MEETING REPORT


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Abstract: Researchers are using the intraductal approach to advance breast cancer risk assessment, prevention, diagnosis, and treatment. Procedures and technologies that can access and interrogate the ductal-alveolar systems include nipple aspiration, ductal lavage and ductoscopy. Ductoscopic papillectomy, ductoscopic margin evaluation, and intraductal therapy are considered promising investigational and innovative treatments. These techniques are used to explore the biology of the normal breast; collect and analyze breast fluid and cells to identify biomarkers that can be used in breast cancer detection and risk assessment; and to identify new ways to find and administer therapeutic and/or preventive agents to the breast tissue. This report summarizes the latest research findings in these areas, presented at The 6th International Symposium on the Intraductal Approach to Breast Cancer in 2009.

Introduction: The human breast is composed of multiple ductal lobular systems that each open onto the surface of the nipple. These ductal systems arborize into ductal trees with associated lobules and are lined with epithelial cells, where most breast cancers are thought to originate. The application of suction to the nipple openings (nipple aspiration) and ductal lavage are used to collect fluid and exfoliated cells from the breast ducts. Many substances associated with the growth, development, and tumorigenesis of the breast have been detected in breast fluids. Cytological techniques have shown that breast cancer-associated abnormalities can be found in these cells [1–6]. In addition, the production and cytological properties of nipple aspirate fluid are associated with breast cancer risk, with long-term follow-up of women undergoing nipple aspiration having shown that breast cancer risk is two to five times greater in women diagnosed with cytologic atypia than it is for women who do not yield fluid [7]. The first International Intraductal Symposium was held in 1999. A biennial event, the Symposium includes discussions and presentations on the anatomy and physiology of the normal and diseased breast, advances in intraductal technologies, findings from genetic, epigenetic, and proteomic analyses of intraductal fluid, and the latest translational research on intraductal approaches to breast cancer risk assessment, diagnoses, and treatment. The 6th International Symposium on the Intraductal Approach to Breast Cancer, sponsored by the Dr. Susan Love Research Foundation and held in Santa Monica, California, on 19–21 February 2009, was attended by more than 100 delegates from 14 countries, including clinicians, epidemiologists, pathologists, basic scientists, translational investigators, and breast cancer advocates. The program included talks by 34 invited speakers and 16 pilot grant applicants in sessions that addressed the etiology of breast cancer; biomarkers of risk in nipple aspirate fluid and ductal lavage; anatomy, ductoscopy, and breast imaging; and intraductal therapy. Delegates also had the opportunity to attend demonstrations of ductoscopy, sonoductography, and nipple aspirate fluid collection on live volunteers.

Minisymposium on a Novel Etiology for Breast Cancer: Inflammation: The first session’s speakers discussed the role that inflammation, nipple aspirate fluid and macrophages may play in breast cancer development. Lisa Coussens (University of California, San Francisco, USA) discussed her research with an MMTV-PyMT mouse model of mammary carcinogenesis that revealed a tumor-promoting role for TH2-CD4 T effector cells. These novel findings suggest the immune system modulates the early onset of cancer development in specific organs, and confirmed epidemiological studies showing that increased macrophage presence correlates with higher tumor grade and decreased survival. Eliminating the T effector cells did not regulate primary disease, and also made it more likely that the cancer would metastasize to the lung, behavior that appeared to be regulated by macrophages. These findings could one day translate into an immune signature that is an independent predictor of recurrence risk, while identifying the pro-tumor mechanisms could lead to new cancer treatments.

Premalignant cell damage in the breast duct lining signals a biochemical cascade that delivers inflammatory proteins to the cell site. Chandice Covington (Texas Tech University Health Services Center, Lubbock, TX, USA) discussed one of these proteins, C-Reactive. CRP is associated with cancer risk, and has...
been shown to be present in increased levels in serum in more advanced breast cancer. Dr. Covington data showed that CRP levels in nipple aspirate fluid were associated with age at first pregnancy, gravidity, wean-time from breast-feeding last baby, percentage body fat, and body mass index and that CRP in NAF significantly and positively related to breast cancer risk as predicted by the Gail model. Several large epidemiological studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) are associated with lower breast cancer risk [8]. Patricia Thompson (Arizona Cancer Center, Tucson, AZ, USA) presented data from her phase Ib dose study investigating the COX dependent and independent effect of sulindac, a non-selective NSAID with COX-independent induction of apoptosis, on NAF biomarkers. Sulindac also may affect EGFR/HER2 downstream molecules. Thirty women with increased breast cancer risk were randomized to 150 mg or 300 mg of sulindac daily for 6 weeks. Nipple aspiration was used to measure levels of sulindac in the breast fluid. Sulindac and sulfide were detectable in 57.7% of NAF samples with sulfone detectable in 11.6%. Serum levels did not correlate with breast fluid levels. COX inhibition in the breast was achieved at 150 mg sulindac, but the higher dose was needed to exhibit activities via COX independent pathways.

Taken together, these preliminary studies show that NSAIDs in general and COX inhibitors, in particular, could be promising agents in the prevention or early treatment of breast cancer. More attention needs to be paid to the pro- and anti-inflammatory nature of the microenvironment of the breast.

**Biomarkers of risk in nipple aspirate fluid and ductal lavage:** Intraductal approaches have the potential to offer new methods of assessing an individual woman's breast cancer risk. Cytology was the first method used to evaluate breast fluid for risk assessment. However, it has been shown to reveal a high degree of prognostically ambiguous atypia. These results, in conjunction with recent technological advancements, have resulted in a growing field of research aimed at identifying biomarkers in breast fluid that could be used for risk assessment as well as determining the best techniques for finding these markers in NAF.

Viruses cause several major human cancers. Gertrude Buehring (University of California, Berkeley, School of Public Health, USA), a prior pilot grant recipient, discussed her research exploring whether oncogenic viruses in NAF mammary epithelial cells can serve as biomarkers to identify women at high risk for breast cancer. She is using in situ polymerase chain reaction (PCR) to detect genomes of bovine leukemia virus, Epstein-Barr virus, and human papilloma virus. Studies have shown that all three of these viruses are found more frequently in breast tissue of women with breast cancer than they are in women with no breast cancer history [9].

Massimo Tommasino (International Agency for Research on Cancer, Lyon, France) presented data from his study using PCR to evaluate HPV in ductal lavage fluid. His data showed that DNA of alpha mucosa and beta cutaneous HPV types are rarely present in the breast fluids of high-risk women, suggesting that a direct role of HPV in breast carcinogenesis is unlikely.

Paul J. van Diest (University Medical Center Utrecht, The Netherlands) discussed his ongoing prospective study, which has high-risk women undergo annual standard screening along with nipple aspiration. Expression of NAF was enhanced in his study with the use of nasal oxytocin spray. Quantitative multiplex methylation-specific PCR (QM-MSP) is used to identify promoter methylation of a selected set of tumor suppressor genes known to be involved in breast carcinogenesis in the NAF. “In five to ten years,” said Dr. van Diest, “some of these high-risk patients will either have an operation because they have breast cancer or decide to have a prophylactic mastectomy, which would permit us to compare methylation data with what is found in surgical tissue to determine if there is a correlation between the number of methylated genes that are present and risk.”

Proteomic profiling may have more value for risk assessment than as a diagnostic screening tool. Savitri Krishnamurthy (MD Anderson Cancer Center, Houston, TX, USA) discussed her research that is using an array of techniques, including ELISA, high-resolution proteomic analysis, SELDI-TOF, and protein microarrays to identify protein signatures in NAF that could be used for risk assessment. The seven proteins her team is currently investigating include insulin-like growth-factor binding protein-3, basic fibroblast growth factor, CRP, erythropoietin, urokinase and prostaglandin E2.

Ercole Cavaliere, (Eppeley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA) discussed his research on DNA adducts, which play a major role in cancer development. Estrogen-purine DNA adducts are shed from cells and end up in the blood and urine. His studies have shown that high levels of estrogen-DNA adducts are associated with breast cancer risk. He noted that “since the reaction of catechol estrogen quinones with DNA is the first critical step in the initiation of cancers, elimination of DNA adduct formation should block the initiation and development of cancer,” this may be a new target for breast cancer prevention and treatment.

Robert Chatterton (Feinberg School of Medicine, Northwestern University, Chicago, IL, USA) presented data from a study investigating the endocrine environment of the breast. Forty-seven premenopausal and 40 postmenopausal high-risk women underwent ductal lavage and NAF collection and also provided a serum sample. Those at high risk were offered tamoxifen as a chemopreventive agent and a repeat ductal lavage 6 months later. The results showed that NAF and ductal lavage fluid contain a rich source of hormones and other markers of risk, but the findings are too preliminary to draw any conclusions.

Ferdinando Mannello (University “Carlo Bo,” Urbino, Italy), a prior pilot grant recipient, presented an update on his research study investigating the role oxidative stress plays in the breast microenvironment and the function of adhesion molecules in the breast cells of the ductal-lobular unit. He has published his findings on protein analyses of NAF in healthy women versus breast cancer patients [10].

Catharina Svanborg (Lund University, Sweden) is studying HAMLET (human a-lactalbumin made lethal to tumor cells), a protein-lipid complex that is found in human milk, and that shows promise as a new anti-cancer agent. HAMLET kills tumor cells and immature cells but spares healthy differentiated leukocytes. HAMLET shows broad anti-tumor activity, and Dr. Svanborg’s studies have identified several death pathways activated by HAMLET in tumor cells, including apoptosis, anoikis, and autophagy [11].

Hypermethylated genes are promising powerful biomarkers of breast cancer detection and perhaps risk assessment. Studies have shown that tumor suppressor gene (TSG) methylation is identified more frequently in random periareolar fine needle
aspiration samples from women at high risk for breast cancer than in women at low risk. David Euhus (University of Texas Southwestern Medical Center, Dallas, TX) investigated whether the finding that would hold true for breast fluid samples obtained via ductal lavage. For this study, 514 samples obtained from 150 women were assessed cytologically and by Quantitative Multiplex Methylation-Specific PCR (QM-MSP) for methylation of cyclin D2, APC, HIN1, RASSF1A, and RAR-beta2. Dr. Euhus reported that the study showed that TSG methylation in ductal lavage samples did not predict marked atypia, and that both methylation and marked atypia were independently associated with highly cellular samples, Gail model risk, and a personal history of breast cancer, suggesting related, but independent, pathogenic pathways in breast epithelium [12].

Mary Jo Fackler (Johns Hopkins University School of Medicine, Baltimore, MD, USA) is also using QM-MSP to identify potential biomarkers. Dr. Fackler presented findings from a pilot study that explored the potential of using ductoscopic washings along with QM-MSP to evaluate spontaneous nipple discharge and that showed that QM-MSP is 3-fold more sensitive than cytology in identifying cancerous cells. She also discussed a prospective pilot study of 54 postmenopausal women who were receiving anastrozole as their sole adjuvant therapy. Thirty-three of the women had an optional contralateral biopsy at baseline and at 6 months after starting treatment. A comparison of these biopsy samples showed significant decreases in methylation for TWIST1, RASSF1A, and RARbeta. Both findings suggest more prospective studies are necessary to evaluate the relationship between changes in methylation and breast cancer incidence.

Gerald Gui (Royal Marsden Hospital, London, UK) presented data from his study comparing measurements of methylation-specific PCR in ductal lavage fluid, breast cancer tissue, adjacent normal breast parenchyma, and plasma in women with early breast cancer. The study showed a positive correlation between tumor tissue and ipsilateral ductal lavage in women with breast cancer (n = 24). Methylation was also significantly higher in tumor and ipsilateral ductal lavage fluid when compared with adjacent normal tissue and contralateral ductal lavage.

Bassem Haddad (Georgetown University, Washington, DC, USA) discussed his research aimed at identifying a panel of biomarkers that could improve the diagnostic value of ductal fluid in detecting pre-malignant breast lesions and early stages of breast cancer in high-risk women. Edward Sauter (University of North Dakota School of Medicine, Grand Forks, ND, USA) discussed his use of two intraductal approaches, ductoscopy and nipple aspiration, to assess response to both pharmacologic and nutritional chemopreventive interventions.

