IDENTIFYING THE MIR FINGERPRINT IN NAF, SERUM, AND TISSUE IN PATIENTS WITH DCIS OR INVASIVE BREAST CANCER

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Study Product: *Intranasal Oxytocin*

Protocol Number: *IRB-AAAL5203*

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List of Abbreviations

DCIS: ductal carcinoma in situ
IM: intramuscular
IV: intravenous
miR: microRNA
NAF: nipple aspirate fluid
PK: pharmacokinetic
SQ: subcutaneous

Study Summary

Title	INTRANASAL OXYTOCIN USE IN IDENTIFYING THE MICRO-RNA FINGERPRINT IN NAF, SERUM, AND TISSUE IN PATIENTS WITH DCIS OR INVASIVE BREAST CANCER
Short Title	Oxytocin, microRNA and breast cancer
Protocol Number	IRB-AAAL5203
Phase	Pilot
Methodology	Pilot proof of principle study
Study Duration	1 year
Study Center(s)	Columbia University Medical Center
Objectives	To determine the feasibility of assessing miR profiles in NAF, serum and tissue of women diagnosed with DCIS or invasive breast cancer
Number of Subjects	40
Diagnosis and Main Inclusion Criteria	Patients with DCIS or invasive breast cancer scheduled for breast surgery at Columbia Medical Center
Study Product, Dose, Route, Regimen	Oxytocin (Syntocinon Spray), 4 IU (1 spray) per nostril, intranasal route, prior to nipple aspirate fluid collection
Duration of administration	Once before NAF is collected
Reference therapy	None
Statistical Methodology	Calculation of presence of miRs compared to normative reference population, binomial test to determine significant differences, Benjamin Hochberg procedure to rule out false positives, and ranking of miRs by p-value to determine most favorable biomarkers.

1 Introduction

This document is a protocol for a human research study. This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

1.1 Background

Breast cancer is the leading cause of cancer death among women. In 2008 alone, 458,400 deaths worldwide were due to breast cancer and more than 1.3 million women were newly diagnosed with breast cancer.[1] Presently, the best and most widely accepted tool that we have to screen for breast cancer is the mammogram. However, this modality remains radiation-based, is uncomfortable for patients, and has a maximum sensitivity of 70-90%. The other main methods of detection include physical exam, ultrasound and MRI, each of which has its own set of limitations. Physical exam requires a palpable lesion, which may hinder early diagnoses. Ultrasound is time-consuming and highly operator-dependent. MRI is costly, lacks specificity and is not covered by insurance for non-high risk patients.

As an alternative, we propose the use of NAF from breast cancer patients as a way to identify microRNA profiles. Considering 90% of breast cancers originate from ductal epithelium, it is possible that cells and secretions collected from such ducts would allow for earlier detection of breast cancer. The collection of NAF is furthermore appealing because it is a non-invasive method of obtaining specimens. Ideally, NAF would be used to identify biomarkers that predict the risk of breast cancer and/or detect breast cancer in its early stages. [2,3,4] To date, cellular atypia, DNA methylation and proteins have all been studied in aspirate fluid.[5,6] Our pilot appears to be the first to successfully extract and analyze miR from NAF.

MiRs are small, 20-nucleotide non-coding RNAs that modulate messenger RNA transcription, leading to changes in tumor suppression and oncogenic expression.[7] They tend to be more stable than protein markers, detectable in small quantities both inside and outside of cells.[8]

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[1] Jemal, Ahmedin, Freddie Bray, Melissa M. Center, Jacques Ferlay, Elizabeth Ward, and David Forman. "Global Cancer Statistics." *CA: A Cancer Journal for Clinicians*61.2 (2011): 69-90.
[2] Calin, G. A. "MicroRNA Profiling Reveals Distinct Signatures in B Cell Chronic Lymphocytic Leukemias." *Proceedings of the National Academy of Sciences*101.32 (2004): 11755-11760.
[3] Calin, G. A. "Human MicroRNA Genes Are Frequently Located at Fragile Sites and Genomic Regions Involved in Cancers." *Proceedings of the National Academy of Sciences* 101.9 (2004): 2999-3004.
[4] Lu, Jun, Gad Getz, Eric A. Miska, Ezequiel Alvarez-Saavedra, Justin Lamb, David Peck, Alejandro Sweet-Cordero, Benjamin L. Ebert, Raymond H. Mak, Adolfo A. Ferrando, James R. Downing, Tyler Jacks, H. Robert Horvitz, and Todd R. Golub. "MicroRNA Expression Profiles Classify Human Cancers." *Nature*435.7043 (2005): 834-38.
[5] Suijkerbuijk, Karijn P.M., Elsken Van Der Wall, Marc Vooijs, and Paul J. Van Diest. "Molecular Analysis of Nipple Fluid for Breast Cancer Screening." *Pathobiology*75.2 (2008): 149-152.
[6] Pavlou, M. P., V. Kulasingam, E. R. Sauter, B. Kliethermes, and E. P. Diamandis. "Nipple Aspirate Fluid Proteome of Healthy Females and Patients with Breast Cancer." *Clinical Chemistry* 56.5 (2010): 848-855.
[7] Croce, Carlo M., and George A. Calin. "MiRNAs, Cancer, and Stem Cell Division." *Cell*122.1 (2005): 6-7.
[8] Mitchell, P. S., R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin, and M. Tewari. "Circulating MicroRNAs as Stable Blood-

Approximately 30 miRs have been found to play important roles in the progression of breast cancer. These functions include differentiation of breast tissue from normal to cancerous cells[9], tumor invasion, metastasis[10,11] and metastatic suppression.[12]

