# Ultrasound Imaging in Animal Models of Human Disease—Is it a Step Toward Early Diagnosis in Humans?

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**Abstract:** Despite extensive research, cancer of the ovaries remains a major medical problem. The main reason is delay in diagnosis and hence, poor prognosis. This is due to issues in screening and a lack of specific symptoms in early disease. Yearly ultrasound examination and measurement of serum CA125 remain the recommended method despite less than ideal results. Animal research plays a major role in medical research, especially in cancer. Many publications describe the use of ultrasound in cancer research in a large variety of animals. B-mode, spectral and color Doppler have been employed and, more recently, ultrasound contrast agents, both for diagnostic and therapeutic purposes. We have demonstrated that ultrasound can be used to detect early ovarian cancer in the egg-laying chicken. The major advantage is that chickens develop spontaneous ovarian cancer, with a tumor histology that is identical to humans. Furthermore, chickens with ovarian tumors have serum anti-tumor antibodies similar to humans. In addition, the first sign that the egg-laying chicken is going to develop cancer is that it stops laying eggs prematurely.* Thus, a strong biological sign exists to categorize the chicken in a very high risk group, allowing sequential examinations at very close intervals. We have also shown that ultrasound contrast agents may be used for visualization of ovarian vascularity, a step, we hope, in the development of better methods for screening and early diagnosis.

**Keywords:** Ovarian cancer, animal models, chicken, ultrasound, Doppler, contrast agents.

## INTRODUCTION

Most research into cancer is carried out using engineered rodent models or non-animal methods such as cell culture, computer modeling or lower organisms such as yeast. However, animal studies remain vital in cancer research to investigate early events, preclinical drug trials and metastatic spread. Breast and prostate cancers receive the most attention and funding from Federal and private sources. There is a breast cancer awareness month, the US Postal service made available a breast awareness stamp, the pink ribbons are ubiquitous and Federal funds for breast research are 15 times those earmarked for ovarian cancer. Regarding prostate cancer, in 1996 General Norman Shwartzkopf was involved with a campaign for prostate cancer screening: "Every man above the age of 50 should be tested". The cost analysis for over 30,000,000 men in the USA for a blood test (PSA, at $40/test) and a digital rectal exam ($60) is 3 billion US dollars. If one adds laboratory costs, physician fee and transrectal ultrasound, the cost becomes a staggering 10 billion US dollars![1]

On the other hand, ovarian cancer in the USA continues to be the 1st cause of death from gynecological cancer in women and the 5th cause of death from any cancer.[2] There are more than 20,000 new cases diagnosed and approximately 15,000 deaths expected each year.[3] The lifetime risk of death is 1:70-100 women. A very important aspect of the disease is that the five-year survival is > 90% in stage I, but only 25-30% in stage III and 5-10% in stage IV; 60% are diagnosed in these two later stages.[4] A further complicating factor for early detection of ovarian cancer is that most cases are sporadic which begs the question: whom to screen and how? In addition the question of monoclonal versus polyclonal origin,[5] as documented by the occurrence of primary peritoneal cancer after bilateral oophorectomy,[6] and the occasional cases of patients with a normal CA-125 value and normal ultrasound and presenting a very short-time later (months) with advanced ovarian or

* Not all hens get ovarian cancer but, as they age, egg production declines. This is similar to menopause in women. Egg-laying is a direct measure of ovulation and ovarian function. Thus, the significance of egg-laying rate is that it is a direct and non-invasive indicator of ovarian function.

peritoneal cancer[7] suggest a single test may not adequately diagnose early ovarian cancer. A further problem with screening for ovarian cancer is its relative low incidence in the general population (17 per 100,000 women) and, thus, currently available screening methods have relatively low sensitivity and specificity which results in a high number of women screening positive who do not have ovarian cancer (false positive screen). Furthermore, a 1991 estimate of the cost of yearly screening for the general population (approximately 43 million women over the age of 45) with transvaginal ultrasound ($275) and CA-125 ($45) was over 13 billion.[8] Consequently, screening is not recommended for the general population.