Catherine Carpenter (University of California, Los Angeles, USA), a prior pilot grant recipient, provided an update on her research investigating the impact of diet and exercise on biomarkers in breast ductal fluid in overweight postmenopausal women. To date, five previously sedentary overweight women have completed the study. Preliminary midpoint data showed a major drop in leptin, DHEA, and estrone in serum that was not mirrored in the lavage fluid. The Gail Risk model, which is widely used to assess breast cancer risk, was developed based on data from Caucasian women and has been found to be less accurate in assessing risk in women of other ethnicities [13]. Lisa Bailey (Carol Ann Read Breast Health Center, Alta Bates Summit Medical Center, Oakland, CA, USA), a prior pilot grant recipient, provided an update on her research exploring the feasibility of an intraductal approach for risk assessment in African American, Asian, and Hispanic women.

Atilla Soran (Magee-Womens Hospital, University of Pittsburgh Medical Center, PA, USA), a prior pilot grant recipient, presented preliminary research findings from his study investigating anti-MUC1 and anti-cyclin B1 antibodies in nipple aspirate fluid in breast cancer patients. He suggested that more sensitive techniques for antibody detection might be needed to identify the low antibody levels in the premalignant lesions, which could boost the usefulness of NAF as the source for this diagnostic assay.

These presentations demonstrated the different types of strategies researchers are using to identify biomarkers associated with genetic, epigenetic, and proteomic abnormalities in breast fluid. These reports add to growing body of published studies that have found that biomarkers appear to be more sensitive than cytologic atypia in predicting breast cancer risk [14–22].

**Anatomy, ductoscopy, and imaging of the breast**: Intraductal approaches have been used to advance our understanding of the anatomy of the breast. They have provided evidence that the breast ducts do not form an even, radial pattern, and shown us that there are both central ducts that go directly back to the chest wall and peripheral ducts that drape over this central group. This knowledge of the anatomy of human breasts will guide future breast cancer research. Intraductal approaches are also utilized in the intraoperative setting in patients with both benign and malignant breast conditions to guide treatment decisions. As James Going (University of Glasgow, Scotland, UK), a prior pilot grant recipient, underscored in his presentation, “Anatomy seems old-fashioned, but a house without a good foundation is a rickety structure and we need to understand that foundation, no matter how good the biology.”

Dr. Going’s presented findings from his research investigating what he calls “the paradox of nipple anatomy.” As he noted, 5–9 ostia can be observed in the lactating breast, and a few of these ostia can be cannulated. Yet 20–30 ducts are observed when the nipple is transected raising the question, “What are these ducts doing?” He found no evidence for his previous two-duct-type hypothesis, but did find evidence that appears to point to ostium sharing – several ducts converging in a common opening in the nipple.

Gerald Gui (Royal Marsden Hospital, London, UK) reported findings from his study investigating the anatomical association between fluid yielding ducts and breast cancer location. This study of 40 patients undergoing mastectomy for breast cancer, used ductal lavage followed by infusion of the mastectomy specimens ex vivo with a colored resin. It showed that the duct system of the cancer affected segment was concordant to a fluid-yielding duct in 58% of cases, a similar finding to previous studies. Dr. Gui said this suggested that cytology studies would likely be limited by duct accessibility whereas protein studies, which could demonstrate a field effect, would not.

Sheldon Feldman (Columbia University New York City, NY, USA) provided a history and overview of the field of mammary ductoscopy. The ability to see ductal pathology and monitor response to therapy in real time along with technological improvements, including better visualization and an interventional approach, are moving the field forward. Current challenges include the absence of an established CPT code and a lack of data from clinical trials evaluating the efficacy and clinical role of ductoscopy or randomized trials evaluating whether more
cancers are found with ductoscopy. Even so, said Dr. Feldman, “We are on the threshold of the future.”

Fatih Balci (Ankara State Hospital, Ankara, Turkey) presented images and data from his study evaluating the therapeutic value of endoscopic papillectomy in patients with pathologic nipple discharge (PND). Breast ductoscopy was performed on 178 of 213 patients with PND enrolled in the study. All visualized papillomas were removed endoscopically with a scope and a grasping basket. Dr. Balci reported that they identified 34 papillomas and 5 cases of DCIS, and that there was a 100% correlation between ductoscopy findings and histopathology. These findings, said Dr. Balci, confirm that ductoscopic papillectomy “is a safe and easy procedure to remove intraductal papillomas from the breast that avoids unnecessary resection of breast tissue” [23].

William Dooley (University of Oklahoma, Oklahoma City, OK, USA) discussed current techniques and uses for ductoscopy. In his clinical practice, Dr. Dooley routinely uses ductoscopy to manage early invasive cancer. As a result, he said, he has a low annual hazard rate for ipsilateral breast cancer recurrence. In the discussion that followed, Dr. Dooley noted that surgeons rely on radiation to improve local control, but that ductoscopy could do the same. “If we can get good enough at doing endoscopically directed lumpectomy,” he said, “we would not need to perform radiation unless there was lymphovascular invasion.” One essential area needed to advance the field of ductoscopy will be carefully planned prospective multi-center studies to address the sensitivity, specificity, false negative and false positive rates of ductoscopy in accurately identifying cancerous versus non-cancerous tissue.

Wai-ka Hung (Kwong Hospital, Kowloon, Hong Kong) discussed the role of mammary ductoscopy in breast surgery, also noting that for the field to move forward good endoscopic-pathologic correlation was necessary. This, in turn, could spare women unnecessary surgery, as it would allow for therapeutic mammary ductoscopy and removal or ablation of a papilloma. In addition, if it could be determined that there was no ductal involvement in the areola, it might also be used to better select appropriate candidates for for nipple-sparing mastectomy.

Debra Strick (University of California, Los Angeles, USA), a prior pilot grant recipient, presented findings from her research on intraductal micromagnetic resonance imaging and spectroscopy. Dr. Strick developed an intraductal radiofrequency microcoil that can reduce the volume of diseased tissue to a cubic-centimeter, allowing MRI spectroscopy, which could distinguish between benign and malignant tissue, and therefore be used for early diagnosis. This microcoil could potentially be used with ductoscopy and conventional MRI to reduce false-positives or for an MRI-guided biopsy.

**Intraductal therapy: background, current and future:** The session on intraductal therapy highlighted studies investigating preclinical testing and early phase human clinical trials.

Dixie Mills (Dr. Susan Love Research Foundation, Santa Monica, CA, USA) presented data from a study exploring the physiology of the resting, or non-lactating, breast. A total of 14 women underwent blood collection, nipple aspiration, and ductal lavage five times over 12 hours. After baseline testing, subjects were given 200 mg of caffeine (NoDoz) and 200 mg of cimetidine (Tagamet). Previous studies have shown that in lactating women caffeine passively diffuses into milk rapidly and reflects serum levels. This study found that in the resting breast, caffeine levels peaked at 6 hours or later. Cimetidine has previously been found to be concentrated in the milk of lactating women, but was not detected in the ductal fluid obtained from the resting breast. These findings reinforce the need for more studies on the resting breast as its properties are clearly different than the lactating breast.

Saraswati Sukumar (The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD, USA) presented new data from her preclinical studies with pegylated liposomal doxorubicin (PLD) in rat and mouse models of breast cancer. Dr. Sukumar reported that in addition to preventing tumor formation for three months or more, PLD appeared to stunt the growth of the mammary glands in mice. Following up on this finding, she looked at the response of the treated mammary gland to a new pregnancy. She reported that that pups were unable to get sufficient milk from the treated duct, suggesting a compromised response in the mammary gland to pregnancy hormones. In addition, pregnancy did not increase the incidence of tumors in PLD treated mice. She also noted that their studies have found that mice receiving intraductal treatment have fewer stem cells than other mice, which has resulted in a new line of investigation [24].

Moving into the clinic setting, Vered Stearns (Johns Hopkins School of Medicine, Baltimore, MD, USA) presented data from a phase I feasibility study of PLD in women awaiting mastectomy. Participants underwent nipple aspiration and ductal cannulation using a dose escalation scheme. Blue dye was injected into the treated duct just prior to mastectomy and tissue was obtained for pharmacokinetic and biomarker analysis. Dr. Stearns reported that doxil was present in the region at both the 5 mg and 10 mg dose, evidence that it can leave the duct. No changes were seen in the stroma surrounding the ducts, however a dose-dependent effect was seen in plasma and in breast tissue. Noting that there is no data on how IV doxil affects breast tissue, Dr. Stearns said her next study would compare the breast tissue of women who receive IV doxil prior to surgery with that of women receiving intraductal doxil.

Susan Love (Dr. Susan Love Research Foundation, Santa Monica, CA, USA) presented data from a Phase I feasibility study conducted in Beijing, China. One of two drugs, PLD or carboplatin, were used intraductally 2–7 days prior to mastectomy, at 3 dose levels, with the highest dose approximating the clinical intravenous dose. Dr. Love reported that PLD stayed in the duct longer and showed a lower serum dosage. At the highest doses, women receiving PLD reported tenderness and erythema, whereas the highest doses of carboplatin resulted in mild nausea and vomiting, a sign that the drug had gotten into the bloodstream. Dr. Love also reported that pathological examination showed the drugs were widely distributed through the ductal systems reaching the terminal duct lobular units. In conclusion, said Dr. Love, this shows us that this approach is feasible and safe.

Jianyu Rao (David Geffen School of Medicine, University of California, Los Angeles, USA) described the histopathological changes observed in the study Dr. Love presented. The pathology was not performed to study the tumor but to assess ductal epithelial cell changes and inflammatory reaction seen in ducts and surrounding fibroadipose tissue in association with duct treatment. Dr. Rao reported that carboplatin resulted in a dose-response increase of both in inflammation and ductal epithelial cell changes. In contrast, PLD resulted in no severe inflammatory changes in any dose group, but there was a significant increase of...
epithelial response. Dr. Rao said this suggests that short-term intraductal treatment may induce some degree of epithelial changes and some inflammatory response, while the long-term effect remains to be determined.

Ellen Mahoney (St. Joseph Hospital, Eureka, CA, USA) presented data from her pilot preoperative study testing the effects of PLD delivered through the affected duct of 30 women with DCIS diagnosed by core needle biopsy awaiting surgery. To date, six patients have undergone treatment. In those who were treated successfully with PLD, the pathology has revealed reactive and reparative changes to the duct. If this proves effective, said Dr. Mahoney, we could develop “a chemical mastectomy for post-breastfeeding women that would eradicate the stem cells and epithelial cells inside the breast duct, thus eliminating the possibility for breast cancer to occur.”

Taken together, the intraductal approach for potential prevention and therapy of breast cancer has been born and is growing as more researchers begin to study and answer the critical questions needed to advance this exciting emerging field.

Pilot grants: The Dr. Susan Love Research Foundation utilizes a unique grant review mechanism to distribute pilot grants at the Symposium. Applicants submit one-page abstracts and responsive proposals are selected for presentation at the meeting. A multidisciplinary peer review committee, comprised of basic scientists, breast cancer activists, and physicians, evaluates both the abstracts and the presentations. At the Symposium, 16 researchers presented proposals. At the close of the Symposium, the Foundation announced that it would be providing $84,000 to support 8 innovative research projects utilizing the intraductal approach.

