EGFR-mutations Identified from Blood-Derived Circulating Tumor DNA for Patients with Advanced Lung Adenocarcinoma

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Background

With emerging mutations throughout cancer progression, prior therapies are no longer effective. Unfortunately, it is not feasible to get repeat invasive tissue biopsies to test for alterations. This is especially true when the cancer metastasizes, and a biopsy at a single location is not enough to provide the full genomic makeup of the cancer. However, with the emergence of circulating tumor DNA isolation technologies, scientists will be able to obtain more information about the patient. ctDNA tests would be obtained by drawing blood, making it cheaper, more efficient, and less invasive than tissue biopsies .[1] With this emerging technology, simple blood tests will now be able to detect resistance mutations via next-generation sequencing.[3] One type of cancer that is especially susceptible to mutations in non-small cell lung cancer (NSCLC).[9] In 2004, epidermal growth factor receptor (EGFR) mutations were shown to be a driver NSCLC. Being able to identify this mutation and match therapies to patients with genetic alterations can lead to an earlier prognosis, increased response rates to therapies, and maximized survival.[9] The objective of this research is to present research on how genomic analysis in blood-derived circulating tumor cell DNA identified by next-generation sequencing can be used to detect epithelial growth factor receptor mutations and identify therapeutic targets in patients with advanced lung adenocarcinoma.

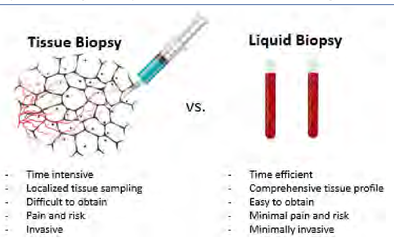

Figure 1: ctDNA analysis (liquid biopsies) has several advantages over a traditional tissue biopsy. *Retrieved from:* Overman, M. J... (2013).[7]

Abstract

Lung cancer is the second most common type of cancer and the leading cause of cancer-related death in the world. Non-small cell lung cancer (NSCLC), which often identified in later stages, is the most common histological variant, making up approximately 85% of all cases. [9,5] Mutations in epidermal growth factor receptor (EGFR), a driver of NSCLC, are correlated with decreased response rate and progression-free survival.[9] Genomic profiling of circulating tumor DNA (ctDNA) is an alternative to repeat invasive biopsies in NSCLC patients with tissue insufficient for genomic profiling. Circulating tumor cells (CTCs) are shed off primary tumors into the bloodstream and release ctDNA after cell death.[8] ctDNA tests require small amounts of blood, are a more affordable option than tissue biopsies, and can identify resistance mutations.[1,3] The utility of ctDNA analysis needs to be tested to facilitate the use of blood-derived liquid biopsies in advanced lung adenocarcinoma. 88 patients with lung adenocarcinoma were followed at UC San Diego Moores Cancer Center. 34 had NGS, 29 had other forms of genotyping, and 25 had no tissue testing due to contraindications. ctDNA was isolated from their plasma, mutations were identified, therapy was matched to the alterations, and therapeutic efficacy was measured. The results indicated that 82% had ctDNA alterations. 27.35% were EGFR mutations. The overall concordance rate for EGFR alterations was 76.5%. Excluding patients with no ctDNA detected, the overall concordance rate was 80.8% for EGFR mutations, and 100% in patients whose time interval between the blood draw and tissue biopsy was less than 1 month. Of the 28.4% of patients who were matched to a therapy, 85% received matched therapy to EGFR mutations, and 73% achieved stable disease. In conclusion, liquid biopsies via ctDNA provides an affordable and effective way to detect resistance genomic mutations that can revolutionize cancer detection methods and improve matched therapy.[9]

Materials and Methods

In the study, 88 consecutively tested patients with lung adenocarcinoma at UC San Diego Moores Cancer Center were reviewed. ctDNA was tests were performed on their plasma from August 2014 until October 2015. Digital sequencing of ctDNA in all of the patients was performed by the Guardant Health. 2 10ml Streck tubes of plasma were drawn from each patient, and 5 to 30 ng of ctDNA was isolated from the plasma sample. Sequencing libraries were prepared with custom barcode molecular tagging. The capture is followed by NGS of critical exons in 70 genes. It reports on all four major types of genomic alterations: point mutations, indels, fusions, and copy-number amplifications. To remove false positives, bioinformatics is used to match complementary strands of each of the DNA fragments that are barcoded. The mutations are measured using the variant allele fraction (VAF), which is the number of mutated DNA molecules divided by the total number of DNA fragments at that allele.

For the detection of EGFR, a 54-gene panel was used in 40 patients, which is utilized to identify potential tumor-related alterations in 54 cancer-related genes (such as copy-number amplifications in EGFR). For 47 patients, a 68-gene panel was used to measure all alterations. For the patients who had ctDNA detected in their plasma, CLI/CAP-accredited next-generation sequencing was performed on tumor tissue. For these patients (who had tissues NGS and plasma ctDNA testing) the concordance rate and corresponding kappa statistics of EGFR alterations was calculated. The analysis was also performed excluding patients with no alterations detected due to lack of ctDNA detection.

Next, treatment was matched and actionability was analyzed. A treatment was considered to be matched if it targeted at least one genetic abnormality or deviant pathway component in the patient's molecular profile. Actionability assumes that the protein product of the genetic abnormality can be changed with the targeted drug. A potentially actionable alteration is an alteration that was targeted, such as the EGFR inhibitor targeting an EGFR mutation. For therapeutic efficacy the following were measured: rate of stable disease (SD)/partial responses (PR)/complete response (CR), progression-free survival (PFS), and overall survival (OS). SD, PR, and CR were determined by the treating physician. PFS was defined as the time from the beginning of the therapy to progression. [9]

Methods and Materials cont.

OS was defined as the time from the beginning of the therapy to death or the last follow-up (for patients who were alive). The date of censoring was set as December 28, 2015. For statistical analysis, $\chi 2$ tests were used to compare categorical variables, nonparametric Mann-Whitney U tests were used to compare two groups with a continuous variable, binary logistic regressions were performed for categorical endpoints, linear regressions were performed for continuous variables, and the Kaplan-Meier method was used to analyze PFS and OS. [9]

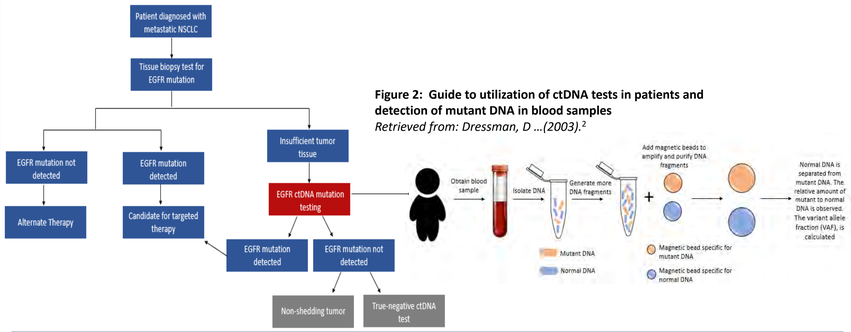

Figure 2: Guide to utilization of ctDNA tests in patients and detection of mutant DNA in blood samples
Retrieved from: Dressman, D ...(2003).[2]

Results

In the 88 tested patients, 27.3% (24 patients) were identified to have EGFR alterations. Patient data was analyzed in smokers compared to nonsmokers. Characterized EGFR alterations were more frequent in non smokers (13% (13/36)) than in smokers (16% (8/50)). 34 patients (38.6%) with EGFR alterations also had a multigene panel NGS tissue testing. The median time interval between the blood draw and the tissue biopsy was 0.8 months. This timepoint was used as a cut point for differentiating between tissue and ctDNA results. The overall concordance rate for EGFR alterations was 76.5%. When tissue testing of non-NGS types were included, the concordance rate was 75%. When the time between the blood draw and tissue biopsy for NGS was less than 0.8 months, the concordance rate was 88.2%. When the time interval was greater than 0.8 months, the concordance rate was 64.7%. 26 patients (with ctDNA alterations) had both ctDNA and a common tissue molecular test. For these patients, the median time interval between the tissue biopsy to blood draw was 1 month. This analysis excluded patients with no ctDNA detected in their plasma. The overall concordance rate for these set of patients was 80.8% for EGFR alterations. Out of 26 patients, 7 patients had positive concordance, 14 had negative concordance, 5 had discordance, 2 were only positive for EGFR alterations in ctDNA, and 3 were only positive for EGFR alterations in the tissue.

Of the 88 patients, 28.4% had an alteration in their ctDNA results that could be matched with a therapy. 80% of these (18 patients) received a therapy directed to EGFR. Targets include activating EGFR mutations in exon 19, L858R in exon 21, G719C in exon18 for first and second-generation inhibitors, and EGFR T790M for third-generation inhibitors. For all 25 patients that received a matched therapy, all received FDA-approved drugs, with exception to the 5 patients who were matched to EGFR T790M, which was being used in a clinical trial at the time (but is now FDA approved). 22 patients were able to be evaluated for response (3 were too early to assess). 16 (72.3%) achieved stable disease (SD) greater than 6 months. 11 achieved partial response. The median PFS for all 25 matched patients was 14.7 months, but was longer in patients carrying EGFR alterations versus not (17.6% compared to 5.1 months). 5 patients were given a third-generation EGFR inhibitor. These patients exhibited a EGFR T790M resistance mutation in their ctDNA results. All five achieved SD greater than 6 months. The PFS were 3.2+, 7,2, 8.1+, 14+, and 15+ months. They all also had a tissue biopsy confirming the EGFR T790M mutation. [9]

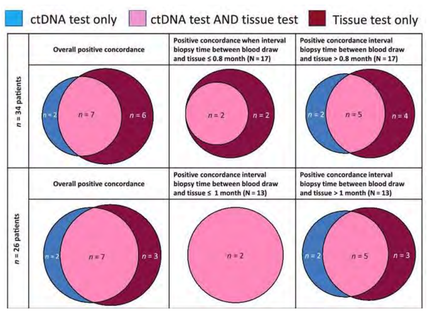

Figure 3: Positive concordance of *EGFR* alterations detected in both ctDNA and tissue tests. For the 34 patients with both types of testing, the median biopsy interval time was 0.8 month. For the 26 patients who had alterations detected in both tests, the median biopsy interval time was 1.0 months.
Retrieved from: Schwaederlé, M. C...(2017) [9] .

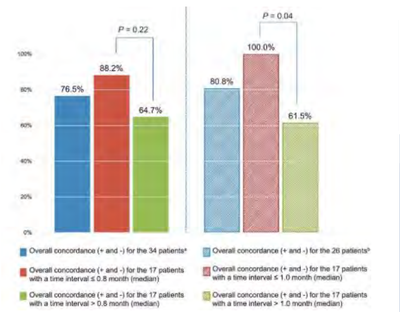

Figure 4: *EGFR* concordance between ctDNA and tissue tests. Included patients with *EGFR* alterations (all *EGFR* alterations considered) that had both ctDNA and tissue tests. Overall concordance included positive (+) and negative (–) concordant cases,
Retrieved from: Schwaederlé, M. C...(2017) [9] .

	Overall concordance**				Concordance for patients with tissue biopsy to blood draw time interval ≤ median[a,b]				Concordance for patients with tissue biopsy to blood draw time interval > median[a,b]				P-value* (≤ versus > median)
	(-)	(+)	Overall %	Kappa (SE)	(-)	(+)	Overall %	Kappa (SE)	(-)	(+)	Overall %	Kappa (SE)	
***N=34 patients*[a]**	N=19	N=7	76.5%	0.471 (0.155)	N=13	N=2	88.2%	0.605 (0.241)	N=6	N=5	64.7%	0.301 (0.224)	0.22
***N=26 patients*[b]**	N=14	N=7	80.8%	0.586 (0.165)	N=11	N=2	100%	1.0 (0.0)	N=3	N=5	61.5%	0.217 (0.269)	0.04

Figure 5: Negative concordance **:** alteration was not detected in both tests. Positive concordance (+): alteration was detected in both tests. Kappa values range from κ = 1 to κ = 0 (perfect to no agreement). Patients had EGFR alterations in both tests. SE=Standard Error.
*2-sided Chi-Square test
Retrieved from: Schwaederlé, M. C...(2017) [9] .

Discussion

The order of most frequent alterations detected in the liquid biopsies showed similar rates to the Cancer Genomic Atlas (TCGA). EDFR made up 27.3% of alterations in the experiment compared to 17% in the TCGA. Reasons for variance could be that TCGA was performed on untreated patients while the patients in the study were previously treated. Additionally, resistance mutations such as EGFR T790M are less likely to be evaluated in the TCGA. Other reasons include tumor heterogeneity, propensity of ctDNA into the blood, and patient population (high Asian population and low smoking rates). It was also observed that characterized EGFR alterations were more common in non-smokers, which was consistent with tissue analysis. Actionability and concordance rate of EGFR alterations for patients with both ctDNA and multigene panel NGS testing were similar to previously recorded rates. It was also noted that concordance rate increased when the time interval between the blood draw and tissue biopsy was shortened. This can be explained by changes in the tumor when the patient is undergoing treatment. An analysis on the positive cases indicated that both tests and could independently detect alterations not found in other tests, showing the clinical value of these techniques. Some alterations were only detected by either tissue or ctDNA. In order to obtain a more accurate concordance rates, the comparison of tissue and plasma results should be sampled during and before treatment to avoid ctDNA suppression. Reasons that results may be positive in tissue but not ctDNA include decreased ctDNA shedding after treatment and not all tumors shed DNA. ctDNA results may be positive when tissue is not because ctDNA reflects the genetic footprint of multiple lesions. Additionally, gene mutations post-treatment may not be found in archived tissue. ctDNA is useful to reduce the need for repeat biopsies during progression. Matched therapy based on ctDNA results of EGFR L858R, exon 19 deletion, and T790M produced similar responses to those based on tissue biopsy assays. All ctDNA-detected EGFR T790M alterations were confirmed in corresponding tissue. For the 95% of patients carrying a EGFR mutation, 73% achieved SD. 37.5% of patients with EGFR mutations carried the EGFR T790M resistance mutation and had all been previously treated with EGFR tyrosine kinase inhibitors. The initial detection of EGFR alterations in patients with lung adenocarcinoma and monitoring during treatment is essential for detecting resistance mutations. Limitations to the study include: small sample size, patients didn't have concurrent tissue and ctDNA biopsies, range of time intervals between ctDNA tests and tissue NGS, existence of patients without detectable ctDNA, and response assessment was performed by different physicians. [9]

In summary, the study reveals that ctDNA can be used as a liquid biopsy. The concordance rate of EGFR alterations confirms that a shortened time interval between blood collection and tissue NGS is preferable. Patients receiving cognate therapies had a higher rate of stable disease. Overall, multiple patients can benefit from ctDNA tests, including those with unclear tissue biopsy results, exhausted tissue from histopathology/immunohistochemistry testing, and no detected actionable alterations. For patients with no ctDNA detected, it is important that they receive tissue biopsies, as not all tissues shed their DNA into the bloodstream. Until they achieve common use, ctDNA tests can be used as a guide to verify biopsy findings and search for possible genomic alterations after a decreased response in therapy.[9]

Application of Biotechnology

While plasma ctDNA was discovered almost 60 years ago, it's utility hadn't been recognized until detection and isolation technologies such as identification through tumor-specific mutations and methylation of DNA.[4] Technology has now allowed for the detection of ctDNA at the molecular level. The emerging methods of enrichment, isolation, and analysis has given rise to a greater understanding of tumor cancer biology. Molecular profiling of ctDNA, using technology such as next-generation sequencing (NGS), allows for the analysis of genomic alterations. [10] The CellSearch system utilizes ferrofluid nanoparticles, which separate epithelial cell adhesion molecule (EpCAM) cells from other blood components after centrifugation. AdnaTest uses antibody-coated beads that are specific to the type of cancer. After collection, an RT-qPCR is run to determine expression patterns. Magnetic cell sorter (MACS) is another enrichment technology based on immunomagnetic separation that uses magnetic nanoparticles conjugated with antibodies. There are also microfluidic-based positive enrichment technologies, such as microarrays. This microfluidic technique utilizes anti-EpCAM antibodies to optimize cell attachment to the antibody-coated posts through the geometric arrangement of microposts. [12]

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Neonatal Outcomes Between IVF/ICSI Singletons and Twins Conceived Naturally and Through Assisted Reproduction

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Objective

The purpose of this poster is to examine the correlation between IVF/ICSI singletons and twins and detrimental outcomes in their future development. It will also discuss the impact of twinning on children conceived with and without assisted reproduction technologies. Twins born through IVF are particularly impacted physically and neurologically in this situation.

Abstract

Record numbers of women are turning to IVF with the hope of giving birth to a child despite infertility. While most IVF centers perform single embryo transfer, some will implant multiple embryos if a women chooses to have twins or wants to have a higher rate of successful implantation.[2] There is a clear difference in health outcomes between those infants conceived without assisted reproduction and those through IVF. A study done by the Groningen ART Cohort compared outcomes between three groups: children born through conventional IVF–ICSI vs. modified natural cycle IVF vs. natural conception. They studied 26 twin infants and 63 singletons, comparing rates of attrition in development after four years.[3] A secondary study done in Denmark compared morbidity between 472 IVF/ICSI twins, 1132 non-IVF/ICSI twins and 634 IVF/ICSI singletons. The Groningen study demonstrated that 4-year-old IVF twins have a significantly lower total IQ, a lower body weight and a smaller height than 4-year-old IVF singletons. Supporting the primary findings, the Denmark study indicates that physical health of IVF/ICSI twins is comparable with that of non-IVF/ICSI twins. However, physical health of IVF/ICSI twins is poorer and the negative implications for the families are stronger compared with IVF/ICSI singletons. This supports that twins born through IVF have significantly decreased abilities in comparison to their peers. They have higher rates of anencephaly and preterm birth, as well as a significantly increased chance of respiratory complications, sepsis, and jaundice in later life.[5] The information gathered in these studies raises concerns about the birth of multiple embryos, and indicate that single embryo transfer may be a better option.

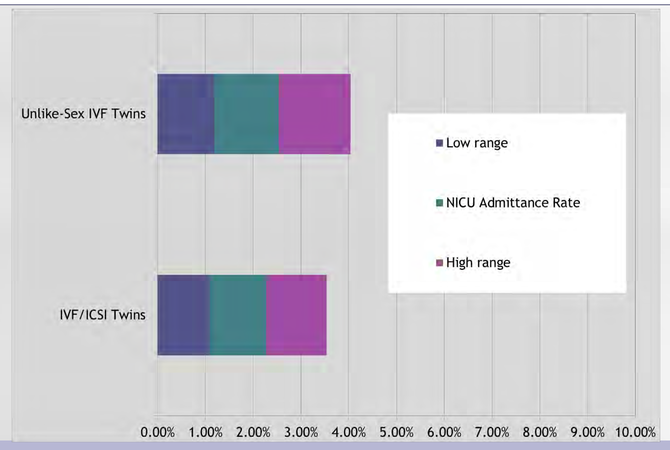

Figure 1: Percent increase from the control twins (not born through ART) to similar and different gender IVF/ICSI twins in relation to NICU admittance rates. Shah, J.S., Nasab, S.H., Chappell, N. (2018) Neonatal outcomes among twins stratified by method of conception: secondary analysis of maternal fetal medicine (MFMU) network database. *Assist Reprod Genet.* 35: 1011.

