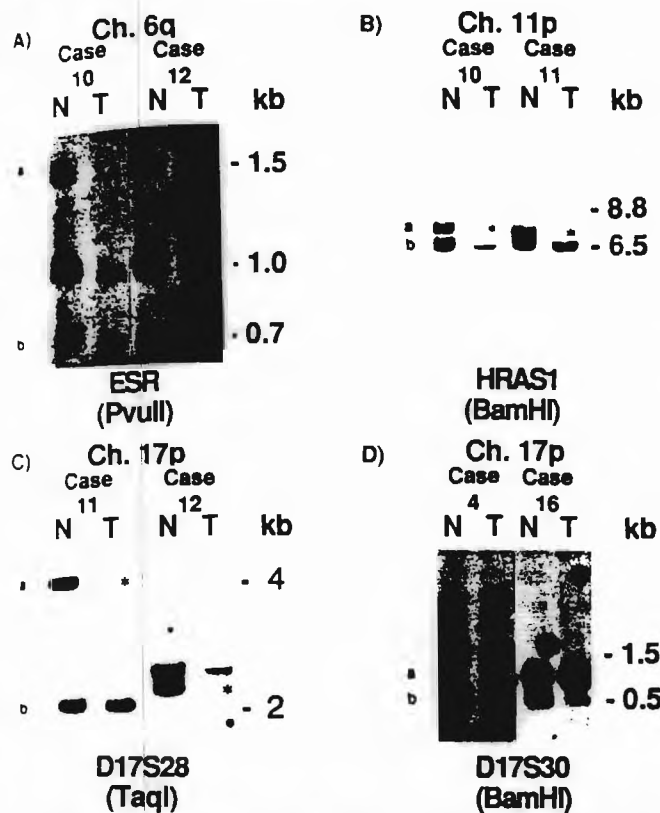


Fig. 1. Southern blot analysis demonstrating loss of heterozygosity on chromosomes 6q (A, cases 10 and 12), 11p (B, cases 10 and 11), and 17p (C, cases 11 and 12; D, cases 4 and 16) in patients with ovarian carcinomas. DNA was isolated from tumors (T) and corresponding normal lymphocytes (N). The loci analyzed and restriction endonucleases used are indicated under each panel, *ESR* (*PvuII*), *HRAS1* (*BamHI*), *D17S28* (*TaqI*), and *D17S30* (*MspI* or *BamHI*), respectively. Allelic forms are designated to the left of each panel: a is the longer and b the shorter fragment. The size in kilobases of each allele is given to the right of each panel. *, the allelic fragment lost in tumor DNA sample in each panel. In this figure, cases 10, 11, and 12 show loss of alleles on more than one chromosome.

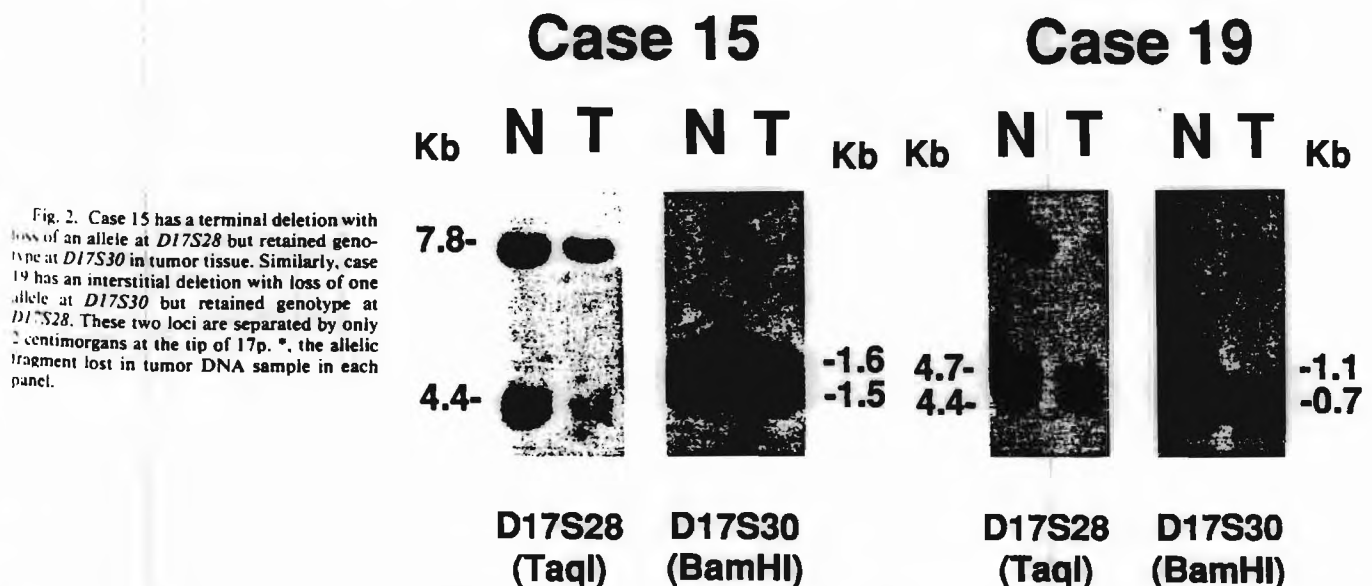
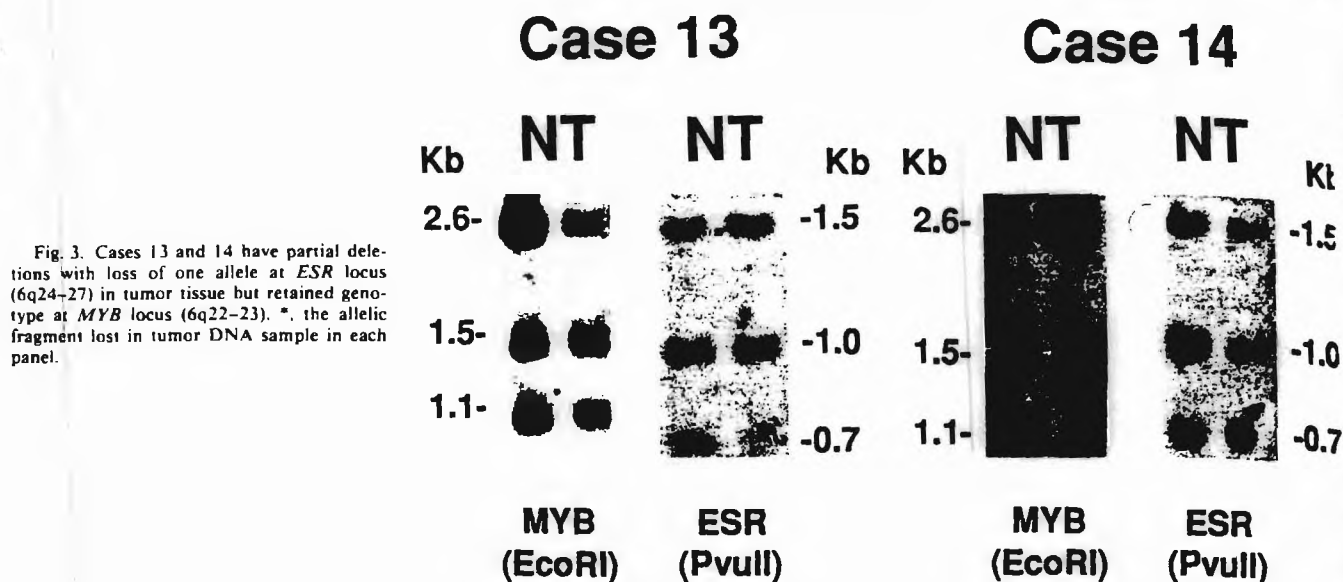


Fig. 2. Case 15 has a terminal deletion with loss of an allele at *D17S28* but retained genotype at *D17S30* in tumor tissue. Similarly, case 19 has an interstitial deletion with loss of one allele at *D17S30* but retained genotype at *D17S28*. These two loci are separated by only 2 centimorgans at the tip of 17p. *, the allelic fragment lost in tumor DNA sample in each panel.



carcinomas and not simply a result of widespread random losses of genetic material, we employed other DNA probes capable of detecting restriction fragment length polymorphisms at 14 additional loci on chromosomes 1, 2, 5, 6, 7, 11, 12, 19, and 22 (see Table 1). Among these, at each chromosomal locus where constitutional heterozygosity was observed, heterozygosity was also maintained in the matched tumor tissue in most cases.

DISCUSSION

The demonstration of loss of heterozygosity at the *HRAS1* locus, as well as other 11p markers, in a variety of human cancers (reviewed in Ref. 40) including ovarian carcinomas (20) suggests that their development or progression may involve loss or inactivation of normal genes on 11p. Previously we reported that tumor-specific allele loss occurs at the *HRAS1* locus on chromosome 11p in 50% of ovarian carcinomas (four primary tumors, eight metastases, and five ascites samples) (20). The current study extends that observation at about the same frequency (46%) to an expanded sample, cases 1-9 in Table 1, which includes five additional primary ovarian carcinomas, and cases 10-19 in Table 1, which includes two additional metastases.

More significantly, our study clearly shows that tumor-specific allele loss occurs in ovarian carcinoma at an even higher frequency for markers on chromosomes 6q (64% at *ESR*) and 17p (75% at *D17S28*, 64% at *D17S30*) than for 11p. This is the first report of tumor-specific allele loss from chromosomes 6 and 17 in ovarian carcinomas, suggesting the potential existence on these chromosomes of tumor- or growth-suppressor genes whose loss promotes this disease.

Deletion of part of chromosome 6q has been observed in several cytogenetic studies of ovarian carcinoma (14-17), which prompted us to investigate the long arm of chromosome 6 at the molecular level. Our finding of such a high incidence of tumor-specific allele loss in both primary and metastatic tumor tissues at the *ESR* gene locus (6q24-q27) (41) suggests that this loss might be a genetic event specific to ovarian cancer. Because 6q deletions have been observed cytogenetically in early gynecological malignancies, including a noninvasive ovarian carcinoma (42) and an ovarian granulosa cell tumor (43) (a borderline malignancy), loss of DNA sequences from 6q might

indicate that inactivation of a tumor- or growth-suppressor locus located there is an earlier event in ovarian tumorigenesis. However, this hypothesis needs to be tested by looking for tumor-specific 6q allele loss in the DNA of such early and borderline cancers before trying to classify such genetic changes as early or late events.

The fact that the more distal *ESR* allelic marker on 6q is lost more frequently (65%), compared to the more proximal *MYB* marker (13%), suggests that the *ESR* locus is closer to the region possibly uniquely lost in ovarian cancer. This notion is supported by the fact that the only tumor sample showing loss of heterozygosity at the *MYB* locus had concordant loss of heterozygosity at the *ESR* locus. Of further interest, other investigators in our laboratory have analyzed breast cancer DNA samples from 15 informative patients and observed an *ESR* gene allelic loss in tumor DNA from only one patient, demonstrating that loss of 6q sequences is not common to all cancer types.³

In addition to the cytogenetic reports of chromosome 6q deletions, an isochromosome for the long arm of chromosome 17 (i[17q]) was also reported in early gynecological malignancies, indicating possible deletion of the short arm of chromosome 17 (42). Our results suggest that the mechanism of allele loss from chromosome 17p resulted from partial or total loss of chromosome 17, because in 6 of 14 (43%) instances there were concurrent allele losses at both short and long arm loci. Because others have shown that tumor-specific allelic loss from chromosome 17 occurs in several different tumor types (12, 44-46) and because our data imply frequent chromosome 17 losses in ovarian cancer, genes on this chromosome might be involved in a cooperative role with other genes on 6q (or 11p) in mechanisms of tumorigenesis or disease progression. Further, the high incidence of concordant allelic losses from chromosomes 6q and 17p in 7 of 14 (50%) patients informative for both loci was, in our study, very impressive. Within the limits of the present study, it would be very difficult to determine the timing of these allelic losses from different chromosomes. The target genes in ovarian cancer may be located on both chromosomes.

In conclusion, whereas allele loss from chromosome 11p has

³ E. H. Koh *et al.*, personal communication.

Table 1. Loss of heterozygosity in ovarian carcinomas.

Location:	1q	2q	5q	6p	6q	7q	11p	11q	12p	12q	13q	17p	17q	22q
Locus:	AT7	D2S1	D2S50	GRL	MYB	ESR	EGFR	EGFR	EGFR	EGFR	EGFR	EGFR	EGFR	EGFR
Restriction enzyme:	PstI	EcoRI	TaqI	BCN	BamHI	EcoRI	PvuII	PvuII	PvuII	PvuII	PvuII	PvuII	PvuII	PvuII
Pt*	Dx	Grade	Site	Stage										
1	AC	3	Ov	III										
2	TCC	2	Ov	III										
3	MAC	3	Ov	III										
4	SAC	3	Ov	III										
5	SAC	3	Ov	III										
6	CAC	3	Ov	IV										
7	EC	3	Ov	IV										
8	AC	3	Ov	III										
9	SAC	3	Ov	III										
10	AC	3	Ov	III										
11	MMMT	3	Om	III										
12	AC	3	Om	III										
13	SAC	3	Om	III										
14	SAC	3	Om	III										
15	CAC	3	Om	III										
16	MC	3	Om	III										
17	SAC	3	Om	III										
18	EC	3	Om	III										
19	SAC	3	Om	III										
Incidence of allele loss														

* Pt, patient; Dx, pathological diagnosis; Ov, ovarian tumor; Om, ovarian metastasis; II, ileal metastasis; RL, rectal metastasis; Ab, abdominal metastasis; AC, adenocarcinoma; TCC, transitional cell carcinoma; MAC, Müllerian adenocarcinoma; SAC, serous adenocarcinoma; CAC, clear cell adenocarcinoma; EC, endometrioid carcinoma; CA, carcinoma; MMT, malignant mixed mesodermal tumor. For each probe, alleles are named with a as the larger restriction fragment and b as the smaller one(s) in each lane. -, a deletion of one allele; blank space, not informative (homozygous state); ND, not done.

been linked to at least eight cancer types and chromosome 17p allele loss to three different neoplasms (see Ref. 40), loss of chromosome 6 sequences has not been associated previously with any cancer. This makes it more likely that the 6q allelic losses uniquely observed here for ovarian cancer indicate the presence on 6q of a putative tumor- or growth-suppressor gene specific to this cancer.

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Cover Illustration The cover shows a generalised scheme for the mechanism of cell intoxication by toxins and immunotoxins. The topic of systemic immunotoxin therapy of cancer is reviewed in this issue by Dr E.J. Wawrzyniak (p. 624).

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GUEST EDITORIAL

Epithelial ovarian cancer: a cytokine propelled disease?

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Ovarian cancer is the commonest cause of death from gynaecological cancer in the developed world (Silverberg *et al.*, 1990; Booth & Beral, 1985). The age adjusted death rates from ovarian cancer have continued to increase since 1955 (Silverberg *et al.*, 1990). A major cause of the high mortality rate from ovarian cancer is the late presentation of the disease in over 60% of patients, with an associated 5-year survival rate of approximately 5% (Ozols *et al.*, 1980).

Several factors have been implicated in the aetiology of ovarian cancer, including a genetic predisposition, reproductive history, smoking, infection with mumps virus, dietary factors, and the use of talc (Piver, 1987). The most convincing factor to emerge as an influence on the development of ovarian cancer is hormonal. Several studies have shown that the risk of ovarian cancer in nulliparous women is 1.5–3.3 times greater than that of parous women (Silverberg *et al.*, 1990; Piver, 1987). The 'incessant ovulation' hypothesis proposed initially by Faithall (1971), and expanded by others (Casagrande *et al.*, 1979) attempted to explain the protective effect of pregnancy as being due to the rest periods afforded by pregnancy from incessant ovulation. The incessant ovulation hypothesis is supported by the more common occurrence of malignant change in the right ovary which ovulates more frequently (Cruickshank, 1990). Cramer and Welch (1983) suggested that whilst incessant ovulation led to the formation of inclusion cysts, excessive and continued gonadotropin stimulation contributed to malignant transformation by trophic effects on ovarian epithelium. Thus, suppression of gonadotropin release by oral contraceptives could explain the decreased incidence of ovarian cancer in women taking oral contraceptives. This hypothesis presents the case for the involvement of another group of peptide cell regulators, the cytokines, in the pathogenesis of ovarian cancer.

Cytokines are low molecular weight (<80 kDa) peptide cell regulatory factors that have wide ranging effects on different cell populations (Balkwill & Burke, 1989). Some cytokines can directly or indirectly inhibit tumour growth and have been used in cancer therapy (Balkwill, 1989). However, inappropriate cytokine activity has been implicated in the pathogenesis of infectious, inflammatory and autoimmune diseases, and there is now increasing evidence that cytokine production by tumours may contribute to the pathophysiology of cancer. For example, interleukin-6 has been shown to be an autocrine growth factor in myeloma, and overproduction of IL-6 causes the systemic symptoms and the immunological abnormalities in Castleman's disease (Kishimoto, 1989). Tumour necrosis factor (TNF) has been implicated in the suppression of haematopoiesis in hairy cell leukaemia, and in bone marrow necrosis in cancer patients (Lindemann *et al.*, 1989; Knuip, Pekala & Vornelius, 1988). Experimental studies also suggest roles for cytokines in cancer cachexia and the hypercalcaemia of cancer (Darling *et al.*, 1990; Sato *et al.*, 1987).

Constitutive production of several cytokines by human ovarian cancer cell lines and fresh tumour biopsy material

has been demonstrated. Naylor *et al.* (1990) have shown TNF mRNA and protein is expressed by ovarian tumour cells in biopsies from patients with epithelial ovarian cancer. In contrast, in the normal rat and bovine ovary immunoreactive TNF has been detected in granulosa- and stromal macrophages, but not ovarian epithelium (R & Terranova, 1989). Constitutive production of macrophage colony stimulating factor (M-CSF) and expression of *c-fms* oncogene (encoding the M-CSF receptor) has been demonstrated in human ovarian cancers and ovarian cancer cell lines (Ramakrishnan *et al.*, 1989; Kascinski *et al.*, 1990) but not in normal ovarian epithelium. Indeed, serum measurements of serum M-CSF in patients with ovarian cancer may be useful in monitoring disease activity (Kacir *et al.*, 1989). A monocyte chemotactic factor produced by ovarian cancer cell lines (Bottazzi *et al.*, 1985), is now known to be the TNF and IL-1 inducible cytokine monocyte chemotactic protein (MCP-1) (Bottazzi *et al.*, 1990). Interleukin-6 production is transiently induced by gonadotropin-primed hyperstimulated ovaries (Moitro *et al.*, 1990), can be detected in the ascitic fluid of ovarian cancer patients (Errori *et al.*, 1989), and is constitutively produced by human ovarian cancers and ovarian cancer cell lines (Wu *et al.*, 1990). Interleukin-1 has also been detected in a proportion of human ovarian cancers by *in situ* hybridisation, immunohistochemistry (unpublished observations). The *in vitro* cell regulatory effects of cytokines at picomolar concentrations *in vitro*, suggest that the expression of cytokines by human epithelial ovarian cancers may have an important role in their *in vivo* pathophysiology.

Tumour necrosis factor has antitumour effects in murine and human tumour xenograft models (Haranaka *et al.*, 1984). We have studied the effect of intraperitoneal TNF administration in intraperitoneal human ovarian cancer xenograft models (Malik *et al.*, 1989). TNF then prolonged survival in two out of three xenograft models. It paradoxically restored the ability of all three non-TNF producing ovarian tumours to form peritoneal tumour implants. Exogenous TNF administration therefore led to a pathological picture reminiscent of metastatic human ovarian cancer in the xenograft models. To study the possible role of tumour-produced TNF on peritoneal implantation and invasion, the behaviour of Chinese Hamster Ovary cells that had been transfected with the human TNF gene (CHO/TNF cells) was compared to CHO cells that contained the transfection vector alone (CHO/NEO cells) (Olliff *et al.*, 1987). CHO/TNF cells showed enhanced implantation to the surface of peritoneum and liver, and also metastasised to the lung. Furthermore, the metastatic capability of CHO/TNF cells could be specifically abrogated by injection of antibodies to TNF (Malik *et al.*, 1990). Other studies in experimental models have shown that TNF can enhance tumour metastasis (Giavazzi *et al.*, 1990). Properties of TNF that could potentially contribute to promotion of metastasis include stimulation of angiogenesis (Fraser-Schroder *et al.*, 1988), enhancement of tumour cell adhesion to host cells (Rice *et al.*, 1988), and induction of tumour cell metalloproteinases (Ito *et al.*, 1990). The known procoagulant activities of TNF (Pober, 1987), and the induction of platelet derived growth factor (Hajjar *et al.*, 1987), could contribute to the generation

of tumour stroma, an important step in the establishment of tumours in metastatic sites (Dvorak, 1986).