Our plan is to study the differences of miR profiles between cancerous breasts and healthy contralateral controls. In addition to the benefits listed above, unique miR profiles would allow clinicians to evaluate treatment response, and provide more individualized, targeted disease management. Thus far, a significant barrier to this strategy has been insufficient NAF yield. Specifically, we were able to collect NAF from 13/40 patients, of which 8 were successfully analyzed for miR. One solution to this problem is the use of intranasal oxytocin, which has been shown to successfully increase milk letdown in both postpartum and non-lactational women.[7,8] Furthermore, both healthy and high-risk groups of non-lactating women were found to express significantly greater levels of NAF following intranasal oxytocin. Compared to a 39-66% success rate of obtaining NAF from healthy female patients, intranasal oxytocin demonstrated success rates as high as 94%.[2,4,9]

1.2 Investigational Agent

Oxytocin is a natural neuropeptide that is synthesized in the hypothalamus and released by the posterior pituitary. It is most commonly known in the United States for its role in promoting uterine contractions during labor. In addition, the oxytocin pathway can also be triggered by suckling, which causes contraction of mammary myoepithelial cells and subsequent milk ejection.[13] Recent studies demonstrate intranasal oxytocin's utility in enhancing the yield of nipple aspirate fluid (NAF) among healthy, non-lactating female patients as well as those at high risk for breast cancer. This capability is crucial for the analysis of various markers associated with breast disease and cancer such as proteins, methylated DNA and miRs.[14,15,16]

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based Markers for Cancer Detection." *Proceedings of the National Academy of Sciences* 105.30 (2008): 10513-10518.

[9] Iorio, M. V. "MicroRNA Gene Expression Deregulation in Human Breast Cancer."*Cancer Research* 65.16 (2005): 7065-7070.

[10] Ma, Li, Julie Teruya-Feldstein, and Robert A. Weinberg. "Tumour Invasion and Metastasis Initiated by MicroRNA-10b in Breast Cancer." *Nature* 449.7163 (2007): 682-688.

[11] Huang, Qihong, Kiranmai Gumireddy, Mariette Schrier, Carlos Le Sage, Remco Nagel, Suresh Nair, David A. Egan, Anping Li, Guanghua Huang, Andres J. Klein-Szanto, Phyllis A. Gimotty, Dionyssios Katsaros, George Coukos, Lin Zhang, Ellen Puré, and Reuven Agami. "The MicroRNAs MiR-373 and MiR-520c Promote Tumour Invasion and Metastasis." *Nature Cell Biology* 10.2 (2008): 202-210.

[12] Tavazoie, Sohail F., Claudio Alarcón, Thordur Oskarsson, David Padua, Qiongqing Wang, Paula D. Bos, William L. Gerald, and Joan Massagué. "Endogenous Human MicroRNAs That Suppress Breast Cancer Metastasis." *Nature* 451.7175 (2008): 147-152.

[13] Molina, Patricia E. "The Hypothalamus & Posterior Pituitary Gland." *Endocrine Physiology*. 3rd ed. New York: Lange Medical /McGraw-Hill Medical Pub. Division, 2006.

[14] Zhang, Liping, Zhi-Ming Shao, Perrin Beatty, Maryam Sartippour, He-Jing Wang, Robert Elashoff, Helena Chang, and Mai N. Brooks. "The Use of Oxytocin in Nipple Fluid Aspiration." *The Breast Journal* 9.4 (2003): 266-268.

[15] Shao, Zhi-Ming, and Mai Nguyen. "Nipple Aspiration in Diagnosis of Breast Cancer."*Seminars in Surgical Oncology* 20.3 (2001): 175-180.

[16] Suijkerbuijk, Karijn PM, Elsken Van Der Wall, Helen Meijrink, Xiaojuan Pan, Inne HM Borel Rinkes, Margreet GEM Ausems, and Paul J. Van Diest. "Successful Oxytocin-assisted Nipple Aspiration in Women at Increased Risk for Breast Cancer." *Familial Cancer* 9 (2010): 321-325.

Intranasal oxytocin ("Syntocinon Spray") had been approved by the United States Food and Drug Administration (FDA) prior to 1982 for initial milk letdown, but was withdrawn from the market in 1995 by its manufacturer, Novartis, for financial reasons.[17] Oxytocin now exists solely as the intravenous (IV) and intramuscular (IM) forms in the US. It is marketed under the brand name "Pitocin" for induction, stimulation or reinforcement of labor; adjunctive therapy in abortion; promotion of uterine contractions during the third stage of labor; and control of postpartum bleeding.[18] The nasal spray remains available outside of the US.

1.3 Preclinical Data

Oxytocin has long been studied in animals, and continues to be prescribed today by veterinarians. The IV, IM and subcutaneous (SQ) forms are indicated for obstetrical use and milk letdown in cows and sows.[19] Many studies over the past several decades support the drug's use in promoting animal lactation. Exogenous oxytocin has been shown to increase milk letdown in animals as well as enhance milk production.[19,20,21] Animal toxicity effects based on a few isolated experiments include fetal growth stimulation and leftward shift of the fetal oxygen dissociation curve.[22] To date, there do not appear to be other significant side effects studied in animals exposed to exogenous oxytocin.