Methods and Materials

In the Groningen ART Cohort study, the group followed up with offspring born through and without IVF to assess their health and development. Initially , they selected both sub fertile and fertile couples. From these, a total of 89 neonates born as a result of ovarian stimulation and IVF–ICSI , 26 twin infants and 63 singletons. The follow-up examination at the age of 4 years was carried out by trained researchers who were blinded to the mode of conception of the children. It consisted of the assessment of neurological and cognitive development and the evaluation of anthropometrics and blood pressure. For neurological development, the Hempel assessment, an age-specific neurological examination, assessed neurological function in five domains: fine motor function, gross motor function, muscle tone and posture, reflexes and visual motor function. To evaluate cognitive development, the Kaufman Assessment Battery for Children was used to test IQ. The assessment of anthropometrics , or physical development, consisted of the measurement of height, weight, triceps skinfold and subscapular skinfold.

	IVF/ICSI Singletons	IVF/ICSI Twins
Weight (kg), mean	18.6 [18.1 to 19.1]	16.9 [16.0 to 17.9]
Standing height (cm), mean	108.8 [107.9 to 109.8]	105.9 [104.0 to 107.7]
SBP, mean [CI] mmHg[a]	101.8 [100.2 to 103.4]	102.3 [99.9 to 104.8]
Subscapular skinfold (cm)	0.558 [0.509 to 0.608]	0.540 [0.451 to 0.629]

Figure 2: Comparison of IVF Singletons and Twins with weight, standing height, standing blood pressure, and subscapular skinfold. [3]

Results

According to the study, twins born through IVF/ICSI have increased implications in relation to neurological, cognitive , and physical development. (See Figure 2 and 3). Twins had a lower birthweight ($P < 0.001$) and shorter gestational age at birth. Furthermore, IVF twins were more often delivered by Caesarean section and born preterm compared to singletons and non-IVF twins. This is supported by their higher admittance rate into the NICU (see Figure 1) and chance of morbidity. Post birth, it was found that the rate of attrition at the age of 4 years was 11% in the Groningen ART cohort study : 7% in singletons and 19% in twins. They had considerable differences between their means in the assessed categories of the study. For IQ, this was −5.4[−9.7 to −1.0]. The difference between weight was −1.7[−2.7 to −0.6], and height was −2.9[−5.0 to −0.8]. Lastly, for blood pressure the mean difference was 0.5[−2.4 to 3.5]. [3] These results align with the Danish study, which showed that compared with IVF/ICSI singletons, more IVF/ICSI twins had surgical interventions (P = 0.03), special needs (P = 0.02), and poorer speech development (P < 0.01). [6]

	IVF/ICSI Singletons	IVF/ICSI Twins
Learning IQ, mean [CI]	98.5 [96.4 to 100.6]	96.6 [93.5 to 99.7]
Knowledge IQ, mean [CI]	111.1 [108.5 to 113.7]	106.4 [101.6 to 111.3]
Total IQ , mean	108.9 [106.7 to 111.1]	103.5 [99.8 to 107.3]
Normal Neurofunction, %	60 (58)	23 (48)

Figure 3: The learning IQ, knowledge IQ, total IQ, and percent of normal neurological outcome between IVF singletons and twins. [3]

Conclusion

Neonatal outcomes are improved for IVF singletons compared with IVF twin births. The overall health of IVF/ICSI twins is poorer and the implications for the families stronger compared with IVF/ICSI singletons. They face more issues immediately, as well as in later life. Though the physical health of IVF/ICSI twins is comparable with that of non-IVF/ICSI twins, there are still some smaller differences between these two groups in relation to preterm birth and weight. However, many of these results found in the studies fall within the non significant value range. Despite this, the values for weight and height between IVF singletons and twins are significant. This indicates that there needs to be a shift away from implanting multiple embryos, which often leads to the birth of twins. Though there is no way to stop an embryo from splitting in utero, it is much less likely to occur and will allow for the birth of a healthier child. Therefore, women should choose single embryo implantation.

Application to Biotechnology

Since IVF is rapidly becoming a commonly used way to treat infertility, it is necessary that couples understand the risks associated with having twins. It is clear that twins conceived through artificial reproduction technologies have worse outcomes; therefore, it is important to increase the amount of singleton births. One way that this can be done is through elective single embryo transfer (eSET). eSET is a procedure in which one embryo, selected from a larger number of available embryos, is placed in the uterus or fallopian tube. eSET helps women avoid several risks to their own health and that of their child that are associated with carrying multiples. There is consensus among experts that the desired outcome of ART is a healthy singleton infant, which is what should be focused on in the future of in vitro fertilization.

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Exploring the Potential of Metformin to Prevent the Occurrence of Endometrial Cancer

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Background

Metformin is a first-line drug, used to regulate the insulin resistance found in type-2 diabetes patients. Recent evidence indicates a correlation between the insulin resistance found in type-2 diabetes patients and the insulin resistance found in endometrial cancer patients. This correlation theorizes metformin's potential to inhibit the proliferation of endometrial cancer cells. The theory is currently a controversial topic because evidence concerning the association lacks large, population-based studies confirming the results. This poster will be overviewing four, recently published studies disproving the association between metformin and a decreased occurrence of endometrial cancer.

Abstract

Metformin is a type-2 diabetes drug theorized to inhibit the growth of endometrial cancer, due to the correlation between the insulin resistance found in type-2 diabetes patients and the insulin resistance found in endometrial cancer patients[6]. Evidence confirming this theory remains controversial because large population-based studies are lacking. The following four studies disprove the potential of metformin to prevent the occurrence of endometrial cancer growth. The first study, a 2016 in-vivo study, proved the inability of metformin to reduce the ki-67 expression and inhibit endometrial cancer proliferation[3]. The second study, a cohort analysis observing the US healthcare claims of 272,422 subjects from 2000-2011, found no association with metformin and decreased development of endometrial cancer[4]. The third study, a case control analysis observing 1,746 subjects from the UK-based General Practice Research Database (GPRD) between 1995-2012, resulted in no lowered risk of endometrial cancer occurrence in subjects taking metformin[1]. A fourth study observing 748 subjects from 1997-2006 utilization databases in Lombardy, Italy, found no association between metformin and decreased proliferation of endometrial cancer[2]. Overall, research indicates that there is insufficient evidence proving the association between metformin and reduced risk of endometrial cancer development. Therefore, further investigation into metformin's potential for preventing endometrial cancer is required to demonstrate its promise.

Methods and Materials

The first study, an in-vivo analysis, involved forty female mice who were divided into two treatment groups at 6 weeks old. Twenty of the mice were fed a high-fat diet (HFD), while the remaining twenty received a low fat diet (LFD). At 10 weeks the two groups were split again. One half of each group received 5 mg/mL of metformin in their drinking water, while the remaining control group received untreated water. At 26 weeks the animals were euthanized. The mice's uterine tissue was scored for degree of the endometrial hyperplasia (EH), and immunohistochemical staining was used to detect ki-67 expression in the endometrial tissue. The second study observed the 2000-2011 US healthcare claims of metformin users with no prior cancer diagnosis, following them until a diagnosis of endometrial cancer. This involved a total of 272,411 subjects. The third study observed the UK-based General Practice Research Database (GPRD) between 1995-2012. This involved the observation of 1,746 subjects. The exposure, duration and long-term use of metformin was used to determine the association of metformin to an altered risk. The fourth study observed the databases in Lombardy Region, Italy between 1997-2006. The study consisted of 748 subjects and the odds ration in relation to metformin was estimated by the conditional logistic regression model.

Results

The research resulted in a variety of evidence disproving metformin's association with the prevention of endometrial cancer. The first study, a 2016 in-vivo study, confirmed the inability of metformin to reduce ki-67 expression and inhibit endometrial proliferation. The second study, a cohort analysis, observing 272,411 subjects from the US healthcare claims between 2000-2011, found no association with metformin and development of endometrial cancer. The third study, a case-control analysis observing 1,746 subjects from the UK-based General Practice Research Database (GPRD) between 1995-2012, resulted in no association between the use of metformin and a lowered risk of endometrial cancer. The fourth study, observed 748 subjects between 1997-2006 from the utilization databases in Lombardy Region, Italy, and also found no association between metformin and endometrial cancer occurrence.

Figure 1.

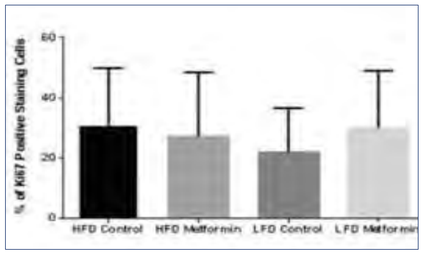


Figure 1. The image above displays no significant difference in cell proliferation between treatment groups, as determined by the percentage of ki-67 positive cells in the endometrium.

Figure 2.

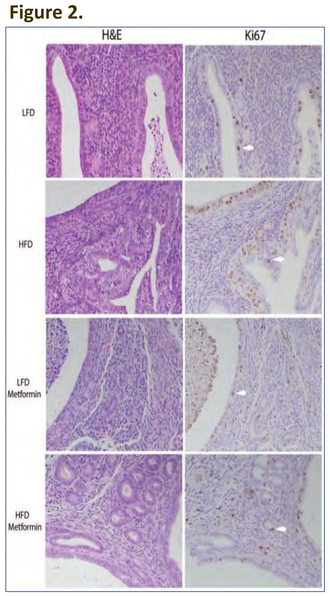
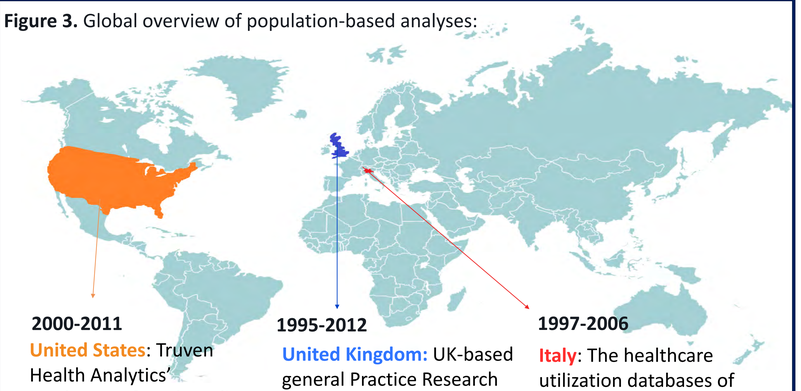


Figure 2. The left column displays endometrial tissue that was observed for the detection of endometrium hyperplasma following H&E staining. The right column displays cellular proliferation by immunohistochemical staining for ki-67 expression. The results of these in-vivo tests indicate that there was no significant decrease in either hyperplasma or ki-67 expression with metformin treatment.

Retrieved from Iglesias et al., 2016.

Figure 3. Global overview of population-based analyses:



2000-2011
United States: Truven Health Analytics' MarketScan& and Medicare supplemental databases.

Total subjects: 272,411

1995-2012
United Kingdom: UK-based general Practice Research Database (GPRD)

Total subjects: 1,746

1997-2006
Italy: The healthcare utilization databases of Lombardy

Total subjects: 748

Discussion

Research indicates that the ability of metformin to prevent the occurrence of endometrial cancer is currently a controversial topic. Evidence regarding this theory is currently lacking large studies confirming the association. The results of both population analysis and in-vivo studies disprove the correlation between metformin and endometrial cancer prevention. This suggests that, evidence confirming the association is insufficient compared to the larger scale of evidence against the association. Therefore, further investigation into the potential of metformin is required.

Application to Biotechnology

The objective of this poster is to present evidence against the association between metformin and endometrial cancer prevention. The evidence against the association was gathered from multiple studies including a recent in-vivo study. The biotechnology used to acquire the results from this study include immunohistochemical staining and H&E staining. The steps to Immunohistochemical staining involve: preparing the test sample, retrieving the antigen, blacking the background of the sample, detecting the target and observing the sample. The steps to H&E staining include: staining rehydrated sections of the tissue with Hematoxylin, washing the sample, staining the sample in Eosin solution, washing the sample a second time, dehydrating and observing the sample. This application of biotechnology reveals, through in-vivo testing, that the association between metformin and the decreased risk of endometrial cancer development requires further testing.

Acknowledgements

First of all, I would like to give Dr. Senegar Mitchell an immense thank you for taking the time out of her busy schedule to mentor the students of Reproductive Oncofertility Science Academy (ROSA). Her hard work and dedication is truly inspiring. I would also like to thank the directors of ROSA who devoted their time and energy into the organization of this academy. I can not express my gratitude enough for acceptance into this prestigious program. I am extremely grateful for the opportunity to be taught by leading scientists, whom I also owe immense gratitude to. This program has truly been an eye-opening experience that has made a lasting impression on my outlook of the medical field.

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Advances in Stem Cell Technology Leading to Increased Live Births Rates in Mice

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Objective

Development of a reliable method for generating adult fertile mice from induced pluripotent stem cells (iPSCs) is desirable in regenerative medicine because it is regarded as the most stringent test for pluripotency.[2,5] However, the same technology has vast implications for reproductive science.

Abstract

New applications of stem cell research, more specifically, the use of induced pluripotent stem cells (iPSCs) may provide another avenue to further the progress of reproductive science when gametes are not an option or desired. In 2006, the Yamanaka lab demonstrated that iPSCs can be generated from somatic cells through transfection of four transcription factors known to affect genetic reprogramming of somatic cells back to full pluripotency.[3] This report will explore the methodology of iPS cell line production and whether the use of a dox-inducible promoter increases the efficiency of live mouse pup births. The Baldwin lab, which utilized the dox-inducible promoter in eight cell lines produced 29 pups from 1,378 injected blastocysts, a 2.1% live birth rate.[2] Their highest producing cell line, iMZ-21, had a 13% live birth rate.[2] The Zhou lab developed six cell lines yielding 27 pups from 1554 blastocysts, a 1.7% rate of live birth.[5] Cell line IP14D-1 being the most productive with a 3.5% yield.[5] Although the methods used by the Baldwin lab indicate an overall higher efficiency, the difference in yields is not statistically significant. However the highly efficient iMZ-21 cell line should be further investigated for other factors that influence efficiency.

Methods and Materials

Generation of iPS cell lines: Mouse embryonic fibroblasts (MEF) were harvested on day 13.5 of pregnancy and prepared for transfection. Retroviral systems that include the Takahashi inscription factors, Sox2, Oct4, c-Myc, Klf4, were used to induce stem cell qualities. Green fluorescent protein (GFP) was used to indicate when the desired traits are expressed. Colonies of fluorescent iPS cells begin to emerge after 10 days, and after 14 days they are isolated for creation of iPS mice.[4]

Generation of iPS mice: 2-cell embryos are fused to 1-cell 4N embryo to create tetraploid blastocysts. iPS cells are injected into the blastocyst which are then transferred to a recipient mouse. After 17.5 days from transfer, observe the results of matured viable pups.[4]

All iPS cells and mice were created using similar methods described above with the following exceptions.

Baldwin lab:

Generation of iPS cell lines: The Takahashi transcription factor gene expression is controlled through the use of dox-inducible promoter which prevents inappropriate expression of these reprogramming factors.[2]

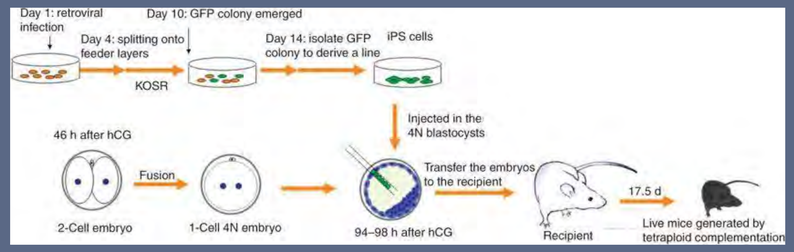

Figure 1. Schematic of developing iPSCs and injecting them into embryos. *Retrieved from Zhao et al.,(2010)*[4]

Results

Overall iPS live pup efficiency is calculated from the number of live mouse pups per injected tetraploid complementation (4N) blastocyst for all iPS cell lines developed by each lab. The results of two labs, Zhou and Baldwin are compared in Tables 1 and 2. The Baldwin lab had an overall efficiency of 2.1% while the Zhou lab had an efficiency of 1.7%.[2,5] A closer look at the efficiency for specific cell lines reveals a more dramatic difference. The highest efficiency produced by the Zhou lab is 3.5%, from the IP14D-1 cell line.[5] However, the Baldwin lab had a 13% efficiency rate iMZ-21 cell line.[2] The Baldwin lab also reports that several of their pups had deformities, often leading to difficulty breathing.[2] The Zhou lab reported that not all of their pups are reproductively competent.[5]

Table 1.
Efficiency of embryos produced by the Zhou lab. *Adapted from Zhao et al.,(2009)*[5]

Zhou Cell Lines	Injected 4N Blastocyst	Live pups (%)
IP14D-1	624	22 (3.5)
IP14D-6	43	1 (2.3)
IP14D-101	181	4 (2.2)
IP20D	204	0
IP40D-19	273	0
IP36D-3	229	0
TOTAL	1554	27 (1.7)

Table 2.
Efficiency of embryos produced by the Baldwin lab. *Adapted from Boland et al.,(2009)*[2]

Baldwin Cell Lines	Injected 4N Blastocyst	Live pups (%)
iMZ-9	172	0
iMZ-9	25	0
iMZ-9	195	7 (3.6)
iMZ-21	140	18 (13)
iMZ-15	257	3 (1.2)
iMZ-11	338	1 (0.3)
iNZ-3	120	0
iNZ-19	131	0
TOTAL	1378	19 (2.1)

Conclusions

Early work established that embryonic stem cells, once injected into blastocysts could produce live mouse pups. Zhou and Baldwin labs were the first to demonstrated that iPS cells could also produce live mouse pups. Advances in the methodology for creating iPS cell lines by the Baldwin lab, that include the use of an inducible promoter system, appear to have a positive effect on the yield of live mouse pups. This is supported by the data (Tables 1 and 2) showing higher overall yield of live pups over multiple cell lines as well as higher efficiency in specific cell lines. The inducible promoter system was used by the Baldwin lab to safeguard against the expression of reprogramming genes that would impede embryonic development. At this early stage of iPSC research it cannot be definitively concluded the differences in efficiency between the two labs is solely due to the dox-inducible promoter system. Other differences in their methods include prolonged valproic acid (VPA) treatment in the Baldwin method and modified cell culture conditions to include knockout serum without antibiotic selection in the Zhou method. Further investigation is required to isolate and test these variables as well as the impact of dox-inducible promoters on a larger scale. Before progressing to other animal models, causes of deformities and infertility needs to be addressed. To best support the goal of reproductive science, iPS technology must establish a reliable method of increasing the live birth rate of healthy, fertile offspring.

Relevant Applications to Biotechnology

Most all of the biological reagents necessary to create the iPS cells and the subsequent live organism generated from these iPS cells are products of the biotechnology industry.[4] As new biological tools become available, stem cell research and applications will continue to advance.

