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An Evaluation of the Molecular Species of CA125 Across the Three Phases of the Menstrual Cycle

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An Evaluation of the Molecular Species of CA125 Across the Three Phases of the Menstrual Cycle

by

Monica Rose McLemore

DISSERTATION

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Dedication

This dissertation is the culmination of almost a decade of my life and represents an evolution of thinking. I have a large number of people that I owe the deepest amount of thanks and gratitude, without having the space to name everyone. I will summarize my remarks to my dissertation committee, my friends, and my family.

I have had the honor of working with the most forward thinking and innovative dissertation committee and this work represents their incredible talent, patience, and guidance. Dr. Lee-may Chen has provided me special clinical insights about ovarian cancer patients and has allowed me to ramble at times when my thoughts and vision were not clear. Her presence has taught me an appreciation for interdisciplinary work, a pattern I will continue throughout my career. Dr. Kathryn Lee, the women's health expert that teaches me to sit with my data and writing until it becomes clear has been a great mentor and colleague. Additionally, she has been a personal advocate of my work and source of intellectual support. I do not have the space to document my respect, appreciation and gratitude for Dr. Christine Miaskowski, also my qualifying exam chair. Chris has taught me to be a careful, ethical, and serious scientist. I will carry the many lessons she has taught me through my career and my interactions with others. Finally, Dr. Bradley Aouizerat has been my constant teacher, mentor, and friend. I have admired his ability to comprehend and understand my thoughts, even when they aren't always clear to me. I will forever be grateful for his financial, emotional, and intellectual contribution to this project.

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and privilege to work at, I am grateful to all of you. Also, to my dearest friend (Selena) and fellow nurses (Carolyn, Debbie, Joan, Nancy, MaryJo, and Rachelle) your support has meant the world to me. My deepest appreciation also rests with Mr. James Evans, you know all that you've done. Finally, to my sister Stephanie and her dear family (The Brays), I am deeply indebted to you and this dissertation is dedicated you and your daughters, Shelby and Lauryn. May you ladies find your existence as young women in the world more fun, more exciting and more mind-blowing than mine which, since I've had a great ride and am planning even more adventures.

Acknowledgements

The text of this dissertation project includes reprints of materials as they appear in Biological Research for Nursing, Oncology Nursing Forum, and Cancer Nursing. The first article published in 2005, "Introducing the MUC16 gene: Implications for Early Detection in Epithelial Ovarian Cancer" in Biological Research for Nursing and the third article "Epidemiologic and Genetic Factors Associated with Ovarian Cancer" in Cancer Nursing in 2009 are comparable to the Literature review section of a standard dissertation. The second article published in 2008, "Rules of Tumor Cell Development and their Application to Biomarkers for Ovarian Cancer" in Oncology Nursing Forum is comparable to the Theoretical framework section of a standard dissertation. The fourth article, currently in press, "A Comparison of the Cyclic Variation in Serum Levels of CA125 across the Menstrual Cycle Using Two Commercial Assays" in Biological Research for Nursing is comparable to a portion of the Methods and Results sections of a standard dissertation. The final article "Differences in the Molecular Species of CA125 across the Phases of the Menstrual Cycle" is being prepared for submission to Cancer

Biomarkers and is included as the second portion of the Methods and Results sections of a standard dissertation. Ms. McLemore contributed 90% of the work to these articles and serves a first author on each paper, her dissertation committee members are listed as co-authors and all members actively participated in the work. We remain grateful to the Bast Laboratory at the MD Anderson Cancer Center for use of the OC125 antibody that Dr. Bast discovered in 1988.

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ABSTRACT

An Evaluation of the Molecular Species of CA125 Across the Three Phases of the Menstrual Cycle

Monica Rose McLemore

CA125, a tumor-associated antigen, is primarily used to monitor epithelial ovarian cancer. However, CA125 alone lacks the sensitivity and specificity necessary for population-based screening in healthy women. Barriers to the development of a screening assay includes the low incidence of the disease, large inter-individual variability in CA125 levels, fluctuations in levels during the phases of the menstrual cycle (i.e., menses, follicular, luteal), ethnicity, menopausal status, and other benign conditions.

Evaluation of the molecular species of serum CA125 is a vital step in understanding the underlying biology of CA125. The specific aims of this study, in a sample of healthy women were: 1) To determine if the molecular species of CA125 differ across the three phases of the menstrual cycle; and 2) To determine if the absolute serum concentrations of CA125 differ across the three phases of the menstrual cycle, using two common commercial CA125 assays.

Healthy, Caucasian women between the ages of 18 and 39 were enrolled using strict criteria to minimize serum CA125 fluctuations. After menstrual cycle regularity was determined using calendars maintained by participants for 3 months, blood samples for analyses of CA125 were collected at three different phases of the menstrual cycle.

Serum levels of CA125 fluctuated across the menstrual cycle, with the highest levels found during menses. The amount of change in CA125 over time (i.e., 0.2 U/ml

per day) was identical using both assays, which suggests that the relative changes in CA125 are consistently measured regardless of assay system.

Additionally Western blot analysis yielded seventeen distinct profiles (patterns of species) of CA125. Demographic characteristics and serum CA125 values are not significantly different for each CA125 pattern or at any time point. Consistent with previous studies of CA125, our data show species ranging from 31 to 460 kDa. Most of the referent bands in our data appear at 117 kDa or higher indicating that CA125 may primarily be a high molecular weight species in healthy women with regular menstrual cycles. Future research needs to determine the clinical importance and the molecular composition of these species of CA125.

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Introduction

A goal of biomarker research is to establish clear reference ranges in well-defined populations (Simon et al., 2009). CA125 is not a well-defined molecular species (Jankovic & Milutinovic, 2007; Jankovic & Milutinovic, 2005; Hollingsworth & Swanson 2004; Maeda et al., 2004; Hamilton et al., 2002; Bidart et al., 1999, Nustad et al., 1998; Jacobs et al., 1989). Variability in serum CA125 levels and CA125 fractionated by gel electrophoresis has been documented since the molecule was first detected in 1988 using the OC125 antibody.

A portion of the variability observed in serum CA125 levels is attributable to epidemiologic factors including differences in the commercial assays (Davelaar et al., 1998; Martin et al., 1997; Clement et al., 1995; Fisken et al., 1989), fluctuations in levels during the phases of the menstrual cycle (Bon et al., 1995; Kan et al., 1992), ethnicity (Pauler et al., 2001; John et al., 1993), menopausal status (Johnson et al., 2008; Pauler et al., 2001), and other benign conditions (Kafali et al., 2007; Bon et al., 1999; Meden et al., 1998) Taken together, these findings suggest, the fluctuations in the absolute concentration of serum CA125 result from a variety of factors.

Additionally, evidence exists for different molecular species of CA125 observed in different tissue types. CA125 is secreted by all of the tissues of the female reproductive tract as a large glycoprotein, which is degraded by proteolysis during transport throughout the circulatory system (Jankovic & Milutinovic, 2007; Jankovic & Milutinovic, 2005; Hollingsworth & Swanson 2004; Maeda et al., 2004; Hamilton et al., 2002; Bidart et al., 1999; Nustad et al., 1998). CA125 degradation is believed to be one of many factors that contribute to the different species measured in serum (Jankovic &

Milutinovic, 2007; Jankovic & Milutinovic, 2005; Hollingsworth & Swanson 2004; Maeda et al., 2004; Hamilton et al., 2002; Bidart et al., 1999, Nustad et al., 1998). Taken together these data suggest that observed differences in absolute concentration and size of CA125 result from several factors that could be controlled, if the participants are carefully screened to include only women who are expected to show minimal fluctuations in CA125 levels. The studies presented in this dissertation represent a first step in determining the molecular species of CA125 in healthy women. The long-term goal of this program of research is to establish clear reference ranges and to characterize the molecular species for CA125 in well-defined, healthy samples. The first chapter entitled: *Introducing the MUC16 gene: Implications for Early Detection in Epithelial Ovarian Cancer*, summarized the known information in 2005 about the recently identified mucin 16 (MUC16) gene that is believed to encode the peptide core of CA125. This discovery was and remains important because it elucidated potential structural and functional domains, which could be used to improve CA125 assay sensitivity and specificity.

The second chapter entitled: *Epidemiologic and Genetic Factors Associated with Ovarian Cancer*, provides a comprehensive review of the epidemiological and genetic factors associated with ovarian cancer. A more complete understanding of the determinants of ovarian cancer may lead to the development of better screening and detection methods for this disease. The first section of this article reviews current literature on screening and early detection of ovarian cancer. The second section reviews the epidemiology of ovarian cancer, specifically highlighting the risk factors

associated with the development of this disease. This article concludes with a discussion of how oncology nurses can apply this information to improve patient care.

The third chapter entitled: *Rules of Tumor Cell Development and their Application to Biomarkers for Ovarian Cancer* serves as the theoretical framework for the entire dissertation project. Grounded in a theory of cellular transformation posited by Hanahan & Weinberg, we applied this theory to biomarkers for tumors.

The fourth chapter entitled: *A Comparison of the Cyclic Variation in Serum Levels of CA125 across the Menstrual Cycle Using Two Commercial Assays* is in press and will appear in Biological Research for Nursing. CA125, a tumor-associated antigen, is primarily used to monitor epithelial ovarian cancer. However, CA125 lacks the sensitivity and specificity necessary for population-based screening in healthy women. The purpose of this study was to determine in a sample of healthy, premenopausal women, if serum concentrations of CA125 differed across the three phases of the menstrual cycle using two commercially available assays for CA125 determination.

Healthy, Caucasian women between the ages of 18 and 39 were enrolled using strict criteria to exclude factors known to contribute to CA125 fluctuations. Menstrual cycle regularity was determined using calendars maintained by participants for 3 months. After cycle regularity was established, blood was drawn at 3 time points for CA125 determination using two commercial assays (i.e., Seimens, Panomics). Regardless of the assay used, CA125 values were highest during menses. CA125 values decreased 0.2 U/ml per day from menses to the end of the same cycle, which resulted in a net decrease of 5.8 U/ml across the cycle. The two commercial assays for CA125 determination demonstrated good concordance in terms of reference ranges

regardless of epitope differences. While CA125 levels changed over the course of the menstrual cycle, these changes may not be clinically significant in healthy women. This study is the first to control for factors known to contribute to CA125 elevations; to quantify a decrease in CA125 levels across the menstrual cycle; and to confirm concordance in the relative decreases in serum CA125 levels across the menstrual cycle between two frequently used commercial assays.

The fifth chapter entitled: *Differences in the molecular species of CA125 across the phases of the menstrual cycle* is being prepared for publication. CA125, a tumor-associated antigen, is primarily used to monitor epithelial ovarian cancer. Despite evidence for different known species of CA125 it is not known if these are present in healthy women during the menstrual cycle and if they are associated with the serum concentrations of CA125. The purpose of this study was to determine if the molecular species of CA125 differs across the three phases of the menstrual cycle in healthy women.

Healthy, Caucasian women between the ages of 18 and 39 were enrolled using strict criteria to exclude factors known to contribute to CA125 fluctuations. Menstrual cycle regularity was determined using calendars maintained by participants for 3 months. After cycle regularity was established, blood was drawn at 3 time points for Western blot analysis.

Western blot analysis yielded seventeen distinct profiles (patterns of species) of CA125. Demographic characteristics and serum CA125 values are not significantly different for each CA125 pattern or at any time point. Different molecular species of CA125 exist in healthy women with regular menstrual cycles. These data provide

additional evidence that CA125 is not a homogeneous molecular species. Future research should evaluate the clinical importance and the molecular composition of these species.

The final chapter explores the clinical and research implications from the dissertation findings and provides recommendations for future research.

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Chapter 1.

Rules of Tumor Cell Development and their Application to Biomarkers for Ovarian Cancer.

McLemore, M.R., Miaskowski, C., Aouizerat, B.E., Chen, L-m, Dodd, M.J. (2008). *Oncology Nursing Forum*, 35, 403-409.

Abstract

Purpose/Objectives: To apply the Hanahan and Weinberg conceptual framework for tumor development to the specific biomarkers observed or expressed in ovarian cancer.

Data Sources: Data-based publications, topical reviews, and book chapters.

Data Synthesis: Articles specific to ovarian cancer were reviewed to examine whether the six rules from the Hanahan and Weinberg conceptual framework were applicable to biomarkers of ovarian cancer. This approach allows for the application of a general framework for the development of solid tumors to the development of ovarian cancer.

Conclusions: The six rules for tumor cell development outlined in the Hanahan and Weinberg conceptual framework are applicable to biomarkers expressed or observed in patients with ovarian cancer.

Implications for Nursing: Oncology nurses can enhance their clinical teaching by integrating this information into their practice. Nurses who conduct research on ovarian cancer can use this framework to guide the selection of biomarker(s) for these studies. Finally, nurse educators can use this framework when teaching students key concepts in the care of patients with cancer.

Key Points:

- The Hanahan and Weinberg conceptual framework attempts to synthesize existing knowledge about tumors and the rules
- The Hanahan and Weinberg conceptual framework is applicable to biomarkers of ovarian cancer.
- The hallmarks of cancer can be distilled to six rules that also apply to biomarkers of ovarian cancer.
- Information about biomarkers of ovarian cancer has increased during recent years, and nurses should integrate this knowledge into their clinical practice.

Introduction

Inherent biologic differences exist among and within various types of human cancer. Although tumors arise and proliferate from a single abnormal cell, they quickly become heterogeneous in their cellular composition, with some more differentiated than others. Historically, experimental work in histology (the microscopic structure of tissues), cytology (structure and function of cells), morphology (form and structure of living organisms), and epidemiology (incidence and prevalence of disease) has provided diverse phenotypes for various cancers. However, as molecular methods improve, synthesizing experimental evidence and thinking conceptually about the underlying rules that govern tumor development have become possible (Hanahan & Weinberg, 2000).

The purpose of this article is to apply the Hanahan and Weinberg (2000) conceptual framework for the development of cancer cells to biomarkers observed or expressed in ovarian cancer. Ovarian cancer was chosen as the tumor type because this framework has not been applied to it and knowledge of biomarkers for ovarian cancer has increased in recent years.

The Rules for Making Tumor Cells and Biomarkers of Ovarian Cancer

The Hanahan and Weinberg conceptual framework attempts to synthesize existing knowledge about tumors and the rules required for malignant transformation. After a review of years of literature in cell biology, biochemistry, genetics, and clinical oncology, six rules were distilled regarding the development of tumor cells (Hahn & Weinberg, 2002; Hanahan & Weinberg, 2000). Hanahan and Weinberg stated that “we foresee cancer research developing into a logical science, where the complexities of the

disease, described in the laboratory and clinic, will become understandable in terms of a small number of underlying principles" (p. 57). The six rules are: (a) self-sufficiency in growth signals, (b) insensitivity to growth-inhibitory signals, (c) evasion of programmed cell death (apoptosis), (d) limitless replicative potential, (e) sustained angiogenesis, and (f) tissue invasion and metastasis (Hanahan & Weinberg). Detailed information about each rule and the exemplars provided in the original article are summarized in Table 1.

All of the rules that govern malignant transformation are grounded in the cell cycle and the proteins involved in the regulation of cellular proliferation. The six rules for malignant transformation are described in the context of the cell cycle; however, the rules do not need to occur in a specific order, and all of the rules of tumorigenesis do not necessarily have to occur in all types of human cancer. Figure 1 provides a glossary of terms that are used throughout this article.

Self-Sufficiency

Normal cells depend on external signals to grow and divide in the context of their neighbors. Tumor cells acquire the ability to replicate, divide, and grow without exogenous growth signals. In addition, tumor cells acquire the ability to alter or generate their own growth signals, rendering them independent from their microenvironment (Hanahan & Weinberg, 2000). Tumor cells also develop alterations in signal transduction that allow them to overexpress growth factors, receptors, and other domains on their cellular surfaces.

The biomarker that best illustrates the self-sufficiency rule is the epidermal growth factor receptor (EGFR). EGFR is part of a large superfamily with many members, including HER2/neu (c-erbB-2), HER3 (erbB-3), and HER4 (erbB-

4).Overexpression of EGFR is observed in a large number of epithelial human tumors, where it initiates a phosphorylation cascade that is responsible for cell proliferation, differentiation, and activation of other signaling pathways that lead to the activation of key transcription factors (Aunoble, Sanches, Didier, & Bignon, 2000).

HER2/neu in ovarian cancer: Amplification of HER2/ neu is found in 19%–59% of ovarian tumors (Aunoble etal., 2000). No correlation has been found among HER2/neu expression and tumor stage, tumor grade, or histologic tumor type. However, overexpression of HER-2/neu in ovarian cancer predicts platinum therapy chemotherapy resistance in 75% of patients (Aunoble et al.).

Insensitivity to Antigrowth Signals

The role of most normal cells in human tissues is to maintain organ homeostasis. Inter- and intracellular signaling helps to maintain the balance among various cells within tissues. Healthy cells use signaling pathways to monitor the external environment in which they find themselves. This monitoring role allows cells to decide to proliferate, remain senescent, or initiate apoptosis (Evan & Littlewood, 1998). Abnormal cells acquire the ability to override external signals and proliferate independent of their environment.

The best characterized biomarker that illustrates the insensitivity rule is tumor growth factor-β (TGF-β). The TGF-β superfamily includes bone morphogenic proteins, growth and differentiation factors, activins, inhibins, and Mullerian inhibitory factors (Chin, Boyle, Parsons, & Coman, 2004). TGF-β functions as a cytokine in the human ovaries, acting as a regulator of epithelial cell growth, differentiation, motility,

organization, and apoptosis (Chin et al.). The inhibins are the best understood member of the TGF-β family in ovarian cancer.

Inhibins and ovarian cancer: Inhibins are produced and secreted by normal ovarian tissue and ovarian tumors; however, increased production and secretion of inhibins are more common with mucinous ovarian tumors. Two heterologous subunits of inhibins exist, namely βA or βB, that form dimmers called activins. These subunits are responsible for tissue development, immunosuppression, and proliferative and antiproliferative activity (Robertson et al., 2004; Tong, Wallace,& Burger, 2003).

Inhibins regulate pituitary follicle-stimulating hormone secretion using a negative feedback loop (Robertson et al.,2004). The primary mode of action of inhibins is to antagonize the activins (in dimer configuration), which renders the inhibins as indicators of ovarian cancer recurrence. Inhibins are measured as biomarkers in conjunction with CA125 levels. Inhibin A (α-βA) and inhibin B (α-βB) are serum biomarkers drawn in conjunction with CA125 to monitor granulose cell ovarian cancer after surgical debulking. The inhibins are markers of altered circulation and considered a characteristic of mucinous ovarian cancer; however, their utility in epithelial ovarian cancer is unknown.

Evading Apoptosis

Apoptosis controls cellular proliferation and attrition in normal cells. Apoptosis occurs in response to DNA damage, during conditions of extreme cellular stress (e.g., heat shock, lack of nutrients), and in response to external injury or damage (Hanahan & Weinberg, 2000). The process of apoptosis can last 30–120 minutes and follows a distinct pattern, beginning with cellular membrane disruption. The cytoplasmic and

nuclear skeletons break down, the cytosol is extruded, the chromosome is degraded, and nuclear fragmentation occurs (Hanahan & Weinberg). The degraded cell is engulfed by a leukocyte and metabolized within 24 hours.

Tumor cells can evade apoptosis and gain the ability to proliferate indefinitely (Evan & Littlewood, 1998; Hahn& Weinberg, 2002). Two well-characterized pathways of apoptosis exist, one that includes the mitochondrial caspases and one that uses the p53 transcription factor to activate the ubiquitin pathways of cellular death. A multitude of biomarkers are associated with both apoptotic pathways, but the best characterized biomarker that illustrates the evading apoptosis rule is p53.

p53 in ovarian cancer: The loss of the tumor-suppressor gene Tp53, which leads to overexpression of the p53 protein, occurs in 30%–50% of ovarian cancers and is specific for serous carcinomas (Aunoble et al., 2000; Fujita, Enomoto, & Murata, 2003). In addition, overexpression of the p53 protein is observed in 26%–62% of ovarian tumors (Aunoble et al.). Loss of the p53 gene occurs through a variety of mutations (e.g., missense, frameshift, splice-site). Patients with ovarian tumors associated with the loss of p53 have a poorer survival when considered with a variety of other characteristics, including International Federation of Obstetrics and Gynecology stage, tumor grade, and histologic type (Aunoble et al.; Wenham, Lancaster, & Berchuck, 2002). The p53 gene is unlike c-myc, k-ras, and HER2/neu. When the p53 gene is lost, the p53 protein is overexpressed but its function may be altered (Hanahan & Weinberg, 2000).

The ability of cancer cells to respond to chemotherapeutic agents has been shown to depend on functional apoptotic pathways (Fraser et al., 2003). In ovarian

cancer, drug-induced apoptosis is governed by the up-regulation of proapoptotic factors, including p53, and modulation of cell survival factors (Fraser et al.). The loss of p53 initiates several cascades that render ovarian cancer cells resistant to cisplatin and paclitaxel (Fraser et al.).

Limitless Replicative Potential (Immortality Rule)

Normal cells, during each replication, lose 50–100 base pairs of DNA at the ends of every chromosome. Telomeres function as specialized DNA that create protective caps on the ends of chromosomes (Hanahan & Weinberg, 2000). Once the telomeres have eroded completely, the chromosomes become susceptible to end-to-end fusion and karyotypic disarray and the cell is marked for death (Hanahan & Weinberg; Matias-Guiu & Prat 1998).

In culture, cells grow until they reach confluency, usually after 60–80 cell generations, at which point they enter a nongrowing yet viable state called senescence (Hahn & Weinberg, 2002). The replicative capacity of a cell directly correlates with telomere length, thus creating a mitotic clock that signals when a cell should enter senescence (Villa et al.,2000). Tumor cells that express telomerase gain the capacity to replicate without limit and can become immortal (Hanahan & Weinberg, 2000). The biomarker that best illustrates the immortality rule is telomerase. Telomerase is a large ribonucleoprotein complex that is expressed only in normal embryonic cells and gametocytes (King & Stansfield, 2002).

Telomere maintenance and telomerase activity in ovarian cancer: Normal adult cells do not express telomerase. Ninety-two percent of ovarian tumors express telomerase (Matias-Guiu & Prat, 1998). Expression of telomerase is equivalent to

cellular resistance to apoptosis (Villa et al., 2000). Increased telomerase expression has been associated with a poorer prognosis in all ovarian tumor types (Wenham et al., 2002). Ovarian cancer cells in culture have been shown to use telomerase to catalyze the synthesis of new repeats during DNA replication (Wenham et al., 2002). Telomere length is related inversely to the degree of chromosomal abnormality in ovarian cancer; however, whether telomerase is activated as a part of tumorigenesis or is a result of subsequent chromosomal instability remains to be determined (Counter, Hirte, Bacchetti, & Harley, 1994).

Sustained Angiogenesis

Angiogenesis is a normal process by which capillaries are formed and blood is supplied to tissues. This process is tightly controlled by a net balance between positive and negative regulators (Mukhopadhyay & Datta, 2004; Ribatti, 2005). Solid tumors are confined within the organ space and cannot grow beyond 2 mm unless they acquire access to the circulatory system. Without access to a blood supply, tumor cells are subject to necrosis and apoptosis (Hanahan & Weinberg, 2000; Mukhopadhyay & Datta). Several factors are involved with angiogenesis; however, the biomarker that best illustrates the sustained angiogenesis rule is the vascular endothelial growth factor (VEGF) family (Ribatti).

VEGF is classified as an endothelial cell-specific mitogen with five known isoforms of the gene named VEGFA, VEGFB, VEGFC, VEGFD, and VEGFE (Ribatti, 2005). VEGF has preferential and specific binding to VEGF receptors (VEGFR), of which three forms are known to exist: VEGF R1 and VEGFR2, which are restricted to endothelium, and VEGFR3, which has preferential binding with lymphatic endothelium

(Ribatti). The VEGF gene is homologous with the platelet-derived and placental growth factor family of genes (Ribatti).

VEGF is responsible for embryonic vasculogenesis and angiogenesis and rarely is expressed in normal adult tissues. When expressed in normal adult tissues, VEGF induces proliferation, sprouting, migration, and tube formation of endothelial cells (Tammela, Enholm, Alitalo, & Paavonen, 2005). Endothelial cells are quiescent except in human ovaries, the uterus, and skin cells (Ribatti, 2005). In normal ovarian tissue, VEGF is responsible for neovascular formation within the corpus luteum and for growth of the ovarian follicle (Sonmezer, Gungor, Ensari, & Ortac, 2004). Within the uterus, VEGF supports the growth of the endometrial vessels during embryo implantation (Ribatti).

