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# RNA Sequencing and Pathway analysis to understand the morbidity of Assisted Reproductive Technology-treated patients with infertility

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**ABSTRACT: BACKGROUND: Assisted Reproductive Technologies (ART) is widely employed as an effective treatment for infertility. Women undergoing infertility treatments, particularly in vitro fertilization (IVF), face a slightly elevated risk of severe maternal morbidity. However, the long-term effects of ART-associated morbidity have not been much studied. There is a crucial need to pinpoint predictors specific to patients and treatments that influence severe maternal morbidity, potentially shaping the choice of treatment offered to a woman. AIM: This study aims to perform RNA Sequencing and pathway analysis of Breast, Ovarian, and Endometrial cancer cases that were previously on Assisted Reproductive Technology Treatment, and to understand the different genes and pathways involved in the development of Cancer. Our study included cases from Ovarian, Breast, and Endometrial who were previously treated with ART. MATERIALS AND METHODS: Samples of Ovarian, Breast, and Endometrial Cancer who were previously on Assisted Reproductive Technology treatment were collected at the Medical Health and Research Institute (MHRI) in Hyderabad. We evaluated these cases via retrospective analysis to understand the long-term morbidity of ART. The study protocol was approved by the Clinical Research Ethics Committee of the MHRT. All the samples were collected after collecting consent from the patients. About 5mL of venous blood obtained from patients was centrifuged at 3,000 rpm for 10min at 40C, then 10,000 rpm for 10min, within 2h of collection. The supernatant (serum) was then stored at −80◦ C before use. Total RNA was isolated using miRNeasy kit (Qiagen, Germantown, MD; Cat# 217004) and submitted for RNA-seq. Samples were processed for transcriptome high through-put sequencing (Sequencer name) and subsequent bioinformatics analyses were carried out. RNA sequencing library preparation and next-generation RNA sequencing were carried out. RESULTS: This is the first study that involved molecular study of different reproductive cancer genes of ART treated patients. Our results showed up-regulation of 10 hub genes namely STAT5B, FOS, BCL2, CXCR4, JAK3, NOTCH1, TGFB1, PIK3CB, PIK3CD, and PTEN and JAK-STAT and PI3K-Akt signaling pathways as the most enriched. CONCLUSION: RNA Sequencing results showed up-regulation of 10 hub genes namely STAT5B, FOS, BCL2, CXCR4, JAK3, NOTCH1, TGFB1, PIK3CB, PIK3CD, and PTEN. We could not completely attribute the development of Cancer to the ART Treatment, but we could identify JAK-STAT and PI3K-Akt signaling pathways as the most enriched in the patients with Ovarian Cancer, Breast Cancer and Endometrial Cancer. This could help in providing personalized treatment to the patients with cancer and previously treated with ART.**

**Keywords:** Assisted Reproductive Technology, Infertility, Morbidity, RNA Sequencing, Bioinformatics

## INTRODUCTION

Every year, the global prevalence of infertility is on the rise, attributed to factors such as environmental pollution, mental and psychological stress, delayed childbearing, and various other influences (Ramya et al., 2023; Ahmed et al., 2019; Bala et al., 2021;

Fisher et al., 2020). Assisted Reproductive Technology(ART) involves the utilization of infertility drugs designed to stimulate the maturation of multiple follicles, subsequently inducing multiple ovulations. Common fertility drugs encompass ovulation-inducing medications like clomiphene citrate (CC) and letrozole, and controlled ovarian stimulation drugs, including Gonadotropin (Gn), human menopausal Gonadotropin (hMG), human chorionic Gonadotropin (hCG), and progesterone (Naz et al., 2024; Fauser, 2019). These medications are administered either individually or in combination as per an ovulation stimulation protocol (Si et al., 2023; Kristensen et al., 2024).

Exposure to these hormones raises apprehensions regarding the long-term safety of ovarian stimulation, specifically concerning the potential risk of ovarian malignancies (Somigliana et al., 2014). Assisted Reproductive Technology has helped many infertile women to conceive and be able to give birth. However, it has many detrimental effects on the mother and foetus also and the safety of ART on the mother and foetus has not been much studied. ART has been associated with maternal and childhood morbidity such as Placenta previa, Placental abruptions, thromboembolism, and excessive bleeding. However, the long-term effects of ART-associated morbidity have not been much studied (Wada et al., 2023). Previous studies have shown a link between infertility-related drugs and the risk of severe Cancers of the Breast and the female reproductive tract (Hanson et al., 2017; Levine et al., 2015)

Studies have shown that women who achieved conception through Assisted Reproductive Technologies (ART) had an elevated risk of serous, endometrioid, clear-cell, and other unspecified forms of ovarian carcinoma when compared to women who did not experience infertility or ART-assisted births (Siez et al., 2023; Spaan et al., 2015). Previous research has shown that ovarian cancer (OC) cells undergo genetic changes as they progress from primary tumors to metastatic tumors. However, the genetic changes that occur during metastasis are limited. This suggests that the microenvironment of the metastatic site plays a role in driving OC progression (Zong et al., 2019).

RNA sequencing using Gene expression profiling and further bioinformatics analysis may provide an insight into malignancies to target the appropriate pathway (Han et al., 2015). The utilization of Assisted Reproductive Technology (ART) treatments has seen a rise in Western countries in recent decades. The use of ovarian hormone stimulation in ART has prompted concerns, particularly given that around 80% of breast cancers are hormone-sensitive (Vassard et al., 2021; Kohler et al.,2015). The potential carcinogenic effects of hormonal stimulation during ART treatment remain unclear. It is crucial to note that the risk of breast cancer is influenced by various reproductive factors, and further research is needed to clarify the relationship between ART treatments, hormonal stimulation, and the risk of breast cancer (Łukasiewicz et al., 2021)

