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14. ABSTRACT Ovarian cancer (OVCA) affects about 25,000 women annually. Most cases are diagnosed at a late stage with less than a 20% probability of survival. We used the egg-laying hen, a spontaneous animal model of OVCA, to test the hypothesis that hens with AOA are more likely to develop OVCA, and therefore that AOA can be used as a predictor of OVCA. We compared the proportion of hens with AOA to those without AOA that developed OVCA. Serum AOA was measured by immunoassay by standard methods. Angiogenesis (new blood vessel formation) is a hallmark of tumor pathology and was measured in serial assessments as blood flow resistance by color Doppler ultrasound. Only hens with anti-tumor antibodies showed significant evidence of angiogenesis, a surrogate marker of early OvCa. We also demonstrated that specific antibodies to mesothelin, a specific antigen in human OVCA, are found in hens with OVCA. Demonstrating that AOA precedes OVCA would expand the number of measurable risk factors for OVCA, revolutionize screening for OVCA, and make it possible to detect OVCA prior to its development, or in its early stages when effective treatments are available.					
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INTRODUCTION

Subject: The objective of this study was to determine if anti-ovarian autoantibodies (AOA) precede ovarian cancer (OVCA) in the laying hen model of OVCA. In humans, AOA are associated with infertility [1, 2]. Infertility is also an epidemiologic risk factor for OVCA that has not been explored [3]. Since the same group of women have both AOA and OVCA risk, AOA may be a risk factor that can be used to predict OVCA, and this will be tested in the hen model.

Purpose: We will test the hypothesis that hens with AOA are more likely to develop OVCA, and therefore that AOA can be used as a predictor of OVCA.

Scope: The study design is longitudinal. We developed ultrasound detection of hen OVCA that permits longitudinal evaluation of early events associated with tumor initiation and progression. Since hens with reduced egg production have declining ovarian function, similar to human infertility, we use egg production as a selection factor, in addition to the presence or absence of AOA. Thus, to test the hypothesis that AOA precede ovarian pathology, we will select hens with low egg production, and with and without AOA, and monitor ovarian pathology by ultrasound in middle aged hens (2.5-3 year old) at intervals for 9 months.

Addendum: At the term of this we project we requested and received an extension (reasons detailed below). This report describes additional progress in the last 12 months. The additional information is underlined.

BODY

Research accomplishments associated with each task in the Statement of Work

The plan was dependent on access to an ultrasound machine; the pilot data was obtained with a z-one (Zonaire Medical Systems) and was loaned by the company for the pilot data that led to the study. However, in the first 3 months, the company that we were collaborating with was closed. We applied for and received an institutional capital equipment grant and purchased a Micromax 2D Ultrasound (Sonosite Inc.). This took about 10 months. Therefore, we have also applied for a no-cost extension for this project (letter submitted to contract officer, Susan.Dellinger@Amedd.Army.Mil). The original progress report represented the 3 months of initial work (June 2008-September 2008) and 4 recent months of work (August 2009-October 2009). The “Addendum” sections report work since the last report and covers one year (November 2009-November 2010). A summary of final activity is included in the Addendum. The tasks associated with the original SOW were accomplished and are summarized below.

Personnel supported by this project include: Judith Luborsky, PhD, Professor (Principal Investigator), Animesh Barua, PhD, Assistant Professor (co-investigator) and Seby Edassery, MS (Research Associate).

SOW Task 1: Select 120 hens for longitudinal study of risk factors for OVCA (months 1-3) by selecting White leghorn hens (*Gallus domesticus*) hens with normal (5-6 eggs/clutch) or low egg production (< 3 eggs/clutch) with normal ultrasound morphology.

1. Perform Gray scale ultrasound to assess morphology for normal follicles and absence of ovarian mass or cysts. *Progress: 117 hens were examined for inclusion in the study. 85 hens were screened by gray scale ultrasound to determine normal morphology; 57 without an apparent mass and several follicles were select for further monitoring. As of this report all morphology by ultrasound is completed.*

2. Perform Doppler ultrasound to assess blood flow indices in comparison to same aged hens with normal egg production. *Progress: 85 hens were screened by color Doppler ultrasound and the Resistive (RI) and Pulsatility Index (PI) measured as an indication of blood flow velocity and tumor associated neo-angiogenesis. As of this report all measurement of angiogenesis by Doppler ultrasound is completed.*

3. Analyze data and determine baseline values. *Progress: Baseline data, summarized below, was analyzed and was presented at the regional Reproductive Biology Symposium (abstract appended). As of this report initial data analysis of ultrasound is completed.*

b. Measure serum AOA by immunoassay and 2D Western blot by standard methods in serum samples. *Progress: Baseline data for AOA was analyzed and was summarized in the attached abstract presented at the regional Reproductive Biology Symposium. Serum AOA measurement was completed.*

c. Form 3 groups as follows: (group A, n= 40) low egg production with AOA; (group B, n=40) low egg production negative for AOA; and (group C, n=40) normal egg production without AOA. Assign random numbers (1 to 40) to hens in each group so that hens in control groups (B, C) will be sacrificed when the same numbered hen is sacrificed in experimental group A. *Progress: Three hen groups were formed, as reported in the attached abstract. The study is not complete as explained above. As of this report hen groups were determined; they may be modified for analysis according to final histological categories as a secondary analysis. The final adjustments are 90% complete.*

SOW Task 2: Follow animals to detect tumor initiation (months 3-12):

a. Measure ultrasound and AOA by immunoassay and Western blot monthly. *Progress: Ultrasound scans were performed; 85 hens were scanned once and 57 hens were scanned twice. Serum was obtained at each scan. Western blot will be completed at the end of the study for those hens with at least three ultrasound scans and serum samples.*

b. Sacrifice animals when Doppler ultrasound indices (RI and PI values reflecting reduced resistance associated with angiogenesis) decline significantly compared to values for normal hens. After the first change, evaluate hens again the next month and if the values remain abnormal, sacrifice hens and collect serum and tissue. *Progress: Hens have been sacrificed or died (n=7). The plan is to continue monitoring the 50 remaining hens in the next 6 months.*

SOW Task 3: Determine if AOA predicts OVCA (months 10-18):

a. Confirm the presence or absence of ovarian tumors in ovarian tissue. For histological evaluation, ovaries will be cut into 8 segments, fixed in formalin, paraffin embedded and sectioned for H and E staining. Every third section (10 μ m) from the entire ovary will be evaluated, photographed and scored. Ovarian histology will be examined for focal lesions exhibiting OVCA histology and for the number of inclusion cysts. Any pathological change observed by ultrasound and confirmed by histology will be scored as OVCA. *Progress: A systematic study of tumor histology was completed and published [4] as a basis for this task (manuscript appended). This task is completed.*

b. Review and compile data and perform statistical analysis to determine if AOA is a significant predictor of OVCA. The proportion of hens with AOA that develop OVCA (group A) will be compared with those without AOA (groups B, C) using Chi square analysis with $p < .05$ considered significant. *Progress: This task remains to be completed. This task remains to be completed.*

The data obtained as of September 2009 was presented at a regional meeting (30th Annual Minisymposium on Reproductive Biology, October 2009). The data is presented in Table 1. In brief, hens (n=50) were assessed initially by ultrasound and color Doppler ultrasound and then again at about 14 months. AOA were determined in serum taken at the same time using a pooled extract of three tumors in immunoassay.

At the second assessment, there were 3 groups of hens: (A) anti-tumor antibody negative (n=20) at both time points, (B) anti-tumor antibody negative at the first assessment and antibody positive at the second assessment (n=18) and (C) anti-tumor antibody positive/borderline at both assessments (n=12). In hens that remained antibody negative (group A), the average blood flow resistance declined non-significantly from 0.52 ± 0.14 (RI) and 0.86 ± 0.37 (PI) to 0.47 ± 0.11 (RI) and 0.68 ± 0.22 (PI) ($p=0.2$ and 0.07 respectively). In group B, hens that transitioned from negative to positive, the average RI and PI values declined significantly ($p=0.04$ and 0.01 respectively). In hens that remained positive (group C), values also declined significantly ($p=0.05$ and 0.02) from 0.55 ± 0.17 (RI) and 0.89 ± 0.38 (PI) to 0.43 ± 0.10 (RI) and 0.57 ± 0.16 (PI). Thus only hens with anti-tumor antibodies showed significant evidence of angiogenesis, a surrogate marker of early OvCa.

Table 1. Comparison of Blood Flow Velocity (angiogenesis) by AOA

Antibody group	1 st scan		2 nd scan	
	RI	PI	RI	PI
negative (n=20)				
average±SD	0.52±0.14	0.86±0.37	0.47±0.11	0.68±0.22
p value (t test)	ref	ref	0.20	0.07
changed (n=18)				
average±SD	0.62±0.19	1.02±0.40	0.51±0.10	0.73±0.21
p value (t test)	ref	ref	0.04	0.01
positive(n=12)				
average±SD	0.55±0.17	0.89±0.38	0.43±0.10	0.57±0.16
p value (t test)	ref	ref	0.05	0.02

Addendum: Overall 119 hens were examined by ultrasound to determine ovarian morphology and evidence of angiogenesis (defined by a decrease of RI and PI values below 0.4). Of these 42 already had OvCa and were excluded, died naturally or were euthanized due to other illness during the course of the study; 77 were monitored further. 52 hens had three or more ultrasound scans and usable data during 16 months. 21 were initially positive for AOA and 13 hens progressed to OVCA (62%); 31 were initially negative for AOA and 12 of these hens progressed to OVCA (39%). However, of the 31 hens that were initially negative, 7 became positive for AOA during surveillance bringing the total AOA negative hens (n=24) that developed OVCA to 5 out of 24 (20%). Therefore we proved our original hypothesis, although further work remains to be completed.

Relationship of the most recent findings with that of previously reported findings

The concept that autoantibodies precede overt signs of carcinogenesis is supported by literature reports; for example, in patients with chronic hepatitis (which predisposes to liver cancer) specific antibodies appeared a year or more before patients went on to develop hepatocellular carcinoma [5]; the same antibodies reappeared before recurrence in these patients. The increase in antibodies occurred either as an increase in titer of a pre-existing antibody or as the appearance of a novel reaction. Similarly, studies suggest that anti-p53 appears before lung cancer develops in groups at high risk [5]. The link between autoimmunity (e.g., rheumatoid arthritis, SLE, Sjogren's syndrome) and non-Hodgkin's lymphoma is well established. Furthermore, a role for B cell hyperactivity in carcinogenesis was suggested by a small study in which the anti-B cell drug rituximab reduced tumor burden in colon cancer [6]. Thus, although the role of antibodies and B cells in cancer etiology is not well understood, the phenomenology is apparent, and supports the concept that antibodies could be used as very early "reporters" of incipient cancer.

The results are consistent with our pilot study which showed that low egg laying hens with and without AOAs followed prospectively for up to 45 weeks using Doppler ultrasound as reported previously [7] (Figure 7). Briefly, we monitored Resistive and Pulsatile Indices generated by the Micromax 2D Ultrasound (Sonosite Inc.) at 15 week intervals in hens (n=9) that were either AOA positive or negative at the initial reading. The blood flow indices reflect ovarian blood flow velocities and detect changes in vascular architecture related to neo-angiogenesis. Neo-angiogenesis is an early change in tumor development. Tumor tissue revealed lower indices reflecting increased vascularization and flow velocity. Significantly, 83% of hens with antibodies developed OVCA and this was accompanied by a characteristic decrease in blood flow resistance (angiogenesis) similar to women with ovarian tumors [8].

This study will be the first to show that AOA predicts OVCA. This suggests the possibility of developing a screening test for early detection of OVCA based on antibody detection. In order to translate these findings to

humans we are submitting applications to repositories with serial serum collections that include pre-diagnostic sera.

Problems in accomplishing tasks

- The plan was dependent on access to an ultrasound machine; the pilot data was obtained with a Zonair and was loaned by the company for the pilot data that led to the study. However, in the first 3 months, the company that we were collaborating with closed. We applied for and received an institutional capital equipment grant and purchased a Micromax 2D Ultrasound (Sonosite Inc.). This took about 10 months. Therefore, we have also applied for a no-cost extension for this project.
- Since the assay we are using for antibodies is an ovarian tumor extract, it may not efficiently monitor all relevant antibodies. During this study we developed additional information on specific antigens in the hen, relevant to this project using Western blots and mass spectrometry [9] and using immunoassays with recombinant proteins to detect antibodies [10]. We found that mesothelin is expressed in hen ovarian tumors with a similar pattern of expression as in humans (manuscript appended). Furthermore, we detected antibodies to recombinant mesothelin in serum from hens with ovarian tumors, similar to humans [10]. Thus we conducted the pilot study and found an increase in mesothelin antibody in two hens before OVCA (Figure 3). This suggests that a positive AOA result may reflect changing antibody specificities during tumor progression since of the two hens one was initially AOA positive and the other was initially AOA negative, but neither had mesothelin antibodies at the outset.

Recommended changes or future work to better address the research topic

We suggest that in addition to using the AOA antibody assay, we will test for several specific antibodies that are relevant to human OvCa to gain additional information on early reactions that would predict OvCa. Currently, it is not clear which antibody reactions might occur early and if the antibody specificity or titer changes with tumor stage, as shown for liver cancer [5]. Thus the serum and data collected for this study could be used to define the timing of specific antibody appearance.

Ultrasound has proven useful for assessing general morphology but selecting hens with reduced egg-laying (reduced ovarian function) leads to lower initial values for blood flow velocity and we may be missing initial angiogenic changes. Other parameters can be assessed.

KEY RESEARCH ACCOMPLISHMENTS

- Hens were selected using ultrasound and AOA immunoassay. Mid-study results show hens with AOA are more likely to show evidence of neo-angiogenesis (used as a surrogate marker of OVCA), consistent with the results of a pilot study. The results were presented at a regional meeting.
- A systematic study of tumor histology and tumor classification was completed as a baseline for evaluation of tumor pathology. The manuscript was published.
- The expression of mesothelin, a relatively specific antigen in OVCA was demonstrated and corresponding circulating mesothelin antibodies were found specifically associated with ovarian tumors in the hen .
- **Addendum: From this study**
 - Hens were followed serially for morphology and angiogenesis by ultrasound and for the presence of AOA up to 20 months
 - 95% of the diagnostic histology is completed
 - pilot data was obtained showing an increase in mesothelin antibody in two hens before OVCA
 - cellular immunology foundation study showed immune cell content in ovaries and tumors and that hens express SIP1 (which regulates lymphocyte trafficking)

REPORTABLE OUTCOMES

- Manuscripts: seven manuscripts were produced
 - Barua, A., et al., *Histopathology of Malignant Ovarian Tumors in Laying Hens, a Preclinical Model of Human Ovarian Cancer*. *Int J Gyn Cancer*, 2009. **19**(4): p. 531-539.
 - Yu, Y., et al., *Mesothelin expression in ovarian tumors of the laying hen model is similar to human ovarian cancer*. revisions submitted December 2010.
 - Barua A, Bitterman P, Bahr JM, Bradaric MJ, Hales DB, Luborsky JL, Abramowicz JS (2010) *Detection of tumor-associated neoangiogenesis by Doppler ultrasonography during early-stage ovarian cancer in laying hens: a preclinical model of human spontaneous ovarian cancer*. *J Ultrasound Med*. 2010 Feb;29(2):173-82.
 - Barua A, P Bitterman, JM Bahr, S Basu, E Sheiner, MJ Bradaric, DB Hales, JL Luborsky, JS Abramowicz (2010) *Contrast Enhanced Ultrasonography Detects Spontaneous Ovarian Cancer at Early Stage in a Preclinical Animal Model*, *J Ultrasound Med*, in press
 - Bradaric, MJ, Barua, A, Penumatsa, K, Yi, Y, Edassery, SL, Sharma S, Abramowicz, JS, Bahr, JM, Luborsky, J (2010) *Sphingosine-1 Phosphate Receptor (S1p1), a Critical Receptor Controlling Human Lymphocyte Trafficking, is Expressed in Hen and Human Ovaries and Ovarian Tumors*, submitted December 2010.
 - Bradaric, MJ; Barua, A; Penumatsa, K; Bitterman, P; Abramowicz, JS; Bahr, JM; and Luborsky, JL (2011) *Analysis of lymphocytes in spontaneous ovarian tumors in the laying hen show altered B cell content in early stage tumors*, in preparation (submission January 2011)
- Abstracts & Presentations: five meeting abstracts were produced
 - J. E. Nam, S.L. Edassery, A. Barua, J. Abramowicz, P. Bitterman, J.M. Bahr and J.L. Luborsky, *Anti-tumor antibodies may precede ovarian tumors in the laying hen model of ovarian cancer*, 30th Annual Minisymposium on Reproductive Biology, Chicago, IL, October, 2009 (poster)
 - J. E. Nam, S.L. Edassery, A. Barua, J. Abramowicz, P. Bitterman, J.M. Bahr and J.L. Luborsky, *Anti-tumor antibodies may precede ovarian tumors in the laying hen model of ovarian cancer*, 30th Annual Minisymposium on Reproductive Biology, Chicago, IL, October, 2009 (poster)
 - Judith L Luborsky, Seby L Edassery, Krishna Penumatsa, M Bradaric, Yi Yu, Karl Eric Hellstrom, Animesh Barua, Pincas Bitterman, Ingegerd Hellstrom, *Common autoantibodies in ovarian cancer (OvCa) and infertility may define biomarkers for OvCa risk*, American Association for Cancer Research (annual meeting), Washington, DC, abstract #2737
 - Yi Yu; Seby L Edassery; Animesh Barua; Pincas Bitterman; Jacques S. Abramowicz; Janice M Bahr; Ingegerd Hellstrom and Judith L Luborsky, *Mesothelin expression in ovarian tumors and serum autoantibodies of the laying hen model is similar to human ovarian cancer*, American Association for Cancer Research (annual meeting), Washington, DC, abstract #3264
 - Bradaric, MJ; Barua, A; Penumatsa, K; Bitterman, P; Abramowicz, JS; Bahr, JM; Luborsky, JL *Lymphocyte content differs in normal ovaries and ovarian tumor stages in spontaneous ovarian cancer in the chicken*. American Association for Cancer Research (annual meeting), Washington, DC, #1918
- Patents and licenses applied for and/or issued; degrees obtained that are supported by this award:
 - none
- Development of cell lines, tissue or serum repositories:

- Hen serum is archived for additional tests.
- Funding applied for based on work supported by this award
 - Awarded: NIH 1R01CA134487-01 (I Hellstrom, PI), J Luborsky (co-invest & PI, subcontract), “Mesothelin as a marker and therapeutic target for ovarian cancer”, 9/15/08-9/14/12. The subcontract objective is to use the hen model for preclinical studies of a vaccine against mesothelin.
 - NIH R01CA143457-01, J Luborsky (PI), “Antibodies as predictors of ovarian cancer and role of B cells in tumor etiology” (submitted February 2009; not funded)
 - NIH (RFA-CA-09-017, The Early Detection Research Network: Biomarker Developmental Laboratories (U01)) , J Luborsky (PI), “Novel cancer risk groups: biomarkers for ovarian cancer risk in infertility,” (submitted October, 2009; not funded)
 - NIH RFA-OD-09-003 for (03) Biomarker Discovery and Validation and specific Challenge Topic, 03-CA-110 Validation of Known Biomarkers”, J Luborsky (PI) “Antibodies to ovarian cancer markers in infertility define ovarian cancer risk” (score 7 percentile; not funded)
 - NIH R01, J Luborsky (PI) “Autoantibodies predict ovarian cancer risk in women with infertility” re-submission February 2011 (Aims based on the Challenge grant with a 7 percentile)
 - Marsha Rivkin Center for Ovarian Cancer Research, “Specific ovarian autoantibodies predict ovarian cancer”, submitted December 2010
- Employment or research opportunities applied for and/or received based on experience/training supported by this award :
 - Dr Barua, co-investigator, was promoted from postdoctoral fellow to Assistant Professor (2008)

CONCLUSION

This study will be the first to show that AOA predicts OVCA. This suggests the possibility of developing a screening test for early detection of OVCA based on antibody detection. A suggested change to future work is to relate the development of ovarian tumors to specific antibodies using recombinant protein antigens for detection. Since we found that many of the antigens associated with human OVCA are also found in hens with OVCA (e.g., mesothelin antibodies) identification of the predictive value of these antibodies in the hen has a direct application to patients at risk of OVCA and development of these tests is a marketable product.

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7. Barua, A., et al., *Anti-ovarian and anti-tumor antibodies in women with ovarian cancer*. Am J Reprod Immunol, 2007. **57**: 243-249.
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9. Barua, A., et al., *Prevalence of anti-tumor antibodies in the laying hen model of human ovarian cancer*. International Journal of Gynecological Cancer, 2009. **18(4)**: 500-507.
10. Yu, Y., et al., *Mesothelin expression in ovarian tumors of the laying hen model is similar to human ovarian cancer*. Gynecol Oncol, 2009. submitted October 2009.

APPENDICES

- Barua A, Bitterman P, Bahr JM, Bradaric MJ, Hales DB, Luborsky JL, Abramowicz JS (2010) Detection of tumor-associated neoangiogenesis by Doppler ultrasonography during early-stage ovarian cancer in laying hens: a preclinical model of human spontaneous ovarian cancer. J Ultrasound Med. 2010 Feb;29(2):173-82.
- Barua A, P Bitterman, JM Bahr, S Basu, E Sheiner, MJ Bradaric, DB Hales, JL Luborsky, JS Abramowicz (2010) Contrast Enhanced Ultrasonography Detects Spontaneous I Ovarian Cancer at Early Stage in a Preclinical Animal Model, J Ultrasound Med, in press
- Bradaric, MJ, Barua, A, Penumatsa, K, Yi,Y, Edassery,SL, Sharma S, Abramowicz,JS, Bahr, JM, Luborsky, J (2010) Sphingosine-1 Phosphate Receptor (S1p1), a Critical Receptor Controlling Human Lymphocyte Trafficking, is Expressed in Hen and Human Ovaries and Ovarian Tumors, submitted December 2010.