1.4 Clinical Data to Date

Oxytocin is a natural neuropeptide that is synthesized in the hypothalamus and released by the posterior pituitary. It is most commonly known in the United States for its role in promoting uterine contractions during labor. In addition, the oxytocin pathway can also be triggered by suckling, which causes contraction of mammary myoepithelial cells and subsequent milk ejection.[13]

Intranasal oxytocin ("Syntocinon Spray") was approved by the FDA prior to 1982 for initial milk letdown, but was withdrawn from the market in 1995 by its manufacturer, Novartis, for financial reasons.[17] Oxytocin now exists solely in the intravenous (IV) and intramuscular (IM) forms in the US. It is marketed under the brand name "Pitocin" for induction, stimulation or reinforcement of labor; adjunctive therapy in abortion; promotion of uterine contractions during the third stage of labor; and control of postpartum bleeding.[18]

Recently, there has been a resurgence of the research involving intranasal oxytocin. Increased NAF yield in the setting of intranasal oxytocin has been recorded among women with premature infants as well as among adoptive mothers.[19,20] In the field of breast oncology, this drug has been safely used to improve NAF collection with the ultimate goal of enhancing biomarker analysis.

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[17] Lietzan, Erika K. "Citizen Petition." Letter to Division of Dockets Management. 8 Feb. 2006. MS. http://www.fda.gov/ohrms/dockets/dockets/06p0068/06p-0068-cp00001-01-vol1.pdf.
[18] "Oxytocin." *Lexicomp Online*. Lexicomp, n.d. Web. 05 Sept. 2012. <http://online.lexi.com/>.
[19] Gorewit, R.C., and R. Sagi. "Effects of Exogenous Oxytocin on Production and Milking Variables of Cows." *Journal of Dairy Science* 67.9 (1984): 2050-2054.
[20] Nostrand, S.D., D.M. Galton, H.N. Erb, and D.E. Bauman. "Effects of Daily Exogenous Oxytocin on Lactation Milk Yield and Composition." *Journal of Dairy Science*74.7 (1991): 2119-2127.
[21] Knight, C. H. "Short-term Oxytocin Treatment Increases Bovine Milk Yield by Enhancing Milk Removal without Any Direct Action on Mammary Metabolism." *Journal of Endocrinology* 142.3 (1994): 471-473.
[22] "Oxytocin - Animal Toxicity Studies." *Oxytocin*. National Library of Medicine HSDB Database, n.d. Web. 05 Sept. 2012. <http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~qYnAi5:1:animal>.

One group at UCLA (Zhang et al., 2003) studied nasal oxytocin in 9 healthy, non-lactating female volunteers between the ages of 19-41. NAF collection before and after 25U oxytocin per nostril showed a significant increase in volume of 1.39 ± 2.2μL (p = 0.0116). Six breasts that did not release NAF initially did so following oxytocin administration. Pregnant women were excluded from the study because of the potential for abnormal uterine contractions; however, no adverse events were noted.[14]

Several important oxytocin experiments were recently completed in Europe by van Diest et al. In the first of a series (Suijkerbuijk, 2007), oxytocin-supported nipple aspiration was successful in 63 of 67 healthy female volunteers. This rate (94%) was overwhelmingly greater than those reported in previous studies not using oxytocin, which ranged from 39-66%.[23,24] A subgroup analysis determined that there was sufficient material for genetic, epigenetic and proteomic characterizations. Again, there were no side effects reported, with 66/67 women who would be willing to repeat the procedure and an average discomfort rating of 1.3 on a scale of 0-10.[25]

A follow-up study was published in 2010 by the same group (Suijkerbuijk, 2010). It demonstrated a similar effect of intranasal oxytocin on 90 patients at high risk for breast cancer. NAF was successfully collected from 90% of the subjects, with a mean discomfort rating of 0.6 (mammography averages 4.9, MRI 2.6). A subgroup analysis among 50 patients showed that quantitative multiplex methylation-specific PCR was able to be performed on 100% of the samples, suggesting the utility of NAF in biomarker identification.[16]

Studies using intranasal oxytocin have also become increasingly common in the field of psychiatry. Intranasal oxytocin has been shown to increase sociability and decrease anxiety in both animals and human subjects.[26,27] Several IND exemptions for Syntocinon Spray have been

[23] Fabian, C. J. "Breast-tissue Sampling for Risk Assessment and Prevention." *Endocrine Related Cancer* 12.2 (2005): 185-213.
[24] Wrensch, Margaret R., Nicholas L. Petrakis, Larry D. Gruenke, Virginia L. Ernster, Rei Miike, Eileen B. King, and Walter W. Hauck. "Factors Associated with Obtaining Nipple Aspirate Fluid: Analysis of 1428 Women and Literature Review." *Breast Cancer Research and Treatment* 15.1 (1990): 39-51.
[25] MacDonald, Elayne, Mark R. Dadds, John L. Brennan, Katrina Williams, Florence Levy, and Avril J. Cauchi. "A Review of Safety, Side-effects and Subjective Reactions to Intranasal Oxytocin in Human Research." *Psychoneuroendocrinology* 36.8 (2011): 1114-1126.
[26] Ross, Heather E., and Larry J. Young. "Oxytocin and the Neural Mechanisms Regulating Social Cognition and Affiliative Behavior." *Frontiers in Neuroendocrinology* 30.4 (2009): 534-547.
[27] Bartz, J. A., J. Zaki, N. Bolger, E. Hollander, N. N. Ludwig, A. Kolevzon, and K. N. Ochsner. "Oxytocin Selectively Improves Empathic Accuracy." *Psychological Science* 21.10 (2010): 1426-1428.

approved in these settings. Other psychiatric diseases with which intranasal oxytocin are being studied include schizophrenia, autism and borderline personality disorder.[28,29,30]

Finally, among all trials found for the purposes of this IND proposal, oxytocin nasal spray side effects were minor and rare. A meta-analysis of 38 randomized controlled trials between 1990-2010 evaluating the safety of intranasal oxytocin found the most common side effects to be increased calmness, euphoria or energy; lightheadedness, drowsiness or headache; and nasal irritation, dry mouth or throat. Other rare adverse reactions included abnormal uterine contractions, allergic dermatitis, anaphylaxis and water intoxication.[25]

This drug is currently marketed outside the United States as Syntocinon Spray. To our knowledge, it has not been withdrawn from marketing for any reasons related to safety or effectiveness.