The other cytokines that are produced by ovarian cancers are intimately associated with TNF in the cytokine network (Balkwill & Burke, 1989). Interleukin-1 and M-CSF both induce TNF in monocytes and mesenchymal cells (Philip & Istein, 1986; Le & Vitek, 1987; Warren & Ralph, 1986). TNF induces the production of TNF, M-CSF, IL-1, IL-6, and MCP-1 in several cell populations (Balkwill, 1989). Thus there exists a potential self-perpetuating cytokine induction network in the vicinity of a tumour cell or a normal host cell generating cytokines which can influence tumour biology. For example IL-1 enhanced metastasis of melanoma cells (Graziani *et al.*, 1990) and promoted the peritoneal implantation of human ovarian cancer xenografts (Malik *et al.*, unpublished data). IL-6 has been reported to enhance the motility of breast cancer cells, a property that is associated with enhanced metastatic potential (Tamm *et al.*, 1989). M-CSF and MCP may contribute indirectly to metastasis by recruiting macrophages to tumour sites. These could be a further source of metastasis enhancing cytokines such as IL-1 and TNF, and secrete other factors that have growth stimulatory effects on ovarian cancer cells (Welanders *et al.*, 1982).

Can reasonable correlations be made between the effects of cytokines in ovarian cancer models with some of the known risk factors and prognostic indicators in ovarian cancer? Figure 1 shows the potential sites of cytokine induced tumour progression and the cytokines that could influence these stages.

The damage involved in ovulation could lead to the local release of cytokines, such as TNF and TGF- β by host macrophages, that contribute to the healing fibrotic response (Postelwaite & Sever, 1990). Both these cytokines are capable of inducing metalloproteinase production by tumour cells, and increasing their invasive potential (Ito *et al.*, 1990; Welch *et al.*, 1990). There is no evidence as yet that cytokines can cause malignant transformation of normal epithelia, although TNF can cause DNA strand damage in cells susceptible to its cytotoxic effects. TNF also activates several putative oncogenes including *c-myc* (Bhomer *et al.*, 1989) which is overexpressed in some human ovarian tumours (De Greve *et al.*, 1990). Overexpression of the *c-erbB-2* oncogene is an adverse prognostic indicator in human ovarian cancer

(Slamon *et al.*, 1989), and the similarity of the *c-erbB-2* oncogene product to the epidermal growth factor receptor suggests that an unidentified cytokine may be involved in promoting ovarian cancer growth. Levels of *c-erbB-2* oncogene expression correlate with resistance to the cytotoxic effects of TNF (Hudziak *et al.*, 1988). Although there is no evidence as yet that TNF regulates *c-erbB-2* expression, one could speculate that *c-erbB-2* positive tumours are TNF resistant tumours that have emerged after prolonged exposure to TNF.

Current evidence indicates that the normal ovarian surface epithelium, unlike epithelial ovarian cancer cells, does not express mRNA or protein for IL-6, M-CSF, or TNF (Rohy & Terranova, 1989; Motro *et al.*, 1990). Dysregulated cytokine production could occur during the transformation of normal epithelium to malignant cells or due to oncogene activation (Demetri *et al.*, 1990), and subsequent constitutive expression of cytokines and cytokine receptors may lead to autocrine growth stimulation of tumour cells or to modulation of their metastatic potential. The presence of M-CSF and *c-fms* like receptor transcripts, particularly in poorly differentiated ovarian tumours suggest that M-CSF may be an autocrine growth factor in some ovarian cancers. M-CSF production can be increased by gonadotropins (Bartocci *et al.*, 1986), and this cytokine may be an important mediator in the putative tumour promoting role of gonadotropins. The protective effect of oral contraceptives could be mediated by decreased M-CSF induction due to feedback inhibition of gonadotropin release. Reproductive hormone/cytokine interaction may also involve the reported inhibitory effects of oestrogens on TNF secretion (Ralston *et al.*, 1990). Loss of oestrogen secretion coupled with high levels of gonadotropin secretion could perpetuate the induction of TNF in the ovary via M-CSF, and explain the increased incidence of ovarian tumours with advancing age. Of relevance to this is the observation that a high percentage of ovarian cancers have specific deletions near to, and possibly involving, the oestrogen receptor locus on chromosome 6 (Lee *et al.*, 1990).

Cytokines released in the vicinity of the tumour may induce tumour cytokine production. For example, TNF has been shown to induce TNF mRNA and protein in epithelial tumours *in vitro* and *in vivo* (Niitsu *et al.*, 1988; Malik & Balkwill, 1990). Prolonged exposure to TNF led not only to the development of resistance to TNF, but induced constitutive secretion of TNF by tumour cell lines (Spriggs *et al.*, 1987). This could be relevant to the observation that tumours from the more frequently ovulating right ovary have a higher rate of metastasis at presentation (Cruickshank, 1990), i.e. these tumours may have acquired the ability to produce TNF by more frequent exposure to TNF released locally during the wound healing process after ovulation. A similar phenomenon could explain the association between the use of talc and occurrence of ovarian cancer in some studies, as talc induces granulomatous lesions which are a source of TNF (Kindler *et al.*, 1989). Alternatively, the invagination of ovarian surface epithelium that leads to inclusion cyst formation, may lead to the epithelium being exposed to intra-ovarian TNF being produced by macrophages and granulosa cells (Rohy & Terranova, 1989).

In conclusion, epithelial ovarian cancers and ovarian cancer cell lines have been shown to inappropriately elaborate a number of cytokines. These cytokines can potentially perpetuate their own biological effects by recruiting cytokine producing host cells and inducing further cytokine production. Recent experimental evidence indicates a potential role for at least two cytokines (TNF and IL-1) in the promotion of ovarian cancer metastasis, and two others (M-CSF and the ligand for the *c-erbB-2* proto-oncogene) as putative autocrine growth factors in ovarian cancer. These regulatory molecules may be acting to create a hormone modulated tumour promoting environment in this disease. If so, future therapies for ovarian cancer may involve negating the effects of these cytokines, either by inhibition of cytokine secretion, inhibition of the recruitment of cytokine producing cells, or treatment with specific cytokine antagonists.

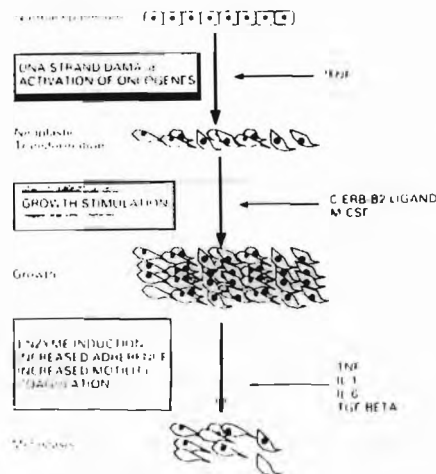


Figure 1 Potential sites of cytokine interactions in ovarian tumours.

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GUEST EDITORIAL

Liver transplantation for malignancy

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Tumour ablation by resection, when feasible, is the treatment of choice for most patients with primary hepato-biliary tumours and secondary malignancies of colo-rectal origin (Iwatsuki & Starzl, 1988a). As a result of the use of tumour markers in the populations at risk and an increased use of accurate non-invasive imaging techniques, improved detection of smaller lesions within the parenchyma or hilum has occurred and this has led to a higher resectability rate (Okuda & Ishak, 1987), although this rarely exceeds 30-40% (Bismuth *et al.*, 1986). Furthermore, a multidisciplinary approach, improvements in patient selection and management, and refined techniques in liver surgery, have contributed both to a lower perioperative mortality and improved long-term survival rates (Tang, 1985).

For small primary hepatocellular carcinomas, follow-up of 144 patients, who underwent resection, showed a 5-year survival of 63.2% (Tang *et al.*, 1989). The best results were found in patients with a tumour of less than 2 cm and those with an encapsulated tumour. Similar results were found in a French-Italian cooperative study of 72 patients with cirrhosis (Franco *et al.*, 1990). However, where the tumour is bilobar or central, where there is vascular invasion, or if the remaining parenchyma is severely compromised from cirrhosis, subtotal hepatectomy may not be an option. In this situation, the median post-operative survival is only 2 to 4 months.

For patients whose disease is not resectable by conventional approaches, a total hepatectomy and subsequent liver replacement may offer the only true chance for survival, although in practice only about one quarter of patients referred to transplant centres will be suitable (Ismail *et al.*, 1990).

Hepatocellular carcinoma (HCC)

The value of liver replacement for HCC is still the subject of much controversy. The European Liver Transplant registry, with results from 32 European centres, reported a 2-year actuarial survival rate of 30% in 217 patients who underwent liver replacement for HCC (Bismuth *et al.*, 1987). Centres in Hannover, King's College/Cambridge and Pittsburgh report similar 3-year survival rates of about 20% and the King's College/Cambridge group report some limited early success with subsequent chemotherapy (Ringe *et al.*, 1989; O'Grady *et al.*, 1988; Koneru, 1988).

Iwatsuki *et al.* (1989) have described the prognostic indicators affecting survival and recurrence after transplantation. In a group of 80 liver recipients with HCC, the tumour size (greater than 5 cm), multiple nodules, vascular invasion and tumour shape (non-circumscribed) were poor prognostic histopathological factors for recurrence in non-fibrolamellar HCC. They and others have confirmed the more favourable prognosis associated with the fibrolamellar variant of HCC, where the 1-year survival rate was 100% with an overall

recurrence rate of 50% (O'Grady *et al.*, 1988; Starzl *et al.*, 1986).

The Hannover group report significant differences in median survival time for different groups classified according to TNM (Ringe *et al.*, 1989). Stage II (pT2 pN0 pM0) patients had a median survival of 120 months compared to 11.8 months for stage III (pT1-3 pN0-1 pM0) and 8.75 months for stage IVA (pT4 pN0-1 pM0). All patients grouped as stage IVB (pT1-4 pN0-1 pM1) died within 2 months. In this series pre-operative serum alpha-fetoprotein levels did not have a significant correlation with post-operative outcome. However tumour-free long-term survivors were noted to have normal or only slightly elevated alpha-fetoprotein levels before liver replacement. For these patients, coexistence of cirrhosis did not influence the long-term survival rate but the 30-day mortality was higher in the cirrhotic patients. However, of the ten patients transplanted for coexistent cirrhosis and HCC in Birmingham, none survived more than 1 year (Ismail *et al.*, 1990).

Carcinoma of the biliary tract

The prognosis following liver replacement for tumours of the biliary tract is uniformly poor. Peripheral cholangiocarcinomas appear incurable by this approach as widespread distant metastases developed in all eight patients surviving more than 30 days in the Hannover series (Ringe *et al.*, 1989). Similarly six of seven patients in the King's College/Cambridge series developed recurrence of tumour (the remaining patient died at 4 months following a cerebro-vascular accident) (O'Grady *et al.*, 1988).

Slightly more encouraging results are found in patients with central lesions. The Hannover group report on 20 patients and found the major influence on survival time was the lymph node status (Ringe *et al.*, 1989). Of the node negative recipients, eight of 13 were alive (median survival 35 months) and the overall 2-year actuarial survival rate was 64.1%. All seven patients with regional lymph node metastases, however, had a much more limited survival span (median survival 7 months). However, of the 13 liver transplants performed in King's College/Cambridge for central cancers, only seven patients survived 3 months and 6 died of recurrence of tumour (median survival 8.5 months). The remaining patient was alive and well at 6.5 years (O'Grady *et al.*, 1988). In a series of nine patients from Pittsburgh, Iwatsuki reported that no patient with bile duct cancer had lived 2 years post-operatively (Iwatsuki *et al.*, 1988a).

There is no doubt that accurate staging of the tumour is vital before transplantation and many centres routinely perform an exploratory laparotomy with lymph node sampling. The value of such a procedure has to be balanced against an increased risk to the patient and the disadvantage of interfering at the site of transplant.

Some hope for these patients can be drawn from reported results of a series of six bile duct carcinomas treated by 'abdominal cluster' operations (Starzl *et al.*, 1989). The operation consists of the removal of the liver, stomach, spleen, pancreas, duodenum, proximal jejunum, terminal ileum, and

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REPRODUCTIVE, GENETIC, AND DIETARY RISK FACTORS FOR OVARIAN CANCER

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A case-control study of 110 women with ovarian epithelial carcinoma and 220 individually age-matched controls was conducted in Hokkaido, Japan, to identify ovarian cancer risk factors. Both the cases and the matched controls were surveyed either from 1980 to 1981 or from 1985 to 1986. Ovarian cancer risk was increased in single women ($p < 0.01$), and in women with a family history of breast, uterine, or ovarian cancer in a mother or sister ($p < 0.001$). Conversely, risk was decreased in women who had experienced a livebirth ($p < 0.001$), an induced abortion ($p < 0.05$), or who had had permanent sterilization by tubal ligation ($p < 0.05$). Each of the reproductive factors remained significant when adjusted for each other using logistic regression analysis. The odds ratio for ovarian cancer decreased significantly with increasing number of livebirths ($p < 0.001$). Furthermore, a significant negative association between anovulatory periods and ovarian cancer risk was noted ($p < 0.01$). No association was observed for any types of contraceptive methods other than tubal ligation, but the prevalence of oral contraceptive use was very low. A significant positive association with daily fish consumption ($p < 0.05$) and a marginally significant negative association with daily milk consumption ($p = 0.05$) were also observed.

anovulation; diet; neoplastic syndromes, hereditary; ovarian neoplasms; reproduction; retrospective studies

The age-adjusted mortality rate of ovarian cancer has been gradually rising in Japan, although it is still among the lowest in the developed countries (1). Japanese women have a number of different reproductive experiences and dietary habits compared with those of other populations. For

example, more than a third of Japanese married women have experienced an induced abortion (2), but less than 5 per cent have ever taken oral contraceptives. By contrast, more than 50 per cent of their partners have used a condom for contraception (2). The amount of daily meat con-

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sumption per capita is lower in Japan than in any of the Western countries (3).

We conducted a case-control study of ovarian cancer in Japan designed to evaluate reproductive (4-17), genetic (5, 7, 9, 12, 17), and dietary risk factors (17-21), all of which have been found to be associated with the occurrence of ovarian cancer in Western countries.

MATERIALS AND METHODS

Cases

All hospitals in Hokkaido which treat ovarian cancer patients were registered for the survey. During the period of the survey, we contacted the chief gynecologist of each of these hospitals. When an ovarian cancer patient was identified, our interviewer visited the hospital within a week. None of the doctors in charge or their patients rejected our request for an interview. The patients were surveyed from November 1980 to June 1981 ($n = 45$) or from July 1985 to February 1986 ($n = 65$) with the use of a uniform questionnaire. All information was collected through direct interviews. The information obtained included the variables shown in table 1.

The 110 patients with pathologically

confirmed primary epithelial ovarian cancer were interviewed at 35 gynecologic wards which cover the entire Hokkaido geographic area. The cases had adenocarcinomas with the following histologic classifications: 52 serous, 20 mucinous, three clear cell, four endometrioid, 16 undifferentiated, and 15 unspecified.

Controls

Two control series were obtained by matching the cases on year of birth (within three years) and the year of the survey. The first control group (control group A) comprised 110 inpatients from the 37 gynecologic wards in hospitals in Hokkaido, mostly from the same wards as the case group. The control patients had gynecologic complaints other than an ovarian tumor. Forty-two (38 per cent) were admitted for myoma uteri, 33 (30 per cent) for cervical carcinoma, 11 (10 per cent) for various gynecologic inflammatory disorders, seven (6 per cent) for prolapsus uteri, and the remainder for various other conditions.

The second group (control group B) comprised 110 women who were identified from outpatients at the Department of Obstetrics and Gynecology, Sapporo Medical College ($n = 39$), from women screened by the Papanicolaou test at three anti-cancer associations in the cities of Sapporo, Asahikawa, and Kushiro ($n = 61$), or from women who attended two senile welfare facilities in the cities of Sapporo or Abashiri ($n = 10$). Women who were diagnosed as having a malignant disorder or ovarian disease had been excluded from this control group. Fifty-four (49 per cent) were disease-free gynecologically, 51 (46 per cent) had various kinds of vaginitis, and the remainder had various benign disorders.

Controls, as with cases, were interviewed in person, and no one who was selected as a control declined our request for an interview.

Statistical analyses

Differences in variables between the cases and the controls were evaluated by

the univariate method of Miettinen (22). That is, the maximum likelihood estimate was used for point estimations of odds ratios, and exact interval estimations of odds ratios were calculated. Multivariate analyses were performed by the conditional logistic regression method (23) to adjust for potentially confounding variables. The paired t test and analysis of variance were used to assess differences between cases and the two control groups for continuous variables.

RESULTS

The mean age at interview for the 110 cases was 51.1 years (standard deviation (SD) = 12.2); for the 110 women in control group A it was 50.6 years (SD = 12.0); and for the 110 women in control group B it was 51.1 years (SD = 12.4). Eighty-seven (79 per cent) of the cases were interviewed within a year from the diagnosis.

Reproductive variables

The average ages at menarche were 14.7 years (SD = 2.2) for cases, 14.8 years (SD = 2.1) for control group A, and 14.5 years

(SD = 1.7) for control group B. The average age at natural menopause of cases (48.9 years, SD = 3.7 ($n = 48$)) was younger than that of either control group A (49.2 years, SD = 4.2 ($n = 46$)) or control group B (49.9 years, SD = 3.6 ($n = 55$)), but the differences were not statistically significant. Seven cases and three controls in group A and five controls in group B had had a hysterectomy.

Table 2 shows odds ratios of variables related to marital status and childbearing. The proportion of cases who had never married was significantly larger than that of controls ($p < 0.01$). The average age at first marriage of cases (23.6 years, SD = 3.8) was similar to that of both control group A (23.1 years, SD = 3.9) and control group B (23.3 years, SD = 2.8).

Analyses for various reproductive characteristics were restricted to women who had ever married. More cases than controls were nulliparous ($p < 0.001$). Frequency of miscarriages did not differ between the cases and controls, but significantly fewer cases had had induced abortions ($p < 0.05$). The average age at first livebirth was similar among cases (24.4 years, SD = 2.8),

TABLE 2

Odds ratios (ORs) (with 95 per cent confidence intervals (CIs)) for ovarian cancer associated with reproductive variables, Hokkaido, Japan, 1980-1986

Reproductive variables	Cases (%)	Comparative group					
		Control group A (%)	OR*	Control group B (%)	OR*	Combined control groups A + B	
						OR*	95% CI†
Never married	8.2	1.8	8.0	1.8	8.0	7.6	1.5-73.4
Ever pregnant	88.8	94.9	0.4	93.9	0.4	0.4‡	0.1-1.2
Ever had a livebirth	81.6	93.9	0.3	92.9	0.3	0.2‡	0.1-0.6
Miscarriage	29.6	29.6	1.0	37.8	0.7	0.8‡	0.5-1.4
Induced abortion	38.8	59.1	0.3	45.9	0.5	0.5‡	0.3-0.9
Tubal ligation	9.1	24.5	0.4	18.4	0.4	0.4‡	0.1-0.8
Condom use by partner	41.8	42.9	1.0	51.0	0.6	0.8‡	0.4-1.4
Had ever breast-fed	86.3	89.0	0.8	90.0	0.7	0.7§	0.3-1.9
Lactation ≥6 months per child	71.2	67.1	1.3	60.3	1.7	1.6§	0.7-3.4
Anovulatory period ≥6 years	6.4	20.4	0.1	17.3	0.3	0.2	0.1-0.7
Anovulatory period ≥6 years	9.6	26.0	0.1	19.2	0.4	0.3§	0.1-0.8

* Maximum likelihood estimates of odds ratio.