Type 1 endometrial cancer stands out as the most common histologic type, and it is characterized as hormone-dependent. The association arises from a strong correlation with prolonged exposure to unopposed estrogen and risk of Endometrial cancer (Mallozzi M et al., 2017; Wan et al., 2016). Notably, progesterone is recognized to exert a protective effect on the endometrium, offering a counterbalance to the proliferative effects of estrogen and contributing to the regulation of endometrial tissue growth (Michalczyk et al., 2021). Clomiphene citrate and Tamoxifen have been used in the treatment of female infertility for decades. There are shared chemical properties between clomiphene citrate (CC) and tamoxifen, the latter of which has been associated with an elevated risk of endometrial cancer. This similarity suggests a potential pathogenetic link between Clomiphene Citrate and endometrial cancer. However, a substantial number of studies have explored this connection, yielding contradictory results (Diakosavvas et al., 2021)

Studies present in the literature have focused more on clinical parameters, but very few have focused on molecular dynamics and RNA sequencing. There are very few studies reported on the association of fertility medications and the development of Ovarian Cancer (Diergaardeb et al., 2014). But there is a lack of evidence related to the complex nature of infertility, the potential long-term effects of these medications, and other risk factors in this patient population. Our study aims to understand the molecular pathways and differentially expressed genes in ART-treated patients leading to morbidity in these patients. Moreover, the advent of next-generation sequencing (NGS) has opened the door to multiple opportunities for understanding the dynamics of molecular pathway activation. NGS can easily find out targetable, novel, and mutation sites to guide precision with the help of bioinformatics tools (Yadav et al., 2023; Gulilat et al., 2019). Bioinformatics, along with the help of cutting-edge technologies like Artificial Intelligence and machine learning, could lead to precise and precision medicine (Ahmed et al., 2020). Performing NGS standard of care and precision is a common place for many malignancies but for other cancers, including ovarian cancer, is still emerging (Govindarajan et al., 2020). Our study included cases of Breast, Ovarian, and Endometrial Cancer, which were confirmed cases of Assisted Reproductive Technology-treated patients. This study aims to perform RNA Sequencing and pathway analysis of Breast, Ovarian, and Endometrial cancer cases that have been Assisted Reproductive Technology-treated, to understand the different genes and pathways involved in the development of Cancer. We could not completely attribute the development of Cancer to ART infertility treatment. However, the study could be useful in providing personalized/tailored treatment to the patients.

## METHODOLOGY

**Study subjects:** This study was conducted at MHRT, a tertiary care center, and referral centers from other hospitals. The research encompassed an examination of 350 cases of infertility spanning from January 2023 to January 2024. This study was carried out in 225 cases of ART-treated patients as shown in Figure 1. The study protocols were approved by the Institutional Ethics Committee of MHRT Hospital & Research Centre, Hyderabad with an ethical clearance number stated as “MHRT 302/03-IV-2024/5”

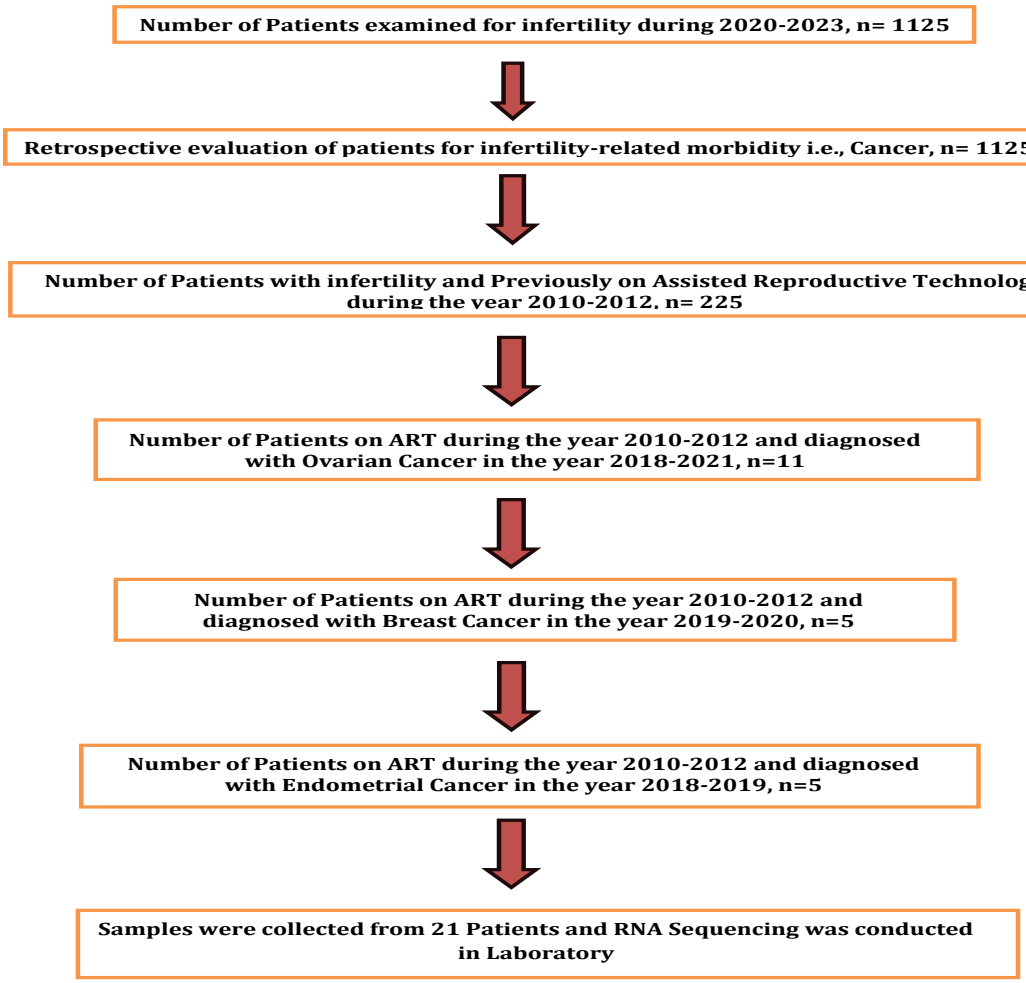


**Figure 1: Study Flow chart/ Study Design**

**Study Design:** We have conducted a **Retrospective study to understand their long-term morbidity** and recruited patients by visiting different Cancer hospitals. Cases were collected during the period from the year 2010 to 2012. This study was carried out in 225 cases of ART-treated patients. Out of which 11 cases were found to be associated with Ovarian Cancer, 5 cases were associated with Breast Cancer, and 5 cases were associated with Endometrial Cancer. These cases were studied further to understand the association of infertility-related morbidity i.e., the development of Cancer.