Vascular endothelial growth factor in ovarian cancer: High degrees of tumor angiogenesis and VEGF expression have been shown to correlate with poorer survival in patients with ovarian cancer (Alvarez-Secord et al., 2004). Although increased serum VEGF levels alone do not provide a clear indication of angiogenesis in ovarian cancer, high levels of VEGF in ascitic fluid are a more accurate predictor of tumor growth and spread (Hsieh et al., 2004). Ascites is the collection of fluid within the pelvis of patients with ovarian cancer that supports the growth of tumor cells in vitro and in vivo (Hsieh et al., 2004). The production of ascites follows impaired lymphatic drainage in a feedback loop that increases the rate of lymph production because of the lack of drainage (Hsieh et al.). VEGF has been implicated as an important mediator of ascites formation. VEGF is an upstream regulator of PI3KCA, a known oncogene in ovarian cancer, and is

associated with ascites formation as an early event in malignant transformation (Shayesteh et al., 1999; Sonmezer et al., 2004; Zhang et al., 2003).

Tissue Invasion and Metastasis

Although immune cells can travel and invade under normal circumstances, tissue invasion and metastasis generally are observed only in tumors. The extracellular matrix is an important component of the process of metastasis because of its role in cellular adhesion. Cellular adhesion involves zonula occludens (tight junctions), zonula adherens (adherens junctions), and macula adherens (desmosomes) (Sundfeldt, 2003). The adherens and tight junctions polarize the cells and participate in condensation during tissue development, compaction during embryogenesis, contact inhibition, wound healing, and tumorigenesis (Sundfeldt).

Crucial to cellular adhesion processes are five classes of molecules: interleukins, immunoglobulins, cadherins, integrins, and extracellular proteases (King & Stansfield, 2002). Cell-to-cell adhesion requires cadherins, selectins, and integrins; cell-to-matrix adhesion requires integrins and transmembrane proteoglycans (Alberts et al., 2002). The best characterized biomarkers that illustrate the tissue invasion and metastasis rule are E-cadherin and CA125.

E-cadherin is a member of the cadherins family, also known as cell adhesion molecules, which are dependent on calcium or magnesium to function (Alberts et al., 2002). Cadherins are glycoproteins that facilitate cell-to-cell adhesion, which creates an integral part of the cell membrane (Alberts et al.). Four members of the family exist, including E-cadherin (the best described, specific to epithelial cells), N-cadherin (found in neurons, heart, skeletal muscle, lens, and fibroblasts), P-cadherin (found in placenta,

epidermis, and breast epithelium), and VE-cadherin (specific to endothelial cells) (Alberts et al.).

CA125 is expressed as part of normal ovarian function the first trimester of pregnancy and during the menstrual cycle (Kafali, Artunc, & Erdem, 2007; Kan, Yeh, Ng, & Lou, 1992; Kenemans, van Kamp, Oehr, & Verstraeten, 1993; Verheijen, van Mensdorff-Pouilly, van Kamp, & Kenemans, 1999). In addition, CA125 is known to be elevated in benign conditions, such as pelvic inflammatory disease, endometriosis, and ovarian cysts (Jacobs & Bast, 1989; Rubin & Sutton, 2004). CA125 may participate in the mechanisms of tissue renewal of the reproductive tract during ovulation (Nustad, Onsrud, Jannson, & Warren, 1998). CA125 is hypothesized to lubricate surrounding tissues and decrease the immune responses to exposed tissue until repair is complete (Auersperg, Ota, & Mitchelle, 2002; Nustad et al.).

During normal ovulation, the ovarian surface epithelium must quickly repair the postovulatory wound created by follicular rupture. This process requires the migration of ovarian surface epithelium stem cells to maintain the tissue until the outcome of fertilization is known (Roskelley & Bissell, 2002). E-cadherin is not expressed in those cells because they do not require adhesion properties. The ovarian surface has an epithelial-mesenchymal phenotype, meaning it has characteristics of epithelial and mesenchymal tissues and is not fully committed to becoming epithelium (Auersperg et al., 2002). Mesenchymal cells are inherently migratory given their cellular function in wound healing. These cells undergo an epithelial to- mesenchymal transformation that maintains tissue integrity (Roskelley & Bissell). However, the epithelial and stromal cells of the ovary express E-cadherin to maintain adhesion.

E-cadherin in ovarian cancer: Approximately 64% of ovarian tumors show a loss of E-cadherin expression (Faleiro-Rodrigues, Macedo-Pinto, Pereira, Ferreira, & Lopes, 2004). In ovarian cancer, decreased expression of E-cadherin is associated with a poorer stage and histologic type and peritoneal metastasis (Faleiro-Rodrigues et al.; Roskelley & Bissell, 2002). In addition, epithelial-to-mesenchymal transformation is strongly associated with a more metastatic phenotype in patients with ovarian cancer (Roskelley & Bissell). Loss of E-cadherin expression is associated with tumor progression.

CA125 in ovarian cancer: CA125 is elevated in 95% of women with serous ovarian tumors and adenocarcinomas (Verheijen et al., 1999). Generally, it is not elevated in women with tumors of mucinous or germ cell origin (Rubin & Sutton, 2004). Elevations in CA125 following total abdominal hysterectomy and bilateral salpingo-oophorectomy or first-line chemotherapy for ovarian cancer suggest disease recurrence or treatment failure. In addition, increases in CA125 are associated with progression from benign to malignant states in cultured cells (Kui Wong et al., 2003; Seelenmeyer, Wegehingel, Lechner, & Nickel, 2003). The acquired function of CA125 in ovarian tumors is to protect ovarian tumor cells from immune system surveillance (Kabawat, Bast, Welch, Knapp, & Colvin, 1983; Tannock, Hill, Bristow, & Harrington, 2005).

Discussion

Although the Hanahan and Weinberg conceptual framework provides a useful model to understand some of the biomarkers for ovarian cancer, it has limitations. First, the visual representation of the original framework appears in Figure 2 as a circular model with interchangeable parts; however, the exact sequence of events that leads to the

expression of various biomarkers for ovarian cancer or other cancers has not been established at the present time. In addition, the framework makes no distinction among which phenomena were observed exclusively in human, animal, or cellular models. Based on available data, knowing which rules are directly applicable to individual patients is not possible.

Even so, understanding the biomarkers for ovarian cancer in the context of these rules provides justification for their use in clinical studies and a useful framework for teaching students about the mechanisms of tumorigenesis. Table 2 lists normal ranges for the various biomarkers highlighted in this article. VEGF, CA125, and inhibin A and B are the only biomarkers that have commercially available clinical assays. The other biomarkers are used in clinical trials and not approved by the U.S. Food and Drug Administration for clinical use. All of the biomarkers highlighted in this article are produced in varying quantities in normal and cancerous cells. How these biomarkers differ in women with and without ovarian cancer needs to be determined.

The application of this framework to the various biomarkers that are expressed or observed in patients with ovarian cancer provides oncology nurses with information that can be used for patient education about the rationale for various diagnostic tests and therapeutic interventions. In addition, nurses who conduct research on ovarian cancer can use this framework to guide the selection of biomarkers for studies. More research is needed on changes in and interactions among these biomarkers throughout the trajectory of ovarian cancer.

Conclusion

The six rules for tumor development outlined in the Hanahan and Weinberg conceptual framework are applicable to biomarkers associated with ovarian cancer. Understanding these biomarkers within the context of the rules provides justification for their use in clinical research and an informative framework for clinician and patient education and begins to integrate biomarkers for ovarian cancer into the broader framework of tumor cell biology.

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Table 1.1 Rules From the Hanahan and Weinberg Conceptual Framework

Table 1. Rules From the Hanahan and Weinberg Conceptual Framework

Rule	Evidence (Exemplar)	Other Names	Other Family Members and Homologues	Loci	Characteristics of the Exemplar
Self-sufficiency	EGFR	HER2/neu	EGFR (erbB1) HER2/neu (c-erbB-2) HER3 (erbB-3) HER4 (erbB-4)	17q21–22	Cell proliferation Differentiation Activation of key transcription factors
Insensitivity to growth-inhibitory signals	TGF	TGF-α or EGF	TGF-α TGF-β	19q	Anti-growth factor Prevention of phosphorylation of retinoblastoma protein Prevent cell cycle at G_1
	c-myc	p64 p67	myc	8p24	DNA-binding proteins Cell proliferation Activate and inactivate cyclins family and p53
Apoptosis	p53	Tp53	N/A	17p13.1	DNA-binding protein Sequence-specific transcription factor Growth arrest Apoptosis Metabolite deprivation, physical damage DNA damage, heat shock, hypoxia Oncogene expression
Immortality (limitless replicative potential)	Telomerase	N/A	N/A	Adds hexanucleotide repeats to ends of telomeric DNA	Large ribonucleoprotein complex Expressed only in embryonic cells and gametocytes
Sustained angiogenesis	VEGF	PDGF-β PIGF	VEGFA VEGFB VEGFC VEGFD VEGFE	VEGFA 6p21.3	Embryonic vasculogenesis and angiogenesis Proliferation Sprouting, migration, tube formation of endothelial cells
Metastasis (tissue invasion)	E-cadherin	N/A	N-cadherin P-cadherin VE-cadherin	16q22.1	Highly conserved cytoplasmic tail complexes with catenins family Cellular cohesion and detachment
	Interleukin-6	IL-6	Cytokine	Unknown	B-cell differentiation Immunoglobulin production Megakaryopoiesis (generation of platelet precursors)
	CA125	MUC16 (gene)	N/A	19p13.2	Ligand for galectin-1 Ligand for mesothelin

EGF—epidermal growth factor; EGFR—epidermal growth factor receptor; N/A—not applicable; PDGF—platelet-derived growth factor; PIGF—placental growth factor; TGF—transforming growth factor; VEGF—vascular endothelial growth factor

Note. Based on information from Hanahan & Weinberg, 2000.

Table 1.2 Normal Ranges for Biomarkers of Ovarian Cancer

Table 2. Normal Ranges for Biomarkers of Ovarian Cancer

Biomarker	Normal Ranges	Ranges in Samples of Patients With Ovarian Cancer	Platform and Assay Manufacturer	References
HER2/neu[a]	Range = 595–1,947 HNU/ml Mean = 1,203 HNU/ml ± 279 HNU/ml	Range = 526–16,332 HNU/ml; 1,761 was chosen as the cutoff for an elevated level (i.e., two standard deviations above the mean).	ELISA	Cirisano & Karlan, 1996; Meden et al., 1994; Meden & Kuhn, 1997
p53[a]	Range = 0–0.50 U/ml	Median = 0.69 U/ml (benign tumor median = 0.32 U/ml)	ELISA	Sorak et al., 2007
Telomerase	Value < 0.2 $A_{450-660}$nm	Mean = 0.795 (± 0.168) $A_{450-660}$nm	PCR, ELISA/ Roche	Sun et al., 2007
VEGF	Range = 10.0–778.8 pg/ml Value = < 300.0 pg/ml	Median = 220.0 pg/ml; range = 134.7–671.2 pg/ml)	ELISA/Quantikine Human VEGF Kit, R & D Systems	Candido Dos Reis et al., 2002; Hazelton et al., 1999
CA125	Value = < 35 U/ml (although some assays report < 21 U/ml)	20–10,000 U/ml (not all ovarian tumors secrete CA125)	ELISA/Centocor Roche, Boehringer	Jacobs & Bast, 1989; Verheijen et al., 1999

[a] Commercial assays for this biomarker do not exist.

ELISA—enzyme-linked immunosorbent assay; PCR—polymerase chain reaction; VEGF—vascular endothelial growth factor

Figure 1.1 Definitions

Amplification: chromosomal changes in tumor cells that allow for more than one copy of a gene to be present; amplification is sometimes referred to as a copy number alteration or aneuploidy (Alberts et al., 2002).

Confluency rule: Cells grown in culture grow exponentially until confluency is reached. Once plated cells have occupied the surface space, they will no longer divide until additional resources and space are provided (King & Stansfield, 2002).

Glycoprotein: a protein containing a small amount of carbohydrate (< 4%), also known as a membrane protein (Alberts et al., 2002; King & Stansfield, 2002)

Immunoglobulins: antibodies secreted by mature lymphoid cells; immunoglobulins (Igs) are y-shaped tetrameric molecules with five known chain classes: IgA (epithelial tissues of respiratory and digestive tracts), IgE (allergies), IgD (unknown function), IgG (most common and responsible for immunologic memory), and IgM (early response to initial antigen exposure) (King & Stansfield, 2002).

Interleukins (cytokines): a group of at least 15 soluble proteins secreted by leukocytes that promote growth and differentiation of immune cells

Mesenchyme: embryonic type of connective tissue, consisting of amoeboid cells with many processes; these cells form loose networks and produce the connective tissue and circulatory system during development of vertebrates (King & Stansfield, 2002).

Mitogen: a compound that stimulates cells to undergo mitosis (King & Stansfield, 2002)

Signal transduction: pathways through which cells receive external signals and transmit, amplify, and direct them internally (King & Stansfield, 2002)

Tumor suppressor genes: antioncogenes (King & Stanford, 2002) that suggest the production of too little gene product through loss of function mutations or allelic loss (Nussbaum et al., 2001) or result in tumorigenesis

Figure 1. Definitions

Figure 1.2 Hanahan and Weinberg Conceptual Framework *Note.* From “The Hallmarks of Cancer,” by D. Hanahan and R.A. Weinberg, 2000, *Cell, 100*, p. 58. Copyright 2000 by Elsevier.

Figure 2. Hanahan and Weinberg Conceptual Framework

Note. From “The Hallmarks of Cancer,” by D. Hanahan and R.A. Weinberg, 2000, *Cell, 100*(1), p. 58. Copyright 2000 by Elsevier. Reprinted with permission.

Chapter 2.

Epidemiologic and Genetic Factors Associated with Ovarian Cancer.

McLemore, M.R., Miaskowski, C., Aouizerat, B.E., Chen, L-m, Dodd, M.J. (2009).

Cancer Nursing, 32, 281-288

Ovarian cancer has the highest mortality rate of all female cancers; more than 50% of the 21,650 women diagnosed with ovarian cancer die annually of this disease. A more complete understanding of the epidemiological and genetic determinants of ovarian cancer may lead to the development of better screening and detection methods for this disease.

Current Screening and Early Detection of Ovarian Cancer Screening

There is inadequate evidence to support screening for ovarian cancer in the general population. However, 2 large, ongoing, prospective, randomized controlled trials (RCTs)[2,3] should provide important information about the efficacy of using transvaginal ultrasound (TVU), CA-125, and screening examinations for the early detection of ovarian cancer. The first RCT, being conducted in the United States, is the Prostate, Lung, Colorectal, and Ovarian screening trial, sponsored by the National Cancer Institute. This study includes 37,000 healthy postmenopausal women between 55 and 74 years of age who were randomized to either an annual CA-125, TVU, and pelvic examination or standard care.[2] The end points are cost and the establishment of important time points for screening. The outcome variable is mortality. Patients will be followed up for 13 years. Based on preliminary analyses, 1,338 participants (4.7%) had abnormal TVU, and 402 participants (1.4%) had abnormal CA-125 (only 34 had abnormal results on both tests).[3] Data from this RCT pertaining to the efficacy of screening are still being analyzed.

The second study, being conducted in the United Kingdom, has enrolled 200,000 postmenopausal women and is called the North Thames Ovarian Cancer Study. This

study is attempting to determine the sensitivity of CA-125 plus TVU versus TVU alone as screening measures for ovarian cancer. Study end points include quality of life, morbidity, and cost.[3,4] Enrollment was completed in 2005, and results should be forthcoming soon. These studies should provide valuable information about the crucial time points and the optimal screening methods for ovarian cancer.

Early Detection

Early detection of ovarian cancer has been a goal of clinical research. However, the underlying biology of the disease and its vague symptom profile have rendered it difficult to diagnose at early stages. A recent consensus statement[5] jointly issued by the Gynecologic Cancer Foundation,[5] Society of Gynecology Oncologists, and the American Cancer Society identified 4 symptoms that are more likely to occur in women with ovarian cancer: (1) bloating, (2) pelvic or abdominal pain, (3) difficulty eating or feeling full quickly, and (4) urinary symptoms (i.e., urgency or frequency). Taken together, these symptoms are called the Ovarian Cancer Symptom Index (OCSI). The release of this statement was not without controversy; the symptoms identified are common in healthy women, and the survey used to identify the symptoms included in the index was not prospectively validated in a sample of healthy women.[6]

Two studies have provided evidence to support the clinical use of the OCSI.[7,8] Goff and colleagues[7] used a 23-item symptom survey to develop the OCSI. Using this survey, the symptoms were collapsed into a single variable if the correlation coefficient among the symptoms was 0.70 or greater.[7] Odds ratios (ORs) were used to compare symptoms of different duration, frequency, and severity. Finally, logistic regression was

used to determine the independent contribution of the symptoms to predict risk for ovarian cancer in a different sample of high-risk women.[7]

The OCSI was considered positive if women reported any of the following 6 symptoms more than 12 times in 1 month but that they were not present for more than 1 year: pelvic/ abdominal pain, increased abdominal size/bloating, and feeling full/difficulty eating. Once the symptom index was developed, the study cohort was divided into an exploratory group and a confirmatory group to test the sensitivity and specificity of the index in the confirmatory sample. The index had a sensitivity of 56.7 to detect early-stage and 79.5 to detect advanced-stage ovarian cancer. The specificity of the index was 90% in women older than 50 years and 86.7% in women younger than 50 years of age.

The second study,[8] from the same research group, combined data from the symptom index with CA-125 values to determine if it improved the sensitivity and specificity of the index. This prospective case-control study enrolled 254 healthy high-risk women (due to family history) and 75 women with ovarian cancer. Cases were defined as women scheduled for surgery for evaluation of a mass suggestive of tumor. Using methods from their previous study,[7] the investigators found that cases were more likely to be older and have elevated levels of CA-125 and a positive OCSI score. Fifty-three percent of the cases had a positive symptom index score and an elevated CA-125; 25.3% had only an elevated CA-125; and 50% of the total group of cases and controls had a positive symptom score (which represented 10.7% of the total case group). Symptom index scores independently predicted an ovarian cancer diagnosis

after adjusting for CA-125 levels (ie, OR, 11.51; 95% confidence interval [CI], 4.62-28.66). However, when used alone, the symptom index score had a lower sensitivity than CA-125 level (64% vs 78.7%). These findings suggest that the combination of CA-125 and the OCSI score improved the sensitivity of detecting ovarian cancer in women participating in a high-risk screening program.

Prevention

Two methods of primary prevention of ovarian cancer are available for high-risk populations, namely, chemoprevention and risk-reducing salpingo-oophorectomy (RRSO).[9-14] Chemoprevention of ovarian cancer has not been studied independently. However, several studies designed to evaluate other outcomes have noted a decreased risk for ovarian cancer in patients taking oral contraceptives (OCPs).[9,12-17] In a large review of 12 case-control studies in the United States,[18] OCP use and ovarian cancer risk had an overall OR of 0.67 (95% CI, 0.37-1.2), an OR of 0.62 (95% CI, 0.24-1.6) in African-American women, and an OR of 0.70 (95% CI, 0.52-0.94) in white women.[19] These data suggest that ever-users of OCPs had a decreased risk for ovarian cancer.[18] The benefit of OCP use remained in women who had used OCPs for 2 to 5 years and leveled off in women who used them for 6 years or longer. These data suggest that 5 years of OCP use confers a 50% decrease in the risk for ovarian cancer with a protective effect that remains for up to 10 years after OCP use is discontinued.[9,18,20] The mechanism by which OCP use protects against the development of ovarian cancer is its progestin effect (ie, decrease in the number of ovulatory events).[13,20]

Risk-reducing salpingo-oophorectomy is defined as the removal of ovaries in women with no documented ovarian disease or with a known increased risk for ovarian cancer.[13] Four studies[9-12] evaluated the use of RRSO in women who were at high-risk for ovarian cancer. Across these studies, RRSO decreased the risk for ovarian cancer by more than 90% and for breast cancer by approximately 50%, with a mean follow-up time of 5 years. While surveillance of high-risk women was evaluated, surgery was considered superior for risk reduction.[11,12] Patients tended to report higher satisfaction [13,21] with surgery and little anxiety or regret.Longer-term follow-up is still needed. Microscopic occult disease has been found in 10% to 15% of women with genetic predisposition for ovarian cancer, and even with negative pathology, these women are still at increased risk for primary peritoneal carcinoma.[13]

Epidemiology of Epithelial Ovarian Cancer

Much of the research on ovarian cancer has focused on the epidemiology of the disease because of its high mortality rate, the lack of experimental models (ovarian cancer is rare in other ovulatory animals), the small numbers of patients impacted by the disease, and the emphasis on hereditary cases. [1,9,13] According to the National Cancer Institute, a woman without a family history of ovarian cancer has a 1 in 55 lifetime chance of developing ovarian cancer.[1] This risk increases 10-fold when known familial/hereditary conditions exist.[1,13,21] Sporadic epithelial ovarian cancer is defined as any ovarian cancer that arises in a woman with no known family history of breast, ovarian, prostate, or colon cancer[13] and is a distinct disease from sex cord-stromal ovarian tumors and germ cell tumors, which are not discussed in this review. Hereditary

ovarian cancer (HOC) may be associated with different genetic defects: (1) hereditary breast cancer and HOC (HBOC/ HOC) is attributable to a germ-line mutation in BRCA1 or BRCA2 and (2) hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome is due to a germ-line mutation in one of several genes associated with DNA mismatch repair (MMR).[22,23]

Risk Factors

A number of epidemiological studies have evaluated a variety of risk factors for ovarian cancer. To date, these risk factors include age, [1,16,20] chronic inflammation, nonsteroidal anti-inflammatory drug (NSAID) use, [24-28] diet, [29-39], ethnicity [9,18,19], hormone replacement therapy (HRT) [9,16,17,40,41], hysterectomy, [17,42] infertility drug use, [43-47] obesity, [48-50] OCP use [13,16,17,20], parity (pregnancy), [13,15,16] smoking, [51-59] and talc use/asbestos exposure. [60-64] The following sections summarize published data on these risk factors for ovarian cancer.

Age

Age as a risk factor for ovarian cancer needs to be placed within the context of other events in a woman's life. Overall, age is considered a risk factor because ovarian cancer is a disease of older women. The annual incidence of ovarian cancer worldwide, regardless of age, is 42 cases per 100,000. [65] The annual incidence of ovarian cancer in women between the ages of 75 and 79 years in the United States is 61.3 per 100,000, higher than any other age group, when age is considered independent of all other risk

factors.[1] When all other risk factors are considered, incidence is highest in women between the ages of 60 and 64 years.[65]

Age at menarche is considered a weak predictor of ovarian cancer risk. A moderate increase in risk for ovarian cancer occurs in women when menarche begins earlier than age 12 years.[1,15] No association was found when menarche begins after age 16 years.[16,20] Age at natural menopause has been studied. Positive associations were found with late age of natural menopause and risk for ovarian cancer. [15-17] Odds ratios for late natural menopause were reported as low as 1.19 and as high as 1.25 (95% CI, 0.95-1.49).[1,16] The ratios did not achieve statistical significance, but late natural menopause was associated with increased risk for ovarian cancer.

Recently, maternal age at last birth was implicated in decreasing the risk for ovarian cancer if the last birth was at age 35 years or older.[16] This finding is important because it compared nulliparous (never pregnant) women with women who had late first-time live births. These findings suggest that regardless of age at first birth, pregnancy still confers a significant risk reduction for the development of ovarian cancer. The protective effects of pregnancy may be associated with the decrease in ovulation associated with higher levels of progestins.

Chronic Inflammation and Nonsteroidal Anti-inflammatory Drugs

Chronic inflammation caused by talc and/or asbestos is a known risk factor for ovarian cancer.[24] In addition, patients with ovarian cancer have a higher incidence of endometriosis and pelvic inflammatory disease.[13,20] These inflammatory processes may contribute to the development of ovarian cancer through a variety of mechanisms. Much

of the available data on inflammation and cancer come from studies that evaluated the effect of NSAIDs on cancer risk.[24-28]

Nonsteroidal anti-inflammatory drugs have strong anti-carcinogenic effects, and experimental evidence suggests that NSAIDs can impact the tumorigenic pathways in cancers of the small and large bowel.[26] Four studies examined the relationship between aspirin use and ovarian cancer.[24-27] In three (Refs. 25, 26, and 28) of the 4 studies, as aspirin use increased, ovarian cancer risk decreased. Mechanistically, NSAIDs have many physiological effects. Three of these may be pertinent to the development of ovarian cancer. First, NSAIDs interrupt the synthesis of prostaglandins, which decreases inflammation. Second, NSAIDs cause apoptosis (i.e., cell death) of human epithelial cancer cells. Finally, NSAID use may reduce the local inflammatory processes associated with endometriosis and pelvic inflammatory disease.[20,28]

Data on the protective effects of NSAID use in relationship to ovarian cancer in premenopausal women are confounded by the high frequency of use of these medications in women of this age. It is difficult to determine the exact mechanisms by which NSAIDs decrease ovarian cancer risk, because NSAID use has never been measured prospectively in patients who later developed the cancer. All of the retrospective studies [25-27] had small samples and did not quantify the exact dose of NSAIDs used. Future research needs to clarify the role of inflammation in the development of ovarian cancer before routine use of NSAIDs as preventative agents can be recommended.