† 95% exact confidence intervals of odds ratio.

‡ Based on 98 sets of a case and two controls excluding never married women.

§ Based on 73 sets of a case and two controls excluding non-parous women.

TABLE 1

Variables for assessing differences between ovarian cancer patients and control women, Hokkaido, Japan, 1980-1986

ologic variables
Body height and weight, Quetelet's index, blood type, sibship size, birth order
Family history
Health status of close relatives, occurrence of cancers among close relatives
Menstrual profile
Menarche, menstrual irregularity, dysmenorrhea, menopause
Reproductive history
Marital status, pregnancy, parity, lactation, contraceptive usage
Dietary variables
Tea, fish, milk, coffee, alcohol, tobacco
Other variables
Residence, birthplace, sports activity, cosmetics usage, occupational history

control group A (24.4 years, SD = 3.1), and control group B (24.9 years, SD = 3.2).

Table 2 shows that a significantly lower proportion of cases than controls had permanent sterilization by tubal ligation ($p < 0.05$). The average age at tubal ligation of cases (31.5 years, SD = 4.1) was similar to control group A (31.2 years, SD = 4.3) and control group B (32.3 years, SD = 4.2). The proportion of cases whose partners used condoms was not significantly different from controls. Only two cases and four controls had ever taken oral contraceptive pills. No difference was observed in the frequency of use of any other types of contraceptive methods.

Nulliparous subjects were excluded when comparisons were made concerning breast feeding (table 2). There was no significant difference in either the proportion of women who had ever breast fed or the period of breast feeding per child.

As shown in table 3, the odds ratio for ovarian cancer decreased significantly with increasing number of livebirths, either crudely (linear trend, $p < 0.001$) or after adjusting for marital status and age at first livebirth (adjusted linear trend, $p < 0.05$). Compared with nulliparous subjects, women with one or two children had a third of the risk of ovarian cancer, women with

three or four children, a fourth the risk, and women with five or more children, a 20th the risk.

To assess the "anovulatory years" model of Casagrande et al. (5), an anovulatory period (in months) was individually calculated as follows: anovulatory months = (total months of pregnancies) + (total months of oral contraceptive use) + (total anovulatory months caused by lactation). Total months of pregnancies in this formula was calculated as follows: (parity * 9) + (total months of each abortion, miscarriage, or ectopic pregnancy). Total anovulatory months caused by lactation were calculated by the following formula provided by Risch et al. (14): total anovulatory months by lactation = $(1.5 + 0.55 * \text{mean lactation months}) * (\text{number of livebirths})$. A significant negative association between anovulatory periods and ovarian cancer risk was observed whether it was examined in the entire group ($p < 0.01$) or restricted to subjects who had ever had a livebirth ($p < 0.01$) (table 2).

Conditional logistic regression analysis was performed using the reproductive variables identified as risk factors in the univariate analyses; they included marital status, number of livebirths, experience of abortion, and tubal ligation. Table 4 shows that the number of livebirths (odds ratio per child = 0.7, $p = 0.002$), experience of ever having an abortion (odds ratio = 0.6, $p = 0.04$), or tubal ligation (odds ratio = 0.5, $p = 0.05$), all remained significantly associated with ovarian cancer risk.

Genetic variables

The occurrence of various cancers among parents and siblings of the cases and controls was examined. As shown in table 5, there were no differences in the occurrence of cancers in parents and siblings of both sexes combined. Although not significant, more cases reported a cancer history in the mother or a sister. Moreover, a family history of breast, uterine, or ovarian cancer in a mother or sister was significantly more

TABLE 4
Adjusted effects of reproductive variables on ovarian cancer, Hokkaido, Japan, 1980-1986

Reproductive variable	Logistic regression			Odds ratio	p value†
	Unit	Coefficient	SE*		
Never married	Yes/No	0.943	0.725	2.6	0.19
No. of livebirths	No.	-0.365	0.117	0.7	0.002
Induced abortion	Yes/No	-0.544	0.270	0.6	0.04
Tubal ligation	Yes/No	-0.764	0.396	0.5	0.05

* SE, standard error of logistic regression coefficient.

† Two-sided probability.

TABLE 5
Odds ratios (ORs) (with 95 per cent confidence intervals (CIs)) for ovarian cancer associated with genetic factors, Hokkaido, Japan, 1980-1986

Genetic factor	Cases (%)	Comparative group					
		Control group A (%)	OR*		Control group B (%)	Combined control groups A + B	
			OR*	95% CI†		OR*	95% CI†
Cancer in parents or siblings	34.5	30.9	1.2	38.1	0.9	1.0	0.6-1.6
Cancer in a mother or sister	20.9	9.1	3.2	16.4	1.3	1.9	0.9-3.7
Breast, uterine, or ovarian cancer in mother or sister	10.9	3.6	3.7	1.8	11.0	5.3	1.6-22.9
Blood type A	45.5	37.3	1.4	34.5	1.6	1.5	0.9-2.5
Blood type O	20.9	30.9	0.7	32.7	0.6	0.6	0.3-1.0

* Maximum likelihood estimates of odds ratio.

† 95% exact confidence intervals of odds ratio.

common in the cases ($p < 0.001$). Three breast cancers, eight uterine cancers and one ovarian cancer were reported by cases; one breast cancer, three uterine cancers, and no ovarian cancers were reported by control group A; and no breast cancers, two uterine cancers and no ovarian cancers were reported by control group B. It was not specified whether the site of uterine cancers was the cervix or endometrium.

A larger proportion of cases had blood type A (odds ratio = 1.5), and a smaller proportion had blood type O (odds ratio = 0.6); however, these results were not significant.

Dietary variables

Table 6 shows the distribution of cases and controls by daily consumption of meat, fish, milk, coffee, alcohol, and smoking prior to diagnosis. There were no significant differences between cases and controls observed in meat, coffee, and alcoholic bev-

erage consumption, or smoking history. However, the association between daily fish consumption and the risk of ovarian cancer was positive and significant ($p < 0.05$). Conversely, there was a marginally significant negative association between daily milk consumption and ovarian cancer ($p = 0.05$), but a dose-response relation was not observed. The odds ratio of ovarian cancer for Quetelet's index ($\text{weight}(\text{kg})/\text{height}(\text{m})^2$) less than 20 was 1.7, but this difference was not significant.

No additional medical history variables investigated in this study revealed significant associations with ovarian cancer.

DISCUSSION

In this study, as with numerous other case-control studies (4-12, 14, 16, 17), the number of livebirths was found to have a strong negative association with the occurrence of ovarian cancer.

We have proposed previously (5) that

TABLE 3

Odds ratios (ORs) for ovarian cancer associated with number of liveborn children, Hokkaido, Japan, 1980-1986

	No. of liveborn children			
	0	1-2	3-4	5+
Cases	29	45	32	4
Control group A	9	52	39	10
Control group B	10	56	31	13
Unadjusted OR (Linear trend, $p < 0.001$)	1.00	0.19	0.17	0.03
Adjusted OR* (Linear trend, $p < 0.05$)	1.00	0.34	0.28	0.05

* Adjusted for marital status and age at first livebirth.

TABLE 6

Odds ratios (ORs) (with 95 per cent confidence intervals (CIs)) for ovarian cancer associated with dietary variables, Hokkaido, Japan, 1980-1986

Dietary variable	Cases (%)	Comparative group					
		Control group A (%)	OR*	Control group B (%)	OR*	Combined control groups A + B	95% CI†
Eats meat daily	27.3	20.9	1.4	21.8	1.3	1.4	0.8-2.5
Eats fish daily	63.6	50.9	1.9	50.9	1.6	1.7	1.0-2.9
Drinks milk daily	35.5	50.0	0.6	46.4	0.7	0.6	0.4-1.0
Drinks ≤ 200 ml milk daily	20.9	20.9	1.0	19.1	1.1	1.1	0.6-1.9
Drinks coffee daily	41.8	32.7	1.6	36.4	1.3	1.4	0.8-2.5
Drinks alcoholic beverages at least once a week	21.8	23.6	0.9	19.1	1.2	1.0	0.6-1.9
Smokes daily	14.5	16.4	0.8	10.9	1.3	1.1	0.5-2.3
Quetelet index $<20\ddagger$	22.7	17.3	1.5	12.7	2.0	1.7	0.9-3.3

* Maximum likelihood estimates of odds ratio.

† 95% exact confidence intervals of odds ratio.

‡ Weight (kg)/height (m^2).

(incessant) ovulation provides the stimulus for division of epithelial ovarian cells, and that ovarian cancer risk is directly related to the frequency of such periods of active cell division. Under this hypothesis, total duration of anovulatory periods would be expected to reduce the risk. The total of anovulatory periods was shorter among ovarian cancer patients than among the controls (even if the analysis was restricted to parous women). This finding, in conjunction with other reports (5, 11, 13), which show a similar association with the total duration of anovulatory period, would suggest that number of livebirths directly reduces the incidence of ovarian cancer.

A tubal ligation was found to be negatively associated with the occurrence of ovarian cancer, even after controlling for marital status, number of livebirths, and experience of induced abortion. A tubal ligation has been reported to reduce serum levels of gonadotropins (24), and Heintz et al. (25) have suggested that inclusion cysts, produced by incessant ovulation (25-28), may be induced to proliferate and transform by high gonadotropin levels. Therefore, it is possible that a tubal ligation may indirectly reduce ovarian cancer risk by diminishing the opportunity of malignant

transformation of inclusion cysts via reduced gonadotropin stimulation.

Family history of breast, uterine, and ovarian cancer was more frequently observed in cases in this study. This result is consistent with the reports of other case-control studies (5, 7, 9) as well as with the known familial aggregation of these cancers (29, 30). Attention must be paid to the possibility that recall bias may be an explanation for this finding. This seems unlikely, given the site-specificity of the results.

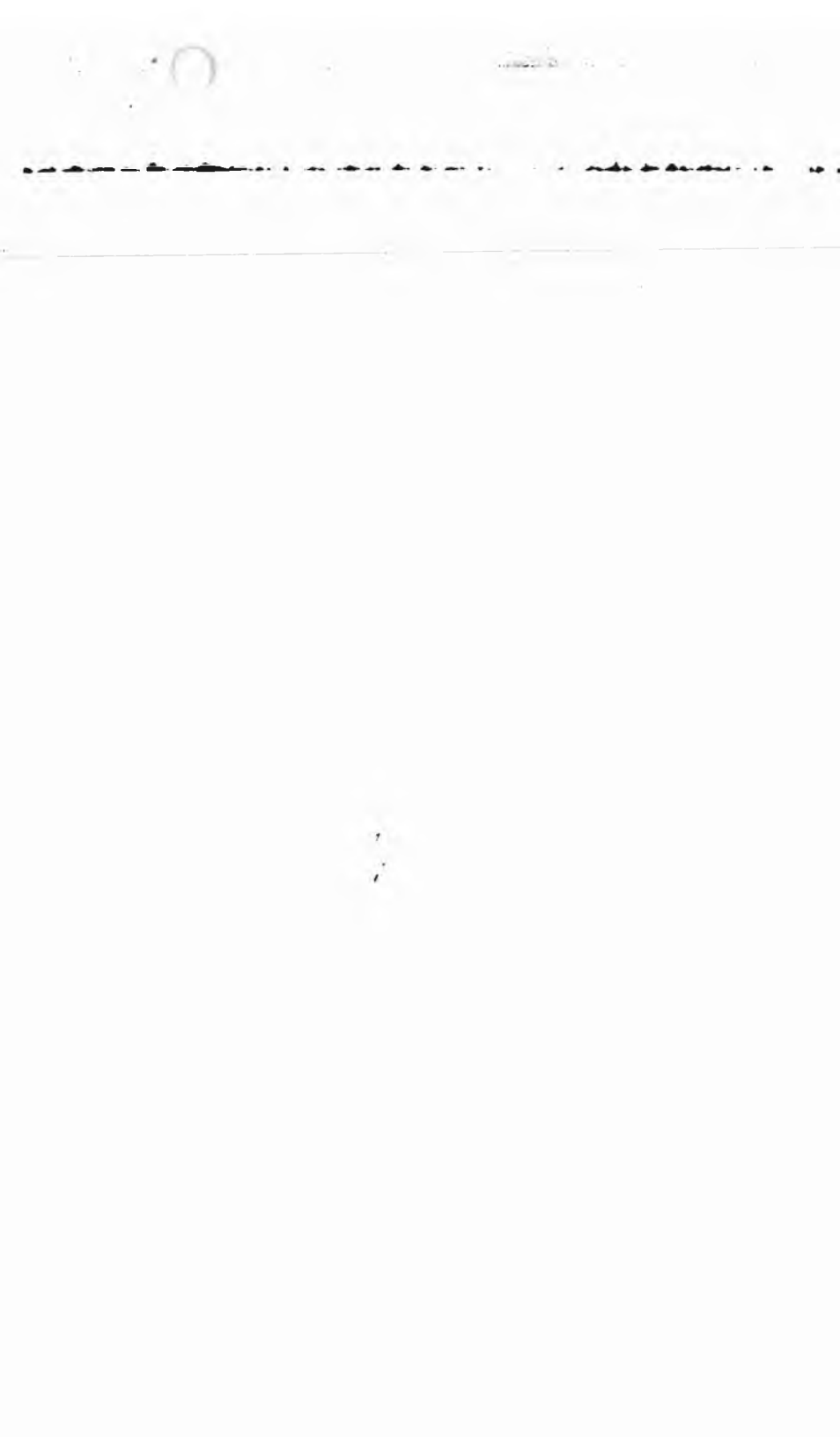
We could not confirm the positive associations of either alcohol consumption (17, 21) or coffee consumption (18) with ovarian cancer, which have been reported previously. Studies on the role of dietary fat have been inconclusive; one report showed a significant association (20) while another did not (19). Our study suggests no strong association. The marginally significant associations of higher fish consumption and less frequent milk consumption observed here require further investigation, but we can propose no mechanism to explain the associations with these specific food items.

Despite the markedly dissimilar sources used for identifying control groups A and B, the distribution of most variables of interest was quite similar between the two

control groups and risk factors tended to be the same using either group for comparison. Since the major sources of both control groups were facilities that diagnosed and treated gynecologic complaints, biased risk estimates might exist for some variables, especially the reproductive factors. Population-based or neighborhood controls are difficult to identify in Japan, because a close protection of privacy is still common among Japanese women. The similar results obtained using either control group offer some reassurance of the validity of our findings.

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Dietary and Other Risk Factors of Ovarian Cancer among Elderly Women

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The age-specific mortality rate of ovarian cancer is increasing among women over 50 years of age, but remaining at a stable level among women under 50. This case-control study of ovarian cancer was undertaken to assess the environmental factors which may increase the mortality of the disease in the elderly. Fifty-six women with primary epithelial ovarian cancer whose ages were 50 years old or over were compared with two age-matched control groups. The results of the Mantel-Haenszel analysis were as follows. More cases were found to have never married than controls ($P < 0.05$), a larger proportion of cases were nulliparous ($P < 0.05$), a smaller proportion of cases had experienced an induced abortion ($P < 0.05$) or had undergone permanent sterilization by tubal ligation ($P < 0.05$), the occurrence of breast or uterine cancer was more common in the mother or sisters of cases ($P < 0.01$), a larger percentage of cases used to eat meat daily ($P < 0.01$) or used to eat fish daily ($P < 0.05$), and a larger proportion of cases weighed under 40 kg ($P < 0.05$). Daily meat consumption was significantly associated with the occurrence of ovarian cancer even after adjusting for reproductive and other risk factors by conditional logistic regression analysis. The attributable risk for ovarian cancer in the elderly was 19.2% for daily meat consumption. The recent change in dietary habits might in part explain the rise of the mortality rate among the Japanese elderly.

Key words: Ovarian neoplasms Retrospective study Risk factor Diet

The age-standardized mortality rate of ovarian cancer is gradually rising in Japan. The age-specific mortality rate of the disease is increasing year after year among women over 50, but this rate remains stable among women under 50 (Fig. 1). This divergence could not be explained only by the difference in the histological origins of primary ovarian cancer in the different age groups. We suspected that some environmental factors might contribute to the increased incidence of ovarian cancer among the elderly in Japan.

We have conducted a case-control study of ovarian cancer in Hokkaido, and reported the reproductive, genetic, and dietary risk factors when all age groups were combined and analyzed.¹⁾ In the present paper, we restrict the analyses to the ovarian cancer patients who were diagnosed at the age of 50 or above in order to better assess the dietary risk factors which might explain the recent increase of the disease among the elderly in Japan.

MATERIALS AND METHODS

One hundred and ten cases with pathologically confirmed primary epithelial ovarian cancer were

interviewed at 35 gynecological wards, covering the entire Hokkaido geographical area. The cases were surveyed using a uniform questionnaire from November, 1980, to June, 1981 ($N = 45$), or from July, 1985 to February, 1986 ($N = 65$), because the main author had gone abroad from 1982 to 1984. All information was collected through direct interviews. The information obtained included the variables shown in Table I. The questions asked about dietary habits and consumption of tobacco and alcoholic beverages are presented in Table II.

The analyses were restricted to 56 cases (50.9%) of 110 ovarian cancer patients, who were diagnosed at or over 50 years of age. Histologically, all the cancers were adenocarcinomas of common epithelial origin; 27 serous, 11 mucinous, 1 clear cell, 1 endometrioid, 8 undifferentiated, and 8 unspecified.

Cases were compared to two control series, individually matched with cases by age (within 3 years) and the year of the survey. The first control series (control group A) consisted of 56 in-patients from the gynecologic ($N = 53$), ophthalmologic ($N = 2$), and dermatologic wards ($N = 1$). Most of control group A were selected from the hospitals where the cases were identified and interviewed, except for 4 women who were selected from two other hospitals. Their primary diagnoses were 25 cervical carcinoma, 12 myoma of uterus, 7

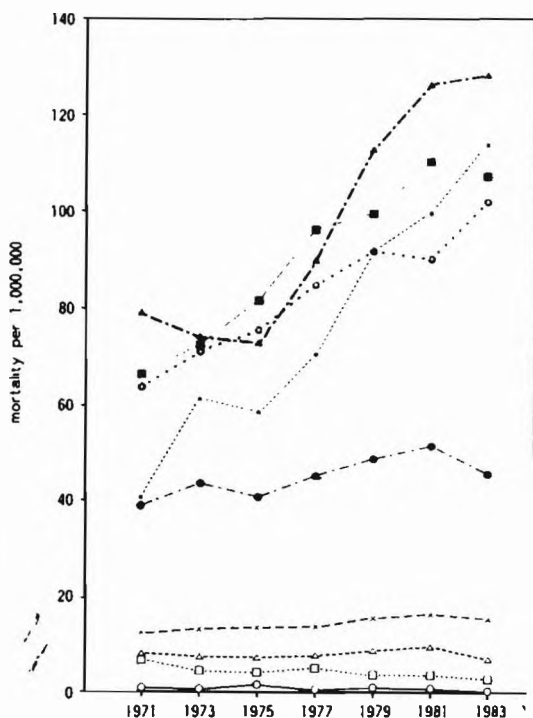


Fig. 1. The age-specific mortality rate of ovarian cancer per 1,000,000 population from 1971 to 1983 in Japan: ○, 0-9 years; □, 10-19 years; △, 20-29 years; ●, 30-39 years; ■, 40-49 years; ▲, 50-59 years; ◆, 60-69 years; ★, 70-79 years; ☆, 80 years. (Data source: Vital Statistics, Japan, Volume 3).

Table I. Variables Surveyed

Biological factors

Age, blood group, height and weight

Family history

Mothers's parity, cause of death of close relatives

Menstrual profile

Menarche, general menstrual, difficulties with menstruation, premenopausal symptoms, menopause

Marital status

Reproductive history

Gravidity, parity, lactation

Contraceptive usage

Medical history

Chronic disease, surgery, X-ray examination, hormonal treatment

Social factors

Address, occupation, tobacco and alcohol, cosmetic usage, food intake

DIETARY RISK FACTORS OF OVARIAN CANCER

Table II. Questions about Dietary Habits and Consumption of Tobacco and Alcoholic Beverages

Could you tell me your dietary habits and consumption of tobacco and alcoholic beverages in several years before you had been notified of the disease?