We have collected history from patients with Ovarian Cancer, Breast Cancer, and Endometrial Cancer and have considered only the ART-treated infertile cases with Cancer. **None of the cases were conceived even after four to six cycles of ART treatment.**

**Table 1: Inclusion of Cancer Patients into the Retrospective Study**

| Cancer Cases Reference | N | Conceived/Not Conceived with ART |
|---|---|---|
| Number of Infertility cases on ART | 225 | Not Conceived with ART |
| No of Cycles (Year- 2010 to 2012) | 4-6 | NA |
| Number of patients reported for Ovarian cancer | 11 | Not Conceived with ART |
| Number of patients reported for Breast cancer | 5 | 1 Conceived with ART |
| Number of patients reported for Endometrial Cancer | 5 | 1 Conceived with ART |

The Number of Infertility cases with ART as a treatment was 225, No of ART Cycles given was between four to six. The number of patients reported and studied for Ovarian Cancer was eleven, the number of patients reported for Breast cancer was five and the number of patients reported for Endometrial cancer was five. These cases were studied further to understand the role of infertility-related long-term morbidity. Out of eleven cases of Ovarian Cancer, four cases were found to be infertile, nulliparous, and have undergone infertility treatment for more than a year with age 56-62years and duration of marriage of 14 to 15 years (Primary Infertility)., two cases were found to be with less parity (Para 1) and with age 35-38 years and 8 to 10 years of duration of marriage (Secondary Infertility). And two were unmarried (Primary Infertility). and three cases were with 2 children (Para 2) (Secondary Infertility). The Five reported cases of Breast Cancer were all nulliparous with ages between 39 to 47 years (Primary Infertility). In

the other five cases with Endometrial Cancer, all were obese, BMI>30. Age between 48-55 years, High BP, Type II DM, with one to two children (Secondary Infertility).

**ART Protocol:** All the cases were treated by Controlled Ovarian Stimulation Protocol + GnRH antagonist protocol as a workup of ART. All the patients were treated with recombinant Follicle-stimulating Hormone (rFSH) with a dose of 200-300 IU FSH for 10 to 12 days. In some cases, Recombinant Luteinizing Hormone (rLH) has been used. The number of Oocytes retrieved was between 5 to 6. All the confirmed cases of Cancer were then further evaluated by molecular analysis (RNA Sequencing and Pathway Analysis) to understand the pathways involved that may be linked to the development of cancer.

**Molecular Gene expression study (RNA Sequencing & Pathway Analysis) of the confirmed cases of cancer reported in ART-treated patients**

**Sample Collection:**
Samples of Ovarian, Breast, and Endometrial Cancer who were previously on ART were collected at the Maternity Health and Research Trust (MHRT) in Hyderabad. The study protocol was approved by the Clinical Research Ethics Committee of the MHRT. All the samples were collected after collecting consent from the patients.

**Serum Preparation:**
To thoroughly remove cell debris, about 5mL of venous blood obtained from patients was centrifuged at 3,000 rpm for 10min at 40C, then 10,000 rpm for 10min, within 2h of collection. The supernatant (serum) was then stored at −80◦C before use.

**RNA Isolation & High-Throughput Sequencing:**
Total RNA was isolated using miRNeasy kit (Qiagen, Germantown, MD; Cat# 217004) and submitted for RNA-seq. Samples were processed for transcriptome high through-put sequencing (Sequencer name) and subsequent bioinformatics analyses were carried out. RNA sequencing library preparation and next-generation RNA sequencing were carried out. The library preparation was done using TruSeq Stranded mRNA HT Sample Prepkit (Illumina, SanDiego, CA;cat#RS-122-2103) according to the manufacturer's protocol and 8- nucleotide barcodes were added for multiplexing. The barcoded libraries were cleaned by bead cut with AMPure XP beads (Beckman Coulter, Atlanta, GA; cat#A63882), verified using Qubit3 fluorometer (ThermoFisher Scientific, Waltham, MA) and 2200 TapeStation bioanalyzer (Agilent Technologies, Santa Clara, CA), and then pooled. The pool was sequenced on NextSeq 500 (Illumina) with NextSeq75 High Output v2 kit (Illumina, cat#FC-404- 2005).

**Bioinformatics analysis**
**Differential expression analysis with DeSEQ2**
Further processing of the count tables was done with R(v3.5.1) making use of tidy verse (v1.2.1). Gene abundance expression read counts obtained were normalized using the sum of all reads mapping to Sequin spikes or RC spikes as size factors in DESeq2 (v1.20.0).

To assess the biological signal in the case/control cohorts, we performed differential expression analysis between the ovarian cancer patient and existing ovarian cancer patient using DESeq2 (v1.20.0). Only mRNAs present in at least 80% of each group (cancer or benign) with at least 7 read counts per sample were included in the analysis. Genes were considered differentially expressed when the absolute log2 fold change> 1 and at $q < 0.05$. Principle component analysis was performed and the first two principal components for the normalized sequencing data were plotted using the website called SR Plot. Significantly differentially expressed genes at 5% FDR with at least two-fold change were called using DESeq2 ver. 1.12.3. Categories based on overlaps among significantly differentially expressed genes between the two experiments (cancer patient versus serous ovarian carcinoma) were analyzed using Metascape (metascape.org). Metascape is a web-based tool that has been under development since 2014. It enables multiple computational analyses of large-scaled at an integrating gene annotation, and membership analysis and has multi-gene-list meta-analysis capabilities. It allows the leveraging of 40 independent databases for studying functional enrichment, interactomes, and gene annotation.

**Gene set Enrichment analysis**
Gene set enrichment analysis was conducted using GSEA Desktop ver. 3.0, build 0160 [68]. Hallmark gene sets from Molecular Signatures Databasever.MSigDB6.1 were used. GSEArot algorithm was used to compute significance of enrichment for each of the hallmark gene sets, taking into consideration that the primary tumors and liquid biopsy from this case study were matched.