The goal of producing iPS cells with true pluripotency is to advance regenerative medicine and reproductive science. Within reproductive medicine iPSCs are a method for cloning organisms or a method for making germ cells.[3]

Acknowledgment

I would like to thank everyone involved with making this academy possible, especially Dr. Ericka Senegar-Mitchell, Mrs. Winter, and Dr. Chang. I would also like to thank all my ROSA sisters and Katie Larratt for their help and encouragement. Lastly I would like to thank my teachers and mother for helping me get into and through this academy.

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Effects of P-MAPA with Cisplatin on Survivorability from Serous Ovarian Carcinomas

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Background

Ovarian cancer (OC) is the leading gynecological cause of death in the US, with 80% of patients that survive it the first time relapsing within 2 years. 90% of OC cases are epithelial ovarian cancers, 50% of which are serous, or fluid-filled, carcinomas.[7] This makes serous epithelial ovarian carcinomas the most common ovarian cancer. Recently, the role of immunity in OC is starting to be studied for development of immunotherapies. Protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride (P-MAPA) is a protein aggregate that is already used in bladder cancer immunotherapy and works by targeting the tumors' Toll-like receptors (TLR) 2 and 4, which are key in innate immunity and initiate apoptosis and arrested growth.[2,6] When P-MAPA was tested against OC in rats, it was successful in reducing tumor size, increasing immune activity, and increasing survival. Response rates were up to 70%, which is substantially higher than the 11-25% response of current OC immunotherapies.[1,2,3] However, dual cancer therapies are historically known to increase rates of survival in many cases. This study explores whether P-MAPA can better increase rates of survival with cisplatin (CIS), a common chemotherapy agent for OC.[5]

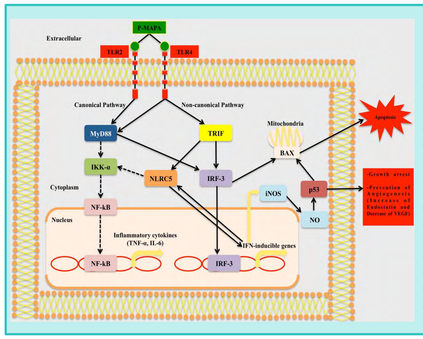

Figure 1. Action of P-MAPA binding to TLRs 2 and 4 and the resulting cascade. Ends in apoptosis and cell growth arrest. (Garcia, P. V., Seiva, F. R. F., Carniato, A. P., de Mello Júnior, W., Duran, N., Macedo, A. M., … Fávaro, W. J. (2016). Increased toll-like receptors and p53 levels regulate apoptosis and angiogenesis in non-muscle invasive bladder cancer: mechanism of action of P-MAPA biological response modifier. *BMC Cancer,* 16, 422.)

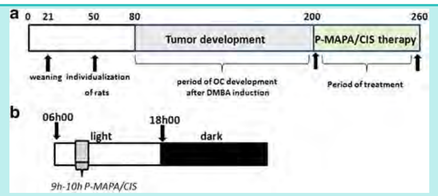

Figure 2. Chronological timeline for experimental design. (De Almeida Chuffa, L. G., de Moura Ferreira, G., Lupi, L. A., da Silva Nunes, I., & Fávaro, W. J. (2018). P-MAPA immunotherapy potentiates the effect of cisplatin on serous ovarian carcinoma through targeting TLR4 signaling. *Journal of Ovarian Research*, 11, 8.)

Abstract

Despite recent oncologic advancements, ovarian cancer (OC) is the leading gynecological cause of death in the US.[7] The role of immunity in OC is being studied for immunotherapies that target tumors' Toll-like receptors (TLR), which contribute to innate immunity, initiate apoptosis, and arrest cell growth.[6] P-MAPA is a protein aggregate that is used in bladder cancer immunotherapy and can been shown to target TLRs in OC rats with almost 70% response rates, compared to 11-25% response with other OC immunotherapies.[1,2,4] This study determines whether P-MAPA can better increase rates of survival from serous epithelial ovarian carcinomas when used in conjunction with cisplatin (CIS), a common chemotherapy agent for OC. Forty rat models were divided into four experimental groups: OC (control), OC+P-MAPA (P-MAPA treatment), OC+CIS (cisplatin treatment), and OC+CIS+P-MAPA (cisplatin and P-MAPA treatment). OC was induced by injecting the ovaries with DMBA and treatment was conducted for 8 weeks, over which numbers of living rats were recorded on Kaplan-Meier Curves. After therapy, the tumors were sectioned and necropsied, massed, stained for Western Blotting, and put through an ELISA assay to analyze immune activity and tumor size.[2] Surprisingly, while the combined therapy caused greater expression of immune components and larger decrease in tumor size, solely P-MAPA therapy led to highest survivorship, with P-MAPA rats and P-MAPA+CIS rats living 65% and 35% longer than the control, respectively.[2,3] Both immunotherapies have substantially greater effects on immunity and survival than current OC therapies, making P-MAPA a promising drug for OC patients.[1,2] Applications could include usage with chemotherapy and in isolation, depending on the clinical situation.[5] P-MAPA also holds great possibilities for use in other cancers.[4] The next step is testing OC response to P-MAPA in animals closer to humans, such as primates, then move to clinical trials in humans, which could eventually revolutionize ovarian cancer treatment.

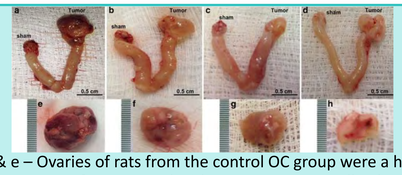

Figure 3. a & e – Ovaries of rats from the control OC group were a hard, solid mass. b & f – Ovaries of rats treated with P-MAPA were smaller, but had a large cyst and serous secretions. c & g – Ovaries of rats treated with CIS were much smaller and composed of softer tissue. d & h – Ovaries of rats treated with P-MAPA+CIS were very small and composed of soft, mobile tissue. (De Almeida Chuffa, L. G., de Moura Ferreira, G., Lupi, L. A., da Silva Nunes, I., & Fávaro, W. J. (2018). P-MAPA immunotherapy potentiates the effect of cisplatin on serous ovarian carcinoma through targeting TLR4 signaling. *Journal of Ovarian Research*, 11, 8.)

Methods and Materials

The study was conducted at the São Paulo State University in Brazil using forty female Fischer rats. Over the course of the study, the rats were provided with control factors of propylene cages, standard rodent food diets, 12 hr light/dark cycles, controlled room temperature, and filtered water. Ovarian cancer was induced in the left ovaries of all forty rats by injection of 100 µg of immunosuppressor 7,12-dimethylbenz(a)anthracene (DMBA) in 10 µL of sesame oil. The right ovaries of each rat received the vehicle for OC induction, sesame oil. The tumors were allowed to develop for 200 days, after which the rats were randomly split into 4 groups of 10 rats each and each group was given a different therapy. The four therapies included control, P-MAPA therapy, CIS therapy, and P-MAPA+CIS therapy, each of which was injected into the mice twice a week for 8 weeks. Dosages of the drugs were 5 mg/kg of body weight dissolved in 0.20 mL of 0.9% (v/v) saline solution, with combined therapy rats receiving that dosage of both drugs. The control rats received only the vehicle for therapy, saline water, throughout the 8 weeks. Samples for other analysis procedures were collected from the euphemized rats after the therapy period.[2]

Over the course of the tumor growth and treatment, the tumor was monitored through ultrasound for approximation of tumor volume. After the treatment was over, the rats were euthanized and the tumors were removed from their bodies for analysis. The tumors were massed for size and the rats' internal reproductive organs (ovaries, uterus) of the mice were dissected in search of general abnormalities. The number of rats living was also monitored daily during the period of OC growth and treatment. Results were charted on a Kaplan-Meier Curve.[2]

A portion of the tumor was frozen and -80^{o}C and sectioned into 5-µm thick slices. The slices were then stained with hematoxylin and eosin, biological dyes, for histopathological analysis. Slices of OC tissue were also tested for immune reactivity through an immunohistochemistry paraffin protocol. The pieces of tumor were deparaffinized and rehydrated using a microwave and 0.01 M sodium citrate buffer. Antigens were retrieved from the tissue and the OC was incubated with 3% bovine serum albumin (BSA) and primary antibodies (rabbit polyclonal anti-TLR2 and mouse monoclonal anti-TLR4). The tissues were then reacted with diaminobenzidine and counterstained with hematoxylin, enabling chromogenic detection of the samples.[2]

Immune activity was also measured through Western Blotting. The frozen tumor samples were fractioned with extraction reagents and cytoplasmic and nuclear protein extracts were obtained. The proteins were lysed with 10% (v/v) triton X-100 and quantified with a Bradford protein assay. 1.5× Laemmli buffer was added to the proteins and they were seperated with 4–12% acrylamide gradient gels. The proteins were then transferred to nitrocellulose membranes and blocked with 3% BSA. They were then incubated with primary antibodies for TLR2, TLR4, MyD88, TRIF, IKK-α, p-IkBα, and NF-kB p65 and then incubated with secondary antibodies for visible blotting.[2]

An ELISA assay was performed on the samples to detect levels of proteins related to cell death and proinflammation. Small, equal volumes of protein were extracted from the OC samples and were reacted with staining antibodies for the proteins IFN-γ, IL-6, and TNF-α. By having an equal amount of total protein, color shades can be compared between the three samples to determine relative amounts of each protein.[2]

One last test to determine immune activity was an immunofluorescence assay, in which isolated OC samples were permeabilized with proteinase K and incubated with polyclonal antibodies NF-kB p65 and anti-IgG conjugated to FITC. The nucleus was stained with 4,6-diamidino-2-phenylindole and florescence was read with a florescence microscope. Relative amounts of florescence were used to compare data.[2]

Results

Mass and Volume

After 30 days of treatment, rats treated with CIS and CIS+P-MAPA therapy reduced OC volume by 24.4% and 20.3%, respectively, from control volume. P-MAPA therapy had no significant effect on volume yet. After 60 days of treatment, P-MAPA, CIS, and CIS+P-MAPA therapy reduced tumor volume by 16.3%, 41% and 32.2%, respectively. At the end of the treatment, CIS and CIS+P-MAPA therapy groups had significant reductions in mass of 30% and 26%, respectively, while P-MAPA had no effect of importance on mass.[2]

Anatomopathology

Large differences were observed between tissue quality in tumors of each group. The control OC group was seen to have tumors of large, dense, solid mass and scattered necrotic spots. The group with P-MAPA therapy had tumors that were smaller, but contained large serous-filled cysts. The CIS and CIS+P-MAPA therapy tumors were very morphologically different, with softer, mobile tissue and very few adhesions.[2]

TLR2 and TLR4 Expression

TLR2 and TLR4 have been related to apoptosis and reduced cell growth.[6] The expression of TLR2 was greatly enhanced from the P-MAPA therapy, but did not seem to be effected by CIS or CIS+P-MAPA therapies. P-MAPA also upregulated TLR4 expression more than CIS therapy, but the combined CIS+P-MAPA therapy did so at the highest extent.[2]

Proteins	OC	P-MAPA	CIS	CIS+P-MAPA
TLR2	+	++	+	+/++
TLR4	+	++/+++	++	++/+++

Figure 4. Levels of TLR2 and TLR4 expression in tumors of different therapies: represented as 0 (absent), + (low), ++ (moderate), or +++ (high). (De Almeida Chuffa, L. G., de Moura Ferreira, G., Lupi, L. A., da Silva Nunes, I., & Fávaro, W. J. (2018). P-MAPA immunotherapy potentiates the effect of cisplatin on serous ovarian carcinoma through targeting TLR4 signaling. *Journal of Ovarian Research*, 11, 8.)

IFN-γ, TNF-α, and IL-6 Levels

The levels of all three molecules (IFN-γ, TNF-α, and IL-6, previously associated with tumor death) were upregulated by the combined CIS+P-MAPA therapy and the P-MAPA therapy.[2,3] CIS therapy increased expression of TNF-α and IL-6, but not IFN-γ. Combined CIS+P-MAPA therapy led to the highest expression of IFN-γ and IL-6, while P-MAPA therapy led to the highest expression of TNF-α.[2]

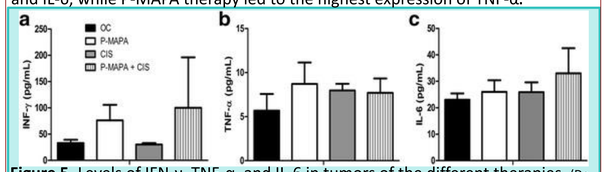

Figure 5. Levels of IFN-γ, TNF-α, and IL-6 in tumors of the different therapies. (De Almeida Chuffa, L. G., de Moura Ferreira, G., Lupi, L. A., da Silva Nunes, I., & Fávaro, W. J. (2018). P-MAPA immunotherapy potentiates the effect of cisplatin on serous ovarian carcinoma through targeting TLR4 signaling. *Journal of Ovarian Research*, 11, 8.)

Survivorship

The OC control group had an extremely short lifespan, with 100% of the rats dying at 280 days after OC induction. The longest survival rate was shown by rats with P-MAPA therapy, living an average of 65% longer than the control rats. CIS therapy had an approximately 35% increased lifespan from the control group and the combined therapy increased the lifespan by about 25%.[2]

Discussion

Overall, surprisingly, the study found that P-MAPA better extends lifespan when used as a therapy on its own than when used in conjunction with cisplatin chemotherapy. However, through this study, it can also be noticed that the combined cisplatin and P-MAPA therapy has more health promoting effects, such as decreasing tumor size and stimulating the immune system, than either cisplatin or P-MAPA therapy.[2] Combined, these results could have revolutionary effects on the treatment of ovarian cancer. Most importantly, they confirm both the P-MAPA and CIS+P-MAPA therapies as valid treatments of ovarian cancer and show both to be far more effective than current OC immunotherapies and chemotherapies alone.[1,2,5] While P-MAPA did prove to best increase length of life, more health benefits can be reaped from the CIS+P-MAPA therapy, which still significantly increased lifespan, while also greatly reducing tumor size and increasing expression of TLR4.[2] So, the combined therapy should be used in most clinical situations for treatment of ovarian cancer. This would help tumor removal go faster, meaning the patient would undergo shorter treatment and would be exposed to less radiation. However, the solely P-MAPA therapy also has extremely promising effects and can be used when patients grow chemoresistant to certain chemotherapy drugs. In these clinical cases, P-MAPA therapy would effectively continue treatment of the tumor, so that the patient is not taking a break from treatment in order to find a new drug, which sometimes allows the tumor to relapse.[5] The next step towards the implementation of P-MAPA as an official ovarian cancer immunotherapy is to research the effects of P-MAPA in animals that are closer to human size and physiology than rats, such as primates. After that, steps would be to move into clinical trials of the drug, both with and without cisplatin, to see if results in humans are different than in mice. Also, considering P-MAPA has previously been successful in bladder cancer, as well, it has an extremely promising future application to many different types of cancer.[4] Its targets, Toll-like receptors 2 and 4, are not organ-specific proteins, which makes P-MAPA applicable to a multitude of cancers.[6] Overall, development of P-MAPA into a viable immunotherapy drug could transform ovarian cancer treatment and be expanded to help patients with other cancers, as well.

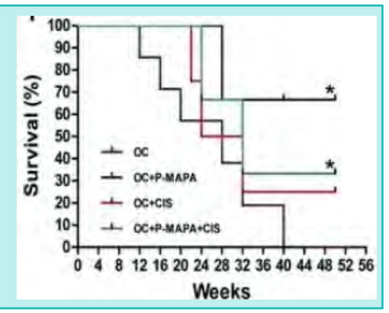

Figure 6. Kaplan-Meier Curve showing percentages of rats surviving in weeks after beginning of treatment. The P-MAPA rats survived best, but the CIS+P-MAPA therapy also significantly increased lifespan. (De Almeida Chuffa, L. G., de Moura Ferreira, G., Lupi, L. A., da Silva Nunes, I., & Fávaro, W. J. (2018). P-MAPA immunotherapy potentiates the effect of cisplatin on serous ovarian carcinoma through targeting TLR4 signaling. *Journal of Ovarian Research*, 11, 8.)

Relevant Applications to Biotechnology

This study was made possible with the use of many advanced biotechnologies. One of these is advanced ultrasound techniques, which were used to monitor the tumors over their weeks of development. An enzyme-linked immunosorbent assay (ELISA), in which an antibody with an affinity to the molecules of interest binds to them, was used to allow scientists to relatively compare amounts of bound antibody and determine the quantity of the molecules. Immunohistochemistry was used to analyze the slices of tumor for immune activity. Lastly, Western blotting techniques, which also involve electrophoresis, aided in measuring protein levels by separation, incubation, and probing of the tissue.[2]

Acknowledgements

I would like to thank everyone that has supported me in this research project and my journey so far at the Reproduction and Oncofertility Science Academy at UCSD. Specifically, I'd like to thank Dr. Ericka Senegar-Mitchell for all of her support and advice and her dedication to this program. I'd also like to thank Dr. Jeffrey Chang for all of his teachings and support and Ms. Patricia Winter for overseeing this program and providing so many girls with such an amazing opportunity. Thank you, Dr. Jamie Schiffer, for being an amazing guiding mentor and for all of your advice and wisdom. Also, thank you to everyone else associated with ROSA that has been involved in helping us learn and grow as students. Lastly, thank you to my ROSA sisters and my family for supporting me through this journey.

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Decreased Expression of MUC1 in Endometrial Tissues Correlates with Recurrent Embryo Implantation Failure

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The Academy of Our Lady of Peace

Background

It has been proposed that females who undergo assistive reproductive procedures and suffer from recurrent implantation failure (RIF) have abnormal endometrial receptivity. During the mid-luteal phase, the adhesion ligands of the endometrium will become pervasive in the endometrium and inhibitory factors will be removed.[5] A number of molecules have been associated with endometrial receptivity. One such molecule, Mucin 1 (MUC1), is a glycoprotein present abundantly in the endometrium during the receptive phase.[2] Its expression varies from fertile to infertile patients. This poster aims to demonstrate the correlation between recurrent embryo implantation failure and the expression of MUC1 in lumina and glandular epithelium.

Abstract

The endometrium plays a critical role in a successful embryo implantation.[1] Disruptions in implantation may account for women suffering from infertility. During the implantation window, approximately 7 days after a surge of luteinizing hormone, the endometrium becomes receptive to the implantation of the embryo.[4] Abnormal endometrial receptivity may contribute to recurrent implantation failures in females experiencing infertility.[5] It has been proposed that certain molecules are markers for endometrial receptivity but the exact molecular changes surrounding this issue are not well understood. MUC1, a member-associated protein expressed in luminal and glandular epithelium, is found at a higher level of expression in fertile patients than infertile.[2] Subjects include 14 women with RIF, 25 with recurrent miscarriage (RM), and 20 fertile controls who participated in endometrial biopsy during the implantation window. The spatial and temporal expression of MUC1 was studied using semi-quantitative immunohistochemistry. It was found that MUC1 expression in both lumina and glandular epithelium in women with RIF were significantly decreased compared to that of RM and control groups. This decreased expression was also not found to be associated with demographics or clinical characteristics.[5] Another study investigating MUC1 expression in females with implantation issues suggested that MUC1 expression in an infertile endometrium differs greatly from fertile and appears to be a component of altered gene expression.[3] Therefore, it can be strongly supported that decreased expression of MUC1 in endometrial tissues correlates with recurrent embryo implantation failure, and could be a potential target for therapeutics for enhancing success rates with implantation.