- A. Did you eat meat almost every day? 1. yes 2. no
- B. Did you eat fish almost every day? 1. yes 2. no
- C. Did you drink milk almost every day? 1. yes () ml/day 2. no
- D. Did you drink coffee or tea almost every day? 1. coffee 2. tea 3. neither
- E. Did you smoke every day? 1. yes () cigarettes/day 2. no
- F. Did you drink alcoholic beverages at least once a week?
1. yes What kind of beverages? () 2. no

gynecological inflammation, 7 prolapsus of uterus, 2 cataract, 1 cytocele, 1 tumor of palate, and 1 tumor of vagina.

The second comparative group (control group B) consisted of 56 women, who were identified from among women screened by means of Papanicolaou's test at three Anti-Cancer Associations in Sapporo, Asahikawa, and Kushiro cities (N=42); from among outpatients at the Department of Obstetrics and Gynecology, Sapporo Medical College (N=9); or from among women who attended two Senior Citizen Welfare Facilities in Sapporo or Abashiri cities (N=5). We thus selected control group B from different institutions in Hokkaido. As a result, 34, 8, and 14 women of control group B were from Sapporo and its adjacent areas, northern Hokkaido, and eastern or southern Hokkaido, respectively, as compared with 33, 10, and 13 women of the case group who were from the respective areas. The disease status of control group B was 29 with no gynecological disorder, 26 with gynecological inflammation, and 1 with cervical polyp.

The controls, like the cases, were interviewed in person. All cases and most controls were interviewed by two medical doctors who had been trained for interviewing, except for 8 women of control group B who were interviewed by public nurses.

Differences in variables between the cases and the controls were evaluated by the univariate method of Mantel and Haenszel.²¹ Multivariate analyses were performed by the conditional logistic regression method²¹ to adjust for potentially confounding variables. Population attributable risks of the main risk factors were calculated by the method of Fleiss.⁴⁾

RESULTS

The mean age at the time of the interview for the 56 cases was 60.2 years (SD=7.6); for

the 56 women in control group A it was 59.6 (SD=7.6); for the 56 women in control group B it was 60.8 years (SD=7.6). The mean age at diagnosis for the cases was 59.7 years (SD=7.2), and 48 cases (85.7%) were interviewed within a year after the diagnosis.

Reproductive Characteristics Table III shows the odds ratios (the estimated relative risks) of variables related to marital status and child-bearing. The proportion of cases who had never married was significantly larger than that of the controls ($P<0.05$). Analyses for various reproductive characteristics were restricted to women who had married. More cases than controls were nulliparous (the odds ratio, OR=3.75, $P<0.05$). Frequency of miscarriages did not differ between the cases and the controls, but significantly fewer cases had experienced an induced abortion (OR=0.39, $P<0.05$). Table III shows that a significantly lower proportion of cases had had permanent sterilization by tubal ligation than controls (OR=0.39, $P<0.05$). The average age at the time of tubal ligation for the cases (33.2 years, SD=4.0) was similar to that of control group A (33.0 years, SD=4.7) and control group B (33.1 years, SD=4.7). There were no significant differences between the cases and the control groups with regard to other contraceptive methods. The prevalence of taking oral contraceptives was rare in both the cases (N=1) and the controls (N=1).

Genetic Factors The occurrence of various cancers among parents and sibling of the cases and the controls was examined. As shown in Table IV, there were no differences in the occurrence of cancers in parents and siblings

Table III Reproductive Factors

Item	Case group	Control group A	OR	Comparative group		Control A + B OR
				Control group B	OR	
Never married	3.6%	0.0%		0.0%		"
Never pregnant ^a	9.3	5.6	1.33	1.9		3.00
Never had a live birth ^a	14.8	5.6	2.67	3.7	7.00	3.75 ^a
Live births ^a ≥ 3	50.0	61.1	0.50	63.0	0.65	0.59
Miscarriage ^a	25.9	29.6	0.83	35.2	0.67	0.74
Induced abortion ^a	31.5	50.0	0.38	48.1	0.40	0.39 ^a
Tubal ligation ^a	11.1	22.2	0.46	25.9	0.33	0.39 ^a
Condom use ^a	25.9	24.1	0.91	29.6	0.82	0.79

a) $P < 0.05$.

b) Analyzed 54 triples excluding never-married women.

OR, odds ratio.

Table IV Genetic Factors

Item	Case group	Control group A	OR	Comparative group		Control A + B OR
				Control group B	OR	
Cancer in parent or sibling	37.5%	37.5%	1.00	44.6%	0.77	0.85
Cancer in mother or sister	28.6	10.7	4.33 ^a	21.4	1.44	2.17
Breast or uterine cancer in mother or sister	10.7	1.8	6.00	1.8		11.00 ^a

a) $P < 0.05$ b) $P < 0.01$ OR, odds ratio.

when a combined analysis was performed. Although not significant, more cases had reported a cancer history in a mother or sister. Moreover, a family history of breast or uterine cancer among a mother or sister was significantly more common in the cases than in the controls (OR 11.0, $P < 0.01$). Two breast and 5 uterine cancers were reported by families of cases; 0 breast and 1 uterine cancer by families of control group A; and 0 breast and 1 uterine cancer by families of control group B. It was not specified whether the site of uterine cancer was the cervix or the endometrium.

Dietary Factors Table V shows the distribution in cases and controls of the daily consumption of meat, fish, milk, coffee, alcohol, and smoking prior to diagnosis. There were no significant differences observed in milk, coffee, and alcoholic beverage consumption,

or even in the smoking history. However, the positive association between daily meat consumption and the risk of ovarian cancer was found to be significant (OR 3.11, $P < 0.01$). Likewise, the positive relation of daily fish consumption to the disease was significant (OR 2.54, $P < 0.05$). A significantly larger proportion of cases weighed under 40 kg than that of the controls (OR 3.50, $P < 0.05$).

Table VI indicates the correlation matrix of food consumption frequency among the women surveyed. There was a significant positive relationship of daily meat consumption to daily fish consumption (Pearson's correlation coefficient $r = 0.25$, $P < 0.01$). A positive correlation was also observed between daily coffee drinking and daily milk drinking ($r = 0.22$, $P < 0.01$), as well as between daily coffee drinking and weekly alcoholic beverage consumption ($r = 0.28$, $P < 0.001$).

DIETARY RISK FACTORS OF OVARIAN CANCER

Table V. Dietary Factors

Item	Case group	Comparative group				Control A + B OR
		Control group A	OR	Control group B	OR	
Eating meat daily	28.6%	8.9%	4.67 ^a	14.3%	2.33	3.11 ^a
Eating fish daily	73.2	60.7	2.40	50.0	2.63 ^a	2.54 ^a
Drinking milk daily	37.5	53.6	0.50	50.0	0.65	0.58
Daily milk $\geq$ 200 ml	8.9	17.9	0.50	19.6	0.40	0.45
Drinking coffee daily	23.2	26.8	0.82	32.1	0.58	0.70
Drinking alcoholic beverages at least once a week	16.1	14.3	1.17	12.5	1.29	1.23
Smoking daily	16.1	16.1	1.00	5.4	3.00	1.67
Body weight $<$ 40 kg	12.7	3.6	3.50	3.6	3.50	3.50 ^a

a) $P < 0.05$. b) $P < 0.01$. OR: odds ratio.

Table VI. Correlation Matrix of Dietary Habits

Item	1.	2.	3.	4.	5.	6.
1. Eating meat daily	1.00					
2. Eating fish daily	0.25 ^a	1.00				
3. Drinking milk daily	0.04	0.02	1.00			
4. Drinking coffee daily	0.07	0.02	0.22 ^a	1.00		
5. Drinking alcohol weekly	0.08	0.03	0.09	0.28 ^b	1.00	
6. Smoking daily	0.16	0.03	-0.03	0.05	0.15	1.00

a) $P < 0.01$. b) $P < 0.001$.

Adjustment of the Dietary Factors To adjust the possible confounding variables with each other, a conditional logistic regression analysis was performed. Six items included in the multivariate formula were all significantly associated with the disease according to univariate analyses; number of live births, experience of induced abortion, status of tubal ligation, familial history of breast or uterine cancer, daily meat consumption, and a body weight of less than 40 kg. Since daily fish consumption was significantly correlated with daily meat consumption, daily meat consumption and daily fish consumption could not be simultaneously involved in the regression model.⁵⁾

Table VII shows the result of the logistic regression analysis when daily meat consumption was included in the model. The association between daily meat consumption and ovarian cancer was still significant ($P < 0.05$) even after adjusting for the other 5 variables, although the estimated relative risk was slightly reduced to 2.7 from 3.1 in the univariate analysis.

Population Attributable Risk of the Main Risk Factors The population attributable risks were calculated for three factors, all of which were found to be significantly associated with the disease by multivariate analysis. As shown in Table VIII, the population attributable risk for ovarian cancer

Table VII. Adjusted Risk Factors

Item	Unit	Logistic regression coefficients (β)	SE (β)	Relative risk	95% CI
Number of live births	Number	0.34	0.15	0.7 ^a	0.5-0.9
Induced abortion	Yes/No	0.74	0.47	0.5	0.8-5.3
Tubal ligation	Yes/No	0.69	0.56	0.5	0.2-1.5
Breast or uterine cancer in mother or sister	Yes/No	2.55	1.19	12.8 ^a	1.1-132.8
Eating meat daily	Yes/No	0.99	0.49	2.7 ^a	1.0-7.0
Body weight > 40 kg	Yes/No	0.03	0.02	1.0	0.9-1.0

a) $P < 0.05$. SE (β): standard error of logistic regression coefficient. CI: confidence intervals.

Table VIII. Population Attributable Risk of Risk Factors

Risk factor	Populations attributable risk	95% confidence intervals
Never had a live-born child	14.0%	0.2-24.4%
Breast or uterine cancer in mother or sister	9.1	0.0-17.3
Eating meat daily	19.2	3.4-32.4

among the elderly women was 14.0% for never having a live-born child, 9.1% for familial history of breast or uterine cancer, and 19.2% for daily meat consumption.

DISCUSSION

A few case-control studies and cohort studies have provided data on dietary factors in ovarian cancer. According to the result of a case-control study conducted by La Vecchia *et al.*⁴¹ in Italy, women with ovarian cancer had a significantly elevated frequency of consumption of meat and higher subjective scores of fat intake. The median age of the ovarian cancer cases was 55 years in their study. Similarly, Cramer *et al.*¹¹ indicated that there was a significant trend for increasing risk in ovarian cancer with increasing animal fat consumption. The average age of the cases was 53.2 years in their study. On the contrary, Byers *et al.*⁴² reported that there was no significant risk of ovarian cancer associated with the consumption of fat.

Hirayama¹¹ reported a significantly positive association between daily meat consumption and ovarian cancer as a result of a 13-year cohort study of 142,857 women aged 40 years or over in Japan. Snowdon¹⁰ noted the preliminary result of a cohort study of 16,190 persons that women who ate fried food five or more days per week had a significantly greater risk (relative risk = 2.9).

The analysis showed a marginally significant association with daily fish consumption but no relationship with meat consumption when all age groups of ovarian cancer patients were combined.¹¹ However, when the analysis was restricted to the ovarian cancer patients who were diagnosed at or over 50 years of age, there was a clear positive association of the disease with daily meat consumption as well as with daily fish consumption. This significant association was noted independently of nondietary risk factors such as decreased number of live births and family history of breast or uterine cancer, as found by the logistic regression analysis.

It is not entirely understood how dietary factors such as high meat consumption and high animal fat intake increase the risk of the disease. Cramer *et al.*¹¹ have proposed that elevated gonadotropin levels brought about by ingesting high levels of animal fat might increase the ovarian cancer risk. However, this hypothesis is not consistent with several serological studies of the dietary-fat effect on gonadotropins. For instance, Armstrong *et al.*⁴³ indicated that postmenopausal vegetarian (low-fat diet) women did not have different plasma gonadotropin levels when compared to non-vegetarian women (high-fat

diet). Furthermore, Hill *et al.*¹¹⁾ reported that in postmenopausal black women in South Africa a Western diet (high-fat diet) decreased both plasma luteinizing and follicle-stimulating hormones as compared to a low-fat diet.

Alternatively, Cramer and Welch¹³⁾ have postulated that in view of the presence of estrogen receptors in epithelial ovarian tumors, diets high in animal fat could promote tumor development by their purported ability to cause an increase of extragenital estrogen. This hypothesis is consistent with the serological study which has shown that postmenopausal vegetarian women have lower urinary estrogen levels in comparison with matched nonvegetarian women.¹¹⁾ Further investigations are needed to clarify the mechanism of the relationship of dietary factors to ovarian cancer by changing the hormonal milieu.

In numerous other case-control studies, like this study, the number of live births was found to have a strong negative association with the occurrence of ovarian cancer. The references and discussions relevant to this finding appeared in our previous report.¹⁾

Family history of breast or uterine cancer was more frequently observed in cases in this study. This result is consistent with the reports of a case-control study by McGowan *et al.*¹⁴⁾ as well as with the known familial aggregation of these cancers.^{15,16)}

About 45% of ovarian cancers among the elderly women in our study was attributed to three risk factors: never having a live-born child (14.0%), family history of breast or uterine cancer (9.1%), and the dietary factor of daily meat consumption (19.2%). In regard to this result, the recent rapid change of dietary habits among Japanese might partly explain the rise of ovarian cancer mortality. A decreasing trend in the number of births may also account in part for the increasing mortality of the disease.

Although we asked the patients about their dietary history before they had been notified of the diseases, recall bias might exist. Furthermore, the results concerning dietary history should be carefully interpreted, because the latent period between dietary exposure and the occurrence of ovarian cancer has not yet been clarified.

Since the major sources of both control groups were facilities diagnosing and treating gynecologic complaints rather than the general population, bias might exist. However, the similarity of the results obtained using the two control groups offers some reassurance as to the validity of our findings.

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A Comparative Epidemiologic Study on Geographic Distributions of Cancers of the Lung and the Large Intestine in Japan

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To examine what kinds of factors could have caused the geographic variation observed in lung cancer morbidity in Japan, a correlation study was performed comparing various regional traits. The same study was also conducted on large intestinal cancer, aiming to distinguish the possible urban factors associated with both cancers. Lung cancer was highly correlated with industrialization-related factors such as localization of manufacturing industries, automobile traffic and air pollution, whereas colon cancer was correlated with the population density of workers in the tertiary industries such as services, trade and government. A multiple regression analysis could not detect any single factor with an exceptionally strong influence on either cancer. The present findings suggest that the hazardous environmental condition of urban areas has, to some extent, contributed to the recent increase of lung cancer cases in this country.

Key words: Lung cancer — Colon cancer — Cancer registry — Geographic correlation — Air pollution

In Japan lung cancer has been rapidly increasing in recent years. The age-adjusted mortality rates were 27.4 in males and 7.7 in females per 10⁵ population in 1985, nearly five times and four times larger, respectively, than those in 1955. It is generally suspected that the growing tobacco consumption in these decades has made a significant contribution to the past increase of lung cancer.¹⁾ However, the incidence rate also shows a notable geographic variation which mainly corresponds to the urban-rural difference. If all geographic districts are classified by their population size, the lung cancer mortality is highest in the largest cities and lowest in the rural areas. This urban-rural difference may be, as has been speculated from epidemiologic findings reported in other countries,¹⁾ ascribable to differential tobacco consumption rates. Nevertheless, it is quite improbable that other kinds of hazardous factors in the urban environment have nothing to do with this disease.

Several authors in other countries have reported that, even after age and smoking rate

were standardized, a significant effect of residence still appeared in the geographic variation of lung cancer mortality.¹⁻⁵⁾ The effect of residence could be due to either some feature(s) of urban life style or environmental pollution. Regarding the association of these latter factors with lung cancer, many epidemiologic studies⁶⁻¹¹⁾ have been carried out and provided apparently positive but inconclusive evidence because other factors such as occupation and smoking rate were not thoroughly standardized.¹²⁻¹³⁾

The present study attempted to obtain some clues concerning the geographic correlation between lung cancer morbidity and those factors inherent to urbanization, especially environmental pollution or localization of its sources. In general, this type of correlation study is rather inferior to the more advanced analytical epidemiologic studies, because it can only demonstrate a correlation between the disease and regional traits. Still it should be useful if the factors to be studied are of area-specific rather than person-specific kinds.

tions of solutions will provide maximal nitrogen sparing and caloric supplementation.

CONCLUSION

Some patients who currently are being supported with central venous TPN could benefit from PNPV with little attendant morbidity. Similarly, many patients who might benefit from protein sparing therapy and caloric supplementation who do not receive TPN because of risk of complications, cost, or complexity of the monitoring and delivery system are good candidates for PNPV. Even those patients who appear to be at low risk for postoperative morbidity might benefit from the infusion of amino acid solutions alone because of their nitrogen sparing properties.

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The Woman at Risk for Developing Ovarian Cancer^{1,2}

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Defining the woman at risk for developing ovarian cancer can identify factors that cause the disease and lead to earlier diagnosis and improved survival. Our 3-year case-control study of 197 women with histologic proven primary ovarian cancer of the epithelial type reveals the typical patient to have a family history of gynecologic cancer, be exposed to rubella in the peripubertal period, be nulliparous or of low parity, experience difficulty in conceiving, have certain exacerbated premenstrual symptoms, and to rarely undergo surgically induced menopause. In women with carcinomas of low malignant potential a later onset of menarche as well as menstrual disorders occur.

During the past three decades the persistently poor survival has made ovarian cancer one of the most common causes of cancer deaths in American women. In 1979 there will be approximately 17,000 new cases of primary ovarian cancer with approximately 11,100 deaths. Ovarian cancer is the fourth leading cause of cancer deaths in American women [2]. Because of our inability to consistently diagnose the disease in its early stages, the usual ovarian cancer patient has widespread disease, clinical stage III, at the time of operation [1,17,21]. Improvements in survival depend, in great measure, upon earlier detection of the disease. Even if we had a near-perfect diagnostic aid for ovarian cancer, we would have to apply it to those women who have the greatest likelihood of developing the disease. In addition, the concept of preventing ovarian cancer or at least recognizing those causative or contributing factors in the development of the disease is one of our more interesting clinical problems.

The purpose of this study was to identify risk factors of presumed etiologic and diagnostic significance for ovarian cancers of the epithelial type.

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MATERIALS AND METHODS

The present study was designed specifically to address the methodological deficiencies of prior epidemiological studies of cancer of the ovary.

Case selection and pathologic confirmation. Our case-control study was conducted from September 1974 to April 1977 in the Washington, D.C., metropolitan area. One hundred ninety-seven cases of pathologically confirmed primary ovarian cancers of the epithelial type were identified and included in the study. Of these 42 (21%) were carcinomas of low malignant potential (LMP) and were analyzed separately.

Operative reports, surgical pathology reports, and representative microscopic slides of the tumor were obtained for each ovarian cancer case and were reviewed by the study's pathologist (H.J.N.). Study eligibility was limited to the epithelial-type primary cancer of the ovary as defined by the World Health Organization [22]. The neoplasms included in this study were mainly serous, mucinous, or endometrioid types, but the clear cell, Brenner, mixed, undifferentiated, or unclassified epithelial tumors [1,19,22] were also identified. Within each type, tumors of borderline malignancy (carcinomas of low malignant potential) as well as malignant tumors were recognized [22].

Control selection. One hundred ninety-seven women were selected as controls and were frequently-matched by age, race, and hospital of origin with the ovarian cancer cases. The control group of women had no history of cancer and were selected from general medical and surgical services so as to exclude obstetric, gynecologic, psychiatric, and cancer patients.

Data collection. Information was collected through direct interviews conducted by women trained as medical interviewers. In an attempt to minimize interobserver variability, only five interviewers were used. The detailed interview was administered in the same manner to all cancer patients and to those women in the control group and took approximately 60 min to complete. The information obtained included the categories of variables seen in Table 1.