Diet

The protective effects of fruits and vegetables have been investigated in other cancers and are of interest to ovarian cancer researchers. The role of milk and other dairy products, meat consumption, fat consumption, carbohydrate intake, and alcohol use have been investigated. However, only a few studies have evaluated the impact of dietary factors on ovarian cancer risk.[29-39]

One of the first studies to examine the association between dietary factors and the risk for ovarian cancer was conducted in northern Italy.[29] This study described the influence of red meat, alcohol, dietary fat, vegetable oil, and butter consumption on ovarian cancer risk. This case-control study compared 455 patients with ovarian cancer with 1,385 age-matched controls. No relationship was found between alcohol use and ovarian cancer risk. Dietary factors that increased the relative risk (RR) for ovarian cancer included meat consumption of more than 7 portions versus less than 4 portions per week (RR, 1.6; 95% CI, 1.21-2.12) and butter versus fat consumption (RR, 1.9; 95% CI, 1.20-3.11).[29] However, disparate measures of body weight, socioeconomic status, parity, and contraceptive use confounded these analyses. Dietary factors that decreased ovarian cancer risk included consumption of whole-grain bread and pasta (RR, 0.60; 95% CI, 0.41-0.88).

A larger cohort study[30] in the United States that enrolled 29,083 postmenopausal women found that ovarian cancer risk was not associated with dietary fat intake, but did find that eggs increased the RR when consumed 2 to 4 times per week (RR, 2.04; 95% CI, 1.23-3.36).[30] This study found that green leafy vegetables were strongly associated with decreased risk for ovarian cancer (RR, 0.44; 95% CI, 0.25-0.79). No consistent

association was found with meats, breads, cereals, and starches.[30] However, this study did find statistically significant associations between increased risk for ovarian cancer and intake of sweets and dairy.

Whereas 6 population-based studies[30,36-39,43,54] have examined the relationship between alcohol intake and ovarian cancer risk, only one found a statistically significant association.[30] However, this study showed that an intake of 10 g/d of alcohol decreased the risk for ovarian cancer by 50%.[30] At the present time, no definitive conclusions can be drawn about the association between alcohol consumption and ovarian cancer risk.

Ethnicity

Only a few studies have reported differences in the incidence of ovarian cancer among ethnic groups, primarily because of their small sample sizes. However, data from 2 meta-analyses[18,19] suggest that the rates of epithelial ovarian cancer are higher in white women (OR, 0.70; 95% CI, 0.52-0.94)[18] compared with black women (OR, 0.62; 95% CI, 0.24-1.6).[19] An examination of potential ethnic differences in ovarian cancer rates and other factors, such as breast-feeding, OCP use, and parity, found that ethnicity explained only a small proportion of why the ovarian cancer rates were higher in white women.[19]

Ovarian cancer seems to be the only gynecologic malignancy in which race does not impact overall survival, as mortality is high across all racial groups. Ethnicity does play a role in incidence, as women of Ashkenazi Jewish descent are at greater risk for

BRCA1/2 mutations, which gives them an overrepresentation in the numbers of HOC cases.[19]

Hormone Replacement Therapy

Several studies[40-42] have tried to determine whether the use of postmenopausal HRT is associated with increased risk for developing ovarian cancer. However, these studies have yielded inconclusive results. Several variables need to be considered when reviewing the literature about HRT including duration of use, type of hormones, circumstances of use (e.g., surgical menopause vs natural menopause), reproductive history, and previous history of cancer in any organ.

Hormone replacement therapy is defined as any hormone orally ingested in combination (estrogen-E4 plus progesterone P2) or E4. Other routes of administration (i.e., creams, injections, patches) are not included in this definition, as these methods of HRT delivery have not been included in research studies that have attempted to understand HRT use and cancer risk.

It was originally thought that postmenopausal women would benefit from the supplemental use of exogenous estrogen and progesterone. Until the Women's Health Initiative,[40] it was believed that the protective effects to the cardiovascular system and the prevention of bone loss outweighed the risks of HRT. However, several meta-analyses have questioned data originally used to support postmenopausal HRT use and risk for ovarian cancer.[40,41]

Hysterectomy

As previously discussed in the prophylactic oophorectomy section, hysterectomy confers a decrease in ovarian cancer risk.[23,40,42] In a large case-control study,[41] risk for \ovarian cancer was decreased by 36% (RR, 0.64; 95% CI, 0.48-0.85), and tubal ligation decreased risk by 39% (RR, 0.61; 95% CI, 0.46-0.85). In high-risk women, hysterectomy has also been studied, which decreases ovarian cancer risk by 50%.[13,22,42]

Infertility Drug Use

Clomiphene was approved in 1967 to treat primary infertility.[60] Several epidemiological studies have questioned if ovarian hyperstimulation mimics ‘‘incessant ovulation’’ and therefore increases the risk for ovarian cancer.[43-47,60] The use and doses of clomiphene and human gonadotropins (e.g., menotropins [Pergonal and Humegon] and urofollitropin [Metrodin]) were not reported consistently in infertility research studies. This inconsistency in reporting makes meta-analytic procedures difficult to use to estimate the true relationship between infertility drug use and ovarian cancer. Initial studies estimated that the risk for epithelial ovarian cancer associated with the use of infertility drugs was as high as 27-fold for nulligravida women (95% CI, 2.3-315.6).[45] A subsequent, larger, and more rigorous study found that the use of clomiphene resulted in a 2.3 increased risk for ovarian cancer in nulligravida women (95% CI, 0.5-11.4).[47] Three studies[45-47] did not find an increased risk for ovarian cancer when clomiphene and gonadotropins were evaluated. These studies were rigorously designed, included an analysis of all hormonal stimulants, and followed up patients through in vitro fertilization for up to 15 years. However, a large Danish study that enrolled 684 cases and 1,721

controls found an increased incidence of cancer in women with a history of infertility.[44] The overall OR in all women in the study with known fertility status for ovarian cancer was 1.54 (95% CI, 1.22-1.95). The unadjusted OR for ovarian cancer in infertile nulliparous women who were not treated for infertility was 3.13 (95% CI, 1.60-6.08). This 3-fold increase was observed even after adjustment for infertility treatment, drug type, and pregnancy outcome (i.e., miscarriage, induced abortion, ectopic pregnancy), where the adjusted OR was 2.71 (95% CI, 1.33-5.52).

A large, retrospective, cohort study[47] was conducted to determine if a true association existed between fertility drug use and increased risk for ovarian cancer. This study enrolled 12,193 women, and data included a survey of death records, registry data, historical medical records, interviews with patients, follow-up of in vitro fertilization clinic data, and mailed surveys. The standardized incidence ratio (IR) was 1.98 (95% CI, 1.4-2.6), where women exposed to clomiphene (i.e., IR, 0.82; 95% CI, 0.4-1.5) or gonadotropins (i.e., IR, 1.09; 95% CI, 0.4-2.8) had a decreased risk for ovarian cancer.

Ovarian hyperstimulating drugs have been used in the United States for only 37 years. Therefore, many women who have used these drugs have not reached the median age of highest-incidence ovarian cancer. Future studies need to follow up these women throughout their menopausal experience to determine the true risk for ovarian cancer.

Obesity

Obesity is a risk factor for ovarian cancer because of its relationship to sex steroid hormones. Obesity is known to increase adrenal secretion of androgens, enhance conversion of gonadal and adrenal androgens to biologically active estrogens, and

reduce sex hormone-binding globulin capacity, which increases the amount of free, biologically active estradiol.[51] Adipose tissue is the primary source of endogenous estrogens in postmenopausal women.[50,51]

Several studies have evaluated the impact of obesity on ovarian cancer risk using body mass index (BMI) as the measure of obesity.[48-51] Body mass index is defined as weight in kilograms divided by height in meters squared. Several studies have reported a 70% increased risk for ovarian cancer in obese patients (OR, 1.7; 95% CI, 1.1-2.7).[48] However, these estimates were based on cutoffs where BMI of less than 19.8 kg/m^2 was considered ‘‘normal’’ weight and BMI of greater than 24.1 kg/m^2 was considered obese. Current guidelines from the National Heart, Lung, and Blood Institute[52] define a normal BMI to be 18.5 to 24.9 kg/m^2; overweight, 25 to 29.9 kg/m^2; obesity class 1, 30 to 34.9kg/m^2; obesity class 2, 35 to 39.9 kg/m^2; and extreme obesity as greater than 40 kg/m^2. Another study using the new guidelines and with a larger sample confirmed the association between obesity and increased risk for ovarian cancer at 70%.[49] In addition, obesity was associated with increased mortality in lesbian women with ovarian cancer.[50]

Oral Contraceptive Use

As previously discussed, OCP use confers significant risk reduction for ovarian cancer. Other routes of hormonal contraception, including the Ortho Evra (Ortho-McNeil-Janssen Pharmaceuticals, Raritan, NJ) patch, Nuva Ring (Organon, Shering-Plough, Kenilworth, NJ), Mirena (Bayer Healthcare Pharmaceuticals, Wayne, NJ) IUD,

and medroxyprogesterone acetate/Depo Provera (Pfizer, NY, NY), have not been studied in terms of risk association in ovarian cancer. Future research needs to determine if other routes of administration of hormonal contraception confer similar protective effects.

Parity

The American College of Obstetrics and Gynecology developed standardized nomenclature to refer to the pregnancy history of women. Gravidity refers to the number of times a woman was pregnant in her lifetime. Parity refers to numbers of births. Parous women have a lower risk for ovarian cancer than nulliparous women (OR, 0.76; 95% CI, 0.63-0.93).[13,16] Women with term pregnancies (OR, 0.87; 95% CI, 0.76-0.91) versus failed pregnancies (OR, 0.93; 95% CI, 0.59-1.48) have lower ORs, with a risk reduction of about 14% for each subsequent pregnancy after the first. Data suggest a 40% decrease in the risk for epithelial ovarian cancer with the first live birth.[13,16] These data support theories that suggest that the hormonal changes associated with pregnancy provide a respite from continuous ovarian exposure to estrogen, a known mitogen.[13]

Smoking

Although cigarette smoking as a causative factor for other gynecologic cancers is well documented,[1,2] the relationship between smoking and ovarian cancer is not as clear. However, metabolites of nicotine, including cotinine and benzo[a]pyrene-DNA (B[a]P-DNA) adducts, have been found in ovarian follicular cells.[53] In addition, polycyclic hydrocarbons such as dimethylbenzanthracene are known to induce ovarian cancer in

rodents.[58] The theoretical mechanisms that may explain how cigarette smoke impacts malignant ovarian transformation include altering steroid metabolism and concentration and impairing ovarian function.[51] Seven studies have examined the relationship between smoking and ovarian cancer risk.[51,54-59] Five of these studies were conducted outside the United States and found a statistically significant relationship between smoking and increased risk for ovarian cancer.[51,53,57-59] However, 2 studies conducted in the United States failed to confirm this finding.[55,57]

Talc Use/Asbestos Exposure

Talcum powder (talc) use was implicated in ovarian cancer risk in the early 1960s when it was found to be biologically similar to asbestos, a known carcinogen.[60] Women exposed to asbestos in their reproductive years have a 2-fold increased risk for ovarian and other cancers of the pelvis in a dose-dependent manner.[63] Several studies [61-64] found a positive association between talc use and increased risk for ovarian cancer. These studies evaluated perineal application; use of talc in sanitary napkins/pads as well as first application at birth, at puberty, or in adulthood; and exposure to asbestos. A meta-analysis of 16 studies that included 11,933 patients examined the effect of talc use and increased risk for ovarian cancer.[61] The use of talc conferred a 33% increased risk for ovarian cancer (RR, 1.33; 95% CI, 1.16-1.45). Two mechanisms are responsible for the increased risk for ovarian cancer in peritoneal talc users. The first mechanism is that the talc particles become entrapped in the ovarian surface epithelium, causing physiological responses similar to incessant ovulation.[62] The second mechanism pos-

tulates that the presence of talc during ovulation allows it to be absorbed into the pelvic cavity where it is found in inclusion cysts. The foreign body in the inclusion cyst ultimately forms a granuloma and initiates an acute inflammatory response.[63] It is this inflammatory response that is thought to lead to DNA damage, beginning the cascade of events necessary for tumorigenesis.[63]

Genetic Risk Factors for Ovarian Cancer

As mentioned previously, the 2 most common hereditary cancer syndromes associated with ovarian cancer include HOBC/ HOC and HNPCC (Lynch syndrome).[22,23] These patients are considered to be at high risk for ovarian cancer, and the distinction between the hereditary types is based on which genetic mutation is involved in tumor development.

Hereditary Breast and Ovarian Cancer

Women who carry disease-specific alleles for BRCA1 and BRCA2 are at significantly higher risk for epithelial ovarian cancer than women in the general population.[1,2,22,23] The BRCA1 gene is an oncosuppressor gene located on chromosome 17q that was identified in 1994.[13] It contains small deletions or insertions that result in premature stop codons that shorten (truncate) its protein product.[13] Alterations in this gene are found in 75% of families with HBOC. The BRCA1 gene participates in chromatin remodeling processes, interacts with the retinoblastoma (Rb) gene, and is a key member of the histone deacetylase complex. This gene participates in crucial steps within the cell cycle. When mutations occur, cellular growth controls are unchecked, which results in tumorigenesis.

The BRCA2 gene, located on chromosome 13q, is found in 10% to 20% of HBOC and was isolated a year after the BRCA1 gene. [11,13,22-23] The BRCA1 and BRCA2 genes share sequence homology, although, relatively speaking, the BRCA2 gene is associated with a higher risk for breast than ovarian cancer compared with BRCA1. BRCA2 is also associated with male breast cancer, as well as prostate and pancreatic cancer.

Although no standard clinical definition of HBOC exists, several general characteristics are used to identify affected families. These familial characteristics include (1) several cases of breast cancer diagnosed before the age of 50 years, (2) one or more relatives with ovarian cancer, (3) one or more relatives with both breast and ovarian cancer, and (4) the presence of a BRCA1 or BRCA2 germ-line mutation. Ovarian cancers associated with BRCA1/2 are typically high-grade serous carcinomas, but with a relatively favorable clinical course.

HNPCC/Lynch Syndrome

Hereditary nonpolyposis colorectal cancer/Lynch syndrome is an autosomal, dominant syndrome, where the mean age of onset of colorectal cancer is 45 years old.[13] Families that exhibit HNPCC/Lynch syndrome including colorectal cancer have an increased risk for endometrial, ovarian, gastric, pancreatic, and biliary tract cancers. [13,22,23]

Unlike HBOC, HNPCC has a standardized clinical definition, termed the Amsterdam II criteria. These criteria include (1) 3 or more relatives with colorectal or other Lynch-associated cancer, one of whom is a first-degree relative to one of the other two; (2) affected members in at least 2 generations; (3) at least 1 Lynch-associated

diagnosis in the family before age 50 years; and (4) exclusion of a diagnosis of familial adenomatous polyposis.

Hereditary nonpolyposis colorectal cancer/Lynch syndrome is a result of mutations in MMR genes that are found on at least 4 chromosomes (2p, 3p, 7p, 2q). These genes form heterodimers, which recognize and repair DNA mistakes during transcription. Mutations in MMR genes are associated with a 9% to 12% increase in the risk for ovarian cancer. Unlike the BRCA1/2-associated tumors, ovarian tumors that develop from this genetic mutation represent all histopathologic types. [13,22]Autosomal dominant mutations such as HNPCC have a 50% chance of being transferred to offspring of the affected parent.[13] In addition, these mutations are highly penetrant, meaning there is a high probability of developing one of the tumors associated with HNPCC at some point during the offspring's lifetime.

Nursing Implications

Nurses can use the information provided in each section of this article to improve care for cancer patients in at least 3 distinct ways. Nurses can integrate this information in patient teaching and clinical care. During routine physical examinations (where most stages I and II ovarian tumors are detected), nurses can assess family histories and construct thorough family pedigrees. Pedigrees are a graphical representation of a family tree or history using standardized symbols that nurses can use to screen for families with potential hereditary syndromes.[13] Data obtained from the pedigree can be used to refer patients for risk assessment and genetic counseling.

Second, nurses who conduct research with healthy women can include key variables in their studies that can contribute to our understanding of the demographic

and epidemiological factors that impact the risk of healthy women developing ovarian cancer. Nurses collect data from women and their families that may contribute to refining the risk factors for ovarian cancer and possibly identifying high-risk subgroups susceptible to these tumors. In addition, nurses can use the OCSI in research studies to determine its sensitivity and specificity in low-risk, healthy women.

Finally, nurses can disseminate this information to their coworkers, family members, and other women in their lives to increase awareness of the potential risk factors for ovarian cancer.

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Chapter 3.

Introducing the MUC16 gene: Implications for Early Detection in Epithelial Ovarian Cancer.

McLemore, M.R. & Aouizerat, B.E. (2005). Biological *Research for Nursing*, 6, 262-267.

Abstract

Over 24,000 women in the United States are diagnosed with ovarian cancer every year, and half of these women die from their disease. Stage 1 ovarian cancer is curable in 95% of cases; however, due to inadequate screening tools and lack of symptoms in early disease, ovarian cancer is generally at stage 3 or 4 when finally diagnosed. CA125 is a tumor antigen used to monitor the progression and regression of epithelial ovarian cancer. When its levels are elevated postsurgery (hysterectomy/salpingo-oophorectomy with or without peritoneal washings and lymph node biopsy) and postchemotherapy it is suggestive of recurrent disease. Due to its similarly elevated levels in some nonmalignant conditions, however, it is not a specific enough to be used for population screening. The CA125 molecule is considered a very large glycoprotein because of its molecular weight and has 3 domains: the carboxy terminal domain, the extracellular domain, and the amino terminal domain. MUC16 is the gene that encodes the peptide moiety of the CA125 molecule. MUC16 domains provide novel opportunities to develop new assays and refine current tools to improve the sensitivity and specificity of CA125 for population-based screening guidelines.

KEYWORDS: Ovarian cancer, CA125, MUC16, genetics, early detection

Glossary

Crystal structure: A 3-dimensional picture of molecules based on how they crystallize when frozen. This gives scientists the ability to see which atoms reside near other molecules and to understand biochemical relationships of these residues to each other.

Dalton: An alternate name for the unified atomic mass unit (u or amu). The dalton is often used in microbiology and biochemistry to state the masses of large organic molecules; these measurements are typically in kilodaltons (kDa).

Denatured protein: Proteins that have lost their folded shape.

Epitope: The antigenic determinant on a molecule that recognizes an antibody.

Genotype: The DNA sequence that determines the type of protein product created and subsequently expressed.

Glycosylation: The addition of single (oligosaccharide) simple sugars to side chains of amino acids.

Native protein: A protein found in its normal state, in 3D structure and folded.

OC125 and M11: Epitopes that specifically identify the CA125 antibody. There are approximately 85 known epitopes to CA125, many of which also recognize other antibodies.

Phenotype: The measurable and external expression of a genotype.

Phosphorylation: The addition of a terminal phosphate group to a serine, threonine, or tyrosine side chain of a polypeptide.

RRRKKEGEY: The protein sequence known for tyrosine phosphorylation: R=Arginine, K=Lysine, E=Glutamic Acid, G=Glycine, Y=Tyrosine.

Residue: A general name for a single amino acid.

Despite advances in genetics and surgical techniques and new chemotherapeutic agents, ovarian cancer remains the most lethal of gynecologic cancers. Over 24,000 women in the United States are diagnosed with ovarian cancer every year and over half of these women die of their disease (American Cancer Society, 2004a). It is the fifth most lethal cancer in women. Though stage 1 ovarian cancer is curable in 95% of cases (National Cancer Institute, 2004), inadequate screening tools and lack of or vague symptoms in the early stages of disease have meant that ovarian cancer is generally diagnosed late at stage 3 or 4.

Current screening for ovarian cancer includes annual pelvic exams. Surveillance for high-risk populations, however, also includes bimanual annual pelvic exam with transvaginal ultrasound and serial CA125 measurements (American Cancer Society, 2004b). CA125 is a tumor antigen used to monitor the progression and regression of epithelial ovarian cancer. CA125 is considered to be normal when reported at <35 miu/ml. Though it is not specific enough to be used for population-based screening due to its elevation in nonmalignant conditions such as menses, first-trimester pregnancy, pelvic inflammatory disease, tuberculosis, cirrhosis, and other pelvic conditions causing inflammation (Bast, 1981), elevations of CA125 postsurgery and postchemotherapy are suggestive of recurrent disease.

The newly mapped MUC16 gene encodes the protein CA125. Variation in the MUC16 gene may cause changes in the CA125 protein because it is a multivalent molecule (i.e., the antibody used to trap and detect it can bind in many portions of the molecule). .In most circumstances, serum laboratory tests detect one molecule and quantify it, with no genotypic information required. However, given the molecular

complexity of the CA125 tumor antigen, patients with different genotypes of MUC16 may display differences in the amount of the CA125 protein product it encodes. Thus the assumption that clinicians are measuring the same biomarker between patients with different genes is subject to question. This is clinically significant as our reference values of CA125 are not relative to which allele of the gene women carry.

If the primary laboratory tool used to detect and monitor ovarian cancer progression is subject to change due to genetic variation between patients, our understanding of ovarian cancer clinical management may be flawed, and treatment decisions may be based on faulty information. A better understanding of the MUC16 gene may provide additional information about the potential lack of consistency in protein measurement across patient populations. This review aims to clarify CA125 use and interpretation and to present clinicians with information about why the nature of this molecule allows for such variation across populations of women. It also justifies the need for experimental and empirical work to clarify the relationship of the MUC16 genotype to CA125 measurement. This information could lead to new assays, technologies, and reference values of CA125 based on the patient's specific genetic makeup and allow for more informed decision making regarding treatment options.

Domains of MUC16

MUC16, found on the long arm of chromosome 19q13.2, is the gene that encodes the peptide moiety of the CA125 molecule. It is named for its homology with the other members of the family of secreted proteins called mucins, which are heavily glycosylated. A cartoon schematic of the MUC16 gene product can be found in Figure 1. By molecular weight, CA125 is a very large glycoprotein with 3 domains, which are

summarized in Table 1: the first is the carboxy terminus, which includes a cytoplasmic tail and a tyrosine phosphorylation site for proteolytic cleavage (O'Brien, 2001). The transmembrane domain is found in the carboxy terminus. The extracellular portion of the molecule is the second domain and is by far the largest. It contains the 156 amino acid repeats, which house the SEA (S=sea urchin, E=enterokinase, and A=agrin) domains and antibody epitope repeats (Yin & Lloyd, 2001). Depending on the splicing variant there can be 7, 20, or 60 of these repeats, increasing the core molecular weight from 200,000 daltons to almost 4 million daltons (Maeda et al., 2003). The final domain is the amino terminal domain, which consists of heavily linked O and N glycosylation sites.

Carboxy Terminus

The carboxy terminus (COOH) is found within the cytosol of the cell. By definition, the carboxy terminus is the "last" amino acid in a protein and has a free COOH group. This group is generally on the end opposite the N-terminus group, though both of these groups can lie on the same side of a cellular membrane. Once this group is seen in protein sequence, the peptide portion of the molecule ends. Any additional protein sequence found after the free carbonyl group is considered a new peptide. CA125 is a type I transmembrane protein, meaning that its carboxy terminus and amino terminus are on opposite sides of the cell membrane. The carboxy terminus of the MUC16 gene product includes a cytoplasmic tail within the cell and a transmembrane domain, which protrudes through the cellular membrane (O'Brien et al., 2001). The hydrophobic residues found in this area of the molecule (L-Lysine) indicate that this portion of the carboxy terminus can both exist within the membrane and span the membrane.

The 9 amino acids (RRRKKEGEY; see Figure 1) above the carboxy terminus comprise a known site for tyrosine phosphorylation. The next 15 amino acids (ending at aspartic acid, labeled “D”) comprise the final portion of the intracellular domain. The transmembrane domain, part of the carboxy terminus, begins with the amino acid L (lysine), is hydrophobic, and spans the cell membrane.

The Extracellular “Repeat” Domain

The extracellular repeat domain begins at a cleavage site right above the transmembrane domain (indicated in Figure 1 by the scissors). What cleaves this portion of the molecule is not known. However, the cleaved extracellular portion of CA125 is what is detected in most laboratory tests. These repeats, represented in Figure 1 by the circles labeled “1” through “60,” are characteristic of mucins and are each comprised of 156 amino acids. Different variants of MUC16 can have 7, 20, or 60 of these repeats. The lines following each circle represent cysteine loops, stabilizing sites in proteins that signal the end of a repeat and the epitopes that recognize OC125 and M11. These sites on amino acids bind with CA125. This binding traps the molecule and allows for CA125 levels to be calculated. Epitope and antibody recognition of CA125 has been conducted since Bast and colleagues developed monoclonal antibodies to the CA125 tumor antigen in 1981. Both native and denatured CA125 are detectable by all commercial assays. Recently, the crystal structure of the repeats were characterized, revealing the presence of varying types of SEA domains (Maedea et al., 2003). The epitopes to OC125 and M11 were found on the β-sheets of the SEA domains, which means that the detection mechanism of most commercial assays for CA125 is found in the extracellular repeat domains.