1.5 Dose Rationale and Risk/Benefits

Based on prior studies in non-lactating women, we plan to administer one spray (4 IU) per nostril 15-30 minutes before NAF collection. The benefits will be greater NAF yield for miR analysis. The risks include rare anaphylactic reactions, common headache, tachycardia or bradycardia, uncommon arrhythmia, very rare hypertension, common nausea and vomiting, rare rash and allergic dermatitis, and uncommon abnormal uterine contractions. Because of the possibility of abnormal uterine contractions, pregnant women will be excluded in the study. *[Frequency: Very common (> 1/10); common (> 1/100 to < 1/10); uncommon (> 1/1000 to < 1/100); rare (> 1/10 000 to < 1/1000); very rare (< 1/10 000), including isolated cases.]*

2 Study Objectives

The main objective of this study is to demonstrate the feasibility of characterizing miR profiles in the NAF, serum and tissue of patients with DCIS or invasive breast cancer. Each set of miRs will be assessed for correlation with tumor presence. Tissue and serum are expected to be acquired from all patients. With the addition of intranasal oxytocin, bilateral NAF collection of adequate volume for analysis is expected from 77% of participants at increased risk for breast cancer.[16]

Analysis of the miR profiles will include comparisons among the different biological samples (tissue, serum, NAF) in order to understand their relationships with one another. We hypothesize that cancer subtypes will be identifiable by unique miR fingerprints, with the goal of creating a breast cancer screening tool.

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[28] Pedersen, Cort A., and David L. Penn. "Oxytocin Treatment of Social Cognitive and Functional Deficits in Schizophrenia - Full Text View - ClinicalTrials.gov."*Oxytocin Treatment of Social Cognitive and Functional Deficits in Schizophrenia - Full Text View - ClinicalTrials.gov*. National Institute of Mental Health, n.d. Web. <http://clinicaltrials.gov/ct2/show/NCT01394471>.

[29] Guastella, Adam J., Stewart L. Einfeld, Kylie M. Gray, Nicole J. Rinehart, Bruce J. Tonge, Timothy J. Lambert, and Ian B. Hickie. "Intranasal Oxytocin Improves Emotion Recognition for Youth with Autism Spectrum Disorders." *Biological Psychiatry* 67.7 (2010): 692-694.

[30] Simeon, D., J. Bartz, H. Hamilton, S. Crystal, A. Braun, S. Ketay, and E. Hollander. "Oxytocin Administration Attenuates Stress Reactivity in Borderline Personality Disorder: A Pilot Study." *Psychoneuroendocrinology* 36.9 (2011): 1418-421.

Our aim is to first demonstrate proof of principle. We would like to extend our initial trial by 1 year since the NAF volumes were inadequate without pharmacologic enhancement. Specifically, we were able to collect NAF from 13/40 patients, of which 8 were successfully analyzed for miR. To remedy this problem, we propose collecting additional specimens in the setting of intranasal oxytocin. This project will establish a foundation from which future miR analysis may be completed.

3 Study Design

3.1 General Design

We propose a pilot proof of principle study to assess and compare the miR fingerprint (or profile) in NAF, serum, and tissue from patients undergoing a lumpectomy or mastectomy for in situ or invasive breast cancer. As this study is a proof of concept there is no power presented. Other than the collection of NAF, treatment will not vary from the standard of care. The collection of all specimen types will be completed within the regularly scheduled operative time period. The surgery will not be prolonged in any way for specimen collection. Forty patients (20 with DCIS, 20 with invasive breast cancer) will be entered into the study. A total of 5 patients will also undergo pharmacokinetic (PK) draws during the time of surgery to assess the baseline oxytocin level. All other subjects enrolled will be enrolled into the "non-PK" cohort. Patients receiving care at the Breast Surgery Department at Columbia who meet criteria will be offered participation in the study. Patients will answer a brief questionnaire preoperatively. At the time of surgery patients will undergo a blood draw as well as collection of NAF (via prior administration of intranasal oxytocin, gentle massage, aspirator, and breast pump from both breasts). The NAF and blood samples will be sent to the pathology lab for analysis of miR profile. At the completion of the case, the breast tissue will be sent to pathology per routine. Once in pathology, 5 paraffin-embedded slices will be created to evaluate for miR profile. Serum, NAF, and tissue will be analyzed for their miR profile.

Once total RNA is extracted from the biological samples it will be processed to fluorescently label the microRNAs species according to the V2-Human microRNA array (Agilent). MicroRNA profiling will be obtained and analyzed using the gene-spring software analysis package. First, expression and cluster analysis will be performed to identify the microRNA that are expressed in our samples and to classify them accordingly to their expression. Then, the predicted targets of the over/down-regulated microRNAs will be identified using target prediction bioinformatics tools (TargetScan, Miranda and Pictarvert). Finally, statistical studies between over/under-represented microRNAs will be performed.

3.2 Primary Study Endpoints

The primary endpoint is feasible miR analysis of NAF as well as serum and tissue collected from study participants.

3.3 Secondary Study Endpoints

The secondary endpoint is sufficient NAF volumes following the administration of oxytocin.

3.4 Primary Safety Endpoints

The primary safety endpoints are anaphylactic reactions or water intoxication secondary to intranasal oxytocin. Refer to 1.5 for complete list of side effects.