A high-risk group has been defined: women with a strong family history of ovarian cancer and/or presence of BRCA 1 or BRCA 2 mutations.[9-12] However, while their risk is very high of developing ovarian cancer, only 5-10% of all diagnosed cases of cancers are found in these women* and even for these women, benefits of screening have not been indisputably demonstrated.[13,14] On the other hand, early diagnosis is also difficult: the ovaries are relatively remote and difficult or even impossible to assess either by a caregiver or certainly by self-examination, as is recommended for the breast. Commercially available non-invasive testing, similar to the Pap smear for cervical cancer does not exist at the moment; symptoms of early ovarian cancer are non specific. As a result, most cases are diagnosed at advanced stages. Currently available tests (CA125, transvaginal ultrasound, or a combination of both) lack the sensitivity and specificity to be useful in screening the general population.[3,15] Different biomarkers have been tested,[16] from CA-125 to lysophosphatidic acid (LPA)[17,18] as well as carcinoembryonic antigen,[19] placental alkaline phosphatase,[20, 21] Lewis X mucin determinant,[22] cytokine macrophage colony-stimulating factor,[23] matrix metalloproteinase 2 and 7,[24,25] kallekrein-6 and -10,[26] mesothelin,[27] osteopontin,[28] prostasin,[29] the interleukins e.g. 6, 8, 10 and 12 to name a few[30-33] and angiogenesis factors[34] as well as combination of markers.[23, 35] Proteomics is a very promising relatively new field with applications in cancer in general[36] and, ovarian cancer in particular.[37-44] Anti-tumor antibodies have been reported in several cancers.[45] They are stable and established markers of several diseases are associated with ovarian cancer in humans and may represent a reliable early marker for ovarian cancer.[13] However, it is not known whether most of these markers are associated with early stage of ovarian cancer. The timing of antibody appearance and the early changes in the ovarian morphology leading to ovarian cancer is currently under study.[46] A relatively new technological application may move the diagnosis of cancer to the early stages of the disease and, possibly, improve screening by visualization of early vascular changes: the use of ultrasound contrast agents.[47-69]

* In other words: 90-95% of ovarian cancers develop in low-risk women.

## ANIMALS IN OVARIAN CANCER RESEARCH

Animal models have been used to understand the etiology, progression, and prevention of various human diseases that are difficult to study in humans, particularly in cancer research.[48, 49,70-73] Advantages of using animals for research include standardization, frequent repeatability, and, naturally, the fact that the patients are always on time for their appointments and don't complain. But how applicable are the findings to humans? There are, in fact, many publications on ultrasound studies of cancer in animals. Generally the cancer is induced and/or the animals are transgenic. This is not necessarily similar to human situations and hence applications to human medicine are not always obvious. Examples of the use of various technologies, particularly ultrasound include lung cancer in pigs,[74] hepatocellular carcinoma in woodchucks[75] and transgenic mice,[76] liver metastases in rabbits[77] and mice,[78] breast (udder) cancer in goats and cats[79] and rats,[80] ultrasound for melanoma in mice,[81,82] 3D ultrasound micro-imaging for prostate tumor in transgenic prostate cancer mice,[83] human pancreatic tumor cells implanted in mice,[84] ovarian tumors in various animals,[85] several cancers in mice,[86] ultrasound contrast agents for malignant gliomas in rats[52] and prostate cancer in dogs[54] and even zebra fish.[87]

Most animals do not spontaneously develop ovarian cancer. Among domestic animals the desired state is pregnancy and/or lactation and most wild animals are pregnant, lactating or sexually inactive. The rodent model, cell lines from human tumors and normal ovarian surface epithelial cells have been used in cancer research but the study of the origin and development of early tumors is limited. Spontaneous ovarian tumors occur in some strains of mice (CBA/J; C3HeB/Fe; HAN:NMRI; SWR/J and more) and in Wistar and Sprague-Dawley rats. But they are of a wide variety of histologic subtypes (tubular adenoma, adenoca, papillary cystadenoma, mesothelioma, granulosa cell tumor and polycystic sex cord/stromal tumor). In addition, the incidence is low and a long time is needed to obtain growth, hence, these are not very useful. In general, in animals, ovarian cancer is not spontaneous, it is non-uniform, it develops over prolonged time periods, is unpredictable, metastatic spread is different from the human and there are no biological early markers. The chicken is very different.