The 2009 recipients were: Robert Chatterton, PhD, and Seema Khan, PhD (Northwestern University), ‘Investigation of factors regulating estrogen uptake and retention by the breast’; Hong Ling, MD (Cancer Institute, Fudan University), ‘Comparison of PH value in nipple aspiration fluid from ductal carcinoma with or without calcification’; Ferdinando Mannello, PhD, and Gaetana Toniti, PhD (University of Urbino ‘Carlo Bo’), ‘Iron-driven inflammation in the breast microenvironment: assessment of iron-overload in breast cancer development’; Atilla Soran, MD (University of Pittsburgh Medical Center), ‘Autofluorescence ductoscopy for the early detection of breast cancer’; Barbara Urban, MD, and Lorriaine Tafra, MD (Anne Arundel Breast Center), ‘Pilot study to identify breast cancer protein biomarkers in nipple aspirate fluid’; Ameae Walker, PhD (University of California, Riverside), ‘A prolactin binding compound in breast ductal fluids’;

Daniel Wreschner, MD (Tel-Aviv University), ‘Monoclonal antibodies and peptides targeting the MUC1 alpha/beta junction of the ablation of human cancer cells via the intraductal approach’; Gang Zeng, PhD (University of California, Los Angeles), ‘A multiplex approach for detecting antibodies in the nipple fluid of breast cancer patients’.

Conclusion: The 6th International Symposium on the Intraductal Approach to Breast Cancer highlighted the advances that have been made in intraductal research and the potential the intraductal approach has to change clinical practice. Delegates in attendance expressed an interest in designing collaborative studies that can provide more data on the effectiveness of ductoscopy and identify new biomarkers in breast fluid. An update on these projects, along with findings from this year’s pilot grant projects, will be presented in 2011 at the 7th International Symposium on the Intraductal Approach to Breast Cancer.

List of abbreviations used: CRP: C-reactive protein; DCIS: ductal carcinoma in-situ; NAF: Nipple aspirate fluid; PLD: Pegylated liposomal doxorubicin; PND: pathologic nipple discharge; QM-MSP: Quantitative multiplex, methylation-specific polymerase chain reaction; PCR: polymerase chain reaction.

Competing interests: Susan Love is a founder, Board member and share holder of Brexys (Windy Hill Medical).

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10. Mannello F, Medda V and Toniti GA: Protein profile analysis of the breast microenvironment to differenti-


MEETING ABSTRACTS

S1
Protumor immunity and breast cancer development
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For decades, it was generally accepted that leukocytic infiltrations in tumors represented a failed attempt of the immune system to eradicate damaged cells. While indeed some aspects of failed anti-tumor immunity exist, what we now appreciate is the fact that multiple protumor immune programs are instead co-opted by nascent tumors, and in so doing significantly enhance tumor development, including breast cancer. Based upon our evaluation of human clinical specimens revealing significant infiltration of breast tumor tissue by both T lymphocytes and macrophages, we asked the question as to whether adaptive immunity was perhaps enhancing protumor properties of macrophages and thereby potentiating breast carcinogenesis. Utilizing the MMTV-PyMT mouse model of mammary carcinogenesis, CD4+ T cell-deficient mice, and an ex vivo three-dimensional organoid co-culture model, we revealed a tumor-promoting role for T\textsubscript{H}2-CD4+ T effector cells that elicit pro-tumor, as opposed to cytotoxic, bioactivities of tumor-associated macrophages (TAMs) and enhancement of pro-metastatic epidermal growth factor (EGF) receptor signaling programs in malignant mammary epithelial cells. These novel findings provide a mechanism explaining how T\textsubscript{H}2-activated TAMs achieve high level expression of EGF necessary for inducing survival, invasive growth and metastatic programs in malignant cells, and together indicate that anti-tumor acquired immunity, mediated by CD4+ T lymphocytes are usurped by engaging cellular components of the innate immune system, and identifies new cellular targets, namely T\textsubscript{H}2-polarized CD4+ T lymphocytes, for anti-cancer therapy.

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S2
Chronic inflammation, C-reactive protein, and breast health: future directions to predict, prevent, personalize and inspire consumerism in the mitigation of breast cancer

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Breast cancer incidence has dropped dramatically with further reductions predictably linked to lifestyle/behavioral and environmental changes. About 98% of malignant breast conditions emanate within the epithelial cell compartment in the breast ductal system. Premalignant cell damage in this lining signals a biochemical cascade that deliver inflammatory proteins to the site. One protein, CRP, is associated with carcinogenic risk. An inflammation cell milieu amenable to efficient, low cost, preventive interventions that impede biochemical/molecular conversion to cancer would offer a ‘disruptive innovation’ with public health implications. Nipple aspirate (NAF), a non-invasively obtained tissue sample, is a rich source of protein markers manufactured by the ductal system. First, Lithgow’s dissertation determined if CRP relates to reproductive, nutrition, and body composition and activity factors. Results were that age at first pregnancy, gravity, wean time from breast-feeding last baby, %fat, and BMI, with p < .05, and serum triglycerides (p = .01), were related significantly to CRP levels in NAF. A model derived from selective factors significantly (p = .05) accounted for the variance in CRP in NAF. Second, Lithgow evaluated if risk factors, as identified by the Gail model, were associated with NAF CRP levels. CRP in NAF significantly (p = .04) and positively related to breast cancer risk as predicted by the Gail model. A third bio-cultural study offers a perspective breast microenvironment that challenges western views of the female breast. Proteins prevalent in grandmother age (35–70 years of age) breast fluids collected in an investigation of the use of re-lactation in response to a goal to reduce infant exposure to HIV transferred in mother’s breast milk found that these grandmothers could re-establish a nutritious/adequate milk supply equivalent with the cell and protein components of human milk. The inflammatory hypothesis, as a potential preventive base to support breast-feeding support for maternal health, systematic testing of NAF, serial expression of NAF, dietary supplements and other innovations taken together promise further reductions of breast cancer incidence. The western view of female breast pathophysiology may benefit from a ‘disruptive innovation’ philosophy applied to the translational model of knowledge development that is predictive, preventive, personalized, and participatory in the mitigation of breast cancer.

S3
The effect of sulindac on growth differentiation factor 15 and 13,14-dihydro-15-keto prostaglandin A2 in nipple aspirate fluid

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Use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated in several large epidemiologic studies with a lower risk of epithelial cancers including breast though no randomized, placebo controlled phase III studies have been conducted to date. NSAID use was associated with a significantly lower risk of breast cancer among participants in a multiethnic cohort, though the nearly 50% reduction in risk was limited to women with estrogen receptor positive tumors; an observation previously reported by Terry et al., and supported by Gierach et al., and findings of Harris et al. for cyclooxygenase-2 (COX-2) inhibitors. These findings in epidemiologic studies are supported by work in human mammary tissues and cell culture where overexpression of COX-2, one of two cyclooxygenase targets of NSAIDs, acts as an early event in the transition of normal breast cells to malignancy. Further, COX-2-associated prostaglandin E2 (PGE2) production has been shown to increase aromatase activity in mammary epithelial cells possibly explaining the observed benefit of NSAID use in hormone receptor positive disease and the recent observation of lower circulating estradiol levels among regular users of NSAIDs. Thus, there exists sufficient rationale for the use of NSAIDs for the prevention of breast cancer.

While the best studied target of NSAIDs is the COX enzymes, the anti-tumor activities of each of the NSAID agents via ‘non COX’ pathways remains unknown. Sulindac, a non selective NSAID with well recognized COX-independent induction of apoptosis has remained a strong candidate for chemoprevention largely because of its broader anti-tumor activity. Growth differentiation factor 15 (GDF-15), a potent proapoptotic molecule in the TGF-β superfamily and potential tumor suppressor is upregulated by several NSAIDs independent of its activity as a COX inhibitor. Sulindac sulfone, a metabolite of sulindac and a COX-independent mediator of apoptosis, has been shown to be a potent inducer of GDF-15 at pharmacologically relevant doses whereas Celecoxib, a COX-2 specific inhibitor, has low GDF-15 induction potency even at toxic doses. Other NSAIDs such as aspirin and indomethacin as well as a number of candidate dietary chemopreventives including resveratrol, indole-3-carbinol, 3,3’-Diindolylmethane (DIM), and lycopene as well as the PPAR agonists troglitazone, and 15-deoxy-Δ12,14-prostaglandin J2 all show potent induction of GDF-15 leading to the suggestion that GDF-15 might serve as a complementary molecular target to COX-2 for cancer chemoprevention and serve as a surrogate biomarker of apoptotic response.
To better understand the effect of NSAIDs in the breast, we conducted a phase 1b dose study using sulindac as a representative drug of the non selective NSAIDs with known activity to induce GDF-15 and inhibit COX-2. To assess sulindac and its metabolites and their effects on a stable COX-2 derived prostaglandins 13,14-dihydro-15-keto prostaglandin A2 [PGEM], and the NSAID inducible growth differentiation factor (GDF-15) in nipple aspirate fluid (NAF), we randomized 30 women at increased risk for breast cancer to 150 mg once daily or twice daily sulindac for 6 weeks. Sulindac and sulfide were detectable in 57.7% of NAF samples with sulfone detectable in 11.6%. Sulindac was associated in a dose independent manner with a non-significant decrease in NAF PGEM levels (p = 0.1). Serum levels of sulindac, but not NAF sulindac, were correlated with a decrease in NAF PGEM levels (p = 0.03). GDF-15, showed a borderline significant trend towards higher levels in NAF with 300 mg daily sulindac (p = 0.07). This study suggests that COX inhibition in the breast may be achieved at 150 mg sulindac once daily while a higher daily dose is needed to exhibit activities via COX independent pathways. The study also suggests that NAF levels of PGEM and GDF-15 could be considered as drug effect biomarkers for future early phase chemoprevention trials.

**S4 Oncogenic viruses in nipple aspirate fluid: biomarkers for breast cancer risk assessment?**

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It would be a considerable advantage to be able to identify women at high risk for developing breast cancer to justify closer follow-up and use of multiple methods to ensure early detection. At present, the only high risk individuals identifiable with a biomarker are those ≈5% with a strong familial history of breast cancer. For the remaining 95% there is a lack of such biomarkers. Three independent epidemiologic studies found that women with mammary epithelial cells (MEC) in their nipple aspirate fluids (NAF) were more likely to subsequently develop breast cancer than women without cells. A possible explanation for this is that early stages of breast carcinogenesis and tumor progression most likely involve MEC hyperplasia triggered by a carcinogenic agent. Viruses cause several major human cancers (e.g. primary liver cancer and cervical cancer). Recent studies found bovine leukemia virus (BLV), Epstein-Barr virus (EBV), and human papilloma virus (HPV) more frequently in breast tissues of women with breast cancer vs. those with no breast cancer history.

The objective in this study is to determine if oncogenic viruses in NAF MEC can serve as biomarkers to identify women at high risk for breast cancer. Specific aims are 1) to test the feasibility of detecting genomes of BLV, EBV, and HPV in MEC from NAF using in situ PCR, which allows localization of the signal to individual cells; 2) to determine if viral presence in NAF correlates with viral presence in matching breast tissue from the same donor; and 3) to determine if viruses are present more frequently in women with breast cancer history than those without. The pilot grant from the Dr. Susan Love Research Foundation has enabled us to tackle some of the technical challenges (specific aim #1). The first challenge was to minimize loss of NAF MEC from the slide during the PCR reaction. We found that neither modifications of fixation nor addition of viscous additives could improve adherence when NAF cells were sparse. Therefore, we will use only samples with at least 100 cells per slide to assure adherence of enough cells to analyze. The second challenge is to screen for 3 viruses using one NAF preparation per donor, since that is all that is available to us. We have worked out a scheme whereby all three viruses could be amplified simultaneously and then detected individually with specific DNA probes in one in situ hybridization reaction. Each probe would have a different label incorporated into its DNA, with the final detection signal fluorescing at different wavelengths. We have begun to test this triple label system using three control cell lines each harboring one of the three viruses. Specific aims #2 and #3 involve analysis of patient samples for the presence of the three oncogenic viruses. With co-funding from the Avon Foundation, we have begun the acquisition of matching tissue sections and NAF (cytospun onto slides) from the same donor, provided by Drs. Krishnamurthy and Kuerer at M.D. Anderson Cancer Center in Houston, TX. Women with NAF cell abnormalities are 4–5 times more likely to develop breast cancer than women with no NAF. This pilot study aims to determine if that risk is related to oncogenic viruses and to evaluate the feasibility of detecting these viruses in NAF. The results could be translational, enabling identification of women at higher risk of developing breast cancer, just as HPV screening of cervical samples is now being used.