4 Subject Selection and Withdrawal

4.1 Inclusion Criteria

Patients meeting the following criteria may be included in the study:

- All female patients with DCIS or invasive breast cancer who are scheduled for a lumpectomy or mastectomy and are receiving surgical care from the Breast Surgery Department at Columbia Medical Center.
- Patients diagnosed with unilateral DCIS or invasive breast cancer

4.2 Exclusion Criteria

Patients meeting the following criteria will be excluded from the study:

- Patients not receiving breast surgery care from the Department of Breast Surgery at Columbia Medical Center.
- Pregnant patients are to be excluded given the risk associated with oxytocin.
- Male patients
- Patients with a prior breast cancer diagnosis
- Patients who have had an adverse reaction to oxytocin

4.3 Subject Recruitment and Screening

Patients with unilateral DCIS or invasive breast cancer who are scheduled for a lumpectomy or mastectomy will be offered participation in this study. A negative pregnancy test is required for all women with child-bearing potential. Post-menopausal women (i.e., women who have not menstruated for at least 1 year) and women who have undergone hysterectomy or bilateral oophorectomy will not need to undergo a pregnancy test. All women of childbearing potential will be tested for pregnancy prior to enrollment. All participants will receive their care at Columbia Presbyterian in the Breast Surgery Department.

4.4 Early Withdrawal of Subjects

Subjects may withdraw at any time during the study.

4.4.1 When and How to Withdraw Subjects

Subjects may withdraw from the study at any time for any reason, and can do so by contacting the study coordinator by phone or e-mail.

4.4.2 Data Collection and Follow-up for Withdrawn Subjects

Withdrawn subjects may continue to follow-up at Columbia Presbyterian, but are not obligated to donate NAF, serum or tissue samples.

5 Study Drug

5.1 Description

Oxytocin is a natural neuropeptide that is synthesized in the hypothalamus and released by the posterior pituitary. It is most commonly known in the United States for its role in promoting uterine contractions during labor. In addition, the oxytocin pathway can also be triggered by suckling, which causes contraction of mammary myoepithelial cells and subsequent milk ejection.[13] Recent studies demonstrate intranasal oxytocin's utility in enhancing the yield of nipple aspirate fluid (NAF) among healthy, non-lactating female patients as well as those at high risk for breast cancer. This capability is crucial for the analysis of various markers associated with breast disease and cancer such as proteins, methylated DNA and miRs.[14,15,16]

Intranasal oxytocin ("Syntocinon Spray") had been approved by the United States Food and Drug Administration (FDA) prior to 1982 for initial milk letdown, but was withdrawn from the market in 1995 by its manufacturer, Novartis, for financial reasons.[17] Oxytocin now exists solely as the intravenous (IV) and intramuscular (IM) forms in the US. It is marketed under the brand name "Pitocin" for induction, stimulation or reinforcement of labor; adjunctive therapy in abortion; promotion of uterine contractions during the third stage of labor; and control of postpartum bleeding.[18] The nasal spray remains available outside of the US.

5.2 Treatment Regimen

The Principal Investigator will administer one spray of intranasal oxytocin, or 4 IU, into each nostril approximately 15-30 minutes before NAF collection. Prior to collection, the nipples will be scrubbed with alcohol swabs to eliminate keratin plugs, breasts will be massaged by the MD investigator for approximately 2 minutes, but no more than approximately 7 minutes. An aspirator will be applied to each breast, and fluid will be collected into capillary tubes. NAF samples will be acquired from study subjects by the MD investigator in the operating room while under anesthesia before the opening incision. The duration of time required to obtain NAF samples will be documented.

5.3 Method for Assigning Subjects to Treatment Groups

All subjects will receive the same treatment.

5.4 Preparation and Administration of Study Drug

Intranasal spray. One spray or 4 IU of oxytocin will be administered into each nostril of each patient approximately 15-30 minutes before NAF collection. Study drug will be administered by the Principal Investigator.

5.5 Subject Compliance Monitoring

The principal investigator will administer the intranasal oxytocin, therefore assuring compliance. Proper documentation will be obtained.

5.6 Prior and Concomitant Therapy

N/A

5.7 Packaging

Intranasal oxytocin is packaged into spray containers by the manufacturer (Novartis).

5.8 Blinding of Study Drug

N/A. All patients enrolled in the study will receive intranasal oxytocin.

5.9 Receiving, Storage, Dispensing and Return

5.9.1 Receipt of Drug Supplies

Upon receipt of the of the study treatment supplies, an inventory must be performed and a drug receipt documented by the appropriate research pharmacy personnel accepting the shipment. It is important that the designated study staff counts and verifies that the shipment contains all the items noted in the shipment inventory. Any damaged or unusable study drug in a given shipment (active drug or comparator) will be documented in the study files.

5.9.2 Storage

The drug must be refrigerated at 35.6 – 46.4 degrees Fahrenheit, and may be used up to 1 month after opening.

5.9.3 Dispensing of Study Drug

The drug will be stored at and dispensed by at the research pharmacy.

5.9.4 Return or Destruction of Study Drug

Empty/used oxytocin spray containers will be returned to the research pharmacy. At the completion of the study, there will be a final reconciliation of drug shipped, drug consumed, and drug remaining. This reconciliation will be logged on the drug reconciliation form, signed and dated. Any discrepancies noted will be investigated, resolved, and documented prior to return or destruction of unused study drug. Drug destroyed on site will be documented in the study files.

6 Study Procedures

6.1 Visit 1

The collection of all specimen types will be completed within the regularly scheduled operative time period. The surgery will not be prolonged in any way for specimen collection. Patients will answer a brief questionnaire preoperatively, after the subject has consented to the trial and prior to administration of oxytocin. Blood pressure will be recorded prior to oxytocin administration and 5 minutes post-administration of oxytocin.