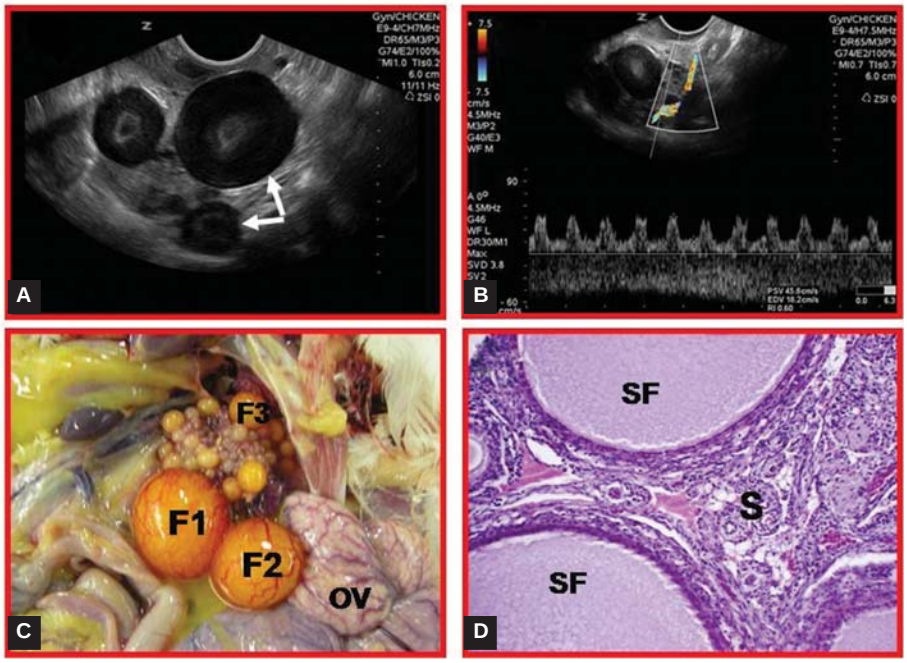
### Is the Chicken Better?

The egg-laying chicken (White Leghorn, commercials strain, *Gallus Domesticus*) mature at 20-22 weeks, lives about 6-7 years, lays about 250 eggs/year and has ovulatory patterns similar to the human female: daily ovulation for 1-2 years (humans: monthly for 10-25 years). Farmers cull hens after 2 years because egg production ceases to be financially profitable. Ovarian cancer in the laying hen resembles human cancer because it is

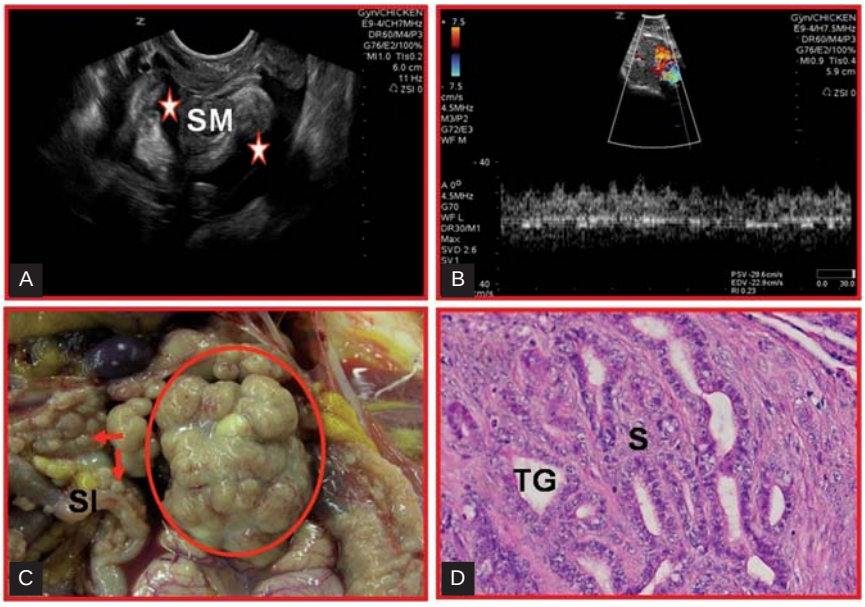
spontaneous and shows an increased incidence with advancing age.[88] The incidence is about 10% at age 2-3 years and 40-60% at age 4-6 and varies by flock.[89] It expresses several histological markers similar to women and has a similar pattern of metastatic spread. The first sign that a chicken is going to develop ovarian cancer is that it (prematurely) stops laying eggs.[90] Not all hens that stop laying get cancer (see abstract footnote). Hence, there exists a biological marker predicting the development of cancer. Hen ovarian cancer is cross-reactive with many antibodies used to detect several antigens in human ovarian cancer.[45,91] The cross reacting anti-human tumor antibodies include Ca125, Ki-67 and PCNA (markers of proliferation), AE1/AE3 and pankeratin (cytokeratins), TGF-a, erbB-2 and EGFR (growth factor, protooncogene and GF receptor), CEA (carcinoembryonic antigen), Tag 72, Lewis Y (oncofetal tumor markers) and Muc1 and 2 (Mucin antigens). Hen ovarian cancers are positive for expression of ovalbumin, an oviductal protein.[92] This is remarkable since the most common ovarian cancer in humans has oviduct-like characteristics. In general, variation in ovarian cancer rates between different strains and there is a 5-fold greater tumor incidence in White Leghorn hens.[89] Hens generally develop tumors characterized by epithelial cell histology, particularly endometrioid and clear cell. In the humans, endometrioid type accounts for 16-30% of all cancers and clear cell, about 5-11% (but 50% in nulliparae).