**S5 Analysis of the presence of cutaneous and mucosal papillomavirus types in ductal lavage fluid, milk and colostrum to evaluate its role in breast carcinogenesis**

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**Background:** The family of the epithelio-tropic human papillomavirus (HPV) comprises approximately 100 different types that have been subgrouped in different genera according to their genomic DNA sequence. In addition, the HPV's can be subdivided in mucosal and cutaneous based on their tissue tropism. Members of the genus alpha, referred to as mucosal high-risk HPV types, have been clearly linked to cervical cancer. Emerging lines of evidence indicate that another group of HPV's belonging to the genus beta may be involved in human carcinogenesis, i.e. non-melanoma skin cancer (NMSC). Several independent studies have suggested the involvement of mucosal HPV types in the aetiology of human breast cancer, while others have reported opposite findings. Here, we have analyzed the prevalence of alpha...
S6 Primary prevention of breast cancer in high-risk women by monitoring epigenetic changes in nipple aspirates

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Breast cancer is the leading cause of cancer death in women in the Western world. In the Netherlands, the incidence is about 12,000 per year, which means that eventually every 9th woman will get breast cancer. The most well-established breast cancer risk factor is the presence of a germline mutation in the BRCA1 or BRCA2 genes, which indicates a life time risk of 45–80% to get breast cancer.

Regular screening by clinical breast examination, mammography and/or Magnetic Resonance Imaging (MRI) is offered to these high-risk women, but one out of four breast tumors are missed by these screening modalities. The most effective form of primary prevention for high-risk women is bilateral mastectomy, which gives a considerable breast cancer risk reduction. As this procedure is highly mutilating, many women opt out and those who decide to undergo prophylactic surgery prefer to postpone it as much as possible. This bears a significant risk of developing invasive breast cancer in the meanwhile. In contrast, the procedure has to be seen as over-treatment in the 15%-55% of BRCA carriers that would never have developed breast cancer. Up to now no procedures are available that accurately predict who of these high-risk women will and who will not develop breast cancer nor, at what age the cancer will occur.

A new primary prevention modality for these high-risk women could very well be found in the analysis of nipple fluid. Nipple fluid, that contains breast epithelial cells, free DNA and proteins secreted by them, is produced in small amounts in the breast ducts of non-lactating women and can be collected in a non-invasive way by vacuum-aspiration. We were the first to prove that intranasal administration of oxytocin enables harvesting nipple fluid in almost all women.

We have set up a clinical trial for genetic monitoring of nipple aspirates as a primary prevention tool for women at high risk of breast cancer. To this end, we once yearly harvest nipple fluid in patients with a hereditary predisposition for breast cancer. In this nipple fluid, we assess promoter methylation of a selected set of tumor suppressor genes known to be involved in breast carcinogenesis. Such epigenetic changes are non-specific, frequent and early changes that are ideally suited for early detection of breast cancer and its precursors. Patients will be monitored until preventive or therapeutic surgery, and epigenetic changes in nipple fluid will be correlated to histopathological findings in the resection specimens. This will allow us to establish in retrospect a specific pattern of epigenetic changes that points to a progression from benign to malignant and therefore indicate the time point for prophylactic surgery in high-risk women. On the other hand, should this pattern never occur, prophylactic surgery could be completely avoided.

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S7 Identification of protein biomarkers in nipple aspiration fluid

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Nipple aspiration fluid (NAF) can be useful for studying relevant changes in breast parenchyma and identification of potential biomarkers for detection, monitoring therapy and risk assessment of breast cancer. NAF has been investigated extensively for identification of protein biomarkers using approaches such as ELISA, immunobassay, western blot for chosen individual protein markers such as prostate specific antigen, basic fibroblast growth factor, prostaglandin E2 etc., or by using comprehensive analysis of protein expression using platforms such as surface enhanced laser desorption ionization-time of flight mass spectrometry. The results of the former studies indicate the potential value of selected markers for detection of cancer which have not been validated in larger studies. The results of majority of studies that performed comprehensive protein analysis indicate that protein expression patterns are highly conserved between cancerous and non cancerous breasts and that protein profiling of NAF may have more value for risk assessment than for detection of breast cancer. Using a novel approach of protein microarray for seven markers with significant differences in protein levels were found between NAF obtained from cancerous and contralateral breast and healthy controls for markers such as cathepsin and...
urokinase. Overall, proteomic studies of NAF indicate the probable value of several protein biomarkers. There is a need for validation of the markers that have been identified so far, to be promising for risk assessment or early detection of breast cancer in large prospective clinical trials.

S8
The unifying mechanism in the initiation and prevention of breast and other human cancers
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Exposure to estrogens is a risk factor for human breast cancer. Experiments on estrogen metabolism, formation of DNA adducts, carcinogenicity, mutagenicity and cell transformation led to and support the hypothesis that reaction of specific estrogen metabolites, catechol estrogen-3,4-quinones, with DNA can generate the critical mutations to initiate breast, prostate and other human cancers. The major initiating pathway is illustrated in Figure 1. Estrone (E1) and estradiol (E2) can be metabolically converted to 4-OHE1(E2) by cytochrome P450 (CYP)1B1. Oxidation of these catechol estrogens leads to the corresponding catechol estrogen-3,4-quinones, which can react with DNA to form very small amounts of stable adducts, which remain in DNA unless removed by repair, and predominant amounts (99%) of depurinating adducts, which detach from DNA, leaving behind apurinic sites. Errors in the repair of these sites can lead to the critical mutations that can initiate breast, prostate and other human cancers.

Cancer biomarkers: The depurinating adducts are shed from cells and tissues into the bloodstream and are excreted in urine. This allows their identification and quantification as biomarkers of risk of developing breast, prostate and other human cancers. High adduct levels have been detected in analysis of urine and serum from women that are at high risk for breast cancer or already have the disease. These analyses are conducted by using ultraperformance liquid chromatography/tandem mass spectrometry, a technology developed in our laboratory. In an initial study of such women, highly significant differences were observed when urine samples from normal-risk women were compared to high-risk women or those with breast cancer. Similar results were obtained in a second, larger study of women with and without breast cancer and at high risk for the disease. In addition, analysis of urine samples from men with and without prostate cancer showed that men with the disease have relatively high levels of estrogen-DNA adducts compared to men without prostate cancer. These results have been confirmed in a second, larger study.

Cancer prevention: Prevention of cancer arises from blocking the first critical step in initiation, reaction of catechol estrogen quinones with DNA. The most appropriate compounds for this task include N-acetylcysteine (NAcCys), which blocks reaction of the quinones with DNA by reacting with the quinones and reducing the semiquinones back to catechols. In addition, NAcCys helps replenish the antioxidant glutathione in cells. Resveratrol has the same chemical properties as NAcCys in reducing the semiquinones to catechols, but also induces the protective enzyme quinone reductase and modulates the activating enzyme CYP1B1. When the MCF-10F human breast epithelial cells were cultured in the presence of 4-OHE2, and resveratrol and NAcCys were added to the culture medium at
different doses, the formation of estrogen-DNA adducts by the cells was greatly inhibited. These two antioxidants also inhibit the transformation of MCF-10F cells and E6 mouse mammary cells to malignant cells. The results of these studies suggest that selected natural antioxidant compounds can reduce formation of estrogen-DNA adducts, presumably decreasing the risk of initiating and, thus, developing breast, prostate and other human cancers.

S9 Relation of hormones in ductal lavage fluid to age, tamoxifen treatment, and breast cancer risk
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Breast ductal lavage provides a source of both cells and fluid from the breast in a non-invasive manner. The fluid contains freely diffusible steroid hormones and other factors that may be secreted or released from the epithelial cells that line the alveoli and ducts of the breast as well as from myoepithelial cells, stomal cells, and immune cells that populate the breast. The purpose of the present study was to determine how perturbations of the system are reflected in ductal lavage fluid (DLF). We examined changes associated with menopausal status, tamoxifen treatment, and Gail model breast cancer risk scores.

Methods: Subjects: Subjects were recruited from the Bluhm Family Program for Breast Cancer Early Detection and Prevention, at the Lynn Sage Breast Center of Northwestern Memorial Hospital. Data on known breast cancer risk factors were used to estimate the 5-year breast cancer risk using the Gail model. Eligible women included (a) unaffected healthy women with a 5-year risk estimate of >1.6 and (b) women with breast cancer who had not received treatment. Only the contralateral breast was sampled in the latter group. The number of patients treated with tamoxifen was 29. A ductal lavage (DL) was performed under local anesthesia in the office setting according to established methods. Women were informed of the cytologic findings and allowed to choose tamoxifen therapy at a dose of 20 mg daily, or observation (OBS) group. None had taken tamoxifen (TAM) or dietary supplements for prevention. All subjects were asked to return for DL 6 months after the first procedure (follow-up lavage). The day of the menstrual cycle was estimated in premenopausal women based on the last menstrual period and the typical length of menstrual cycles among women who had regular menstrual cycles of 26 to 32 days.

Samples and laboratory analyses: The lavage effluent was collected in Cytolyte (Cytyc) and was made up to a final volume of 20 ml. Of this 10 ml was taken for analysis of hormones and other analytes in the fluid. The methanol component was removed in a centrifugal evaporator and sample volume was reduced to 4 ml by lyophilization. Unconjugated steroids were extracted into ethyl acetate-hexane (3:2) and the aqueous fraction was kept for steroid sulfates and proteins. Estrogens were separated from non-phenolic steroids by solvent partition. All analytes were measured by immunoassays. TAM and 4-hydroxytamoxifen concentrations in plasma and NAF were determined by liquid chromatography-tandem mass spectrometry. Data are expressed in terms of the content of the lavage.

Statistical analysis: All data except 5-year breast cancer risk estimates were transformed to their natural logarithms. This provided adequate normalization for parametric analyses. The means, ranges, standard deviations were calculated for pre- and postmenopausal groups and for TAM and control treatments. The intraclass correlation coefficients (ICCs) were calculated for values between subjects (average of both breasts) between breasts and between ducts (both breasts) for the baseline samples. Relationships between DLF analytes and breast cancer risk estimates were determined by multiple stepwise backward regression procedure with probability of entry or removal of 0.1 using the SYSTAT v11 statistical package, Richmond, CA.

Results: Variability estimates: Variation among data obtained between breasts and between visits was determined for first visits (prior to treatment) of all patients. The ICCs between visits (six months apart) were generally as low as those between breasts, indicating stability of measurements over time. The between-subject variance represented, on average, approximately 50% of the total variance and was not different between pre- and postmenopausal women.

Pre- and postmenopausal comparison: A comparison of the geometric mean contents of the analytes in DLF from pre- and postmenopausal women (initial visits) was made. Values of androstenedione, DHEA, and DHEA sulfate in postmenopausal women were highly significantly lower than those of premenopausal women (Bonferroni adjusted P values were all <0.001). The mean values for E2 and estrone sulfate were lower in postmenopausal than in premenopausal women (17.4% and 10.7%, respectively) but not significantly so. Differences in other analytes were not significant. The range of natural log of E2 was 0.45 to 6.91. The natural log of E2 increased significantly with age in DLF of the premenopausal women. The equation with its standard errors was: Age = 1.02 ± 0.48 (ln E2) + 41.6 ± 2.2 years (regression, P = 0.04).

TAM concentrations: Tamoxifen concentrations in plasma were approximately 20-times greater than that of 4-OHT. The ratio of 4-OHT to TAM in the DLF was 4-times higher than that of 4-OHT. The ratio of 4-OHT to TAM in the DLF was 4-times higher than that in plasma.