All patients will undergo a blood draw as well as collection of NAF (via prior administration of intranasal oxytocin, gentle massage, aspirator, and breast pump from both breasts). All biological samples will be collected by a qualified clinician. The NAF and blood samples will be sent to the pathology lab for analysis of miR profile. At the completion of the case, the breast tissue will be sent to pathology per routine. Once in pathology, 5 paraffin-embedded slices will be created to evaluate for miR profile. Serum, NAF, and tissue will be analyzed for their miR profile.

Five patients who are participating in the PK cohort of the trial will also undergo a PK blood draw at the time of surgery prior to oxytocin administration. This will be used to assess the baseline oxytocin level and will be done in conjunction with the pre-oxytocin blood draw to assess miR. These patients will have a second blood draw approximately 30 minutes after the administration of oxytocin to assess oxytocin levels in comparison to the baseline. All blood draws will be done by a qualified clinician.

Once total RNA is extracted from the biological samples it will be processed to fluorescently label the microRNAs species according to the V2-Human microRNA array (Agilent). MicroRNA profiling will be obtained and analyzed using the gene-spring software analysis package. First, expression and cluster analysis will be performed to identify the microRNA that are expressed in our samples and to classify them accordingly to their expression. Then, the predicted targets of the over/down-regulated microRNAs will be identified using target prediction bioinformatics tools (TargetScan, Miranda and Pictarvert). Finally, statistical studies between over/under-represented microRNAs will be performed.

6.2 Visit 2/End of Study Visit

One blood draw will be performed at the first standard of care, post-surgical outpatient follow-up visit. A final adverse event assessment will also be performed at this visit and documented. Any unresolved, oxytocin related adverse events will be followed until resolution. Any adverse events deemed unrelated to oxytocin administration will be considered resolved at this time. Any applicable standard of care interventions to address non-oxytocin related adverse events will be handled as per standard clinical practice. No further contributions to the study will be required after this visit.

6.3 Study Calendar

	Pre-Study	**Visit 1**	**Visit 2/End of Study Visit**
Informed consent	X		
Demographics	X		
Medical history	X		
Oxytocin Administration		X	
NAF collection		X	
Adverse event evaluation	X	X	X[e]
Blood Pressure	X	X[b]	
B-HCG[a]	X[a]		
Patient Questionnaire		X[d]	
Serum Blood Draw		X	X
Pharmacokinetic Blood Draw		X[c]	
Tissue from On-Study Surgery		X	
a: Only for subjects who are of childbearing potential b: Blood pressure recordings will be taken prior to oxytocin administration, and approximately 5 minutes post-administration of oxytocin.			

c: PKs will be drawn prior to the administration of oxytocin, and then a post-administration PK will be drawn approximately 30 minutes post oxytocin administration.
d: Questionnaire can be administered any time after the subject signs the informed consent, so long as it is captured prior to the administration of oxytocin.e. Adverse events will be recorded at visit 2/End of Study visit, if there are any adverse events that are attributed to oxytocin, they will and must be followed until resolution. All other adverse events that are not attributable to oxytocin will be considered closed out at this visit, and will be followed/addressed via standard of care clinical/medical interventional procedures.

7 Statistical Plan

7.1 Sample Size Consideration

Contralateral breast without disease will serve as the control. In a sample of 40 patients with unilateral disease, we expect 60% of the patients to show significantly altered expression of miRNAs in the diseased breast as compared to the breast without disease for the test to be of clinical relevance. Ideally a detectable difference would be the difference between a null hypothesis of 0% and an alternative of 60% or a second alternative of 35%. Since this study is the first of its kind, we will consider the test to be not clinically relevant if less than 35% of patients show significantly altered expression miRNA in the diseased breast as compared to the breast without disease.

Using a two sample z-test, type I error of 0.05, and a two-sided significance level, a sample size of 40 will have greater than 80% power to detect a difference between a null hypothesis of 35% and an alternative hypothesis of 60%.

7.2 Statistical Methods

The primary analysis will be conducted to determine the presence and expression levels of 800 miRNA's among DCIS and invasive breast cancer patients. Based on the resulting profiles, we will determine whether or not its expression for each miRNA from the tumor side differs from that of the normal side.

We will interrogate 800 miRNAs. The vast majority will not be expressed in any sample. A minority will be measurable/detectable in some samples and a smaller minority will be detectable in all samples.

The presence of miRNA can be as follows:
a. Present in tumor and absent in normal
b. Present in normal and absent in tumor
c. Present at high level in tumor and low level in normal
d. Present at high level in normal side and low level in tumor.

Of these a and c are most important and relevant for our analysis. If even a single miRNA is absent in tumor and present in 30% of cancers or if there is at least one miRNA out of these 800 that is present at low levels in all “normals” and high levels in 30% of tumors that would be a "success". We will dichotomize to low and high levels of miRNA to calculate frequencies and the associated risk estimates and 95% confidence intervals. The comparison of proportions will be performed using two-sample z-tests.

7.3 Subject Population(s) for Analysis

Patients diagnosed with DCIS or invasive breast cancer.

8 Safety and Adverse Events

8.1 Definitions

Adverse Event

An ***adverse event*** (AE) is any symptom, sign, illness or experience that develops or worsens in severity during the course of the study. Intercurrent illnesses or injuries should be regarded as adverse events. Abnormal results of diagnostic procedures are considered to be adverse events if the abnormality:

- results in study withdrawal
- is associated with a serious adverse event
- is associated with clinical signs or symptoms
- leads to additional treatment or to further diagnostic tests
- is considered by the investigator to be of clinical significance

Serious Adverse Event

Adverse events are classified as serious or non-serious. A ***serious adverse event*** is any AE that is:

- fatal
- life-threatening
- requires or prolongs hospital stay
- results in persistent or significant disability or incapacity
- a congenital anomaly or birth defect
- an important medical event

Important medical events are those that may not be immediately life threatening, but are clearly of major clinical significance. They may jeopardize the subject, and may require intervention to prevent one of the other serious outcomes noted above. For example, drug overdose or abuse, a seizure that did not result in in-patient hospitalization, or intensive treatment of bronchospasm in an emergency department would typically be considered serious.