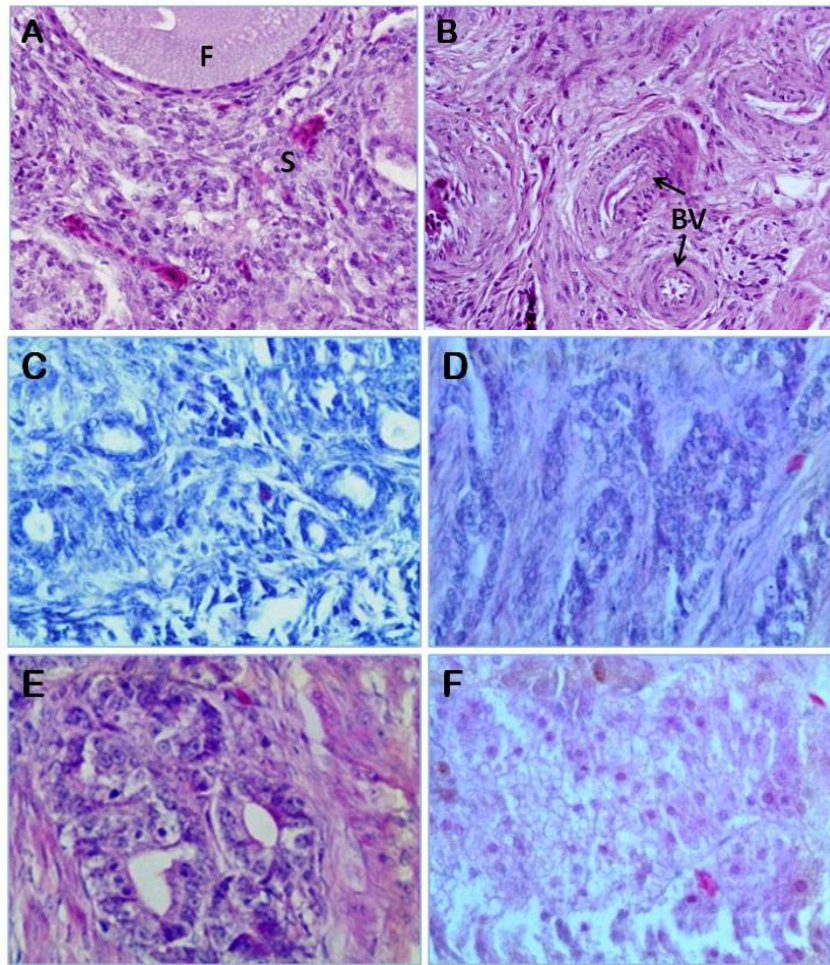


Figure 1. Examples of the diagnostic histology of hen ovaries showing normal ovaries (A-B) and ovarian tumors (C-F). (A) Normal ovary showing follicle (F) and stroma (S). (B) Normal ovary showing the medullary (M) region with large central blood vessels (BV). (C) Endometrioid ovarian tumor (D) Serous ovarian tumor (E) mucinous ovarian tumor (F) clear cell ovarian tumor. Original magnification = 40x.

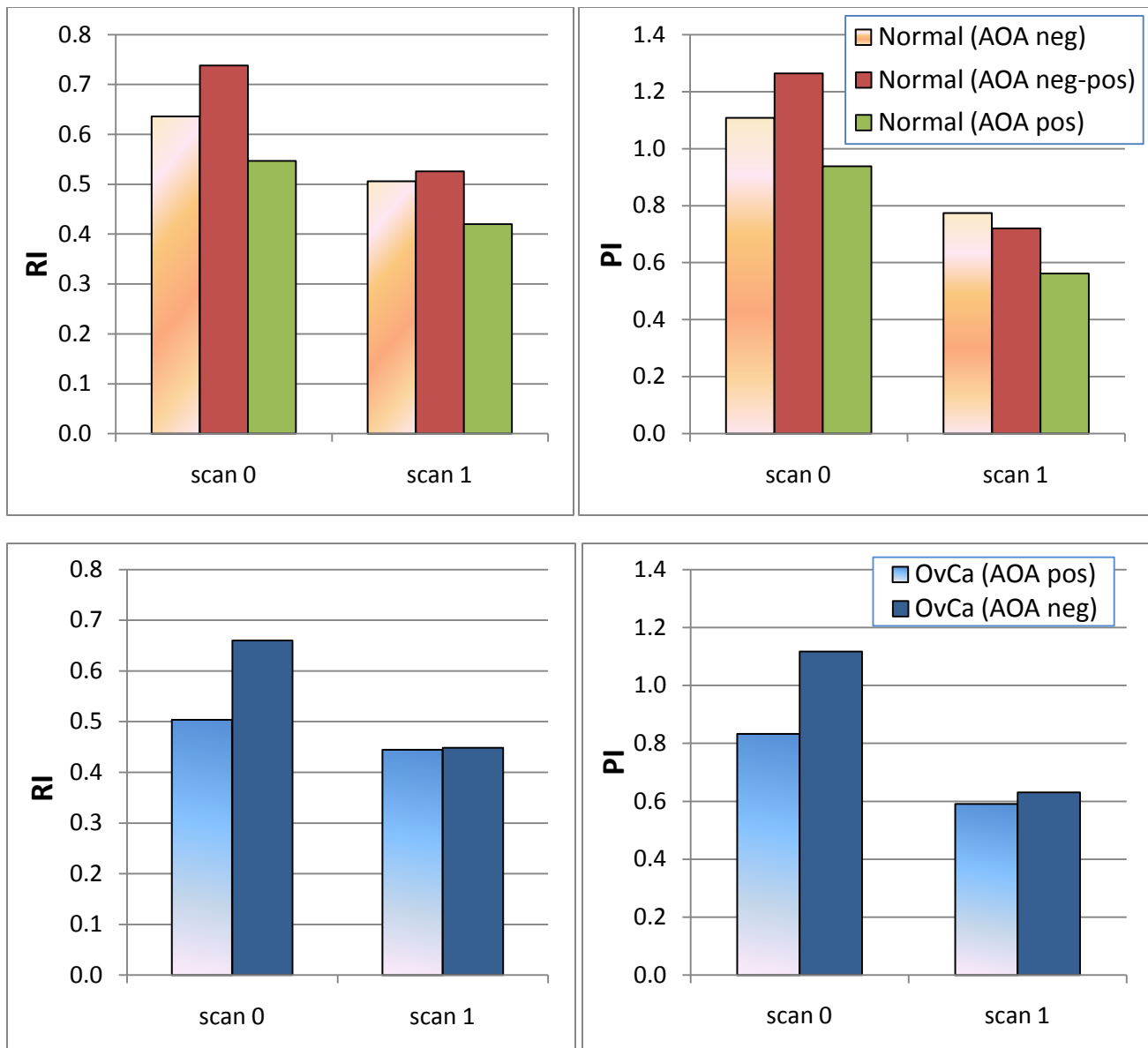


Figure 2. Comparison of changes in angiogenesis by histology and AOA status. Histological analysis was performed at sacrifice which occurred from 4-6 months after the second ultrasound scan.

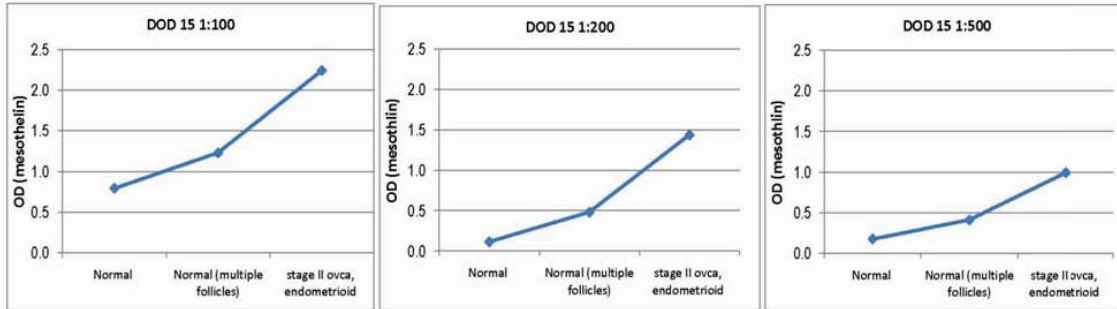
The top panel shows RI and PI values as an indicator of angiogenesis for normal ovarian histology; groups are based on the AOA at the first scan (scan 0) and second ultrasound scan (scan 1) which occurred within an interval of 10 months. AOA either remained negative (AOA neg; n=5), changed from negative to positive (AOA neg-pos; n=5) or remained positive (AOA pos; n=6). The RI/PI values were unchanged in AOA negative hens. The key decline in RI/PI values occurred in the group that switched from negative to positive, with a similar but smaller decline in the AOA positive group suggesting neo-angiogenesis may occur about the same time as the appearance of AOA in hens with normal ovaries.

The bottom panel shows RI and PI values as an indicator of angiogenesis for hens with ovarian tumor histology. Hens either remained AOA positive (AOA pos; n=11) or AOA negative (AOA neg; n=11). Overall the RI and PI values are lower than for hens with normal histology which is consistent with previous data that angiogenesis is an early indicator of tumors. The RI/PI values did not change for AOA positive hens with OvCa, while AOA negative to positive hens showed evidence of neo-angiogenesis from the first to the second ultrasound scan ($p=0.002$ from 1st to 2nd ultrasound scan; Wilcoxon signed rank test). This is a critical group in which negative hens with normal RI/PI values converted to positive with evidence of angiogenesis and ovarian tumors.

LONGITUDINAL COMPARISON OF BLOOD FLOW VELOCITY & MESOTHELIN SERUM ANTIBODY

DOD 15

SCAN DATE	DIAGNOSIS BY MORPHOLGY	blood flow velocity		serum dilution		
		RI	PI	1:100	1:200	1:500
scan1, 7/30/08	Normal	0.46	0.7	0.79	0.12	0.18
scan2, 7/29/09	Normal (multiple follicles)	0.28	0.33	1.23	0.48	0.41
scan3, 9/22/09	stage II ovca, endometrioid	0.56	0.84	2.24	1.44	1.00



DOD 26

SCAN	DIAGNOSIS	blood flow velocity		serum dilution		
		RI	PI	1:100	1:200	1:500
scan1,7/30/08	Normal	0.54	0.84	0.39	0.01	0.12
scan2,7/01/09	normal (multiple follicle)	0.64	1.04	0.94	0.04	0.44
scan3,9/22/09	stage II ovca, endometrioid	0.65	0.97	2.07	1.59	1.03

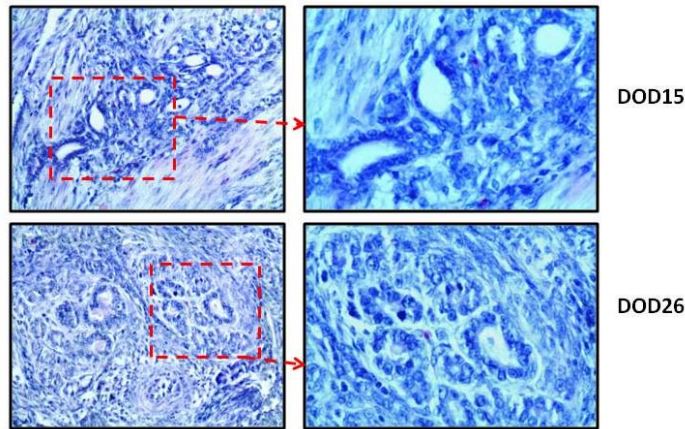
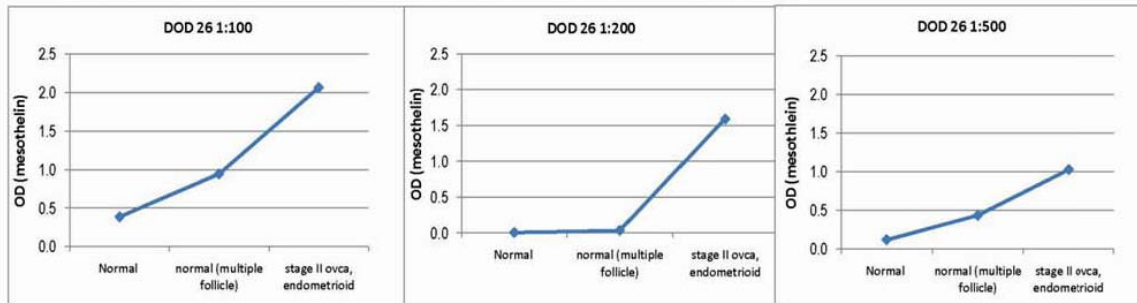


Figure 3. Longitudinal Comparison of Changes in Mesothelin antibody and Angiogenesis during ovarian tumor appearance. Two examples of hens that developed tumors expressing mesothelin determined by RT-PCR are shown (Yi, 2010). Hens were assessed for mesothelin antibody using a previously described immunoassay (Hellstrom et al, 2008). Serum was also assessed for anti-ovarian antibodies in the same sera by a previously described immunoassay (Barua et al, 2008). Angiogenesis (RI value below 0.4) was assessed using color Doppler ultrasound and serum was obtained before each reading. Hen DOD15 was initially positive for anti-ovarian antibodies and mesothelin antibodies began to appear after evidence of angiogenesis (decreased RI). Hen DOD26 was initially negative for anti-ovarian antibodies and developed both anti-mesothelin and anti-ovarian antibodies before evidence of angiogenesis or tumor morphology. Both hens developed mucinous ovarian tumors.

Detection of Tumor-Associated Neoangiogenesis by Doppler Ultrasonography During Early-Stage Ovarian Cancer in Laying Hens

A Preclinical Model of Human Spontaneous Ovarian Cancer

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Abbreviations

AI, angiogenesis index; CA-125, cancer antigen 125; DUS, Doppler ultrasonography; OVCA, ovarian cancer; PI, pulsatility index; RI, resistive index; TAN, tumor-associated neoangiogenesis; TVUS, transvaginal ultrasonography; VEGF, vascular endothelial growth factor

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Objective. Tumor-associated neoangiogenesis (TAN) is one of the earliest events in ovarian tumor growth and represents a potential target for early detection of ovarian cancer (OVCA). Because it is difficult to identify patients with early-stage OVCA, the goal of this study was to explore a spontaneous animal model of in vivo ovarian TAN associated with early-stage OVCA detectable by Doppler ultrasonography (DUS). **Methods.** White Leghorn laying hens were scanned transvaginally at 15-week intervals up to 45 weeks. Gray scale ovarian morphologic characteristics and Doppler indices were recorded. Hens were euthanized at diagnosis for ultrasonographic morphologic/vascular abnormalities or at the end of the study (those that remained normal). Ovarian morphologic and histologic characteristics were evaluated. Vascular endothelial growth factor (VEGF) and $\alpha_v\beta_3$ -integrin expression was assessed by immunohistochemical analysis. Doppler ultrasonographic observations were compared with histologic and immunohistochemical findings to determine the ability of DUS to detect ovarian TAN. **Results.** Significant changes in ovarian blood flow parameters were observed during transformation from normal to tumor development in the ovary ($P < .05$). Tumor-related changes in ovarian vascularity were identified by DUS before the tumor became detectable by gray scale imaging. Increased expression of VEGF and $\alpha_v\beta_3$ -integrins was associated with tumor development. Ovarian TAN preceded tumor progression in hens. **Conclusions.** The results suggest that ovarian TAN may be an effective target for the detection of early-stage OVCA. The laying hen may also be useful for studying the detection and inhibition of ovarian TAN using various means, including the efficacy of contrast agents, targeted molecular imaging, and antiangiogenic therapies. **Key words:** Doppler ultrasonography; early detection; ovarian cancer; ovarian neoangiogenesis; spontaneous animal model.

Ovarian cancer (OVCA) remains a fatal malignancy of women and is responsible for the third highest rate of mortality due to gynecologic cancers, after breast and cervical cancers.¹ In contrast to OVCA, the availability of early-detection tests for other gynecologic malignancies, including breast and cervical cancers, has contributed in part to the gradual decline in death rates due to these malignancies.¹

Because of the lack of an effective screening tool or an early diagnostic method, it is difficult to detect OVCA at an early stage, and, in most cases it is detected at late stages, when the 5-year survival rate of patients is less than 10% compared with greater than 90% when detected at early stages.^{2,3} The nonspecificity of OVCA symptoms at early stages reduces the accessibility to a patient's specimens. It is therefore difficult to validate potential targets for early diagnosis and to establish an effective method to detect those targets.⁴ Consequently, this represents one of the main obstacles to the development of an early-detection test for OVCA.

Substantial studies have been described, involving 3 different approaches to develop an effective early-detection test for OVCA, including a serum marker (cancer antigen 125 [CA-125]), traditional transvaginal ultrasonography (TVUS), and their combination.^{5,6} Although circulating levels of CA-125 have proven useful in determining the prognosis and response to treatment for patients undergoing chemotherapy, elevated CA-125 levels are also associated with many benign gynecologic conditions. In addition to mucinous OVCA, in which elevation of CA-125 levels is less frequent, 20% of patients with OVCA do not show elevated CA-125 levels.⁷ On the other hand, the effectiveness of the Doppler indices (resistive index [RI] and pulsatility index [PI]) from TVUS scanning has been tested extensively to detect early-stage OVCA in humans without any success because of limited resolution and overlap between normal and abnormal indices. Improvement in the overall level of OVCA detection was not observed even with the use of TVUS together with CA-125 because no imaging target in the ovary (indicative of early OVCA-associated morphologic or cellular changes) corresponding to the serum CA-125 level has been defined. Therefore, appropriate changes in the ovarian morphologic, cellular, or molecular architecture associated with early-stage OVCA need to be identified.

Tumor-associated neoangiogenesis (TAN) is one of the earlier events in tumor development. Similar to cancer of other organs, neoangiogenesis is an early event in ovarian malignancy and is a critical determinant of tumor growth, invasion, progression, and metastatic potential.^{8,9}

Therefore, ovarian TAN represents a potential target for early detection of OVCA, and noninvasive diagnostic techniques to assess *in vivo* TAN are needed to detect OVCA at an early stage. Ultrasonography is the currently recommended noninvasive method for the evaluation of ovarian abnormalities, but the difficulty in diagnosing early-stage OVCA impedes conduction of studies to improve the ability of Doppler ultrasonography (DUS) to detect early-stage OVCA in humans. Animal models have been useful for preclinical testing of diagnostic paradigms,¹⁰ but the often-used rodent models of OVCA do not develop OVCA spontaneously, and the histopathologic characteristics of induced OVCA differs from those of spontaneous OVCA in humans.^{10,11} Laying hens (*Gallus domesticus*) develop OVCA spontaneously^{12,13} with similarities to human OVCA in histopathologic characteristics^{12,13} and expression of several markers. The objective of this study was to examine the suitability of ovarian TAN as an *in vivo* detection target of early-stage OVCA by DUS and to further explore the feasibility of the laying hen as a preclinical model to elucidate the ovarian vascular changes relative to OVCA development.

Materials and Methods

Animals

A flock of 110 commercial strains of White Leghorn laying hens (*G domesticus*) was reared under standard poultry husbandry practices with the provision of feed and water *ad libitum*. Egg production and mortality rates of hens (if any) were recorded daily. On the basis of their egg-laying rates and ultrasound scans, 3-year-old apparently healthy hens with low or irregular egg-laying rates (n = 15) were selected from the flock to monitor prospectively. The incidence of OVCA in laying hens of this age group is approximately 15% to 20% and is associated with low or complete cessation of egg laying. A low egg-laying rate indicates reduced ovarian function, and hens that lay 50% less eggs than their peers are considered to have abnormal ovarian function. The normal egg-laying rate for a commercial laying hen is more than 250 eggs per year, and a rate of less than 50% of the normal laying rate is considered a low egg-laying rate.

Ultrasound Scanning

Hens were scanned by TVUS (both B-mode and Doppler) as reported previously,¹⁴ and hens with apparently normal health without any ultrasonographic ovarian abnormality were selected for the study. All hens with suspected ovarian abnormalities on gray scale ultrasonography were excluded from prospective monitoring and euthanized immediately, and ovarian tissues were processed for routine histologic and immunohistochemical evaluation. All procedures were performed according to Institutional Animal Care and Use Committee–approved guidelines. After initial scans, hens were monitored by gray scale ultrasonography and DUS at 15, 30, and 45 weeks using an instrument equipped with a 5- to 7.5-MHz endovaginal transducer (z.one ultrasound system; Zonare Medical Systems, Inc, Mountain View, CA). Briefly, each hen was immobilized by placing the breast up with the legs gently restrained by an assistant. Transmission gel was applied to the surface of the transducer, which was then covered with a probe cover, and gel was reapplied to the covered probe to ensure uninterrupted conductance of the sound waves. The transducer was inserted transvaginally approximately at a 30° angle to the body and 3 to 5 cm into the cloaca (place of vaginal opening). Two-dimensional transvaginal gray scale ultrasonography and color and pulsed DUS were performed. Young egg-laying hens (the ovaries of these hens contain more developing eggs compared with old hens) were used as standard controls for mechanical adjustment to reveal and characterize the fully functional normal ovary. The laying hen contains one functional ovary (left ovary), and the region surrounding the ovary was scanned. Once the ovary was located, the transducer was swept through the entire area for complete scanning of the ovary. Gray scale B-mode morphologic evaluation of the ovary was performed with attention to the follicular development and the presence of any abnormality, including cysts, septations, papillary projections or solid areas, and variation in echogenicity.

After morphologic evaluation, the color Doppler mode was activated for identification of vascular color signals. If blood flow was detected, it was shown as either “peripheral” (color signals in the ovarian periphery) or “central” (blood flow

detected in septa, papillary projections, or solid areas). Once a vessel was identified on color Doppler imaging, the pulsed Doppler gate was activated to obtain a flow velocity waveform. The RI ($[(\text{systolic velocity} - \text{diastolic velocity}) / \text{systolic velocity}]$) and PI ($[(\text{systolic velocity} - \text{diastolic velocity}) / \text{mean}]$) were automatically calculated from at least 2 consecutive samples, and the average RI and PI values were used for analysis. In the stroma of hens with normal ovaries, high blood flows with prominent vascular areas were observed on the surface of developing preovulatory follicles, and data from these areas were excluded from the study. In addition to the blood flow patterns mentioned above, abnormal ovarian Doppler patterns (suggestive of ovarian TAN) were characterized by evaluating intratumoral blood flow and RI and PI values. Hens with higher intratumoral blood flow as well as lower RI and PI values than their normal counterparts were predicted to have ovarian TAN. All images were processed and archived for future reference.