**Gene Ontology (GO)**
GO provides structured terms to describe gene product attributes based on cellular components (CC), molecular functions (MF), and biological processes (BP), is widely used to explore information about biological pathways involving DEGs. We used Metascape (https://metascape.org/) web-based tool designed for experimentalists to interpret large-scale datasets (NIH . (n.d.). *Metascape A Gene Annotation & Analysis Resource*. Metascape. https://metascape.org/gp/index.html#/main/step1). Minimum overlap ≥3 performs GO annotation and KEGG pathway enrichment analysis.

Metascape is effective and efficient and p-values <0.01 were considered statistically significant. The GO annotations of the selected transcriptomic genes were queried from the String database. The protein-protein interactions between each transcriptomic feature

and its first neighboring protein counterpart with a number less than 20 were downloaded from the STRING database with a total confidence greater than or equal to 0.7.

**Pathway Network Enrichment Analysis.**
Pathway network of the disease-related genes shows a network among different genes related. Similar to the Tree tab, this interactive plot also shows the relationship between enriched pathways. Two pathways (nodes) are connected if they share 20% (default) or more genes.

**Pathway enrichment analysis KEGG**
Different genes cooperate to play corresponding biological functions in the body. The pathway-based analysis contributes to further understanding of the biological functions of genes. Here, we used David (version 6.8) and Metascape software to analyze the pathway involved in upregulated genes and the important pathway was selected for key analysis, which is conducive to understanding the pathogenesis of the disease, and also can provide a reliable target for the research of new drugs.

**Interaction network analysis of genes significantly enriched in pathways**
According to the results of DAVID database analysis, genes enriched in the Disease pathways and genes appearing on the pathway with frequency ≥ 9 were input into the String database (http://string-db.org/)toconstructprotein-protein interaction network model and the species was set as "HOMA sapiens"(*Welcome to string*. STRING. (n.d.). https://string-db.org/ ). To ensure the reliability of the data, the minimum protein interaction threshold was set to "highest confidence"(> 0.9). The protein interaction was screened, and the results were saved in a format.

**Protein-Protein Interaction Construction**
Core disease genes were identified by constructing a PPI. There are various databases related to PPIs, and the STRING database (https://string-db.org/) covers the greatest number of species and contains more interactive information (40). In this study, the core disease targets were submitted to STRING, with the species limited to Homo sapiens and the confidence score limited to those > 0.4, to obtain PPI data. The value of "degree", the number of correlations of the node in the whole network, was used as a reference to the importance of the core target."Molecular Complex Detection" (MCODE: a plug-in of Cytoscape) was applied to determine the relationships between network clusters to identify hub genes with a high degree of connectivity (41). According to previous literature, advanced options were set with a degree cutoff = 2, a node score cutoff = 0.2, and a K-Core = 2 (42–44).

## RESULTS

**Differential expression analysis with DeSEQ2**
A total of 1588 genes were differentially expressed in the primary tumor versus Ovarian cancer patient with a significance level of false discovery rate (FDR) < 5% and absolute fold change ≥ 2 (Figure 1), whereas 404 genes were significantly up regulated and 1184 genes were down-regulated in patient samples with a significance level of FDR < 5% and absolute fold change ≥ 2, heat maps were generated for up-regulated genes (Figure 1).

**Gene Set Enrichment Analysis**
Gene set enrichment analysis (GSEA) was done for the hallmark gene sets to identify the pathways during OC progression. 15 gene sets were negatively correlated with serious ovarian cancer versus Ovarian cancer patients at FDR < 25% as shown in Figure 3 while four gene sets were positively correlated at FDR < 25% as shown in Figure 4. The most significantly correlated gene sets in primary tumors versus a patient with metastasis were E2f transcription factor (E2F) targets(enrichment score(ES)= -0.573),G2MCheckpoint(ES= -0.563)and MYC targets V1 (ES = -0.407) myogenesis (ES=-0.419), epithelial–mesenchymal transition (EMT) (ES=-0.708), Hedgehog signaling (enrichment score (ES) = -0.544). Taken together, these results indicate that during the process of carcinogenesis and progression of diseases a proliferative phenotype where cell cycle regulation is affected along with the activation of oncogenic pathways. EMT transition (ES = -0.708), myogenesis (ES = -0.419) E2f transcription factor (E2F) targets (ES = -0.573), are enriched in the metastasis (Figure 2A), highlighting the importance of invasiveness and contractility during the process of metastasis.

The primary tumors and patient with metastasis revealed additional gene sets that positively correlated with metastasis including, allograft rejection, interleukin 6 (IL6) JAK signal transducer, and activator of transcription 3 (STAT3) SIGNALING (enrichment score (ES) = 0.346), interleukin 2 (IL2)—signal transducer and activator of transcription 5 (STAT5) signaling and inflammatory response. Similarly, those that were negatively correlated were MTORC1 signaling, protein secretion, androgen response, and MYC targets.

**Functional enrichment analyses for three gene lists:**
In the GO enrichment analysis of intersection genes, we observed several enriched gene sets. The biological process (BP) was mainly enriched in neutrophil degranulation, T-cell activation, and regulation of cell adhesion. For the result of the cellular component (CC), it was revealed that these genes were mainly involved in secretory granule membrane, cytoplasmic vesicle lumen, and membrane raft. Moreover, in the molecular function (MF) analysis, cytokine receptor activity, cytokine binding, and immune receptor binding structural constituent were indicated to be related to the intersection genes as shown in Figure 6.

**Pathway networking analysis**

Pathway networking analysis of disease-related genes observed major pathways responsible for cancer progression and metastasis. **JAK-STAT signaling pathway, PI3K-Akt Pathway, and Ras/Raf/MAPK pathway** were upregulated in patient transcriptome profile which are important cascades of signal transduction for multiple growth factors and cytokines, that regulate gene expression and cell activation, proliferation, and differentiation as seen in Figure 6 and 7. Moreover, pathways that promote metabolism, proliferation, cell survival, growth, and angiogenesis in response to extracellular signals were observed in pathway networking analysis.