All adverse events that do not meet any of the criteria for serious should be regarded as ***non-serious adverse events***.

Adverse Event Reporting Period

The study period during which adverse events must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up. For this study, due to the short half life of oxytocin, the study treatment follow-up period for adverse event reporting will be considered completed at the Visit 2/End of Study visit as outlined in Protocol Section 6.2.

Preexisting Condition

A preexisting condition is one that is present at the start of the study. A preexisting condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

General Physical Examination Findings
At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event must also be recorded and documented as an adverse event.

Post-study Adverse Event
All unresolved, oxytocin related adverse events should be followed by the investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit, the investigator should instruct each subject to report any subsequent event(s) that the subject, or the subject's personal physician, believes might reasonably be related to participation in this study. The investigator should notify the study sponsor of any death or adverse event occurring at any time after a subject has discontinued or terminated study participation that may reasonably be related to this study. The sponsor should also be notified if the investigator should become aware of the development of cancer or of a congenital anomaly in a subsequently conceived offspring of a subject that has participated in this study.

Abnormal Laboratory Values
A clinical laboratory abnormality should be documented as an adverse event if <u>any one of the following</u> conditions is met:

- The laboratory abnormality is not otherwise refuted by a repeat test to confirm the abnormality
- The abnormality suggests a disease and/or organ toxicity
- The abnormality is of a degree that requires active management; e.g. change of dose, discontinuation of the drug, more frequent follow-up assessments, further diagnostic investigation, etc.

Hospitalization, Prolonged Hospitalization or Surgery
Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a serious adverse event unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an adverse event if the condition meets the criteria for and adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an adverse event in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition. Surgery should ***not*** be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.
- Hospitalization or prolonged hospitalization required to allow efficacy measurement for the study.
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

8.2 Recording of Adverse Events

At each contact with the subject, the investigator must seek information on adverse events by specific questioning and, as appropriate, by examination. Information on all adverse events should be recorded immediately in the source document, and also in the appropriate adverse event module of the case report form (CRF). All clearly related signs, symptoms, and abnormal diagnostic procedures results should recorded in the source document, though should be grouped under one diagnosis.

All adverse events occurring during the study period must be recorded. The clinical course of each event should be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. Serious adverse events that are still ongoing at the end of the study period must be followed up to determine the final outcome. Any serious adverse event that occurs after the study period and is considered to be possibly related to the study treatment or study participation should be recorded and reported immediately.

8.3 Reporting of Serious Adverse Events

8.3.1 Study Sponsor Notification by Investigator

A serious adverse event must be reported to the study sponsor by telephone within 24 hours of the event. A Serious Adverse Event (SAE) form must be completed by the investigator and faxed to the study sponsor within 24 hours. The investigator will keep a copy of this SAE form on file at the study site. Report serious adverse events by phone and facsimile to:

Sheldon Feldman, MD Phone 212-305-9676 Fax 212-305-1522

At the time of the initial report, the following information should be provided:

- Study identifier
- Study Center
- Subject number
- A description of the event
- Date of onset
- Current status
- Whether study treatment was discontinued
- The reason why the event is classified as serious
- Investigator assessment of the association between the event and study treatment

Within the following 48 hours, the investigator must provide further information on the serious adverse event in the form of a written narrative. This should include a copy of the completed Serious Adverse Event form, and any other diagnostic information that will assist the understanding of the event. Significant new information on ongoing serious adverse events should be provided promptly to the study sponsor

8.3.2 EC/IRB Notification by Investigator

Reports of all events (including follow-up information) that meet the definition of an unanticipated problem posing risk to subjects or others must be submitted to the IRB within one week (5 business days) following the occurrence of the unanticipated problem or the principal

investigator's acquiring knowledge of the unanticipated problem in accordance with IRB policy. Additionally, the sponsor-investigator will submit a summary of all unanticipated problems that occurred since the beginning of the study at the time of continuing review. Copies of each report and documentation of IRB notification and receipt will be kept in the Regulatory binder.

8.3.3 FDA Notification by Sponsor

The Columbia University Medical Center Sponsor-Investigator, as holder of the IND, will be responsible for all communication with the FDA. Columbia University Medical Center Principal Investigator will report to the FDA, regardless of the site of occurrence, any adverse event that is serious, unexpected and there is evidence to suggest a causal relationship between the drug and the adverse event. These must be reported to the FDA and any affiliate sites as soon as possible, but in no case later than 15 calendar days after the sponsor determines that the information qualifies for reporting. The Sponsor-Investigator will also submit an IND annual report to the FDA in accordance with 21.CFR 312.33.

The Columbia University Medical Center Sponsor Investigator must report to the FDA and any affiliate site investigators as follows:

- Any unexpected fatal or life-threatening event must be reported as soon as possible, but no later than 7 calendar days after the sponsor investigator initial receipt of the information
- Any findings from epidemiological studies, pooled analysis of multiple studies, or clinical studies, whether or not conducted under an IND, and whether or not conducted by the sponsor-investigator, that suggest a significant risk in humans exposed to the drug must be reported as soon as possible but no later than 15 calendar days after the sponsor-investigator determines that the information qualifies for reporting
- Any findings from animal or in vitro testing whether or not conducted under an IND, and whether or not conducted by the sponsor-investigator, that suggest a significant risk in humans exposed to the drug must be reported as soon as possible but no later than 15 calendar days after the sponsor-investigator determines that the information qualifies for reporting
- Any clinically important increase in the rate of a serious suspected adverse reactions over that listed in the protocol or Investigator Brochure
- Expected SAEs and AEs will be included in the IND Annual Reports.