Methods and Materials

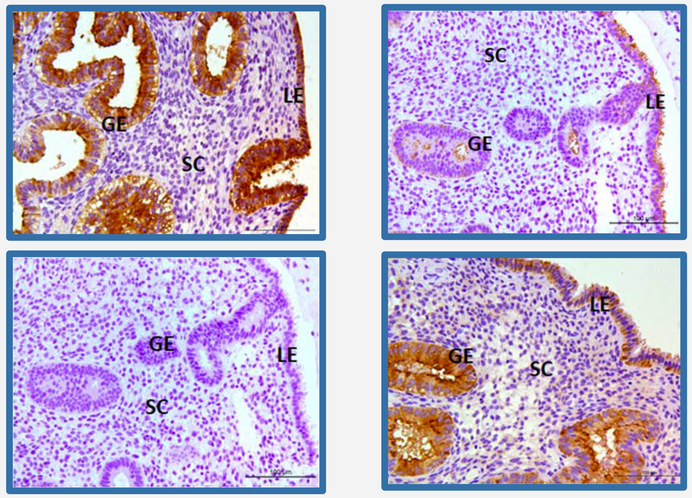
The subjects of this study included 59 women <40 years with regular menstrual cycles, typical body mass index, and no use of hormonal contraception within 3 months prior to the study. Women with endometrial or uterine pathology were excluded. 14 women were identified with RIF, meaning they failed to achieve a clinical pregnancy post 4 embryo transfers in 3 or more transfer cycles. 25 women were categorized with RM for 3 or more miscarriages prior to 20-week gestation. 20 fertile controls (at least 1 live birth in the last 1-2 years) also participated. All subjects had daily urine tests from day 9 of the cycle onwards to pinpoint the LH surge. Also, an endometrial biopsy was obtained using a Pipelle sampler (Prodimed) or Pipet Curet (Cooper Surgical) at LH + 7 day, during the implantation window. The specimens were then immediately washed in phosphate-buffered saline (PBS, pH= 7.4) and divided in half. One part was fixed in 10% neutral buffered formalin for immunohistochemistry and the other was examined pathologically for endometrial dating. Samples diagnosed as chronic endometritis were excluded.[2] The expression of MUC1 in the samples was analyzed through two methods: immunohistochemistry and H-score analysis.[5]

Immunohistochemistry-

Endometrial specimens were embedded into paraffin wax and cut to a thickness of 4 µm after overnight formalin fixation and serial ethanol dehydration. MUC1 spatial expression in the sample was determined by standard immunohistochemistry: sections were dewaxed in xylene, rehydrated by descending ethanol to PBS, and quenched in 3% hydrogen peroxide in methanol for 20 min. The antigens were then retrieved in a microwave oven with 10mmol/L sodium citrate buffer (pH=6.0). The samples were then washed in PBS and blocked in 1% bovine serum albumin buffer for 1 hour at room temperature and incubated at 4 degrees C overnight with mouse monoclonal anti-human MUC1 antibody. Next, the sections were washed in 0.5% PBST. The antibody binding was visualized by peroxidase substrate 3,3'-diaminabenzidene tetrhydrochloride and counterstained with hematoxylin. The sections were then dehydrated, put in synthetic resin DPX, and examined under light microscopy.[5]

H-score analysis-

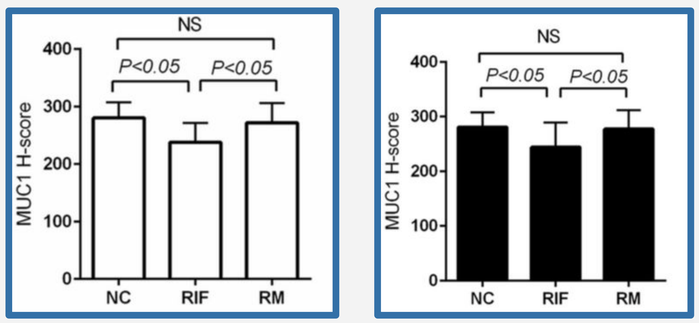
MUC1 expression intensity was quantified through the equation H-score = ∑Pi, where i was referred as staining intensity (0 = negative; 1 = weak; 2 = moderate; 3 = strong) and Pi was referred as percentage of cells stained at each intensity (0–100%). H-score was obtained in 5 qualified 400x visual fields per sample and scored by two independent observers.[5]



➢ Figure 1. MUC1 staining in endometrium. Magnification: X 200. Scale bar: 100µm. Top left- Control Group. Bottom left- Negative. Top right- RIF. Bottom right- RM. (Wu, F., Chen, X., Liu, Y., Liang, B., Xu, H., Chiu Li, T., & Chiu Wang, C. (2018). Decreased MUC1 in endometrium is an independent receptivity marker in recurrent implantation failure during implantation window.)[5]

Results

The results of the experiment effectively demonstrated the correlation between decreased expression of MUC1 in endometrial tissues and recurrent embryo implantation failure. Demographic details such as age, number of previous pregnancies, number of live birth and previous miscarriage, BMI, and menstrual cycle length seemed to have no significant correlation to MUC1 expression. MUC1 demonstrated strong immunoreactivity in both the luminal and glandular epithelial cells of the control group. However, it was much less intense in the RM subjects and extremely low in the RIF subjects. H-score analysis results demonstrated that the expression of MUC1 in luminal and glandular epithelium in RIF subjects was significantly below that in the control and RM subjects. A multivariate linear regression model of demographic and clinical characteristics shows data as follows: MUC1 H-score in luminal epithelium post-hoc statistical power ($P<0.05$) was 0.99. MUC1 H-score in glandular epithelium post-hoc statistical power ($P<0.05$) was 0.97. Residual score in the luminal epithelium was 0.61 whereas in the glandular epithelium it was 0.57. The R square score in the luminal epithelium was 0.37 and in the glandular epithelium it was 0.32. In the luminal epithelium, the adjusted R square score was -0.64. In the glandular epithelium, the adjusted R square score was -0.77. The probability of the MUC1 H-score in luminal epithelium was 0.90 and in the glandular epithelium it was 0.94. The beta coefficient in the luminal epithelium was 235.52 with a 95% confidence interval of -370.43-841.48. On the other hand, the beta coefficient in the glandular epithelium was 226.85 with a 95% confidence interval of -402-48-856.19. The probability in the luminal epithelium was 0.36 and in the glandular epithelium it was 0.40. [5]



➢ Figure 2. Differentiated expression of MUC1. Left- expression in luminal epithelium. Right- expression in glandular epithelium. Compared by one-way ANOVA among groups. NC: control group. RIF: recurrent implantation failure. RM: recurrent miscarriage. NS: no significant. (Wu, F., Chen, X., Liu, Y., Liang, B., Xu, H., Chiu Li, T., & Chiu Wang, C. (2018). Decreased MUC1 in endometrium is an independent receptivity marker in recurrent implantation failure during implantation window.)[5]

Conclusion

In summary, this study showed that MUC1 expression was significantly decreased in both the luminal and glandular epithelium in females with RIF, but not in women with RM or fertile controls.[5] Additionally, when compared to demographic characteristics, MUC1 expression was not found to be related and therefore is an independent marker of endometrial receptivity in women with RIF. It has been suggested that MUC1 is an anti-adhesive protein because of is physiochemical hindrance mediated by its long ectodomain. It may potentially inhibit the attachment of the blastocyst to the endometrium. The results of this study highly suggest MUC1 contributes to the unexplained reproductive failure in patients suffering from RIF and may be a potential target for therapeutics for enhancing success rates with implantation.[5] Further studies on the functionality and intervention of MUC1 are necessary to better understand its role in embryo implantation failures.

Applications to Biotechnology

A variety of applications of biotechnology influenced and were employed during this study. The hypothesis of the study investigates the correlation of MUC1 expression with recurrent implantation failure. Embryo implantation is a crucial part of a assisted reproduction techniques that involves the injection of a fertilized egg directly into the uterus in hopes that it will adhere to endometrial lining and begin to develop. Unfortunately, during this process, some women suffer from recurrent implantation failure. This leads to an undeveloped blastocyst and lack of a pregnancy. This study attempts to explore a potential cause of RIF by investigating MUC1 expression in the luminal and glandular epithelium. The intensity of MUC1 staining was evaluated using two biotechnological-related techniques. Immunohistochemistry is an important process that involves specific stainings and microscopic examination. Overall, this process assists in the diagnosis of neoplastic tumors and is also used to study biomarkers and differentially expressed proteins in biological tissue.[2] H-score analysis allows researchers to further analyze the test results from the immunohistochemistry test. It measures staining intensity amongst a variety of other factors.[5] This H-score is easily calculated by computers allowing scientists to gain access to a wide analysis of data in a matter of seconds. Such technologies bring medical professionals one step closer to helping women who suffer from recurrent implantation failure and hopefully assist them in having a successful live birth. In the future, this research on the correlation of deceased MUC1 in endometrial tissues and recurrent embryo implantation failure may lead to the development of therapeutic modalities and/or technology that would allow patients' MUC1 levels to be quickly tested prior to implantation.

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Using the Overexpression of RHAAM as a Prognostic Marker for Ovarian Cancer

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UC San Diego Health Sciences

Objective

About 20,000 women will be diagnosed with ovarian cancer this year, while around 14,000 will die at the hands of it. Currently, CA-125 tests are the only prognostic marker used in the detection ovarian cancer, however this test only works 50% of the time and gives false positives for benign gynecological diseases causing most physicians to shy away from using it.[3] Due to the inadequacies of current methodologies, ovarian cancer is oftentimes detected at later stages, decreasing potential survivorship by over 60%.[1] The niche of reliable diagnostic markers for ovarian cancer has yet to be filled until now, with the analysis of hyaluronan mediated motility receptor (RHAAM) overexpression. This novel research can catapult the creation of a prognostic marker and has the potential to become routine at clinical checkups, helping save thousands of lives every year.

Abstract

Ovarian cancer (OC) is the fifth leading cause of death in women and the leading gynecological disease that results in death.[1] This is due to inadequate diagnostic markers for women with OC, coupled with late detection and minimal early symptoms. If detected in early stages, OC patients have a 90% or greater chance of survival, whereas in late stages, the survival rate dwindles to a mere 30%.[1] Additionally recurrence rate of ovarian cancer detected at stage I is only 10% while at stage 3 it is a whopping 90 percent. The identification of a potential prognostic marker provides key information aiding early detection. Currently, CA-125 tests are occasionally used for early detection, however have proven unreliable due to recurrent false positives and 50% success in early stages- creating the necessity of a novel early detection marker.[3] RHAAM overexpression has been correlated with metastasis as well as the promotion of an invasive phenotypic expression in certain cancers, including OC.[4] In normal tissue, RHAAM has been known for its role in mitosis and cell growth. RHAAM expression has been studied through urinalysis, ELISA, and tissue staining in normal patients and OC patients.[3] RHAAM antibodies were used in order to aide and assist in detection of the protein through the aforementioned tests in tissue and urine samples. The results found that patients with OC exhibited elevated tissue RHAAM levels, while staining intensity increased with escalating cancer stage and tumor grade. Additionally, urinary RHAAM levels were elevated in OC patients. In all control patients, urinary RHAAM levels were undetectable, while 6/9 OC patients exhibited elevated RHAAM levels.[1] Furthermore, urinary RHAAM in women with benign gynecological disease was significantly lower than OC patients. When measured in OC cell lines, RHAAM levels were 20–70 times higher versus control ovarian cell lines.[1] Using RHAAM as a prognostic marker for OC is highly effective and exhibits increased reliability to current methodologies. The use of RHAAM as a prognostic marker can revolutionize early detection, as it is noninvasive, reproducible, and relatively inexpensive.[3]

Methods and Materials

Experiment 1: First, researchers sought to determine the potential correlation between RHAAM expression and ovarian cancer. To do so they analyzed RHAAM levels in tissue samples from a 27 patient cohort as well as used cell lines to confirm the results. The patient cohort included ovarian cancer patients varying from stages I-IV as well as normal patients. Tissue samples of ovarian cancer came from patients who had tumor excision through surgery and controls were used from women who had their ovaries removed or oophorectomy due to unrelated pathology. Patient samples were from varying stages of ovarian cancer. Additionally, excised tissue was examined and reviewed to confirm diagnosis by cross checking with the guidelines published through the International Federation of Gynecology and Obstetrics. The tissue was formalin fixed and embedded in paraffin in order to increase storage potential. Subsequently, immunohistochemical studies were performed on the tissue samples. The samples were immunostained with Rabbit Anti-Human CD168 antibody, dried, and mounted. Staining intensity was then monitored and localized to the specific regions in the cell. [1]

Figure 1. Layout of Different Experiments Adapted from Buttermore et al.,2017

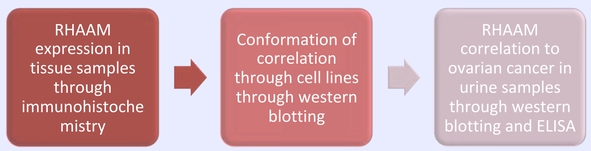


Figure 2. Summar of Patient Cohort for Tissue Samples Adapted from Buttermore et al., 2017

Patients	N=27
Normal	5
Ovarian Cancer	22
Stage 1	2
Stage 2	3
Stage 3	9
Stage 4	8

Experiment 2: The link between ovarian cancer and RHAAM overexpression was then analyzed in cell lines to confirm the results. Cell lines were cultured in complete media and western blotting was used to analyze expression of RHAAM protein. OC cell line was compared to HIOSE cell line which originates from normal human surface epithelium. Anti β-actin was used as a control because of its expression in all eukaryotic cells while RHAAM antibody was used at a 1:1000 dilution and protein bands were monitored.[1]

Experiment 3: After correlation was found between RHAAM overexpression and ovarian cancer, urinary RHAAM levels were found through western blotting and enzyme linked immunsorbent assay (ELISA) of normal and control patients. To conduct the western blot analysis, patient urine samples were spun down at 16000xg, gel electrophoresed, and transferred to a nitrocellulose membrane. The membrane was incubated in monoclonal rabbit anti-CD168 RHAMM antibody overnight in four degrees celsius. Sandwich ELISA was conducted after bringing samples up to room temperature (23 degrees celsius) and spun down in order to remove particulates-dirt or debris. The sandwich ELISA was conducted by putting urine samples on plates coated with RHAAM antibody. In the presence of RHAAM, the antigen would bind to the antibody and the plate would change color accordingly.[1]

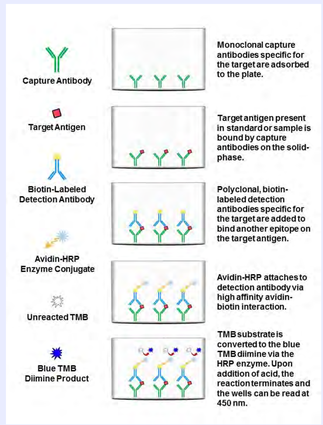
Figure 3. Summary of Patient Cohort for Urinary Western Blots Adapted from Buttermore et al., 2017

Patients	N=19
Normal	10
Ovarian Cancer	6

Figure 4. Summary of Patient Cohort for Urinary Sandwich ELISAs Adapted from Buttermore et al., 2017

Patients	N=209
Normal	29
Ovarian Cancer	150
Benign Disease	30

Figure 5. Explanation of Sandwich ELISA Test Adapted from Buttermore et al., 2017

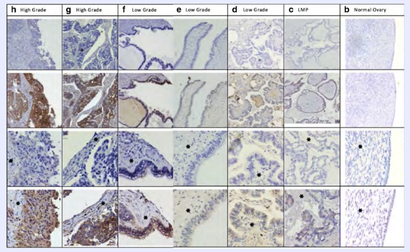


The capture antibody, in this case Rabbit Anti-Human CD168 antibody is used to coat the plate while RHAAM (target antigen) present in urinary samples binds to the antibody and causes the respective color change.

Results

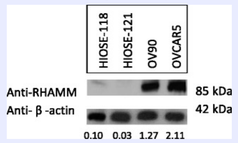
Experiment 1: Through immunohisterochemistry as depicted above, 91% (20/22) of ovarian cancer stained positive for RHAAM. 0% (0/5) normal surface tissue stained positive for RHAAM. RHAAM was expressed in 5/5 early stage (I and II) ovarian cancer. Additionally, localization of the RHAAM expression was in the cytoplasm in all ovarian cancer samples that RHAAM expression was found (20/22). Furthermore, it was found that staining intensity increased with escalating tumor grade.[1]

Figure 6. Immunohistochemical Staining of Patient Tissue Samples Adapted from Buttermore et al., 2017



Experiment 2:

Figure 7. Cell Line Western Blot Analysis Adapted from Buttermore et al., 2017



Western blotting in cell lines found that there was 12-40 times higher expression of RHAAM in the OV90 (ovarian adenocarcinoma) and a 20-70 times higher expression in OVCAR5 (high grade ovarian serous adenocarcinoma) cell lines, proving a correlation between RHAAM expression and ovarian cancer in a cell line model.[1]

Experiment 3: Through western blotting analysis it was found that urinary RHAAM was elevated in 66% (6/9) urine samples while in was undetectable in 10/10 of normal patients. Through ELISA testing it was found that urinary RHAAM was 15 times higher that in normal controls. Additionally, the average RHAAM levels in normal patient urine was 8.16 pg/ml while in ovarian cancer patients it averaged around 116.66 pg/ml. In women with benign gynecological disease, RHAAM levels averaged 12.47 pg/ml which was not statistically significant ($p<0.0001$). [1]

Figure 8. Urinary Western Blot Analysis Adapted from Buttermore et al., 2017

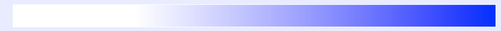
Figure 9. Breakdown of Urinary ELISA Results Adapted from Buttermore et al., 2017

Patients	N=209	Average Urinary RHAAM (pg/ml)
Normal	29	8.16
Benign Disease	30	12.84
Ovarian Cancer	150	116.66
Stage 1	11	157.63
Stage 2	4	105.34
Stage 3	45	162.35
Stage 4	6	85.83
Stage 5	11	87.58
No Family History	6	139.62
Family History	15	154.67

Relevant Applications to Biotechnology

The relevance and significance of this research lies in its potential as a prognostic marker. Similar to a pregnancy test, a RHAAM test could be formulated by using the RHAAM antibody LS-C164940. This antibody was used, with success, in all western blots and ELISAs in this study. When soaked in patient urine, the test would use immunoaffinity chromatography and change color depending on RHAAM levels present. This would work through RHAAM binding to the antibodies present. This can revolutionize early screening and early detection methods for ovarian cancer as it makes detection accessible and affordable.