Differences in risk factors between cases and controls were statistically evaluated using the Students *t* test and the χ^2 for univariate comparisons. The risk of ovarian cancer associated with various reproductive and menstrual characteristics was calculated and evaluated for statistical significance using the Mantel-Haenszel method [15,23].

RESULTS

Sociodemographic Characteristics

Ovarian cancer patients and their controls were comparable in age, race, and sex due to the frequency-matching criteria utilized for control selection. The mean age of women with malignant ovarian tumors was 52.1 years with a mean age of 52.2 years for their controls. Women with carcinomas of low malignant potential and their controls were considerably younger with a mean age of 40.9 and 40.4 years, respectively. These age distributions are expected [1]. White women with malignant ovarian tumors comprised 87.1% of the group and blacks 12.9%; identical racial proportions were achieved in the control group. Among the women with

TABLE I
CATEGORIES FOR EVALUATING DIFFERENCES IN OVARIAN
CANCER PATIENTS AND CONTROL WOMEN

Sociodemographic data
Age, race, religious preference
Residence patterns
Years of completed education
Marital status
Socioeconomic status
Occupation
Family-relatives
Anthropometric measures
Menstrual profile
Menarche
General menstrual
Difficulties with menstruation
Premenstrual symptoms
Menopause
Reproductive history
Sexual history
Contraceptive usage
Past history
Benign cysts
Chronic disease history
Diet and weight
Exposure to toxic substances
Family cancer history
Hygiene
Infectious diseases
Medication usage
Substances abuse
Surgical history

carcinomas of low malignant potential and their controls, 76% were white and 24% black.

The majority of women (92.9%) with malignant ovarian tumors had been married at least once as compared with 87.1% of their controls. This high percentage of "ever-married" women was also observed among patients with borderline malignant ovarian tumors and their controls, 78.5 and 83.3% respectively.

Women with ovarian cancer and their controls were also observed to be comparable with regard to religious preference, social class (as measured through use of the Hollingshead Social Index), and educational level. The majority of women with ovarian cancer and their controls lived in the metropolitan Washington, D.C., area for at least 10 years prior to their diagnosis or inclusion in this study.

Because of the small number (42) of low malignant potential cases included in the study, statistical analyses could not be carried out for all variables. Statistical data for this group will therefore be presented for selected variables only.

Reproductive History

The reproductive experience of the study participants was divided into measurements of gravidity (the state of a woman after conception and until termina-

tion of the gestation) and measurements of parity (the state of having given birth to an infant or infants, alive or dead).

Pregnancy (gravidity). Comparative analyses of women with ovarian cancer and their controls were carried out for the following variables: mean and categorical number of pregnancies, person-years at risk of conceiving, number of pregnancies per 1000 persons-years at risk of pregnancy, and perceived difficulty in conceiving.

Analyses for differences between cases and controls as related to various reproduction characteristics were restricted to women who were ever married (including all subjects who were currently married, widowed, divorced, or separated). This allowed for the comparison of women whose risk of pregnancy was considered to be more homogeneous. Confining the analyses to women who were ever married did not alter the comparability of the case and control groups as this subgroup was noted to be similar with respect to race, religious preference, age, area of residence, and current marital status.

Ever-married women with ovarian cancer were found to have significantly fewer pregnancies than their respective controls (Table 2). A comparison of the mean number of pregnancies, although a useful general indicator of decreased gravidity, disguises the fact that a large proportion of ever-married controls had five or more pregnancies, whereas a similarly large proportion of ovarian cancer cases were nulligravidas (see Table 3). The magnitude of difference in pregnancy experience between the women with malignant ovarian tumors and their controls is reflected in the strength of the *P* value ($P = 0.008$), a finding not likely to be produced by an artifact secondary to small sample size (Table 3).

Calculations of relative risk indicated that nulligravidas were 2.45 times more likely to develop malignant ovarian tumors and 2.9 times more likely to develop carcinomas of low malignant potential than women who were pregnant three or more times. The risk of ovarian cancer was greatly reduced among women who had at least one pregnancy (see Table 4).

Gravidity measurements are, of course, highly influenced by a number of factors in addition to those which have been controlled for in frequency matching. A number of pregnancy risk estimates have, therefore, been developed for purposes of the present analysis and include: (1) Married risk which refers to a composite person-years at risk of pregnancy while married. This index is computed by subtracting the subject's age at marriage from the subject's age at termination of marriage, menopause (surgical or natural), or adoption of permanent birth control measures (e.g., tubal ligation in self or vasectomy in husband)

TABLE 2
MEAN NUMBER OF PREGNANCIES AMONG EVER-MARRIED WOMEN AND CONTROLS

Malignant ovarian tumors <i>N</i> = 144	Controls <i>N</i> = 135	Low malignant potential <i>N</i> = 33	Controls <i>N</i> = 35
2.2	3.0	1.9	2.8
$P < 0.01$		$P < 0.05$	

TABLE 3
GRAVIDITY AMONG EVER-MARRIED CASES AND CONTROLS

	Times pregnant				Total No. of ever married
	0	1-2	3-4	5+	
Women with malignant ovarian tumors	35	47	47	15	144
Control group	17	44	43	31	135
	$P = 0.008$				

depending on which of these three events occurs first. (2) Adjusted married risk which is computed by subtracting the total number of married years in which birth control methods were used from the married risk index as described above.

Analysis of married risk and adjusted married risk indices indicates that the low gravidity reported by both case groups cannot be attributed to fewer years at risk of pregnancy as compared with their controls (see Table 5). Data presented in Table 5 also indicate that a large percentage of the nulligravidity among controls can, perhaps, be attributed to significantly decreased (as compared with cases) person-years at risk of pregnancy. Furthermore, when two additional indices (number of pregnancies per 1000 years of married risk and number of pregnancies per 1000 years of adjusted married risk) are computed, women with ovarian cancer were found to have significantly fewer pregnancies per 1000 years of married risk and adjusted married risk than their controls (Table 6). The actual degree of difference, however, appears to be stronger in the study group comprised of women with low malignant potential ovarian tumors and their controls.

We compared gravidity of cases and controls who were equally "unprotected" from the risk of pregnancy and the number of pregnancies among subjects who reported that they had never used birth control measures of any kind. Thirty-three percent (47/144) of the ever-married patients with malignant ovarian tumors and 28% (38/135) of their respective controls stated that they had never used birth control. The average age of these subjects and years of married risk (or pregnancy) were comparable.

Women with malignant ovarian tumors who had never used birth control methods had significantly fewer pregnancies than controls who also abstained from the use of birth control ($P < 0.02$).

TABLE 4
RELATIVE RISK ASSOCIATED WITH NUMBER OF PREGNANCIES
AMONG EVER-MARRIED WOMEN

No. of pregnancies	Women with malignant ovarian tumors	Women with carcinomas of LMP
None	2.45*	2.9
1-2	1.27	1.02
3+	1.00	1.00

* Significant at 0.05.

TABLE 5
PERSON-YEARS OF MARRIED RISK BY NUMBER OF PREGNANCIES

No. of pregnancies	Mean years of married risk		Mean adjusted years of married risk*		Mean years of married risk		Mean adjusted years of married risk*	
	Women with malignant ovarian tumors	Control group	Women with malignant ovarian tumors	Control group	Women with carcinomas of LMP	Control group	Women with carcinomas of LMP	Control group
0	12	8	9	7	12	3	12	1
1-2	19	16	12	10	13	10	5	6
3-4	21	19	12	8	18	19	9	8
5+	20	19	11	11	—	—	—	—

* Adjustment of mean: Married years at risk minus married years using any form of birth control.

TABLE 6
NUMBER OF PREGNANCIES PER 1000 PERSON-YEARS AT RISK OF PREGNANCY

Risk status	Women with malignant ovarian tumors	Control group	Women with carcinomas of LMP	Control group
Married (Total)	126	183	130	208
(Adjusted)*	213	314	208	419

* Married years at risk unprotected by any method of birth control.

The categorical pregnancy history among non-birth control users is presented in Table 7 and further indicates that cases and controls have significantly different pregnancy profiles, a difference which cannot be explained on the basis of unequal pregnancy risk.

In order to explore the possibility that infertility per se may be associated with the development of ovarian cancer, the subjects were asked whether or not they: (1) ever had difficulty conceiving, (2) consulted a physician for fertility-related disorders, and (3) whether or not the nature and source of the infertility problem was known. Table 8 reveals that women with ovarian cancer report that they had experienced difficulty in conceiving significantly more often than their controls.

Women with malignant ovarian tumors who stated they had difficulty in conceiving reported significantly fewer pregnancies than their controls (Table 9). The relative risk of developing ovarian cancer based on equal risk for three or more pregnancies among those subjects stating difficulty in conceiving suggests that nulligravidity may be highly associated with ovarian cancer development.

Among women with malignant ovarian tumors and their controls who had reported difficulty in conceiving, slightly more controls attributed this difficulty to themselves rather than to their spouses (65 versus 50%). However, this finding may be the consequence that considerably more cases than controls indicated that the source of the difficulty was unknown. Similar proportions of women with tumors of low malignant potential and their controls attributed their apparent infertility to themselves. More controls than cases reported that their infertility had been confirmed by a physician.

TABLE 7
NUMBER OF PREGNANCIES AMONG EVER-MARRIED SUBJECTS USING NO BIRTH CONTROL

No. of pregnancies	Women with malignant ovarian tumors		Control Group		Women with carcinomas of LMP		Control group	
	N	%	N	%	N	%	N	%
0	18	38	11	29	4	44	0	—
1-2	14	30	10	26	4	44	2	29
3-4	13	28	7	18	1	11	3	43
5+	2	4	10	26	0	—	2	29
	P < 0.03				P < 0.05			

TABLE 8
ACKNOWLEDGED DIFFICULTY IN BECOMING PREGNANT AMONG WOMEN WITH
OVARIAN CANCER AND THEIR CONTROLS

Difficulty in becoming pregnant	Women with malignant ovarian tumors		Control group		Women with carcinomas of LMP		Control group	
	N	%	N	%	N	%	N	%
Yes	66	43	35	23	19	45	8	19
No	68	43	89	57	13	31	29	69
Unsure or unknown	21	14	31	20	10	24	5	12
Total	155	100	155	100	42	100	42	100
	$P < 0.005$				$P < 0.005$			

Age at pregnancy. A comparison of the mean age of first pregnancy shows women with malignant ovarian tumors to report their first conception at a somewhat older age than their controls (24.4 versus 23.7 years). Women with carcinomas of low malignant potential demonstrate a similar trend (23.7 versus 22.9 years). The age differences noted above were not statistically significant in either study group. However, a significantly smaller proportion ($P < 0.05$) of the women with malignant ovarian tumors were pregnant for the first time before age 25 (see Table 10). The reported age at first sexual intercourse was approximately the same for cases and controls.

Parity. When the mean number of pregnancies producing a live birth was computed for ever-married subjects, women with ovarian cancer clearly demonstrated a marked decrease in the number of live births relative to their controls (see Table 11). Analysis of the pregnancy history by number of pregnancies reveals that ever-married women with malignant ovarian tumors reported nulliparity almost twice as often as their control group of women and the overall distribution of the number of live births is highly significant (see Table 12). This difference in distribution of live birth pregnancies failed (due to small numbers of subjects) to

TABLE 9
NUMBER OF PREGNANCIES AND RELATIVE RISKS AMONG EVER-MARRIED WOMEN
WITH ACKNOWLEDGED DIFFICULTY IN CONCEIVING

No. of pregnancies	Women with malignant ovarian tumors		Control group		Relative risk
	N	%	N	%	
Never pregnant	23	34.8	8	23.5	1.64
1-2	22	33.3	14	41.2	0.897
3-4	16	24.2	4	11.8	1.00
5+	5	7.6	8	23.5	
Total	66	100.0	34	100.0	
	$P = 0.05$				

TABLE 10
DISTRIBUTION BY AGE AT FIRST PREGNANCY

Age at first Pregnancy	Women with malignant ovarian tumors		Control group	
	N	%	N	%
Less than 20	16	10	18	12
20-24	35	23	53	35
25+	57*	37	47	31
Never pregnant	45	30	34	22
Total	153	100	152	100

* $P < 0.05$.

reach significance in those women with carcinomas of low malignant potential and their control group of women although the same trend was present.

Relative risks computed on the basis of equal risk for three or more live births indicated that the risk of ovarian cancer was elevated for nulliparous women (see Table 13). Women who were married and had at least one live birth were at similar levels of risk of developing ovarian cancer.

Women with ovarian cancer did not have more spontaneous abortions, stillbirths, or defective children than their controls. Therefore, although the women with ovarian cancer demonstrated a significantly lower mean number of live births as compared with their controls, this difference can be attributed to decreased gravidity rather than to decreased fetal viability. There was also no dissimilarity between "ever-pregnant" cases and controls with regard to problems during their pregnancy.

We attempted to determine whether nulligravida among women with ovarian cancer was in fact planned and therefore a reflection of conscious preference to remain childless or unplanned and therefore suggestive of underlying infertility. Twenty-seven percent of the cases with malignant tumors (39) and 15% of controls (20) reported that they were childless; of this group of subjects, 21% of the cases and only 11% of their controls stated that their childlessness was unplanned. The difference in unplanned childlessness was statistically significant ($P = 0.02$) and indicates that childlessness might be ascribed to fertility-related disorders (in self or spouse) more often among ovarian cancer patients than among their controls.

TABLE 11
MEAN NUMBER OF LIVE BIRTHS AMONG EVER-MARRIED
WOMEN AND THEIR CONTROLS

Women with malignant ovarian tumors N = 144	Control group N = 135	Women with carcinomas of LMP N = 33	Control group N = 35
1.56	2.25	1.57	2.20
$P < .05$			

TABLE 12
NUMBER OF LIVE BIRTH PREGNANCIES AMONG EVER-MARRIED WOMEN

No. of pregnancies	Women with malignant ovarian tumors		Control group	
	N	%	N	%
0	45	31	23	17
1-2	55	38	64	47
3-4	40	29	29	22
5+	4	3	19	14

$P = .0003$

Menstrual Profile

Menarche. The mean age at menarche was 12.8 years for women with malignant ovarian tumors and 12.7 years for their controls. For women with carcinomas of low malignant potential and their controls, the mean age at menarche was 12.7 and 12.2 years, respectively. Thus, the age at menarche was similar for all study groups and parallels general menarche statistics available for the United States [13].

When relative risks based on equal risk for menarche between the ages of 11 and 13 were computed, no increased risk of ovarian cancer was found in subjects who reached menarche at age 10 years or less or 14 or more years of age. In the group of women with carcinomas of low malignant potential, those subjects who reported their menarche at age 14 or older experienced a 3.8-fold risk of ovarian cancer over subjects who reported menarche between the ages of 11 and 13 ($P = 0.05$).

General menstrual characteristics. A comparison of a variety of menstrual characteristics revealed that the average length of the menstrual cycle among ovarian cancer cases and their controls was quite similar (28.1 versus 27.8 days). Women with malignant ovarian tumors reported an average of 4.9 days of menstrual flow as compared with their controls who reported an average duration of 5.2 days of menstrual flow.

Menstrual molimen. The reported occurrence of specific types of premenstrual symptoms including breast engorgement, headache, weight gain, mental cloudi-

TABLE 13
RELATIVE RISK ASSOCIATED WITH NUMBER OF LIVE BIRTHS
AMONG EVER-MARRIED WOMEN

Number of live births	Women with malignant ovarian tumors	Women with carcinomas of LMP
0	2.13*	3.00
1-2	0.937	1.17
3+	1.00	1.00

* Significant at 0.05.

TABLE 14
REPORTED INCIDENCE OF PREMENSTRUAL SYMPTOMS

Positive history of irritability				Positive history of malaise				Positive history of irritability				Positive history of malaise			
Women with malignant ovarian tumors				Women with ovarian tumors				Women with carcinomas of LMP				Women with carcinomas of LMP			
N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
65	42	45	29	65	42	45	29	21	50	21	50	21	50	11	26
$P < 0.05$				$P < 0.05$				$P < 0.05$				$P < 0.05$			

ness, and insomnia was similar in women with ovarian cancer and in their controls. Women with ovarian cancer, however, reported a history of marked irritability and generalized malaise preceding their menstrual flow significantly more often than did their controls (Table 14). These symptoms appeared to be highly correlated as an equal number of subjects reported a history of both conditions.

Difficulties with menstruation. Women with ovarian cancer and their controls reported a history of menstrual difficulties such as dysmenorrhea, menorrhagia, and amenorrhea with approximately the same frequency. However, women with carcinomas of low malignant potential who gave a positive history of dysmenorrhea and menorrhagia reported that they experienced these disorders for significantly longer periods of time than did the controls who also reported a history of such disorders (see Table 15).

Menopause. Examination of data regarding menopause indicated that an equal proportion of women with ovarian cancer and their controls experienced a natural menopause prior to their cancer diagnosis or, in the case of the controls, study interview. The mean age at which they experienced their natural menopause was also very similar. When computation of the percentage of subjects who were menstruating at the time of cancer diagnosis (or at the time of the interview) was performed, it was found that significantly more women with ovarian cancer than controls were menstruating despite similarities in age distribution (see Table 16). This difference was due to the fact that 28% of the controls versus 1% of the women with malignant ovarian tumors had undergone surgical menopause. None of the women with carcinomas of low malignant potential versus 21% of their control group had undergone surgical menopause. The surgical menopause as discussed here means only hysterectomy and not removal of the ovaries.

Exogenous Hormones

There were no statistically significant differences in the incidence or duration of use of oral contraceptives between women with ovarian cancer and controls;

TABLE 15
INCIDENCE AND DURATION OF DYSMENORRHEA AND MENORRHAGIA

	Women with carcinomas of LMP		Control group	
	N	%	N	%
Percentage positive history of menorrhagia	23	55	20	48
Mean duration		16 years		7 years ($P < 0.02$)
Percentage positive history of dysmenorrhea	16	39	19	46
Mean duration		17 years		9 years ($P < 0.02$)

TABLE 16
MENOPAUSAL STATUS

	Women with malignant ovarian tumors		Control group		Women with carcinomas of LMP		Control group	
	N	%	N	%	N	%	N	%
Natural menopause	69	45	64	41	9	21	9	21
Surgical menopause	2	1	43	28	0		9	21
Other menopause	3	2	2	1	0		1	2
	$P < 0.01$							
Still menstruating*	81	52	46	30	33	79	23	55
	$P < 0.005$				$P < 0.05$			

* Percentage of subjects menstruating at time of diagnosis (for women with malignant ovarian tumors and women with carcinomas of low malignant potential) or at time of interview (for control group of women).

however, women with ovarian cancer did report use of oral contraceptives less frequently than their controls. Women who did not use oral contraceptives were 1.4 times more likely to develop malignant ovarian tumors and 1.78 times more likely to develop carcinomas of low malignant potential than those women who used oral contraceptives. The mean duration of oral contraceptive use among ever-married birth control users was approximately 40 months for both cases and controls.

The reported use of hormones for purposes other than birth control was quite similar between women with ovarian cancer and their controls.

Family Pregnancy History

The average age at pregnancy for mothers of women who subsequently developed a malignant ovarian tumor was 27.7 years and for the control group, 28.3 years. Similar means were noted among the mothers of women with carcinomas of low malignant potential (28.3 years) and their controls (27.4 years).

To determine whether or not the low gravidity noted among the cases could be attributed to certain sociocultural factors which influence the size of a woman's family, the mean number of siblings was determined for cases and controls. We found that cases and controls came from families of the same average size which indicates that the low-parity cases are not imitating low-parity mothers.

Family Cancer History

A family history of specific cancers such as ovarian, breast, and cervical was not reported with any more frequency among women with ovarian cancer as compared with their control groups. However, women with malignant ovarian

tumors did report significantly more cancer of the "female reproductive organs" in their relatives than did their controls (14/155 versus 2/155) ($P = 0.01$). Many women could not recall the specific gynecologic organ affected by the cancer, consequently the term "female reproductive organs" was used.