SEA domains are so named for the entities in which they were first found: S=sea urchin, E=enterokinase, and A=agrin (Bork & Patthy, 1995). This domain is believed to comprise sites for proteolytic cleavage, implying that detection of the CA125 molecule is dependent upon and can be manipulated by the number of SEA domains encoded for within the MUC16 gene (Maeda et al., 2003). A wireplot of the most common SEA domains of MUC16 based on the crystal structure deposited to the protein data bank appears in Figure 2.

A wireplot is a linear representation of a 3-D structure. The letters represent the individual amino acids in their linear order. The numbers of the amino acids in the linear configuration are also provided. This wireplot represents 132 amino acids. The arrows represent β-sheets. In the 3-D conformation, β-sheets run in a parallel or anti-parallel fashion. The spirals represent α-helices in the 3-D conformation. Deletions or changes within α or β structures can be responsible for disease. Recently deleted sections of proteins were reported to contribute to misfolding and neurodegenerative disorders (Legname et al., 2004). The epitopes to OC125 and M11 are marked β on the wireplot. This information provides researchers with the specific amino acid residues that are needed for CA125 detection.

Unfortunately, SEA-domain analysis within MUC16 has been difficult because, unlike the well-characterized member of the mucin family MUC1 (breast cancer antigen), the SEA domains within MUC16 are not all similar, nor do they have the proteolytic domain sequence typical of most mucins (GSVVV= glycine, serine, valine, valine, valine), with cleavage occurring between the S and V of the sequence. The SEA domains of MUC16 are more similar to each other than to other mucins; future work will

need to distinguish which SEA domains within MUC16 serve as sites of cleavage and where they lie within the molecule. If each SEA domain serves as a site to cut the protein, then theoretically it is possible to have as many phenotypes of disease as there are possible number of SEA domains present in the gene.

The properties of SEA domains include the re-association of cleaved subunits as well as cleavage. Consistent with these properties, CA125 appears to function as a calcium-dependent protease with autoproteolytic activity (Whitehouse & Solomon, 2003), which means that it has the capacity to degrade other proteins. Although the function of CA125 remains unknown, recent studies have shown that CA125 binds to mesothelin, another cell-surface molecule that is expressed in many of the tissues that serve as lining for cavities (Rump et al., 2004). The binding to mesothelin may allow cellular adhesion activity, thus enabling tumors secreting CA125 to evade surrounding tissue and organs (Rump et al., 2004).

The Amino Terminal Domain

Finally, the amino terminal domain, shown as the rectangle in Figure 1, is attached to the repeat region. This domain is heavily glycosylated and has many side chains of simple sugars. The amino domain, also known as the N-terminus, is marked by an NH_2 amino domain and, as mentioned above, is located at the end of the protein furthest from the carboxy terminal domain. This domain is of particular interest in mucin proteins as it is where the heavy glycosylation occurs in the Golgi apparatus during posttranslational modification. Glycosylation of peptides renders them extremely greasy and therefore able to lubricate surfaces and modulate cellular adhesion (Alberts et al., 2002). CA125 in tumor cells is implicated as a mediator of cellular adhesion, acting as a

lubricant as well as a binder of mesothelin, a known cell-surface molecule that is expressed in the mesothelial lining of various body cavities (Rump et al., 2004).

CA125 and Serum Circulation

Release of CA125 from the cell into the bloodstream, where it is detected, is regulated by phosphorylation (Fendrick et al., 1997). Phosphorylation is the transfer of a terminal phosphate group of an ATP molecule to the hydroxyl group on a serine, threonine or tyrosine side chain (Alberts et al., 2002). The addition of the phosphate group generally creates a conformational change, which switches the protein from its inactive state to its active state. CA125 is phosphorylated when it is in the cell and dephosphorylated prior to its release from the cell. The signaling release of CA125 is thought to be modulated upstream within the epidermal growth factor pathway, but this has not been experimentally verified (Fendrick et al., 1997). In the case of CA125, phosphorylation identifies the molecule in its membrane-bound state (i.e., in the cell) and dephosphorylation identifies the molecule for cleavage into the secretory pathway (i.e., outside the cell) (O'Brien et al., 1997).

Conclusion

Interpretation of rising CA125 levels in diagnosed patients continues to be a topic of conversation among clinicians (Tuxen et al., 2000). An understanding the domain structure of MUC16 provides researchers with many possible explanations for the discrepancies in measurement of this tumor marker. Many ovarian-cancer patients make important decisions based on this tumor marker, and clinicians use it to monitor chemotherapeutic response, success of surgery, second-look surgery and initiation of palliative care. If variations within this tumor marker could be identified and related to

genotypic data, it might be possible to change reference values of the tumor marker based on genotype to allow for population-based screening and more precise decision making.

Various proteolytic fragments of CA125 are detected in the commercially available assays, and it is known that CA125 is a multivalent molecule. If it becomes possible to group and categorize patients based on which copy of MUC16 they carry (paying close attention to the number and quality of SEA domains in their extracellular repeat region), it might be possible to adjust the quantitative measurement of the marker to increase its sensitivity and specificity. As our knowledge about this and other genetic and molecular tools continues to expand, it should lead to more appropriate management of disease and have a positive impact on survival and quality of life in ovarian cancer patients.

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Table 2.1 Description of CA125 Gene Domains

Table 1. Description of CA125 Gene Domains

Domain	Included in Domain	Amino Acid Positions (Linear)
Amino terminal domain	N-glycosylation sites[a] O-glycosylation sites[a]	1-1,648
Repeat domain	Epitopes to OC125 and M11 "SEA" domains	1,649-11,438 (9,789 total)
Carboxy terminal domain	Cytoplasmic tail S/T phosphorylation site (serine/threonine/tyrosine) Transmembrane domain	11,439-11,723 (284 total)

a. Linkages of oligosaccharides are named for the residues on which they are bonded. When hydroxyl (-OH) groups are added to the R-group of a serine or threonine, the sugar is considered O-linked. When amino groups ($-NH_2$) are added to the R-group of asparagines, these sugars are considered N-linked.

Figure 2.1 Cartoon schematic of the MUC16 gene product

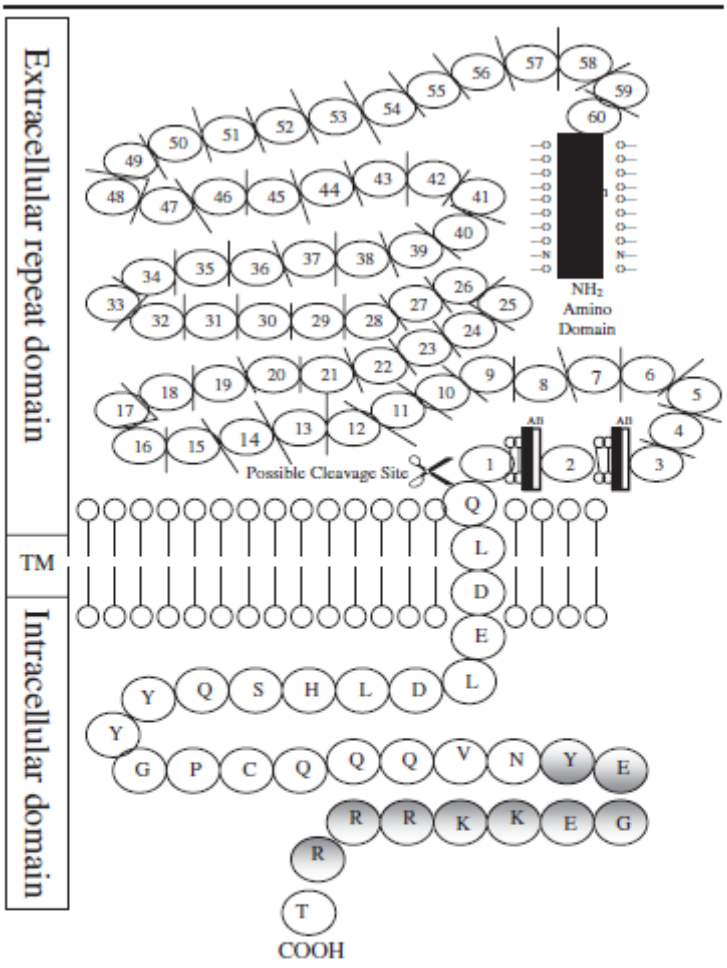

Figure 1. Cartoon schematic of the MUC16 gene product. COOH = carboxy terminus; TM = transmembrane domain; A = alpha subunit; B = beta subunit. Based on a written description from O'Brien et al. (2001).

Figure 2.2 Wireplot of the most common SEA (S = sea urchin, E = enterokinase, and A = agrin) domains of MUC16 based on the crystal structure deposited to the protein data bank (Ho, 2003).

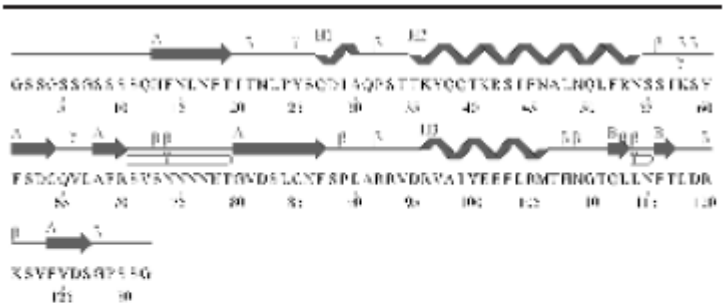

Figure 2. Wireplot of the most common SEA (S = sea urchin, E = enterokinase, and A = agrin) domains of MUC16 based on the crystal structure deposited to the protein data bank.
SOURCE: He et al. (2003).

Chapter 4.

A Comparison of the Cyclic Variation in Serum Levels of CA125 across the Menstrual Cycle Using Two Commercial Assays

McLemore, M.R., Aouizerat, B.E., Chen, L-m., Cooper, B., Tozzi, M., Miaskowski, C. (in press). *Biological Research for Nursing*.

Introduction

CA125, a tumor-associated antigen, is used to monitor epithelial ovarian cancer (Bast et al., 2005; Rubin & Sutton, 2004; Jacobs and Bast, 1989). However, CA125 alone lacks the sensitivity and specificity necessary for population-based screening in healthy women. Currently, the use of CA125 in combination with other modalities is being evaluated as a screening tool for ovarian cancer, as part of longitudinal studies of healthy and high-risk women (NCI, 2009; Johnson et al., 2008; Anderson et al., 2008; Buys et al., 2005; Duffy et al., 2005).

One barrier to the development of a sensitive and specific assay to screen for ovarian cancer is the low incidence of the disease in the population (Crump et al., 2000). In addition, large inter-individual variability in CA125 levels has been attributed to differences in the commercial assays (Davelaar et al., 1998; Martin et al., 1997; Clement et al., 1995; Fisken et al., 1989), fluctuations in levels during the phases of the menstrual cycle (Bon et al., 1995; Kan et al., 1992), ethnicity (Pauler et al., 2001; John et al., 1993), menopausal status (Johnson et al., 2008; Pauler et al., 2001), and other benign conditions (Kafali et al., 2007; Bon et al., 1999; Meden et al., 1998). These factors make it difficult to interpret any one individual's CA125 levels. Another major challenge in the interpretation of CA125 levels is whether variability in the epitopes used in the serum assays themselves are responsible for some of the reported differences in serum CA125 levels across the phases of the menstrual cycle (Davelaar et al., 1998; Martin et al., 1997; Clement et al., 1995; Fisken et al., 1989).

Previous studies conducted to understand fluctuations in CA125 levels in healthy women included convenience samples of women without controlling for factors known

to contribute to CA125 fluctuations (Kafali et al., 2006; Crump et al., 2000; Bon et al., 1999; Pauler et al., 1999; Meden et al., 1998; Kan et al., 1992; Lehtovirta et al., 1990). In this study, strict enrollment criteria were used to control for these factors in a sample of healthy pre-menopausal women. To our knowledge, this study is the first to attempt to control for the known factors associated with CA125 fluctuations.

Additionally, CA125 levels in healthy women fluctuate which is inconsistent with the assumptions that underlie traditional linear regression analysis. Sixteen previous studies that attempted to evaluate CA125 fluctuations using multiple assays, used linear statistical procedures that require normal Gaussian distributions (Kafali et al., 2007; Davelaar et al., 2003; Bon et al., 1999; Yan et al., 1999; Davelaar et al., 1998; Martin et al., 1997; Bonfrer et al., 1997; Bon et al., 1995; Kenemans et al., 1995; Clement et al., 1995; Bonfrer et al., 1994; Van Kamp et al., 1993; Kenemans et al., 1993; Yedema et al., 1992; Lehtovirta et al., 1990; Fisken et al., 1989). This study is the first to use more conservative statistical procedures that account for wide inter-individual differences in CA125 levels.

Given the methodologic limitations in previous studies, the purpose of this study was to determine in a sample of healthy, premenopausal women, if the absolute serum concentrations of CA125 differ across the three phases of the menstrual cycle using two commercially available assays for CA125 determination (i.e., Panomics CA125 enzyme-linked immunosorbent assay (ELISA) assay and Siemens CA125 ELISA assay).

Methods

Sample Selection

Between June 2007 and February 2009, 226 women were approached and 79 enrolled in this study (i.e., 35% response rate). The major reasons for refusal were: scheduling conflicts (n=74), fear and/or refusal of blood draw (n=8), or a desire to become pregnant or begin hormonal birth control (n=65). Twenty women did not complete the study because they did not establish a regular menstrual cycle (n=11); a first-degree relative developed cancer (n=6); or they became pregnant or started hormonal birth control (n=3). Fifty-nine women completed the entire study and one woman completed all but the final blood draw.

The study inclusion criteria were: able to read, write and understand English; Caucasian between the ages of 18 and 39; able to tolerate a venipuncture; and available for 3 consecutive venipunctures. In addition, women had to have a regular menstrual cycle, defined as no less than 25 days, but no more than 35 days, with at least 3 days of bleeding for 3 consecutive months (Fehring et al., 2006; Lee et al., 1990). Participants had to have a body mass index (BMI) between 18.5 and 29.9, which is consistent with the National Heart, Lung, and Blood Institute's guidelines for "normal". Increased risk for ovarian cancer and increased serum levels of CA125 were reported in samples of obese women (Rodriguez et al., 2002; Runnebaum et al., 2001). Women with diagnosed infertility (without hormonal treatment) who met all of the other inclusion criteria were eligible to participate.

Exclusion criteria were selected because these factors could influence CA125 levels (McLemore et al., 2009; Johnson et al., 2008): having a first-degree relative or a personal history of ovarian, endometrial, lung, or colorectal cancer; currently pregnant or post-partum for less than 6 months; lactating; or had an abortion or miscarriage

within the last 3 months. Women with endometriosis, ovarian cysts, polycystic ovarian syndrome, or a history of pelvic inflammatory disease, and any women participating in infertility treatment or ovum donation or who were taking hyperstimulatory medications or gonadotropins were excluded. Women with any chronic illness that required routine non-steroidal anti-inflammatory drug (NSAIDs) use (i.e., defined as daily, around the clock use for greater than 3 days prior to the onset of menses to the end of bleeding) and women who used systemic hormonal contraceptives were excluded. Women taking black cohosh and red clover supplements were excluded.

Study Procedures

Women within a 75-mile radius of the San Francisco Bay Area were recruited using flyers and two internet email lists. Eligible participants provided written informed consent. All procedures were approved by the Institutional Review Boards at the University of California, San Francisco and San Francisco General Hospital (SFGH).

Demographic and menstrual cycle data collection procedures.

Demographic data included age, height, weight, age at first menses, pregnancy and contraceptive history, and current medications. Participants were given a study booklet with 6 calendars and a monthly demographic sheet and were instructed to fill in the booklet for each month they were enrolled in the study.

Menstrual cycle regularity was determined from calendars completed by participants. Participants marked an “X” on any day that they had bleeding and an “S” on any day that they had spotting. Participants maintained these calendars throughout the study and were excused from the study if regularity could not be determined within 6 months of enrollment.

Blood sample procurement and processing. Blood samples for ELISA of CA125, estradiol (E2), and progesterone (P) levels were collected at three different phases of the menstrual cycle: menses (T_1), follicular (T_2; on day 10 of the cycle, +/- 3 days), and luteal (T_3; on day 20 of the cycle, +/-3 days). These samples were transported to a Clinical Laboratory Improvement Amendment (CLIA) approved facility within 12 hours of collection. Whole blood samples collected at the same time points were transported to a research laboratory for additional CA125 analysis. Plasma was isolated and aliquots were placed in -80°C within 48 hours of the blood draw.

Measures

CA125 determination. CA125 levels were determined using two common commercial CA125 assays: Panomics CA125 EIA assay and the Siemens CA125 ELISA assay. These assays were chosen based on their analytic precision and they are two of the most common assays currently in clinical use. Of thirty-five possible assays, these two commercial assays had the smallest published coefficients of variation, (i.e., 3% and 7%, respectively) as well as the smallest intra- and inter-assay variability. The Siemens CA125 ELISA assay (cat. no. 01678114) was completed in the CLIA approved facility. The Panomics CA125 EIA assay (cat. no. BC-1013) was completed in a non-CLIA approved research laboratory.

The Siemens CA125 assay was run in quadruplicate with any outliers removed. Both assays were performed according to the manufacturer's instructions. Samples measured using the Panomics EIA CA125 assay were assayed in triplicate. The reference values were < 35 U/ml and < 21 U/ml for the Seimens and Panomics assays, respectively. For the Seimens assay, the intra-assay coefficient of variation (COV) was

3% to 7%. For the Panomics assay, the intra-assay COV was 11.3% to 15.2% with a mean COV of 13.1%. Inter-assay COV ranged from 12.5% to 14.9% with a mean COV of 13.7%.

Two of the standard dilutions used in the Panomics assay with known CA125 values of 15 U/ml and 50 U/ml were run using the Seimens assay. Mean CA125 values reported using the Seimens assay were 13 U/ml and 52 U/ml. Standard dilutions from the Seimens assay were not made available from the CLIA laboratory to run on the Panomics assay.

E2 determination. Serum E2 levels were determined using the Abbott Architect chemiluminescent immunoassay in the CLIA approved facility. E2 levels were drawn to confirm the participant's phase in the menstrual cycle. Serum E2 levels slowly increase from 100 pg/ml up to 400 pg/ml during the follicular phase and fall to less than 100 pg/ml in the first days of the luteal phase (Speroff et al., 2005).

If E2 levels at T_2 were not between 50 pg/ml and 450 pg/ml, participants were instructed to keep their calendar for an additional month. Similarly, if E2 levels at T_3 were less than 10 pg/ml, participants were instructed to keep their calendar an additional month and repeat the lab work the next month. Two participants needed to maintain the calendar for an additional month due to low E2 at T_2.

P determination. Serum P levels were determined using the Roche Modular E170 electro-chemiluminescent immunoassay in a CLIA approved laboratory. P levels were drawn to confirm the participant's phase of the menstrual cycle. P levels of 0.3 ng/ml to 1 ng/ml occur in the follicular phase, are between 6 ng/ml to 20 ng/ml during the luteal phase, and are less than 1ng/ml during menses (Frasier et al., 1998).

If P levels at T_1 were greater than 1 ng/ml, participants were instructed to keep the calendar for an additional month and repeat the lab work the next month. If P levels at T_2 or T_3 were not between 1 ng/ml and 28 ng/ml, participants were instructed to keep the calendar an additional month and repeat the lab work the next month. Two participants needed to maintain the calendar for an additional month due to elevated P values at T_1. These participants were not the same individuals with abnormal E2 values.

Statistical Analyses

Data were analyzed using SPSS Version 13.0 and Stata Version 9. Descriptive statistics were generated on demographic characteristics and biomarkers. Menstrual cycle length was determined for each participant and phase of cycle was confirmed using E2 and P levels. Random effects negative binomial regression was used to evaluate for changes in CA125 over the course of the menstrual cycle. Paired t-tests were used to compare mean values of CA125 obtained from the two assays at each point in the menstrual cycle. A p-value of <0.05 was considered statistically significant.

Results

Demographic characteristics

As shown in Table 1, the mean age of the sample was 32 years (SD=4.5 years). Only 15 participants (25%) had ever been pregnant. Menstrual cycle length ranged from 25 to 35 days (mean=28 days, SD=1 day) and bleeding during menses ranged from 3 days to 9 days (mean=5 days, SD=1.5 days). Over half of the women (56.7%) reported a medium flow, while 20% described their flow as light, 11.7% described their flow as heavy, and 11.7% stated their flow varied from month to month. On average, 25% of the

women reported spotting (requiring a panty liner) at some time during the month other than menses. Forty-two percent of the women used NSAIDs for menstrual pain.

Changes in E2 and P across the menstrual cycle

E2 and P levels at each phase of the menstrual cycle are summarized in Table 2. Random effects negative binomial regression was used to determine the change in E2 and P across the menstrual cycle. As shown in Table 3 and illustrated in Figure 1, E2 and P levels changed significantly across the menstrual cycle.

Changes in CA125 levels across the menstrual cycle

As shown in Table 2, mean values of CA125 using the Seimens assay ranged from 12.2 U/ml to 15.9 U/ml. CA125 levels using the Panomics assay ranged from 22.7 U/ml to 27.2 U/ml. The percentage of participants above the reference values (i.e., CA125 values of >35 U/ml and >21 U/ml using the Seimens and Panomics assays respectively), was highest (i.e., 19%) at (T_1) using the Panomics assay which represented a third of the sample.

As shown in Table 4 and illustrated in Figure 1 for both assay systems, significant changes in CA125 levels were found across the menstrual cycle. When these data were transformed back to the original CA125 scale (U/ml), for every day of the menstrual cycle from menses to the start of the next cycle, CA125 decreased by 0.2 units using both assays. In other words, CA125 levels decreased 5.8 U/ml from the start of one menstrual cycle to the last day of that cycle.

Differences in CA125 between the two assay systems at each time point in the menstrual cycle

As shown in Figure 2, using paired t-tests, significant differences in CA125 levels

were found between the two assay systems at each phase of the menstrual cycle. All values were plotted as means ± standard deviations (all * are $p<0.0005$). At every time point, CA125 levels were lower (average of 12 U/ml) using the Seimens compared to the Panomics assay.

Discussion

Consistent with previous reports (Kafali et al., 2006; Bon et al., 1999; Meden et al., 1998; Kan et al., 1992; Lehtovirta et al., 1990), serum levels of CA125 in this sample of healthy women fluctuated across the menstrual cycle, with the highest levels found during menses. Changes in E2 and P values over time were also consistent with previous reports (Speroff et al., 2005). The use of negative binomial regression to account for the unexplained between-subject differences in CA125 levels allowed for more precise estimates of the changes in CA125 levels over time.

Prior data on changes in CA125 across the menstrual cycle were derived from studies of peri-menopausal women or women with infertility problems (Kafali et al., 2007; Pauler et al., 2001; Crump et al., 2000; Bon et al., 1999; Bon et al., 1995), women who underwent tubal ligation or hysterectomy for benign disease (Kafali et al., 2006; Bon et al., 1999; Bon et al., 1995), or women at high risk for ovarian cancer (Johnson et al., 2008; Pauler et al., 2001) all conditions that are known contribute to CA125 variability. This study is the first to enroll a homogeneous cohort of healthy community-based women, who met strict inclusion and exclusion criteria for study participation that controlled for these factors.

Mean CA125 values using both assays fluctuated in a narrow range under the reference values of < 35 U/ml and < 21 U/ml. However, the percentage of participants

(19%) with CA125 values above the reference range of < 21 U/ml at T_1 using the Panomics assay is higher than the reported percentages of 5.2% to 13.5% in previous studies (van Kamp et al., 1993; Kenemans et al., 1993). Three possible explanations for these findings exist. First, it is necessary to note that most participants in this study with values above 21 U/ml using the Panomics assay had CA125 values of 40 U/ml or less with only one participant having a value of 99 U/ml which skewed the group mean percentage. Second, the coefficients of variation for the Panomics assay were 5-fold higher than the Seimens assay indicating wider intra-assay variability. Finally, the Seimens assay was run in quadruplicate and mean values for three results were reported from the reference lab. In contrast, the Panomics assay was run in triplicate and mean values for two results were reported when outliers were present.

The amount of change in CA125 over time (i.e., 0.2 U/ml per day) was identical using both assays, which suggests that despite the differences in reference values (< 35 U/ml versus < 21 U/ml) the relative changes in CA125 are consistently measured regardless of assay system. This observation is consistent with the similar CA125 values obtained when the Panomics kit standard dilutions of 15 U/ml and 50 U/ml were run on the Seimens assay where the reported results were 13 U/ml and 52 U/ml. This observation is important because the assays used in this study employ different epitopes (i.e., antigenic regions) recognized by antibodies (Nustad et al., 1998). The Seimens assay uses the OC125 antibody and Panomics uses the M11 antibody. However, without the reciprocal data (i.e., Seimens standards run on the Panomics kit), it is not possible to determine which kit is more accurate in determining serum CA125 levels.