**Pathway enrichment analysis KEGG**

Further downstream analysis by the KEGG database digs deeper to show differentially expressed up-regulated genes in the major pathway. Molecular signature and hub genes that are upregulated in major pathways are highlighted in red. Major disease-related pathways show significant upregulation of genesin multiple pathways by KEGGdb.

**Protein-Protein Interaction Construction**

We constructed the PPI network of intersection genes using the STRING database, and based on the screening conditions of minimum required interaction score >0.7 (Figure 9). The hub genes selected from the PPI network using the MCC algorithm of the CytoHubba plugin are shown in Figure 8. According to MCC scores, the top 10 highest-scored genes were selected as hub genes. Subsequently, we constructed the PPI network of DEGs, screening hub genes by the MCC algorithm of CytoHubba plugin.

**According to MCC scores, the top 10 highest-scored genes were selected as hub genes (Figure 9). The top 10 hub gene lists including STAT5B, FOS, BCL2, CXCR4, JAK3, NOTCH1, TGFB1, PIK3CB, PIK3CD, and PTEN were observed that could be potential targets for precise and precision medicine.**

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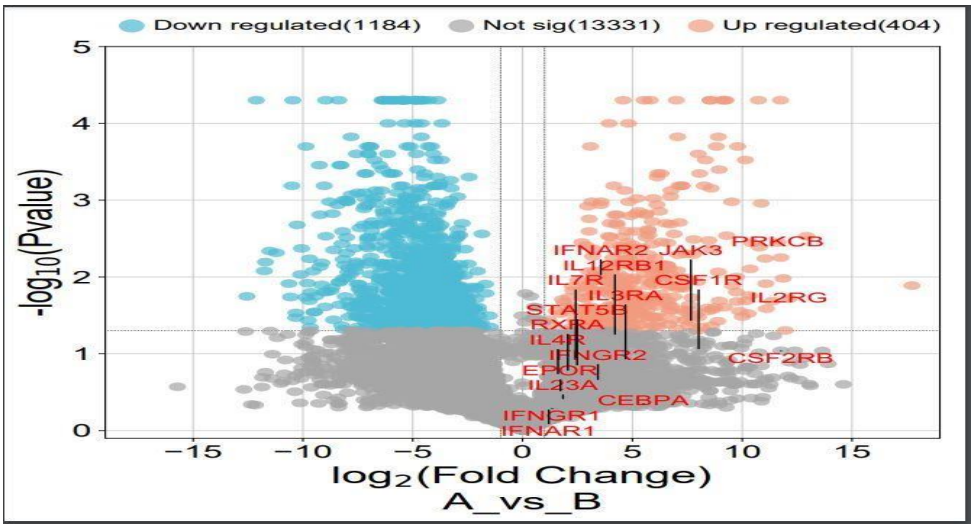



**Figure 2.** Volcano plot of DEGs in Ovarian Cancer treated individuals versus Patient. The horizontal line indicates FDR = 0.05, and the vertical line indicates |log2FC| = 0.59. Blue, grey and Orange dots represent down- regulated, non- significantly differentially expressed and up- regulated genes, respectively.

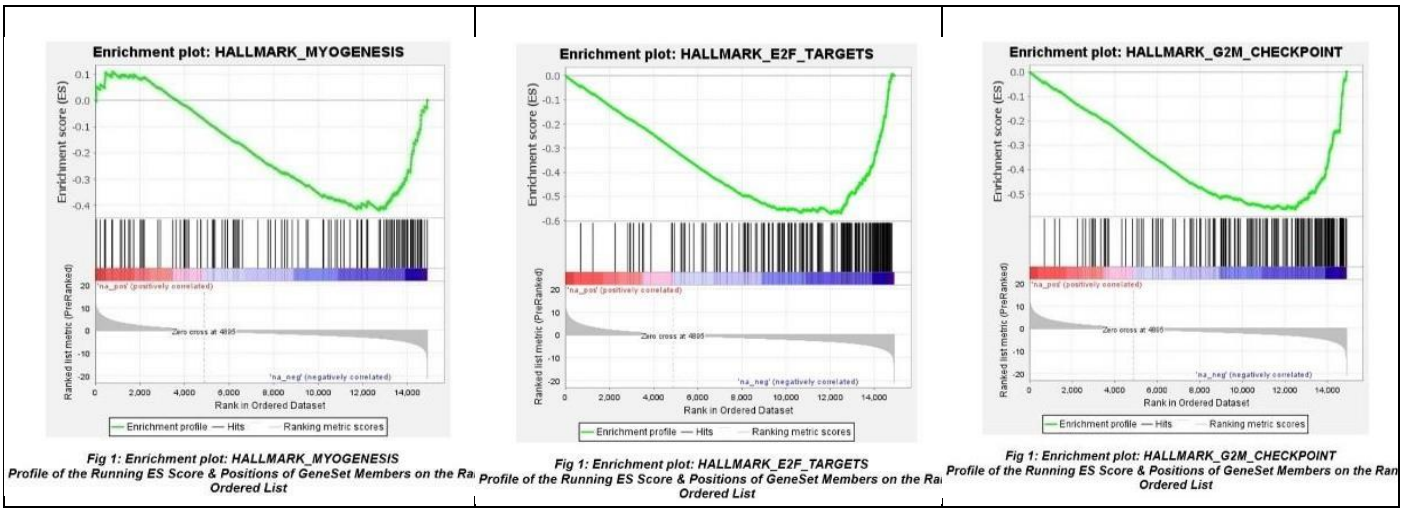



*Fig 1: Enrichment plot: HALLMARK_MYOGENESIS Profile of the Running ES Score & Positions of GeneSet Members on the Ra Ordered List*

*Fig 1: Enrichment plot: HALLMARK_E2F_TARGETS Profile of the Running ES Score & Positions of GeneSet Members on the Ra Ordered List*

*Fig 1: Enrichment plot: HALLMARK_G2M_CHECKPOINT Profile of the Running ES Score & Positions of GeneSet Members on the Ran Ordered List*