Ovarian Morphologic and Histologic Evaluations

All hens were euthanized at diagnosis of ovarian TAN or at the end of the study, and gross ovarian morphologic specimens were examined, recorded, compared with the ultrasonographic evaluations, and photographed. Normal ovarian function was judged on the basis of the number of large preovulatory follicles (described in detail previously¹⁵) without any ovarian abnormality such as a cyst or solid mass. Abnormal ovaries were characterized by the presence of cysts, a shrunken ovarian volume, or a higher number of bloody, discolored, involuted, or atretic small and pearlike follicles. Tumor staging was performed as reported previously,¹⁵ and the early stage of ovarian tumors was characterized by a detectable solid tissue mass within the ovary, followed by histologic observation. Ovarian tissues were fixed in buffered formalin or optimal cutting temperature compound and snap frozen as reported previously. To detect microscopic OVCA lesions in any part of the ovary, the whole ovary was sampled. The tissue blocks were sectioned (5 μm for paraffin and 10 μm for frozen). Sections from each tissue block (paraffin or frozen) were stained with hematoxylin-eosin and observed

under a light microscope to determine the microscopic features, including tumor lesions and types, and compared with the ultrasonographic evaluations.

Immunohistochemical Analysis

Tissue expression of TAN markers, including vascular endothelial growth factor (VEGF) and $\alpha_v\beta_3$ -integrins, was examined by routine immunohistochemical analysis to assess angiogenesis using rabbit polyclonal antihuman VEGF (Abcam, Inc, Cambridge, MA) and mouse anti-human $\alpha_v\beta_3$ -integrins (CD51/CD61, clone 23C6; BioLegend, San Diego, CA) according to the manufacturers' protocols. The densities of VEGF- and $\alpha_v\beta_3$ -integrin-positive microvessels were counted from the tumor vicinity or stroma of normal hens (excluding the follicular areas) using a light microscope attached to digital imaging stereologic software (MicroSuite version 5; Olympus Corporation, Tokyo, Japan) and were expressed as the number of positive vessels in a 20,000- μm^2 area of the normal or tumor ovary. The index for TAN (angiogenesis index [AI]) was calculated for hens with early- and late-stage OVCA as a ratio of the frequency of VEGF-expressing vessels to that in normal ovaries (number of ovarian VEGF-expressing microvessels in each tumor hen/average of ovarian VEGF-expressing microvessels in normal hens; the AI for a normal ovary is considered 1).

Statistical Analysis

Differences in ultrasonographic measurements between two scans of the same group of hens were analyzed by paired sample *t* tests. Differences between two groups (normal and OVCA) of hens (for ultrasonographic indices and the number of microvessels positive for angiogenic markers) were analyzed by 2-sample *t* tests and Mann-Whitney tests. All reported *P* values are 2 sided, and *P* < .05 was considered significant. Statistical analyses were performed with SPSS version 15 software (SPSS Inc, Chicago, IL).

Results

Gray Scale Ultrasonography

No significant changes were detected in the ovarian morphologic characteristics of hens at the

second scan (15 weeks from the initial scan) on gray scale ultrasonography. Similar to the initial scan, all hens had a normal-appearing ovary containing 2 or 3 preovulatory follicles with developing eggs at the second scan (Figure 1A). At the third scan, 30 weeks from the initial scan, substantial changes in the gray scale ovarian morphologic characteristics were observed in 4 of 15 hens (Figure 1C), including the absence of preovulatory large follicles with the appearance of solid tissue masses. However, no such changes were observed in the ovaries of the remaining 11 hens. At the final scan (45 weeks from the initial scan), 5 of the remaining 11 hens developed ovarian abnormalities with no detectable large preovulatory follicles.

Doppler Ultrasonography and Detection of Ovarian TAN

The blood flow patterns at the initial scan were located in ovarian "periphery," mostly on the surface of large preovulatory follicles and small growing follicles in the stroma of hens with normal ovarian morphologic characteristics selected for prospective monitoring. Confluent blood flow in areas surrounding the small developing follicles and the walls of the larger preovulatory follicles was observed in these hens. Although no detectable change in ovarian morphologic characteristics was identified (by gray scale imaging mentioned above), blood flow patterns changed from peripheral to a mixture of peripheral (on the surface layer of large ovarian follicles) and central at the second scan (after 15 weeks; Figure 1B) in 6 hens. In 9 hens, RI (mean \pm SD, 0.42 ± 0.03) and PI (0.54 ± 0.07) values at the second scan were significantly lower compared with the initial scan (mean RI, 0.52 ± 0.05 ; mean PI, 0.70 ± 0.1 ; *P* < .001 for both RI and PI based on paired sample *t* tests; Table 1). At the third scan, with the changes in gray scale morphologic characteristics (including the reduction in the number of detectable follicles and appearance of solid tissue masses), flow patterns changed from mixed to central in these hens (Figure 1D). The RI and PI in these hens decreased further from the second to third scan (mean RI, 0.32 ± 0.09 ; mean PI, 0.40 ± 0.11 ; *P* < .008 and .005 for RI and PI, respectively, based on paired sample *t* tests; Table 1). The

ovaries of the remaining 6 hens had a normal appearance on DUS throughout the monitoring period (mean RI, 0.56 ± 0.08 ; range, 0.47–0.69; mean PI, 0.84 ± 0.2 ; range, 0.63–1.21). Thus, 9 of 15 hens showed changes in their ovarian blood flow patterns and were suspected to have ovarian TAN by 45 weeks from the initial scan.

Ovarian Morphologic and Histologic Characteristics

Gross ovarian morphologic findings were consistent with the ultrasonographic observations. No detectable tissue mass was found in 6 hens

predicted to have normal ovarian morphologic characteristics and vascularity on gray scale ultrasonography and DUS, respectively, at the final 45-week scan (Figure 2A). In contrast, hens with the diagnosis of ovarian TAN and tumor-related morphologic characteristics during or at the end of the study on ultrasonography had tumor-related solid tissue masses (Figure 2C). These hard and solid tissue masses were found to be limited in 1 or 2 areas of the ovary only, although other parts were normal. In addition, no accompanying ascites was found at euthanasia.

Figure 1. Changes in ovarian morphologic characteristics with blood flow patterns leading to tumor development in laying hens. **A**, Gray scale sonogram of a normal hen ovary at the second scan (15 weeks after first scan). The presence of multiple preovulatory follicles (asterisks) of various sizes with no solid mass indicates the functionally normal ovary. **B**, Doppler sonogram of the same ovary imaged in **A** showing blood flows on the follicular wall of the larger preovulatory as well as small stromal follicles, suggesting active follicular growth in the ovary. **C**, Gray scale sonogram of the same hen ovary shown in **A** at the third scan (after 30 weeks from first scan). No developing follicle is seen, and the ovary appears to have solid tissue masses, indicating abnormal morphologic characteristics suggestive of an ovarian tumor. **D**, Doppler sonogram of the same ovary shown in **C**. A central pattern of blood flow is seen on the solid ovarian mass, suggesting the presence of an ovarian tumor. EDV indicates end-diastolic velocity; PSV, peak systolic velocity; and S, stroma.

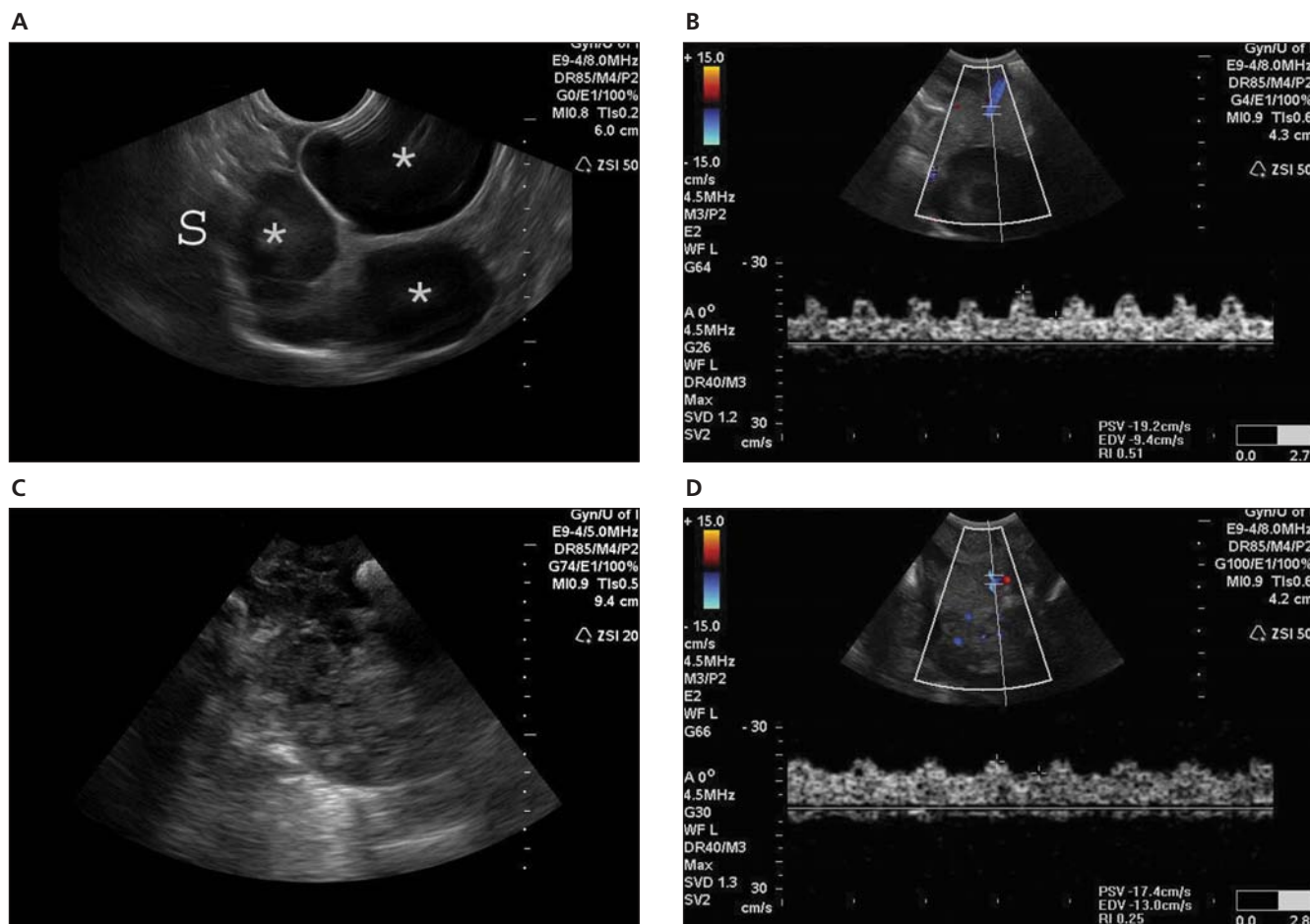


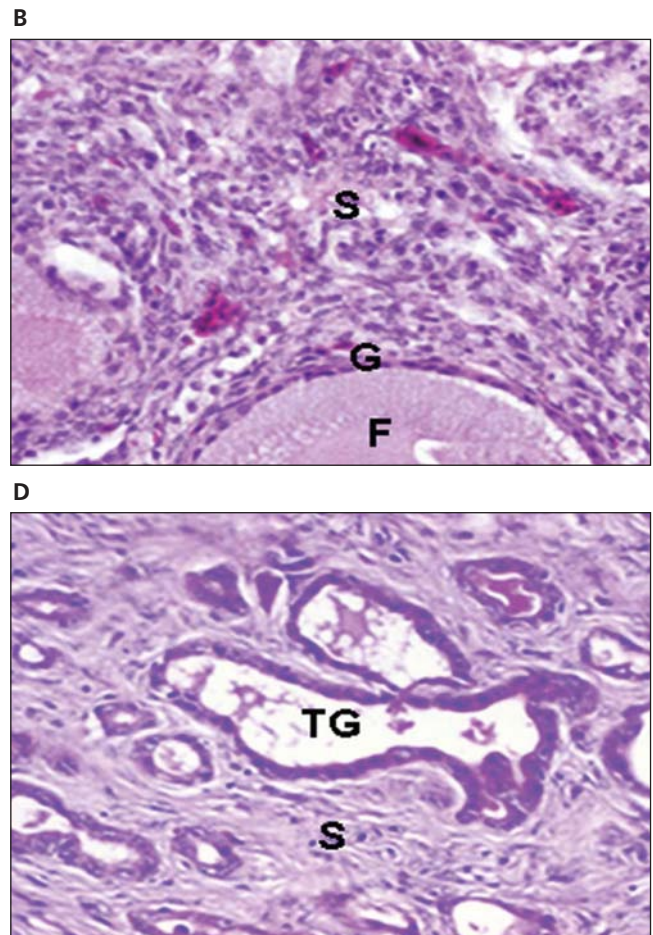
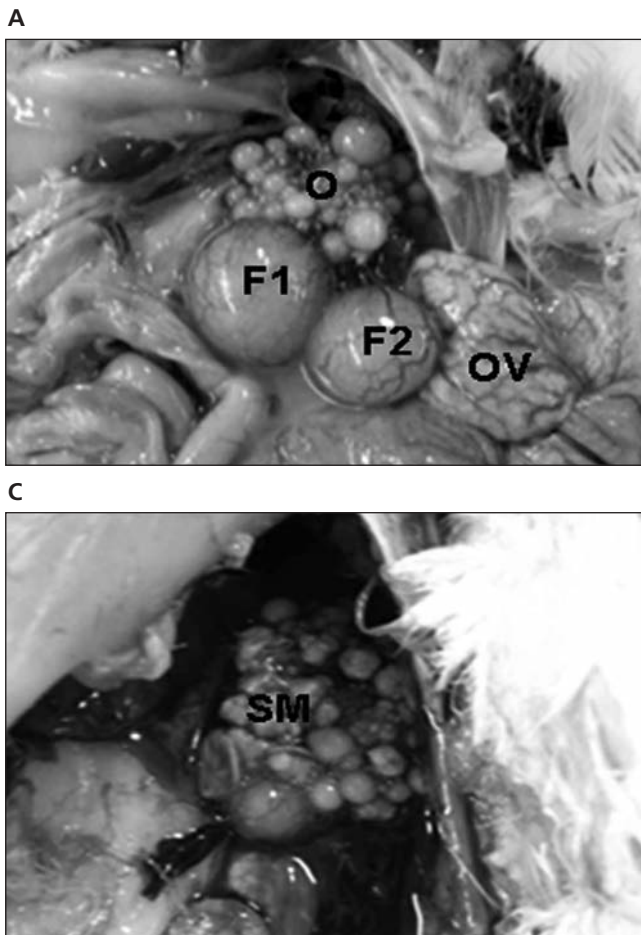
Table 1. Changes in DUS Indices in Association With the Development of Ovarian TAN in a Laying Hen Model of Spontaneous OVCA (n = 9 Hens)

Parameter	1st Scan (Initial Scan)		2nd Scan (After 15 wk)		P (Between 1st and 2nd Scans)	3rd Scan (After 30 wk)		P (Between 2nd and 3rd Scans)
	Range	Mean ± SD	Range	Mean ± SD		Range	Mean ± SD	
RI	0.43–0.60	0.52 ± 0.05	0.36–0.46	0.42 ± 0.03	<.001	0.18–0.48	0.32 ± 0.09	<.008
PI	0.56–0.84	0.70 ± 0.1	0.42–0.62	0.54 ± 0.07	<.001	0.21–0.54	0.40 ± 0.11	<.005

Histologic evaluation confirmed the presence of ovarian tumors (Figure 2D) in 9 hens suspected to have ovarian TAN and tumor-related hardened tissue masses as detected by DUS and gross morphologic evaluation. Glandular tumor structures containing a single layer of epithelial cells or lacelike papillary projections or glands with

secretory ciliated goblet cells as well as many blood vessels of different sizes were seen in hens with the diagnosis of ovarian TAN (Figure 2D). However, such tumor-associated microscopic features, together with hyperplasia and dysplasia, were also found by histologic examinations in 3 additional hens that were not suspected to

Figure 2. Gross and microscopic appearance of a normal ovary and an early-stage ovarian tumor in laying hens. The predicted presence of ovarian TAN by ultrasonography was confirmed by gross and histopathologic examination after euthanasia. **A**, Normal hen ovary showing preovulatory follicles (developing eggs). **B**, Ovarian stroma of the same hen containing embedded follicles (paraffin section stained with hematoxylin-eosin; original magnification ×100). **C**, Ovary showing a solid tumor mass (early stage without ascites). **D**, Corresponding paraffin section stained with hematoxylin-eosin showing confluent tumor glands in the stroma (original magnification ×100). F indicates follicle; G, granulosa layer; O, ovary; OV, oviduct; S, stroma; SM, solid mass; and TG, tumor gland.



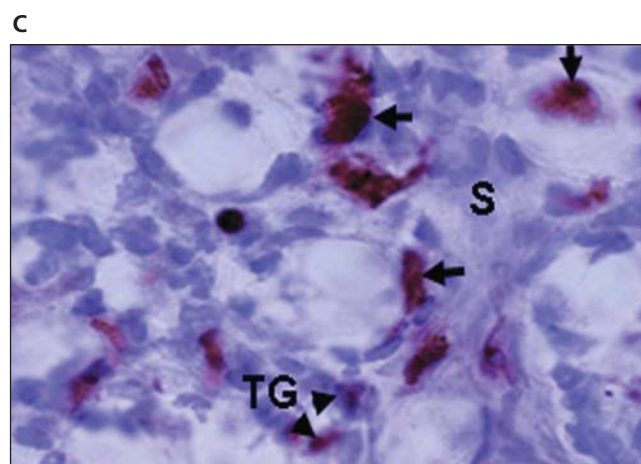
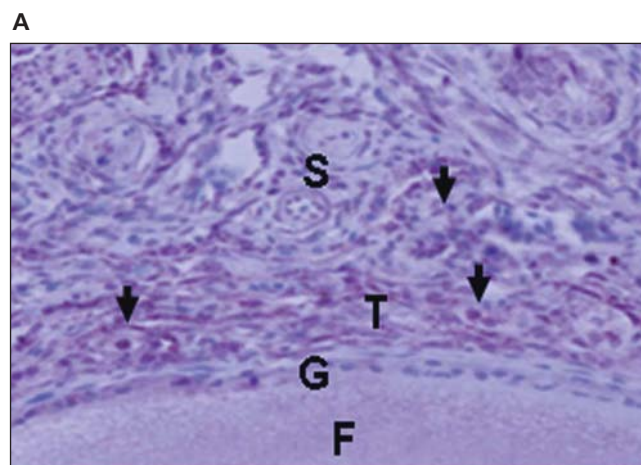
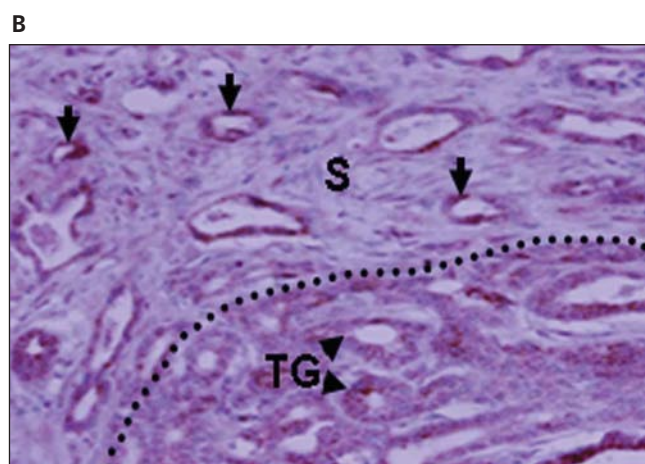
have TAN by ultrasonography. Thus, of 15 hens, 9 had DUS-detectable ovarian TAN and small tumor masses, and 3 had microscopic tumor lesions that remained undetectable by DUS monitoring up to 45 weeks. In contrast, no such change was detected in 3 of the remaining 6 hens that appeared normal at the end of 45 weeks of monitoring (Figure 2B).

Expression of Angiogenic Markers

Intense staining for VEGF was observed in the tumor epithelium and microvessels at the tumor vicinity in hens suspected to have ovarian TAN with a subsequent diagnosis of early-stage OVCA by histologic evaluation (Figure 3B, dotted line). In addition to the tumor vicinity, many microves-

sels expressing VEGF were also seen in the stroma preceding tumor glands (Figure 3B). Similar patterns were also observed for $\alpha_v\beta_3$ -integrin expression in tumor glands as well as stromal blood vessels (Figure 3C). These immunopositive microvessels appeared to be leaky without any well-organized continuous smooth muscle layer surrounding them and stained intermittently. In normal ovaries, immunopositive vessels were seen in and around the developing stromal follicles with very few in the stroma outside the follicles (Figure 3A). The frequency of ovarian VEGF-expressing microvessels was significantly higher ($P < .002$, Mann-Whitney exact test) in hens with TAN (mean, 3.7 ± 0.58 , 6.89 ± 0.73 , and 9.8 ± 1.1 microvessels/20,000- μm^2 area of ovarian

Figure 3. Expression of neoangiogenic markers in hen ovaries predicted to be normal and with ovarian TAN by ultrasonography. **A**, Section showing normal vasculature with few VEGF-stained vessels in the follicular theca and stroma of a hen monitored prospectively by ultrasonography up to 45 weeks (from the hen shown in Figure 2A; original magnification $\times 40$). **B**, Ovarian section of a hen suspected to have ovarian TAN by ultrasonography (from the hen shown in Figure 2C; original magnification $\times 40$). In contrast to **A**, many microvessels and the tumor epithelium expressed VEGF with high intensity. More VEGF-expressing microvessels are seen in the tumor vicinity and stroma preceding tumors (separated by an imaginary dotted line). The numbers of VEGF-expressing microvessels in hens suspected to have ovarian TAN are negatively correlated to their ovarian Doppler indices (RI and PI values; see "Materials and Methods" for detail analysis). Furthermore, the pattern of localization of VEGF-positive vessels in TAN-diagnosed hens suggests that ovarian TAN precedes tumor progression and is associated with the decline in Doppler indices. **C**, Frozen ovarian section of a hen with an ovarian tumor (shown in Figure 2C) immunostained for $\alpha_v\beta_3$ -integrins (original magnification $\times 100$). $\alpha_v\beta_3$ -Integrins are expressed in the microvessels. F indicates follicle; G, granulosa layer; S, stroma; T, theca layer of the stromal follicle (F); and TG, tumor glands. Arrows indicate examples of immunopositive microvessels (**A–C**), and arrowheads indicate examples of immunopositive tumor glands (**B and C**).



tumor tissues in microscopic, early-stage, and late-stage OVCA, respectively) than normal hens (mean, 2.4 ± 0.51 microvessels/20,000 μm^2 area of ovarian tissues). Similar trends were also observed in the frequency of ovarian $\alpha_v\beta_3$ -integrin-positive vessels with respect to the different OVCA stages. The AI for early-stage OVCA was significantly higher than in hens with normal ovaries (2.83 versus 1; $P < .01$, Mann-Whitney exact test). The AI was highest in hens with late-stage OVCA (4.08 versus 1; $P < .04$, for hens that had a diagnosis of late-stage OVCA at the first scan and were euthanized immediately instead of prospective monitoring). Tumor volumes were not examined in this study; hence, no correlation between tumor size and Doppler intensity was evaluated. However, Doppler signals were significantly lower with advancing tumor stages. Moreover, we evaluated the correlation between abnormal versus normal Doppler patterns and immunohistochemical findings, and tissue expression of angiogenic markers and AI values were inversely correlated ($r = -0.90$ and -0.88 for RI and PI, respectively) to Doppler indices (the higher the AI, the lower the RI and PI), indicating that establishment of ovarian TAN is associated with an increase in blood flow to the tumor vicinity.