Effect of TAM on hormone concentrations: The median age of women who chose TAM was 54 yr; the median BMI was 27.1 kg/m², not significantly different from those who chose OBS. The mean Gail score was also not different between the groups. TAM significantly increased the amount of androstenedione and DHEA in the lavage fluid (Bonferroni adjusted P values are 0.040 and 0.037, respectively). The unadjusted P value for cathepsin D was 0.039 but after adjustment for the number of comparisons, it was not significant. A paired comparison of 10 subjects who had samples from both initial and 6 months following TAM visits revealed a 3-fold increase (Bonferroni adjusted P = 0.02) in E2 in DLF during TAM treatment. Other analytes were not different from the group comparisons. Comparisons between first and second visits in the OBS group resulted in no significant differences in any analytes.
Relation of analytes to risk estimates: The association between biochemical DLF factors and Gail breast cancer risk estimates were evaluated by multiple stepwise backward regression. In premenopausal women, DLF analytes accounted for 47.1% of the variability in breast cancer risk estimates. Among the variables, cathepsin D was most significantly related to risk in a negative relationship and DHEA sulfate was next in significance as a positive factor. In postmenopausal women 28.7% of the variability in breast cancer risk scores was accounted for by the analytes in the multiple regression model. Here androstenedione was significantly positively related to Gail scores and DHEA was negatively related.

Relation of estrogen precursors to E2 levels: Androgens and estrone sulfate are potential precursors of E2 in the breast. Overall the squared multiple R value was less for pre- than for postmenopausal subjects, 0.269 vs 0.405, respectively. In premenopausal women DLF estrone sulfate was most highly associated with DLF E2; the association with individual androgens was lower. In postmenopausal women the overall association was higher; androstenedione had the highest association and DHEA sulfate contributed as well.

Summary: These analyses of ductal lavage fluid provide us with a picture of the endocrine environment of the breast which is similar to that provided in our previous studies of nipple aspirate fluid, but we do not see any significant advantages of DLF over NAF in the measurement of these endocrine parameters. However, they support the concept that the local breast environment is a rich source of markers of risk, and measures of the efficacy of preventive interventions which in the future will provide the underpinnings of biologically targeted breast cancer prevention.

S10
Searching new biomarkers in nipple aspirate fluid: peroxidation status and adhesive molecules to early identify breast cancer
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During the last two years (with the support of the DSLRF Grant Award 2007), we have mainly focused our attention on both 1) the involvement of oxidative stress in breast micro-environment and 2) the function of adhesion molecules in the breast cells of the ductal-lobular unit.

1) Starting from our previous evidence that active lipid peroxidation may have a physiologic role in the normal mammary gland and that lower levels of isoprostanes in NAF of BC patients suggest altered arachidonate metabolism (Mannello et al., Int J Cancer 2007;120:1971–6), we have recently discovered that in NAF Phospholipase A2-IIa activity (key enzyme regulating arachidonate metabolism) may play a dual role in breast microenvironment during cancer development and progression.
It shows a beneficial role releasing low Arachidonate levels in physiologic status, while its up-regulation has detrimental functions allowing the conversion of high arachidonate levels into leukotrienes and prostaglandins, promoting BC progression. Noteworthy, the inhibition of this pathway through COX-inhibitors leads to induction of apoptosis and tumor growth reduction (Figure 1).

2) Based on our hypothesis that peculiar junctional complexes may differently seal breast ductal cells leading them to different evolution (Mannello et al., Breast Cancer Res Treat 2007;102:125), we have recently discovered that higher levels of soluble form of P-cadherin were found in NAF from cancer patients. We found a positive correlation of sP-cad with disease stages and tumor grade, and an inverse relationship with estrogen/progesterone receptor status. High levels of sP-cad in cancer NAF suggest its release via proteolytic processing, favouring cancer cell detachment from breast duct.

3) Moreover, we have found that breast epithelial cells floating in NAF of cancer patients contain higher concentrations of Erythropoietin which may be shed in NAF, triggering intracellular signal cascade with subsequent breast cancer initiation. These results may explain the controversies of preclinical/clinical studies about the use of Epo, (i.e., beneficial role improving patient’ survival and negative impact promoting tumor growth progression).

4) We overviewed how Diet nutrients may regulate in breast microenvironment both NAF protein expression and cancer etiology. We also reviewed how the Intraductal approaches assessing metabolic changes in breast microenvironment may help to identify early breast cancer biomarkers in NAF. Finally, we critically overviewed and discussed how the Protein analyses of NAF (mirroring the breast microenvironment) may differentiate healthy women from breast cancer patients.

S11
HAMLET, a tumoricidal molecular complex from human milk
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HAMLET (Human α-lactalbumin made lethal to tumor cells) is a protein-lipid complex that kills tumor cells and immature cells but spares healthy, differentiated cells. The complex is formed from partially unfolded α-lactalbumin and oleic acid, both of which are abundant constituents of human milk. The folding change occurs after removal of Ca²⁺ from native α-lactalbumin, and the partially unfolded protein exposes new fatty acid binding domains, which fit oleic acid. The fatty acid is needed for the tumoricidal activity of the complex, as the unfolded protein alone does not kill tumor cells. HAMLET shows broad anti-tumor activity (>40 different lymphoma and carcinoma cell lines in vitro), suggesting that very basic cell death pathways are activated in tumor cells. The mechanism of cell death is complex but our studies have identified several death pathways activated by HAMLET in tumor cells, including apoptosis, anoikis and autophagy. The complex is internalized and causes rapid mitochondrial destruction. After translocation to the nuclei, HAMLET impairs the transcriptional machinery by high affinity binding to histones and nucleosomes.

We have compared the transcriptomes of carcinoma cells and healthy cells of the same tissue origin and the differences in cellular responses to HAMLET will be discussed. The resistance of healthy, differentiated cells to HAMLET is paradoxical, but healthy cells take up little HAMLET, there is no detectable translocation of HAMLET to the nuclei or evidence of DNA damage. New tumour treatments should aim to destroy cancer cells without harming healthy tissues. Few molecules possess such selectivity, however, and due to their toxicity, current cancer therapies often cause severe side effects. In vivo studies have shown that HAMLET delays the progression of human brain tumor xenografts in a rat model. HAMLET was shown to efficiently remove/reduce human skin papillomas in a placebo-controlled clinical study. Finally, intra-vesical injection of HAMLET killed human bladder cancer tissue, while sparing surrounding healthy tissue. HAMLET thus shows great promise as a new anti-cancer agent. The possible usefulness in breast cancer will be discussed.

S12
Ducts in the human nipple: structure, epithelial immunophenotypes, and relationships with clear cells of nipple epidermis (toker cells)
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It has been suggested that there are structurally distinct ‘type A’ and ‘type B’ ducts in human nipple. If so, properties such as their relative numbers, accessibility at the nipple surface, and relationships with the breast lobes they communicate with could have biological significance and practical relevance for the intraductal approach to breast cancer diagnosis and treatment. To investigate the two-duct-type hypothesis further we examined duct structure and duct epithelial immunophenotypes in the nipples of cancer mastectomies. Relationships between major nipple ducts and clear cells of nipple epidermis (Toker cells) were also explored.

Sections through the nipple duct bundle at the base of the papilla in 10 mastectomy nipples were immunostained for oestrogen and progesterone receptor; proliferation markers Ki-67 and mcm-3; Cox-2; E-cadherin; basal epithelial markers p63, CD10, keratins 5, 14, 17 and luminal epithelial markers keratins 7/8 and 19. In 30 further cases sagittal (and some coronal) sections were immunostained for oestrogen and progesterone; proliferation markers Ki-67 and mcm-3; Cox-2; E-cadherin; basal epithelial markers p63, CD10, keratins 5, 14, 17 and luminal epithelial markers keratins 7/8 and 19. In 30 further cases sagittal (and some coronal) sections were immunostained for keratins 7/8, 14 and progesterone receptor. Duct perimeters were measured for all ducts in transverse sections of the nipple duct bundle in 15 cases.

We did not find unambiguous evidence supporting the two-duct-type hypothesis. Duct perimeters at the base of the papilla were variable but did not apparently define separate populations of larger and smaller ducts; nor could separate duct populations be recognised on their epithelial immunophenotype. However, while some ducts open directly into a funnel-shaped infundibulum, others apparently taper to a pinpoint lumen, and may cluster around a common opening.

Clear cells of nipple epidermis (Toker cells) were present in variable numbers in 31/40 cases, usually around duct openings on
the nipple surface, sometimes show features suggesting possible locomotion, and are occasionally present in keratin plugs. Similarities between clear cells of nipple epidermis and mammary precursor cells have been noted previously; their location and possible motility suggest new avenues for research.

S13
Anatomical association of fluid yielding ducts with location of the breast cancer affected segment in screen detected and symptomatic breast cancer
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Introduction: The concept of the intraductal approach to evaluating the breast microenvironment assumes direct access to the breast cancer containing duct. Previous studies on duct anatomy infusing a dye or contrast found fluid-yielding ducts to be associated with the cancer containing duct in approximately 50% of fluid-yielding breasts with cancer. A concordant anatomical relationship between accessible ductal systems and the cancer-affected lobe is essential if cytology or other cell markers are to be successfully identified as indicators of cancer or of early cancerous change. The concordant relationship is less important if field change effects are considered to predictors of malignant change. The aim of this study was to determine how often duct lavage effluent drains the breast cancer affected segment.

Methods: 40 patients undergoing therapeutic mastectomy for breast cancer were studied (31 symptomatic and 9 screen-detected). Following successful ductal lavage, the mastectomy specimens were infused ex vivo with coloured polyurethane elastomer resin (VasQtec, Zurich). The extent of specimen infusion with resin and the direct anatomical relationship to the cancer affected segment were recorded.

Results: The median number of successful ducts cannulated per cancer affected breast was 2 (range 1–3). 23/40 (58%) therapeutic mastectomy specimens showed successful tracing of the cancer-affected duct system. 5/38 (13%) resin infusions traced duct systems unaffected by cancer and the remaining 12/40 (30%) infusions extravasated. Of the 23 successful tracings, 16 mastectomy specimens contained symptomatic cancer and the remaining 7 were screen-detected nonpalpable cancers. All 12 extravasated infusions occurred in specimens with symptomatic cancer. 7/23 successful infusions showed abnormal cytology concordant with the cancer affected segment.

Conclusion: Breast duct systems of both symptomatic and non-palpable cancer were equally accessed at successful ductal lavage. The duct system of the cancer affected segment was concordant to a fluid yielding duct patent in 58 percent of cases, a similar finding to previous studies. Mastectomy specimens containing clinically impalpable disease were more likely to remain patent than those with palpable lesions, implying distal duct collapse following duct obstruction by larger tumours impedes access to the cancer affected lobe. Future studies that depend on direct access to the cancer affected segment (e.g. cytology) are likely to be limited by duct accessibility while studies that demonstrate field change effects (e.g. protein studies) are less dependent on duct patency.

S14
Mammary ductoscopy: past, present, future
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Mammary ductoscopy is a powerful modality with enormous potential to help improve patient care through enhancing the diagnosis and treatment of patients with breast disease. The ability to see ductal pathology and monitor response to therapy real time has enormous potential to expand our understanding of disease processes. Technological improvements involving optics, scope design, ductal access and interventional ductoscopy becoming feasible will help move this field forward. Historical developments, current applications, personal experience, and future directions will be discussed.

S15
Therapeutic value of a new scarless intervention, ductoscopic papillomectomy, in patients with pathologic nipple discharge
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Background: Efforts have been made to develop new methods to improve the diagnosis of intraductal lesions and their surgical removal. We evaluated the therapeutic value of a new scarless operation, endoscopic papillomectomy. (EP) in patients with pathologic nipple discharge (PND).

Methods: Breast ductoscopy was performed on 213 female patients with PND. These patients underwent a variety of appropriate ductoscopy-assisted (DA) endosurgical interventions, combined with cytologic examinations. Success was determined by recurrence of PND and by standard radiological examinations.