Previous studies have reported varying relative differences in CA125 values using different assay systems with different epitope-antibody recognition sites (Davelaar et al., 2003; Yan et al., 1999; Hornstein et al., 1996; Clement et al., 1995; Bonfrer et al., 1994; Kobayashi et al., 1993; van Kamp et al., 1993; Lehtovirta et al., 1990). All of these previous studies used first-generation kits for CA125 that are no longer in use. Three of these studies (Davelaar et al., 2003; Yan et al.,1999; Kobayashi et al., 1993) reported higher relative differences in CA125, than our data. Five of these studies (Hornstein et al., 1996; Clement et al., 1995; Bonfrer et al., 1994; van Kamp et al., 1993; Lehtovirta et al., 1990) reported relative differences of CA125 using multiple assay systems that were similar to the patterns observed in our data. However, it is important to note that the findings from this study suggest that menstrual cycle and kit variability in CA125 levels persist even when the participants are carefully screened to include only women who were expected to show minimal fluctuations in CA125 levels.

Finally, controversy exists in the CA125 literature about appropriate reference ranges for healthy pre-menopausal women. Several studies have suggested that the upper limit for CA125 be increased to 65 U/ml in healthy premenopausal women particularly if phase of the menstrual cycle is known (Pauler et al., 2001; Crump et al., 2000; Nguyen et al., 1998; Bon et al., 1995). All of these studies have included mixed samples of pre- and post-menopausal women, used a single CA125 assay different than the ones used in our current study which are no longer in clinical use, and none controlled for factors known to contribute to fluctuations in CA125 levels.

The relative differences in CA125 levels observed in three of these studies (Crump et al., 2000; Nguyen et al., 1998; Bon et al., 1995) are similar to our data.

Crump and colleagues reported mean CA125 values of 24.7 U/ml and their median was 16 U/ml with a maximum value was 3600 U/ml (Crump et al., 2000). Nguyen and colleagues reported mean CA125 values of 19.3 U/ml (SD ±15.6 U/ml) in premenopausal women and a statistically significant difference was observed between CA125 values at menses (21.4 U/ml ± 19.3 U/ml) and T_2 where mean values were 14.0 U/ml ± 9.1U/ml. Bon and colleagues reported mean CA125 values of 16 U/ml with a median of 13 U/ml and a maximum value of 113 U/ml. Despite the statistically significant decreases observed in CA125 from menses in all of these studies including ours, we can only conclude that the menstrual cycle does not appear to be a strong modifier of CA125 levels in healthy women, given these fluctuations generally occur within the normal reference ranges for each assay. Findings from this study need to be replicated in larger samples across several menstrual cycles to confirm the 25% decrease in CA125 levels at menses before any recommendations can be made about changes in “normal” reference ranges.

Limitations

Three limitations need to be acknowledged. First, only two commercial assays were run, neither of which are the original assays used in previous studies. This difference does not allow for a direct comparison of relative CA125 results. However, second generation assays using similar antibodies were chosen with comparable COV values and analytical precision. In addition, the assays examined herein are the two most common assays employed in research and clinically for the monitoring of CA125 in ovarian cancer patients. Second, while our sample size had sufficient power to detect changes in CA125 levels over time and differences between the two assays, future

samples should be run in quadruplicate to ensure a similar number of data points and to minimize the impact of outliers. Third, these findings cannot be generalized to more heterogeneous samples because of this study's inclusion and exclusion criteria.

Conclusion

The use of CA125 as a part of a comprehensive screening approach for ovarian cancer would be ideal if the sensitivity and specificity of single and serial measurements were improved. Knowledge of usual patterns of CA125 levels in healthy women is vital to understanding and interpreting these values. Additional research is warranted to determine the appropriate cut-off values for CA125 in healthy women based on multiple assay platforms.

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Table 3.1 Demographic Characteristics of Study Participants (n=60)

Characteristic	Mean ± Standard Deviation	Range
Age (years)	32.0 ± 4.5	21-39
Age at menarche (years)	12.6 ± 2.0	8-19
Height (inches)	64.7 ± 2.6	58.5-70.0
Weight (pounds)	139 ± 17.5	105-179
Body Mass Index (BMI)*	23.2 ± 2.4	17.5-29.2

*BMI calculated as kg/m^2

Table 3.2 Serum values for CA125, Estradiol and Progesterone during the Phases of the Menstrual Cycle

		N	Mean	SD	Minimum	Maximum	% above cut-off
Estradiol (pg/ml)	T1	60	43.4	26.7	10	189	
	T2	60	93.5	69.8	50	430	
	T3	59	90.7	42.3	10	226	
Progesterone (ng/ml)	T1	60	0.85	1.0	0.1	1.0	
	T2	60	1.4	2.4	1.2	12.0	
	T3	59	5.8	5.5	1.2	26.3	
CA125 Seimens (U/ml)	T1	60	15.9	8.2	6	37	2%
	T2	60	12.2	5.4	5	34	0%
	T3	59	12.2	5.7	6	40	3%
CA125 Panomics (U/ml)	T1	60	27.2	13.5	8	99	19%
	T2	60	22.7	12.4	0	86	9%
	T3	59	22.7	12.9	5	67	11%

Abbreviations: ml, milliliters; ng, nanograms; pg, picograms; SD, standard deviation; T1, Menses (Day 1 of bleeding-Day 7); T2, Follicular (Day 1-Day 14); T3, Luteal (Day 15-Day 28); U, Units

Table 3.3 Random Effects Negative Binomial Regression Models for Estradiol, Progesterone and CA125 across the Menstrual Cycle (in days)

		Coef.	SE	Z	95% Confidence Interval		p-value
E2	Days	0.016	0.004	3.85	0.008	0.024	0.0001
	Const.	0.936	0.128	7.34			
P	Days	0.063	0.0129	4.86	0.038	0.088	0.0001
	Const.	-0.292	0.1943	-1.52			

Abbreviations: Coef., Coefficients (log scale); Const., Constant; E2, Estradiol; P, Progesterone; SE, standard error

Table 3.4 Random Effects Negative Binomial Regression Models for Changes in CA125 across the Menstrual Cycle (in days)

		Coef.	Std. Error	Z	95% Confidence Interval		p-value
Siemens	Days	-0.013	0.025	-5.19	-0181	-0.0081	0.0001
	Const.	16.683	561.42	0.03			
Panomics	Days	-0.007	0.003	-2.13	-0.014	-0.0006	0.03
	Const.	2.556	0.200	12.73			

Abbreviations: Coef., Coefficients (log scale); Const., Constant

Figure 3.1 Change over the Phases of the Menstrual Cycle in Serum Levels of Estrogen (picograms/milliliter), Progesterone (nanograms/milliliter), and CA125 (units/milliliter).

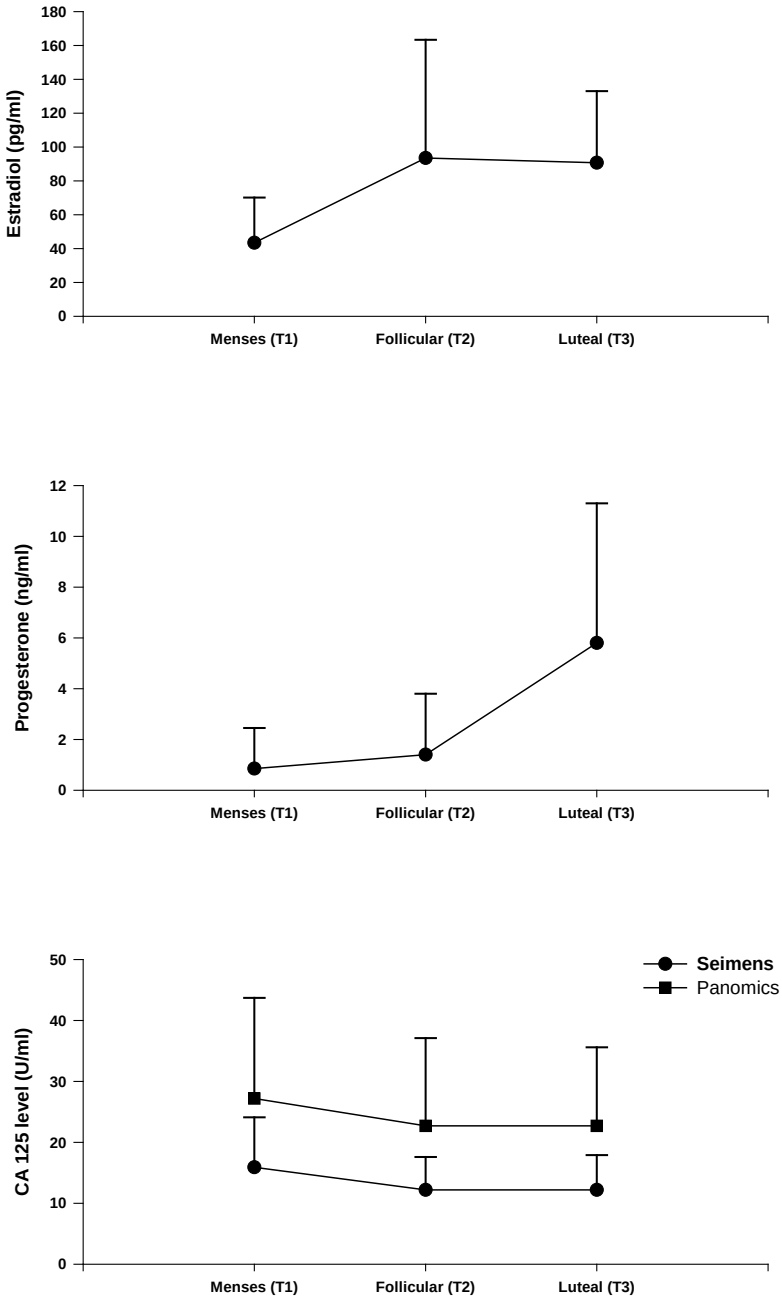
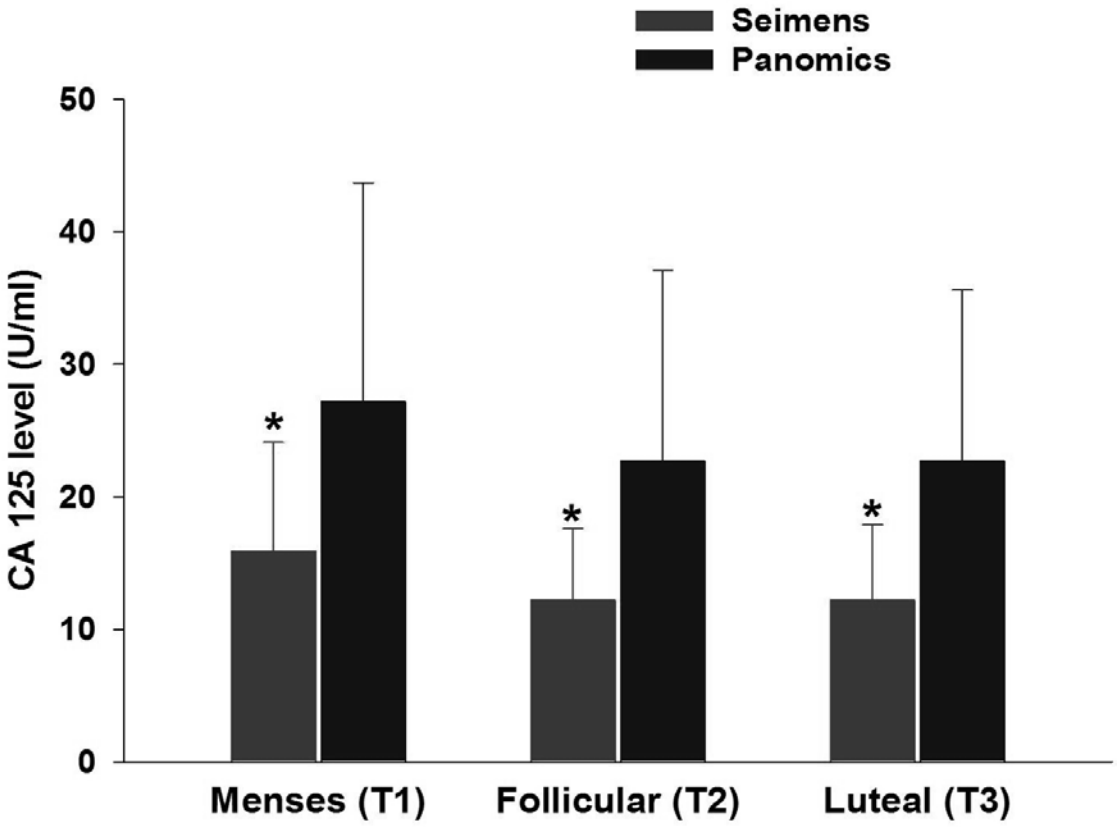


Figure 3.2 Differences in mean CA125 values by assay system at each phase of the menstrual cycle



Chapter 5.

Differences in the molecular species of CA125 across the phases of the menstrual cycle

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Abstract

Background

CA125, a tumor-associated antigen, is primarily used to monitor epithelial ovarian cancer. Despite evidence for different known species of CA125 it is not known if these are present in healthy women during the menstrual cycle and if they are associated with the serum concentrations of CA125. The purpose of this study was to determine if the molecular species of CA125 differs across the three phases of the menstrual cycle in healthy women.

Methods

Healthy, Caucasian women between the ages of 18 and 39 were enrolled using strict criteria to exclude factors known to contribute to CA125 fluctuations. Menstrual cycle regularity was determined using calendars maintained by participants for 3 months. After cycle regularity was established, blood was drawn at 3 time points for Western blot analysis.

Results

Western blot analysis yielded seventeen distinct profiles (patterns of species) of CA125. Demographic characteristics and serum CA125 values are not significantly different for each CA125 pattern or at any time point.

Conclusions

Different molecular species of CA125 exist in healthy women with regular menstrual cycles. These data provide additional evidence that CA125 is not a

homogeneous molecular species. Future research should evaluate the clinical importance and the molecular composition of these species.

Keywords: CA125, molecular species, menstrual cycle

Abbreviations

1. Antibody	Ab
2. Anti-mouse	xMs
3. Dots per inch	DPI
4. Electrochemiluminescent	ECL
5. Electrochemiluminescent sandwich assay	ELISA
6. Methanol	MeOH
7. Micrograms	µg
8. Microliters	µl
9. Milliliters	ml
10. Ovarian carcinoma cell line	OVCAR
11. Polyacrylamide Gel Electrophoresis	PAGE
12. Polyvinylidene Fluoride	PVDF
13. Portable network graphics	png
14. Sodium dodecyl sulphate	SDS
15. Tris-buffered magnesium chloride	TBMC
16. Tris-buffered saline	TBS
17. Tween	Tw
18. Units	U
19. Water	H_20

1.0 Introduction

Molecular speciation is defined as the classification of phenotypically distinct groups of similar entities [1]. The criteria for distinct molecular species includes that each species: requires at least a common precursor [2]; exhibits differences in electrophoretic mobility [2]; shows different responses to the same experimental conditions [3]; or contains a different number of residues or nucleotides [2-3]. These criteria continue to be valuable for current molecular speciation studies [4-11].

Molecular speciation studies of prostate-specific antigen (PSA) have allowed clinicians and researchers to distinguish which species of PSA are associated with prostate cancer, benign conditions of the prostate, or normal metabolic changes associated with aging [12-13]. PSA values were rescaled when the different molecular species of PSA (i.e., PSA, free PSA, and percent free PSA) were identified in the serum of healthy men and survivors of prostate cancer [12-14]. The primary objective in rescaling PSA values was to decrease the number of unnecessary prostate biopsies, diagnostic scans, as well as anxiety in men with elevated PSA levels that were reflective of benign conditions [13]. CA125 is a tumor-associated antigen used to monitor epithelial ovarian cancer (OC) [15-17]. However, CA125 is not a well-defined molecular species with established molecular weights.

CA125 is secreted by all of the tissues of the female reproductive tract as a large glycoprotein, which is degraded by proteolysis during transport throughout the circulatory system [8, 9, 11, 18-21].

Tumor-derived CA125 is degraded in serum, ascites, and in the supernatant of tumor cells lines which results in subunits of varying sizes [11, 17]. The oligosaccharides of tumor-derived and pregnancy-associated CA125 are added to the peptide core of CA125 as post-translational modifications, which results in differences in size and glycosylation patterns [9, 22]. MUC16, the gene that encodes the peptide core of CA125 [23, 24], is alternatively spliced in OC tumor samples, ascites, and supernatant from tumor cell lines [19, 23- 26].

Serum CA125 levels fluctuate during the menstrual cycle in healthy women, with the highest levels observed during menses [27-29]. We hypothesized that these fluctuations in CA125 levels might be due to different species present at each phase of the menstrual cycle. Additionally, it is unknown if structural differences in CA125 species contribute to differences in serum CA125 levels. PSA sensitivity was increased through knowledge of the different molecular species of PSA because the proportion of free PSA to total PSA decreases the risk for prostate cancer (i.e., bound PSA is associated with prostate cancer and free PSA is not [12, 13].

Despite evidence for different known species of CA125 in OC and pregnancy [8-9, 11, 17, 22], it is not known if these species are 1) present in healthy women; 2) present at specific time points in the menstrual cycle; and 3)

associated with the serum concentrations of CA125. Therefore, the purposes of this study was to determine if the molecular species of CA125 differ across the three phases of the menstrual cycle within and across healthy women with regular menstrual cycles and to examine for difference in demographics and menstrual cycle characteristics as well as serum levels of CA125 by CA125 pattern.

2.0 Materials and Methods

2.1 Study Design and Participants

Healthy, Caucasian women between the ages of 18 and 39 were enrolled using strict criteria to exclude factors known to contribute to serum CA125 fluctuations. Eligible participants provided written informed consent. All procedures were approved by the Institutional Review Boards at the University of California, San Francisco and San Francisco General Hospital (SFGH). Menstrual cycle regularity was determined using calendars maintained by participants for 3 months. After cycle regularity was established, blood samples for Western blot analysis of CA125 were collected at three different phases of the menstrual cycle: menses (T_1), follicular (T_2; on day 10 of the cycle, +/- 3 days), and luteal (T_3; on day 20 of the cycle, +/-3 days) and transported to the research laboratory. CA125 ELISA values for all samples were obtained using a common commercially available assay (i.e., Seimens, cat. no. 01678114). Plasma was isolated and aliquots were placed in -80°C storage within 48 hours of the blood draw. Plasma samples from an ovarian cancer patient with a CA125 ELISA

(Seimens assay) value of 2700 U/ml were obtained from the UCSF Cancer Center Ovarian Cancer Tumor Bank and used as a positive control.

2.2 Measurements/Materials

The primary antibody (Ab), X75, was purchased from Novus Biologicals (Littleton, CO, cat. no. NB600-1468) and the secondary Ab, conjugated to horse radish peroxidase for visualization, is a goat-anti mouse antibody purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, cat. no. 115-035-062). Immobilon-P 45 µm Polyvinylidene Fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, cat. no. TM151-1). HiMark pre-stained high molecular weight protein standard (ladder) was purchased from Invitrogen (Carlsbad, CA, cat. no. L5699). Super Signal West Femto electrochemiluminescence (ECL) reagent was purchased from Thermo Scientific (Rockford, IL, cat. no. 34096).

2.3 Gel electrophoresis and immunoblotting

For each participant (N=60) at each of the three time points, 0.5 µl of plasma was mixed with 2 µl of sample buffer and 5 µl of H_20 and heated for 30 minutes at 65°C. After 10 minutes of cooling under non-reducing conditions, sample mixtures were loaded onto the gel in the following order: molecular weight standard, positive control sample, empty lane, participant 1 sample at T_1, empty lane, participant 1 sample at T_2, empty lane, participant 1 sample at T_3, empty lane until the gel was filled.

Twenty-six well, 3% to 8% gradient Tris-Acetate Criterion XT pre-cast gels, XT-Tricine Buffer, and 4X-XT sample buffer were purchased from Bio-Rad (Hercules, CA, cat. no. 345-0131). Gels were subject to electrophoresis at 100 volts until the dye front exited the bottom of the gel (average time was 115 minutes). Gels were removed from cassettes and equilibrated in Tris-Glycine, 20% MeOH transfer buffer for 10 minutes. Proteins were transferred to the PVDF membrane for 1.5 hours at 400 milliAmps at 4°C. After labeling, the blots were immediately immersed in 100% MeOH and placed on clean filter paper to dry for a minimum of 30 minutes.

Blots were rehydrated prior to immunoblotting in 100% MeOH and rocked for 5 minutes in Tris-buffered Magnesium Chloride (TBMC). Blots were blocked using 5% milk (generic powdered, non-fat) in 25 mL tris-buffered saline (TBS) and 0.1% Tween. Blots were incubated with primary antibody (1°Ab) at 4°C overnight. The 1°Ab preparation was X75 at a 1:1850 dilution (i.e., 8.11 µl in 5% milk in 15ml TBS and 0.1% Tween). After incubation, blots were washed quickly in TBS-Tween twice and then 3 times on a rocking apparatus for 5 minutes. Once washed, the blots were then incubated with the secondary Ab for 45 minutes at room temperature. The secondary Ab preparation was Goat xMs IgG at a 1:500,000 dilution in 5% milk in 25 mLs TBS and 0.1% Tween. Blots were rinsed quickly in TBS and 0.1% Tween twice and then 3 times on a rocking apparatus for 15 minutes. Blots were immediately treated with ECL reagents per

the manufacturer's instructions and were subjected to four different film exposures: 30 seconds, 1 minute, 4 minutes, and 10 minutes.

2.4 Image acquisition

The radiographic films of all autoradiogram exposures of the Western blots were scanned using an Epson Perfection 4990 flatbed photo scanner. The resolution for all scans was 300 DPI and all files were imported into Fireworks 4^{TM} software and converted into 24-bit, portable network graphic images (.png). Radiographic films were annotated using the ladder included on each blot, allowing the visible bands to be documented at a kilodalton (kDa) level resolution. All visible bands were included in the annotation, and the darkest band was chosen as the referent band for each film.

2.5 Pattern recognition

Scanned images were compared on screen by referent bands and initial banding patterns were documented using the location of the referent band. An iterative process was used to determine distinct patterns and a template was created (Figure 1). The primary investigator (MM) performed the initial pattern assignment with final adjudication conducted by two investigators (MM and BE). This process was repeated until consensus was reached. Samples that represented the exemplars of each pattern were selected and used as a referrant to confirm pattern assignment for each sample. Final adjudication of the banding patterns was conducted with the research team using the digital and printed images of each pattern.

3.0 Statistical Analyses

Data were analyzed using Stata Version 11. Descriptive statistics were generated on demographic characteristics. Frequencies for each CA125 species were calculated. Analysis of variance (ANOVA) was used to examine differences in age, BMI, menstrual cycle length and pregnancy status by CA125 pattern. Negative binomial regression models were used to examine differences in mean CA125 levels at each time point by CA125 patterns, due to the skewed distribution of serum CA125 values and high dispersion. A p-value of <0.05 was considered statistically significant.

4.0 Results

4.1 Demographic characteristics

As shown in Table 1, the mean age of the sample was 32 years (SD=4.5 years). Only 15 participants (25%) had ever been pregnant. Menstrual cycle length ranged from 25 to 35 days (mean=28 days, SD=1 day) and bleeding during menses ranged from 3 days to 9 days (mean=5 days, SD=1.5 days). The mean values of CA125 using the Seimens assay ranged from 12.2 U/ml to 15.9 U/ml.

4.2 Patterns of CA125 Species

As shown in Figure 1, Western blot analysis yielded seventeen distinct profiles (patterns of species of CA125) in healthy women with regular menstrual cycles. Five profiles occurred in more than one participant. Figure 2 provides the frequencies of the five distinct profiles, where the first three species (i.e., A, B, C)

comprise over 65% of the samples. The 12 samples with unique patterns represent 20% of the overall sample.

4.3 Differences in demographics and serum CA125 values by pattern

Age at enrollment, age at menarche, BMI, menstrual cycle length, and ever pregnant status were not significantly different for each CA125 pattern. Additionally, serum CA125 values determined using the Seimens assay, were not significantly different at any time point for each pattern.

5.0 Discussion

Distinct banding patterns of CA125 were identified using Western blot analysis in healthy women with regular menstrual cycles. This study used CA125 banding patterns (i.e., kDa size) as a proxy for molecular species. Consistent with previous studies of molecular species, our data had a common precursor (i.e., serum CA125 levels were available for all participants), exhibited reproducible differences in electrophoretic mobility (i.e., banding patterns), and showed different responses to the same experimental conditions (i.e., a standard Western blot protocol was used during a 2 year time period using 20 independent runs of the same commercially available antibodies, gels and reagents).

Previously, we hypothesized that genetic variation in MUC16 might contribute to the different species of CA125 [30]. However, the experiments reported in this paper were needed to determine if species of CA125 could be detected before examination for variation. Future work needs to determine if the differences in these banding patterns are directly attributable to different

residues, nucleotides, or amino acid sequences. Recent work from other groups has shown that certain polymorphisms in MUC16 do not contribute significantly to differences in serum CA125 levels [4].