Discussion

To our knowledge, a study reporting the detection of ovarian TAN during the early stage of the disease by gray scale ultrasonography and DUS in an animal model of human spontaneous OVCA has not been reported previously. This study revealed that ovarian TAN is initiated after neoplastic cellular transformation in the ovary even before the tumor becomes detectable by traditional TVUS. The results of this study suggest that ovarian TAN can be a potential target for the detection of early-stage OVCA by noninvasive ultrasonography if its current detection limit can be improved. Because of the difficulty in identifying patients with early-stage OVCA, the laying hen model can be used to study methods to enhance the detectability of early-stage OVCA on ultrasonography.

The fatality of OVCA is due in part to the lack of a reliable and effective early-detection method. A plethora of serum tumor markers have been

reported, and given the heterogeneity of OVCA in different women, none of these markers are routinely effective in detecting early-stage OVCA.⁷ Moreover, the association of any of these markers with early tumor-related changes in ovarian morphologic characteristics has not been reported. Furthermore, despite extensive efforts, the use of CA-125, with or without ultrasonography, to detect early-stage OVCA remains ineffective.⁷ Use of a morphologic index of the architectural features of ovarian neoplasms on pelvic ultrasonography to predict a probability of ovarian malignancy without the use of CA-125 has been shown to have positive predictive values of up to 0.45.¹⁶ However, this morphologic index requires information on tumor parameters, including tumor size and the presence of ascites (which in most cases is associated with advanced OVCA), to effectively predict tumor malignancy. Consequently, this method is unable to detect early-stage OVCA. Previous studies reported the expression of VEGF and $\alpha_v\beta_3$ -integrins in tumor epithelia and blood vessels in patients with OVCA.⁹ Similarly, these markers of ovarian TAN were also detected in hens with early- and late-stage OVCA. In addition, in hens with early-stage OVCA (including hens in which tumors were not detected by DUS), the expression of VEGF and $\alpha_v\beta_3$ -integrins in many blood vessels in the tumor vicinity (Figure 3, B and C) as well as in the stroma adjacent to the tumor suggests that ovarian TAN precedes tumor progression. Thus, ovarian TAN represents an effective target for the detection of early-stage OVCA if it can be detected. Because it is difficult to study patients with early-stage OVCA, laying hens can be used to determine the ultrasonographic parameters indicative of early-stage OVCA-related neoangiogenesis.

Doppler ultrasonography is related to the vascular architecture of a tissue and is currently an accepted method for in vivo imaging of tissue vascularity. In this study, we monitored healthy laying hens with reduced ovarian function by DUS methods until they developed ovarian TAN or up to 45 weeks at 15-week intervals. Hens with reduced ovarian function compared with their peers of a similar genetic background and reared under similar conditions are considered to be at risk of developing OVCA, as reported earlier.^{14,15}

Detection of changes in ovarian vascularity before the tumor became detectable by gray scale ultrasonography in 9 of 15 hens suggests that ovarian TAN can be detected by prospective monitoring of hens. However, this study also confirmed some limitations of DUS in detecting early-stage OVCA-related ovarian TAN. Although DUS detected 9 hens with early-stage OVCA (hens with a small solid ovarian mass and a larger area of the ovary that remained normal at detection), it failed to detect ovarian TAN in 3 hens with microscopic OVCA without any detectable mass. We believe that had we continued to monitor beyond 45 weeks, we would have been able to detect OVCA in these hens also. This failure of DUS reveals its limited capability in detecting ovarian TAN related to microscopic OVCA. It is known that Doppler sensitivity is limited for distinction of OVCA, but in this study, we analyzed changes in the indices, each hen serving as its own control. Although Doppler indices obtained by traditional ultrasonography do not confirm the *in vivo* detection of microvessels, immunohistochemical detection of VEGF expression indicates the presence of an increased number of microvessels in hens with OVCA compared with normal hens. Ultrasonographic contrast agents have been used to enhance the detection limits of DUS in other organs, and few studies of patients with advanced stage OVCA have shown improvement in the detection of ovarian tumor-related vascularity.^{17–21}

The lack of a suitable animal model of spontaneous OVCA is also a reason for fewer studies with contrast agents and presents a considerable barrier to developing effective contrast media for diagnosing early-stage OVCA. With our imaging methods, a laying hen can be scanned repeatedly with DUS in a short interval without affecting it physiologically. This animal model can also be useful in testing which contrast agents show early-stage ovarian tumor-related vascularity better than others. Because ovarian tumors in laying hens also express VEGF and $\alpha_v\beta_3$ -integrins, this spontaneous animal model of OVCA can be used to determine the efficacy of molecularly targeted imaging using microbubbles conjugated with anti- $\alpha_v\beta_3$ -integrins or anti-VEGFR-2 against ovarian $\alpha_v\beta_3$ -integrins and VEGFR-2.

Similarly, it may also be useful in determining the efficacy of *in vivo* imaging-guided, ovary-targeted antiangiogenic therapy in the future.

This was a pilot study, and the small sample size was a limitation. On the basis of our results, however, we conclude that neoangiogenesis may be detected by DUS in the laying hen model of spontaneous OVCA. Expression of relevant markers is associated with the detection of very early ovarian tumors, suggesting that neoangiogenesis may be a feasible target for the detection of early-stage OVCA. Furthermore, this study showed that progression from normal to abnormal ovaries could be followed in the hen model, supporting its utility as a preclinical model of OVCA.

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1 **Contrast Enhanced Ultrasonography Detects Spontaneous Ovarian Cancer at Early Stage**
2 **in a Preclinical Animal Model**

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30 **Objective.** Our goal was to examine the feasibility of using laying hens, a preclinical model of
31 human spontaneous ovarian cancer (OVCA), in determining the kinetics of ultrasound contrast
32 agent indicative of ovarian tumor associated neoangiogenesis (TAN) at early stage OVCA.

33 **Methods.** Three years old White Leghorn laying hens with decreased ovarian function were
34 scanned before and after intravenous injection of OptisonTM at a dose of 5 μ L/kg body weight.

35 Gray scale morphology, Doppler indices, arrival time, peak intensity and Wash-out of contrast
36 agent was recorded and archived on still images and video clips. Hens were euthanized thereafter
37 and ultrasound prediction was compared at gross examination and ovarian tissues were collected.

38 Archived clips were analyzed to determine contrast parameters and Doppler intensities of
39 vessels. A time-intensity curve per hen was drawn and area under the curve (AUC) was derived.

40 Tumor types and the density of ovarian microvessels were determined by histology and
41 immunohistochemistry and compared with ultrasound predictions. **Results.** Contrast agent

42 significantly ($P<0.05$) enhanced the visualization of microvessels which was confirmed by
43 immunohistochemistry. Contrast parameters including *Time of Wash-out and AUC* were

44 significantly different ($P<0.05$) between ovaries of normal hens and hens with OVCA and
45 correctly detected OVCA at early stages than the *time of peak*. **Conclusions.** The laying hen may

46 be a useful animal model to determine ovarian tumor associated vascular kinetics diagnostic of
47 early stage OVCA using a contrast agent. This model may also be useful to test the efficacy of
48 different contrast agents in a preclinical setting.

49 **Key words:** Contrast enhanced ultrasonography; early detection; ovarian cancer; ovarian tumor
50 associated neoangiogenesis; spontaneous animal model.

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53 Neovascularization, preceded by malignant cellular transformation, is an early event during
54 tumor development [1, 2]. Tumor associated neo-angiogenesis (TAN) has been shown to be a
55 critical requirement for the progression and metastasis of malignant tumors of the ovary as well
56 as other organs [1, 3, 4]. Therefore, ovarian TAN represents the potential to be an early detection
57 target for ovarian cancer (OVCA), a fatal disease responsible for the highest death rate of women
58 from all gynecological malignancies[5]. Previous reports on the immunohistochemical
59 localization of tumor microvessels in post operative surgical specimens have shown a positive
60 relationship between tumor progression and tumor associated neo-angiogenesis and suggested
61 the utility of ovarian TAN as a tool for the detection of OVCA at an early stage [6-8]. Therefore,
62 an *in vivo* non-invasive method of detecting ovarian TAN relative to early stage OVCA is highly
63 desirable.

64 Gray scale and Doppler ultrasound imaging are the non-invasive and currently recommended
65 modalities for the evaluation of gynecologic abnormalities including ovarian tumors. Although
66 Doppler ultrasound can detect large intratumoral blood vessels, in many cases including early
67 stage OVCA, it cannot detect neoangiogenic smaller vessels [9, 10]. Failure to distinguish benign
68 from malignant ovarian tumors and detecting OVCA at early stages are the two main limitations
69 of current Doppler ultrasound imaging [11, 12]. This failure of detection is either because of the
70 smaller sizes of tumor associated microvessels or the Doppler shifts in these vessels are too small
71 or too weak to be detected by the current detection limit of the ultrasound scanner [13].

72 Therefore, Doppler ultrasound may be an effective method for early detection of OVCA if its
73 current detection limit can be improved to detect ovarian TAN. A moderate number of studies
74 have shown the efficacy of ultrasound contrast agents (UCAs) to enhance the sensitivity of
75 traditional Doppler ultrasound to visualize blood vessels and distinguish malignant tumors from

76 benign ones on the basis of intratumoral microvascular architecture [13-16]. There is limited
77 literature evaluating contrast enhancement in adenexal masses and, to our knowledge, most of
78 the reported studies on the ovarian imaging by contrast enhanced ultrasound were based on
79 patients with late stage OVCA as difficulty to obtain access to patients with early stage OVCA
80 may be one of the reasons [13, 14, 17, 18]. Thus observations on contrast enhanced ultrasound
81 imaging of these studies may not be useful as-such for the early detection of OVCA. As the 5-
82 year survival rates of OVCA patients is remarkably high when the disease is detected at early
83 stage, studies on the contrast enhanced ultrasound imaging with early stage OVCA cases are
84 needed to establish an early detection method for OVCA. Animal models serve as surrogates to
85 elucidate information on the etiology and mechanism of human diseases especially for those
86 which are difficult to access in patients including OVCA [19]. However, the lack of a feasible
87 spontaneous model of human OVCA represents one of the principal barriers to the understanding
88 of early ovarian tumor development and its detection. Rodents do not develop OVCA
89 spontaneously and the histopathology of induced OVCA in these animals does not resemble that
90 of spontaneous OVCA [19, 20].

91 The laying hens (*Gallus domesticus*) have been shown to be the only widely available
92 animal that develop OVCA spontaneously[21] with similar histopathology [22] and expression
93 of several cellular and molecular makers common to human ovarian tumors [23-26]. Moreover,
94 the incidence rate increases with age (10-40% between 2.5 to 6 years of age)[21]. The high
95 ovulation rate in commercial strains of laying hens (a hen lays an egg almost every day with an
96 ovulatory cycle of about 24-26 hours during its reproductive life) also mimics the *incessant*
97 *ovulation theory* (a condition referring to the frequent ovulation resulting in repeated trauma as
98 *well as wear and tear of the ovarian surface epithelium*)[27]) a much accepted risk factor for

99 OVCA in humans (a detailed description of the reproductive physiology of laying hens can be
100 found in ref nos. 20, 25). Recently, a transvaginal Doppler ultrasound imaging method for hen
101 ovarian tumors using a transducer similar to that used for humans has been reported [28]. As in
102 humans, tissue expression of vascular endothelial growth factor (VEGF), a marker of ovarian
103 TAN, has also been shown to be increased in association with tumor progression in hens [4, 26].
104 In addition, laying hens are easily accessible especially because commercial poultry farms
105 maintain large population of hens which they usually cull when hens get older. Therefore, the
106 laying hen represents a potential feasible surrogate to explore information related to the contrast
107 enhanced ultrasound imaging diagnostic of OVCA at early stages which is difficult to perform in
108 humans.

109 The goal of this pilot study was to examine the feasibility of using laying hens to
110 determine the kinetics of ultrasound contrast agents indicative of ovarian TAN at early stage
111 OVCA. We hypothesized that the ultrasound contrast agent Optison™ would enhance the
112 visualization of ovarian vascularity in the laying hen relative to OVCA.

113 **Methods and Materials**

114 *Animals*

115 Approximately 150 three years old (commercial strains of) White Leghorn laying hens (*Gallus*
116 *domesticus*) were maintained in individual cages with standard poultry husbandry practices
117 including the provision of feed and water *ad libitum*. Egg laying rates (an indicator of ovarian
118 function; low egg laying rate indicates decreased ovarian function) of hens were recorded on
119 daily basis. The normal rate of egg laying by a commercial laying hen is >250 eggs/year and
120 <50% of normal laying rate is considered as low egg laying rate [4, 29]. Hens (n=46) with low,

121 irregular egg laying rates or those stopped egg laying and with or without any abdominal
122 distention (a sign of possible ovarian tumor associated ascites) were selected randomly from a
123 layer flock for ultrasound scanning. The incidence of OVCA in laying hens of this age group was
124 reported to be approximately 15-20% and is associated with the low or complete cessation of egg
125 laying[22, 30].

126 ***Preparation of Optison™ contrast agent***

127 Optison™ (GE Healthcare, St Louis, MO) is a sterile non-pyrogenic suspension of microspheres
128 of human serum albumin with perflutren Protein-Type A of 3.0-4.5 μm in size that create an
129 echogenic contrast effect in blood. Optison™ was prepared prior to injection according to the
130 manufacturer's direction. Briefly, the vial containing Optison™ suspension was inverted and
131 gently rotated to resuspend the microspheres completely. The suspension was transferred from
132 the vial by an injection syringe with a 19 gauge needle to a angiocatheter (small vein infusion
133 set, female luer, 12" tubing, needle of 27 gauge, Kawasumi Laboratories, FL) containing 100 μL
134 of 0.9% sodium chloride previously inserted into the left wing vein (brachial vein) of the hen and
135 followed by the reloading of 1mL 0.9% sodium chloride solution. The loading of sodium
136 chloride solution pre and post injection of Optison™ helped to maintain the vascular patency and
137 air tight condition, in addition to the flushing of the Optison™ from the hen's circulation. The
138 dosage of Optison™ was determined after prior attempts with several dosages using 5 laying
139 hens with fully functional ovaries and 5 $\mu\text{L}/\text{kg}$ body weight was considered optimal for better
140 resolution. With the dosage higher than 5 $\mu\text{L}/\text{kg}$ body weight, the initial arrival of Optison™
141 caused an excessive broadening of color areas (blooming; due to increase in flow signal strength)
142 [13, 31, 32]. Blooming effect was detected when gray scale pixels change to a color display in
143 regions where no flow, in fact, exists. Although in few hens, 5 $\mu\text{L}/\text{kg}$ body weight dosage, the

144 exact border of enhanced vessels often vanished blooming was not so intense that it would
145 disturb the counting of the vessels number. Blooming effects were also reported in previous
146 studies.

147 *Ultrasound scanning*

148 Pre-contrast scanning

149 All procedures were performed according to Institutional Animal Care and Use Committee
150 approval. Ultrasound scanning was performed in a continuous pattern before and after the
151 injection of Optison™ [4, 28] (GE Healthcare, St Louis, MO) with the mechanical set up
152 reported previously. Briefly, all hens were scanned using an instrument equipped with a 5- to
153 7.5-MHz endovaginal transducer (MicroMaxx; SonoSite, Inc, Bothell, WA). Each hen was
154 immobilized by placing hen with the breast up with legs gently restrained by an assistant.
155 Transmission gel was applied to the surface of the transducer; a probe cover was applied; and gel
156 reapplied to the covered probe to ensure uninterrupted conductance of the sound waves. The
157 transducer was inserted approximately at a 30° angle to the body, 3 to 5 cm into the cloaca
158 (transvaginal), and 2-dimensional transvaginal gray scale sonography as well as pulsed Doppler
159 sonography were performed. Young egg-laying hens (as the ovaries of these hens contain more
160 developing follicles compared to old hens) were used as standard controls for mechanical
161 adjustment to reveal and characterize the fully functional normal ovary of hens. The area of a
162 tumor to be imaged was determined according to three conditions: (1) the whole tumor should be
163 seen on the image, if possible; (2) the sectional plane should contain the solid part (wall, septae
164 and papillae) of the tumor; and (3) the most vascularized area was selected. For normal ovaries,
165 ovaries without any detectable tumor or atrophied ovaries, the region surrounding the ovary was
166 scanned and the transducer was swept through the entire area for complete scanning of the ovary.

167 Gray scale morphologic evaluation of the ovarian mass was performed with attention to the
168 number of large preovulatory follicles, the presence of abnormal-looking follicles, bilaterality,
169 septations, papillary projections or solid areas, and echogenicity. After morphologic evaluation,
170 the color Doppler mode was activated for identification of vascular color signals. If blood flow
171 was detected, it was shown as either “peripheral” (color signals in the wall or periphery of a
172 follicle or a suspected mass) or “central” (blood flow detected in septa, papillary projections, or
173 solid areas). Once a vessel was identified on color Doppler imaging, the pulsed Doppler gate was
174 activated to obtain a flow velocity waveform. The resistive index (RI: [systolic velocity –
175 diastolic velocity]/systolic velocity) and the pulsatility index (PI: [systolic velocity – diastolic
176 velocity]/mean) were automatically calculated from at least 2 consecutive samples (two separate
177 images from the same ovary), and the lower RI and PI values were used for analysis. All images
178 were processed and digitally archived.

179 Post-contrast scanning

180 Post-Optison™ injection scanning was performed in a similar and continuous manner with
181 identical mechanical settings as described above. The same pre-contrast imaged area was imaged
182 after Optison™ injection in post-contrast scanning. All images were archived digitally in still
183 format as well as real time clips (6 min for each hen) in single sided recordable digital video discs
184 (DVD + R format, Maxell Video/Data Media, Maxell Corporation of America, Fair Lawn, NJ)
185 readable with personal computer later.

186 *Evaluation of contrast agent effect*

187 The effect of Optison™ contrast agent was evaluated: a) visually during the examination and b)
188 afterwards from reviewing the archived video clips. The time of arrival of contrast agent [time

189 interval in seconds from administration of the contrast agent to its visual observation (in
190 seconds)] in the normal or tumor ovarian vessels was recorded on a real time basis. After review
191 of complete clip, the region of interest (ROI) was selected by drawing around the area of solid
192 tissue containing the largest number of vessels with minimal artifacts similar to those reported
193 earlier [14, 17]. For normal or apparently normal (without detectable tumor mass at gray scale)
194 ovaries, stomal but not follicular vessels were counted. A colored area separated from others
195 with a distinct wall typical of blood vessel was assessed before and after the injection of the
196 contrast agent. Therefore, post-contrast appearing of blood vessels in this study could be either a
197 new vessel or a branch of a vessel already recognized before administration of the contrast agent.
198 Analysis of pixel intensities of blood vessels was carried out with a computer assisted software
199 program (MicroSuite version 5; Olympus Corporation, Tokyo, Japan). Using the software, the
200 intensity of the ROI (sum of the pixel values within the ROI) was measured before contrast agent
201 administration and at intervals of 5 seconds after the arrival of contrast agent up to 6 minutes.
202 Then the peak intensity, the time of peak intensity (secs), intensity at wash-out and the time of
203 wash-out (secs) were determined. Total number of blood vessels from each ROI was counted
204 when the intensity was at peak. In addition, the resistive and pulsatility indices (RI and PI,
205 respectively) were calculated before and after the contrast injection using the formula reported
206 earlier [28].

207 The data were then transferred to a computerized spreadsheet program (Excel 2003,
208 Microsoft, Redmond, Washington) and a time-intensity curve was derived for each hen. The
209 time-intensity curves were analyzed to calculate area under the curve (AUC). The AUC
210 diagnostic of OVCA in hens was derived from the arrival of the contrast agent and the end of the
211 wash-out period minus the area under the baseline in time-intensity curve.

212 ***Ovarian morphology***

213 Following ultrasound scanning, all hens were euthanized and examined for the presence of solid
214 tumor mass in the ovary and/or in any other organs, ascitic fluid, large preovulatory follicles or
215 atrophy of the ovary as reported previously[22]. Gross ovarian status was recorded and compared
216 with sonographic evaluations, and photographed. At gross examination, an ovary is considered
217 normally functional if it contains viable large preovulatory follicles for laying hens (for more
218 detail information on the hen ovarian physiology, see references (20, 26) or atrophy of ovary
219 without any large follicles or visible lesions in hens out of egg laying. In contrast to the normal
220 ovary, the abnormal ovary was characterized by the presence of cysts, bloody, discolored,
221 involuted or atretic large preovulatory, small and pear-shaped follicles. Tumor staging was
222 performed based on gross metastatic status and histological observation as reported previously
223 [22]. Briefly, early OVCA (stage 1 or 2) was characterized by detectable formation of a solid
224 tumor in the ovary or extensive tumors but still restricted to the ovary. Late stages of OVCA
225 were characterized when the tumor metastasized to distant organs with moderate to extensive
226 ascites.