Results: Ductoscopy was successfully performed in 178 patients. Of these 178 patients, 34 had solitary papillomas (SP), 8 had multiple papillomas (MP), 19 had intraductal debris and 5 had ductal epithelial surface abnormality with positive cytology. Of the 34 polyoid lesions (cytology negative), 22 were excised endoscopically (endoscopic papillomectomy). Patients with MP underwent DA-microdochectomy. These 42 patients (34 with SP, and 8 with MP) had histopathology results consistent with intraductal papillomas or papillomatosis. Five patients with positive cytology underwent DA-terminal ductal lobular unit excision after their histopathology reports showed had DCIS. Except in one patient, all discharges disappeared. After a mean ± SD follow up time of 14.4 ± 5.2 months (range, 1–27 months), there were no recurrences of nipple discharge and no radiological results suggestive of malignancy. Thus, the therapeutic efficacy of EP in our study was 95.4% (21/22).
**Conclusion:** Ductoscopy is not only a diagnostic procedure, but is also therapeutic for breast papillomas. EP is a new scarless treatment option for patients with PND (1,2). With technical improvements in ductoscopy and endoscopic sampling instruments, a greater degree of therapeutic efficacy will be achieved.

**S16**

**Ductoscopy technique – using ductoscopy anatomically direct breast conservation surgery**

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Ductoscopy began as a way to identify the primary lesion in pathologic nipple discharge. The technology evolved to allow fairly easy access and adequate visualization for this task in a number of centers worldwide. The next step would have been to excise or substantially biopsy the lesions through the scope or with scope localization and not require open surgical biopsy. Cytologic samples could be obtained adequately but the size and diagnostic capability of the tiny histologic samples was rapidly found to be at about the limits of our technical ability. As a second observation, it was found that in small breast cancers, the duct containing the tumor could often be identified by expressable fluid and ductoscopy could be used to aid in complete removal of associated intra-luminal proliferative lesions. Some of these lesions, in the same ductal tree, seem to account for multi-focality. In a large series at Johns Hopkins and University of Oklahoma, I was able to show dramatic reductions in hazard rate for local failure with a 9 fold fall in local failure when the lumpectomy had been guided by ductoscopy. Many of the lesions identified and excised along with the cancers were proliferative but would not have normally been completely excised following histologic criteria for adequate lumpectomy. Ductoscopy segregated breast cancers into two groups: 1. Those with extensive associated proliferative disease requiring large segmentectomy to remove all intra-luminal disease, and 2. Those with minimal associated proliferative disease requiring minimal volume resection. For this last group, we have investigated minimal access excision devices as an alternative approach to their excision with early success which will be shown. Current scopes allow for mapping of the layout of the breast ducts but still fail in abilities to navigated tight turns and get direct view multiple and adequate histologic biopsy. Pilot engineering approaches to address these issues and develop a more useful imaging scope with better biopsy and working channel capabilities will be compared and discussed.

**S17**

**Impact of mammary ductoscopy in breast surgery**

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**BMC Proceedings 2009, 3(Suppl 5):S17**

Mammary ductoscopy (MD) allows visualization of the lactational duct and the morphology of various ductal pathologies can be identified. It provides a new approach for clinical research.

The main impact of MD is on the modification of the extent of surgery. MD was used to guide the extent of duct excision in patients with pathological nipple discharge. Multiple duct lesions detected by MD can be excised completely. MD was also used to guide resection in breast-conserving surgery for breast cancer. There was less proximal margin involvement with the use of MD. MD also helps to spare unnecessary surgery. In patients with nipple discharge, duct excision can be avoided in those with a normal ductoscopy. Nipple-sparing mastectomy (NSM) is cosmetically superior to traditional mastectomy but there are concerns about the oncological safety with nipple-areola preservation. MD can be employed to exclude major duct involvement and this may help to select patients for NSM. We are currently studying the role of MD in identifying major duct involvement in potential patients undergoing NSM. MD has technical limits. MD cannot examine the distal duct and biopsy facility is limited. No therapeutic facility is available at present but these technical limits will be resolved when the next generation of MD is available.

In conclusion, MD has made a significant impact on breast surgery. The technology is developing and with continued research, the role of MD in breast surgery will continue to expand.

**S18**

**Intraductal micro magnetic resonance imaging and spectroscopy**

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**BMC Proceedings 2009, 3(Suppl 5):S18**

The majority of breast cancers originate in the mammary ducts. As such many diagnostic techniques introduce mm-diameter devices into the ducts through the nipple. Established intraductal techniques include ductal lavage, and white-light or fluorescence endoscopy. While reported values vary, these techniques typically suffer from low sensitivity and specificity. Similarly, Magnetic Resonance Imaging (MRI) for breast oncology suffers a high rate of false positives. Magnetic resonance (MR) spectroscopy is highly effective in distinguishing between benign and malignant tissue. However, clinical relevance is limited to late-stage of disease due to a required cubic-centimeter volume for diagnosis. Small-volume sensitivity for spectroscopy and imaging is possible through use of a miniature radio-frequency (RF) coil. An intraductal RF microcoil reduces the required volume of diseased tissue to a cubic-millimeter, bringing spectroscopy to early stages of early diagnosis. Micro-scale images from an intraductal microcoil may enable high-precision localization of tumors and tumor margins. The microcoil catheter was designed with an approximate diameter of 1 mm for intraductal use and for...
possible integration with ductoscopes. Experimental verification of the first-generation coil design was achieved through ex vivo MR imaging of tissue. As expected, the microcoil provided microscale images. While 3-T (128 MHz) MRI typically provides 1 to 30 voxels per cubic mm the MRI microcoil can provide hundreds, and even thousands of voxels in the same volume. The first generation microcoil consisted of a 1-mm-diameter solenoid with 4.5 turns, spaced 0.05 mm apart, leading to 25-mm-long parallel leads, with one of the leads passing through the center of the solenoid. A crucial requirement for optimal MR coil design is a homogenous magnetic field generated in the coil. Unfortunately, the central conductor inside the solenoid of the original microcoil greatly disturbed the homogeneity of the magnetic field. The parallel leads of the first generation device correspond to a popular catheter-coil geometry, a single-loop coil. The parallel leads therefore collect unwanted signal, in other words noise. The new design overcomes both of these flaws. Turn-spacing of the first microcoil design was intended for optimizing the signal collected from the center of the solenoid. However, since the target tissue is at the tip of the solenoid, the new design is optimized for our target tissue with ten turns separated by the thin insulation of the wire. The turn-spacing of the first generation microcoil was controlled by a thick layer of insulation that also provided a good barrier from the body. Due to the lack of a thick insulator, the second generation-design required a new configuration for isolating the coil from the body to prevent degradation of the performance over time. We analyzed the stability of the new microcoils by soaking 5 microcoils in body temperature saline and measuring the impedance at 128 MHz over time. The impedance of the microcoils remained constant for nearly 2 hours, and increased only slightly after 4 hours. Preliminary heating experiments indicate that little to no heating occurs during use of the microcoil as an MR transceiver. Initial imaging results confirm that the second generation microcoil improves greatly on the first generation coils, with significantly less imaging artifacts despite having twice the lead length.

Recent success by Dr. Love with intraductal chemotherapy application intensifies the need for early diagnosis. A microcoil may provide spectroscopic evidence of cancer. Microcoil images may show if the malignancy has breached the ductal lining. The open lumen of the intraductal microcoil enables clinicians to target sub-branches of ducts with chemotherapy.

S19 Hypermethylated genes as biomarkers of breast cancer
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Hypermethylated genes are emerging as some of the most promising, practical and powerful biomarkers for breast cancer detection and perhaps even of risk assessment. We have assembled a panel of methylated gene detection markers and tested them by Quantitative Multiplex Methylation-Specific PCR (QM-MSP) in breast cancer. Here we two pilot studies: 1) detection of cancer in DNA from spontaneous nipple discharge (SND) and 2) assessing risk, and its possible reduction in contralateral breast tissue of women undergoing treatment with aromatase inhibitors.

SND is most frequently caused by intraductal papilloma (66%), but in a minority of women (~10%) it is caused by ductal carcinoma in situ (DCIS). Therefore essentially all women with SND are screened for breast cancer. All the current methods used for screening exhibit low sensitivity and specificity for detection of cancer. Mammary endoscopy (ductoscopy) appears to improve localization of lesions in patients with SND and allows retrieval of intraductal cells for diagnostic purposes. Development of a non-surgical method to reliably diagnose cancer would offer the possibility of diagnosis without surgery for the majority of women with SND who have a very low likelihood of significant neoplasia. Further, a reliable diagnostic test of intraductal pathology will enable in-situ ablation of benign lesions with either endoscopic techniques or the intraductal administration of anti-neoplastic agent. In a pilot study we investigated whether quantitative assessment of gene promoter hypermethylation could enhance detection of breast cancer in women with spontaneous nipple discharge (SND) when used in conjunction with ductoscopy. Ducts with significant visualized lesions were surgically resected (36 ducts in 33 women) and those with minimal findings were not (28 ducts in 16 women). QM-MSP data of DNA from cells in ductoscopic washings were compared to ductoscopy findings, cytology, and tissue histology. Cells from ducts with significant ductoscopic findings had higher levels of methylation while cells found in ducts with minimal findings had minimal methylation; methylation was higher in cells from ducts with malignant lesions compared to low methylation in ducts bearing benign lesions such as papilloma. RASSF1A, TWIST1, and HIN1 cumulative gene methylation accurately distinguished cells washed from ducts with cancerous vs. benign lesions (100% sensitivity, 72% specificity and AUC of 0.91 according to ROC analyses). Using QM-MSP the positive predictive value of ductoscopy more than doubled because QM-MSP has three-fold higher sensitivity than cytology in evaluation of ductal cells. This study demonstrates the potential benefit of targeting surgical ductal excision to ducts that have both high methylation and significant abnormalities on ductoscopy. Future large-scale studies to validate this approach are needed.

Women with a history of breast cancer are at increased risk to develop a contralateral breast (CLB) cancer. Since gene methylation occurs early in tumorigenesis, and is frequently higher in normal tissues adjacent to a breast tumor, we hypothesized that women with a prior breast cancer would harbor higher levels of methylated genes in the CLB and that treatment with anastrozole, an aromatase inhibitor that reduces the risk of CLB cancer, would decrease methylation levels. We conducted a prospective, single-arm study in 54 postmenopausal women with hormone receptor-positive stage 0–III breast cancer who had completed local therapy, had an intact CLB, and would receive anastrozole as their sole adjuvant therapy. Of those, 33 women underwent an optional CLB biopsy both at baseline and 6 months after initiating Anastrozole. At baseline, 84% of paired samples had measurable cumulative methylation of the 6 gene panel; TWIST1, RASSF1A, and RARB were most frequently methylated. After 6 months of anastrozole, we observed significant decreases in
methylation for TWIST1, RASSF1A, and RARβ, among patients with methylation identified at baseline. These preliminary findings emphasize the need for prospective evaluation of the relationship between changes in methylation and incidence of breast cancer in high-risk women.

**S20**

A direct comparative study of methylation-specific PCR in ductal lavage fluid, breast cancer tissue, normal breast parenchyma and plasma in women with early breast cancer

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**Introduction:** Breast duct lavage for analysis by methylation-specific PCR is a novel but established method for detecting the presence of cancer and may contribute additional information about prognosis and response to treatment. The aim of this consecutive series of breast cancer patients was a case-control study to evaluate qualitative methylation of five published tumour suppressor genes in breast cancer tissue, adjacent normal breast parenchyma, duct lavage fluid and plasma.

**Methods:** Breast cancer tissue and adjacent normal parenchymal tissue was obtained at the time of surgery in 24 women. Normal breast tissue from 22 patients undergoing breast surgery without breast cancer were used as controls. In all patients, tissue cores, matched plasma, ipsilateral and contralateral duct lavage fluid were obtained for methylation-specific PCR. DNA was purified from microdissected tissue, plasma and breast duct biofluids using the QUIAGEN DNeasy kit. Purified DNA was then modified (EZ Zymo methylation kit) for detection of methylated products by methylation-specific PCR, with a significant difference in rates of DNA methylation in duct lavage of cancer patients versus controls. These differences were unrelated to plasma methylation profiles, limiting blood testing as a biomarker of breast cancer progression. The inclusion of other tumour suppressor genes in future studies may increase the sensitivity and specificity of breast-specific biofluids in subclinical cancer.