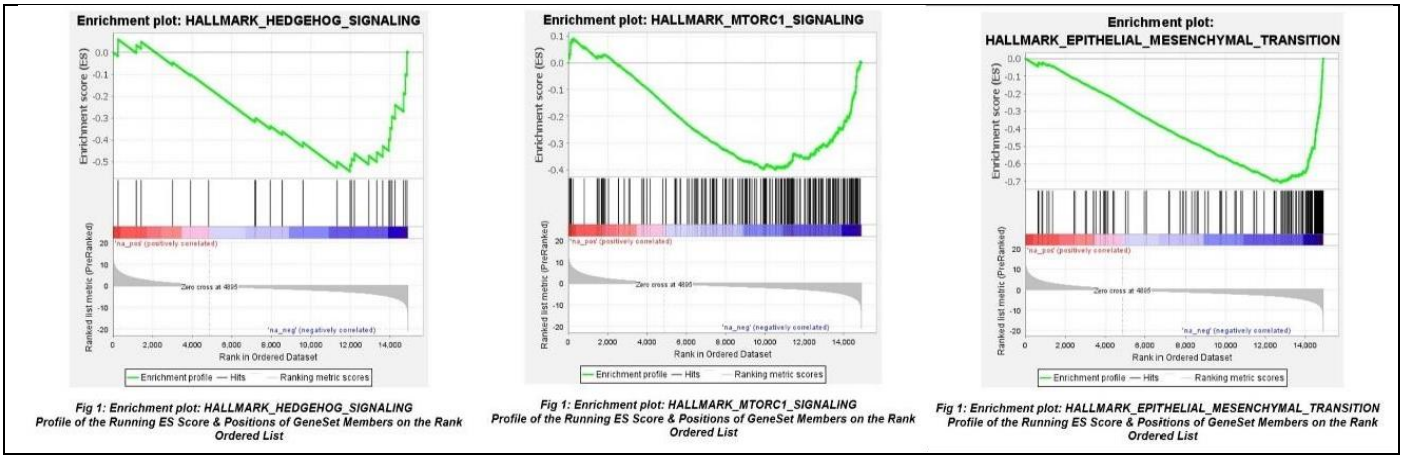



*Fig 1: Enrichment plot: HALLMARK_HEDGEHOG_SIGNALING Profile of the Running ES Score & Positions of GeneSet Members on the Rank Ordered List*

*Fig 1: Enrichment plot: HALLMARK_MTORC1_SIGNALING Profile of the Running ES Score & Positions of GeneSet Members on the Rank Ordered List*

*Fig 1: Enrichment plot: HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION Profile of the Running ES Score & Positions of GeneSet Members on the Rank Ordered List*

**Figure 3**. Gene set enrichment analysis (GSEA) in Ovarian cancer-treated individuals versus control patients (negatively correlated).

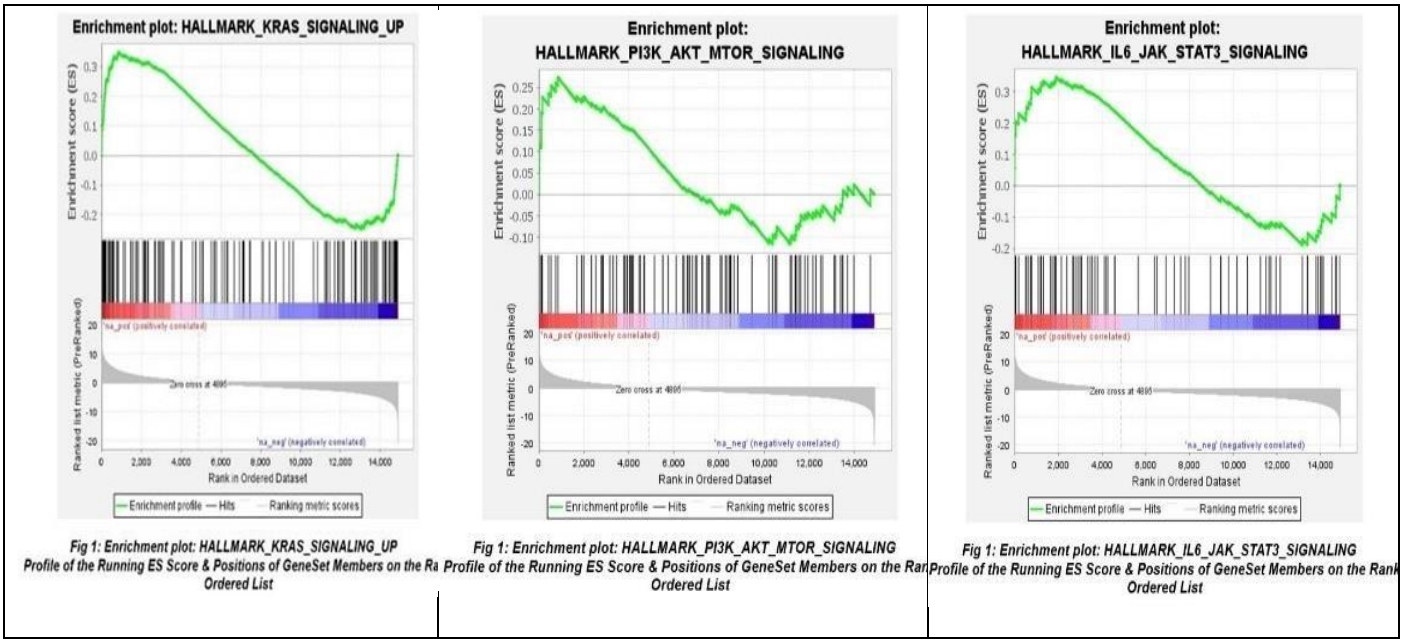



*Fig 1: Enrichment plot: HALLMARK_KRAS_SIGNALING_UP Profile of the Running ES Score & Positions of GeneSet Members on the Ra Ordered List*

*Fig 1: Enrichment plot: HALLMARK_PI3K_AKT_MTOR_SIGNALING Profile of the Running ES Score & Positions of GeneSet Members on the Rar Ordered List*

*Fig 1: Enrichment plot: HALLMARK_IL6_JAK_STAT3_SIGNALING Profile of the Running ES Score & Positions of GeneSet Members on the Rank Ordered List*

**Figure 4.** Gene set enrichment analysis (GSEA) in Ovarian cancer-treated individuals versus Controls (positively correlated).