227 ***Ovarian histopathology and immunohistochemical detection of ovarian microvessel density***

228 Representative portions of a solid ovarian mass or the whole ovary (in case of atrophied or
229 apparently normal appearing ovaries) were divided into several blocks, processed for paraffin
230 sections and stained with hematoxylin & eosin (H&E). Microscopic tumor lesions in any part of
231 the ovary were detected by routine histology with H&E staining and tumor types were
232 determined by light microscopy as reported previously [22].

233 Following histopathological examination, paraffin sections (5 μ m thick) of normal and tumor
234 ovaries of all stages and types were processed for routine immunohistochemistry to assess the
235 tumor associated microvessel density using monoclonal anti- α smooth muscle actin antibodies
236 (primary antibody, Invitrogen, Carlsbad, CA) according to manufacturer's protocols. The
237 densities of immunopositive microvessels were counted from the tumor vicinity or stroma of
238 normal hens (excluding the follicular areas) as reported earlier [33, 34] using a light microscope
239 attached to digital imaging stereologic software (MicroSuite version 5; Olympus Corporation,
240 Tokyo, Japan) with a little modification. Briefly, immunostained slides were examined at low
241 power magnification (X10 objective and X10 ocular) to identify the areas of maximum
242 neovascularization of the tumor. Vessels with thick, regular and complete muscular wall as well
243 as vessels with large lumina were excluded from the count as reported previously [34]. Brown
244 stained leaky smaller vessels with discontinuous or incomplete vessel wall, clearly separated
245 from adjacent microvessels, tumor cells or other connective tissue elements were considered to
246 be a single, countable vessel. In each section, 5 most vascular areas were chosen. The number of
247 microvessels in a 20,000- μ m² area was counted at X40 objective and X10 ocular magnification.
248 The averages of these counts were expressed as the number of immunopositive microvessels in
249 20,000- μ m² area of normal or tumor ovaries. Tumor histology and immunohistochemical
250 observations were compared to the ultrasound predictions.

251 *Statistical analysis*

252 Descriptive statistics for contrast parameters were determined and statistical analysis was
253 performed by SPSS 15 (SPSS, Chicago, IL) statistical software. The differences between the
254 power Doppler intensity changes before and after contrast injection were analyzed by the paired
255 t-test and the (exact) sign test. The differences in the contrast parameters and the density of

256 immunopositive microvessels between the normal and tumor ovaries were analyzed by the two-
257 sample t-test and the (exact) Mann-Whitney test. The χ^2 test was used to investigate the
258 association between the contrast parameters including the Wash-out time or AUC (area under the
259 curve) with the presence or absence of tumor lesion. *P* values less than 0.05 were considered
260 significant. All reported *P*-values are two-sided.

261 **Results**

262 *Ultrasonography*

263 Optison™ injected at a dosage of 5μL/kg body weight was well tolerated and none of the hens
264 showed any adverse condition.

265 *Normal and tumor ovaries in hens*

266 Normal ovaries in healthy hens (n = 24) were found to have multiple preovulatory follicles with
267 many small growing stromal follicles by gray scale ultrasound imaging. Blood vessels were
268 detected in the ovarian stroma by Doppler ultrasound (Figure 1A). In addition, confluent blood
269 flows were detected on the surfaces of the large preovulatory follicles. As compared to pre-
270 contrast scan, Optison™ injection enhanced visualization of ovarian blood vessels in the same
271 area of the ovary (Figure 1B). Solid ovarian masses with or without projected septa and papillary
272 structures and/or accompanied ascites were observed in the ovaries of 15 hens by gray and
273 Doppler ultrasound scan and these hens were “predicted to have ovarian tumors” (Figure 1C).
274 No large preovulatory follicles were observed in these hens. Out of these 15 hens, 11 had solid
275 masses in the ovary together with profuse ascites and were categorized to have late stage OVCA.
276 In the remaining 4 hens, solid masses were found to be limited to a part of the ovary without any
277 detectable ascites and they were categorized provisionally as early stage OVCA (final diagnosis

278 was performed upon gross examination). Optison™ injection remarkably enhanced the
279 visualization of ovarian tumor associated vascular network in all hens predicted to have OVCA
280 (Figure 1D).

281 The ovary in few hens (n=7) appeared regressed with only one or no large preovulatory
282 follicles. Although no detectable solid ovarian mass was observed at gray scale ultrasound in
283 these hens, their RI and PI values were lower than the hens with normal ovary. These hens were
284 termed as “hens with abnormal ovarian morphology” as their diagnosis was not conclusive by
285 ultrasound scanning (Figure 2A-D) and subsequent histopathological examinations (detailed
286 below) showed the presence of microscopic malignant tumor lesions in a portion of the ovary of
287 these hens. Thus these hens were finally categorized as “hens with microscopic OVCA”. The
288 pre- and post-contrast RI and PI values were significantly lower in hens suspected to have
289 OVCA compared to their normal counter parts ($P < 0.01$ from Exact Mann-Whitney test) (Table
290 1).

291 *Contrast parameters*

292 *Number of blood vessels*

293 Overall, as compared with pre-contrast, the number of detectable blood vessels in the normal as
294 well as in the ovaries with tumor was significantly increased following the injection of
295 Optison™ ($P < 0.05$) (Table 1). As compared to normal hens, significantly more blood vessels
296 were observed in hens predicted to have ovarian tumors ($P < 0.05$) and their frequencies
297 increased as the tumor progressed from microscopic to later stages (Table-1).

298 *Doppler intensity and contrast kinetics*

299 Optison™ injection, as compared with the pre-contrast, significantly enhanced the power
300 Doppler intensity signal to more than two-fold in all hens irrespective of their pathological status
301 ($P < 0.05$) (Figure-3). Compared to hens with normal ovaries, the intensity of post-contrast
302 Doppler signals was highest in hens predicted to have ovarian tumors ($P < 0.05$) (Figure-3).

303 Although the time of arrival and the time to reach peak intensity of contrast agent were
304 tended to be faster in hens predicted to have ovarian tumors than normal hens, the difference was
305 not statistically significant (Table 2). The wash-out of contrast agent had two phases: a fast initial
306 decrease and a second was slower decrease approximately to the baseline. The wash-out of
307 Optison™ was shorter in normal hens and was significantly longer in hens predicted to have
308 ovarian tumors ($P < 0.05$) (Table 2). As compared to hens with normal ovary, significantly greater
309 AUC was observed in hens predicted to have ovarian tumors ($P < 0.05$) (Table 2). Differences
310 were not observed among hens of different tumor types (serous, endometrioid, mucinous and
311 sero-mucinous mixed) with respect to their contrast parameters.

312 ***Histopathology and Microvessel Density***

313 Gross examination of hens at necropsy confirmed ultrasound prediction. Ovarian tumors, their
314 stages and types were confirmed at necropsy and by routine histology with H&E staining,
315 respectively (Figure 4A-D). As observed in ultrasound scanning, late stage OVCA (n = 11 hens,
316 4 serous, 5 endometrioid, 1 mucinous, 1 sero-mucinous mixed) was associated with moderate to
317 profuse ascites and metastasis to distant organs. Early stage OVCA (n=4 hens) was limited to the
318 ovary (1 serous, 1 endometrioid, 2 sero-mucinous mixed) with little or no ascites. The seven hens
319 initially diagnosed as “hens with abnormal ovarian morphology” without any grossly detectable
320 solid ovarian mass during gray scale ultrasound had microscopic malignant ovarian lesions upon

321 H&E staining in one or more areas of the ovary and thus were classified as hens with
322 microscopic OVCA.

323 Immunopositive microvessels were detected in both normal ovaries and ovarian tumors
324 (Figure 5). In normal hens most of the immunopositive ovarian vessels had thick, complete and
325 continuous vessel walls with intense staining. In contrast, most of the vessels in ovarian tumors
326 were leaky, discontinuous or incomplete with thin vessel walls (Figure 5). In normal hens, most
327 of the immunopositive microvessels were localized in the follicular theca with few positive
328 blood vessels in the ovarian stroma. The densities of ovarian immunopositive microvessels were
329 significantly higher ($P < 0.01$, Mann-Whitney exact test) in hens predicted to have ovarian TAN
330 at contrast enhanced ultrasonography (mean, 8.0 ± 0.53 , 13.0 ± 0.84 , and 19.0 ± 1.3
331 microvessels/20,000- μm^2 area of ovarian tumor tissues in microscopic, early-stage, and late-
332 stage OVCA, respectively) than normal hens (mean, 4.0 ± 0.55 microvessels/20,000 μm^2 area of
333 ovarian tissues). In addition to immunopositive microvessels, smooth muscle fibers surrounding
334 the tumor glands but not the tumor epithelium were also stained positively. However, differences
335 were not observed among different tumor types with regard to their immunopositive microvessel
336 densities. Thus increased number of ovarian microvessels in ovaries with tumor compared to
337 normal ovaries confirmed the *in vivo* prediction of ovarian tumor associated neoangiogenesis by
338 contrast enhanced ultrasonography.

339 ***In vivo detection of ovarian tumor associated neoangiogenesis***

340 Pathological status of hens including tumor staging (by histopathology) and tissue expression of
341 ovarian microvessels (by immunohistochemistry) were compared with contrast parameters. Data
342 on 3 contrast parameters including the *Peak post-contrast Doppler intensity*, *Wash-out time of*
343 *contrast agent* and *the AUC* were used retrospectively to determine the efficacy of contrast

344 parameters in detecting *in-vivo* ovarian tumor associated neo-angiogenesis. Two cut-off values
345 for each of these 3 contrast parameters namely, the mean + 2 standard deviations (SD) and the
346 mean + 3 SD of peak intensity value and Wash-out time or AUC of normal (control) hens were
347 used to examine the detectability of ovarian TAN at early stage of OVCA. All three parameters
348 with both the cut-off values detected in all hens with OVCA at early stage (tumor detected at
349 gross examination and limited to the ovary) or late stage. The cut-off value of *Peak post-contrast*
350 *Doppler intensity* with 2 SD detected 86% of (6 of 7) hens with microscopic tumor lesion
351 (without any detectable tumor by gray scale or gross at euthanasia) whereas those with 3 SD
352 detected only 43% of (3 of 7) these hens (Figure 3). **On the other hand, cut-off value for AUC**
353 **with 3 SD detected 86% of hens with microscopic OVCA (Table 2).** However, *Wash-out time of*
354 *contrast agent* detected all hens with microscopic OVCA using both cut-off values (Table 2 and
355 **Figure 6**). Thus, the AUC and the Wash-out time of contrast agent were found more efficient in
356 the detection of microscopic OVCA than other contrast parameters.

357 **Discussion**

358 This is the first study on the improvement of *in vivo* detection of ovarian tumor associated
359 vascular architecture by the ultrasound contrast agent OptisonTM contrast agent in laying hens, a
360 spontaneous animal model of human OVCA. Our goals were to test the efficacy of a contrast
361 agent in enhancing the visualization of ovarian tumor associated blood vessels in laying hens and
362 to determine the contrast kinetics associated with early stage OVCA in laying hens. The results
363 of the present study suggest that the contrast agent enhanced the detection of the hen ovarian
364 vascular network. Our results also suggest that laying hens provide a new and valid platform to
365 study kinetic parameters relative to early stage OVCA using a contrast agent. The translational
366 significance of this study is that the laying hen may be a suitable preclinical model of

367 spontaneous human OVCA for the study of contrast enhanced ultrasound imaging to establish an
368 early detection test for OVCA. This animal model of spontaneous OVCA may also be useful to
369 test *in vivo* the efficacy of different contrast agents in a preclinical setting.

370 In the present study, as compared to pre-contrast scanning, significant enhancement in the
371 detection of ovarian microvessels by power Doppler ultrasound using a contrast agent without
372 any adverse physiological effect suggests the utility of this contrast agent in hens as reported in
373 humans. The time of washout of the contrast agent and the AUC were significantly different
374 between hens with ovarian tumors and hens with normal ovaries. Similar patterns were also
375 reported in OVCA patients [13, 14]. Ovarian RI and PI values decreased in hens with normal or
376 tumors ovaries following the contrast agent injection irrespective of their disease status.
377 Although the precise reason(s) is not known, it is possible that the increase in the amount of
378 recognizable vessels as well as increased blood flow may be the factors for such decrease in RI
379 and PI values following the injection of contrast agent. Lower RI and PI values in hen ovarian
380 tumors than in hen normal ovaries also support previous findings that lower RI and PI values are
381 associated with OVCA [4, 28] and thus these indices may be used together with the contrast
382 parameters to distinguish malignant ovarian vasculature from that of the normal ovary.
383 Decreased RI and PI values in association with increased number of blood vessels
384 (immunohistochemical observation) confirmed the enhanced detection of ovarian tumor
385 associated vascular network by contrast parameters using OptisonTM. Thus the tolerance as well
386 as the enhanced detection of ovarian blood vessels by contrast agent in hens as in humans
387 suggests the suitability of laying hens as a preclinical animal model to study the contrast kinetics
388 diagnostic of early stage OVCA.

389 Ovarian TAN is the hallmark of tumor development and progression. Contrast parameters
390 including the time of wash-out of the contrast agent and the AUC, as observed in the present
391 study, may be useful in distinguishing normal ovarian physiological angiogenesis from the
392 ovarian tumor associated neo-angiogenesis at the early stage of OVCA. An increase in ovarian
393 leaky and immature microvessels as observed in this study is one of the characteristics of ovarian
394 TAN [1, 35]. These increased microvessel densities were associated with contrast parameters
395 predictive of ovarian tumors. Recently, we have reported that ovarian tumor progression in hens
396 is associated with increased frequency of VEGF-expressing ovarian blood vessels [4]. Thus an
397 increase in ovarian microvessel frequency (as observed in this study) together with the increased
398 expression of VEGF by these microvessels [4] may suggest that VEGF also plays a critical role
399 in ovarian tumor progression in laying hens as in humans. Therefore, VEGF represents an
400 additional marker of OVCA. Elevated concentrations of circulatory VEGF have been reported to
401 be associated with OVCA in humans. Although not determined in the current study, a serum
402 VEGF concentration may identify which hens are at risk of developing OVCA and may be
403 followed prospectively with ultrasound monitoring for detecting the OVCA related
404 morphological changes in the ovary. Because VEGF is not specific for ovarian TAN only, it is
405 possible that circulatory VEGF (marker of TAN) in association with other techniques like
406 contrast enhanced ultrasound imaging may constitute an effective early detection test for OVCA.
407 The laying hen may be useful in establishing this early detection test. Recently, we have
408 generated anti-chicken VEGF antibodies to detect the serum concentrations of VEGF by
409 immunoassay in chickens. Thus, the findings of the current study may facilitate future studies
410 with the elevated circulatory VEGF concentrations to monitor hens prospectively to detect the

411 development of OVCA at an early stage and to establish an early detection test in this preclinical
412 model.

413 The small number of hens (total = 46 hens, 24 normal, 7 microscopic, 4 early stage and
414 11 late stages of OVCA) used is a limitation of the current study. We have used both the paired t-
415 test and its nonparametric equivalent, the exact sign test for before and after comparison of
416 Optison effects. Similarly, we have used both the two-sample t-test as well as, its nonparametric
417 equivalent, the exact Mann-Whitney test for comparing normal versus tumor ovaries. The
418 reported significant results were typically found in both the t-test (paired or two-sample, as
419 appropriate) as well as the nonparametric test (sign test or the Mann-Whitney test). The p-values
420 for the exact sign test and exact Mann-Whitney test are computed by numerical methods without
421 any large sample approximations and are valid in small samples. Thus the remarkable increase in
422 the intensity of ovarian vascular architecture as well as the number of blood vessels following
423 contrast agent injection may form the foundation for a larger study to establish an effective early
424 detection test for OVCA. Furthermore, laying hens may also be useful in developing the ovarian
425 TAN targeted molecular imaging and in monitoring the effects of anti-angiogenic drugs related
426 to the recession or relapse of ovarian tumors.

427 Few reports have described that Doppler imaging using contrast agent to assess tumor
428 enhancement has low contrast and resolution, substantial background noise, and considerable
429 operator dependence and suggested that these limitations can be addressed by new sonographic
430 imaging methods, such as pulse inversion harmonic (PIH) imaging. [11, 14, 35]. Although we
431 have not used PIH method, we adjusted the dosage of OptisonTM for optimal contrast and
432 resolution with minimum background noise in our initial studies in this model.

433 In conclusion, the results of this pilot study showed that the laying hen, a pre-clinical
434 spontaneous animal model of human OVCA may be useful to understand and determine ovarian
435 tumor associated vascular kinetics during early and late stage OVCA using contrast enhanced
436 ultrasonography. Information on contrast enhanced ultrasound imaging for the detection of early
437 stage OVCA in laying hens will also provide the foundation for clinical studies.

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547 **Table 1:** Changes in Doppler indices and the number of blood vessels in hen ovaries
 548 following the injection of Optison™.

Ovarian pathology	RI values		PI Values		Number of blood vessels	
	Before	After	Before	After	Before	After
Normal	0.68 ± 0.05 (0.60-0.80)	0.56 ± 0.05 (0.49-0.74)	1.05 ± 0.21 (0.83-1.46)	0.80 ± 0.11 (0.68-1.12)	3.29 ± 1.04 (2.00-5.00)	6.17 ± 1.31 (4.00-9.00)
Microscopic OVCA	0.59 ± 0.04 (0.54-0.66)	0.47 ± 0.03 (0.43-0.53)	0.88 ± 0.10 (0.74-1.08)	0.65 ± 0.08 (0.58-0.78)	5.14 ± 0.90 (4.00-6.00)	13.71 ± 3.90 (7.00-18.00)
Early stage OVCA	0.43 ± 0.01 (0.42-0.44)	0.33 ± 0.04 (0.29-0.38)	0.56 ± 0.02 (0.54-0.58)	0.44 ± 0.07 (0.36-0.53)	8.50 ± 1.00 (7.00-9.00)	18.50 ± 5.20 (15-26)
Late stage OVCA	0.36 ± 0.04 (0.30-0.40)	0.25 ± 0.04 (0.18-0.32)	0.46 ± 0.07 (0.34-0.56)	0.33 ± 0.07 (0.22-0.41)	12.91 ± 2.43 (10.00-18)	24.09 ± 4.61 (20-32)

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573 **Table 2:** Kinetics of contrast agent relative to stages of ovarian cancer in hens.

Ovarian pathology	Time of arrival of contrast agent (secs)	Time to reach peak intensity (secs)	Time of wash-out (secs)	Percentage of hens with OVCA detected by Time-of-Washout*	Area under the curve (AUC)	Number of hens with OVCA detected by AUC*
Normal	15 ± 0.82 (14-16)	21 ± 0.81 (20-22)	51 ± 2.44 (48-56)	Ref	5496.94 ± 1439.80	Ref
Microscopic OVCA	14 ± 1.21 (12-15)	20 ± 2.04 (17-22)	72 ± 4.24 (69-81)	100% (<i>P</i> <0.01)	15310.86 ± 2771.64	86% (<i>P</i> <0.05)
Early stage OVCA	14 ± 0.96 (13-15)	19 ± 1.29 (17-20)	120 ± 11.15 (108-135)	100% (<i>P</i> <0.01)	49404.00 + 10957.71	100% (<i>P</i> <0.05)
Late stage OVCA	13 ± 1.12 (11-14)	18 ± 0.94 (17-19)	150 ± 4.94 (142-156)	100% (<i>P</i> <0.01)	80607.00+ 15247.76	100% (<i>P</i> <0.05)

574 Ref = reference

575 * above the cut-off value (mean of normal + 3SD) for Time-of-Washout and AUC.

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596 **Figure Legends:**

597 **Figure 1:** Pre- and post-contrast Doppler ultrasonograms of hen ovaries with or without tumors.
598 Scanning of hens was performed in a continuous manner before and after the injection of the
599 OptisonTM. Images including still and movie clips were digitally archived. Contrast kinetics
600 namely, the time of arrival, time to reach peak intensity and time of wash-out as well as pre-and
601 post-contrast Doppler indices (RI and PI values) were recorded. A) Pre-contrast sonogram of a
602 normal appearing ovary showing a preovulatory follicle and few small follicles without any solid
603 mass and B) is the corresponding post-contrast sonogram of the same hen. As compared to pre-
604 contrast, more vessels are seen in the post-contrast sonogram. C) Pre-contrast sonogram of a
605 hen predicted to have ovarian tumor. Solid tumor-like mass is seen in the ovary and a central
606 blood flow pattern with a few vessels is detected in and around the mass. D) Post-contrast
607 sonogram of the ovary shown in C). The Contrast agent remarkably enhanced the intensity of
608 blood vessels as well as visualization of more blood vessels. As compared to pre-contrast, many
609 blood vessels are seen in the post-contrast scan. Ultrasound predictions were confirmed at gross
610 (euthanasia) and histopathological examination. S = Stroma of ovary, SM = Solid tissue mass in
611 the ovary.

612 **Figure 2.** Contrast agent (OptisonTM) enhanced the ability of Doppler ultrasound to detect
613 microscopic ovarian tumor associated vasculature in laying hens. A) Pre-contrast sonogram of an
614 ovary without detectable solid ovarian mass or abnormality. Only a few blood vessels are seen in
615 this ovary with no preovulatory follicle. B) Corresponding sonogram of the same ovary showing
616 the arrival of contrast agent OptisonTM. As compared to pre-contrast, the number of detectable
617 blood vessels is increased and as compared to pre-contrast, the vessels appear more dilated in the
618 post-contrast sonogram. C) Post-contrast sonogram of the same ovary showing peak level of

619 enhancement. As compared to pre-contrast and post-contrast at arrival of Optison, more vessels
620 are detected at peak enhancement. Although no solid ovarian mass is detected but the central
621 pattern of vascular arrangement indicates a potential ovarian abnormality. D) Gross appearance
622 of the same ovary at euthanasia. As predicted, neither large preovulatory follicle nor detectable
623 solid mass is seen. However, subsequent histopathological examination showed the presence of
624 an endometrioid tumor lesion (termed as microscopic ovarian tumor) confirming the prediction
625 of contrast enhanced ultrasound scanning. Scanning of hens and their subsequent processing
626 were similar to those mentioned in Figure 1. S= Stroma; Dotted circle indicates the ovary.