Follow-up information to a safety report should be submitted as soon as the relevant information is available. However, if the results of a sponsor's investigation show that an adverse drug experience not initially determined to be reportable are so reportable, the sponsor investigator must report such experience as soon as possible, but no later than 15 calendar days after the determination is made.

All other serious unexpected experiences associated with the use of the study treatment will be reported to FDA as soon as possible but in no event later than 15 calendar days after initial receipt of the information.

8.3.4 DSMC Reporting by the Sponsor-Investigator

Serious adverse events not constituting unanticipated problems are to be reported to the HICCC DSMC. Reporting should occur within 24 hours of knowledge of the SAE occurring at our institution or affiliate sites.

8.4 Unblinding Procedures

N/A. Subjects are not blinded.

8.5 Stopping Rules

The study will be stopped immediately if the drug intervention causes more harm than benefit to the patients enrolled.

8.6 Medical Monitoring

It is the responsibility of the Principal Investigator to oversee the safety of the study at his/her site. This safety monitoring will include careful assessment and appropriate reporting of adverse events as noted above, as well as the construction and implementation of a site data and safety-monitoring plan (see section 9 Auditing, Monitoring and Inspecting). Medical monitoring will include a regular assessment of the number and type of serious adverse events.

8.6.1 Internal Data and Safety Monitoring Board

The principal investigator, Dr. Sheldon Feldman, is responsible for monitoring the data and safety related to this study. All adverse events (AE), including serious adverse events (SAE), and unanticipated problems (UP) will be reviewed in an ongoing manner by the principal investigator.

8.6.2 Independent Data and Safety Monitoring Committee

As per the Study Monitoring Plan (see Section 10.1), all monitoring reports generated by the Quality Monitor will be sent for review to the HICCC DSMC upon completion.
In addition, semi-annual Safety Reports will be submitted to the HICCC DSMC for review. The Safety Reports will include, but is not limited to, the following information: enrollment status, toxicity information, unanticipated problems, and serious adverse events.

9 Data Handling and Record Keeping

9.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act (HIPAA). Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject

authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the subject is alive) at the end of their scheduled study period.

9.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

9.3 Case Report Forms

Case Report Forms will be completed for each subject enrolled into the clinical study through the CTMS. It is the investigator's responsibility for ensuring that all clinical and laboratory data entered on the corresponding CRFs are complete, accurate and authentic.

9.4 Records Retention

Study records that identify participants will be kept confidential as required by law. Federal Privacy Regulations provide safeguards for privacy, security and authorized access. Except when required by law or otherwise authorized, name, address and telephone number will not identify participants or any other direct personal identifier in study records disclosed outside of Columbia University Medical Center. The file that links names and other personal information and code numbers will be kept secure at our site. Apart from the health care personnel involved in this study, study data will be available only to representatives of the Institutional Review Board (IRB) at the Columbia University Medical Center.

10 Study Monitoring, Auditing, and Inspecting

10.1 Study Monitoring Plan

This study will be monitored at least twice a year by the departmental Quality Monitor who is under the supervision of the department's Director of clinical trials. The monitor will review the regulatory binder, and regulatory submission to the IRB and the FDA to endure compliance. She will review study files to assess protocol compliance. The monitor will verify data accuracy through reconciliation of case report forms with source documents. A copy of the monitoring report will be filed in the regulatory documents. All monitoring reports will be forwarded to the DSMC for review. The investigator will allocate adequate time for such monitoring activities. The Investigator will also ensure that the monitor or other compliance or quality assurance reviewer is given access to all the above noted study-related documents and study related facilities (e.g. pharmacy, diagnostic laboratory, etc.), and has adequate space to conduct the monitoring visit. The HICCC-DSMC may conduct additional monitoring visits if deemed necessary.

10.2 Auditing and Inspecting

The investigator will permit study-related monitoring, audits, and inspections by the EC/IRB, the sponsor, government regulatory bodies, and University compliance and quality assurance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.). The investigator will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.).

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable University compliance and quality assurance offices.

11 Ethical Considerations

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted independent Ethics Committee (EC) or Institutional Review Board (IRB), in agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the EC/IRB concerning the conduct of the study will be made in writing to the investigator and a copy of this decision will be provided to the sponsor before commencement of this study. The investigator should provide a list of EC/IRB members and their affiliate to the sponsor.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. See Attachment B for a copy of the Subject Informed Consent Form. This consent form will be submitted with the protocol for review and approval by the EC/IRB for the study. The formal consent of a subject, using the EC/IRB-approved consent form, must be obtained before that subject is submitted to any study procedure. This consent form must be signed by the subject or legally acceptable surrogate, and the investigator-designated research professional obtaining the consent.

12 Study Finances

12.1 Funding Source

Funded by private donors.

12.2 Conflict of Interest

Any investigator who has a conflict of interest with this study (patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, etc.) must have the conflict reviewed by the Columbia University Conflict of Interest Committee with a Committee-sanctioned conflict management plan that has been reviewed and approved prior to participation in this study. All Columbia University investigators will follow the University conflict of interest policy.

12.3 Subject Stipends or Payments

There will be no stipend or payments for study subjects.

13 Publication Plan

Neither the complete nor any part of the results of the study carried out under this protocol, nor any of the information provided by the sponsor for the purposes of performing the study, will be published or passed on to any third party without the consent of the study sponsor. Any investigator involved with this study is obligated to provide the sponsor with complete test results and all data derived from the study.