Benign Cysts

Only 7 out of 155 women with malignant ovarian tumors reported a (nonsurgically confirmed) history of benign ovarian cysts as compared with 21 of 155 controls. This finding was significant at the 0.005 level. The reported incidence of benign ovarian cysts among family members of women with malignant ovarian tumors and women with carcinomas of low malignant potential was higher than among relatives of their respective controls (e.g., 12/155 versus 5/155 and 6/42 versus 0/42, respectively).

In addition, women with malignant ovarian tumors reported a significantly higher prevalence of breast cysts among female relatives than did those women who were their controls (26/155 versus 12/155) ($P = 0.01$).

Surgical History

There was no difference between women with ovarian cancer and the control women with regard to a past history of uterine suspension, cervical repair, tubal ligation, cesarean section, or thyroidectomy.

Stress

Women with ovarian cancer and their controls also reported similar histories with regard to exposure to stressful situations or duration of stress.

Douching and Hygiene

Women with ovarian cancer and their controls did not differ in their reported use of douches, consistency of use, average age first began douching, or average number of years of douching (see Table 17).

TABLE 17
DOUCHING HISTORY

	Women with malignant ovarian tumors		Control group		Women with carcinomas of LMP		Control group	
	N	%	N	%	N	%	N	%
Percentage ever used	97	78	112	72	23	70	36	86
Consistent use only	61	49	70	45	14	42	18	43
Total	125	100	155	100	33	100	42	100
Average age began douching	22.4		21.8		21.5		20.0	
Average number of years douched	23.3		22.8		17.1		15.7	

TABLE 18
AGE OF RUBELLA INFECTION

Age (years)	Women with malignant ovarian tumors		Control group	
	N	%	N	%
Less than 12	35	54.7	54	75
12-18	17	26.6*	6	8.3
19+	12	18.8	12	16.7
Total	64	100.0	72	100.0

* $P < 0.02$.

No differences with regard to circumcision among male partners were noted among cases and controls.

Infectious Diseases

The subjects' experience with a variety of childhood illnesses and other infectious diseases was ascertained. Women with malignant ovarian tumors reported having rubella infection during the 12- to 18-year age interval significantly more often than the controls (see Table 18). The study subjects who reported infection with rubella during the ages 12 to 18 were 3.9 times more likely to develop ovarian cancer than women who were infected during their early childhood years (Table 19). The prevalence (i.e., whether or not the subject ever had the disease) of rubella infection is not a distinguishing risk factor but the reported age of the infection does appear to be associated with risk of ovarian cancer development.

Perhaps secondary to the uncertainty in distinguishing rubella from measles for these study subjects, measles demonstrates a similar trend of changing risk with age. The reported age of mumps infection was not found to be strongly associated with the development of ovarian cancer but does repeat the age-risk trend noted for rubella and measles. This raises the question as to the specificity of the association. Perhaps more than one virus may be contributing to later ovarian neoplastic development.

The potential etiologic significance of the timing of the rubella infection and the menarche to ovarian cancer prompted us to cross-tabulate age of rubella infection and age of menarche (Table 20). Among women who experienced menarche at age

TABLE 19
RISK OF MALIGNANT OVARIAN TUMORS ASSOCIATED WITH
SELECTED INFECTIOUS DISEASES

	Age		
	6-11	12-18	19 +
Mumps	1.08	1.35	0.90
Measles	0.82	2.67	0.82
Rubella	0.02	3.90*	1.38

* Significant at 0.05.

TABLE 20
ASSOCIATION OF AGE OF MENARCHE AND RUBELLA WITH OVARIAN CANCER

Age of Rubella infection	Age at menarche							
	11 or younger				12 or older			
	Women with malignant ovarian tumors		Control group		Women with malignant ovarian tumors		Control group	
	N	%	N	%	N	%	N	%
11 or younger	9	69	10	83	26	51	44	75
12 or older	4	31	2	17	25	49	15	25
Total	13	100	12	100	51	100	59	100
	$P = 0.72$				$P = .02$			

11 or younger, the age of rubella infection was not found to be associated with malignant ovarian tumors ($P = 0.72$). In women experiencing menarche at age 12 or older, however, the age of rubella infection was associated with ovarian cancer ($P = 0.02$).

In addition to the aforementioned, data analyses found cases and controls similar in general identifying characteristics and in the majority of variables under study (see Table 21).

TABLE 21
COMPARABILITY BETWEEN WOMEN WITH OVARIAN CANCER AND CONTROL WOMEN

Sex, age, race
Religious preference
Marital status
Socioeconomic status
Years of completed education
Residence patterns
Age at first coitus
Age at menarche and menopause
Length of menstrual cycle
Spontaneous abortions and stillborns
Problems during pregnancy
Pregnancy/live birth ratios
Defective children
Married years at risk of pregnancy
Mother's age when pregnant with her daughter who later developed ovarian cancer
Douching history
Exposure to stress
Operative procedures such as cesarean section, tubal ligation, uterine suspension, cervical repair, and thyroidectomy
Circumcision in male partners

DISCUSSION

Reproductive History

Significant differences were consistently noted between cases and controls with respect to both gravidity and parity. Women with ovarian cancer had significantly fewer pregnancies even when controlling for age, race, socioeconomic status, residence, religion, marital status, number of years at risk of pregnancy, and use of birth control measures; were more frequently nulligravida; reported more difficulty conceiving; and tended toward a later age at first pregnancy than their controls. Calculations of relative risks indicated that nulligravida per se is more influential in establishing risk than the actual number of pregnancies.

In married, sexually active women decreased or zero gravidity could be attributed to ovarian-mediated infertility (either relative or complete), nonovarian-mediated infertility, the conscious use of contraceptive techniques, or an infertile husband.

Certain results of the present study tend to support the hypothesis advanced by Fathalla [5] that "extravagant" and incessant ovulation in the woman may be an inciting factor for ovarian cancer development of the epithelial type. It was noted in our study that: (1) Cases reported use of oral contraceptives less often than their controls, (2) decreased gravidity in cases seemed not to be attributable to anovulatory cycles, and (3) cases reported a history of surgical menopause significantly less often than the controls. These three observations seem to indicate that the cases ovulate for a greater proportion of their reproductive years than their controls. Fraumeni *et al.* have observed that in white Catholic nuns (whose nearly universal nulligravida cannot be ascribed to infertility) there was a higher frequency of ovarian cancer than in the national white female population [8]. Furthermore, the experimental acceleration of ovulation and egg production in the domestic fowl with the early appearance of ovarian adenocarcinoma and no tumors developed in control hens under normal conditions lends supportive evidence that continued ovulation may be associated with ovarian cancer development.

Women with ovarian cancer demonstrate a significantly lower mean number of pregnancies resulting in live births than their controls. This decreased parity can, however, be attributed to decreased gravidity generally as there were essentially no differences in the number of spontaneous abortions and voluntary interruptions of pregnancies among cases and controls. Based on our study data it would seem that women with ovarian cancer do not demonstrate excessive reproductive handicaps if they do become pregnant.

Exogenous Hormones

Our study data found that women with ovarian cancer used oral contraceptives less often than their controls. This finding is interesting in relation to the hypothesis suggested by Stadel [24] of an association between increased pituitary gonadotrophins and the subsequent development of ovarian cancer. The incidence of ovarian cancer increases rapidly after age 40 which is also when significant numbers of women began reporting natural menopause [14]. The menopause is

associated with a decline in estrogen production and a rise in pituitary gonadotropins. This association has been noted by McGowan and Davis as well as others in the experimental production of ovarian neoplasms in animals [18]. The current widespread use of estrogens for treatment of menopausal symptoms or estrogens in oral contraceptives may decrease the risk of ovarian cancer in women by suppressing pituitary gonadotrophin production. However, the present study did not reveal decreased use of estrogen for menopausal symptoms among cases as compared with their controls.

Family History of Cancer

The observation that significantly more cases than controls reported a family history of "gynecologic cancer" may indicate a genetic predisposition toward the development of ovarian cancer of the epithelial type. The literature has established the occurrence of certain "familial cancers," among them gynecologic cancers [6,7,11,12,16].

Viral Diseases

Extensive information regarding childhood diseases was collected in the present study. It must be emphasized that these data represent the patients' subjective reporting of their illness and therefore are limited by the adequacy of recall, completeness of the woman's knowledge of her disease experiences, and lack of confirmation such as with serology.

A previous study had noted the negative association between mumps and ovarian cancer (i.e., ovarian cancer patients reported a history of mumps significantly less often than their controls) [25]. As previously stated, no differences were noted in this study among cases and controls as to the reported incidence of any of the childhood diseases. The strong association between reported age at rubella infection and increased risk of ovarian cancer noted in the present study is, however, of particular interest.

Children with congenital rubella harbor virus in the nasopharynx, lungs, and spinal fluid for at least six months after birth. In a few children, the virus persists until they are about a year old. If cataracts are removed from patients with congenital rubella at age 3 to 4, the virus can often be cultured from the lens tissue [20]. The rubella virus may lay dormant in the body for 12 to 15 years before it manifests itself as a chronic progressive panencephalitis [4,20]. With the ability of the rubella virus to persist for years after the initial infection, one could theorize that a change in ovarian function or morphology is produced which, although it does not interfere with ovulation, might alter cell surfaces making impregnation difficult or impossible.

It is also of interest that a trend toward increased risk of ovarian cancer in association with a reported history of measles and mumps in the 12- to 18-year age range was also present among our study population. The measles virus has also been associated with many years of dormancy [9]. Although these findings in our study did not carry the same strength of statistical association noted for rubella infections, the possibility exists that the incidence of a variety of common childhood viruses in the peripubertal years may increase the risk of ovarian cancer.

In this country required immunization for rubella for all children under 12 is being even more rigidly enforced. In Sweden and elsewhere in Europe rubella vaccination is carried out in girls, ages 11 to 14 years, without prior serologic tests [13]. So far most of the cases of subacute sclerosing panencephalitis have involved congenital rubella, although a case that was reported by Lebon and Lyon may have resulted from acquired rubella [10]. The establishment, maintenance, and periodic review of immunization records by all responsible parties in this very interesting and possibly serious link between rubella infection in the teenage girl and ovarian cancer development are essential.

The importance of this study's findings to the clinician lies in our increased ability to define the woman at risk of developing ovarian cancer of the epithelial type. Although some of our results are tentative and require confirmation through further study, a number of potentially important risk characteristics have been identified. These are: A family history of gynecologic cancer and cystic ovaries; exposure to an infectious disease such as rubella during the peripubertal period; zero or low gravidity; stated difficulty in conceiving; premenstrual symptoms; lack of exposure to oral contraceptives; menopause usually not surgically induced; and for women with carcinomas of low malignant potential, a later onset of the menarche as well as menstrual disorders.

Certainly, the era of preventive oncology is upon us.

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Chemotherapy of Squamous Cell Carcinoma of the Vulva: A Review

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A review of published experience with chemotherapy for the treatment of recurrent or metastatic squamous cell carcinoma of the vulva found data for nine different drugs used as single agents. Only 75 patients have been treated. Bleomycin produced responses in 19 out of 31 patients, adriamycin in 4 out of 6 patients, and methotrexate in 2 out of 5 patients. Seven patients with squamous cell carcinoma of the vulva were treated with five different combination chemotherapy regimens. Responses only occurred with regimens which contained bleomycin and methotrexate.

INTRODUCTION

Squamous cell carcinoma of the vulva accounts for 3-4% of all primary malignancies of the genital tract. Radical vulvectomy and bilateral groin dissection with or without pelvic lymphadenectomy is the standard method of therapy employed in over 80% of all cases.

There is to date no published review of results in treating squamous cell carcinoma of the vulva with cytotoxic chemotherapy. This modality has rarely been employed in treating squamous cell carcinoma of the vulva, because patients are often considered to be too old with diminished performance status. Recently new dosage schedules and newer drugs have been employed with a satisfactory risk-benefit ratio for these elderly patients. Chemotherapeutic agents can have a beneficial role in the management of selected patients with recurrent or metastatic squamous cell carcinoma of the vulva, and reexamination of this treatment modality by gynecologic oncologists is now appropriate. A controlled comparison of the most effective regimens may be feasible.

METHODS

A literature review was undertaken to discover which chemotherapeutic agents have been used in the treatment of metastatic and/or recurrent squamous cell

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REVIEW

The Epidemiology of Ovarian Cancer¹

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The descriptive and analytical epidemiology of ovarian cancer is reviewed, starting from the substantial geographical differences, with high rates in North America and Europe and low rates in developing countries and Japan, although, on a worldwide scale, almost 50% of 140,000 total cases occur in developing countries. Over the past decades, incidence and mortality rates have remained approximately stable in high-risk areas, but have generally tended to rise in low-risk areas. In the past, ovarian cancer was more common in higher social classes, but sociocultural differences seem to have flattened off over recent decades. In etiological terms, the protection afforded by multiparity and oral contraceptive (OC) use is well established, with relative risks (RRs) of the order of 0.5 for multiparae and OC users. There is also consistent evidence that risk increases with late age at menopause. Less consistent and weak, if any, are the effects of age at menarche and first birth, although there is hint that a first birth over age 35 is not protective. It is conceivable that diet can play an important role in ovarian carcinogenesis, but only scattered data are available to suggest that high fat consumption may represent an indicator of risk. Available knowledge on ovarian cancer epidemiology is also discussed in relation to models of carcinogenesis and implications for prevention and public health. © 1991 Academic Press, Inc.

INTRODUCTION

In developed areas of the world ovarian cancer is a common neoplasm, being among the five leading sites of incidence and mortality [1,2]. On a worldwide basis the total number of cases has been estimated to be approx-

imately 140,000, thus representing over 4% of all cancers in women and the seventh leading site of incidence [3].

Besides the observation that ovarian cancer is a disease of affluent societies, a few aspects of its epidemiology are well understood, such as its inverse relationship with parity and oral contraceptive (OC) use. These factors explain, however, only a minor proportion of the substantial differences in worldwide ovarian cancer incidence and mortality. A role for other menstrual, reproductive, and hormonal factors, as well as the potential influence of diet, has been suggested in some investigations, but is far from established or well quantified.

In the present review, therefore, available knowledge on the epidemiology of ovarian cancer is summarized and discussed. In addition to a brief mention of histopathological characteristics, the article includes four subdivisions: histopathology, descriptive epidemiology, risk factors, and conclusions and etiopathogenetic factors.

HISTOPATHOLOGY

Three chief groups of tumors originate from the ovary: epithelial, sex-cord mesenchymal, and germ-cell tumors.

According to Scully [4], epithelial forms represent two thirds of all primary ovarian tumors and 90% of these are malignant. Germ-cell tumors account for about 25-30% of all ovarian tumors (dermoid cysts and dysgerminomas are the more common varieties in this group) and sex-cord and stromal tumors account for an additional 6%, approximately.

The most common epithelial tumors derive from the surface epithelium of the ovary, the descendant of the ectodermic epithelium of the embryonic gonadal ridge (i.e., Mullerian ducts). When a neoplasia develops from the surface epithelium, it acquires the potential of its em-

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byonic neighbor to differentiate into epithelia resembling those of various Mullerian duct derivatives. Serous tumors histologically resemble fallopian tubes, endometrioid tumors the endometrium, mucinous tumors the endocervix, and clear-cell tumors the endometrium during pregnancy.

Since germ cells also are totipotent, a wide variety of neoplasms may develop from them too. Indeed germ-cell tumors recapitulate stages of embryonic development: dysgerminomas (from primitive germ cells), endodermal sinus tumors (from yolk sac development), and various kinds of tumors composed of or containing cytotrophoblastic cells. Teratomas also belong to this group of tumors: they are solid and predominantly contain mature tissue.

Finally, sex-cord mesenchyme tumors derive from the sex cord and stromal component of the developing gonad. The embryonic sex cord develops into Sertoli cells in the testis and granulosa cells in the ovary. The stroma develops into Leydig cells in the testis and stromal lutein cells and fibroblasts in the ovary.

Population-based data on the histologic distribution of ovarian cancer are scanty. In the United States Third National Cancer Survey (1969-1971) [5] all newly diagnosed cases of ovarian cancer in nine areas of the United States were identified and reported according to age and histologic type. Nonepithelial types predominated in younger women and their incidence did not change with increasing age, whereas epithelial types rose rapidly with age, overtaking in frequency nonepithelial types at 15-24 years of age. The most common epithelial subgroups were serous tumors (36.3%), followed by mucinous (13.8%), endometrioid (2.0%), and clear-cell tumors (0.6%). Sex-cord mesenchymal and germ-cell tumors accounted for about 2% of all ovarian tumors. The White/Black ratio was above unity in epithelial types but below unity in nonepithelial tumors. Also in this study, however, a large number of "not otherwise specified" carcinomas, adenocarcinomas, and cystadenocarcinomas was recorded (i.e., approximately 1/3 of the total). Other published material dealing with the relative frequency of individual histologic types mostly derives from hospital-based series, where, however, substantial discrepancies may derive from chance or different referral patterns with respect to age.

Another source of heterogeneity within epithelial ovarian cancers is the presence of estrogen receptors (ER) and progesterin receptors (PGR). Although the ovary is not a primary target tissue for estrogens, ER and PGR have been found in benign and malignant ovarian neoplasms [6-8]. Extracts of more than 50% of epithelial ovarian cancers contain at least 20 fmol ER per milligram soluble cytosol protein, or at least 500 fmol ER per gram tissue wet weight, in contrast to the much smaller quantities of ER observed in normal ovarian tissue [6,7]. The

content of ER and PGR in ovarian carcinomas has not been correlated consistently with the histologic type and grade of the carcinoma. High levels of ER and PGR were found to affect favorably survival in some reports [8] but not in others [9]. Therefore it has not been established convincingly whether a correlation between ER or PGR content and clinical prognosis comparable to that found in breast cancer exists in ovarian cancer.

The considerable variety among and difficulty in diagnosing histologic types of ovarian tumors has always hampered the interpretation of etiological data. Most epidemiological work, including the present review, however, simply refers to ovarian cancer by and large, or, in most recent analytical studies, epithelial ovarian cancer.

DESCRIPTIVE EPIDEMIOLOGY

Geographical Differences

There is a large variation in the incidence of ovarian cancer in different countries (Tables 1 and 2). The highest risk areas are European (especially Nordic countries and the United Kingdom) and North American countries, with a ratio of approximately 4 between the highest and the lowest incidence countries worldwide. Lower rates are generally observed in South European areas. Developing countries and Japan have the lowest incidence rates although registration deficiencies should always be considered in the interpretation of these findings (Table 2).

With respect to the incidence of ovarian cancer as a percentage of all incident cancer cases in women, less marked differences between various countries are observed. Ovarian cancer, in fact, accounts for about 4-6% of all incident cancer cases in women in most areas. It seems, therefore, that ovarian cancer is a relevant public health problem, especially in Westernized societies or developed countries, but more than 50% of new cases diagnosed every year worldwide occur in developing countries [3].

A possible bias in the interpretation of international difference is the influence of the number of ovaries at risk. Where the rate of hysterectomy is high and bilateral oophorectomy tends to be performed routinely also in relatively young patients, such practice may affect perceptibly the denominator and lead to a certain degree of underestimation of ovarian cancer rates.