627 **Figure 3.** Detection of ovarian tumor associated neo-angiogenesis by post-contrast Doppler
628 intensity of blood vessels. A flock of 150 hens were monitored for their ovarian function and
629 their egg laying rates were recorded on a daily basis. Hens with low egg laying rates (n=46) were
630 selected for sonography. Based on gray scale sonography, gross and histopathological
631 examinations, hens were grouped to have normal ovary (n=24 hens), microscopic OVCA (n=7),
632 early stage OVCA (n=4) and late stage OVCA (n=11). Post-contrast Doppler intensities of
633 ovaries or ovarian tumors were measured by power Doppler sonography following the injection
634 of OptisonTM. Cut-off lines [mean peak Doppler intensity values of normal hens with 2 or 3
635 standard deviation (SD)] indicate the detectability of OVCA by post contrast peak Doppler
636 intensities.

637 **Figure 4.** Gross and histological sections of ovarian tumors in hens scanned with contrast
638 enhanced ultrasound. Following contrast enhanced Doppler ultrasound, hens were euthanized
639 and examined grossly for the presence of tumor-related solid ovarian mass which was confirmed
640 by routine histology with hematoxylin and eosin (H&E). A) Ovary of a hen with early stage of
641 ovarian cancer (OVCA). Few small solid tumor masses with no preovulatory follicles are

642 present. The tumor is limited to a part of the ovary. B) Section taken from a normal and
643 uninvolved portion of an ovary in which a tumor was detected in the other part of the ovary
644 (early stage OVCA). Stromal follicles are embedded in the normal appearing stroma. No
645 invasion of tumor cells is seen in this portion of the ovary. C) An ovarian tumor in hens with late
646 stage of OVCA. The solid tumor mass appears like a cauliflower. The tumor metastasized to
647 distant organs and was associated with extensive ascites. D) Section of a serous ovarian tumor
648 from a hen with late stage OVCA. The tumor has a compact sheath of tumor cells with
649 pleomorphic nuclei and tumor glands are surrounded by fibromuscular layers (H&E). All
650 sections are of 20X magnification; F = follicle, G = granulosa layer of follicle, S = ovarian
651 stroma, SM = solid tumor mass, T = theca layer of follicle, Tu = tumor

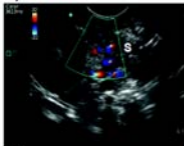
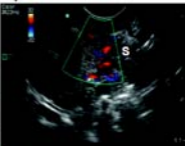
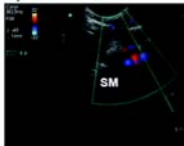
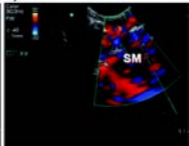
652 **Figure 5.** Immunohistochemical detection of ovarian microvessels in laying hens with or without
653 ovarian cancer (OVCA). Paraffin sections were immunostained with monoclonal anti- α smooth
654 muscle actin. A) Section of a hen's normal ovary. Immunopositive microvessels are seen in the
655 follicular theca and few in the adjacent stroma. Most of the immunopositive vessels have a thick
656 vessel wall. B) Section of hen's ovary with early stage OVCA [ovary shown in Fig 4(A)]. As
657 compared to the normal ovary, more immature immunopositive microvessels with leaky, thinner,
658 incomplete or discontinuous vessel walls are present between tumor glands. The fibrous
659 connective tissue of the tumor glands are also stained positive. C) Section of an ovarian tumor
660 from a hen with late stage OVCA [ovary shown in Fig 4(C)]. As compared to normal and early
661 stage OVCA, many leaky and immature microvessels with discontinuous or attenuated staining
662 are seen in vicinity of the tumor. Arrows indicate examples of immunopositive microvessels. F
663 = follicle; G = granulosa layer; S = stroma; T = theca layer. Dotted circles indicate examples of
664 tumor glands.

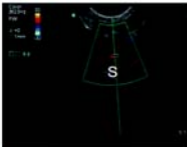
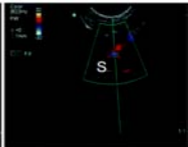
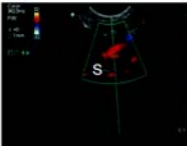
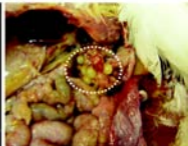
665 **Figure 6.** Detection of ovarian tumor associated neo-angiogenesis by Wash-out time of contrast
666 agent. Other information is similar to the legend of Fig 3. Cut-off lines [mean of Wash-out time
667 (seconds) of normal hens with 2 or 3 SD] indicate the detectability of OVCA by the Wash-out
668 time of contrast agent.

669
670 **Abbreviations**

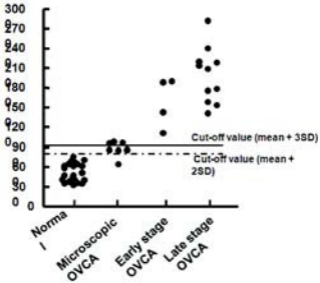
671 AUC, area under the curve; OVCA, ovarian cancer; PI, pulsatility index; RI, resistive index;
672 TAN, tumorassociated, neoangiogenesis; VEGF, vascular endothelial growth factor

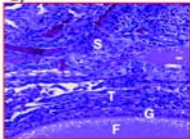
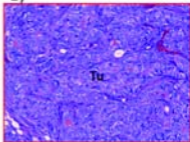
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A)**B)****C)****D)**

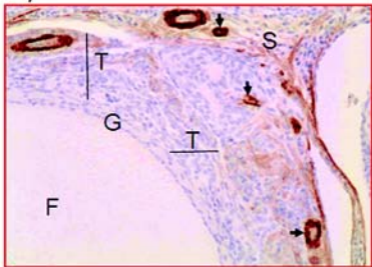
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Post-contrast Doppler peak intensity (pixel values)

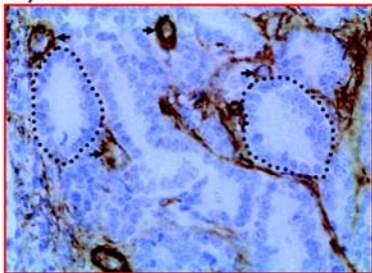


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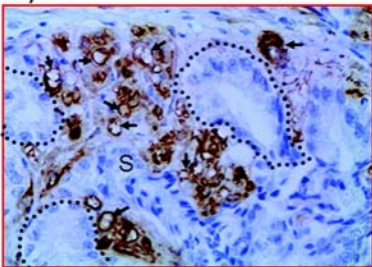
A)



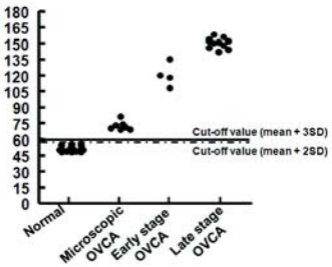
B)



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Wash-out time (seconds)
of contrast agent



Sphingosine-1 Phosphate Receptor (S1p1), a Critical Receptor Controlling Human Lymphocyte Trafficking, is Expressed in Hen and Human Ovaries and Ovarian Tumors

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1 **ABSTRACT**

2

3 **Background.** Sphingosine-1 receptor 1 (S1P1) plays a major role in regulating lymphocyte
4 egress from peripheral lymph tissue. Lymphocyte trafficking is potentially a critical response to
5 tumors and to tumor vaccines. Also, the receptor has been shown to influence metastasis.
6 However, there is little information on its expression in the aged ovary or ovarian tumors. As a
7 basis for further studies in the laying hen model of spontaneous ovarian cancer, the objective of
8 this study was to determine if S1P1 is expressed in hens, and if the morphological distribution of
9 S1P1 is similar in hen and human ovary and ovarian tumors.

10 **Methods.** S1P1 mRNA was ascertained in hen tissue by RT-PCR using hen specific primers.
11 S1P1 protein expression and localization was evaluated in hen and human tissue with a human
12 S1P1 antibody by Western blot and immunohistochemistry.

13 **Results.** S1P1 mRNA was expressed in all hen tissues examined. Protein was detected in
14 human and hen ovary and ovarian tumors at 47, 72 and 108 kDa in Western blots. S1P1 was
15 similarly expressed on endothelial cells, lymphocytes and surface epithelial cells in normal ovaries
16 and tumor-containing ovaries. In addition, S1P1 distribution was heterogeneous in ovarian
17 tumors by immunohistochemistry.

18 **Conclusion.** The results show that S1P1 is expressed in the hen and human ovary as well as in
19 ovarian tumors. These findings support the use of the hen in further studies of the role of S1P1 in
20 metastasis and immune cell trafficking in ovarian tumor development.

21

22

23 **Key words:** ovary, ovarian cancer, chicken, human, S1P1 (EDG-1).

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31 **Background**

32

33 Sphingolipids acting through sphingosine-1-phosphate receptors are involved in
34 embryogenesis, angiogenesis, vascular homeostasis and immune cell trafficking [1;2]. There are
35 five isoforms of sphingosine receptors (S1P1 – S1P5) [3]. Sphingosine receptors are members
36 within a larger family of G-Protein Coupled Receptors (GPCR) that are expressed on leukocytes
37 and on vascular endothelial cells. The ligand, sphingosine-1 phosphate (S1P), binds to several of
38 the sphingosine 1-phosphate receptors with higher affinity to the S1P1 and S1P3 isoforms [4].
39 The S1P1 regulates lymphocyte egress from lymphoid organs [5;6] and is necessary for
40 lymphocyte recirculation from thymus and peripheral lymphoid organs. In addition to a critical
41 role in regulating immune cell trafficking, activation of S1P1 can promote or inhibit apoptosis of
42 immune cells depending on the balance of cytokines [7]. Knockout of S1P1 (LP(B1)/EDG-1) in
43 mice is embryologically lethal [8]. S1P1 also has a role in inflammatory disease such as graft
44 versus host disease and multiple sclerosis [9]. The drug FTY720 binds to S1P1 as a high affinity
45 agonist and causes down-regulation and internalization of S1P1. This drug has been used as
46 novel immunosuppressive agent to inhibit S1P1-mediated immune cell migration from lymph to
47 sites of inflammation and is of particular interest in transplant and in treatment of autoimmune
48 diseases such as multiple sclerosis [9] and more recently, cancer.

49

50 The endogenous ligand (S1P) was recently shown to play an important role in ovarian
51 cancer invasiveness and ovarian tumor cell migration [10;11]. It also appears to protect ovaries
52 from the effects of chemotherapy [12] and radiation [13] and therefore is potentially a therapeutic
53 target to preserve fertility in patients undergoing therapy for cancer. While there are several
54 studies of S1P involvement in ovarian cancer models and ovarian tumor-derived cell lines there is
55 no information on the expression of its receptor, S1P1, in normal human (aged) ovary or in
56 naturally occurring ovarian tumors in humans or animal models.

57

58 We [14-18] and others [19-21] reported that the laying hen, which spontaneously develops
59 ovarian tumors [22] is useful for studies of ovarian cancer. The normal hen ovary has been used

60 extensively to understand ovarian physiology [23;24] because it shares many features of normal
61 human ovary including similar cyclic hormone regulation of follicle development and ovulation
62 [25]. Like human ovaries, hen ovaries express receptors for follicle stimulating hormone (FSH)
63 and luteinizing hormone (LH) and produce inhibins, estrogen, and progesterone in response to
64 FSH and LH [24]. One difference between human and hen ovarian function is the lack of
65 post-ovulatory development of a progesterone-secreting corpus luteum and the events that lead to
66 implantation because eggs are laid externally.

67

68 Likewise, naturally occurring hen ovarian tumors are similar to human tumors [17;22].
69 Commonly, hen ovarian tumors exhibit epithelial cell histology including serous, endometrioid,
70 clear cell and mucinous histology [17] and less frequently tumors of germ cell origin [22] which is
71 typical of the histology seen in humans [26]. The incidence of both hen and human ovarian
72 tumors increases with age [22;27]. In hens, which are pure bred (rather than inbred), the
73 incidence of ovarian tumors is also strain and flock dependent [20] which suggests a genetic
74 component associated with ovarian cancer, as in humans [28]. As well, many of the same
75 proteins are expressed in human and hen tumors such as CA125 [29], E-cadherin [30], COX [19],
76 p53 [28], SBP-1 [31], mesothelin [32] and several others [21]. Interestingly, progesterone
77 reduced the incidence of ovarian carcinoma in hens which parallels the reduced risk of ovarian
78 cancer associated with oral contraceptive use in women [33]. Recently, we developed the use of
79 ultrasound to assess ovarian morphology and tumor-associated angiogenesis [18] in order to
80 facilitate the selection of hens for studies of ovarian cancer and to be able to monitor hens
81 longitudinally.

82

83 A further advantage of the hen as a model for studies of immune mechanisms in ovarian
84 cancer is the well established knowledge of the immune system. In fact, the two different types of
85 immune cells (T and B cells) were first described based on the differences in lymphocytes in the
86 thymus and bursa of Fabricius [34;35]. Also, the first successful anti-tumor vaccine was
87 developed for chickens to prevent Marek's disease, a virally-induced lymphoid neoplasm [36].
88 Moreover, humans [37;38] and hens [16] develop spontaneous ovarian autoimmunity and
89 circulating anti-ovarian antibodies associated with prematurely reduced ovarian function.

90

91 Our future objective is to examine the role of immunity in ovarian tumor development and
92 progression through modification of lymphocyte trafficking. Although the expression and role of
93 S1P1 has been demonstrated in humans, there is little information on its expression in the human
94 or hen ovary. Therefore, the specific objective of this study was to determine if S1P1, a major
95 receptor that regulates lymphocyte trafficking in humans, is expressed in hens, and if the
96 morphological distribution of S1P1 is similar in hen and human ovary and ovarian tumors.

97

98 **Methods**

99

100 **Animals:** White leghorn hens (2-3 years old, strain W/96) were housed at the University of
101 Illinois at Urbana-Champaign (UIUC) at the Poultry Research Farm affiliated with the Department
102 of Animal Science. Food and water were given *ad libitum* and hens were maintained on a 17:7
103 hour light: dark schedule. Hens this age were used in our study because the proportion of hens
104 with ovarian tumors is about 10-15%, based on our experience. Animals were selected for study
105 based on normal or abnormal ovarian ultrasound as described previously [15;17;18]. Hens were
106 sacrificed at UIUC by cervical dislocation and organs removed. Hen tumors were histologically
107 staged and typed by a pathologist using criteria similar to human tumor type and staging as
108 described previously [17]. All procedures were approved by the University of Illinois
109 Institutional Animal Care and Use Committee (IACUC).

110

111 **Human Ovarian Tissues:** Normal ovaries and ovarian tumors were obtained from the
112 gynecologic oncology clinics at Rush University Medical Center and John Stroger Hospital
113 (Chicago, IL) according to Institutional Review Board (IRB) approved protocols. Normal
114 ovaries were obtained at hysterectomy (n = 5; mean age 54 ± 8 years). Ovarian tumors were
115 obtained from patients with malignant tumors (n = 18; mean age 64 ± 15 years). The tumor
116 histology and tumor grade were determined by a pathologist using standard FIGO criteria [17].
117 The criterion for inclusion in the study was women ≥45 years old. The criteria for exclusion were
118 previous history of any cancer and prior chemotherapy or radiation treatment. Of the five ovarian

119 tumors shown in this report, three were serous and two were endometrioid.

120

121 **Tissue preparation:** Hen ovary (n = 20), spleen (n = 5), and ceecal tonsils (peripheral
122 lymphoid organ, n = 4) and brain (n = 2) were cut into three equal portions. Human (normal)
123 ovary (n = 4) and ovarian tumors (n = 4) were similarly prepared. One portion was fixed in 10%
124 PBS-buffered formalin and embedded in paraffin for histology and immunohistochemistry [17].
125 A second portion was frozen (-80°C) for cryostat sections for immunohistochemistry. The final
126 portion was washed with cold 1.5mM Tris HCl, homogenized (100mg wet weight tissue/100mL of
127 40mM Tris HCl, 5 mM MgSO₄ buffer), centrifuged (1,000 x g, 10 minutes, 4°C) and the
128 supernatant stored at -80°C for Western blot analysis [16;31]. In addition, to enrich for S1P1
129 receptors, the supernatant was centrifuged again (18,000 x g, 40 minutes, 4°C) and the pellet was
130 suspended in sample buffer (Bio-Rad Laboratories, Hercules, CA) for one-dimensional gel
131 electrophoresis (1D-PAGE). Rat brain was used for control and was a gift from Dr. Amanda
132 Mickiewicz (Rush University, Chicago).

133

134 **Reverse transcription-polymerase chain reaction (RT-PCR):** To assess S1P1 mRNA
135 expression, RT-PCR was performed as reported previously [38]. Briefly, total RNA (n=40,
136 ovaries = 30, other organs (lymph, muscle, liver) from at least four hens) was extracted using
137 Trizol reagent (Invitrogen, Carlsbad, CA). The RNA content was measured at an optical density
138 (OD) of 260 nm and the purity evaluated using an OD 260/ 280 nm absorbance ratio ≥ 1.7 . RNA
139 was treated with DNase (Invitrogen, Carlsbad, CA) to remove trace amounts of genomic DNA
140 before the first strand synthesis. First strand synthesis was performed using 500 ng of RNA
141 according to the manufacturer's protocol (37°C, 1 hour; High Capacity cDNA RT Kit, (Applied
142 Biosystems, Carlsbad, CA). The PCR amplifications were carried out in a 25 μ l reaction volume
143 containing 25 ng of cDNA using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA)
144 according to the manufacturer's recommendation. The PCR cycle consisted of a primary
145 denaturation at 94°C (3 minutes) followed by 35 cycles of denaturation at 94° C (30 seconds) and
146 54° C (30 seconds) to anneal and 72 ° C (1 minute) for extension followed by a final extension at
147 72° C (10 minutes) in a programmable Peltier Thermo Cycler (PTC-200, MJ Research Inc.,
148 Ramsey, MN). Hen-specific S1P1 primers were designed using Oligoperfect Designer software

149 (Invitrogen, Carlsbad, CA) using the S1P1 sequence from the NCBI [GeneBank: XM_422305.2].
150 The forward primer was CCCCAGGAGCATTAAACTG and the reverse primer was
151 CTGCTGACCACCTCACTG located between exons 1 and 2. β -actin was used as the
152 endogenous control with a forward primer of TGCGTGACATCAAGGAGAAG and a reverse
153 primer of ATGCCAGGGTACATTGTGGT. The expected base pair size for the S1P1 amplicon
154 was 226 bp and for β -actin was 300 bp. PCR amplicons were visualized in a 2% agarose gel
155 (Pierce/Thermo Fisher, Rockford, IL USA) in T.A.E. buffer (4.84g Tris Base, 1.14mL acetic acid,
156 2.0 mL 0.5M EDTA/L of buffer) and stained with ethidium bromide. The image was captured
157 using a ChemiDoc XRS system (Bio-Rad, Hercules, CA). Amplicon from a positive sample
158 (endometrioid carcinoma of the ovary) was used for sequence analysis after purification using the
159 Quia-Quick PCR Purification System (Qiagen, Valencia, CA USA) according to manufacturer's
160 instructions. The purified DNA was sequenced at the DNA sequencing facility at the University
161 of Illinois at Chicago using an ABI 3100 Genetic analyzer (Applied Biosystems, Foster City, CA).

162
163 **One-dimensional (1D) Western Blot:** Ovarian tissue samples (n= 15) were
164 homogenized according to a previous protocol [39] and stored at -80°C. Proteins (10 μ g/lane) were
165 separated by 1D gel electrophoresis using 10% gradient Tris-HCl gels (Bio-Rad, Hercules, CA)
166 using standard procedures [31]. MagicMark XP Western blot standards (Invitrogen, Carlsbad,
167 CA) were used to estimate molecular weight. Rat brain (n=3) was used as a positive control
168 (recommended by Cayman Chemical website). Proteins were transferred (18 Volts, 30 minutes)
169 to a nitrocellulose membrane (0.45 μ m; Bio-Rad, Hercules, CA). Blots were blocked in 10 x
170 Blocking Buffer (diluted to 1x; Sigma St. Louis, MO) containing 0.05% Tween-20 (4°C; 16 hours;
171 Sigma, St. Louis, MO), rinsed in Wash buffer (0.15M NaCl in 10mM Tris containing 0.05%
172 Tween-20, pH7.5) and incubated in rabbit anti-S1P1 polyclonal antibody (1:200; Cayman
173 Chemical, Anne Arbor MI) diluted in blocking buffer containing 0.05% Tween-20. The
174 nitrocellulose membrane was washed three times in cold Wash buffer followed by goat anti-rabbit
175 immunoglobulin-HRP (Horseradish-Peroxidase; Pierce/Thermo Fisher, Rockford, IL). The
176 reaction was developed in Super Signal West Dura substrate (Pierce/Thermo Fisher, Rockford, IL)
177 and digital images acquired using a ChemiDoc XRS system (Bio-Rad, Hercules, CA). Digital
178 images were analyzed by Quantity One software (Bio-Rad, Hercules, CA).

179

180 Because there are currently no commercially available antibodies against avian S1P1, we
181 used a commercially available polyclonal antibody against human S1P1 for Western blotting and
182 immunohistochemical experiments. There are two serine (S) to threonine (T) substitutions in the
183 chicken S1P1R, within amino acids 241-253 of the epitope, and a high degree of homology (> 85%
184 based on sequence comparisons) between the two proteins.

185

186 **Immunohistochemistry:** Hen ovarian tissue (n = 40) was cut into two equal pieces for
187 paraffin and frozen tissue processing. Ovarian tissue was embedded in paraffin and sectioned (6
188 μm). Sections of formalin-fixed, paraffin-embedded tissue stained with Hematoxylin and Eosin
189 (H/E) were examined by a pathologist to determine the histological type and stage. For cryostat
190 sections, tissue was washed in cold phosphate buffered saline (PBS, pH 7.0) and placed in 30%
191 sucrose overnight at 4°C. Tissues were washed once more in PBS the following morning,
192 embedded in OCT Compound (Tissue Tek, Sakura, Japan) and flash frozen in dry-ice cooled
193 methanol and stored at -80°C until use.