Figure 10. Example of Chromatography Gradient with Increased RHAAM



Discussion

In all, RHAAM has proven to be correlated with ovarian cancer progression from the earliest to the latest stages. RHAAM expression has been elevated in ovarian cancer patients through tissue as well as urinary analysis. In the future, similar studies need to be conducted in larger patient cohorts to confirm the results. Western blotting and ELISA tests have proven reliable methods to test elevated RHAAM and protein levels in urine allowing it to be used for prognostic testing in hospital labs around the nation. Furthermore, CA125 tests have proven unreliable on their own with a low detection rate, and high rate of false positives, inducing unnecessary stress and procedures on patients. However, when the study looked at elevated RHAAM and elevated CA125 together to detect ovarian cancer, they had success in 28/29 patients, while sole RHAAM testing worked in 26/29 patients and sole CA125 worked for only 19/29 patients showing that RHAAM detection helps to optimize early detection.[1] This research lays the groundwork for a prognostic marker to be created through the use of a RHAAM antibody and immunoaffinity chromatography for more accessible use, allowing the face of early detection and ovarian cancer treatment to be transformed.

Acknowledgements

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Incidence of Ovarian Hyperstimulation Syndrome in IVF Patients Using Kisspeptin-54 for Ovulation Induction

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Background

Human chorionic gonadotropin (hCG) is commonly used in *in vitro* fertilization (IVF) to induce ovulation, but its long half-life contributes to ovarian hyperstimulation syndrome (OHSS), a serious condition of which symptoms can include enlarged ovaries, ascites, renal failure, and death.[2] Women with increased ovarian follicle count, such as those with polycystic ovarian syndrome (PCOS), are especially vulnerable to severe OHSS resulting from exposure to higher amounts of sex hormones after superovulation.[7] Such symptoms of OHSS can be mitigated by using kisspeptin as an alternative trigger for superovulation. Instead of directly introducing gonadotropins into patients, kisspeptin is a more physiological trigger that stimulates the ovulatory mechanism upstream of the ovaries.[8] Therefore, it is essential that there be a safer, effective option made available for women with these risk factors. This poster directly compares the viability of kisspeptin and that of hCG in terms of OHSS incidence rates and birth outcomes.

Abstract

Ovarian hyperstimulation syndrome (OHSS) is a serious condition that occurs in up to 6% of *in vitro* fertilization (IVF) patients, but it is four times more likely to manifest in women with abnormally high antral follicle count (AFC). OHSS is commonly attributed to the use of human chorionic gonadotropin (hCG) as an ovulation trigger.[1] While hCG induces ovulation at the ovarian level, the hypothalamic protein kisspeptin stimulates the preovulatory luteinizing hormone (LH) surge upstream of hCG.[8] Kisspeptin can induce ovulation in patients with normal ovarian reserve,[6] but the magnified threat of OHSS with current hCG treatment necessitates an investigation of whether kisspeptin can safely and successfully effect oocyte maturation in those with high AFC.[2] Subjects were selected for high risk of OHSS based on an AFC > 23. After a standard FSH/GnRH antagonist treatment, researchers administered kisspeptin-54 subcutaneously at varied doses (single bolus 3.2–12.8 nmol/kg; split dosing 19.2 nmol/kg over 10 hours), and oocytes were retrieved after 36 hours. Following follicular aspiration, OHSS severity was determined through ultrasound examination and patients' self-reported symptoms. Typical IVF protocol proceeded in subjects given kisspeptin-54, as well as in those given hCG or GnRH agonist.[1] For subjects who received kisspeptin-54, there was a 45% live birth rate per embryo transfer, comparable to an average 46.5% for non-kisspeptin IVF patients.[3] Only 3 out of 60 women developed mild symptoms of OHSS, but none were high-risk prior to IVF nor did anyone develop life-threatening symptoms.[2] Another study has directly compared the efficacy of kisspeptin to that of hCG, revealing a severe OHSS incidence rate of 15% with hCG versus 0% with kisspeptin.[1] Thus, kisspeptin is a reliable, more suitable trigger for ovulation in patients predisposed to OHSS.

Methods and Materials

Participant Selection

Subjects of this study (n = 261) were all women seeking IVF treatment due to infertility in 2013–2016 at Hammersmith Hospital in London, UK. The initial cohort of patients receiving the experimental kisspeptin treatment in the 2013–14 randomized control trial (RCT) were selected for the criteria outlined in Figure 1.[2]

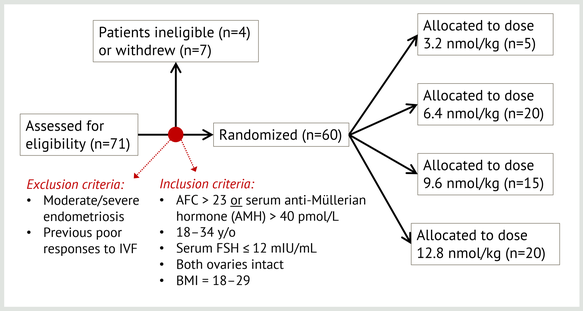

Figure 1. A schematic detailing the process of selecting subjects for one of the kisspeptin clinical trials (2013–14). The first 15 patients were randomly assigned to dosages of 3.2, 6.4, or 12.8 nmol/kg (each group n = 5), and then the following participants were then randomly assigned to dosages of 6.4, 9.6, or 12.8 nmol/kg (each group n = 15). Adapted from Abbara et al., 2015.

IVF Protocol

Superovulation protocol. All patients receiving kisspeptin trigger were first given the short protocol: daily subcutaneous (sc) injections of GnRH antagonist cetrotrelix or ganirelix (Cetrotide or Orgalutran, 0.25 mg) and recombinant follicle-stimulating hormone (FSH; Gonal-f, 112.5–250 IU) to prevent premature ovulation through controlled ovarian stimulation. Patients receiving hCG (choriogonadotropin alpha; Ovitrelle, 0.25 mg) were given either the short or long protocol (GnRH agonist buserelin; Suprefact, 0.2–0.5 mg), and those receiving GnRH agonist trigger (GnRHa; buserelin acetate; Suprecur, 2 mg) were all given the short protocol.[1]

Ovulation trigger. Injections of kisspeptin-54 were administered sc once pelvic ultrasounds confirmed at least three follicles ≥ 18 mm in diameter. Kisspeptin-54 was either administered in a bolus (3.2, 6.4, 9.6, or 12.8 nmol/kg) or split over a period of 10 hours (19.2 nmol/kg). The timing of drug dosages is indicated in Figure 2. hCG and GnRH were dosed according to the amounts above.[1]

Post-trigger. All kisspeptin patients were measured for serum LH, FSH, estradiol, and progesterone levels just prior to injection, as well as 12 and 36 hours after, to monitor sex hormone release.[2] 36 hours after kisspeptin administration, transvaginal oocyte retrieval (TVOR) was performed under ultrasound guidance. Intracytoplasmic sperm injection (ICSI) was then carried out and select embryos (≥ 6 cells, intact) were incubated to five days after oocyte retrieval, after which they were assessed for developmental quality. 1–2 high-quality embryos were then transferred into the uterine cavity for implantation 3–5 days post oocyte retrieval.[1]

Evaluation of OHSS

Screening for OHSS in patients ensued in two parts: early OHSS (3–5 days post retrieval at embryo transfer) and late OHSS (11 days post embryo transfer). In a screening, patients reported their symptoms associated with OHSS (abdominal pain, abdominal bloating, nausea, vomiting, diarrhea, reduced urine output), and transvaginal ultrasound was used to measure ovarian size and note any apparent ascites. Severity of OHSS (mild, moderate, or severe) was determined subjectively according to the criteria outlined below (Table 1).

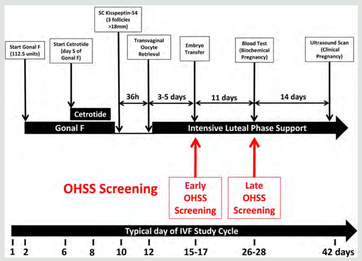

Figure 2. (left) Timeline of treatment for individuals participating in kisspeptin–IVF clinical trials. Retrieved from Abbara et al., 2018.

Table 1. (below) The three grades of OHSS that indicate degree of severity based on presenting symptoms. This is an objective grade created for the purpose of analyzing this study's results. Adapted from Abbara et al., 2018.

OHSS Grade	Criteria
Mild	At least one OHSS symptom, ovarian volume (either one) of 65–903 ml (approx. 5–12 cm in diameter)
Moderate	Meets mild OHSS criteria, 50 ml ascitic fluid
Severe	Meets moderate OHSS criteria, 50 ml pleural fluid

Results

The retrospective study was the first of its kind in directly comparing the efficacies of kisspeptin, hCG, and GnRHa as oocyte maturation triggers in IVF treatment. Kisspeptin was proposed to successfully induce ovulation in patients undergoing IVF, only with reduced incidence of OHSS in those with high AFC (> 23), and the results support this hypothesis. The rate of OHSS in patients was significantly lower in that of kisspeptin patients (Table 2). The kisspeptin-treated group had a drastically lower frequency of moderate to severe OHSS at 0% (0/122) while hCG had a moderate frequency of 23% (9/40) and severe frequency of 15.0% (6/40). Although the GnRH treatment group also did not have any cases of moderate to severe OHSS, the proportion of normal patients (those without any symptoms of OHSS) was only 67% (66/99) compared to 88% in kisspeptin patients (107/122). The mild OHSS rate was also the lowest for kisspeptin (12%; 15/122), compared to that of GnRH (30%; 30/99) and hCG (45%; 18/40). Symptoms of OHSS were also measured by early OHSS screening (see Figure 2), revealing a mere 12% of kisspeptin patients experiencing abdominal pain versus 69% of hCG patients (Figure 3A). The rate at which other symptoms—including bloating, diarrhea, and nausea—were presented is also relatively reduced in kisspeptin patients (Figure 3B–F).

N	Normal	Mild OHSS	Moderate OHSS	Severe OHSS
hCG (n = 40)	7 (18%)	18 (45%)	9 (23%)	6 (15.0%)
GnRH (n = 99)	66 (67%)	30 (30%)	0 (0%)	0 (0%)
Kisspeptin (n = 122)	107 (88%)	15 (12%)	0 (0%)	0 (0%)

Table 2. Rates of early OHSS (at the time of embryo transfer, 3–5 days post oocyte retrieval) diagnosis in each treatment group, categorized into no symptoms presented or mild, moderate, severe symptoms. Again, the mild, moderate, and severe degrees of OHSS were chosen subjectively, as well as the defining criteria in Table 1. Adapted from Abbara et al., 2018.

Mean ovarian volumes (compared to baseline levels from before controlled ovarian stimulation) were 20 times higher for hCG, 8 times higher for GnRHa, and 5 times higher for kisspeptin ($P < 0.0001$). It can then be concluded that kisspeptin, more so than hCG and GnRHa, minimizes vascular endothelial growth factor A (VEGF-A) release from the ovary, which in turn reduces the dangerous loss of fluid to the extravascular space. Almost all patients receiving kisspeptin-54 in one clinical trial (95%) had at least one mature oocyte retrieved, and the oocyte yield of all 60 patients for the same 2013–14 trial was 95% (Table 3). In another 2014 study, the oocyte yield was 36%, 76%, and 103% for 3.2, 6.4 and 12.8 nmol/kg, respectively, substantiating kisspeptin's ability to stimulate oocyte maturation. Since not all patients chose to proceed with embryo transfer and implantation, live birth rates were calculated as a percent per transfer performed (Table 3).[1] The live birth rate for all doses was 45.1%, remarkably close to the mean 46.5% figure reported for all traditional IVF treatments in the US in 2015.[3]

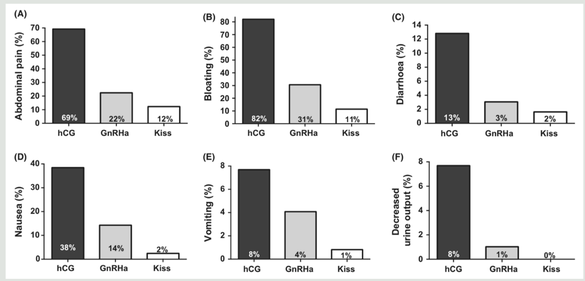

Figure 3. A comparison of select OHSS symptom frequencies between hCG, GnRHa, and kisspeptin patients. The rate of symptoms is 7–71% higher in patients treated with hCG versus those treated with kisspeptin. Adapted from Abbara et al., 2018.

Outcome Measures	Kisspeptin-54 Dose (nmol/kg)				
	3.2	6.4	9.6	12.8	All Doses
n	5	20	15	20	60
Oocyte yield (%) [a]	53	86	86	121	95
No. of patients w/ embryo transfer	4 (80%)	17 (85%)	13 (87%)	17 (85%)	51 (85%)
Live birth rate per transfer (%)	25.0	52.9	61.5	29.4	45.1

Table 3. The IVF outcome measures of kisspeptin-54 for the 2013–14 clinical trial shown as number of patients (% n) and percentages. [a] Oocyte yield is defined as the percent mature oocytes obtained from the number of follicles ≥ 14 mm in diameter on ultrasound immediately before kisspeptin injection. An oocyte yield > 100 is caused by eggs retrieved from follicles slightly less than 14 mm in diameter. [5] Adapted from Abbara et al., 2015.

Conclusion

In summary, exogenous kisspeptin reduces the incidence of OHSS symptoms in high-risk patients. In contrast with hCG's interruption on the body's functioning hypothalamic–pituitary–gonadal axis, kisspeptin's physiological mechanism significantly minimizes the consequences of hyperstimulation following superovulation.[6] Kisspeptin's efficacy has been confirmed in the aforementioned stage 2 trial, but there still needs to be focused experimentation for safety and toxicity in order to progress towards eventual approval for commercialization and widespread clinical application. So far, kisspeptin has exclusively been tested in a small experimental population in London, but it would be greatly beneficial in places like the United States, where over 200,000 IVF cycles were performed in 2015 alone.[3] Moving forward, further examination of kisspeptin analogs should be carried out to possibly improve potency and cost-efficiency,[4,6] subsequently increasing IVF accessibility. Kisspeptin boasts great potential as a valuable tool for assisted reproduction even beyond IVF, opening the doors for those with AFC to explore fertility options with ease and reassurance.

Applications to Biotechnology

In all patients, IVF requires specialized machinery and other assisted reproductive technology (ART). IVF physicians and imaging technicians used both pelvic and transvaginal ultrasound extensively in order to ensure proper follicular development following pretreatment, inspect their AFC, and screen for ovarian swelling. Most importantly, ultrasonography allowed for the extraction of egg cells by aspirating follicular fluid through the vaginal wall, a technique known as transvaginal ultrasound-guided oocyte retrieval (TVOR). Intracytoplasmic sperm injection (ICSI) was incorporated into the IVF process for fertilization, which was especially vital in the case of treatment for male factor infertility. Recombinant FSH follitropin-α (Gonal-f, Merck Serono, Geneva, Switzerland) used in controlled ovarian stimulation had origins in transfected nonhuman ovarian cell lines. Kisspeptin-54 is also shorter, synthetic form of the kisspeptin naturally occurring in humans. As an oocyte maturation trigger, kisspeptin can preclude the major complications of OHSS, making it a candidate for not only IVF enhancement but also egg and embryo cryopreservation, among other ART that involve the use of multiple mature ova.

Acknowledgements

I want to thank Dr. Ericka Senegar-Mitchell and all my other ROSA sisters for inspiring me in ways beyond science and making my summer so much more than I could have imagined. Thank you to Dr. Jeffrey Chang, Dr. Irene Su, and our other guest lecturers for taking the time to speak to us and for giving us unique insights into their work and careers. Also, thank you to Dr. Kellie Church for her mentorship and expertise in the poster-making process. Thank you Mrs. Patricia Winter and everyone else at the Oncofertility Consortium for making this entire program happen and allowing me to have this amazing opportunity. And of course, thank you to my family and friends for their endless support and encouragement.

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Teratogenic Effects of Immunosuppressive Drugs Given to Expectant Mothers after Organ Transplantation

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The Academy of Our Lady of Peace

Objective

Each year, over 10,000 transplantation surgeries are performed on women. While these transplantations save and improve an individual's quality of life, they also require the patient to take immunosuppressive drugs, which help to reduce chances of organ rejection, for the rest of their lifetime. This poster will evaluate whether there is an increased risk of congenital defects and other serious complications as a result of immunosuppressive drugs, such as antiproliferative agents, given to women during their pregnancies.

Abstract

Absolute uterine factor infertility affects approximately one in every 500 women. For years, the only options for these women were adoption and gestational surrogacy. Recent advances in uterine transplantation offer the opportunity for infertile women to carry and give birth to infants.[3] However, uterine and other organ transplantations necessitate immunosuppressive drugs (ISDs) in order to minimize graft rejection. ISDs can result in intrauterine growth restriction, congenital defects, and higher miscarriage rates of up to 48%.[6] In order to evaluate the risk of these fetal anomalies, data was collected from several studies measuring the extent of impact ISDs given to expectant mothers have on developing fetuses. Studies tested the exposure of different ISDs, particularly antiproliferative agents, at various points in the pregnancy of both women and animal models. Researchers separated the drugs into categories of high, medium, low, and unknown risk, and detailed each ISD's embryolethality, teratogenicity, and effect on fertility.[5] Mycophenolate (MMF) presented an increased risk of miscarriage (32-45%, comparable to the general population risk of 15-20%) and birth defects (26%, comparable to the general population risk of 3%). Out of 77 patients exposed to MMF, 25 reported miscarriages and 14 structural malformations.[4] Azathioprine (AZA) and 6-mercaptopurine (6-MP), however, were considered to be generally safe; 155 pregnant women exposed to these drugs were surveyed, and there was found to be no statistical difference in conception failures, birth defects, or spontaneous abortion, though researchers strongly suggest additional ultrasound monitoring in these pregnancies.[5] Dosages of immunosuppressive medications should be tailored for conception plans in order to maintain efficacy while minimizing fetal risk.[2] The results from these studies can be applied toward helping women who have undergone organ transplantation, allowing the increase in possibility for infertile women to carry and give birth to children through uterine transplantation.

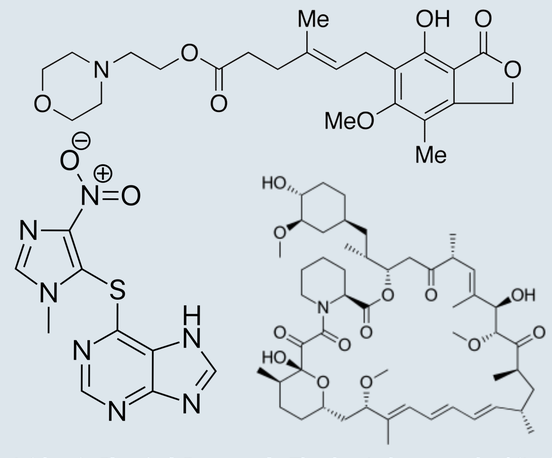

Figure 1 *(above)***: Chemical Compounds.** The chemical compounds of (*in clockwise rotation*) mycophenolate mofetil, sirolimus, and azathioprine are illustrated. Adapted from PubChem.

Materials & Methods

Researchers reviewed the health effects of antiproliferative ISDs on pregnant women and developing fetuses. Animal models and retrospective observational studies were utilized out of ethical concerns. 77 pregnancies exposed to MMF, 155 pregnancies exposed to AZA and 6-MP, and 16 pregnancies exposed to sirolimus (SLM) were monitored, in addition to animal models. Individual cases of exposure were also reviewed, including that of one pregnant women to 50,000 mg of MMF and later miscarriage. These ISDs were evaluated for gametogenesis, mutagenesis, teratogenesis, and deleterious effects on pregnancy; researchers calculated the percentage rate of each risk for each drug. Based on these results, the drugs were sorted into categories of high, medium, and low risk.