**Results:** Methylated DNA products were qualitatively scored and a positive correlation identified between tumour tissue and ipsilateral duct lavage in the breast cancer group (n = 24): HIN-1 (58 and 50%), RIL (63 and 42%), RASSF1A (71 and 54%), CDH13 (42 and 33%) and RARβ2 (38 and 25%). Methylation was significantly higher in tumour and ipsilateral duct lavage fluid when compared with adjacent normal tissue and contralateral duct lavage. (See Table 1).

Methylation was less when scored as accumulated methylation events in benign breast tissue and duct lavage of 22 non-cancer patients: HIN (4% and 4%), RIL (8% and 0%), RASSF1A (13 and 0%), CDH13 (13 and 4%), RARβ2 (0%). No difference was found in plasma methylation of cancer patients versus controls.

**Conclusion:** Our data demonstrates the feasibility to modify purified DNA from cellular breast duct fluid for detection of methylated products by methylation-specific PCR, with a significant difference in rates of DNA methylation in duct lavage of cancer patients versus controls. These differences were unrelated to plasma methylation profiles, limiting blood testing as a biomarker of breast cancer progression. The inclusion of other tumour suppressor genes in future studies may increase the sensitivity and specificity of breast-specific biofluids in subclinical cancer.

**Table 1 (abstract S20)**

<table>
<thead>
<tr>
<th>CANCER (n = 24)</th>
<th>CONTROLS (n = 22)</th>
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</thead>
<tbody>
<tr>
<td><strong>Tumour tissue</strong></td>
<td><strong>Normal tissue</strong></td>
</tr>
<tr>
<td>HIN-1</td>
<td>14</td>
</tr>
<tr>
<td>RIL</td>
<td>15</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>17</td>
</tr>
<tr>
<td>CDH13</td>
<td>10</td>
</tr>
<tr>
<td>RARβ2</td>
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</tr>
</tbody>
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Ip = Ipsilateral; Contra = Contralateral; DL = ductal lavage.
Mammary epithelial cells destined to progress to cancer may have accumulated a number of premalignant genetic changes, prior to the onset of cancer. Therefore, it may be possible to improve upon cytologic assessment of these cells by simultaneously screening for genomic aberrations. This provides a realistic opportunity to improve the detection of premalignant breast changes prior to the onset of cancer. There have been several reports showing the feasibility of studying individual, or a combination of two biomarkers, to assess the ductal fluid. However, no studies have been performed where a panel of multiple biomarkers were evaluated simultaneously, using the same fluid sample. This is particularly significant since it is not clear which biomarker(s) will be most useful for early detection of breast cancer, making a panel of markers more desirable than an individual one. We believe that we can significantly improve the diagnostic value of ductal fluid by maximizing the information one can derive from each specimen.

S22
Intraductal assessment to determine response to chemopreventive interventions
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One of the difficulties that translational scientists encounter in evaluating agents to prevent breast cancer is the assessment of efficacy in real time. Classically, one evaluates chemopreventive efficacy based on whether the intervention leads to a lower number of cancers in the treatment than in the control group(s), but these studies require a large sample size and/or take years to complete in order to obtain a sufficient number of events for statistical comparison. We have used intraductal evaluation of breast nipple aspirate fluid (NAF) and mammary ductoscopy (MD) to assess response to both pharmacologic and nutritional chemopreventive interventions. Specifically, we have measured expression of specific proteins in NAF and changes in DNA methylation in MD specimens both before after the interventions. I will review our findings using these strategies, and discuss both the strengths and limitations of the approach.

S23
Impact of diet and exercise intervention on breast ductal fluid among overweight postmenopausal women
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Obesity and adulthood weight gain, operating through production of estrone in adipose tissue, inflammation, and obesity-related growth factors, increase breast cancer risk after menopause. Many studies have determined the systemic effects of excess adipose tissue on plasma and serum-based biomarkers, but few studies have determined the localized impact on biomarkers contained in breast ductal fluid.

We are currently conducting a pilot 12-week diet and exercise weight-loss intervention among overweight postmenopausal women to evaluate changes in ductal fluid as a result of the intervention. To date, five women completed the study. We successfully obtained breast ductal fluid both before and after the intervention from four of the five women. Fluid was transferred into tubes and frozen in a −80° freezer. We are currently conducting assay measurements of hormones and biomarkers contained in ductal fluid.

We observed changes in body composition and exercise parameters as a function of the intervention. Specifically, a 12% decrease in BMI (body-mass index), a 14% decrease in body fat mass, a 28% increase in aerobic fitness and 28% increase in muscle-strength occurred compared to baseline intervention measurements, among the women who completed the study. Our plans are to enroll five additional women, finish analyzing the ductal fluid biomarkers, and apply for a larger grant to expand the study.

S24
An intraductal approach to breast cancer risk assessment and screening in a diverse population
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The Gail Risk model was developed based on data from Caucasian women. Little information is available regarding breast cancer risk assessment in women of other ethnicities. Our study invited asymptomatic women of color presenting for screening mammography, to be interviewed with a risk questionnaire and to undergo aspiration of nipple fluid (NAF) for cytology. The HALO® system was used to aspirate the nipple fluid.

Thirty three patients participated in the study, including 24 African American, 7 Asian, and 2 Hispanic women all of whom completed the questionnaires. Twelve patients had NAF; and three of those patients had bilateral NAF aspirations, a total of 15 specimens. Ten of the twelve patients with NAF were African American, and all three of the patients with bilateral NAF were African American. The ages of the 33 patients in the study ranged from 40 to 72 years, with an average age of 53, while the patients with NAF were ages 40 to 60 years with an average age of 51 years.

The age of menarche and prior use of oral contraceptives were similar in the NAF and Non-NAF groups. Only 7 of the 33 patients had a history of using any type of HRT. Only two patients had a history of fertility medications. No patients had DES exposure. Fourteen patients had a history of cigarette smoking, including 6 of the NAF patients and 8 of the non-NAF patients. Twelve patients had a family history of breast cancer, 5 with NAF and 7 without NAF, with a higher rate in the NAF group (71%) vs. the non-NAF group (50%). There were 20 patients who stated that they were postmenopausal, and 15 of those patients were in the non-NAF group.

No patients with NAF had abnormal cytology. Eleven of these samples had no ductal epithelial cells present, and the other
4 samples had rare or scant numbers of epithelial cells present. All of the samples had Thin Prep Concentration A used for slide preparation, but only three samples had adequate fluid available for cell block. Three patients (one with NAF and two without NAF) had abnormal mammograms and are having additional imaging.

We have shown that it is feasible to engage women of color in a study of breast cancer risk factors and aspiration of nipple fluid for cytology. Oakland, California is a very diverse community with multiple ethnic groups living in the city. One challenge with the Hispanic and Asian women was related to language barriers, and in a larger study a greater effort will be needed to be able to involve more patients from these communities. We were disappointed in the low number of patients with NAF samples and the adequacy of the samples, and are evaluating the issues that contributed to that problem.

**S25**

**The importance of MUC1 and cyclin B1 antibodies in nipple aspiration fluid (NAF): preliminary results**

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MUC1 glycoprotein is produced by normal breast epithelial cells and cyclin B1 is involved in the transition from G2-to-M phase of the normal cell cycle. In cancer cells, including breast cancer, MUC1 and cyclin B1 are overexpressed and aberrantly expressed leading to their recognition by the immune system and production of specific antibodies detected in the serum of cancer patients. Because MUC1 and cyclin B1 are expected to be deregulated early in disease, we hypothesized that monitoring these antibody responses may be of diagnostic or prognostic value in breast cancer (BC). Furthermore, we wanted to test if NAF can be an alternative source of these antibodies.

**Objective/hypothesis:** The hypothesis is that NAF would contain anti MUC1 and anti-cyclin B1 antibodies in patients with BC and premalignant lesions in comparison with healthy women.

**Aim:** The aim of this project was to test NAF for the presence of anti-MUC1 and anti cyclin B1 antibody in patients with BC, premalignant lesions and healthy women.

**Materials and methods:** We collected the pretreatment NAF of patients with BC, non invasive tumor, premalignant lesions (atypical ductal-, and lobular hyperplasia), and also healthy women. Anti MUC1 and anti cyclin B1 antibody levels were measured by an enzyme linked immunosorbent assay (ELISA). NAF samples were taken from breasts with disease and the contralateral healthy breast, and breasts of healthy women operated upon for benign breast diseases. The mean level of the antibody levels of patients and healthy controls in NAF were compared by a t-test. The discrimination of the antibody levels of patients and healthy controls in NAF were assessed by calculating the area under (AUC) the receiver operating characteristic curve (ROC).

**Results:** A total 82 NAF samples from 50 patients were collected; 35 NAF samples were collected from breasts with invasive cancer, 12 samples with ADH and ALH, 5 samples with DCIS and LCIS, 2 samples with benign lesions and 28 samples from healthy breasts. There were no statistically significant difference between the invasive tumor, high-risk lesion and control groups, but AUC under the ROC was 0.78 and 0.69 in anti MUC1 and anti Cyclin B1 IgM groups between non-invasive tumor group and controls; it is generally accepted that AUC values 0.7–0.8 represent good discrimination.

**Conclusion:** This is the first study investigating anti-MUC1 and anti cyclin B1 antibodies in NAF of BC patients. Even though the sample size is still small and additional samples are being accumulated, finding the tumor specific IgM in the NAF of the non invasive patients is encouraging. Development of more sensitive techniques for antibody detection may allow detection of low antibody levels in the non invasive tumor and premalignant lesions and boost the usefulness of NAF as the source for this diagnostic assay. Combining NAF biomarkers with clinical parameters such as breast density and scoring systems for the clinical judgment of breast tumors may be clarified by future studies.

**S26**

**Preliminary exploration into the physiology of the resting breast**

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**Background:** Epidemiological and animal data are clear that early first pregnancy decreases subsequent breast cancer risk. The mechanism for this decrease however is less clear and several hypotheses abound. One possibility that has not received any attention is the physiology of fluid secretion in the breast. The ductal systems have secretions which can be accessed either through nipple aspiration or ductal lavage. It is not clear whether the mechanisms identified for absorption, secretion, concentration and local synthesis that have been described in the lactating breast are consistently available to the non lactating breast or whether there is a difference in nulliparous and parous women. It is not clear whether the known changes to the ductal-alveolar system with the first pregnancy remain permanent or have long lasting ramifications. The hypothesis tested was that the first pregnancy permanently changes the physiology of the ductal epithelial membrane transport. Two known drug transport mechanisms in lactating women were tested using caffeine and cimetidine.

**Methods:** A total of 14 women were recruited for this IRB-approved prospective study to undergo blood collection, nipple aspiration and ductal lavage five times over 12 hours. Of these women, 5 were postmenopausal and 9 were premenopausal; 8 were parous and 6 nulliparous. Subjects were asked to abstain from caffeine and cimetidine for 24 hours prior to participation. After a baseline was recorded; subjects were then given 200 mg of caffeine (NoDoz) and 200 mg of cimetidine (Tagamet) and the procedure was repeated at set time points 4 more times over 12 hours. Samples were sent to a central laboratory for analysis where caffeine and cimetidine were quantified in serum, nipple aspirate fluid (NAF) and lavage.
Results: There were significant and intriguing results regarding the differences between the “resting” or non lactating breast and the lactating breast. In lactating women caffeine passively diffuses into milk rapidly and reflects serum levels. In resting breasts caffeine levels generally peak at 6 hours or later after ingestion. Cimetidine, on the other hand, is known to be concentrated in milk in the lactating woman but was not detected in ductal fluid from the resting breast. Since cimetidine is known to be actively transported in the lactating woman, this pattern is consistent with a transporter protein which is transcribed only during lactation. The concentrations and time course of drugs in NAF and DL also seem to differ suggesting some physiological difference other than dilution. There was a significant difference between parous and nulliparous women in terms of caffeine concentrations and uptake. Finally preliminary analyses of injected mannitol into breast ducts are still undergoing investigation to better understand the bidirectional transfer of drugs.