194

195 Ovarian sections were incubated with rabbit anti-S1P1 (Cayman, Ann Arbor, MI) diluted
196 1:200 in PBS containing 1% BSA (bovine serum albumin; Fisher, Waltham, MA). The primary
197 antibody was omitted as a control for non-specific antibody binding. Other primary antibodies
198 for immune cell markers include Bu1a (chB6; Abcam, Cambridge, MA) and T cell antibodies
199 (CD3, CD4, and CD8; Southern Biotech, Birmingham, AL). As a control for antibody specificity
200 the anti-S1P1 was pre-absorbed with blocking peptide (Cayman, Ann Arbor, MI) (1:1, v/v; 45
201 minutes, 22°C). The absorbed, control anti-S1P1 was diluted to the same concentration as the
202 untreated S1P1 antibody (1:200) in blocking buffer (Sigma, St. Louis MO) supplemented with
203 0.05% Tween-20 and incubated with sections. Sections were washed and incubated with goat
204 anti-rabbit immunoglobulin-HRP (Pierce/Thermo Fisher, Rockford, IL) (1:10,000 in Sigma
205 Blocking Buffer containing 0.05% Tween-20; 1 hour; 22°C; Sigma, St. Louis, MO). Color was
206 developed with 3, 3-diaminobenzidine (DAB) substrate (Vector Labs; Burlingame, CA). Slides
207 were washed in running water (15 minutes) and counterstained with hematoxylin followed by
208 dehydration with graded alcohol series (70 -100%) and xylene. Sections were examined with an

209 Olympus light microscope (BX41, Tokyo, Japan) and an Olympus U-CMAD3 camera with Micro
210 Suite #5 software.

211

212 **Results**

213

214 **S1P1 mRNA is expressed in hen tissues.** The mRNA for S1P1 was detected at the
215 predicted amplicon size of 226 bp in hen tissue (Figure 1). Four normal ovaries (no evidence of
216 cancer) and four tumor ovaries with endometrioid, serous and mucinous histology had S1P1
217 mRNA (Figure 1A). Other tissues, including muscle, oviduct, liver and kidney also contained
218 S1P1 mRNA (Figure 1B). The expression of S1P1 mRNA was confirmed by sequence analysis at
219 University of Illinois at Chicago DNA Services Facility (DNAS). Human tissue was not
220 evaluated for S1P1 mRNA expression because it was demonstrated previously [40].

221

222 **S1P1 protein is expressed in hen tissues.** S1P1 protein was expressed in human and hen
223 ovaries and ovarian tumors with bands at 47, 72 and 108 kDa detected by Western blot (Figure 2).
224 There were variations in the intensity of bands at each molecular size from different preparations
225 in both hen and human tissues. A membrane-enriched fractionation (18,000 x g) did not result in
226 a consistently enhanced 47 kDa band in either the hen tissues or control rat brain. Hen brain
227 showed the same bands as the positive control. Spleen was expected to express S1P1 because it is
228 a major lymphocyte processing organ and the Western blot reactions were the same as the rat and
229 hen brain. The band intensity was reduced using anti-S1P1 antibody pre-absorbed with blocking
230 peptide and was absent when the primary antibody was omitted.

231

232 **S1P1 localization in hen ovaries and ovarian tumors by immunohistochemistry.**

233 S1P1 was expressed in normal hen ovaries in blood vessels in the stromal (Figure 3A and B) and
234 medullary regions (Figure 3 E) of the ovary. S1P1 was also found in mature follicles, but not in
235 early stage follicles (Figure 3A). Within mature follicles, S1P1 was expressed exclusively in the
236 theca externa (Figure 3A). Surface epithelial cells of the ovary also showed intense S1P1
237 expression (Figure 3C). Atretic follicles (Figure 3D) had S1P1+ immune cells (insert) but S1P1

238 staining was absent in follicle remnants. The endothelial cells but not the smooth muscle cells of
239 blood vessels were S1P1+ (Figure 3E, insert).

240

241 Hen ovarian tumors had varied S1P1 staining (Figure 4). A mucinous ovarian tumor had
242 S1P1 staining associated with mucin-secreting glandular structures (Figure 4A and C). An
243 example of a serous ovarian tumor shows light stromal cell cytoplasmic S1P1 staining but intense
244 staining of the surface epithelium (Figure 4E and 4F). Endometrioid (Figure 4K and 4L) ovarian
245 tumors had similar S1P1+ staining within the tumor; the most intense staining being associated
246 with surface epithelial cells and the area immediately adjacent to it (Figure 4L). Most of the
247 S1P1+ cells associated with clear cell carcinomas were outside the tumor (Figure 4G), while blood
248 vessels in the uninvolved stroma adjacent to the tumor were S1P1+ (Figure 4I).

249

250 **S1P1 localization in human ovary and ovarian tumors by immunohistochemistry.**

251 The staining patterns of S1P1 in human ovarian cancers were heterogeneous, similar to the hen
252 ovarian tumors. Normal ovaries (post-menopausal women, age: 58) had some endothelial cell
253 S1P1 staining around blood vessels as well as light staining of the ovarian stroma (Figure 5A and
254 C). Serous ovarian tumors had some S1P1 staining in the stroma but not the epithelium (Figure
255 5B). Endometrioid structures were not stained, but surrounding stroma was S1P1
256 immuno-stained (Figure 5D).

257

258 **S1P1 expression associated with immune cells in ovaries of hens.**

259 Serial frozen sections of ovarian tissue were stained with hen specific antibodies against Bu1a (antigen specific
260 for avian B cells) and CD3 to determine if S1P1 expression was associated with immune cells
261 (Figures 6 and 7). In normal ovaries (Figure 6), S1P1 was expressed on cells both with and
262 without B or T cell markers in the ovarian stroma and was primarily expressed on blood vessels.
263 The B and T cells were found in close proximity to S1P1 stained blood vessels. In tumors (Figure
264 7) staining patterns were less organized. S1P1 staining occurred in serous tumor cells. While
265 CD4 T cells were more often found scattered around the tumor glands, CD8 T cells and Bu1a+
266 staining was localized throughout the tissue and in tumor glands.

267

268 **Discussion**

269

270 This is the first study reporting the expression of S1P1 in ovarian tissues in the adult laying
271 hen. Although chicken specific primers were used to detect S1P1 mRNA and an anti-human
272 S1P1 antibody was used to detect S1P1 protein, the expression of S1P1 mRNA and protein were
273 correlated. Similarly, S1P1 was detected by immunohistochemistry in tissue positive for S1P1
274 mRNA and protein. This is consistent with the high degree of amino acid similarity (> 85%)
275 between avian [GenBank ACC#: XP_001231780.1] and human [GenBank ACC#: NP_001391.2]
276 S1P1 protein. Furthermore the location of S1P1 positive cells was similar in hen and human. In
277 normal ovaries and ovarian tumors, S1P1 was expressed in endothelial cells of blood vessels and
278 immune cells. In follicle cells of normal hen ovary, theca externa cells but not ovarian stroma nor
279 other follicular structures were stained. Follicles in normal human ovary were not observed in
280 this study because tissue from post-menopausal women was used and thus it was not possible to
281 compare them with the hen follicles. Tumor cells and surface epithelium in ovaries with tumors
282 were variably stained. Overall the expression of S1P1 in hen and human ovaries and in the
283 ovarian tumors examined was remarkably similar.

284

285 Previous reports of S1P1 detected in Western blots indicated various molecular sizes [41],
286 although the expected size is 47 kDa [2]. We observed a 47 kDa species by Western blotting in a
287 membrane-enriched fraction, although it was often faint or undetectable. However, there were
288 two predominant higher molecular weight species (72 and 108 kDa); these are not usually
289 described although they are evident in some reports [42]. Notably, the same molecular sizes were
290 observed in hen and human ovaries and ovarian tumors, hen spleen, and hen and rat brain.
291 Because bands react with S1P1 antibody, the larger size bands may represent aggregates in
292 dimmers or trimers [43]. Alternatively, S1P1 receptor may also be differentially glycosylated,
293 [44] although this would not usually produce proteins double the smaller 50kDa size. When
294 tissues were subjected to deglycosylation enzymes after homogenization (EndoH, New England
295 Bio-Labs, Ipswich, MA USA), the molecular sizes did not change (data not shown) suggesting
296 either the protein is not glycosylated or, more likely, is resistant to EndoH cleavage. Nonetheless,

297 similar protein bands were detected in the human and hen ovary, demonstrating a similar
298 expression pattern.

299

300 The immunohistochemical pattern of S1P1 staining was common to both hen and human
301 ovaries. Normal hen ovary expressed S1P1 in surface epithelial cells, theca cells of the follicle,
302 endothelial cells of blood vessels in the stroma and medullary region, as well as in immune cells
303 such as infiltrating immune cells of atretic follicles. The expression of S1P1 was not confined to
304 immune cells. Because the human ovaries used in this study were from older women, they did not
305 have any follicles for comparison. However, S1P1 was similarly expressed in surface epithelial
306 cells, endothelial cells and immune cells. Likewise, in hen and human ovarian tumors S1P1 was
307 expressed in endothelial cells and immune cells. In addition, tumors cells expressed S1P1 and the
308 expression was dispersed throughout the cytoplasm. Furthermore, S1P1 expression varied
309 among ovarian tumors. This may have been due to variations in expression among tumors or
310 among tumor types or to sampling of individual tumors.

311

312 **Conclusion**

313

314 In summary, S1P1 is expressed on immune cells in the hen. S1P1 is also expressed in
315 ovarian tissues of the laying hen with a distribution in the ovary that is similar to human ovaries.
316 The chicken embryo contains both sphingosine-1 phosphate (ligand for S1P1) and sphingosine
317 kinase; the enzyme responsible for the conversion of sphingosine to sphingosine-phosphate which
318 occurs in the blood [45]. Similarly, chicken embryonic amacrine cells were recently reported to
319 express S1P1 [46], indicating that this receptor can be found in both embryonic and, as our study
320 shows, the adult tissues of the chicken.

321

322 We also show, for the first time, that S1P1 is expressed in both hen and human ovarian
323 tumors. S1P (the ligand for S1P1) has been implicated in the trafficking of immune cells [5].
324 Immune cells are reported to be involved in the progression of tumors of various organs [47].
325 While the role of infiltrating immune cells in ovarian cancer progression is not clearly defined [48]

326 there clearly is a relationship of infiltrating T cells and survival [48-50]. The hen provides an
327 alternative animal model to engineered rodent models for studies of ovarian cancer. Further
328 studies addressing immune cell infiltration into tumors and the role S1P1 plays in regulating
329 immune cell infiltration into ovarian tumors would be facilitated by use of the hen because all
330 stages of spontaneous tumors in the hen can be readily observed.

331

332 **Disclosure Statement**

333 The authors have no conflict of interest.

334

335 **Author Contributions**

336 MJB carried out the molecular and immunohistochemical studies, participated in the sequence
337 alignment, assisted in tissue collection, and drafted the manuscript. AB classified hen ovarian
338 tumors, collected all tissue, prepared tissue for immunohistochemistry and maintained databases.
339 YY reproduced the RT-PCR experiments. KP assisted in Western blotting experiments and
340 maintained the tissue inventory. SLE designed PCR primers, prepared the sequence comparisons
341 and assisted in the molecular biology experiments and their design. SS provided human ovarian
342 tissue used in this study and participated in discussions of the experimental design. JSA assisted
343 AB in detecting potential ovarian tumors with ultrasound. JMB maintained hens, collected tissue
344 with AB and MJB and contributed expertise in avian physiology. JLL conceived the study,
345 participated in its design and coordination, data analysis and preparation of the manuscript with
346 MJB. All authors read and approved the final manuscript

347

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351 Cancer Institute (AB) and Sramek Foundation (AB). Also, the generous effort and support of
352 Chet and Pam Utterback and Doug Hilgendorf at the UIUC Poultry Farm is acknowledged.

353

354 **FIGURE LEGENDS**

355

356 **Figure 1. S1P1 mRNA expression in hen tissues.**

357 (A) S1P1 mRNA (226 bp) is expressed in both normal and tumor ovaries. Examples of mRNA
358 in tumors with endometrioid (En), serous (Sr), and mucinous (Mc) histology are shown. (B)
359 Examples of other hen tissues that express S1P1 mRNA (226 bp) include liver (LVR), kidney
360 (KDNY), skeletal muscle (MSCL), oviduct (OVDT) and spleen. Normal ovary and spleen are
361 from the same hens (1-4). β -actin (300 bp) was used as a loading control.

362

363 **Figure 2. S1P1 protein expression in hen and human tissue.**

364 S1P1 immunoreactions are similar in hen and human ovaries and ovarian tumors. Three bands at
365 47, 72, 108 kDa were observed. The band at 47kDa was faint, while bands at 72 and 108 kDa
366 were consistently present in all tissues but vary in intensity. The 47kD band was not significantly
367 enhanced using a membrane enriched (18,000xg pellet) fraction. The pattern of immunoreactive
368 bands was identical in the positive control recommended by the manufacturer (rat brain) and in hen
369 brain and spleen. The bands were absent in control incubations in which the primary antibody
370 was pre-adsorbed with a blocking peptide or in which the primary antibody was omitted.

371

372 **Figure 3. Localization of S1P1 receptor protein expression in normal hen ovary.**

373 (A) S1P1+ cells (black arrows) in theca of a mature follicle (F) and within small blood vessels
374 (BV; white arrows) in ovarian stroma (100 x). Primordial follicles (f) have comparatively little
375 S1P1+ expression. (B) Endothelial cells of blood vessels (BV) in the theca externa (TE) of a
376 follicle (F) and ovarian stroma are S1P1+ (200 x). (C) Surface epithelial cells (EpC) showing
377 intense S1P1+ expression (400 x). (D) An atretic follicle (af) with characteristic infiltrating
378 S1P1+ immune cells (100 x). Inset: High magnification of (D) showing an S1P1+ immune cell
379 (1000 x). (E) Well developed blood vessels (BV) in the medullary region of the ovary also
380 contain S1P1+ endothelial cells (400 x) but staining is lighter and more diffuse than in stromal
381 blood vessels seen in (A) and (B). Inset: high magnification (800 x) shows detail of smooth

382 muscle cells (SmC) and endothelial cells (EnC). (A-C) are frozen tissues; (D) and (E) are
383 paraffin-embedded.

384

385 **Figure 4. Localization of S1P1 receptor protein expression in hen ovarian tumors.**

386 (A) An example of a mucinous ovarian tumor with S1P1+ staining associated with surface
387 epithelium (black arrow) and mucin-secreting structures (black arrow) (100 x). (B) H&E stained
388 section (100 x) serial to that in (A) shows mucinous histology. (C) Higher magnification of (A)
389 showing S1P1+ mucin-secreting glandular (MG) structures (black arrows) (400 x). (D) H&E
390 stained section of a serous ovarian tumor (100 x). (E) Serial section showing minimal stromal
391 cell stain for S1P1+ but intense surface epithelial cell staining (black arrow) and lighter more
392 diffuse S1P1+ sub-epithelial cells (white arrow) (100 x). (F) High magnification (of box)
393 showing S1P1+ surface epithelial cells (EpC) (600 x). (G) Clear-cell ovarian tumor (T; left of
394 dotted red line) with negligible S1P1+ in tumor and S1P1+ cells in adjacent uninvolved stroma
395 (100 x). (H) H&E-stained serial section from the same tumor region in (G) shows cellular detail
396 of clear cell carcinoma (400 x). (I) Higher magnification of box in(G) showing stromal blood
397 vessels (BV) with S1P1+ endothelial cells and stromal cells (S) (200 x). (J) H&E stained section
398 of late stage endometrioid tumor (100 x). (K) S1P1+ is highly expressed in cells in the tumor
399 periphery and to a lesser extent in tumor stroma (100 x). (L) High magnification of box in (K)
400 showing cytoplasmic staining of endometrioid tumor cells (400x). All images are from
401 paraffin-embedded tissue, except clear cell carcinoma (G-I) which is a frozen section.

402

403 **Figure 5. S1P1 receptor expression in human ovarian carcinomas.**

404 (A) Normal ovary showing diffusely stained S1P+ stromal cells (white arrows) and a blood
405 vessel (BV) with intensely stained S1P1+ endothelial cells (black arrow) (200 x). (B) Serous
406 ovarian tumor with S1P1+ stroma (white arrows) and unstained surface epithelium (200 x). (C)
407 Normal ovary (BV, arrows) are positive (600 x). (D) S1P1+ endometrioid ovarian tumor with
408 patches of intense stain (white arrows) (400 x). (A-D) are paraffin-embedded sections.

409

410 **Figure 6. In normal hen ovary S1P1 expression was observed in areas of immune cell**
411 **infiltration. (Row A)** Immune cells (Bu1a+ and CD3+) are adjacent to the follicle (f) in the ovarian
412 stroma near a transverse blood vessel (arrow in column S1P1). Cells lining the vessel near the follicle (f)
413 are S1P1+ (row A, S1P1) (original magnification 100 x). **(Row B)** High magnification (see red boxes in
414 row A) showing B and T cells clustered within the stroma near S1P1 stained vascular endothelium (arrow).
415 Some immune cells are also S1P1+ (open arrow) (original magnification 400 x). **(Row C)** Cross-section
416 of a large blood vessel shows Bu1a and CD3 positive cells are clustered near the blood vessel. The apical
417 surfaces of endothelial cell express S1P1 (original magnification 100 x).

418

419 **Figure 7. In ovarian tumors S1P1 expression was observed near T and B cells.** Alternate
420 serial sections of a serous ovarian tumor of the hen showing **(A)** Bu1a+ cells in the stroma
421 (arrows) and diffuse tissue stain (open arrow), **(B)** CD4 T cells around tumor glands (arrows),
422 **(C)** S1P1 expression on the epithelium of tumor glands (arrow) and **(D)** CD8 T cells (arrows) in
423 the stroma (original magnification 100 x).

424

425

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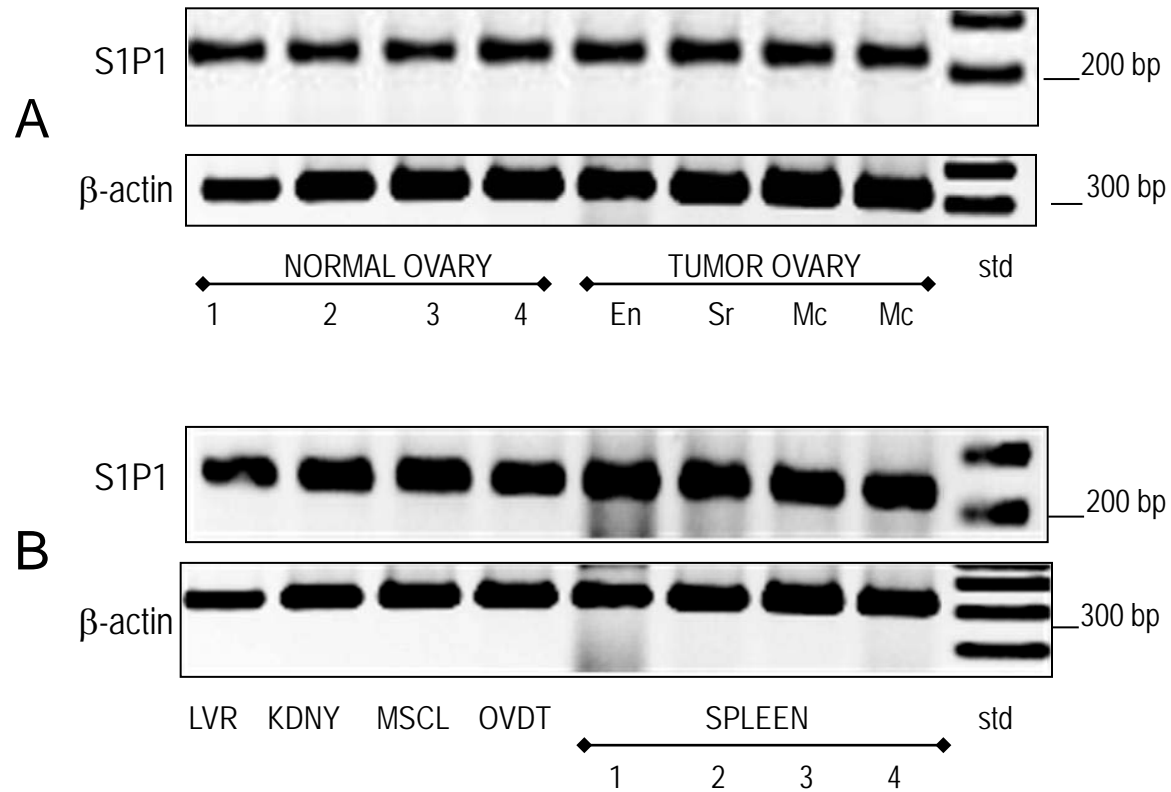


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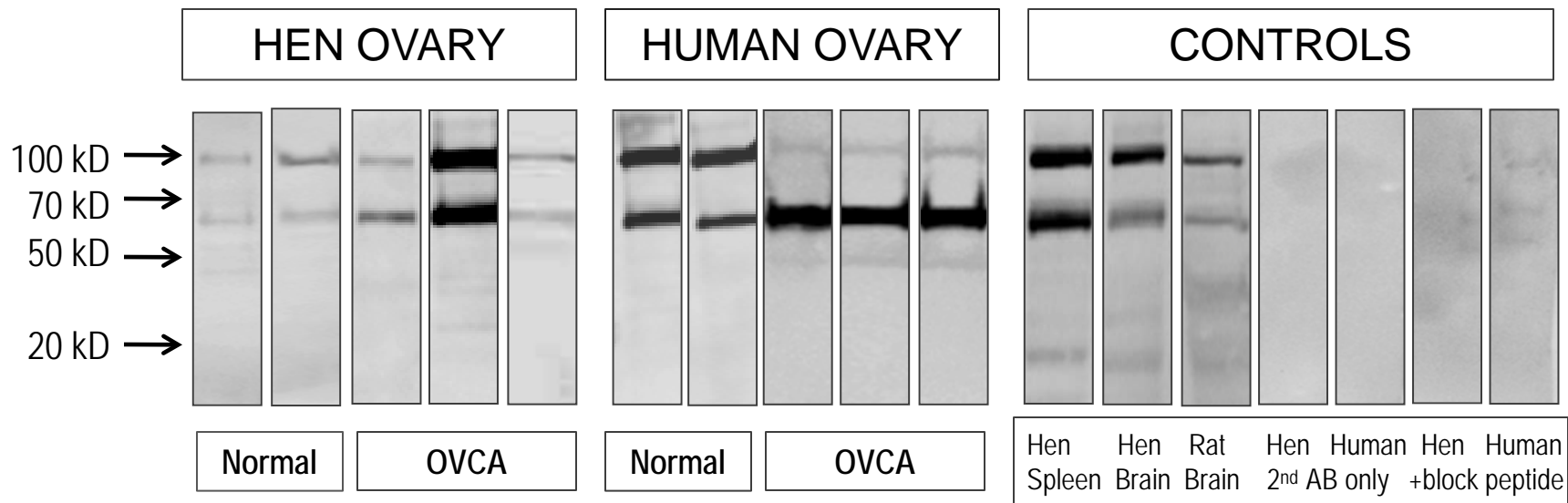