Results & Interpretation

Immuno-suppressive	Gametogenesis	Mutagenesis	Teratogenesis	Pregnancy
Mycophenolate	In rats: no effect on fertility	Mutagenic *in vivo* in rats	Clasto- carcino-teratogenic: multiple craniofacial anomalie	Crosses placenta ++ +
Azathioprine	No presented risk	Mutagenic	Carcinogenic and teratogenic in animals	No presented risk
Sirolimus	No presented risk	No presented risk	3 miscarriages and one child with multiple malformations (+mycophenolate)	No presented risk

Immuno-suppressive	Risk Factor
Mycophenolate	High
Azathioprine	Low
Sirolimus	Medium

Figure 2 *(above)* **: Outcomes of the Meta-Analysis of Antiproliferative Immunosuppressive Drugs**. The table above shows the study's findings on the effects of mycophenolate, azathioprine, and sirolimus. Adapted from tables by: *Leroy, C., Rigot, J.-M., Leroy, M., Decanter, C., Le Mapihan, K., Parent, A.-S., … Vantyghem, M.-C. (2015).*

Figure 3 *(left)***: Evaluation of Risk of Deleterious Effects on Fetal Development.** The table demonstrates the relative risk of harmful effects such as miscarriage or birth defects of immunosuppressive agents on developing fetuses. Adapted from results by: *Leroy, C., Rigot, J.-M., Leroy, M., Decanter, C., Le Mapihan, K., Parent, A.-S., … Vantyghem, M.-C. (2015).*

The data showed that fetuses exposed to MMF had greater risk of mutagenesis, teratogenesis, and spontaneous abortion. MMF posed an increased risk of miscarriage (32-45%) and multiple craniofacial congenital malformations (26%). Out of the 77 pregnancies, 25 were reported to be miscarriages and another 14 of the fetuses were found to have structural malformations. Data suggested that such risk was both cumulative and based on timing. Researchers originally used animal models to study the health implications of AZA and 6-MP without putting any mothers or fetuses at risk; both drugs were found to be teratogenic in animal models, as fetuses developed chromosomal abnormalities on circulating lymphocytes. However, no such risk was presented in later retrospective human studies. The 155 pregnancies exposed to AZA and 6-MP showed no increase in fertility issues, miscarriage, or fetal abnormalities. Research on SLM proved to be inconclusive as there was insufficient data and test subjects. However, of the 16 pregnancies exposed to SLM, 3 resulted in miscarriage and 1 in fetal malformations (although this subject also received MMF early in pregnancy). Thus, it was tentatively determined that, although SLM proved no high risk in teratogenesis, it was embryo- and foetotoxic.

Discussion

In evaluating the risks of antiproliferative ISDs, increases in gametogenesis, mutagenesis, teratogenesis, miscarriage, and infertility indicate high risk factors. Drugs with high risk (like MMF) should be contraindicated in the pre-conception period (the 3 1/2 months before pregnancy) and during pregnancy. Drugs considered to be medium risk (such as SLM) should be used with caution; even low risk drugs (like AZA or 6-MP) should only be taken with additional ultrasound monitoring and if completely necessary. Continuing high-risk ISDs can result in negative health impacts on both child and mother. The management of patients using ISDs who wish to become pregnant should involve consultations to change drug dosages in order to minimize maternal-fetal risks, patient-doctor planning of the pregnancy, and careful monitoring. It is crucial that the patient be advised of the risks and for the patient to employ effective contraception until pregnancy is desired.

Relevant Applications to Biotechnology

Over the past twenty years, over 730,000 organ transplantations have been completed. Many of these transplant patients can go on to lead healthy lives. Understanding the risks of ISDs on pregnancies can help with these patient's future success. Additionally, the past year has seen many advances in uterine transplantation. Although it is still in its developing stages, uterine transplantation would allow previously infertile women to naturally carry and give birth to children. Since immunosuppressive medications are an automatic risk in these women's future pregnancies, it is imperative to better understand possible pregnancy and fetal development complications in order to ensure favorable outcomes in uterine transplantation.

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I would like to thank Dr. Ericka, Dr. Chang, Ms. Winter, and the many other doctors and professionals who dedicated their time and efforts in ensuring this program's success; Dr. Church, Katie Larratt, and my ROSA sisters for their support and assistance; and my family and friends for their love and encouragement.

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Using GnRH Agonists With Chemotherapy to Prevent Premature Ovarian Failure

Emma Ramaley San Marcos High

Objective

Since cancer survival rates are increasing each year, there are a larger amount of infertile women as a result of gonadotoxic chemotherapy treatment. A common condition linked with infertility is premature ovarian failure (POF), which is the loss of normal ovarian function before age 40. Currently, there are some ways this devastating condition can be treated, but it would be more beneficial to patients' quality of life if it could be prevented. There have been promising results for the use of the drug gonadotropin-releasing hormone agonist (GnRHa). By suppressing ovarian function while chemotherapy is received, primordial follicles could be saved from being damaged, which preserves fertility for women.

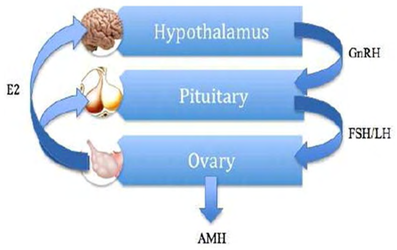

Figure 1. This flow chart shows how the hormones interact in the hypothalamus-pituitary axis and how GnRH begins this process. Bedoschi, G., Oktay, K., & Turan, V. (2013). Utility of GnRH-agonists for fertility preservation in women with operable breast cancer: is it protective?. Current Breast Cancer Reports, 5(4), 302–308.

Abstract

Many chemotherapy drugs can be extremely damaging to fertility, especially in a woman's ovaries.[2] The following research offers insight on a feasible way for premature ovarian failure (POF) to be prevented, ultimately preserving fertility while undergoing chemotherapy. Gonadotropin-releasing hormone (GnRH) is produced in the hypothalamus that signals to the pituitary gland to make FSH and LH, which are sent to the ovaries to produce estrogen and progesterone and control follicular recruitment.[1] The agonist of this hormone, which is a synthetic peptide, has potential to protect the ovaries during chemotherapy, by temporarily suppressing ovarian activity. When the ovaries are not growing follicles, chemotherapy drugs are not able to damage this process to a full extent.[4] In one study, 146 patients were given GnRHa along with chemotherapy, and in a control group, 71 patients were just given chemotherapy. Two years later, it was observed that only 13% of the group given GnRHa suffered POF, whereas in the control group, 51% suffered POF.[3] Also, in the GnRHa group, there were 123 healthy newborns, compared to 40 in the control group, and higher rates of spontaneous conception and retainment of cyclic ovarian function. These results show that not only could GnRHa prevent POF, but also cause normal ovarian function to resume following chemotherapy. Although this is not the most common form of fertility preservation, the use of this drug should be considered for women who are receiving chemotherapy and interested in having children later on. Using GnRHa during chemotherapy would eliminate the need for expensive techniques such as oocyte or embryo cryopreservation.[7] Another trial that would test various dosages would be needed to identify an ideal dosage which would balance the protection of ovarian function yet not disrupt treatment efficacy.

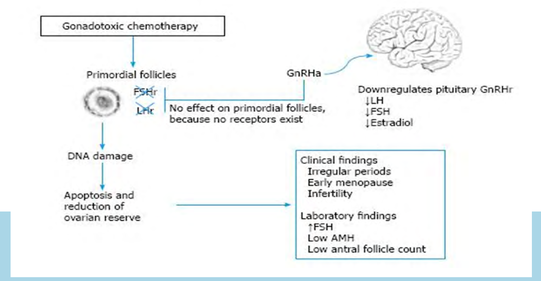

Figure 2. This illustrates how gonadotoxic chemotherapy directly damages primordial follicles, leading to a decrease in the ovarian reserve and eventually premature ovarian failure. But, if GnRHa is administered with chemotherapy, there is no effect on the primordial follicles and the hormones are controlled so that damage to the follicles is less likely.[8] Oktay et al. *J Clin Oncol* 2016; 34: 2563-2565

Materials and Methods

There have been several trials done in the last decade that suggest that GnRHa could prevent POF. Each included women of relatively normal ovarian function and who were premenopausal when given chemotherapy. Also, each patient was included only if they did not have previous chemotherapy, radiotherapy, or other cancers. Both of the following trials were performed to understand the extent of fertility preservation that the administration of GnRHa has, which included the prevention of POF.

Trial 1: This randomized phase 3 trial was conducted at 16 Italian health centers in 2009. It included 281 breast cancer patients, of varying stages, who were of median age 39. Each patient was randomly assigned to a group, making 148 patients in the group that would receive GnRHa with chemotherapy and 133 in the group that would receive chemotherapy alone. The GnRHa group was given an intramuscular dose of 3.75 mg at least one week before the start of chemotherapy and then every four weeks for the duration of treatment. The type of chemotherapy used was cyclophosphamide. Menstrual activity and FSH and estrodial levels were assessed for one year after the end of chemotherapy.[5]

Trial 2: Another similar study was conducted in 2011 by the American Society of Clinical Oncology. This consisted of 129 Hodgkin or non-Hodgkin lymphoma patients, with a median age of 25.6 years, within a multicenter setting. Patients were randomly assigned to the group that would receive GnRHa with chemotherapy, which was 65 women, or the group that would receive only chemotherapy, which was 64 women. Patients in the GnRHa group were given 11.25 mg of GnRHa by intramuscular injection every 12 weeks, with doses beginning 10 days before the start of chemotherapy. The treatment included alkylating agents containing chemotherapy and patients had to be receiving at least 8 cycles of chemotherapy in order to produce high levels of gonadotoxicity for the trial. The goal was to assess the rate of POF in each group after one year of follow-up, in addition to levels of FSH and estradiol levels.[6]

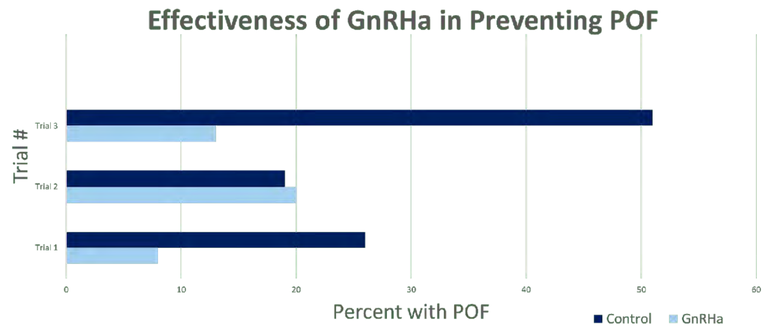

Chart 1. Representation of how effective GnRHa can be in preventing POF, based on 3 trials.[3,5,6] (Trial 3 located in Abstract).

Results

After analyzing these studies, it is possible that GnRHa could be used to prevent POF and preserve fertility for women undergoing chemotherapy. Although, some results were inconsistent, so further trials are needed.

Trial 1: In this group, 21 patients were considered unevaluable due to unrelated conflicts with the chemotherapy administration. Of the remaining 260 women, in the GnRHa group, only 8% suffered POF, whereas in the control group, 26% suffered POF. In the GnRHa group, also, rates of early menopause were lower and resumption of menses was higher, along with twice as many pregnancies as the control group.[5]

Trial 2: By the one year follow-up, there were 45 patients in the GnRHa group and 39 in the control group. There were two patients who faced severe adverse effects from the GnRHa group, but there is no confirmed correlation. Fortunately, most patients had recovered normal ovarian function following treatment, except for 20% and 19% of women in the GnRHa group and control group, respectively, who had developed POF. Additionally, two patients in the GnRHa group had pregnancies. The proximity of this trial's results raises awareness that GnRHa may have inconsistent effects, although this form of treatment may have a long-term benefit on fertility that allows women to maintain normal ovarian function, but it has not been concluded.[6]

Discussion

To conclude, GnRHa has demonstrated that it could become a common solution for preventing POF in women receiving chemotherapy. Numerous trials have shown that GnRHa decreases rates of POF in various ages of women and diseases, making it a versatile option. This could lead to the administering of GnRHa becoming an accepted technique in the field and if the quality of the drug could be approved to increase outcomes of POF prevention, it could be used instead of embryo cryopreservation or other current techniques. Although, some results have been inconsistent and the long-term maintenance of ovarian function as a result of this drug is not certain, so further studies will be needed to prove whether GnRHa could eventually be used routinely. Trials consisting of various diseases, ages, and dosages would be most beneficial to see how this drug varies in each case, because much of that information is not known. If this option of fertility preservation could be perfected, women would not have to use expensive techniques in order to have children following chemotherapy.

Applications to Biotechnology

In the emerging field of oncofertility, there are many exciting discoveries being made using new techniques and procedures. This specific research would not be possible without the engineering of GnRHa, a peptide, which is able to influence the endocrinology and target GnRH receptors in patients. By using recombinant DNA technology, quality drugs, such as GnRHa, are being made to improve the health and quality of life of many patients. Revolutionary technology like this is also making the manufacturing of drugs more efficient by identifying problems that could cause them to fail early on, even before the clinical stage. Because of this, drugs like GnRHa could be improved and prevent infertility caused by gonadotoxic chemotherapy. These advancements are changing the way patients are being treated and given access to new medications, leading to a more promising future of fertility.

Acknowledgements

First of all, I would like to thank Dr. Ericka Senegar-Mitchell for her encouragement through this experience and for inspiring me to be a strong woman in science. Also, thank you to Dr. Chang for teaching us about this amazing field of medicine, Ms. Winter for making this program such a life-changing experience, and especially my OSA sisters for being there for me through it all. Lastly, I am so grateful for my wonderful parents who show their endless love and support for me always.

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Investigating the Efficacy of 5T4 as a CAR T-Cell Target To Increase Ovarian Cancer Survival

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Background

One in every 78 women will develop ovarian cancer in her lifetime, and even with efforts to improve chemotherapy and surgery options, existing treatments have low long-term success rates. [6] However, recent advances in immunotherapy, particularly with chimeric antigen receptor (CAR) T-cells, have restored hope for these women. This method could ultimately be a superior alternative to chemotherapy, as it harnesses the power of the patient's immune system to target cancerous cells exclusively, so limited damage is done to healthy tissues.[5,2] While CAR T-cell therapy in hematologic cancer has been successful, results with solid tumors have been underwhelming, in part due to the on-target, off-tumor effect, where healthy tissue is damaged due to its expression of the target antigen.[1] Thus, there is a need for an antigen to be identified that is overexpressed in cancerous tumors and underexpressed in healthy tissue. This poster will explore the efficacy of the antigen 5T4 as a target for CAR T-cells used to treat ovarian cancer.

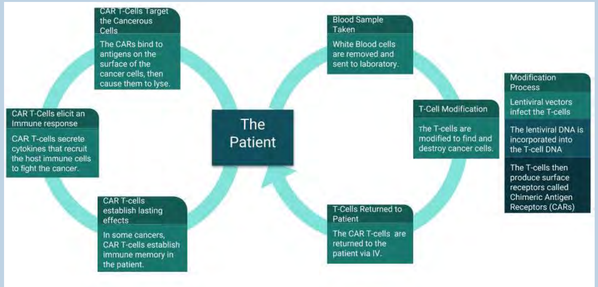


Figure 1: Basic mechanism of action for CAR T-cell therapies. *Adapted from Owens et al., 2018* [5]

Abstract

In 2018, there are projected to be more than 22,000 new cases and 14,000 deaths due to ovarian cancer (OC) in the US.[6] Even with efforts to improve current treatment options, 80% of patients relapse within 18 months of completing their first treatment.[4] Chimeric antigen receptor (CAR) T-cell therapy is a promising new OC treatment, as it harnesses the power of the patient's immune system to target cancerous cells exclusively.[5,2] While the therapy's success with hematologic cancers gives hope for solid tumors, unique problems such as on-target, off-tumor effects must be addressed.[1] 5T4-antigens may be a target for CAR T-cells as they are overexpressed in solid tumors, but have limited expression on normal tissues. The goal of this study was to conduct a series of tests to determine the utility of 5T4-antigen as a target for CAR T-cells in OC. Recently, scientist transduced two anti-5T4 CAR constructs, varying in affinity to 5T4, into T-cells taken from 12 patients. Then, they were co-cultured them with their autologous ovarian tumor. After 24 hours the supernatant was collected and INFγ and IL-2 levels assessed. Results suggested a correlation between amounts of INFγ and IL-2 secreted and 5T4 expression, with all r values ≥.65, indicating immune activation. In another experiment, NSG mice were injected with SKOV-3 cells. At day seven, those with tumors received varying doses of anti-5T4 CAR T-cells, mock-transduced T-cells, or saline, then had their tumor size monitored at regular intervals. Mice given ≤.3 × 10^7 H8-CAR or a placebo died within 90 days while Mice given 1 × 10^7 H8-CAR had a 100% survival rate past 100 days.[5] This establishes 5T4 as a promising target for CAR T-cell therapy in OC, and potentially for other solid tumors that express 5T4. However, before advancing to clinical trial, 5T4's on-target, off-tumor effect must be confirmed in further studies.

Materials and Methods

Two 5T4-specific monoclonal antibodies, a higher affinity H8 CAR construct and a lower affinity 2E4 CAR construct, were used in this study. Affinity was determined by Biacore, which measures the concentration of the antigens on a sensor surface as they bind and dissociate.[5]

SCOV-3 and OVCAR-3 ovarian cancer cell lines, transfected to express luciferase (for use with bioluminescence imaging later), were also obtained for the experiments. Both of the cell lines express 5T4, >90% and >70%, respectively. The cell lines were cultivated in a single layer and routinely checked for contamination.[5]

To create the 5T4-specific CAR T-cells, T-cells were isolated from patient blood samples using a centrifuge. 1.5 × 10^6 cells were put into a 24-well plate. The cells were then co-cultured with one of three lentiviral vectors: 3 µL of H8-CAR, 4 µL of 2E4-CAR, or no vector. The T-cells were counted every other day, beginning on day three, and divided for 14 days.[5]

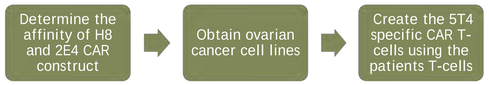


Figure 2: An overview of preparation for the experiments. *Retrieved from Owens et al., 2018.*[5]

Experiment 1: To test the level of expression of 5T4 in ovarian cancer, 12 ovarian tumors were collected from ovarian cancer patients undergoing surgery at St Mary's Hospital, Manchester, UK. The samples were taken with informed consent. Indirect immunohistochemistry was used to test for 5T4 expression. Refine Detection Kit was used to identify the bound antibodies. Next, the tissues were evaluated for proportion and intensity of antigens with a modified H-score. Finally, using GentleMACS, the tissue samples were disaggregated into single-cell suspensions, and then had cell viability tested using a trypan blue stain, for use in experiment 2.[5]

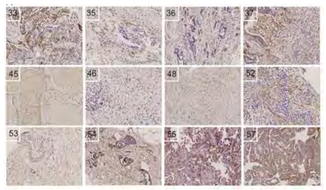


Figure 3: A light microscopy (x20) of each patient's tumor after staining for IHC. *Retrieved from Owens et al., 2018.*[5]

Experiment 2: To test the functional reactivity of both H8 and 2E4 against 5T4 ovarian cancer cell lines and primary tumor cells, both constructs were co-cultured either with the tumors taken from patients or with the 5T4 expressing cell lines. The co-culture was at a 1:1 ratio of 1 × 10^5 T cells to 1 × 10^5 autologous tumor cells in a 96-well plate. For a negative control, T-cells were cultured alone, and for a positive control they were cultured with 50 ng/mL phorbol 12-myristate 13-acetate and 1 µg/mL ionomycin. The cultures were incubated at 37 degrees Celsius for 24 hours, and then the supernatant was collected and IFNγ and IL-2 levels assessed to measure immune activation via enzyme-linked immunosorbent assay kits (ELISA). [5]

Experiment 3: An NSG mouse model was used to test whether 5T4 CAR T-cell therapy conferred a survival advantage *in vivo,* and if so, at what dose. The study was approved by a local ethics committee. The mice were all given identical cages and kept on a 12/12 light dark schedule. They were also all fed the same type/amount of food (Teklad global 19% protein extruded rodent diet). All mice were challenged via the intraperitoneal route with SKOV-3 cancer cells. 7 days later the mice that had developed peritoneal tumors were injected with varying doses of H8-CAR T-cells. The doses ranged from .03 × 10^7 to 1 × 10^7 total T-cells. The control mice were treated either with 1 × 10^7 Mock-transduced T-cells (positive control) or saline (negative control). The mice's tumors were assessed via bioluminescence on day 6 and at regular intervals for 100 days.