Consistent with previous studies of CA125 that included Western blot analysis of CA125 determinants [5, 9, 11, 23-24, 31], our data show species ranging from 31 kDa to 460 kDa. Most of the referent bands in our data appear at the level of 117 kDa or higher indicating that CA125 may primarily be a high molecular weight species in healthy women with regular menstrual cycles. In this study only two of the sixty participants exhibited visibly detectable changes in pattern across the menstrual cycle. In other words, across the five distinct profiles, while different species of CA125 were found among the 5 distinct profiles, within each profile the species were consistent, across the menstrual cycle. The lack of significant differences in demographic and menstrual cycle characteristics as well as serum levels of CA125 among the CA125 pattern groups needs to be interpreted with caution because of the small number of participants within each pattern group.

It is important to note the major difference between our data and other published studies that investigated the molecular species of CA125: the type of tissue used. This study used samples derived from human participants, including our positive control from an ovarian cancer patient prior to treatment (CA125 value of 2,700 U/ml). Only two prior studies used samples derived from healthy human participants [5, 9]. The first study [5] used cervical mucus collected during

the menstrual cycle, and the second [9] obtained samples from first trimester, human placentas between 6 and 12 weeks gestation. While ELISA values were not reported for the cervical mucus samples, distinct bands were noted between 50 kDa and 300 kDa across all participants and banding patterns changed within each woman across the menstrual cycle [5]. ELISA values were reported for the placental samples which averaged at 6000U/ml and a single broad band was noted at 205 kDa [9]. Our data include bands ranging from 41 kDa to 460 kDa. Despite the use of healthy human samples, each study used a different tissue type, which could explain some of the variation in the observed species.

Ascites, abdominal fluid removed from ovarian cancer patients during tumor reduction surgery, was used as the source material in both O'Brien studies [23-24]. Only the extracellular repeat region of CA125 was blotted by O'Brien and colleagues in 2001 [24]. This analysis, using three antibodies (i.e., OC125, K95, M11) yielded similar bands of varying intensities at 32 kDa. In 1998, all of the isolated CA125 was blotted and bands were observed at 200 kDa, between 60 and 55 kDa, and at 30 kDa. Ascites is not generally present in healthy women and the use of the non-gradient gel could account for the differences in CA125 banding patterns across studies.

Two studies used cell culture medium for CA125 analysis. The first study used media from the WISH cell line [11]. Gel filtration was initially used to separate CA125, which resulted in fractions of 750, 210, 55, and 15 kDa [11]. This analysis showed banding patterns of CA125 above 200 kDa with no banding

occurring under 200 kDa. The second study used cell culture media from the HOC-I cell line [31]. Species with masses ranging from 100 to 400 kDa were found.

6.0 Limitations

Two limitations need to be acknowledged. First, two of the patterns identified in this study (D and E) contain less than 10 participant samples which required more stringent statistical procedures (i.e., bootstrapping of standard errors), therefore decreasing the statistical power to detect potential differences of demographic variables by species. Second, it is unknown what the differences in the species represent and it is necessary to determine if these species have different residues, nucleotides, or amino acids.

7.0 Conclusions

Different molecular species of CA125 exist in healthy women with regular menstrual cycles. These data provide additional evidence that CA125 is not a homogeneous molecular species. Future research needs to evaluate the clinical importance of these species, the molecular composition of these species, and the relationship of these species with the serum ELISA values of CA125.

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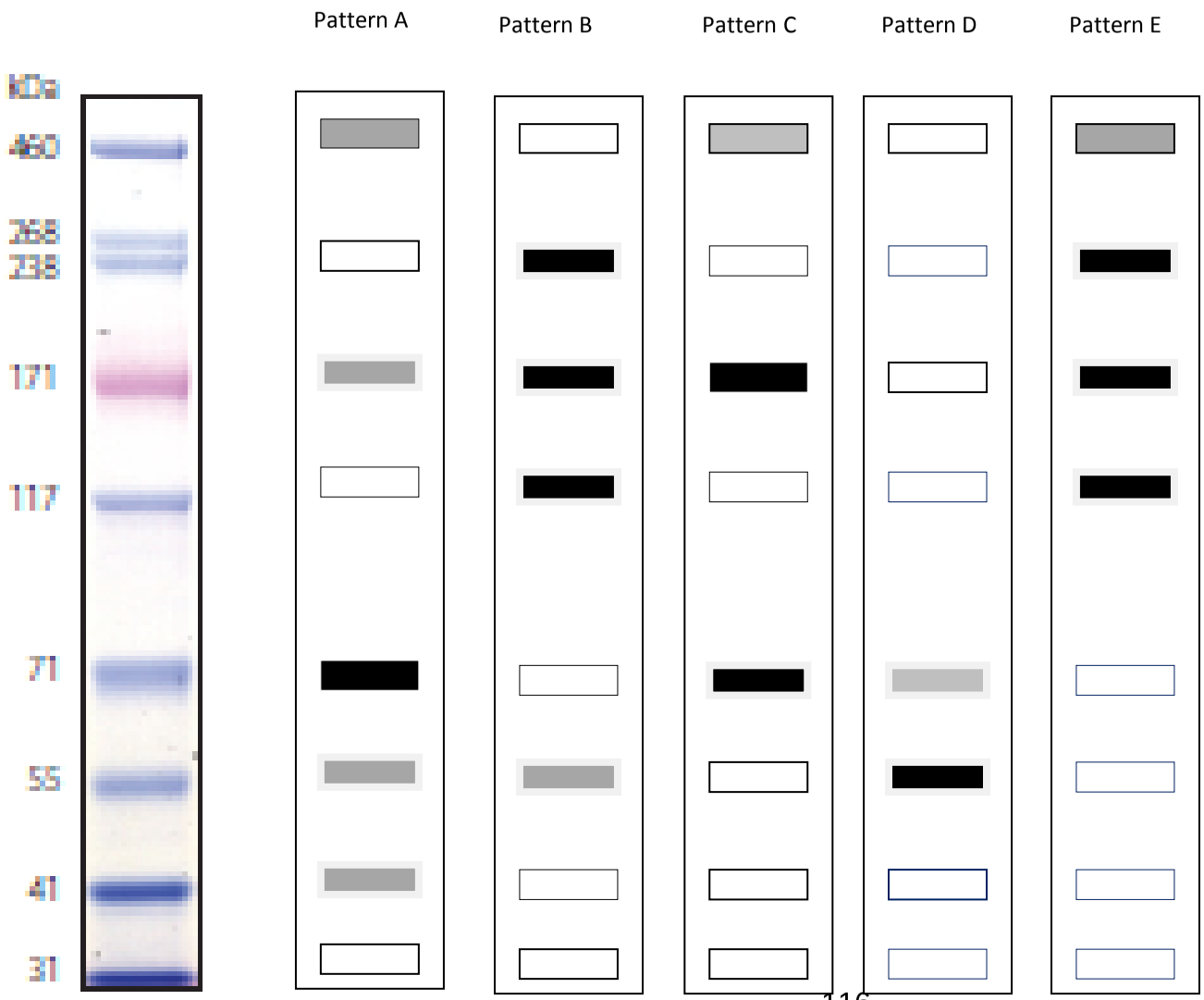
Table 4.1. Demographic characteristics of study participants (n=60)

Characteristic		Mean ± Standard Deviation	Range
Age (years)		32.0 ± 4.5	21-39
Age at menarche (years)		12.6 ± 2.0	8-19
Menstrual cycle bleeding (days)		5.0 ± 1.5	3-9
Menstrual cycle length (days)		28.0 ± 1.0	25-35
Height (inches)		64.7 ± 2.6	58.5-70.0
Weight (pounds)		139.0 ± 17.5	105-179
Body Mass Index (BMI)*		23.2 ± 2.4	17.5-29.2
CA125 (Units/ml)			
Seimens	T_1	15.9 ± 8.2	6-37
	T_2	12.2 ± 5.4	5-34
	T_3	12.2 ± 5.7	6-40

*BMI calculated as kg/m^2

Figure 4.1 Patterns of the species recognized by OC125 Antibody



Patterns (Black = Dark, Grey = Faint)

Pattern A (exemplar: Sample #9)

Faint band at 460 kDa

Faint band at or near 171 kDa

Referent Prominent dark band at or near 71 kDa

Faint, but visible band at 55

Optional: Faint band at 41 kDa

Pattern B (exemplar: Sample #57)

Doublet at 268 kDa and 238 kDa

Dark band at 171 kDa

Referent Doublet or dark band at or near 117 kDa

Faint bands at or under 55 kDa

Pattern C (exemplar: Sample #3)

Faint, but visible band near 460 kDa

One large dark band at or near 171 kDa

Referent Dark band at or near 71 kDa

Pattern D (exemplar: Sample #47)

No distinct bands above 268 kDa

Faint, but visible band at 71 kDa

Referent Dark band at 55 kDa

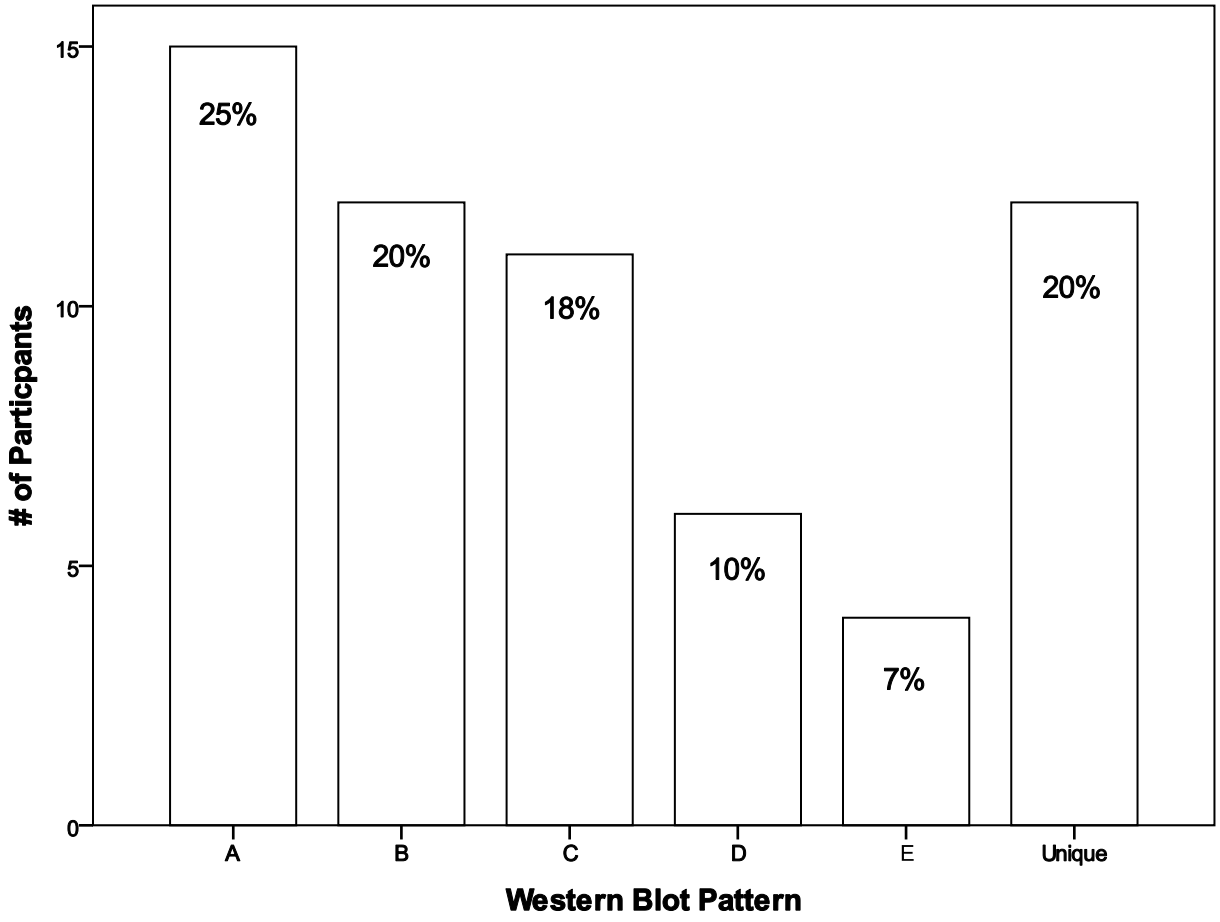
Pattern E (exemplar: Sample #64)

Doublet or triplet between 460 kDa and 171 kDa

Multiple bands between 238-171 kDa

Referent Dark band at 117 kDa

Figure 4.2 Frequency of CA125 patterns



Footnote. All samples in Pattern U had unique patterns, n=12

Conclusions

The studies conducted for this dissertation contribute to our understanding of the behavior of CA125 in healthy, pre-menopausal women. Additionally, there are several clinical and research implications to this work.

Findings and Clinical Implications

First, serum CA125 levels decreased by 0.2 units for every day of the menstrual cycle from menses to the start of the next cycle. Thus, CA125 levels decreased 5.8 U/ml from the start of one menstrual cycle to the last day of that cycle. This change in CA125 over was identical using both assays, which suggests the relative changes in CA125 are consistently measured regardless of assay system. Despite these statistically significant decreases observed in CA125 from menses, we concluded that the menstrual cycle does not appear to be a strong modifier of CA125 levels in healthy women, because these fluctuations generally occur within the normal reference ranges for each assay. However, for ovarian cancer patients who have at least one ovary and continue to have regular menstrual cycles, these data may provide some assistance in interpreting fluctuations in serum CA125 levels. Finally, different molecular species of CA125 exist in healthy women with regular menstrual cycles. These data provide additional evidence that CA125 is not a homogeneous molecular species.

Taken together, the data from these specific aims suggest fluctuations in CA125 levels and patterns of CA125 may be due to other biological mechanisms and that perhaps epidemiologic factors are only a part of the variation that is observed. The clinical implications of these data are: 1) incremental fluctuations in CA125 levels in healthy women may not be an indicator of disease states and a thorough history in

addition to the CA125 level should provide guidance if additional testing is warranted, and 2) the CA125 assays used in this study are able to consistently measure changes in CA125 over time, but given the differences in reference ranges, the same CA125 assay should be used if consecutive values are required.

Directions for future research

Findings from these studies need to be replicated in larger samples across several menstrual cycles to confirm the 25% decrease in CA125 levels at menses before any concrete clinical recommendations can be made. Additionally, the patterns of CA125 found in this study should be validated across several menstrual cycles to determine if these patterns are stable across menstrual cycles.

Future research needs to evaluate the clinical importance of the different molecular species and patterns, the molecular composition of these species, and the relationships among these species with the serum ELISA values of CA125. Given the variability of CA125 in this well-controlled sample, future work should also include genotyping of the samples of these participants to determine if genetic variation is responsible for some of the differences observed.

Analysis of these samples using mass spectroscopy may provide some insights into the molecular composition of these species or the glycosylation patterns associated with CA125 from healthy women (Li et al., 2008; Helleman et al., 2008; Su et al., 2007; Andersch-Bjorkman et al., 2007; Zhang et al., 2006; Kozak et al., 2005). This approach is currently being used to develop a glycoproteomic index to aid in the determination of differences in CA125 among ovarian cancer patients (Li et al., 2008; Leiserowitz et al.,

2008; Helleman et al., 2008; Su et al., 2007; Andersch-Bjorkman et al., 2007; Zhang et al., 2006; Kozak et al., 2005).

Finally, taken together these dissertation data provide evidence to support previous observations that CA125 is not a well-defined molecular species (Jankovic & Milutinovic, 2007; Jankovic & Milutinovic, 2005; Hollingsworth & Swanson 2004; Maeda et al., 2004; Hamilton et al., 2002; Bidart et al., 1999; Nustad et al., 1998 Jacobs et al., 1989). This point needs to be considered when one attempts to generalize CA125 research findings to broad populations. Therefore, we suggest that these data are representative of the healthiest of healthy women and future work should continue to consider controlling for the factors known to contribute to fluctuations in serum CA125 until the relationship between the species and serum CA125 levels can be determined.

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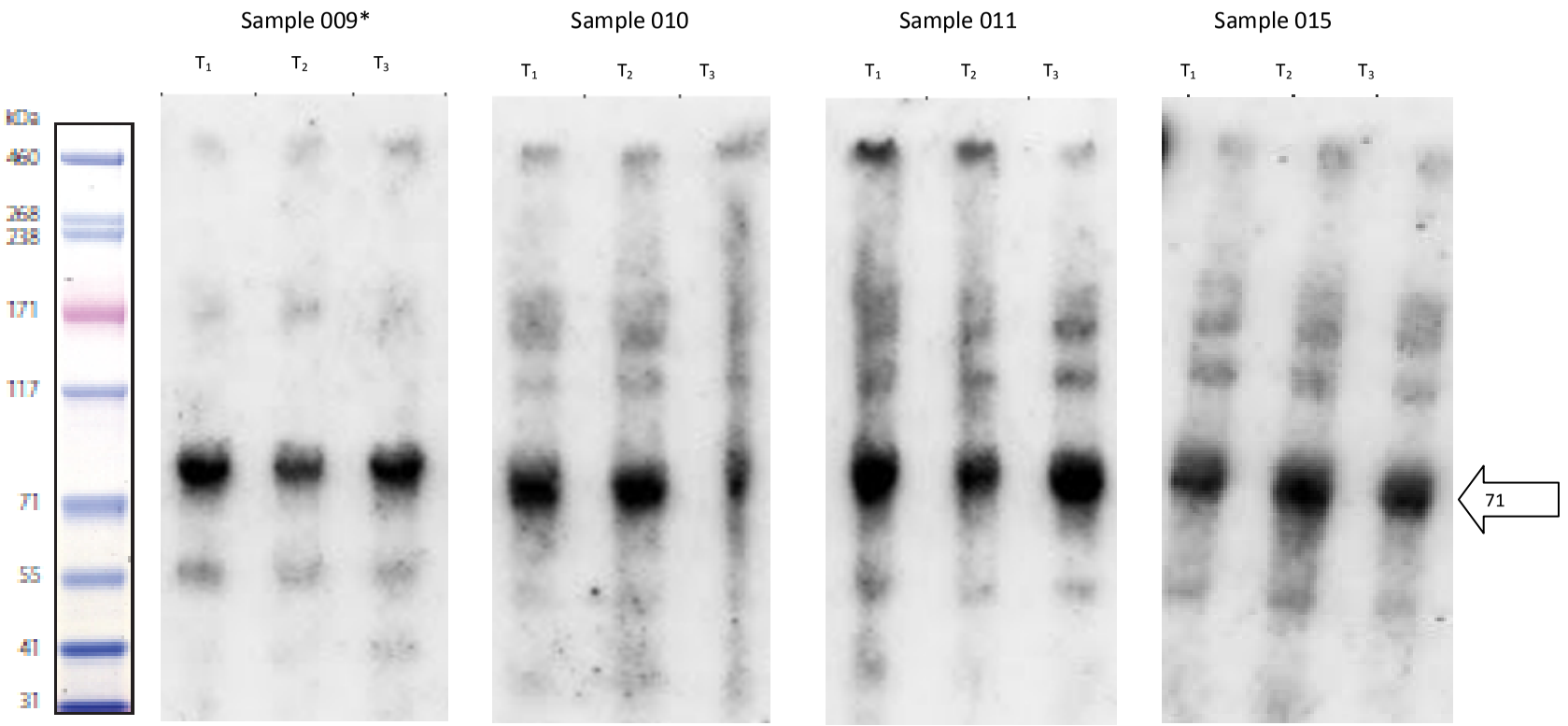
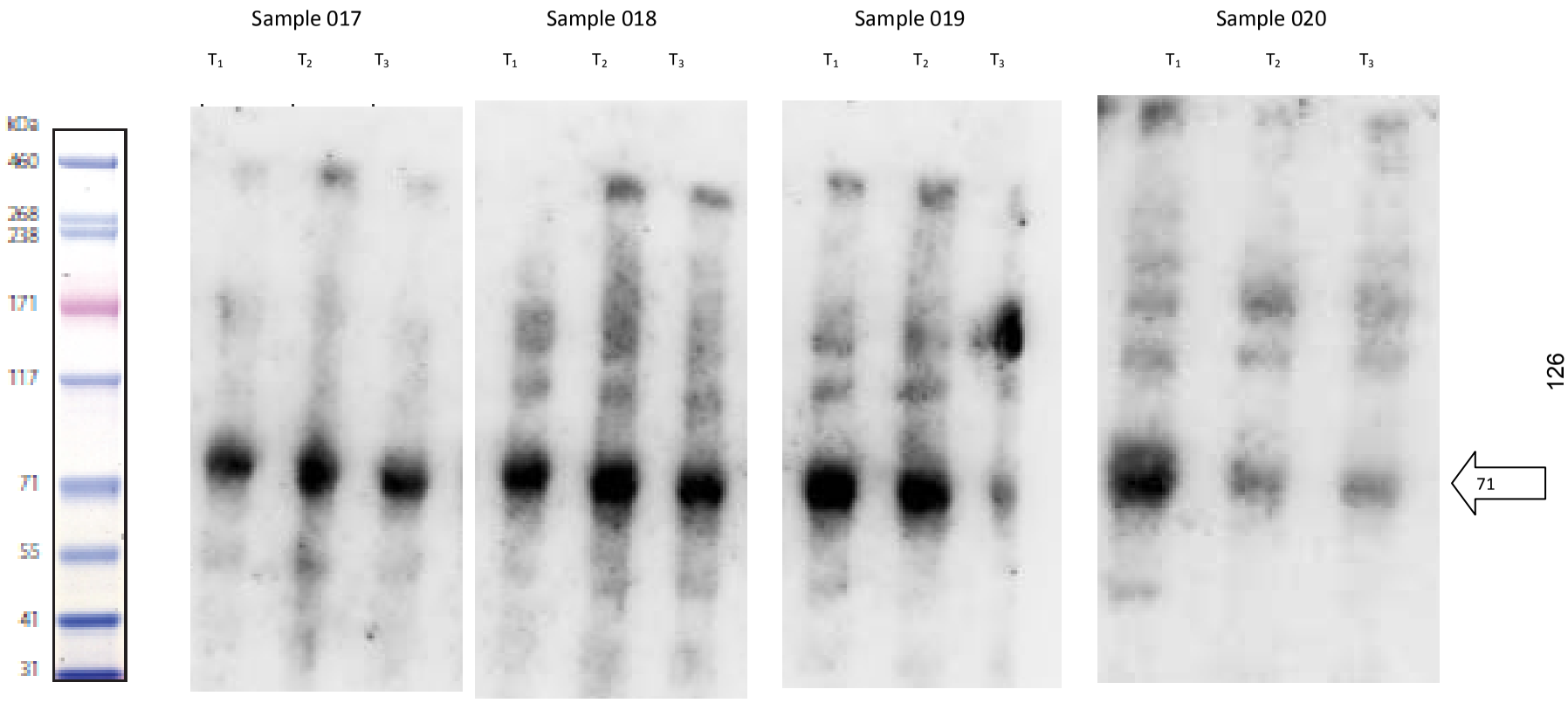
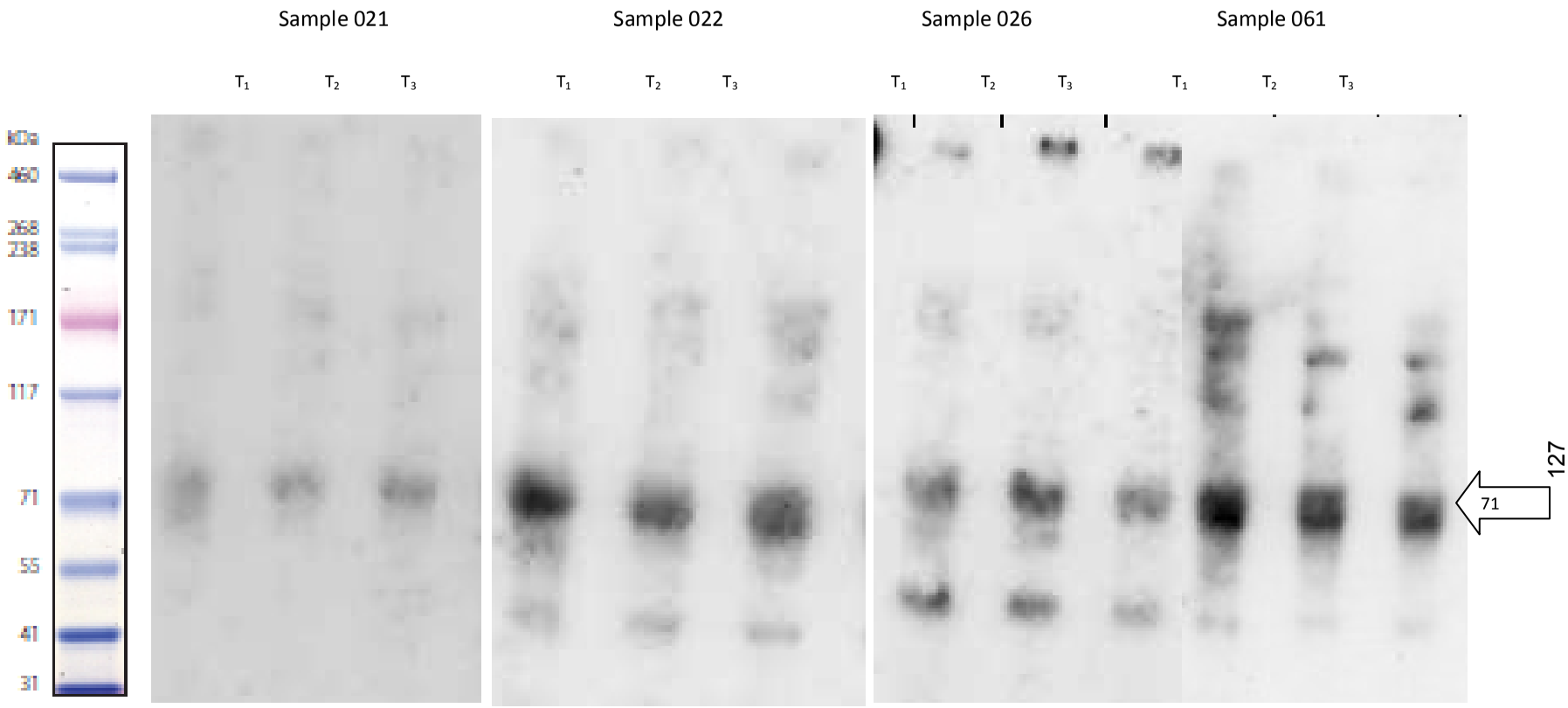
Appendices

Supplemental Figure 1. Pattern A of CA125, n=15



* The first sample panel on the left is the exemplar.