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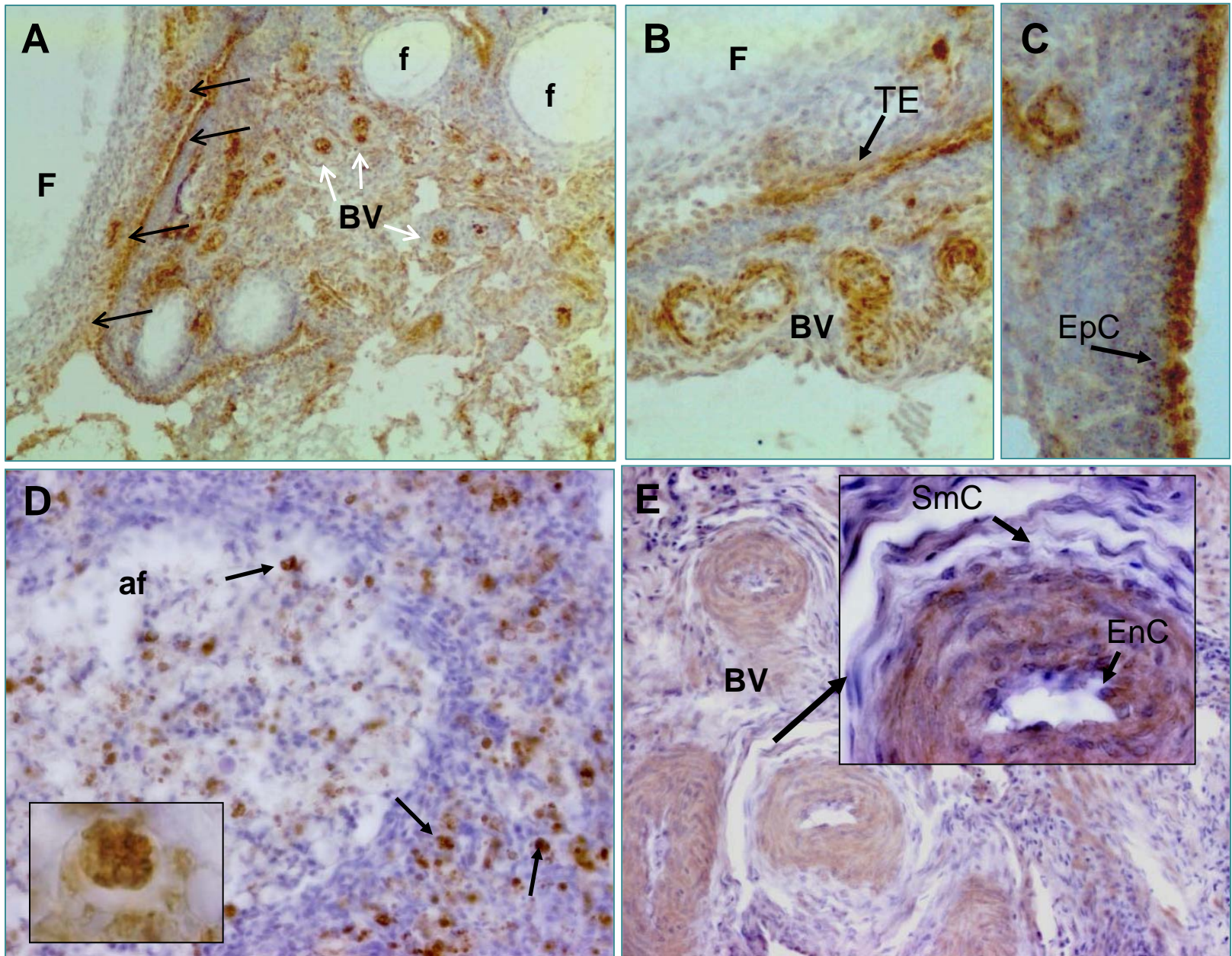


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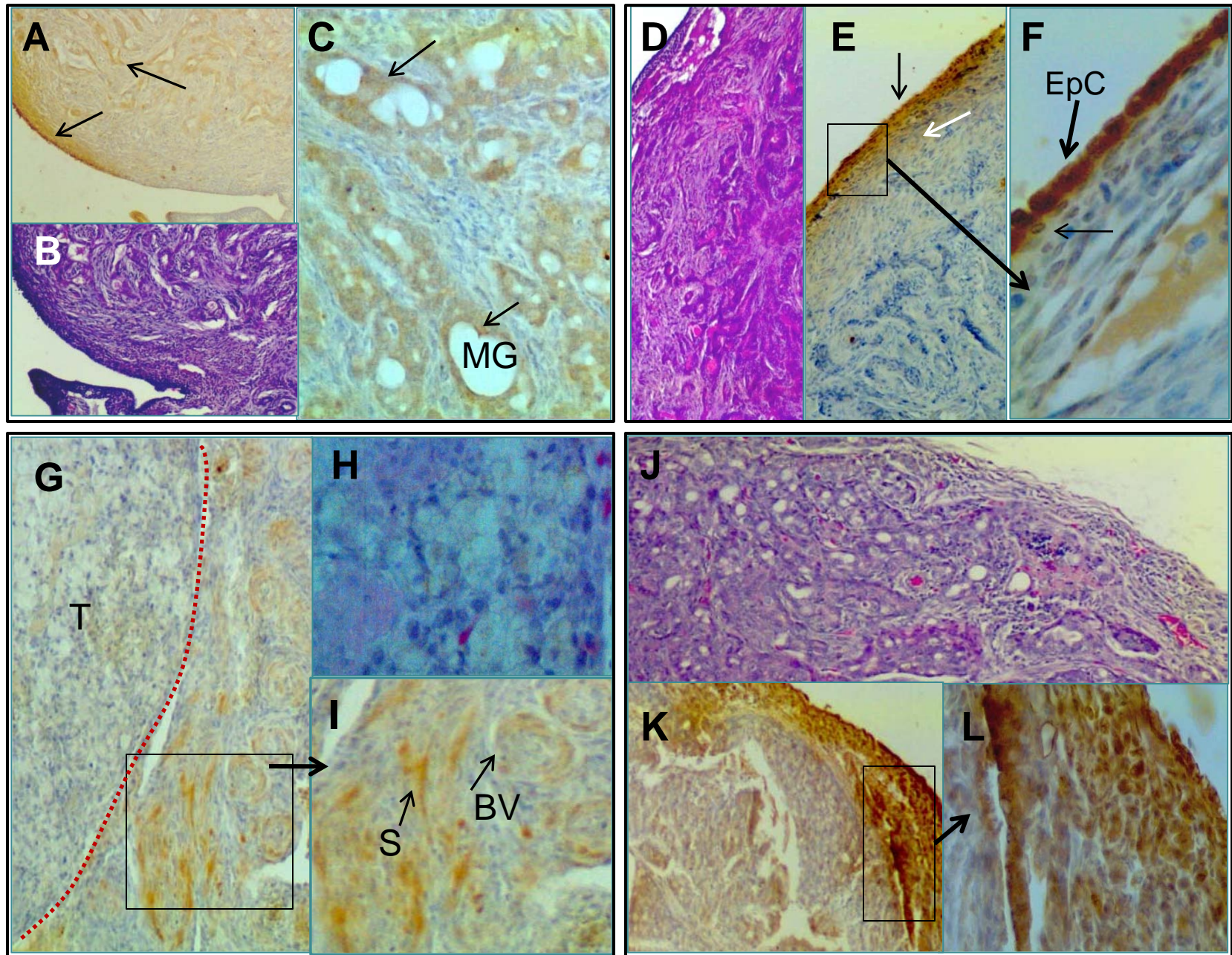


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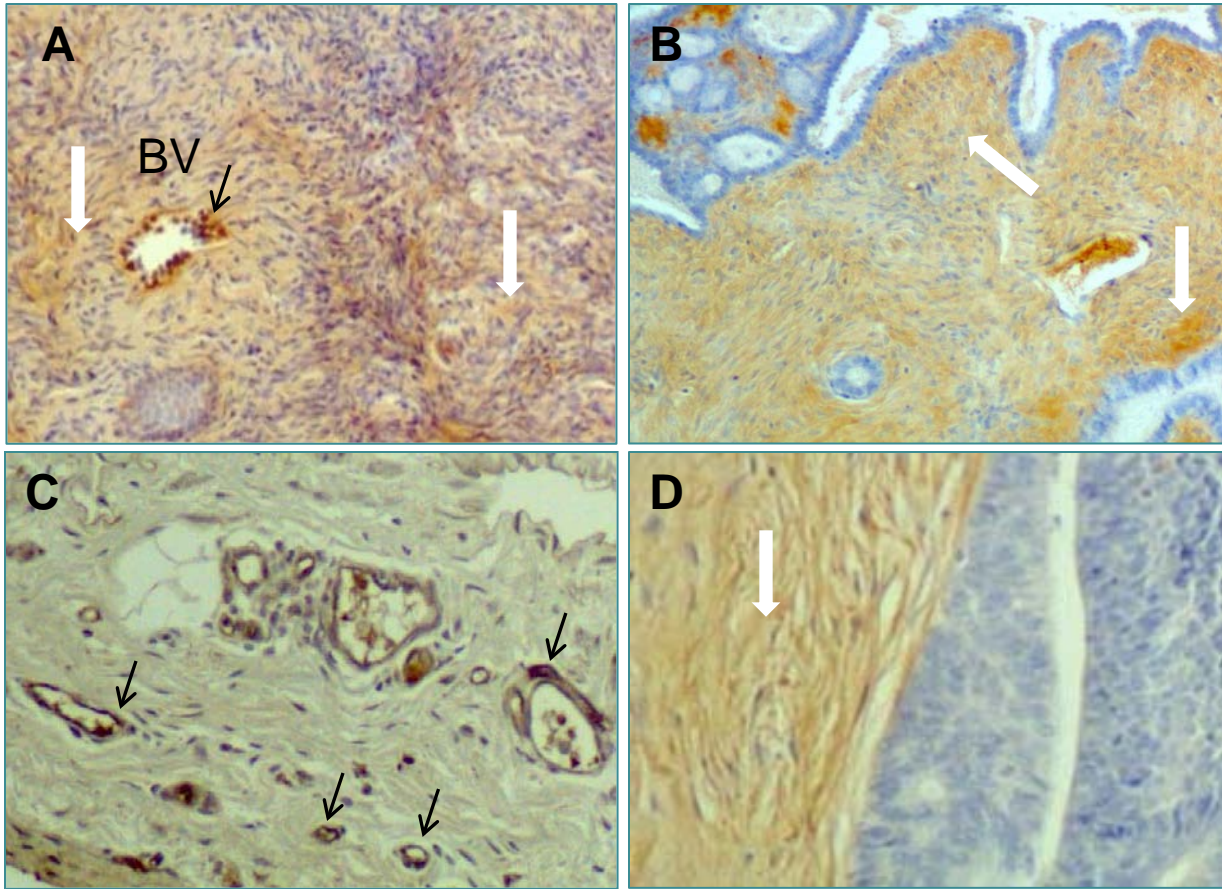


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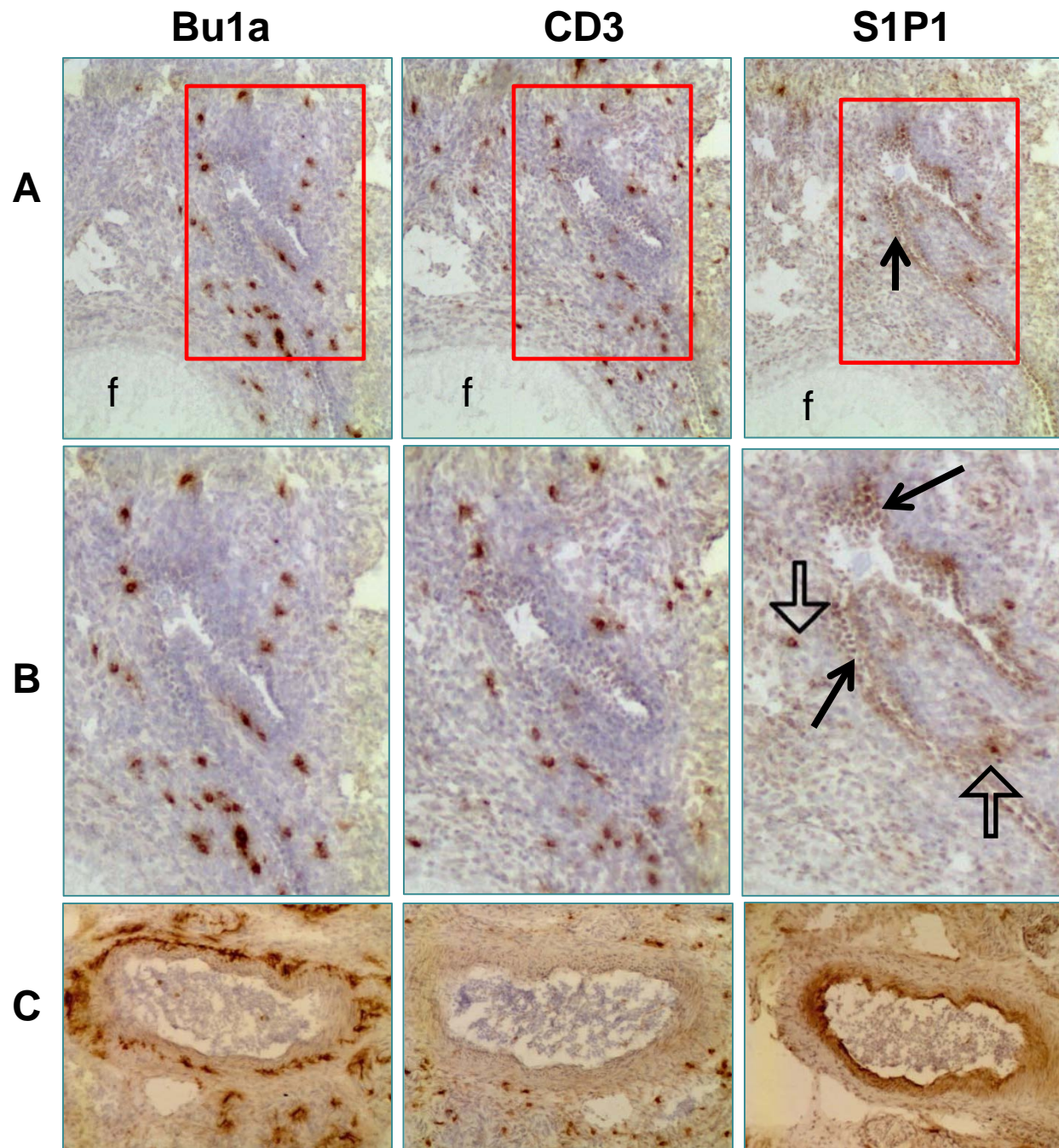


Figure 6

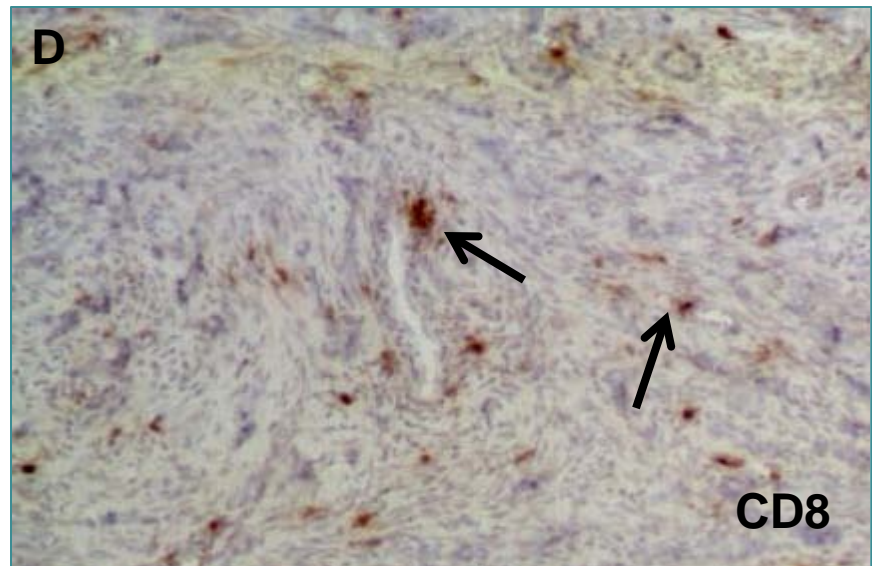
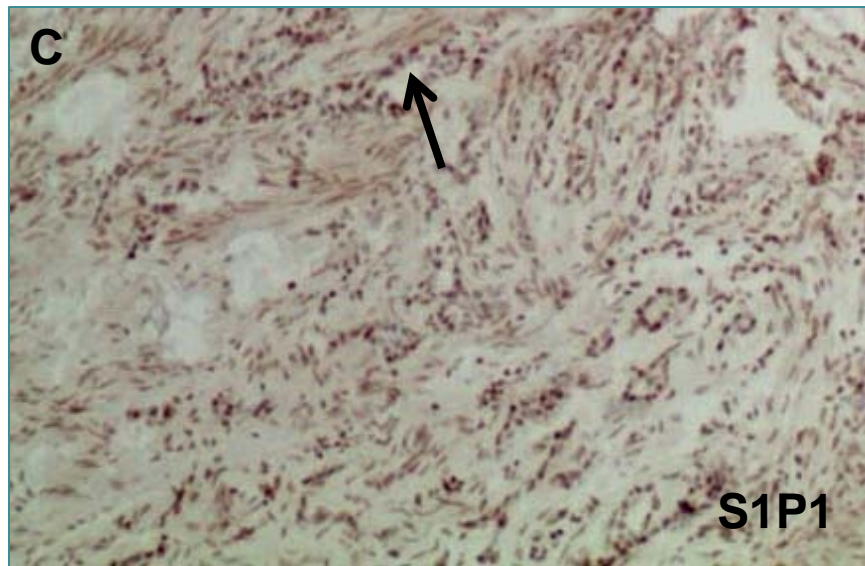
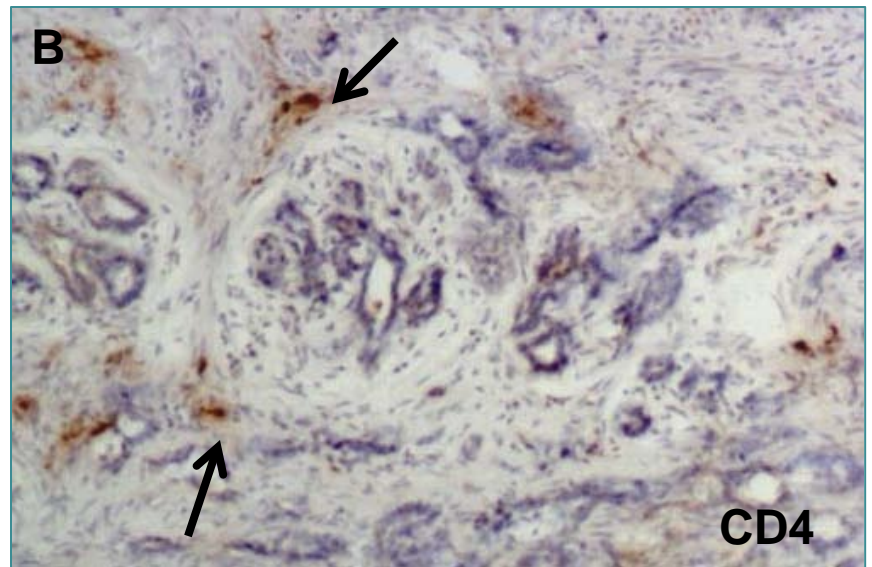
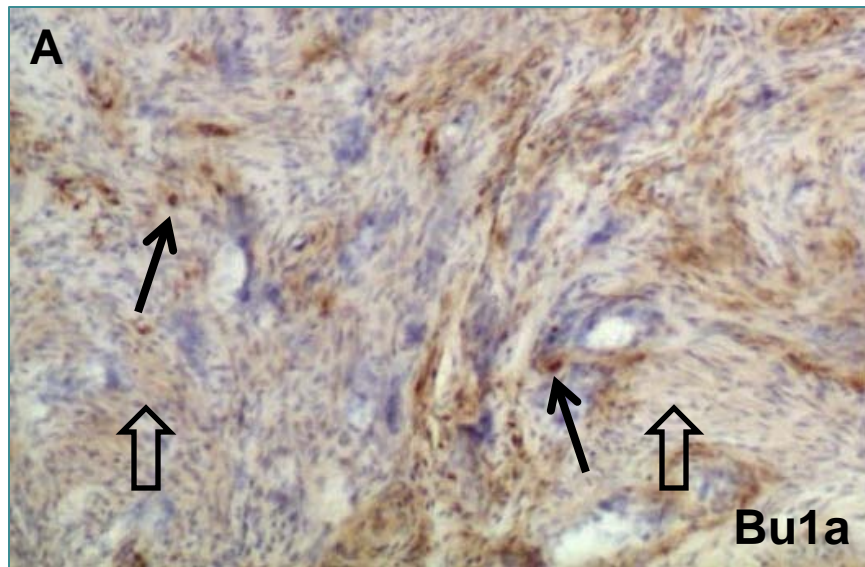


Figure 7