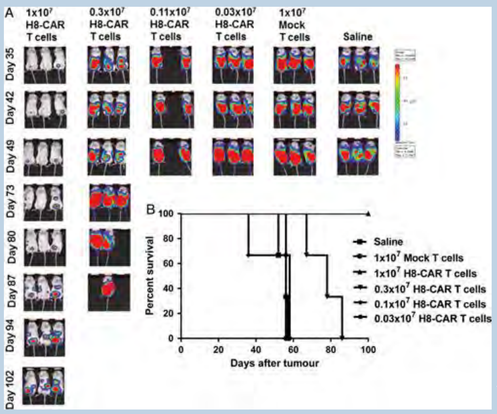


Figure 5: Mouse tumor burden was assessed via bioluminescence and is pictured on the left, while survival is measured and compared on the bottom right. *Retrieved from Owens et al., 2018.*[5]

Results

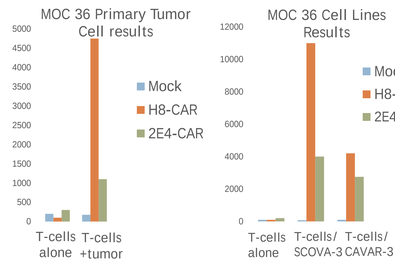
Taken together, these three experiments demonstrate the efficacy of 5T4 as a target antigen for CAR T-cell therapy. The first experiment supports the notion that there is a high level of expression of 5T4 in ovarian cancer cells. The second study suggests that the higher affinity H8 construct may be the most effective at eliciting an immune response and that a patient's own T-cells can be used to target autologous tumors. Finally, the third study demonstrates that the H8 CAR construct confers a significant survival advantage to mice with ovarian cancer tumors. [5]

Experiment 1: The 12 patient tumors all proved to be positive for 5T4 expression via immunohistochemistry. This suggests there is consistent expression of 5T4 expression in ovarian cancer. However, the intensity of 5T4 expression varied between patients. Overall, 50% of EpCAM+ Cells (25.12% (±24.89%) of the total cell population) expressed 5T4. The intensity of expression was measured by H-score post immunohistochemistry *(see below).*[5]

Patient	MOC 33	MOC 35	MOC 36	MOC 37	MOC 45	MOC 46
H-Score	13	5.3	5	11.7	4	4.3
Patient	MOC 48	MOC 52	MOC 53	MOC 54	MOC 55	MOC 57
H-Score	5.7	11	7	10.3	14	16

Figure 4: H-score data calculated post-immunohistochemistry. *Adapted from Owens et al., 2018.*[5]

Experiment 2: When patient-derived peripheral T cells with both H8 and 2E4 CAR constructs were co-cultured with SKOV-3 and OVCAR-3, significant levels of IFNγ were produced. In comparison with 2E4, the H8 construct produced significantly more IFNγ. When the primary tumor cells were co-cultured with the H8 and 2E4 CAR constructs, they produced more IFNγ than the mock-transduced T-cells. The T cells from patient MOC 52 failed to grow, so no tests were performed on this tissue. For 6 of the remaining tumor samples, the H8-CAR construct outperformed the 2E4-CAR construct, producing significantly more IFNγ. In the OC cell lines, IL-2 secretion was moderate, with a mean of 344±56.8 pg/mL for H8-CAR and 131±35.4 pg/mL for 2E4-CAR. In the autologous tumors, there was even less IL-2 secretion.[5]



Figures 5/6: Patient MOC 36's results from experiment 2 *adapted from Owens et al., 2018.*[5]

Experiment 3: The ability of 5T4-specific CAR T-cells to treat ovarian cancer was assessed in a NSG mouse model (figure 5). Mice given ≥.3 × 10^7 total T-cells showed a significant (P<.05) survival advantage compared to the mice treated with 1 × 10^7 Mock-transduced T-cells or saline. While all mice given ≤.3 × 10^7 or a control died before 90 days, all mice treated with 1 × 10^7 survived past 100.[5]

Discussion

This study shows for the first time that 5T4-specific CAR T-cells can produce an immune response in autologous tumors. 5T4-specific CAR T-cells were also shown to confer a statistically significant survival advantage on affected mice, especially in doses ≥1 × 10^7. This study also confirms previous finding that show that 5T4 antigens are highly expressed in the majority of ovarian tumors, and suggests that it may be able to minimize on-target off-tumor effects. When taken together, these experiments strongly suggest the validity of 5T4 antigen as a target for CAR T-cell therapy in ovarian cancer.[5] 5T4-specific CAR T-cell therapy could also eventually be expanded to other solid tumors that also express 5T4. However, there are some important steps that must be taken before this treatment can advance to clinical trial. First, the results of these experiments should be replicated in rabbits, which have more human-like immune systems.[4] A larger *in vitro* study, with a 100 ovarian tumor biopsies, should also be performed, in order to obtain more statistically reliable estimates of the effect size.

More than one hospital should also be included, as all the tumors in this study were taken from the same hospital, limiting the generalizability of the findings. The discrepancy between IL-2 and IFNγ secretion should also be explored further as well. Finally, further studies must be conducted on the potential on-target, off-tumor effects of 5T4. This should include an *in vitro* experiment to measure the expression of 5T4 on regular tissues compared to ovarian cancer tissue biopsies. Both types of tissue would be treated with the 5T4-specific CAR T-cells, and the levels of IFNγ produced would be measured via ELISA. An *in vivo* study in rabbits with ovarian tumors would follow to determine the extent of the on-target, off-tumor effect of 5T4 CAR T-cell therapy, as well its effect on survival. While there are several steps to take before moving this treatment to clinical trial, 5T4-specific CAR T-cell has shown immense promise as a new ovarian cancer therapy, and may have profound implications for the future of ovarian cancer treatment.

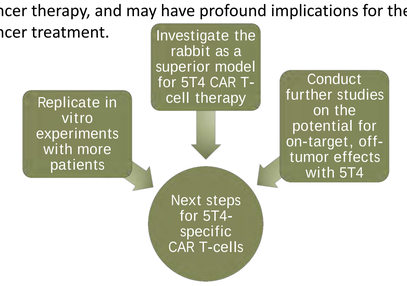


Figure 7: Next steps for 5T4-specific CAR T-cells before clinical trial.

Application of Biotechnology

Many different technologies were used to perform these experiments. In experiment 1, immunohistochemistry was used to test for 5T4 expression. Immunohistochemistry works by co-culturing the tissue sample with the antibody of the antigen of interest. The primary antigen is then introduced to the culture and binds to the mouse antigen. Next, the secondary antigen is applied to bind to the first. Finally, the bound antigens are all stained by 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB), to visualize the binding. This indirect staining method provides a greater sensitivity to the stain. Experiment 2 used enzyme-linked immunosorbent assay (ELISA) to measure amounts of IFN***γ*** and IL-2 produced by each sample. Finally, experiment three used biotechnology to help measure tumor burden in mice. Before the experiment began, the SCOVA-3 cells were transduced with a deactivated virus carrying the information for luciferase expression, allowing for tumor size to be assessed via bioluminescence.[5]

Acknowledgements

I would like to thank Dr. Ericka Senegar-Mitchell for her mentorship, support, motivation, and constant enthusiasm for science. A big thanks to Dr. Schiffer for helping me focus my research and actualize my dreams for this poster. I would also like to acknowledge Dr. Chang, Dr. Su and all our other guest lecturers for so generously donating their time to this program. Finally, thanks to all of my ROSA sisters whose support and love has made this summer one to remember.

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The Correlation between Insured Women and Access to Assisted Reproductive Technologies

Hallelujah Temesgen
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Objective

12% of people in the US are struggling with infertility; the solution is Assisted Reproductive Technology (ART), which uses different technologies to try and help couples conceive a child. Some of the methods used include: In Vitro Fertilization (IVF), surrogacy, and donor eggs. However, many of these techniques are very costly; for example, IVF can cost on average about $11,500. Many couples can not afford to pay for assisted reproductive care because it is not covered by health insurance, due to the cost of the procedure and drugs. The goal of this research is to determine why insured women do not continue their IVF treatment.

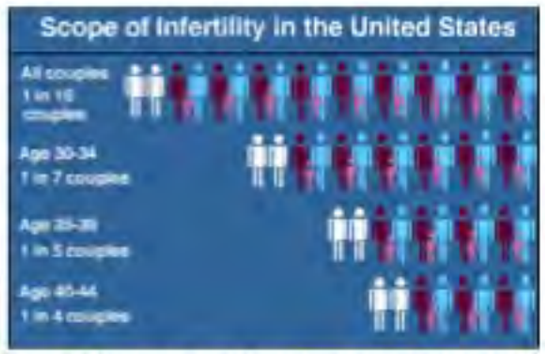


Figure 2 Statistics on infertility in the United States
Human Reproduction, Seminars: Infertility, Overview. (n.d.).

Abstract

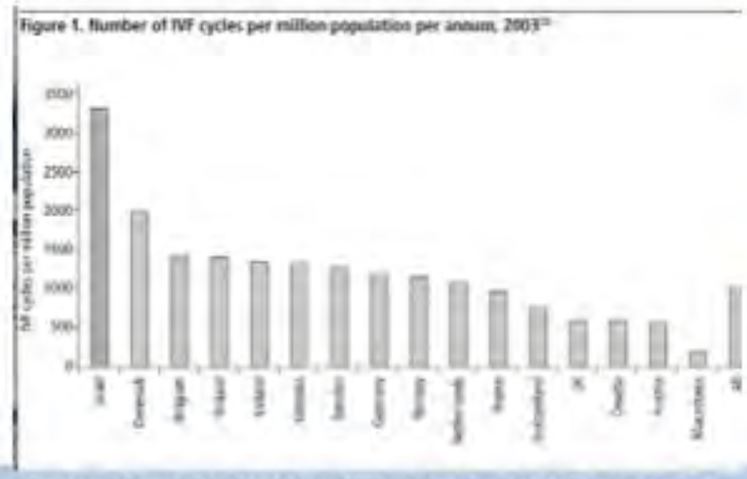
12% of people in the US are struggling with infertility and are trying to find answers. The solution is Assisted Reproductive Technology (ART), which uses different technologies to try and help couples conceive a child. Some of the methods used include: In Vitro Fertilization (IVF), surrogacy, and donor eggs. However, many of these techniques are very costly; for example, IVF can cost on average about $11,500. Many couples can not afford to pay for assisted reproductive care because it is not covered by health insurance, due to the cost of the procedure and drugs. In a study with 893 infertile and insured women who were seeking IVF treatment only 312 women completed the survey, demonstrating that two-thirds of them stopped their treatment and did not seek further care. Almost 50% of the patients stopped treatment due to financial issues.[3] If we look at different healthcare systems around the world, we can find solutions. For example, Israel has national healthcare coverage and it covers IVF costs for up to two kids.[1] New Zealand also follows a similar model where they cover the full cost, if the women is at a certain age and BMI. In Australia, health insurance covers most of the IVF costs. If we apply the different ART coverage methods used in countries, like Israel, Australia and New Zealand, to the US ART can be more accessible to all. [2]

Methods and Materials

The patients from this study went to a private academically affiliated infertility center called Boston IVF for their IVF treatment. There were 893 patients, from the ages of 18-42, that were all insured, completed one IVF cycle but didn't return for at least one year, and did not achieve a live birth. 312 women were emailed an online survey which asked them about whether they had continued their IVF treatment, if they continued elsewhere, why they discontinued the treatment, and what could have made their experience better.[3]

Results

In this study with 893 infertile and insured women who had finished one cycle of IVF, only 312 women completed the survey, demonstrating that two-thirds of the participants stopped their treatment and did not seek further care. When they were asked why they discontinued their treatment 40.2% said that the process was too stressful, 25.1% said that they couldn't afford the costs, and 24.6% said that they had lost their health insurance coverage, almost 50% of participants stopped treatment due to financial issues. In addition, it has been shown that infertile people often have psychological issues, like depression and anxiety. When participants were asked for feedback almost 40% said that they wished they had a mental health professional to help them through this process.[3]



Birenbaum-Carmeli, D.,& Dirnfeld, M. (2008). In Vitro Fertilisation Policy in Israel and Women's Perspectives: The More the Better?Reproductive Health Matters, 16(31), 182-191.

Conclusions

IVF is not accessible to everyone due to the price of the procedure and the medications needed. There are many different models in other countries that offer solutions to this issue, for example, Australia, New Zealand, and Israel. Australia uses public health insurance to make ART more affordable. The cost of ART is about $10,000 but if you have Medicare, Australia's national health insurance, they offer a partial reimbursement of $6,000-$7,000. New Zealand offers access to free ART, however, it depends on the women's age and body mass index (BMI).[2] Israel also follows a similar model because they have a national health insurance which covers the full IVF cost for women under the age of 45 until she has up to two kids with her current partner.[1] Although, Israel covers the full cost of IVF, fertility rates are declining, in 2007 18% of Israeli women achieved a live birth through IVF, and three years later it became 14.8%.[5] However Australia and New Zealand have more successful live birth rates, during the first cycle the cumulative live birth rate was at 32.7% and by the eighth IVF cycle 54.3% of women achieved a live birth.[2] If we apply the different ART coverage methods used in countries, like Australia, New Zealand, and Israel, to countries struggling with infertility, ART can be available to all. Even though, not all of the women in these countries achieve a live birth, they all have the equal opportunity to conceive.

Application to Biotechnology

Assisted Reproductive Technology uses technology to help a women conceive a child. IVF is a type of ART that uses biotechnology because it involves extracting egg and sperm and physically fertilizing the egg on a perti dish. In order to extract the eggs, the physician uses ultrasound and guides the needle to the ovary where it retrieves mature egg from the surface of the ovary.

Acknowledgments

I would like to thank Dr. Ericka for giving me an opportunity to be a part of this program and investing her time, and energy into me. I would also like to thank the Oncofertility Consortium for investing their resources into the next generation of females in medicine.

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Congenital Malformations in Children Conceived by In Vitro Fertilization and Intra-cytoplasmic Sperm Injection

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Poway High School

Objective

Children conceived through IVF and ICSI have an increased chance of many chronic and/or life-threatening illnesses and congenital malformations. The goal of this research is to emphasize the importance of finding causes and preventions of these abnormalities during fetal and infant development.

Abstract

About 10% of women and 17% of men have infertility. In Vitro Fertilization (IVF) and Intra-cytoplasmic Sperm Injection (ICSI) are the two most common methods in assisted reproduction. The first successful birth through IVF occurred July of 1978 and the first successful birth through ICSI was January of 1992.[6] IVF and ICSI have helped many couples with fertility and conception problems, as well as other health issues. Five million babies have been born through IVF and ICSI, but not all of the children were born without complications and health issues. IVF and ICSI increase the chances of the child being born with congenital malformations, chronic illnesses, and developmental issues by 4.2%.[5] In a study in Belgium of 5,884 infants born through IVF and ICSI, cardiac malformations were found to be the most common.[1] Out of 281 IFV and ICSI born children in a study in the United States, 1.27% had septal heart defects, such as pulmonary stenosis (obstruction of blood flow from right ventricle to pulmonary artery) and ventricular septal defect (abnormal opening in the heart between the lower ventricles).[4] In a meta-analysis of twenty-four studies, 74,644 children born through IVF and ICSI had a 2.01% increased risk of malformations in the nervous system, 1.66% in the digestive system, and 1.64% in the cardiovascular system.[3] These studies demonstrate the risk of IVF and ICSI and the chance of abnormalities and chronic illnesses in children conceived through these two processes. A significant cause of the malformations in the children has not been identified and no processes have been found that prevent these abnormalities.

Materials and Methods

In the Netherlands, data on congenital malformations in children conceived naturally and through IVF were obtained from three national professional perinatal and neonatal registers: the National Perinatal Database for Primary Care, a register of midwife-assisted births; the National Perinatal Database for Secondary care, register of obstetrician-assisted births; and the National Neonatology Database, carried out by pediatricians. In the National Perinatal Database, all birth records of children conceived through spontaneous pregnancies were selected to constitute a control group (n=314,605) by excluding all pregnancies where the use of assisted reproduction was coded. The IVF study population consisted partially of a cohort of 1925 IVF children, 9% of which were born through ICSI, born in 1995 and 1996. Detailed information on the congenital malformations was collected through specific questionnaires addressed to both the mothers of the IFV children and obstetricians involved in pregnancy and delivery care, The questionnaires were completed within ~2 months after birth. Because no unique identification marker is available in the Netherlands, a statistical matching procedure was applied that searched for the following variables: birth date of the child and mother, gender of the child, birth order for multiple births, birth weight, and gestational age. Using this matching technique, 89% of the children for the IVF cohort were found (n = 1716). In 79% of the traced records, the conception method was correctly coded as "IVF" Furthermore, all other birth records with the coding "IVF" as a conception method (n = 2508) were added to the IVF study population. Therefore, the total study population was 4224 children. Possible differences in registration and classification of congenital malformations were investigated through the National Perinatal Database.

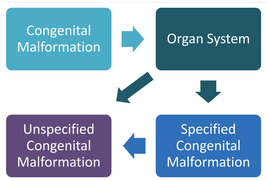

Figure 1. National Perinatal Database Classification of Congenital Malformations

Materials and Methods (contd.)