Conclusion: Our study has reinforced our opinion that the physiology of the “resting” breast is understudied and the transport mechanisms of drugs, vitamins or other nutrients in the non-lactating breast may be very different than the known mechanisms in a lactating woman. While different transport mechanisms, including passive diffusion, carrier-mediated and transcytosis have been identified in the lactating breast no one that we are aware of has studied these routes in the female “resting” breast. Our pilot data with three drugs has lead to more questions than answers that we plan on probing further. This information is not only critical for potential understanding of systemic drug delivery to the breast for prevention or early intervention but also for the growing field of intraductal therapy.

Acknowledgements
Grant funded by the Avon Foundation.

S27 Intracutal pegylated liposomal doxorubicin may achieve long term protection in HER2/NEU transgenic mice by restricting mammary gland outgrowth
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BMC Proceedings 2009, 3(Suppl 5):S27

This work is based on the hypothesis that intraductal injection can treat existing lesions and prevent future breast cancers originating in the epithelial cells lining the breast ducts. We have previously demonstrated our ability to access the entire mammary gland through the tear in mouse and rat mammary tumor models. Further, we showed that several other common chemotherapeutic drugs such as 5 fluorouracil, carboplatin, and methotrexate were effective, but not to the same extent as PLD. Thus, intraductal injection of PLD has potential in the prevention and neo-adjuvant therapy of breast cancer.

Recently, we observed that in addition to rendering the mammary glands tumor free for more than 3 months after all of the control animals had developed multiple tumors, mammary glands of PLD treated mice were stunted in their growth. Instead of the florid growth with multiple branches and side branches studded with terminal end buds, akin to a “spring tree” seen in normal glands the PLD treated mammary gland presented the aspect of an “autumn tree”. In the transgenic mice, the epithelial cells in the entire mammary gland express the MMTV-Her2/neu transgene and at even higher levels during pregnancy. This raised the concern that stimulation of proliferation of these cells, for example, by pregnancy, may result in a higher incidence of spontaneous mammary tumors in the PLD treated mice.

What then is the response of this treated mammary gland to a new pregnancy? To test this concept, we induced pregnancy in PLD treated mice. Her2/neu mice treated with intraductal PLD, like their untreated controls, had normal deliveries, with pups of normal weight and number in the litter. However, unlike control mice, their pups survived for less than one week. Long term follow-up showed that pregnancy did not increase the incidence of tumors in PLD treated mice. Their mammary glands showed a very poor proliferative response to the pregnancy hormones and remained stunted in their growth. It is likely that PLD not only affected the eradication of preneoplasias but also resulted in a depletion of normal mammary gland stem cells. This concept is being tested by determining the difference in stem cell content between untreated and PLD treated glands by transplantation of serial dilutions of epithelial cells from sham and PLD treated glands into cleared mouse fat pads, examination of markers of stem cells, such as ALDH, etc. If proven to be the case, these findings raise the possibility that long term protection may be achieved by intraductal injection of PLD to women at high risk of developing breast cancer.

S28 A phase I study assessing the feasibility and safety of intraductal pegylated liposomal doxorubicin (PLD) in women awaiting mastectomy
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Background: Our preclinical data have demonstrated that intraductal administration of PLD decreases tumor volume, prevents the development of new lesions, and eradicates pre-malignant disease. We initiated a phase I study to determine the feasibility, safety, and maximum tolerated dose of PLD administered to women awaiting mastectomy.

Methods: Women 18 or older with a known breast cancer awaiting a mastectomy were eligible. Neoadjuvant chemotherapy was allowed. Women with T4 features, prior breast irradiation, or procedures that in the opinion of the investigator may have altered the breast ductal system were excluded. Participants underwent nipple aspiration and ductal cannulation using a dose escalation schema. The first 3 women received 5 mL intraductal dextrose only. We determined serial doxorubicin and doxorubicin concentrations in plasma and nipple aspirate fluid using LC/MS/MS. We injected blue dye into the treated duct just prior to mastectomy and obtained tissue for pharmacokinetic and biomarker analysis.

Results: From 02/06 to 09/08, 14 women entered the study, and 12 underwent study procedures successfully. We completed all dose levels up to 10 mg PLD per one duct without serious
adverse events or surgical delays. Pharmacokinetic and representative histopathological data will be presented.

**Conclusion:** Intraductal administration of PLD is feasible and can be safely administered both in women with and without prior chemotherapy awaiting mastectomy. Studies to evaluate other agents administered to one or more ducts are required.

**S29 Local drug delivery to the breast: a phase I study of breast cytotoxic agent administration prior to mastectomy**

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**Background:** Intraductal administration of cytotoxic agents has been shown to inhibit the development of breast cancer in Her-2/neu over-expressing mouse and MNU rat models. This dose escalation study was performed to demonstrate the safety of this approach in women prior to mastectomy.

**Methods:** The study was performed in Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China where the standard of care includes a long preoperative hospital stay prior to mastectomy. Two drugs, pegylated liposomal doxorubicin (PLD) and carboplatin (C) were administered at 3 dose levels (PLD: 10, 20, 50 mg and carboplatin 60, 120, 300 mg) with the highest dose approximating the clinical intravenous dose. There were five subjects in each group with 15 subjects treated with each drug. Study drug was administered once per subject. On obtaining informed consent, subjects underwent a local nipple block and cannulation of 5–8 ducts with intraductal instillation of the drug. Venous blood samples were obtained for pharmacokinetic analysis. The total dosage was divided by the number of cannulated ducts to yield a dose per duct. The breast was removed surgically as planned 2–5 days post treatment and the treated ducts were marked to enable identification on pathological evaluation.

**Results:** Intraductal administration was generally well-tolerated with mild, transient breast discomfort upon administration associated with the rate of infusion. Clinically significant laboratory adverse events were limited to decreases in hemoglobin following mastectomy, consistent with blood loss. Neither leucopenia nor thrombocytopenia were observed in the study. In the carboplatin arm, three women at the 300 mg dose experienced mild nausea and vomiting. In the PLD arm most women had mild erythema and swelling of the breast over the 72 hours following the drug administration while the women receiving the highest dose experienced local erythema until the time of surgery.

Pharmacokinetic analysis showed that carboplatin rapidly entered systemic circulation with an early peak time (tmax~30 min) with a resultant PUF AUC (area under the curve) consistent with the Calvert Formula using estimated GFR. Total plasma doxorubicin had delayed peak concentration times (tmax >36 hours) with a linear dose response and peak concentrations substantially lower than expected from equivalent IV dosing. No doxorubicin metabolite was detected in the plasma.

Pathological examination showed the drugs were widely distributed throughout the ductal systems reaching terminal duct lobular units, and there was a significant although variable dose-related epithelial cell loss in ducts with dye indicating drug effect. While dye was seen in or near the cancer areas, the effect of drug treatment on the disease could not be distinguished.

**Conclusion:** This study demonstrates that cytotoxic drugs can be easily administered into breast ducts with minimal toxicity.

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**S30 Histopathological responses to a short term intraductal cytotoxic agent treatment: results of a feasibility study**

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**Background:** Previous animal model studies from Sukumar and others demonstrated the effect of intraductal administration of cytotoxic agents in preventing the occurrence of breast cancer. To determine the feasibility of the approach in humans, a preliminary study was performed in patients who were diagnosed with breast cancer and were waiting for mastectomy. Two drugs, carboplatin and Pegylated Liposomal Doxorubicin (PLD) were tested. These drugs were selected based on the results of preclinical studies. This report presents findings of histopathological examination of breast tissue taken from patients received intraductal administration of these two agents.

**Methods:** This was an uncontrolled observational dose escalation study. A total of 31 subjects undergoing mastectomy for breast carcinoma were included in this study. There were 15 subjects in the Carboplatin arm, and these 15 were divided into 3 dosage groups (60, 120, and 300 mg), with 5 subjects per dosage group. There were 16 subjects in the PLD arm (one patient was withdrawn because of a central lesion which precluded duct cannulation) and they were also divided into 3 dosage groups (10, 20, 50 mg). The 30 subject’s age ranged from 25.9 years to 75.6 years. After intraductal injection of drugs into patients for at least 24 hours (up to 5 days), mastectomy was performed and specimens were processed in the pathology laboratory. Specific attention was paid to compare the cannulated ducts (dye stained ducts) with non-cannulated ducts (no-dye stained ducts) for inflammatory response and ductal epithelial changes (eosinophilic cytoplasm, nucleiolli, loss of epithelium), each scored as none, mild, moderate, to severe using routine HE sections. The examination was performed without specific knowledge of dose levels for each drug by two pathologists (JYR and HYY).

**Results/conclusion:** For Carboplatin group, there was a dose-response increase of inflammatory response at the levels of mild to moderate degree. There was also a dose-response increase in the ductal epithelial cell changes (P < 0.05 for both). For PLD group, no severe inflammatory changes were seen in any dose

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group, and a non-significant trend (P > 0.05) of increased for mild to moderate inflammatory response was seen in nipple, in dye stained ducts, and stromal tissue. There was a significant increase (P < 0.05) of epithelial response to the PLD treatment in ducts with dye versus ducts without dye in any given dose. However, the low dose level appeared to have more “mild” to “moderate” degree of change whereas the high dose group showed more “severe” epithelial cell change. No changes were seen in ducts without dye in the high dose group.

Together, short term intraductal treatment of cytotoxic agents may induce some degree of epithelial changes and some inflammatory response. However, the long term effect remains to be determined.

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**S31**

**Intraductal therapy of DCIS with liposomal doxorubicin: a preoperative trial in rural California**

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Thirty women with ductal carcinoma-in-situ (DCIS) diagnosed by minimally invasive biopsy techniques are being recruited for an IRB approved study testing the effects of neoadjuvant pegylated liposomal doxorubicin (Doxil) delivered through the affected duct on histology and imaging. Pre-procedure MRI and mammography are obtained on consenting women with a core biopsy demonstrating DCIS. The affected duct is cannulated and a ductogram performed to document both absence of perforation and presence of dye in the diseased duct. 20 mg (10 cc) of Doxil is then instilled into the duct. Patients are observed for one hour, and examined in about 24 hours. They have continuous access to study staff to report symptoms, and periodic contact is made with them to verify their status. Four to six weeks later, just prior to surgery, the mammogram, MRI and CBC are repeated. At operation, ductoscopy is performed and the duct identified for the pathologist. India ink gel is instilled immediately after the tissue is removed in order to identify the treated duct on histology. Three of the thirty patients will be randomized to receive normal saline instead of Doxil in a blinded fashion.

To date, six women have consented to the study. The first was not treated because of technical difficulties with the ductogram. Of the remaining five women two sustained perforated ducts and were not treated; two received the full dose of drug into the correct duct and one had a smaller dose into an adjacent duct. The treatment has been very well-tolerated. One fully treated patient had an inflammatory reaction in the treated area three weeks after Doxil administration. The episode lasted about 24 hours and responded to anti-inflammatory medication. Subsequent histology at the time of lumpectomy surgery 6 weeks later verified the presence of inflammation, squamous metaplasia and fat necrosis.

This study, sponsored by the Dr. Susan Love Research Foundation (DSLRF) and funded by the California Breast Cancer Research Program (CBCRP), is taking place in an isolated, economically-challenged and medically-underserved area. The presence of high-level original research in the local area has been a source of pride to the community as a whole, and the work validates the ideals of the CBCRP and the DSLRF. Support from local clinicians has been substantial, and the work maintains high visibility.

In summary, this research is feasible in a community setting. We are testing the concept of using the ductal system itself as a drug delivery system to affect the natural history of a disease confined to the duct. We are testing our ability to correctly identify the orifice of the affected duct by inspection of the mammogram. The treatment appears to be well-tolerated by the patients, and does not appear to affect the mammographic appearance of the breast despite documented cell death.