## SUMMARY

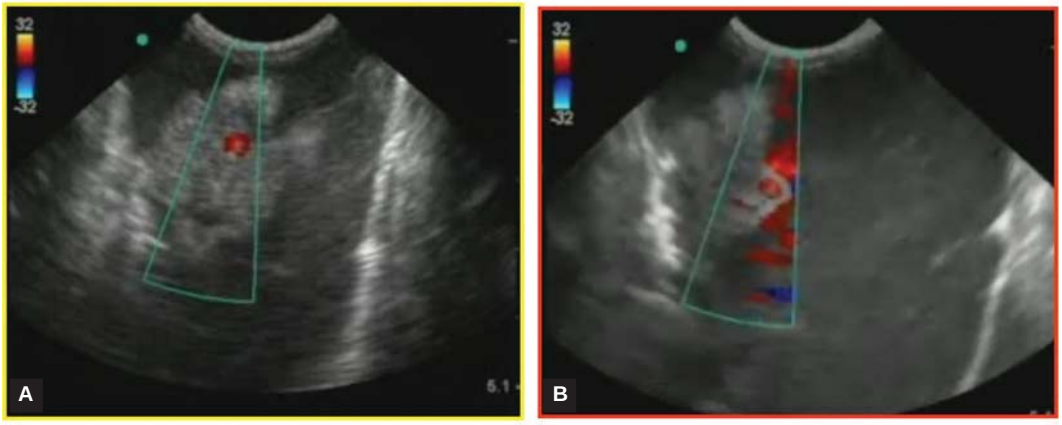
Histological features of ovarian cancer in hens and humans are similar, metastatic spread and development of ascites are also analogous. Immunoreactive ovarian antigens are similar in humans and hens with ovarian cancer and the cancers in hens are associated with serum anti-ovarian tumor antibodies as seen in human patients. Despite the published research on ovarian cancer in chicken, information on the early morphologic changes associated with ovarian cancer in hen is not available. Ultrasound has been described for imaging of chicken ovaries[93] but only for normal physiology. We, therefore, decided to evaluate the usefulness of ultrasound in the diagnosis of ovarian cancer in hens.[94] Transvaginal ultrasound was performed with commercially available instruments (Z.One, Zonare; Accuvix V10, Medison and MicroMax, Sonosite) in unanesthetized chicken, manually gently restrained on their backs. The ovary (egg-laying chicken have only one [left] ovary) was visualized in every case (Fig. 1). B-mode, grey-scale images, as well as color and spectral Doppler were obtained. The region surrounding the ovary was scanned, and once a follicle had been located, the transducer was swept through the entire area to obtain a complete image of the ovary. Gray scale morphologic evaluation was performed (Fig. 1A) with attention to the number of developing hierarchical follicles, the presence of abnormally looking follicles, bilaterality, septations, papillary projections or solid areas, and echogenicity. After morphologic evaluation, color Doppler was activated for identification of vascular signals. If blood flow was detected, it was defined as either "peripheral" (color signals in the wall or periphery of a follicle or a suspected mass) or "central" (blood flow detected in septa, papillary projections, or solid areas). Once a vessel was thus identified, pulsed Doppler was activated to obtain a flow velocity waveform (Fig. 1B). The hens were separated in 2 groups: normal ovarian status (3-5 developing preovulatory follicles) and abnormal status. The first group served as a control group, allowing us to determine a normal range of Doppler resistance indices (resistive index, RI defined as peak-systolic velocity minus end-diastolic velocity over peak-systolic velocity and pulsatility index, PI, defined as peak-systolic velocity minus end-diastolic velocity over cycle mean velocity). A second group of hens with lower RI and or PI than normal hens were defined as having abnormal ovarian status. Hens were also grouped by age: young hens (12 months old) were used as controls. The study group consisted of older hens with or without egg-laying. We also attempted to associate ultrasound prediction of cancer with gross and microscopic appearance of the tumors as well as serum analysis. Sera were collected before the ultrasound scan and tissues were collected after (animals were euthanized according to Institutional Animal Care and Use Committee protocols). The following were performed: gross examination, ELISA for detection of serum antibodies, histology (H and E staining), proteomics (two-dimensional Western Blot) to identify the immunoreactive ovarian proteins and their similarities to humans.[95,96] Blood flow velocity was detected in all hen ovaries irrespective of their gray scale sonographic appearances. Normal hens with multiple developing hierarchical follicles had confluent blood flow around areas where small growing follicles were located and along the follicular walls. Blood flow in the ovary of abnormal hens with cystic ovarian architecture was variable from the center to the periphery, whereas central blood flow was observed in hens with solid tissue masses (Figs 2A and B). The mean RI ± SD (0.27 ± 0.07; range, 0.16–0.38) and PI (0.347 ± 0.06; range, 0.28–0.42) values of hens with ovarian cancer were significantly ($P < 0.001$) lower than those of normal hens.[94] Overall, the gray scale and color Doppler evaluations for ovarian tumors (Figs 2C and D) as well as normal ovarian morphologic characteristics (Fig. 1C-D) matched their corresponding gross observations (100% accuracy). Although this appears to be excellent, all cancers were advanced stage. Figure 2A is a sonographic image of a large malignant tumor, seen macroscopically in Figure 2C. We further prospectively followed chickens at risk for ovarian cancer over a period of 45 weeks. Doppler velocimetry demonstrated a clear difference between chickens who eventually developed cancer and those who did not with a significant downward slope in chickens who became affected (presented at ISUOG 2009 Annual Meeting in Chicago and manuscript in preparation). In addition, we have shown