Intracountry Variability

The suggestion that ovarian cancer risk is directly associated with modern Western life-style is further confirmed by the consistent intracountry variation in incidence rates. Considering the incidence data from 20 regions in the world where it has been possible to distinguish urban and rural areas [1], higher rates of ovarian

TABLE 1
Incidence of Ovarian Cancer (and Percentage of All Incident Cases in Women) in Selected Developed Countries (World Population Age-Standardized Rates), 1978-1982

Country	Incidence rate/100,000	Percentage of all incident cancer cases		Country	Incidence rate/100,000	Percentage of all incident cancer cases	
		in women				in women	
Europe							
Norway	15.3	7.2		Switzerland, Vaud	9.0	4.5	
Sweden	15.2	6.8		Ireland, Southern	9.0	5.0	
Denmark	14.5	6.0		France, Dombes	9.0	5.3	
Iceland	13.9	6.0		France, Calvados	8.2	5.1	
UK, North East Scotland	13.2	6.1		Italy, Ragusa	7.9	5.5	
FRG, Hamburg	12.8	6.0		France, Isere	7.8	4.6	
UK, Oxford	12.6	6.2		Romania, County Cluj	6.6	4.9	
UK, Southeast Scotland	12.1	5.1		Spain, Tarragona	6.5	4.2	
GDR	12.1	6.5		Spain, Navarra	6.4	4.2	
Switzerland, Geneva	12.0	5.4		Hungary, Szabolcs	5.8	5.1	
Poland, Cracow	12.0	6.6		Spain, Zaragoza	5.4	4.2	
Poland, Nowy Sacz	11.9	9.5		North America			
UK, Trent	11.8	5.7		Canada			
UK, South Western	11.8	5.9		Manitoba	12.8	4.9	
UK, South Thames	11.7	6.2		Ontario	12.0	5.0	
Italy, Parma	11.7	5.8		Quebec	11.4	4.8	
Netherlands, Heindhoven	11.6	5.7		Alberta	11.1	5.3	
Switzerland, Neuchatel	11.3	5.0		British Columbia	10.6	4.7	
UK, North Western	11.2	5.5		United States			
France, Bas Rhin	11.2	5.5		Los Angeles (Whites)	13.5	4.9	
UK, East Scotland	11.1	4.9		New York State (except City)	12.6	5.0	
UK, Birmingham	11.1	5.9		Iowa	12.4	5.2	
UK, West Scotland	11.0	5.0		Detroit (Whites)	12.3	4.6	
UK, Mersey	10.8	5.0		Seattle	12.1	4.6	
Switzerland, Zurich	10.5	5.2		Connecticut (Whites)	12.0	4.6	
Poland, Warsaw	10.5	6.6		Japan			
Italy, Varese	10.2	5.0		Nagasaki	6.9	4.1	
Finland	9.8	5.6		Osaka	4.4	2.9	
Yugoslavia, Slovenia	9.5	6.0		Miyagi	4.2	2.9	
UK, North Scotland	9.5	4.6		Australia			
Hungary, Vas	9.4	5.7		New Zealand (Maori)	10.9	3.7	
Czechoslovakia, Slovakia	9.2	5.6		New Zealand (non-Maori)	8.9	4.3	
Switzerland, Basel	9.1	4.4					
FRG, Saarland	9.1	4.3					

Source: Muir et al. [1].

cancer in urban populations were reported in 14. The excess was about 20-50%, irrespective of whether the region was in a low- or high-risk area (Table 3).

In the United States the incidence of ovarian cancer was approximately 50-70% higher among Whites than among Blacks in all seven areas covered by the Surveillance Epidemiology and End Results (SEER) Programs (Table 4) and in both premenopausal and postmenopausal women [1, 10].

Trends in Ovarian Cancer Incidence

Trends in ovarian cancer rates over the last 20 years in selected areas are presented in Table 5. Rates in the highest risk areas, for example, the United States, Can-

ada, Scandinavia, and the United Kingdom, remained over the period 1958-1982 almost constant while rising values were observed in low-risk areas such as Japan, India, and Singapore (Chinese population) [1, 10-13].

Besides improvements in diagnostic and registration accuracy, which might have produced artifactual increases, especially in less developed areas, an increased frequency of oophorectomy (often performed together with hysterectomy in peri- or postmenopausal women), especially in developed countries, should be taken into account in the interpretation of temporal trends.

An additional difficulty stems from the presence of complex cohort effects. In several developed countries younger women tend to show substantial declines in ovarian cancer risk, possibly due to the protection afforded

TABLE 2

Incidence (World Population Age-Standardized Rates) of Ovarian Cancer and Percentage of All Incident Cases in Women in Selected Developing Areas, 1980

Country	Incidence rates/100,000	Percentage of all incident cancer cases in women
Africa		
Middle Africa	5.3	5.0
Western Africa	4.6	7.8
Northern Africa	4.1	4.7
Eastern Africa	3.6	4.7
Southern Africa	2.4	3.1
Central/South America		
Temperate South America	8.7	3.6
Tropical South America	5.7	3.5
Caribbean	5.2	4.3
Central America	1.9	2.4
Asia		
USSR	9.3	5.2
Melanesia	5.5	5.8
South Eastern South Asia	5.4	6.1
Micro/Polynesia	4.6	5.3
Western Asia	4.1	4.5
China	4.0	3.7
Southern Asia	3.5	4.4
Other East Asia	2.8	2.4

Source: Parkin *et al.* [3].

by OCs [14]. At older ages, conversely, upward trends, which are probably partly real and partly due to improved diagnosis or death certification, are still evident.

RISK FACTORS

Sociocultural Indicators

Education, as a sociocultural indicator, is examined in Table 6. In 5 out of 10 studies, cases were more educated than controls, and in 1 study, conducted in China [15], the difference was statistically significant (Table 6). Such a sociocultural gradient seems, however, to have flattened off over more recent years, especially in studies from developed areas of the world [16,17].

In other investigations different social indicators such as annual income or social class were examined, with rather inconsistent results. For instance, Slattery *et al.* [18] reported a nonsignificant decrease in ovarian cancer risk in the upper level of income, whereas Beral *et al.* [19] and Booth *et al.* [20] found that cases belonged more frequently to the higher social class. Generally the higher proportion of women of upper social class among cancer cases as compared to controls is interpreted in terms of lower fertility in this group, as the number of children and the economic level appear to be in most instances inversely related.

TABLE 3

Ovarian Cancer Incidence Rate/100,000 Women in Urban and Rural Populations in Selected Countries (World Population Age-Standardized Rates)

Country	1975		1980	
	Urban	Rural	Urban	Rural
Japan, Fukuoka	3.2	2.9		
Japan, Miyagi	4.2	2.6	4.9	3.3
Japan, Osaka	3.5	2.1		
Czechoslovakia, Slovakia	12.1	7.7	12.2	8.8
FRG, Saarland			10.2	6.9
France, Calvados			7.9	8.8
France, Doubs	7.2	5.7	9.9	7.1
Hungary, Szabolcs	9.0	6.0	7.2	5.4
Norway	14.8	14.2	15.9	14.8
Romania, County Cluj			6.4	7.1
Switzerland, Vaud	12.6	14.9	6.9	10.3
UK, England and Wales			11.1	10.9
Austria, North South West	9.2	7.9	9.2	7.8
Finland	11.8	9.8		
France, Bas Rhin	11.3	10.7		
Poland, Cieszyn	9.6	7.7		
Poland, Katowice	8.0	7.9		
Poland, Warsaw	11.9	9.2		
Spain, Zaragoza	5.2	5.3		
UK, North Western	10.3	6.7		

Source: Waterhouse *et al.* [10]; Muir *et al.* [1].

Menstrual History

Age at menarche. The role of age at menarche in ovarian cancer risk has been extensively investigated. The majority of the studies [17, 20–22] found a moderately increased risk in women with early menarche. The estimated RRs were of the order of 1.2 to 1.5 in women whose menarche occurred below age 12 as compared to that of those with menarche at age 15 or more (Table 7).

TABLE 4

Age-Adjusted Ovarian Cancer Incidence Rates/100,000 Women in Whites and Blacks in Selected SEER Areas: United States (World Population Age-Standardized Rates)

Area	1975		1980	
	Whites	Blacks	Whites	Blacks
Alameda	14.0	8.7	12.1	10.5
Bay Area	14.4	8.5	12.9	9.0
Los Angeles	13.9	9.6	13.5	8.0
Connecticut			12.0	7.3
Atlanta	12.2	6.9	12.6	8.4
New Orleans	9.3	5.5	11.0	7.2
Detroit	12.8	9.8	12.3	8.8

Source: Waterhouse *et al.* [10]; Muir *et al.* [1].

TABLE 5
Trends in Incidence of Ovarian Cancer in Selected Areas, 1960-1980 (World Population Age-Standardized Rates)

Country	Calendar period*				
	1958-1962	1963-1967	1968-1972	1973-1977	1978-1982
Canada					
Newfoundland	9.0	9.9	11.7	9.2	8.8
Manitoba	15.4	14.3	11.6	10.8	12.8
Quebec	—	7.5	9.6	9.2	11.4
Alberta	9.3	10.0	9.8	10.3	11.1
Saskatchewan	10.0	10.3	11.0	10.7	11.9
British Columbia	—	—	14.0	12.2	10.6
United States					
Hawaii					
Caucasian	11.4	14.8	13.7	9.4	11.0
Japanese	7.5	9.4	6.9	7.0	8.0
Chinese	—	13.1	5.8	7.3	7.8
Hawaiian	15.9	16.6	11.6	11.1	14.1
Filipino	—	4.8	9.0	4.6	8.8
Alameda					
White	—	12.6	13.5	14.0	12.1
Black	—	10.4	10.3	8.7	10.5
Connecticut	13.2	11.3	12.5	12.1	12.0
New York State	10.2	—	11.5	13.0	12.6
Colombia, Cali	12.8	9.2	8.0	10.1	7.8
Jamaica	7.4	8.3	8.6	7.0	—
Puerto Rico	4.9	5.1	5.3	5.3	5.9
Japan, Miyagi	2.2	1.9	2.8	3.4	4.2
India, Bombay	—	6.1	4.8	7.2	7.2
Singapore					
Chinese	3.2	—	6.0	5.7	8.6
Israel					
All Jews	11.9	11.4	13.0	12.6	11.9
FRG, Hamburg	17.6	—	11.5	13.3	12.8
Slovenia	8.1	8.6	10.1	10.7	9.5
GDR	—	11.3	12.1	12.2	12.1
Hungary					
Szabolcs	—	5.6	5.6	6.6	5.8
Vas	—	9.0	9.0	8.6	9.4
Poland					
Warsaw	—	11.2	11.4	11.9	10.5
Cracow	—	6.3	9.1	11.4	12.0
Denmark	13.1	13.5	15.1	14.6	14.5
Finland	9.0	10.1	10.4	10.9	9.8
Norway	11.5	11.5	14.2	14.6	15.3
Sweden	13.1	14.4	15.1	15.9	15.2
United Kingdom					
Oxford	—	10.1	11.7	11.4	12.6
Liverpool	9.7	10.4	10.1	9.9	10.8
South Metropolitan	10.6	—	—	—	—
Birmingham	10.1	10.8	11.3	11.0	11.1
New Zealand					
Non-Maori	—	11.9	11.4	11.3	10.5
Maori	12.6	11.7	13.3	12.7	10.9

*Data are not necessarily for the whole 5 years.

Source: Doll *et al.* [11]; Doll *et al.* [12]; Waterhouse *et al.* [13]; Waterhouse *et al.* [10]; Muir *et al.* [1].

A stronger inverse relation emerged from a study in Shanghai, China [15], where very late menarche (i.e., at age 18 or over) was not unusual. Finally, no apparent

relationship was found in three other studies from the United States [23-25] and one from Norway [26].

Available evidence thus indicates that age at menarche

TABLE 6

Percentage Distribution of Ovarian Cancer Cases and Controls in the Upper Educational Level (>12 Years of Education) in Selected Case-Control Studies

Author [Ref.]	Ovarian cancer	Control
West [44]	11.3 (30)*	8.8 (18)
Wynder <i>et al.</i> [23]	25.0 (38)	15.0 (44)
Franceschi <i>et al.</i> [21]	21.0 (34)	20.0 (113)
Cramer <i>et al.</i> [49]	26.0 (38)	27.0 (38)
Tzonou <i>et al.</i> [29]	17.0 (26)	19.0 (48)
CASH Study [16]	47.0 (231)	52.2 (2207)
Harlow <i>et al.</i> [53]*	50.9 (59)	61.4 (97)
Cramer <i>et al.</i> [72]	60.4 (142)	51.9 (124)
Shu <i>et al.</i> [15]*	14.5 (25)	2.9 (5)
Parazzini <i>et al.</i> [17]	14.7 (93)	15.6 (254)

* The corresponding numbers of cases and controls are shown in parentheses.

* Borderline tumors.

* $P < 0.01$.

has very little if any effect on ovarian cancer risk in developed countries. The possibility that very late menarche may exert some protection deserves further attention in studies from developing countries.

Age at menopause. At least 12 studies [15,17,20-24,26-30] considered the relationship between age at menopause and ovarian cancer risk (Table 8). In seven [15,17,20-22,24,29] a direct relationship was found.

Such direct association with age at menopause tends to be long lasting, being present also among older women [17]. The strength of this association, however, seems to vary across different studies, ranging from 1.4 [22] to 4.6 [21] in the highest category of age at menopause. In four

TABLE 7
Relative Risk Estimates of Ovarian Cancer and Age at Menarche

Author [Ref.]	Age at menarche			
	<12	12-14	>14	>17
Wynder <i>et al.</i> [23]	1*	1.5	1.1	
Hildreth <i>et al.</i> [24]		Not significant		
Franceschi <i>et al.</i> [21]	1.5	1.3	1*	
Tzonou <i>et al.</i> [29]	1*	1.2	1.5	1.9
Kvale <i>et al.</i> [26]*	1*	1.0	1.0	
Wu <i>et al.</i> [22]	1*	0.9	0.6	
Booth <i>et al.</i> [20]	1.3	0.9	1*	
Parazzini <i>et al.</i> [17]	1.1	1*		
Shu <i>et al.</i> [15]	4.4	3.4	3.2	1*
Gwinn <i>et al.</i> [25]	0.9	1*	1.0	

* Reference category.

* Cohort study.

TABLE 8

Relative Risk Estimates of Ovarian Cancer and Age at Natural Menopause

Author [Ref.]	Age at natural menopause				
	<40*	41-45	46-49	50-54	≥55
Wynder <i>et al.</i> [23]		No association			
Annegers <i>et al.</i> [28]		1.1			1*
Hildreth <i>et al.</i> [24]	1*	2.0	2.3		2.9
Franceschi <i>et al.</i> [21]	1*		2.9		4.6
Tzonou <i>et al.</i> [29]	1*	0.7	1.6		1.5
Harlow <i>et al.</i> [30]		No association			
Kvale <i>et al.</i> [26]*	1*		1.0	1.0	1.0
Wu <i>et al.</i> [22]*	1.1		1.3		1.4
Booth <i>et al.</i> [20]	1*		2.0		2.5
Parazzini <i>et al.</i> [17]	1*		1.3	1.5	1.7
Shu <i>et al.</i> [15]		1*	2.8	1.6	2.1

* Reference category.

* Cohort study.

* Reference category: premenopausal women.

other studies [23,26,28,30] no association emerged between age at natural menopause and ovarian cancer risk.

Such large differences are partially explainable by means of potential methodological problems, including definition of menopause or type of menopause, different age distribution, or adjustment for age and other covariates in the analysis. The overall evidence tends to weigh in favor of a real, although moderate, direct relationship between age at menopause and ovarian cancer risk.

Reproductive Factors

Number of pregnancies. Nulliparity and, even earlier, single marital status (see Wynder *et al.* [23]) have been very consistently related to high risk of ovarian cancer. A strong inverse association between completed family size and mortality from ovarian cancer in different countries and for successive cohorts in the same country has been reported [19].

The most debated issue is whether nulliparity and low parity per se or difficulty in conceiving facilitates the development of ovarian cancer. Unfortunately, infertility cannot be measured accurately in most epidemiological studies [31]. Many infertile women do not seek medical care for this problem, and even more women receive only an incomplete and inconclusive evaluation of the causes of their infertility and/or are unable to report accurately on them.

Some studies suggest, however, that pregnancies per se reduce the risk of ovarian cancer. Of at least 20 studies that have examined the question [21-27,29,32-43], 16 [21,25-27,29,32,33,35-43] showed a further decline in risk associated with full-term pregnancies beyond the first,

TABLE 9
Relative Risk Estimates of Ovarian Cancer and Parity and Age at First Birth

Author [Ref]	Parity				Age at first birth (years)					
	1	2	3	4	15	16-20	20-24	25-29	30-34	35
Wynder <i>et al.</i> [23]	1*	0.8	0.8	1.2	0.8					
Joly <i>et al.</i> [32]		2.1		1.3	1*	1*	1.4		2.3	
Casagrande <i>et al.</i> [33]		1*								
McGowan <i>et al.</i> [34]		1*		1.6	0.3*	1*	0.7		1.4	
Hildreth <i>et al.</i> [24]	1*	0.6	1.0		0.5*	1*	1.9	3.0*		2.5*
Cramer <i>et al.</i> [36]										
Unadjusted	1*			0.5*	0.5*	1*		1.1		1.2
Adjusted	1*			0.5*	0.5*	1*		1.0		1.0
Risch <i>et al.</i> [37]				0.8						
La Vecchia <i>et al.</i> [38]										
Unadjusted	1.9			1.7	1*	1*	2.7*	3.2*		4.0*
Adjusted	1.3			1.3	1*	1*	2.5*	2.8*		3.3*
Nasca <i>et al.</i> [39]	1	1.0	1.0		0.8	1	0.7	0.6	0.6	1.1
Tzonou <i>et al.</i> [29]		1*			0.7					
Lexher <i>et al.</i> [40]										
Unadjusted	1*	0.9	0.7		0.6	1*	0.8	1.2		1.2
Adjusted	1*	0.9	0.7		0.5	1*	0.8		1.1	0.9
Voight <i>et al.</i> [41]										
Unadjusted	1*	0.9	0.7		0.8	1*	1.0	0.8		1.2
Adjusted	1*	0.8	0.7		0.6	1*	0.9	0.6		1.0
Kvale <i>et al.</i> [26]										
Unadjusted	1.5	1.0	0.9	0.8	0.6*	0.7	0.9	0.9	1.4	1.0
Adjusted	1.4	1.0	0.9	0.9	0.7*	0.8	1.0	0.9	1.3	0.8
Mori <i>et al.</i> [43]		1*	1.1		0.4					
Wu <i>et al.</i> [22]	1*	0.9		0.9		1*	1.3	1.0		1.3
Gwinn <i>et al.</i> [25]	1	0.8	0.7*	0.4*	0.4*	1	0.8	0.7		0.5

* Reference category.

* 95% confidence interval does not overlap 1.

* Adjusted for parity or age at first birth, as appropriate.

* Cohort study.

thus suggesting that additional risk reduction was conferred by events accompanying each pregnancy.

Table 9 summarizes RRs from studies allowing an assessment of the effect of number of pregnancies and age at first pregnancy in parous women only [29,21-24,26,27,29,31-41,43]. It is apparent that the reduction in risk deriving from pregnancies beyond the first is weak and, partly on account of limited study size, it reaches statistical significance in only four investigations [24-26,36].

Age at first pregnancy. The suggestion that early age at first pregnancy may also protect against ovarian cancer came from four case-control studies [24,32,34,38]. Since multiply pregnant women usually start reproduction early in their life, independent effects of age at first pregnancy and number of pregnancies are difficult to establish. An Italian study [38] found a significant excess risk associated with age at first birth after adjustment for parity. Conversely, in three case-control studies [36,40,41] and one prospective investigation [26], no relationship with

age at first birth remained after adjustment for parity. Several other investigations did not find a consistent relationship between risk of ovarian cancer and age at first pregnancy [25,36,29], but in at least two further studies [20,29] the RR was elevated in women in whom first birth had occurred at age 35 or more. Furthermore, in these two studies, nulliparous women were not at increased risk over those who first bore a child at age 35 or more.

In conclusion, the effect of age at first pregnancy on ovarian cancer is probably weak, if any, but there are various suggestions that the protective effect of full-term pregnancy depends on the age at which it takes place, and that this protection may be weak, or absent, above age 35.