Sample 017
Sample 018
Sample 019
Sample 020
T1
T2
T3
kDa
460
268
238
171
117
71
55
41
31
71


Sample 021
Sample 022
Sample 026
Sample 061
T1
T2
T3
kDa
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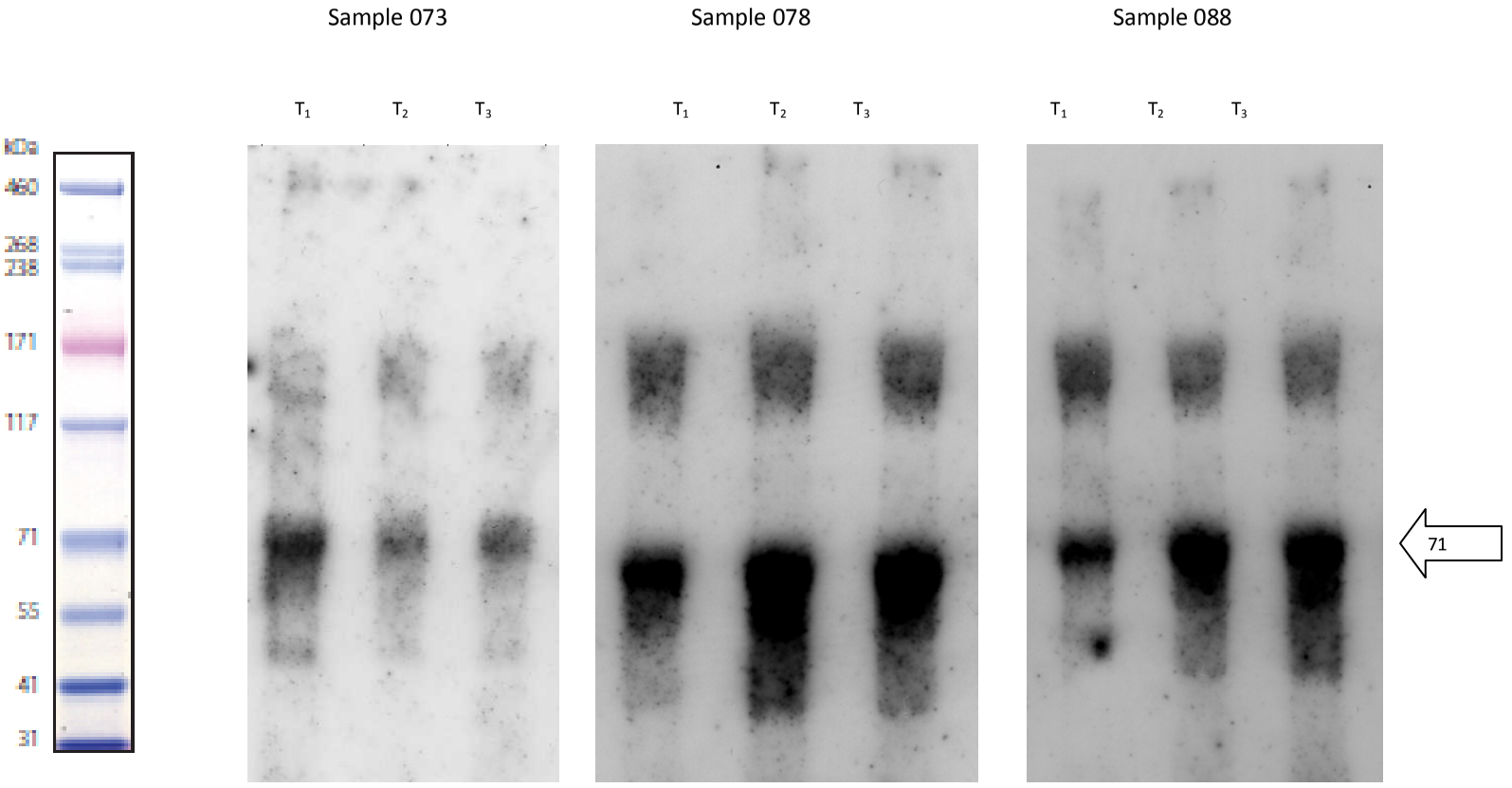
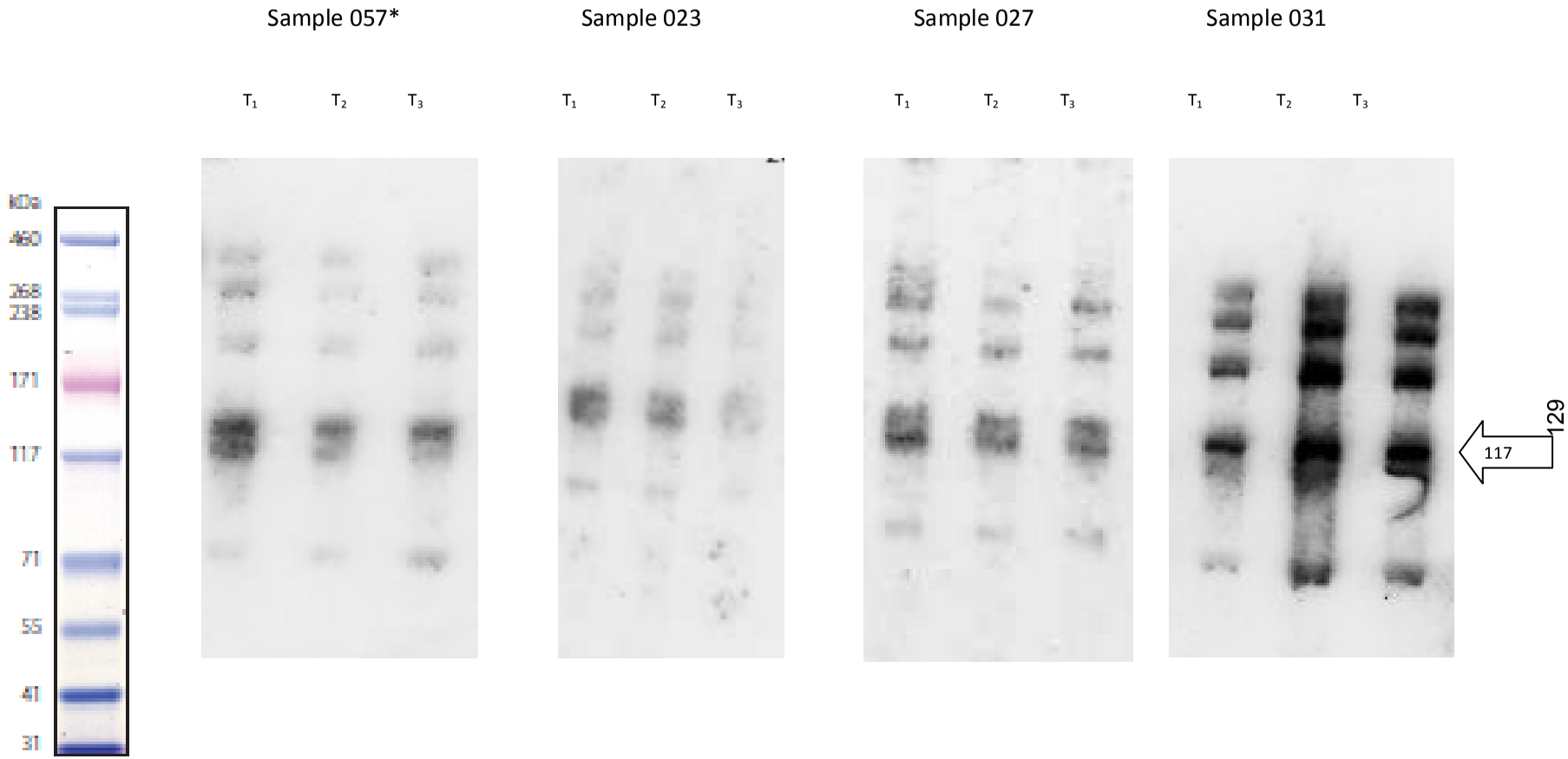
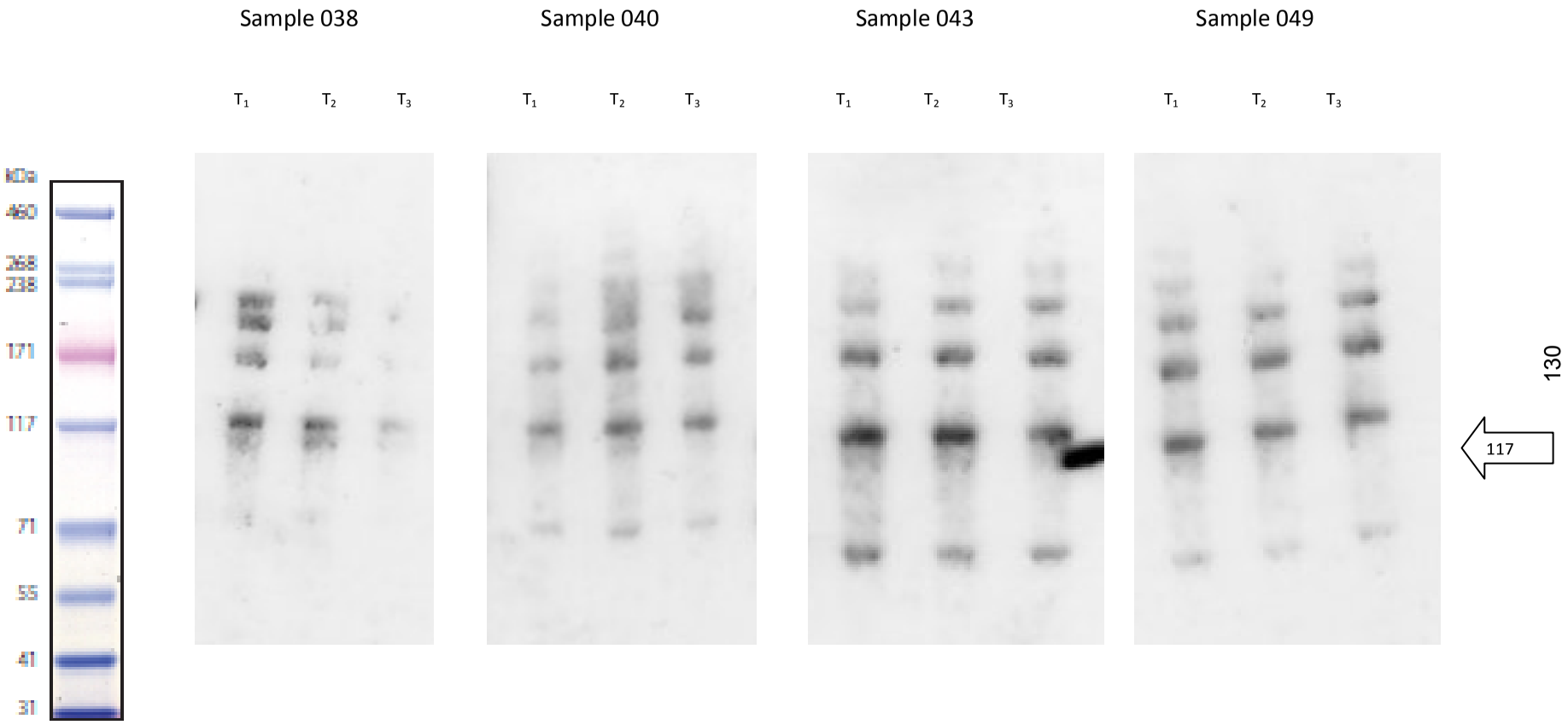
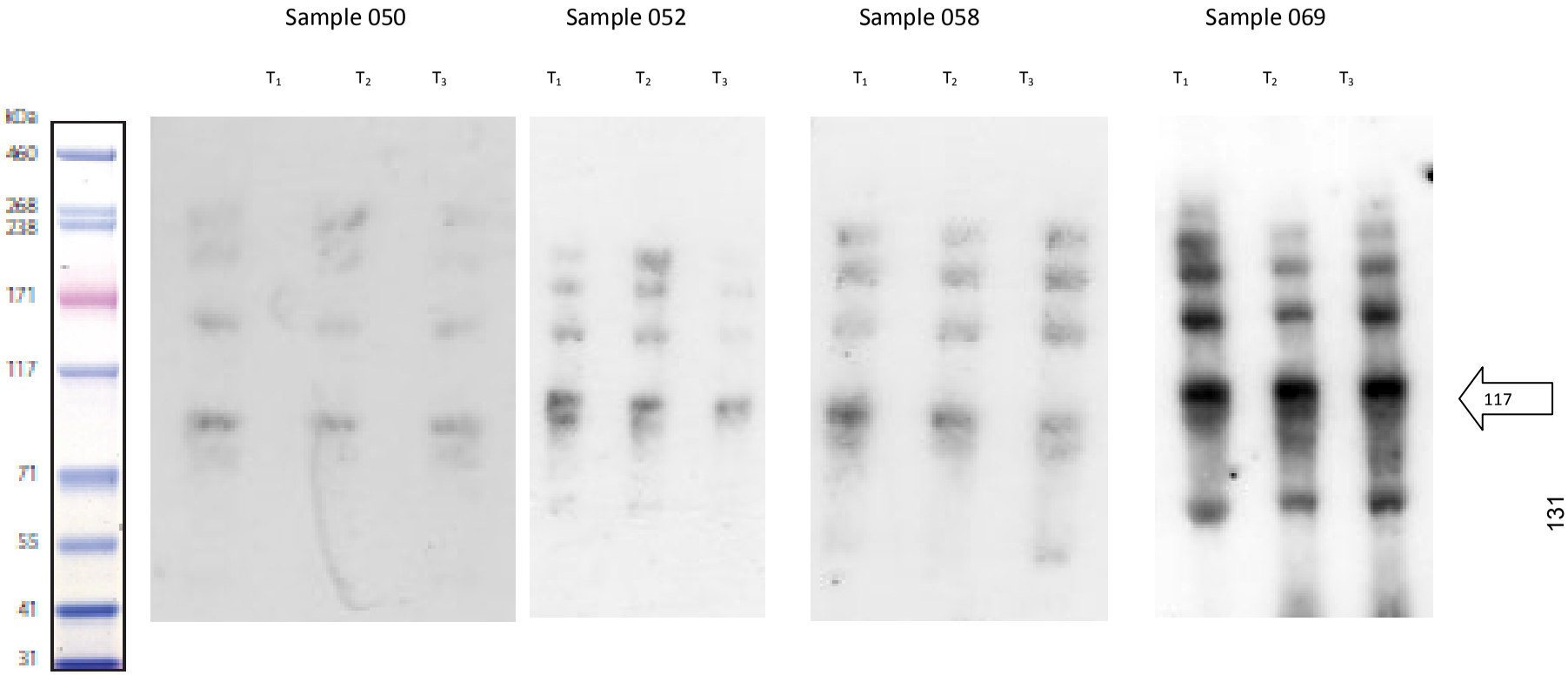
268
238
171
117
71
55
41
31
127
71


Sample 073
Sample 078
Sample 088
T1
T2
T3
kDa
460
268
238
171
117
71
55
41
31
71

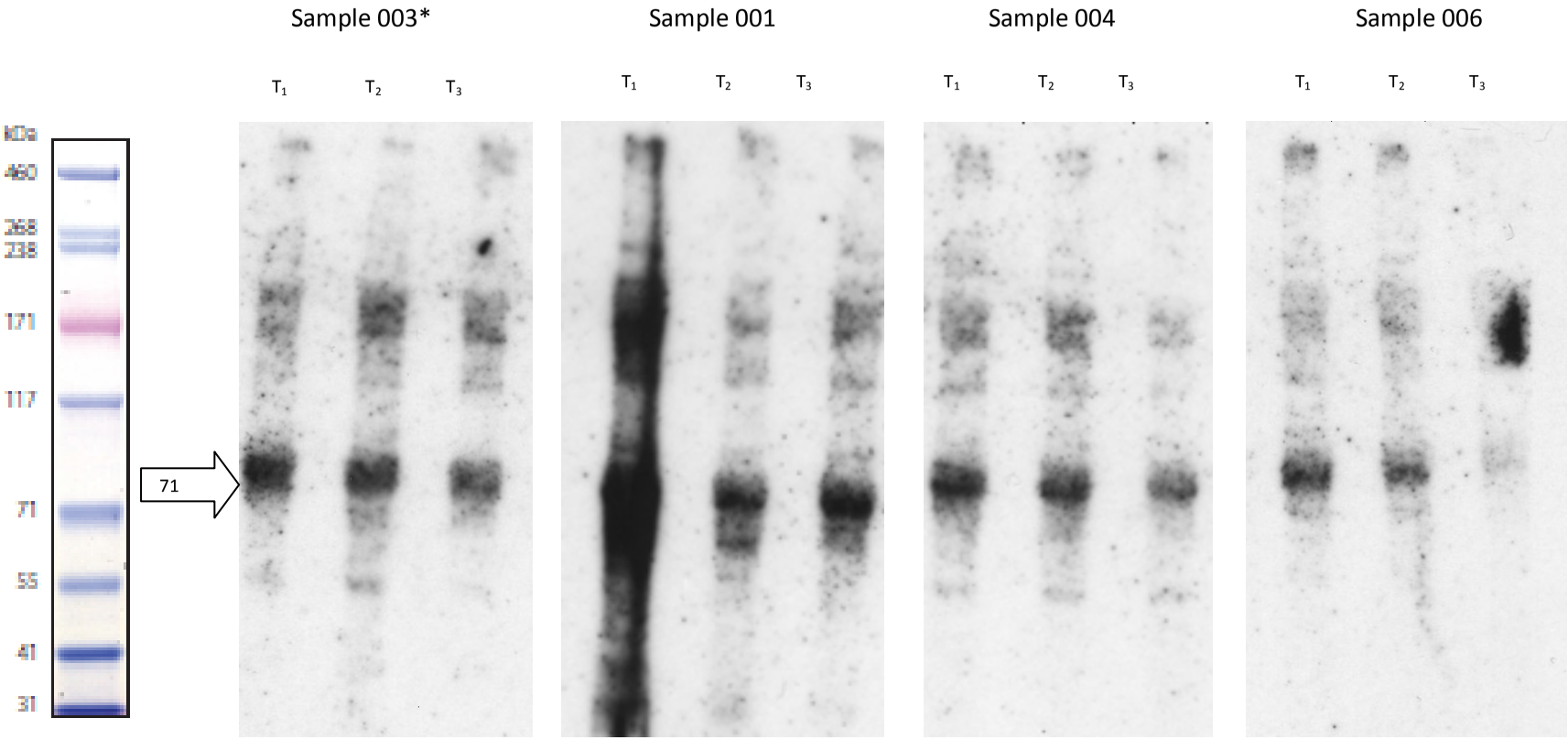
Supplemental Figure 2. Pattern B of CA125, n=12

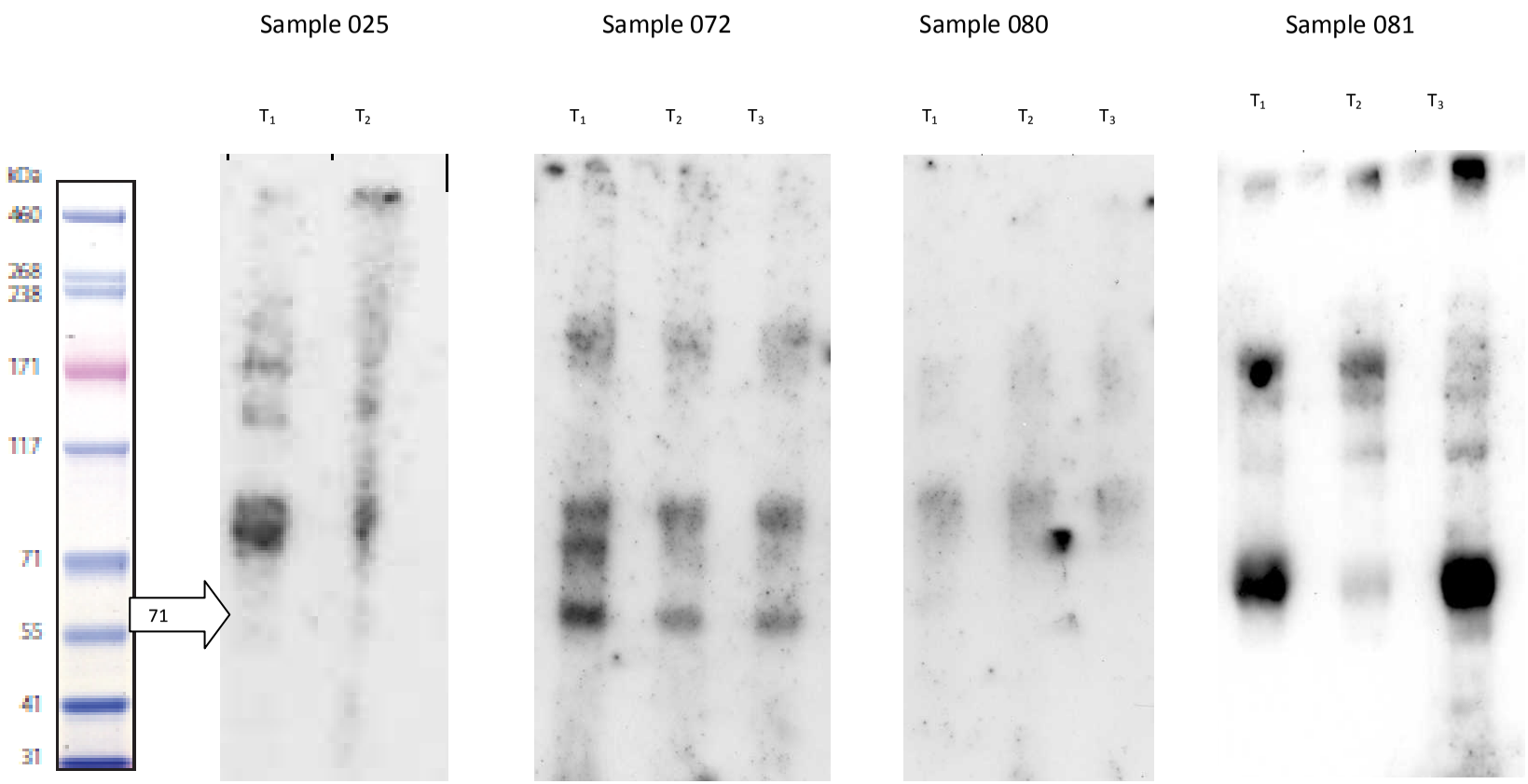



Sample 038
Sample 040
Sample 043
Sample 049
T1
T2
T3
kDa
460
268
238
171
117
71
55
41
31
130
117


Sample 050
Sample 052
Sample 058
Sample 069
T1
T2
T3
kDa
460
268
238
171
117
71
55
41
31
117
131