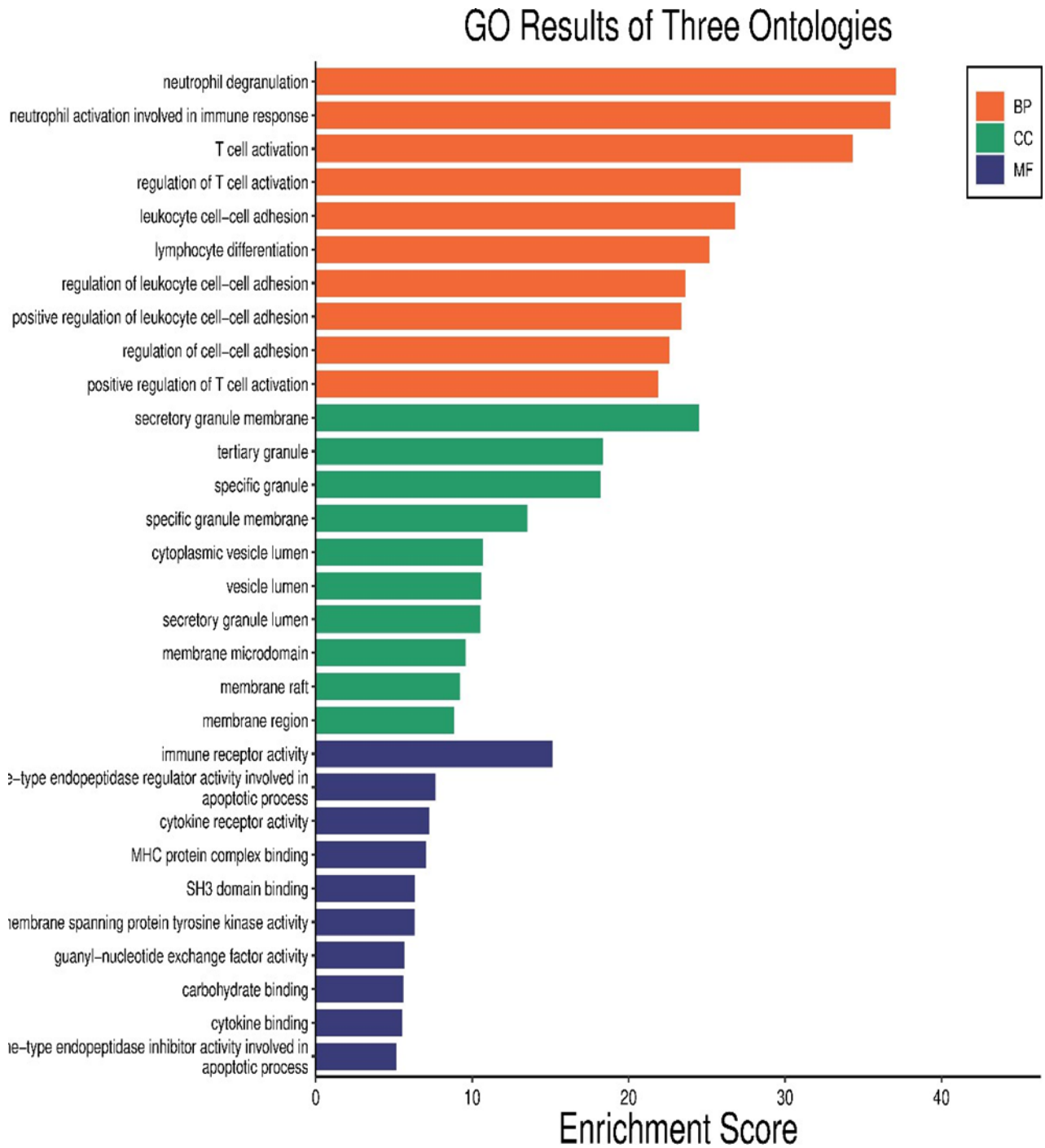



**Figure 5**: KEGG pathway and GO term analysis of three ontologies. BP, biological process; CC, cellular component; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function.