Congenital malformations are coded by organ system: there are eight different organ systems distinguished with 51 specified and 20 unspecified categories of congenital malformations (Figure 1). This classification was also used with the questionnaires to code the malformations reported. All specific congenital malformations, the total number of congenital malformations per organ system, and the overall incidence of all congenital malformations were compared for the IVF study population and control population. In this study, a distinction was also made between major and minor congenital malformations based on the severity of the malformation. Calculated differences in malformation rates were expressed using odds ratios (OR) and 95% confidence intervals (CI). The X^2 (Chi Squared Test) was used to test for any significant differences in the malformation rate ($P < 0.05$). The Fischer Exact Test was used when numbers were very small and the logistic regression model was used to correct the estimated OR for the overall number of malformations for distribution of maternal characteristics "age of mother," "parity," and "ethnicity" by introducing them into the model as covariates. Statistical analyses were performed in Statistical Package for the Social Sciences (SPSS) version 10.[2]

	IVF/ICSI Born (n=4224)	Naturally Born (n=314605)
Central Nervous System	15	934
Cardiovascular System	32	1531
Digestive System	17	1029
Respiratory System	5	349
Urogenital System	30	1904
Skin and Abdominal Wall	11	934
Muscular and Skeletal System	34	1836
Chromosomal, syndromal and other malformations	16	1471

Table 1. Total number of children with one or more congenital malformations in the different organ system.[2]

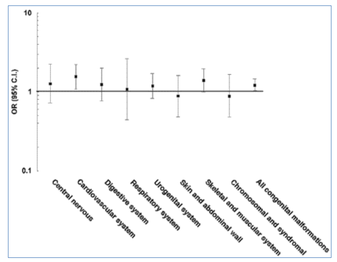

Figure 2. Calculated OR for different organ systems, for the IVF study population versus the NC control population.[2]

Results and Interpretation

Congenital malformations were observed in 137 IVF children (3.2%) and 8526 of the NC control group children (2.7%). The overall OR for the risk of any malformation for IVF children compared with NC control group children was 1.20 (95% CI: 1.01-1.43). Similar ORs were found for children with major, minor, and unspecified congenital malformations. These ORs were, however, no longer significant due to the smaller number of congenital malformations in these different subcategories. After correction the confounding factors of "maternal age," "parity," and "ethnicity," the OR was 1.03 (95% CI: 0.86-1.23). Further investigation of congenital malformations occurring in the different organ systems was performed (Figure 2). Except for "skin and abdominal wall malformations" and "chromosomal and syndromal malformations", the ORs for IVF were slightly higher for every specific organ system (Table 1). The difference only reached statistical significance for cardiovascular malformations (OR=1.56, 95% CI: 1.10-2.22). Further exploration of the cardiovascular system malformations showed that all specific cardiovascular malformations were more frequently reported in the IVF study group, with the ORs ranging from 1.32-1.38.

Results and Interpretation (contd.)

However, only differences in occurrence of "single umbilical artery" reached statistical significance (OR=1.93, 95% CI:1.11-3.35). Neural tube defects did not occur more frequently in the IFV study group. Although the ORs of all specific central nervous system malformations ranged between 1.10-5.73, none reached statistical significance. The only malformations that occurred significantly more frequently in IVF were relatively minor: "single umbilical artery," "inguinal hernia," "club foot," and "other unspecified skeletal and muscular malformations." These findings could, however, be chance findings due to many categories of congenital malformations compared between the IVF study population and the control population. In the 1716 children, a total of 95 congenital malformations were coded.[2]

Conclusions

IVF costs $12,000 and an additional $8,000 for medications. Although few IVF and ICSI children are being affected by congenital malformations, the malformations can be serious and lead to chronic illnesses. Many of the malformations require medications and/or immediate surgery. If the malformations are not covered by insurance, parents may be burdened with the costs of medical care. With studies and research, health issues and possible financial problems due to IVF and ICSI can be significantly reduced, if not eliminated. Parents will be more hopeful their children can be born without congenital malformations.

Relevant Applications to Biotechnology

Advancements in biotechnology have allowed Assisted Reproductive Technology (ART) to grow and become an option for people with infertility and other health issues. ART continues to become more precise and a wider variety of options within ART are available. Research will be conducted to improve the lives of the children affected by the causes of their malformations and chances of congenital malformations will be lowered and eventually eradicated.

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Letrozole as a Potential First-Line Drug Treatment for PCOS Women by Providing Higher Pregnancy Rates and Maintaining a Healthier Endometrium

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Objective

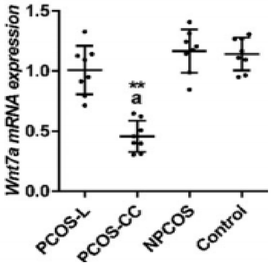
Polycystic ovary syndrome (PCOS) is a common cause of infertility, affecting 6-8% of women in their reproductive age causing infertility in over 80% of cases.[1] PCOS is a constellation of symptoms in which hirsutism, irregular menstruation or anovulation, and polycystic ovaries are presented. The goal of this meta-analysis is to compare the effects and benefits of Clomiphene Citrate (CC) and Letrozole (LE) therapy for inducing ovulation in women with PCOS, demonstrating the potential of using aromatase inhibitors for treatment. Replacing the current first-line agent for ovulation induction with Letrozole will prevent the antiestrogenic effects of CC such as thinning the endometrial lining, proving the promise of aromatase inhibitors.

Abstract

Polycystic ovarian syndrome is one of the most common causes of female infertility that affects about 5% of women and is characterized by irregular menstrual bleeding, exaggerated hair growth, excess androgens in the body, and polycystic ovaries.[5] Studies and trials were conducted to explore the effects and benefits of Letrozole (LE) and Clomiphene Citrate (CC) for ovulation induction and infertility treatment in PCOS women by measuring endometrial health and pregnancy rates. LE is an aromatase inhibitor used to inhibit the growth of estrogen-dependent breast cancer cells to prevent recurring, metastatic or advanced breast cancer.[5] In contrast, Clomiphene Citrate (CC), the primary treatment for infertility, is a selective estrogen receptor modulator that stimulates the release of follicle stimulating hormone and luteinizing hormone. In a double-blind randomized controlled trial, 149 PCOS women between 18-39 years old were given either 50 mg of CC (74) or 2.5 mg of Letrozole (75) until pregnancy or for up to 6 ovulatory cycles.[1] Patients were monitored with ultrasound follicle tracking and progesterone serum measurements to assess endometrial parameters, ovulation, pregnancy and live birth (LB) rates.[1,5] Pregnancy rates of LE and CC were 61.2% and 43%, respectively, and LB rates of LE were 48.8% and 35.4% in CC. Ovulation rates in LE were 83.8% compared to 79.7% in CC-women.[1] There was no significant difference in the number of miscarriages, anomalies, multiple or loss in pregnancies. CC showed lower endometrial protein and gene expression which are primarily controlled by estradiol and responsible for endometrial proliferation, resulting in a higher percent of CC-women having a thin endometrium.[3] Possible explanations for the superiority of Letrozole over Clomiphene Citrate include a lower estradiol level during the luteal phases and higher progesterone levels from the Letrozole.[1] Overall, Letrozole lacks the adverse antiestrogenic effects that Clomiphene Citrate possesses, allowing for higher pregnancy rates and a thicker endometrium, and therefore should be considered as a potential primary ovulation induction treatment for PCOS women.

Methods and Materials

To assess the benefits of Letrozole (LE) over Clomiphene Citrate (CC), a study was conducted in which 149 women between 18-39 years old who met at least 2 of the 3 criteria requirements (oligo-/anovulation, hyperandrogenemia, and appearance of polycystic ovaries by sonography) for PCOS were enrolled. The clinical trial was double-blind, therefore all patients, investigators and assessors were blind to the allocation of the drugs to the patients, and both drugs were encapsulated in identical capsules. 74 women received 50 mg of CC and 75 women were treated with 2.5 mg of LE. Treatment was administered orally for 5 days beginning on day 3 until pregnancy or for up to 6 ovulatory cycles.[1] If ovulation was not achieved, the dose was increased. Patients were monitored with ultrasound for follicular development and assessed for progesterone serum levels during mid-luteal phase. Ovulation was defined by a progesterone level above 25 nmol/L and a follicular diameter greater than or equal to 17mm.[1] Pregnancy was diagnosed by visualization of the gestational sac using ultrasonography. To distinguish the effects of both agents on endometrial receptivity, women between 21-40 years old were monitored for the immunoexpression of gene markers important for implantation: LIF (leukemia inhibitory factor), dickkhopf homolog 1 (DKK-1), fibroblast growth factor-22 (FGF-22).[5] Women used an urine ovulation detection test to determine ovulation and performed luteal-phase endometrial biopsies 7 days later. Blood was also analyzed for estrogen and progesterone hormone levels. The endometrial tissue was put in a buffered formalin solution to preserve the tissue and then the RNA was extracted from the tissue using phenol chloroform and transcribed into complementary DNA (cDNA). The resulting cDNA was exposed to real-time polymerase chain reaction (PCR) with LIF, LIFR, DKK-1 and FGF-22 gene primers. Lastly, gel electrophoresis was performed to interpret purity and size of each product in separate reactions.[5] To determine differences in ligand expression of glycoproteins as a result of both treatments in PCOS women, another trial was put in place to monitor women 20-35 years old. They were randomly given either 5mg of LE or 100mg of CC per day, starting on day 3 for 5 days, and were compared to PCOS women with no treatment and normal ovulatory women with male factor infertility. All women were analyzed for endometrial thickness by transvaginal sonography, hormone (FSH, LH, E2, P4) levels through blood tests, and protein expression by endometrial biopsy. Similar to gene extraction, the RNA was extracted from the biopsy, evaluated with gel electrophoresis, and then amplified carried out with specific primers: Wnt3, Wnt8b, and Wnt7a.[3] Wnt3, Wnt7a, and Wnt8b are crucial in reforming the endometrium during the menstrual cycle, proliferating and tumorigenesis. The study especially addresses the Wnt7a protein, which is specifically responsible for uterine gland formation, endometrial receptivity and implantation.



The graph shows the increased expression of Wnt7a proteins in women treated with LE. Wnt7a is a major ligand responsible for endometrial development, proliferation, and is required for interaction between stroma and epithelium, gland formation and differentiation. Studied groups include: PCOS-Letrozole (PCOS-L), PCOS-Clomiphene Citrate (PCOS-CC), PCOS no-treatment (NPCOS) and normal women (Control).

Retrieved from Mehdinejadiani et al., 2018.

Results

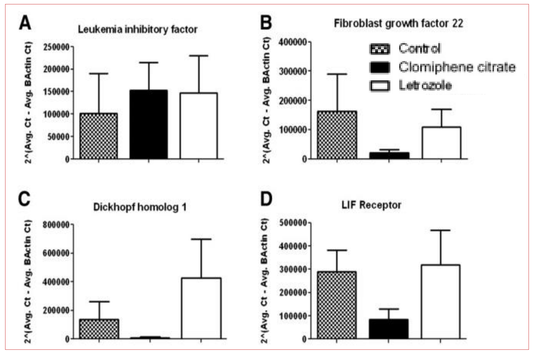
The studies concluded that Letrozole yields a statistically significant higher pregnancy rate of 61.2% compared to 43.0% in Clomiphene Citrate treated women (p=0.022). Live birth (LB) rates of LE and CC were 48.8% and 35.4%, respectively. Although there was not a major difference in LB rates (p=0.089), a trend of higher LB rates in LE-treated women is demonstrated. Additionally, ovulation rates for LE were 83.8% and 79.7% in CC-treated women.[1] Between both groups, demographic, clinical and endocrine characteristics--such as age, hormone levels, BMI, and race, were similar, which eliminates the effect of any confounding variables. Moreover, Letrozole yielded higher pregnancies per cycle (p=0.036) and ovulations per cycle (p=0.045) in comparison to Clomiphene Citrate, allowing for better chances for mono-ovulation.[1,2] Likewise, the pregnancy rate per ovulating women was much higher in LE treated women compared to CC (p=0.024). Furthermore, time to pregnancy for LE and CC were 4 and 6 treatment cycles, sequentially. However, there were no significant difference in fetal, maternal or neonatal complications between both agents. Reported side effects of Clomiphene Citrate include: 2 cases of haemorrhagic cysts (one in each arm), acute cholecystitis, which required hospitalization, elevated liver enzymes, hot flushes, migraines, and cyst formation. Recorded effects of Letrozole included: cyst formation, diarrhea, nausea, vomiting, headache, and neck pain.[1] Yet, the risks and adverse effects of Letrozole are much less severe than those attributed to CC treatment.

Outcome	Letrozole (*N* = 80)	CC (*N* = 79)
Pregnancy rate	49/80 (61.2%)	34/79 (43.0%)
Live birth rate	39/80 (48.8%)	28/79 (35.4%)
Ovulation rate	67/80 (83.8%)	63/79 (79.7%)
Pregnancies per ovulating patient	47/67 (70.1%)	32/63 (50.8%)
Pregnancies—strata 1 (BMI <30)	37/54 (68.5%)	25/53 (47.2%)
Pregnancies—strata 2 (BMI 30–35)	12/26 (46.2%)	9/26 (34.6%)
Live births—strata 1 (BMI <30)	29/54 (53.7%)	20/53 (37.7%)
Live births—strata 2 (BMI 30–35)	10/26 (38.5%)	8/26 (30.8%)
Pregnancies per cycle	49/261 (19.0%)	34/278 (12%)
Live births per cycle	39/261 (15%)	28/278 (10%)
Ovulation per cycle	196/261 (75%)	187/278 (67%)
Mono-ovulation*	80/94 (85.1%)	64/77 (83.1%)

Table 2 demonstrates the outcomes of Letrozole versus Clomiphene Citrate therapy for PCOS women in the intention-to-treat analysis. LE has significantly higher pregnancy, ovulation and live birth rates.

Retrieved from Amer et al., 2017.

Results in endometrial health in regards to gene expression favored Letrozole as well. Analysis of variance statistical models were used to differentiate endometrial expression of LIF, LIFR, DKK-1 and FGF-22 between CC and LE. Letrozole increased LIF (immunostained in glandular epithelial and endometrial stromal cells) immunoexpression compared to the controls and CC group (p=.006 and p=.005, respectively). Additionally, immunoexpression was reduced in the CC group compared to the control (p=.039). In regards to DKK-1 (stained in endometrial stromal cells), women in the control group had an increased immunoexpression compared to the women treated with CC (p=.007), and LE increased the expression compared to CC (p=0.015). LIFR mRNA expression was reduced in CC compared to both LE and the controls. Lastly, FGF-22 (stained in glandular epithelial and endometrial stromal cells) expression was significantly reduced in the CC group in comparison to LE (p=.003) and the control (p=.011). Although not significant, (p=.114) LE was reported to increase FGF-22 immunoexpression in endometrium compared to the control.[5]



The graph demonstrates the effects of CC and LE on the expression of (A) LIF; (B) FGF-22; (C) DKK-1; and (D) LIFR messenger RNA expression in the endometrium. Overall, RNA expression is higher in LE groups, which results in a healthier and thicker endometrium.

Retrieved from Wallace et al., 2011.

Higher protein expression also supported Letrozole for being the first-line treatment for PCOS. Like the other trials, baseline characteristics of the patients were similar and comparable. The endometrial thickness of LE-women was increased compared to CC-women ($p<0.05$) and released higher progesterone levels than the non-treated PCOS women (NPCOS) and CC groups ($p<0.001$). The NPCOS women has the highest expression of Wnt3, whereas CC expressed the lowest amounts of Wnt3 and Wnt7a compared to NPCOS, LE and controls ($p<0.001$). Clomiphene Citrate expressed Wnt7a ($p<0.001$) and Wnt8b ($p<0.05$) as extremely low levels compared to the other 3 groups.[3] The lower expression of proteins responsible for endometrial proliferation in CC-treated women can be the cause of poor endometrial development and low pregnancy rates.

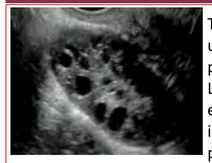
Discussion

Analysis of the effects of Clomiphene Citrate and Letrozole has determined that LE improves ovulation, implantation, endometrial gene expression, shortened time-to-pregnancy, and safety data presents no increase in fetal or neonatal anomalies. Clomiphene Citrate works by blocking the negative feedback of endogenous estrogen to stimulate the production of FSH and LH, however up to 55% of women are CC-resistant and the agent has many adverse effects.[2] A possible explanation for the success of Letrozole is that LE results in a lower mid-luteal estradiol serum level and higher progesterone levels perhaps caused by aromatase inhibition.[1] Because LE does not expend estrogen receptors like CC, normal feedback occurs and estrogen levels increase, leading to FSH suppression and atresia of smaller follicles, allowing for mono-ovulation.[2]

In regards to gene expression, the discrepancy in pregnancy rates and endometrial health between both therapies is attributed to the differences in endometrial gene expression. Even though both CC and LE increase mRNA expression of LIF in the endometrium, only LE increases its protein expression as well. A low mRNA or protein expression of LIF is correlated to infertility; therefore, LIF can be used as a predictor for implantation, subsequent pregnancy and regulates endometrial receptivity because of its influence on the trophoblast (layer of tissue that later becomes the placenta responsible for nourishing the embryo). Another possibility suggests that even though CC increased the gene expression of LIF, it did not increase protein expression because of the reduction of LIF gene receptors in the endometrium. Similarly, the reduced DKK-1 expression in CC women is responsible for frequent implantation failure and the increased expression of FGF-22 in LE women is responsible for stimulating more endometrial proliferation by increasing blood flow throughout the endometrium.[5]

After analyzing results for the Wnt pathway, which is vital in proliferation, implantation, and placental development, it was concluded that Clomiphene Citrate results in poor endometrial health and development in PCOS women. Up-regulation of Wnt3 and Wnt8b are involved in the proliferation phase which justifies the thicker endometrium in women treated with LE. Moreover, the Wnt pathway is mostly estrogen-dependent; CC exhausts the estrogen receptors which causes the reduction in expression. The major protein involved in the interaction between stroma and gland formation and differentiation is Wnt7a. The study deduced that the lower expression of these glycoproteins in CC-women is causing reduced endometrial thickness and poor estrogen regulation, ultimately resulting in less pregnancy rates.[3] Although, Clomiphene Citrate is the current first line therapy for women with PCOS, clinical trials and studies have provided convincing evidence of Letrozole superiority as the primary agent for treating PCOS; LE shows credible promise for yielding higher pregnancy rates and maintaining the health of the woman's endometrium.

Relevance To Biotechnology



Transvaginal ultrasound of a polycystic ovary; several immature follicles and cysts are seen supporting the diagnosis of PCOS

Throughout the studies conducted on PCOS women, vaginal ultrasound and ultrasonography are important for monitoring follicular growth, appearance of polycystic ovaries, and pregnancy. Blood tests are vital for measuring E2, P4, FSH, and LH levels to distinguish the effects of LE and CC as well as predict ovulation. Preserving endometrial tissue and extracting DNA are important for examining gene expression, in which several buffer solutions are used. Gel electrophoresis, used to extract proteins and genes from endometrial biopsies, and equipment are necessary to normalize separate reactions, assess purity and size of real-time polymerase chain reaction (PCR) products--PCR is used for amplifying a segment of DNA with gene primers shown to be regulated differently based on the agent--after the biopsy. Lastly, mRNA analysis and the pipelle suction curette, for performing endometrial biopsies, are necessary to determine the genes expressed with LE and CC therapy.

Retrieved from Lee et al., 2012.

Acknowledgments

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