Supplemental Figure 3. Pattern C of CA125, n=11




Sample 025
Sample 072
Sample 080
Sample 081
T1
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T3
kDa
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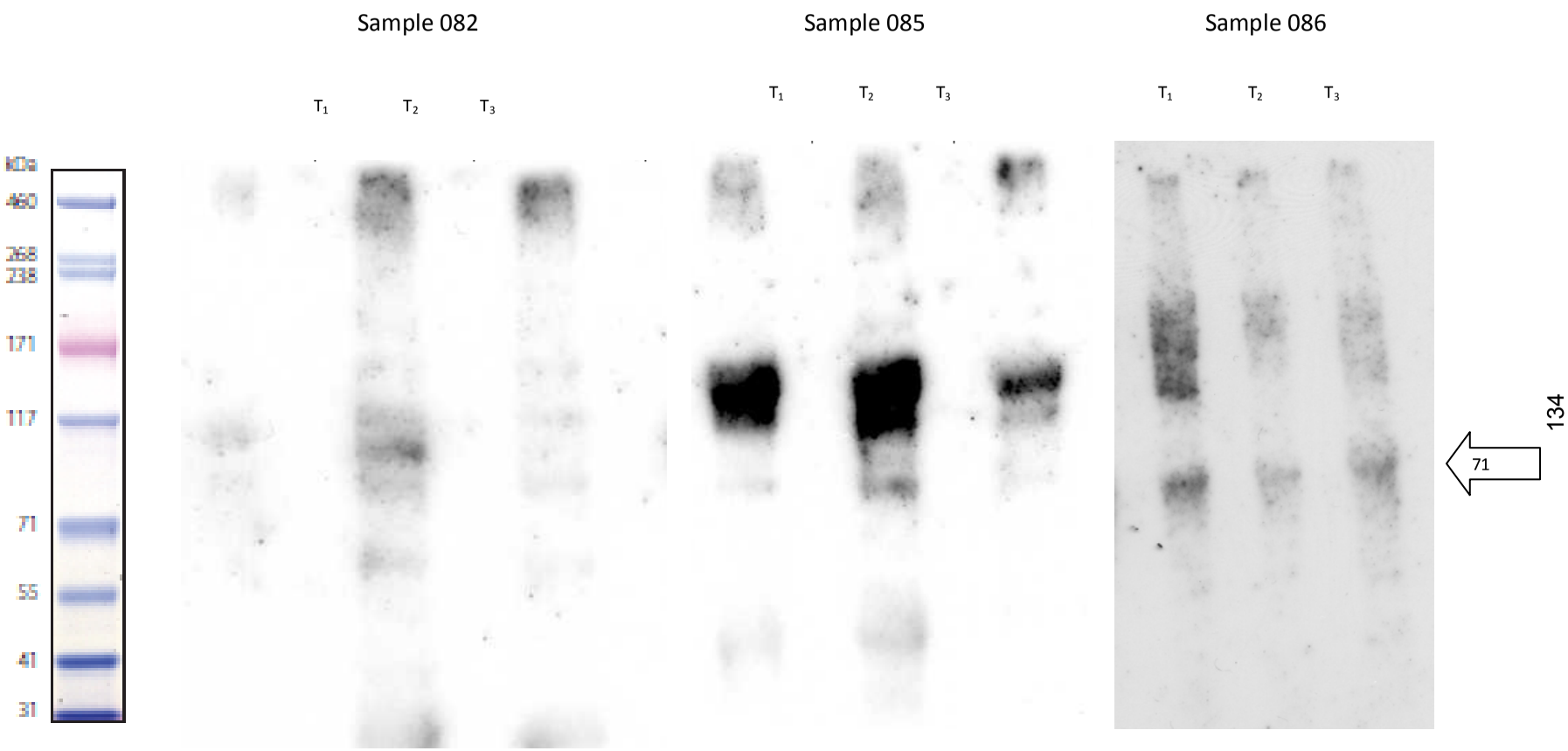
268
238
171
117
71
55
41
31
71


Sample 082
Sample 085
Sample 086
T1
T2
T3
kDa
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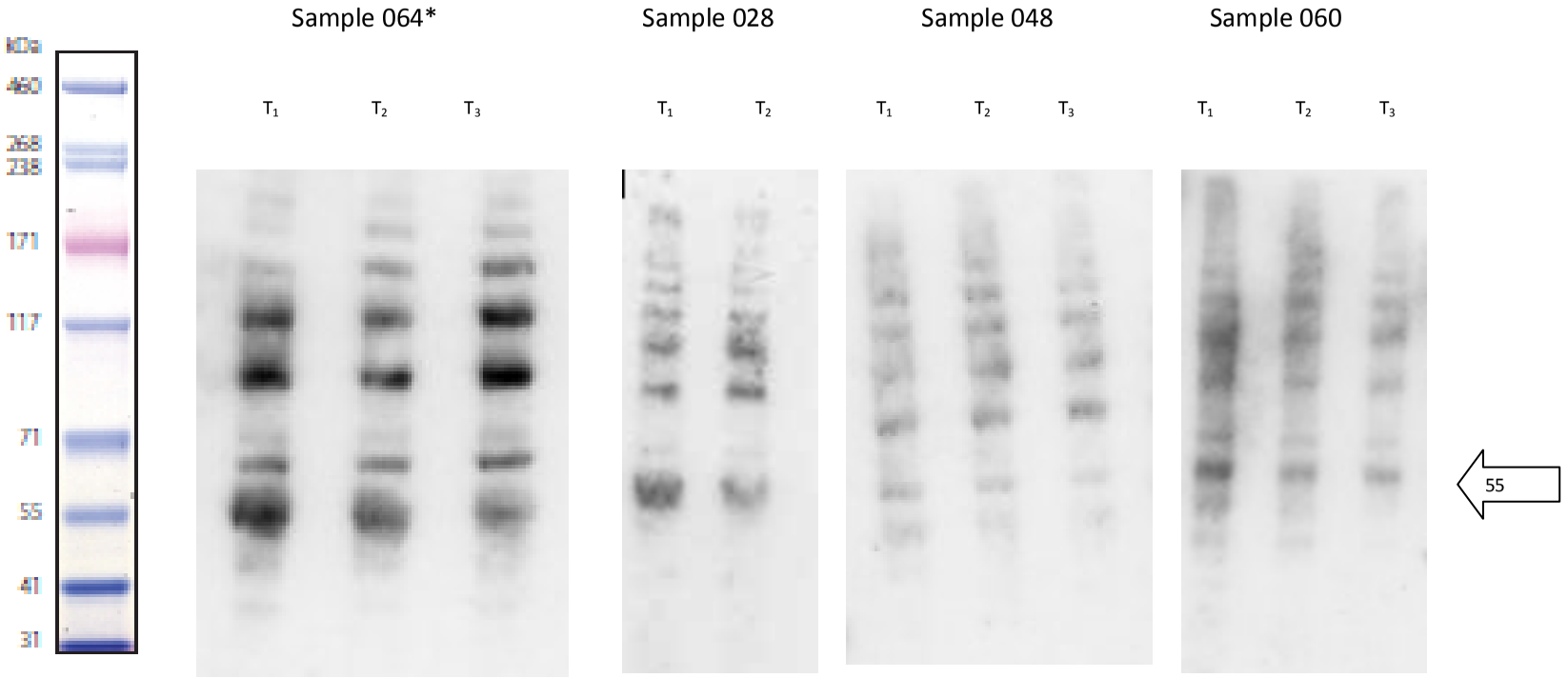
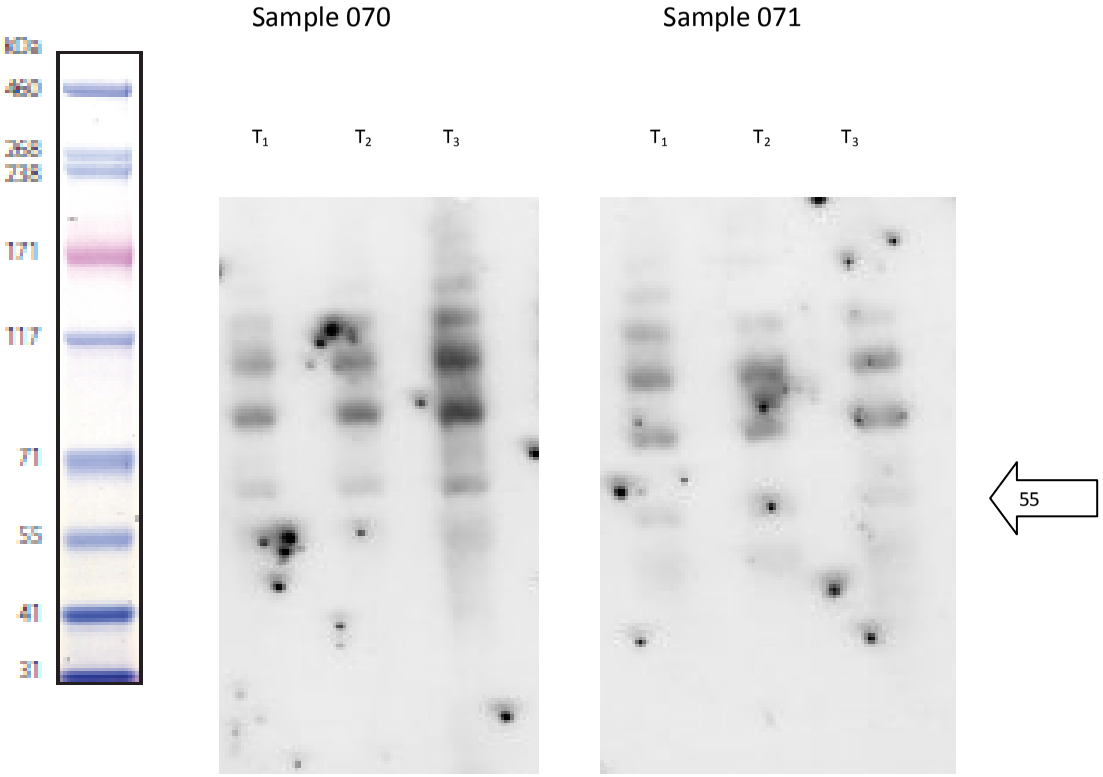
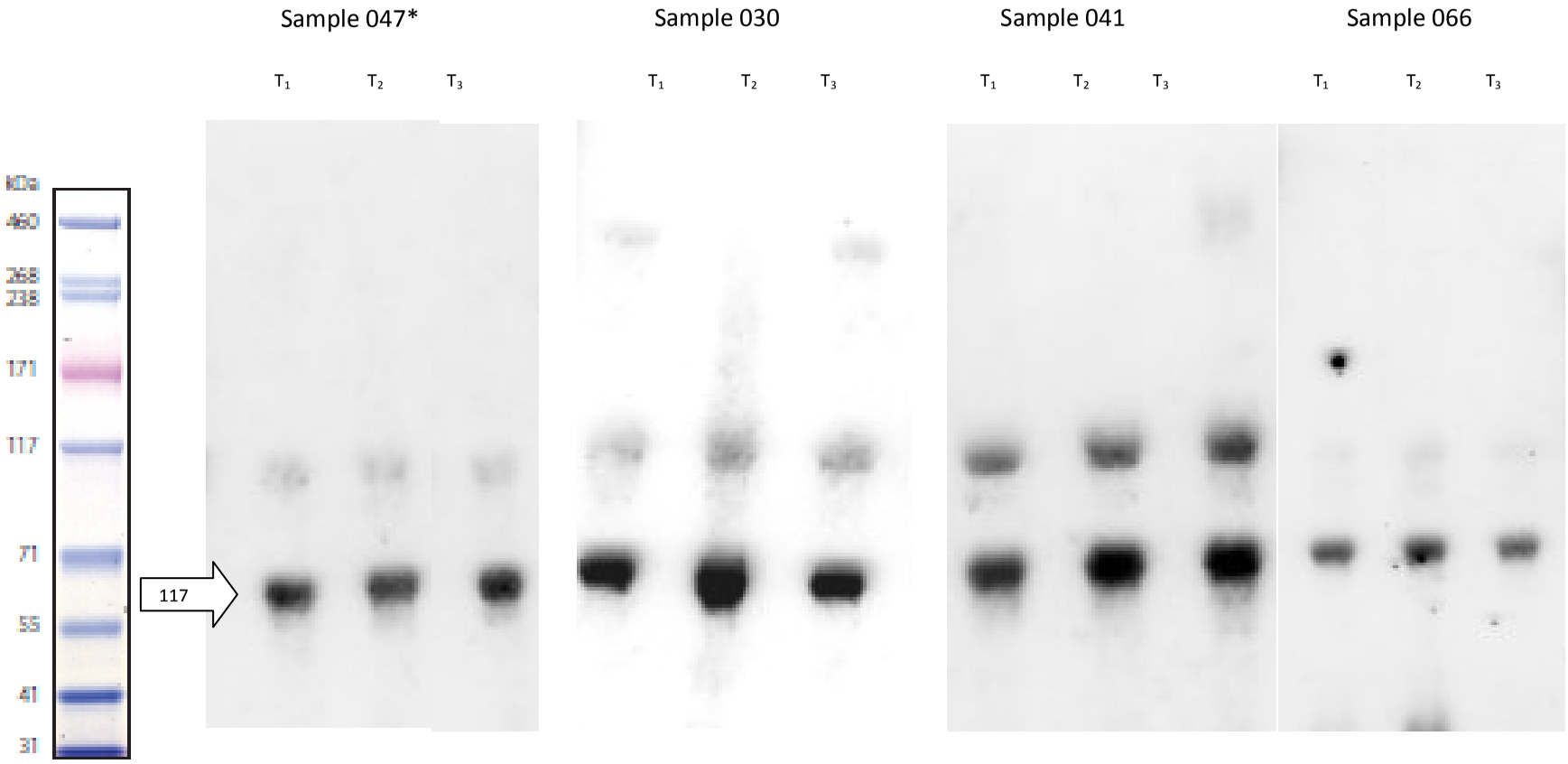
268
238
171
117
71
55
41
31
134
71

Supplemental Figure 4. Pattern D of CA125, n=6

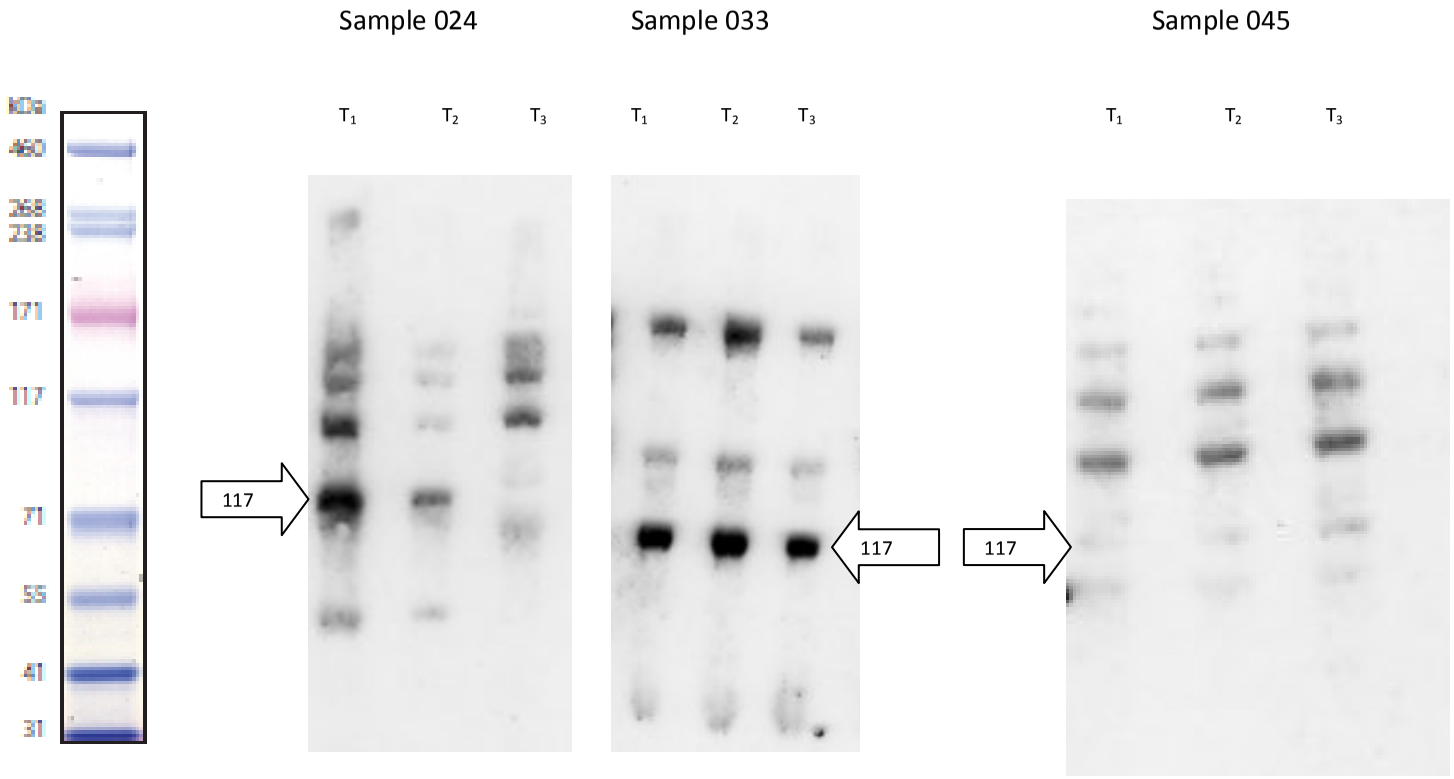


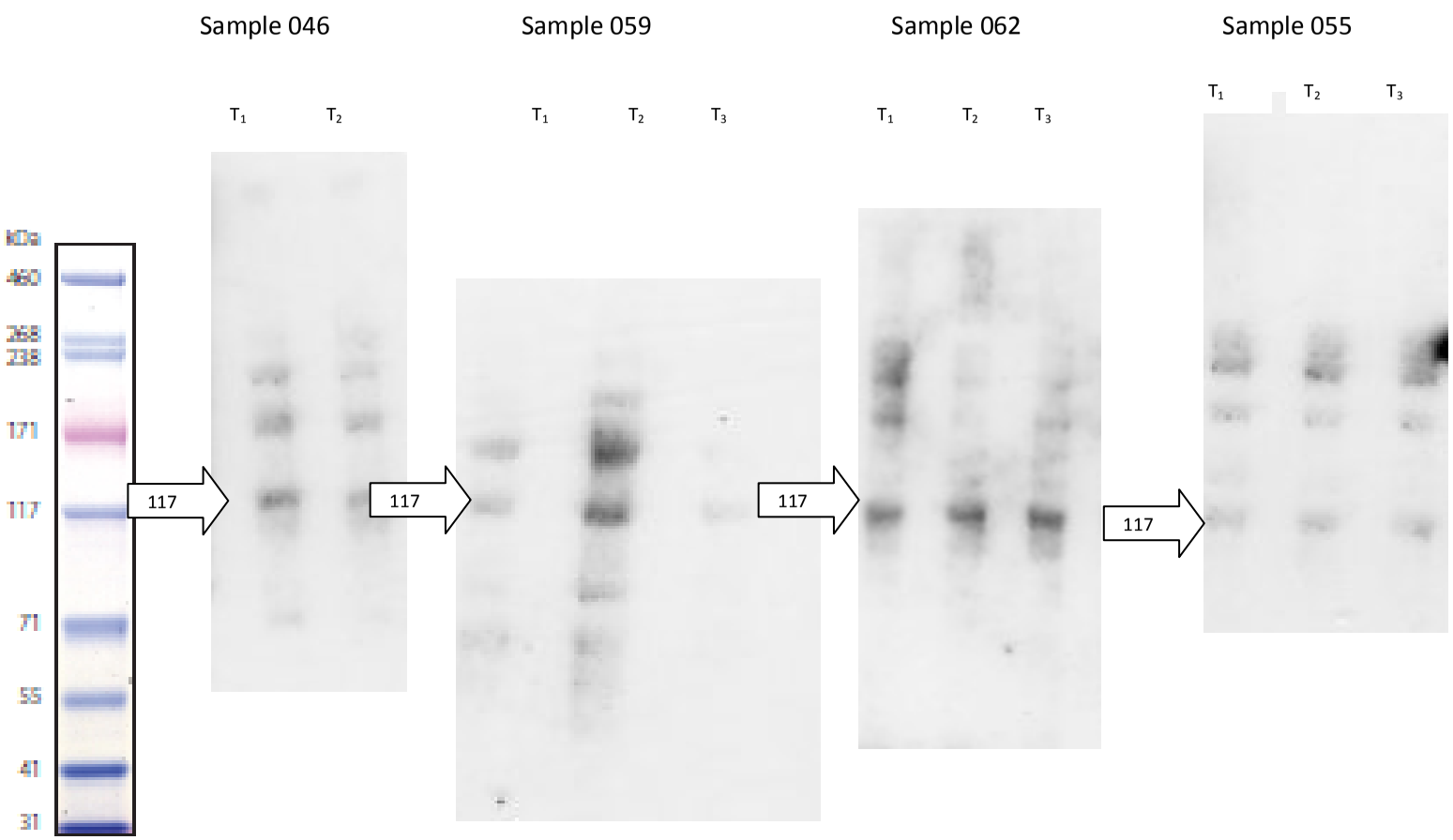

Sample 070
Sample 071
kDa
460
268
238
171
117
71
55
41
31
T1
T2
T3
T1
T2
T3
55

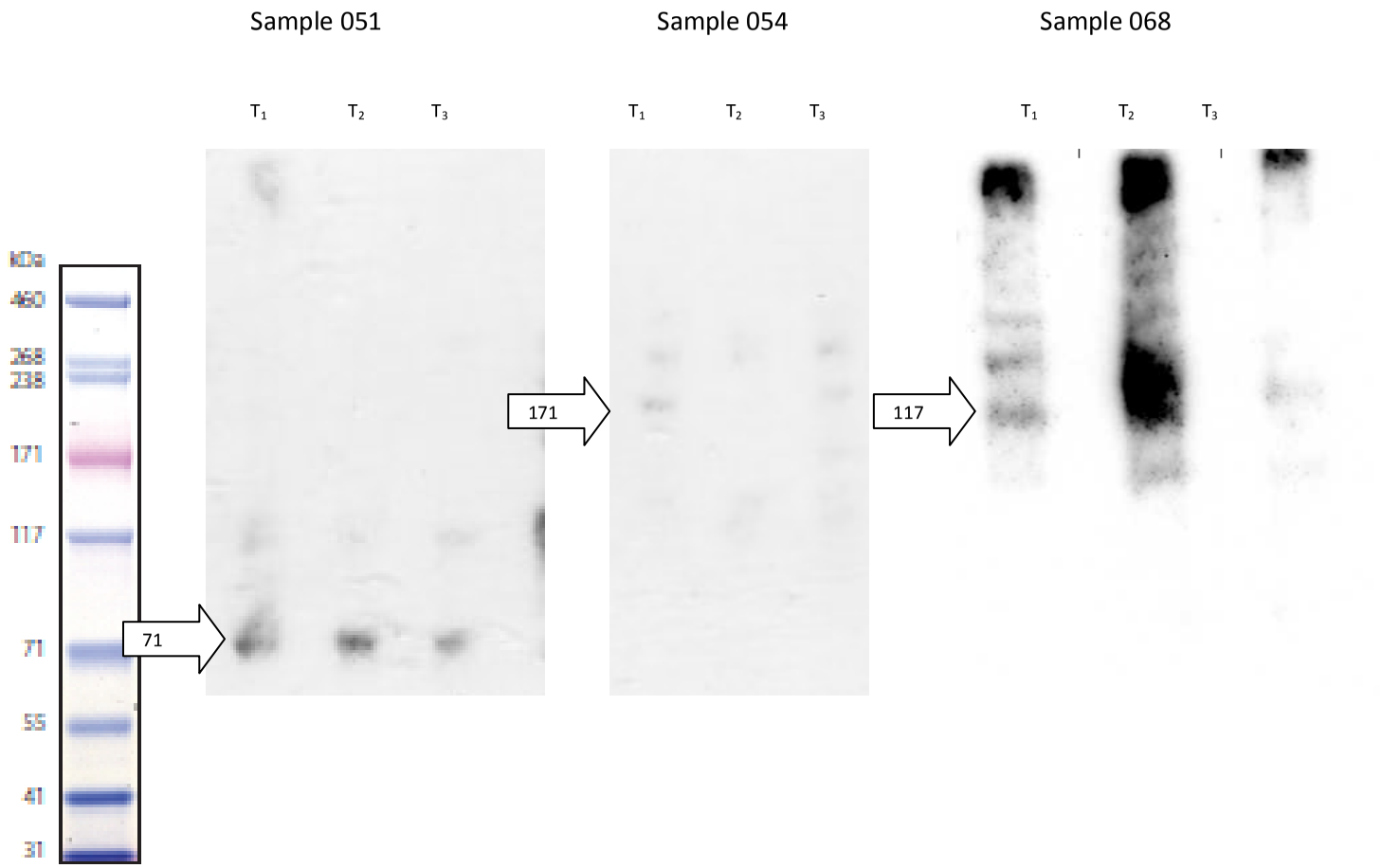
Supplemental Figure 5. Pattern E of CA125, n=4



Supplemental Figure 6. Pattern U of CA125, n=12




Sample 046
Sample 059
Sample 062
Sample 055
T1
T2
T3
kDa
460
268
238
171
117
71
55
41
31
117
117
117
117


Sample 051
Sample 054
Sample 068
T1
T2
T3
T1
T2
T3
T1
T2
T3
kDa
460
268
238
171
117
71
55
41
31
71
171
117

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