Incomplete pregnancies. Studies that provided information on ovarian cancer risk according to different outcomes of pregnancies are shown in Table 10. Seven investigations [17,20,26,29,33,37,43] showed that the decrease in ovarian cancer risk deriving from incomplete pregnancies is not appreciably different from that conferred

TABLE 10
Relative Risk Estimates of Ovarian Cancer and Different
Pregnancy Outcomes

Author [Ref.]	Abortions		Stillbirths
	Induced	Spontaneous	
West [44]	No difference		—
Wynder <i>et al.</i> [23]	≥ 1.1	≥ 1.8	—
Joly <i>et al.</i> [32]	—	No difference	No difference
Casagrande <i>et al.</i> [33]	—	0.1 1:0.54 ≥ 2.0	—
McGowan <i>et al.</i> [34]	—	No difference	No difference
Cramer <i>et al.</i> [36]	—	≥ 1.0	—
Risch <i>et al.</i> [37]	—	≥ 1.0	—
Nasca <i>et al.</i> [39]	—	0.1 1:1.1 2:1.1 ≥ 3.1	—
Tzonou <i>et al.</i> [29]	—	0.1 1:0.9 ≥ 2.0	—
Kvale <i>et al.</i> [26]	—	≥ 1.0	—
Mori <i>et al.</i> [43]	≥ 1.0	≥ 1.0	—
Wu <i>et al.</i> [22]	No difference		—
Booth <i>et al.</i> [20]	—	0.1 1:1.0 ≥ 2.0	—
Hartge <i>et al.</i> [46]	—	0.1 1:1.0 2:1.0 ≥ 3.1	—
Parazzini <i>et al.</i> [17]	—	0.1 1:0.9 ≥ 2.0	—
Shu <i>et al.</i> [15]	0.1 1:0.7 ≥ 2.0	0.1 1:1.3 ≥ 2.1	—
Whitemore <i>et al.</i> [45]	No difference		—

* Cohort study.

* 95% confidence interval does not overlap 1

by full-term pregnancies. The comparability with a few other studies in which no association emerged [15,22,32,34,44-46] and others [23,39], which showed nonsignificant inverse associations, is hampered by substantial differences in data analysis, such as inclusion of ever pregnant women only [23,32], choice of hospital controls with benign ovarian tumors or other gynecological and obstetrical complaint or hormone-dependent cancers [32,44], inclusion of stillbirths [15,39], and, by and large, intention to assess the role in cancer risk of predisposition to miscarriages as a possible outcome of pregnancy rather than pregnancy per se.

Oral contraceptives The protection afforded by combined OCs against subsequent ovarian cancer risk is one of the best established and most important, on a public

health scale, characteristics of epithelial ovarian cancer. Available results on this relationship are shown in Table 11. Nineteen out of twenty-one studies [16,20,22,24,25, 27,29,33-35,47-55] found relative risks below unity, and the sole apparent outlier was a study conducted in China [15] that included only 21-pill-users among the cases and 12 among the controls. The overall estimate of protection is approximately 40% in ever users of OCs and such protection increases with duration of use [56].

The favorable effect of OCs on lowering ovarian cancer risk seems to persist for some time (at least 15 years) after OC use has ceased, but the data do not provide definitive RR estimates for longer periods or in relation to latency or recency of use. Nulliparity (and perhaps infertility) increases the risk of ovarian cancer and, therefore, the possibility that nonusers tend to be more frequently infertile than OC users has been claimed to hamper the interpretation of the inverse association between OC use and ovarian cancer [54].

Along this line, OCs were reported to have a more limited protective effect in nulliparous than in parous women in two investigations [34,51]. However, in six other studies [22,29,46,47,49,55] no relations were found between the magnitude of the protective effect of OC use and strata of parity or marital status. Furthermore, no association emerged in published studies between ovarian cancer and barrier methods of contraception or IUD use

TABLE 11
Relative Risk of Ovarian Cancer and Oral Contraceptives

Author [Ref.]	Relative risk (95% CI) (ever vs never users)
Newhouse <i>et al.</i> [27]	0.6(0.3-1.1)
Casagrande <i>et al.</i> [33]	0.7(0.4-1.1)
McGowan <i>et al.</i> [34]	0.7(nonsignificant)
Hildreth <i>et al.</i> [24]	0.5(0.2-1.7)
Weiss <i>et al.</i> [47]	0.6(0.4-1.0)
Willett <i>et al.</i> [48]	0.8(0.4-1.5)
Cramer <i>et al.</i> [49]	0.4(0.2-1.0)
Rosenberg <i>et al.</i> [35]	0.4(0.4-0.9)
La Vecchia <i>et al.</i> [50]	0.6(0.3-1.0)
Tzonou <i>et al.</i> [29]	0.4(0.1-1.1)
CASH Study [16]	0.6(0.4-0.9)
Vessey <i>et al.</i> [51]	0.3(0.1-0.7)
Beral <i>et al.</i> [52]	0.6(0.1-2.3)
Harlow <i>et al.</i> [53]	0.4(0.2-0.9)
Wu <i>et al.</i> [22]	0.7(0.5-1.1)
Booth <i>et al.</i> [20]	0.5(0.3-0.9)
Hartge <i>et al.</i> [46]	1.0(0.7-1.7)
Shu <i>et al.</i> [15]	1.8(0.8-4.1)
WHO Study [54]	0.8(0.6-1.0)
Gwinn <i>et al.</i> [25]	0.5(0.4-0.7)
Parazzini <i>et al.</i> [55]	0.7(0.5-1.0)

* Relative mortality from a cohort study.

* Borderline tumors.

[20,55] (although a nonsignificantly reduced risk in IUD users was reported in a Chinese study [15]).

Since the incidence of ovarian cancer is already appreciable in middle age and survival is low, the protection attributable to OC use corresponds to a far from negligible number of deaths (i.e., probably several hundred per year in countries like Britain [51], where OC use has been frequent for a long time).

Hormonal replacement therapy. After a first report in the late 1970s [57], several studies examined the relationship between hormone replacement therapy (in particular estrogens) and risk of ovarian cancer (Table 12). Two studies [28,46] showed a lower but not significant risk in women who have ever used estrogens in menopause, whereas five studies [20,29,36,58,59] found a moderate increase in risk that was significant only in subgroups (i.e., women who underwent hysterectomy or in some histological subgroups).

Some authors reported a significant increase in risk only in tumors of endometrioid type [58,60], but this finding was not confirmed by others [30,59].

One study [15] investigated the association between other female hormones and risk of ovarian cancer: elevated RRs emerged in women who used medroxyprogesterone (2.8), diethylstilbestrol (5.4), or other hormones for the treatment of fertility problems (2.1), but these associations should again be discussed in relation to the possibility of indication bias.

Dietary Factors

Most of the links between ovarian cancer and diet are based on international differences or correlational studies. Positive correlations were observed with fats, proteins, and total calories and are in the same direction as those of endometrial and breast cancer [61,62].

Information from follow-up studies in selected populations is scanty and controversial: Seventh-Day Adventists, many of whom are lactovegetarians, showed ovarian cancer mortality 40% lower than that of the population of California [63], but Mormons in Utah had a standardized incidence ratio of 1.7 compared to the general population of the United States [64]. In a cohort of British nuns with very low intake of meat and fats, no reduction in risk was, however, apparent [65].

The results of case-control studies are somewhat more consistent (see, as a review, [66]). Among four studies [15,67-69] that considered various measures of fat intake (Table 13), three [15,68,69] found significant direct associations. In one of these [15], which was based on a Chinese population with wide ranges of consumption of various foods and was able to provide estimates of intake of several nutrients, the effect of fats persisted after adjustment for total calories, whereas, after allowance for

animal fat intake, no significant associations with caloric or proteins remained.

Within Seventh-Day Adventists, it has been reported that women who ate eggs or fried foods more frequently had an approximately threefold elevated risk of ovarian cancer [70]. An increased risk also emerged for daily meat consumption from a case-control study of 56 cases aged above 50 conducted in Japan [43].

Studies in Italy [69] and North America [69a] showed reduced risk with more frequent consumption of green vegetables and carrots and greater vitamin A intake. Vitamin C and total vegetables were protective also in a Chinese study [15]. Conversely, according to Slattery *et al.* [18], calories, fat, protein, fiber, and vitamin A and C did not appreciably alter the risk of developing ovarian cancer, whereas high intake of β -carotene appeared to confer protection. The mechanism of action of these nutrients is, however, far from being understood.

Since worldwide ovarian cancer risk is strongly correlated with per capita milk consumption and lactase persistence (i.e., the ability to digest lactose after infancy [71]), it has been hypothesized that lactose consumption rather than animal fat, may be a dietary risk for ovarian cancer and low transference activity a genetic marker for increased susceptibility [72]. The observation of hypergonadotropic hypogonadism in rodents fed with lactose-rich diet and in galactosemic women or carriers with reduced transference [72] provides some biological basis for the aforementioned associations, which however was not supported by the results of an epidemiological investigation [69a].

Alcohol and coffee consumption. Another component of diet which has been found to be protective in a large case-control study from the United States is ethanol [73]. This observation finds a possible biological interpretation in the reduced gonadotropin levels caused by high dose of alcohol consumption with consequent anovulation and hence protection against ovarian carcinogenesis [33,74]. The results of other studies are, however, largely inconsistent. A similar suggestion of a direct association between alcohol and ovarian cancer emerged from a large American hospital-based study [75], but in three other investigations from the United States [44,67,76], no significant association was found. In two European studies [29,77], the relationship between alcohol consumption and ovarian cancer risk was above unity with a significant positive trend with amount of alcohol ingested.

These apparent discrepancies could simply be discussed as the results of random fluctuation, but also could be viewed as reflections of a complex series of a socioeconomic, reproductive, dietary, or other correlates of a cohort drinking in different countries.

Findings from epidemiological studies on the relation

TABLE 12
Relative Risk Estimates of Ovarian Cancer and Estrogen Replacement Therapy

Author [Ref]	Relative risk (95% CI) (ever vs never users)	Comment
Annegers <i>et al.</i> [28]	0.5(0.3-0.9)	No trend in risk with duration of use
Hildreth <i>et al.</i> [24]	0.9(0.5-1.6)	No trend in risk with duration of use
La Vecchia <i>et al.</i> [60]	0.8(0.4-1.3)	
Endometrioid	2.3(1.3-5.3)	
Weiss <i>et al.</i> [58]	1.3(0.9-1.8)	No trend in risk
Endometrioid	3.1(1.0-9.8)	
Cramer <i>et al.</i> [36]	1.6(0.9-2.9)	RR for cyclic fashion, 2.5 (95% CI = 1.0-6.4); RR for duration of use longer than 5 years, 2.8 (borderline significance)
Tzonou <i>et al.</i> [29]		
Postmenopausal women	1.6	
Harlow <i>et al.</i> [53]	0.9	Borderline tumors; no trend in risk with duration of use
Harige <i>et al.</i> [30]	0.6(0.4-0.8)	No trend in risk with duration of use
Endometrioid type	0.5(0.3-0.8)	
Wu <i>et al.</i> [22]	0.9 (n.s.)	No trend in risk with duration of use
Booth <i>et al.</i> [20]		
Postmenopausal women	1.5(0.9-2.6)	
Women reporting hysterectomy	10.9(1.7-69.0)	
Nonhysterctomized women	1.2(0.7-2.3)	
Kaufman <i>et al.</i> [59]	1.2(0.8-1.9)	No trend in risk with duration of use
Endometrioid	0.9(0.3-3.0)	

ship between coffee consumption and risk of ovarian cancer are shown in Table 14. The risk estimates tended to be systematically (although moderately) above unity, and a pooled analysis based on crude data reached an RR of 1.3, with 95% confidence interval from 1.1 to 1.5, for regular coffee users versus nonusers [78]. Although confounding factors did not seem to be a major problem in these studies, and significance was formally obtained with such large numbers of cases, it is not possible to make an inference of causality with such a moderate risk elevation.

Family History

There are several clinical observations suggesting familial aggregations of ovarian cancer. Almost 150 kindreds in which two or more women were affected by

epithelial ovarian cancer have been published [79-81]. Besides the clustering of ovarian cancer, an excess of

TABLE 14
Relative Risk Estimates of Ovarian Cancer and Coffee Consumption

Author [Ref]	Coffee consumption (cups/day)	Relative risks* (95% CI)
Harige <i>et al.</i> [96]	<2	1.0(0.5-2.2)
	2-3	1.8(0.9-3.6)
	≥4	1.4(0.6-3.0)
Byers <i>et al.</i> [67]	<3	1.3
	≥3	1.0
Cramer <i>et al.</i> [68]	Regular drinkers	1.1
La Vecchia <i>et al.</i> [97]	≤1	1.5(0.9-2.5)
	2-3	1.9(1.2-3.0)
	≥4	2.2(1.2-3.9)
Tzonou <i>et al.</i> [29]	0.5-1	0.9
	1.5-2	1.6
	2.5-3	0.9
	≥3.5	1.5
Miller <i>et al.</i> [98]	1	1.3
	2	1.2
	3	1.3
	4	1.7
	≥5	1.1
Whitemore <i>et al.</i> [89]	1	2.4(1.2-5.1)
	2-3	2.3(1.1-4.7)
	≥4	2.1(1.0-4.4)

* Reference category

* Never drinkers were the reference category.

TABLE 13
Relative Risk Estimates of Ovarian Cancer and Fat Intake

Author [Ref]	Level of fat intake			
	1*	2	3	4
Byers <i>et al.</i> [67]	1	1.3	1.2	—
Cramer <i>et al.</i> [68]	1	1.4	1.9	1.8
La Vecchia <i>et al.</i> [69]	1	1.2	2.1	—
Shu <i>et al.</i> [15]	1	1.1	1.8	1.9

TABLE 15
Main Results from Selected Case-Control Studies on Family History of Ovarian and Breast Neoplasms and Risk of Ovarian Cancer

Author [Ref.]	Family history of ovarian cancer	Family history of breast cancer
Wynder <i>et al.</i> [23]	No association	No association
Casagrande <i>et al.</i> [33]	7 cases and no control reported a family history of ovarian cancer	RR 1.1 for a family history of breast cancer
McGowan <i>et al.</i> [34]	14 cases and 2 controls reported a family history of cancer of the "female reproductive organs" (one-sided, $P = 0.01$)	
Hildreth <i>et al.</i> [24]	RR 18.2 (95% CI, 4.8-69.0) for a family history of ovarian cancer	RR 1.0 (95% CI, 0.4-2.5) for a history of breast cancer in mother or sister
Cramer <i>et al.</i> [36]	RR 11.3 (95% CI, 8.9-10.0) for a family history of ovarian cancer	RR 1.5 (95% CI, 0.8-2.9) for a family history of breast cancer
Mori <i>et al.</i> [43]	1 case and no controls reported a family history of ovarian cancer	3 cases and 1 control reported a family history of breast cancer (RR 6.1, 95% CI, 0.1-107.3)
Schildkraut and Thompson [84]	RR 3.6 (95% CI, 1.8-7.1) for a history of ovarian cancer in first- and second-degree relatives	
Koch <i>et al.</i> [85]	RR 2.6 for a history of ovarian cancer in first- and second-degree relatives	RR 1.5 for a history of breast cancer in first- and second-degree relatives
Parazzini <i>et al.</i> [86]	RR 1.9 (95% CI, 1.1-3.8) for a history of ovarian cancer in first-degree relatives	RR 1.6 (95% CI, 1.1-2.3) for a history of breast cancer in first-degree relatives

breast cancer (breast-ovarian cancer syndrome) or more general excesses of several cancers (including colon and endometrium, "cancer family syndrome" [82]) have been described, also. These hereditary patterns are consistent with an autosomal dominant gene with variable penetration, transmitted through men and women, probably accounting, however, for an extremely limited proportion of cases [83].

Most case-control studies that included data on family history have confirmed this association [23,24,33,34,36,43,84-86] and have provided a quantitative evaluation of risks related to belonging to families prone to ovarian cancer (Table 15). The estimated RRs range from 2 to 10. It must, however, be borne in mind that, in the absence of a reliable genetical marker of susceptibility, a "positive family history" includes both true genetically determined familial cases and cases occurring by chance, thus resulting in an underestimation of the actual importance of familial predisposition.

Discrepancies in investigations on family history can be at least in part explained by chance (the confidence limits largely overlap) and by selective publication of positive results, particularly in earlier studies. Furthermore, potential differences in baseline genetic characteristics of various populations and in the prevalence of "hormonal" risk factors should be considered. Hormonal factors can, in fact, play a role in the expression of an inherited enhanced susceptibility of ovarian tissue toward carcinogenesis. Notably, no relationship emerged in a survey conducted in Japan [43] and in a study on borderline malignancies [84].

Despite convincing evidence of an increased risk of ovarian cancer in women with a positive family history, the percentage of ovarian cancer cases explained by family history is limited and probably smaller than the proportion for other female tumors. For example, less than 1% of ovarian cancers were attributable to the "family risk factors" in a case-control study in Italy [86] (as compared for instance, with the 4% estimated for breast cancer).

Epidemiological data on the relationship between ovarian cancer risk and family history of breast or endometrial cancer are scanty and not completely consistent. An increased risk of ovarian cancer in women reporting familial breast cancer cases was found in four [36,43,85,86] out of seven case-control studies [23,24,33,36,43,85,86], and an association with a family history of endometrial cancer was found in two [24,43] out of four studies [24,36,43,86] (Table 15).

Medical History and Other Miscellaneous Factors

The relationship between childhood infectious diseases and ovarian cancer was investigated in several studies [27,34,36,44]. Their results are not consistent, since some, but not all, showed, among cases, more frequent past history of mumps [36,44] or occurrence of rubella in adolescence [34].

The rationale for suspecting an association between talc and ovarian carcinogenesis comes from the similarity and the frequent contamination of talc with asbestos, an important factor in carcinogenesis of pleural and peritoneal mesotheliomas. Case-control studies have, however, failed to provide convincing evidence [87-89].

CONCLUSION AND ETIOPATHOGENETIC HYPOTHESES

The incidence of ovarian cancer is higher in more developed countries, although in the past years steady increases have been observed in the areas where incidence was low, while a flattening off of upward trends in more recent cohorts has been recorded in several developed countries.

Many factors may be at the origin of such between-country variations and their gradual decrease over the past years. Nulliparity or low parity and late age at first birth, oophorectomy, and OC use are, for instance, more common in industrialized, highest risk areas, but some of these characteristics or medical procedures are increasingly being adopted by other countries.

Besides OC use and parity (the two best ascertained and quantified protective factors for ovarian cancer) case-control and cohort studies have shown, or suggested, that late menopause and lifelong regular menstrual periods [17] increase the risk of the disease. In terms of mechanisms, these factors are thought to act on ovarian cancer risk by affecting lifetime number of ovulations. Along this line, Casagrande *et al.* [33], following the suggestion of Fathalla [90], proposed that "incessant ovulation" could be the relevant exposure that defines the incidence of the neoplasm. Ovulation exposes ovarian epithelium to recurrent minor trauma and contact with follicular fluid and, according to Zajicek [91], may induce epithelial inclusions of the surface epithelium, relevant to cancer development. Animal data have shown that ovarian epithelium proliferates rapidly after ovulation and mitotic figures are concentrated near the site of ovulation [92]. The subsequent model based on "ovulatory age" (i.e., period from starting to cessation of menses minus "protected time" constituted by pregnancies and OC use) is not, however, at least at its present state [33], satisfactory, perhaps on account of different proportions of ovulatory years at different ages.

Others [36] have suggested that pregnancy, as well as OCs, may protect against ovarian cancer by reducing total exposure to pituitary gonadotropins (which stimulate the growth of cell lines derived from human ovarian carcinomas [93]). The lack of protection from estrogen replacement therapy [37] provides, however, evidence against the importance of gonadotropin stimulation.

Among the nonreproductive or hormonal factors, diet is likely to be the most relevant; it is still, however, very poorly quantified. It may well explain part of the substantial international variability in the incidence of the disease and, therefore, remains a priority in epidemiological research on ovarian cancer, especially from a public health viewpoint, since it is more amenable to modification than reproductive and menstrual factors.

Despite the large number of epidemiological studies, no clear associations between viral factors, chemical agents, and ovarian cancer risk have emerged, thus confirming that the mechanisms of ovarian carcinogenesis should be essentially searched in the hormonal and dietary (together with genetic) fields.

Finally, relatively few epidemiological studies have been able to subdivide reliably epithelial ovarian carcinomas according to histological types. Also, the epidemiology of borderline tumors and benign cysts has seldom been considered [53,94,95], suggesting, however, differences in risk factors between benign and malignant ovarian tumors. Future epidemiological research should take advantage of improvements in diagnostic standards in order to cast more light on potential epidemiological differences between ovarian tumors of different histological types and degree of malignancies and, hopefully, to clarify carcinogenetic pathways.

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