**Figs 1A to D:** Transvaginal ultrasound scanning of ovaries in laying hen. (A) B-mode, gray scale image showing 3 preovulatory hierarchical follicles of different sizes (arrows) without any abnormality suggestive of normal functional ovary. (B) Corresponding Doppler image of the ovary showing a peripheral pattern of blood flow on the follicular walls. (C) Corresponding ovary (gross) at sacrifice showing hierarchical follicles of different sizes (F3-F1) protruded from the ovarian surface (see the text for detailed description) with numerous small developing follicular stock confirming the ultrasound prediction. (D) Paraffin section of the corresponding ovary showing stromal embedded follicles without any hyperplastic of dysplastic structure (40X). F1-F3 = largest to 3rd largest preovulatory hierarchical follicles. S = stroma; SF = stromal follicle; OV = oviduct




**Figs 2A to D:** Transvaginal ultrasound scanning of ovarian tumors in hen. (A) B-mode, gray scale image of hen ovary predicted to have tumor. The solid ovarian mass accompanied with profuse ascites (*) suggesting late stage ovarian cancer. (B) Corresponding Doppler image of the ovary showing a central pattern of blood flow on the solid tissue mass characteristic of ovarian cancer. (C) Gross appearance of the scanned ovarian tumor confirming the ultrasound prediction. The tumor appeared like a cauliflower (circle) and metastasized to the abdominal organs including intestine (arrows). (D) Paraffin section of the corresponding ovarian tumor showing confluent back to back well developed glandular structures characteristics of endometrioid ovarian cancer. Hematoxylin and eosin stain (40X). S = stroma; SI = small intestine; SM = solid mass; TG = tumor gland




**Figs 3A and B:** Contrast enhanced ultrasound scanning of hen ovaries. Hens were injected with Optison® contrast agent @10 ul/kg body weight at wing vein followed by washing with saline. Scanning was performed before, during and after injection of Optison® in a continuous manner using all the imaging parameters as reported previously. (A) Only few vessels are seen in the ovary prior to Optison® injection. (B) In contrast, more blood vessels are seen along the ovarian mass 15 s after Optison® injection suggesting significant enhancement in the visuality and detectability of small vessels

that ovarian vascularity can clearly be demonstrated with the ultrasound contrast agent Optison® (Figs 3A and B). Further research is ongoing on delivery of anti-angiogenic agents to ovarian tumors in chicken as this appears to be a very promising technology.[97]

## CONCLUSIONS

The egg-laying chicken appears to be an excellent model for ovarian cancer. Histology and serology are identical to human cancer. Imaging with ultrasound is feasible. Tumors are visualized with grey scale, color and spectral Doppler clearly demonstrate abnormal vascularization and ultrasound contrast agent allow better visualization of the vasculature, hopefully opening the way to earlier diagnosis and, hopefully, screening.

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