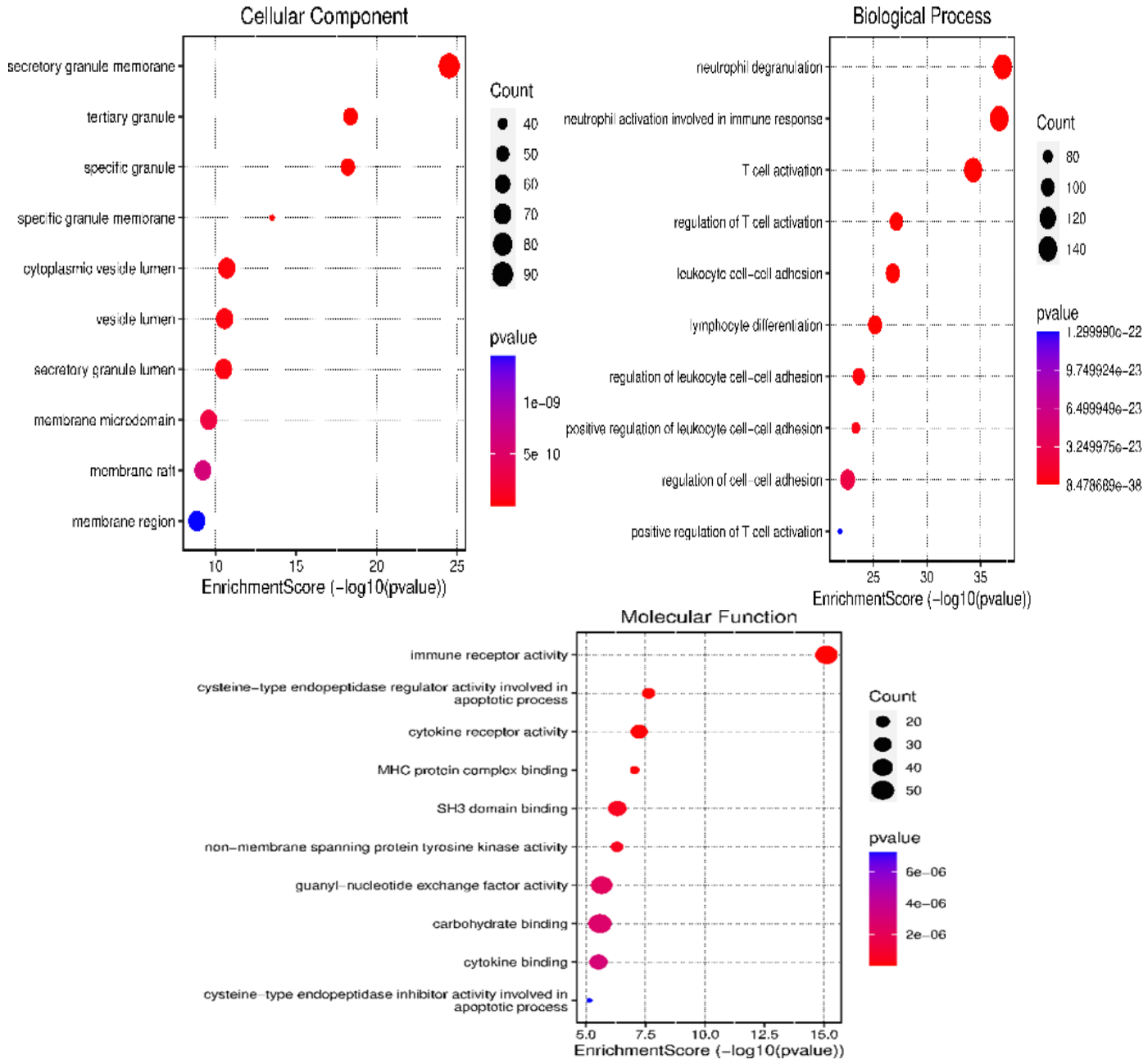



**Figure 6**: Enriched GO and KEGG pathways of Up-DEGs by using SR Plots. The X-axis serves as the proportion of relevant genes, and the Y-axis serves as the GO and KEGG terms. Each bubble means a term. The size of the bubble implies the account of relevant genes. Lighter colors suggest the smaller p-values. The red dots present low P value, while blue dots present high p value.

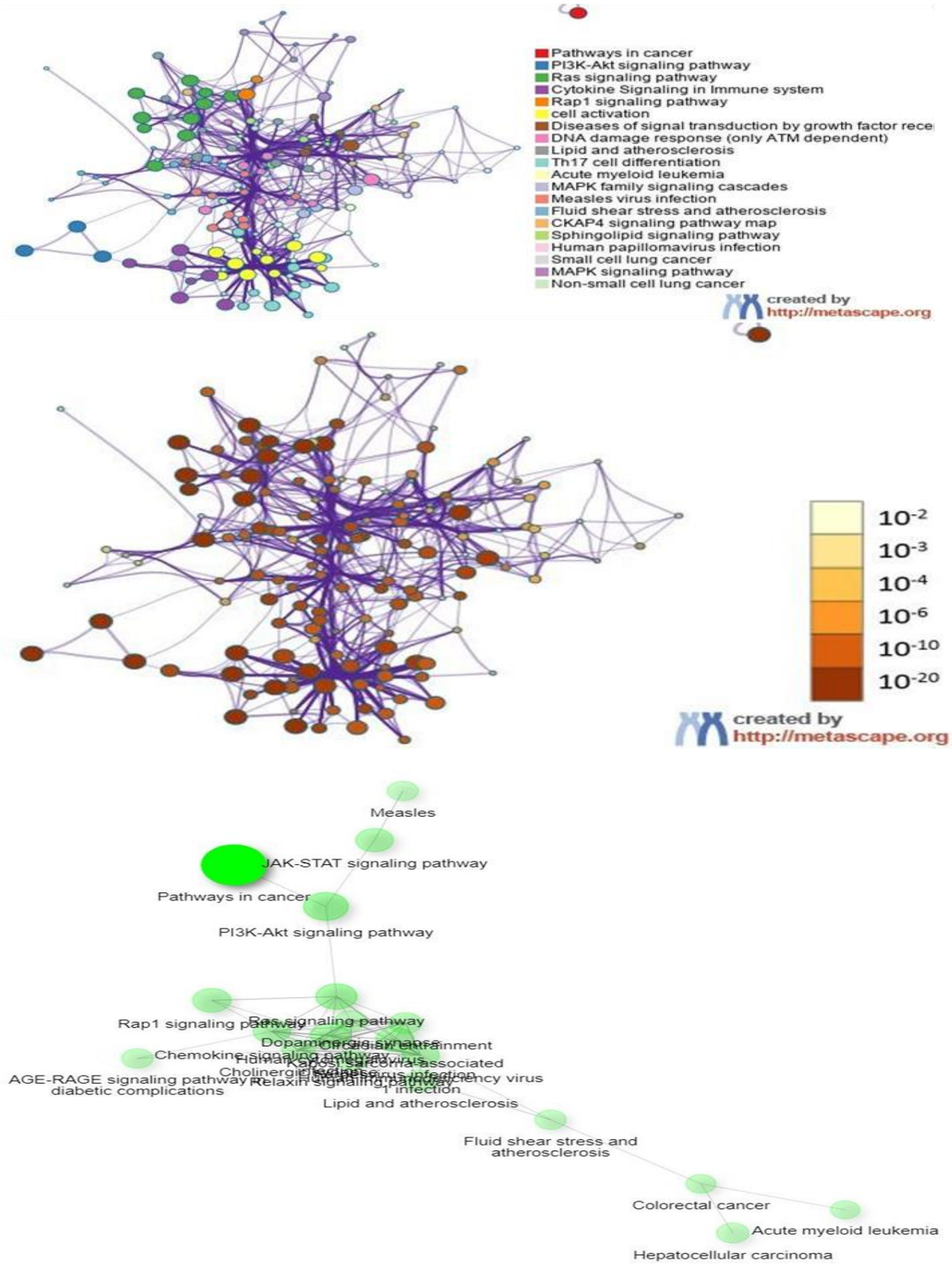
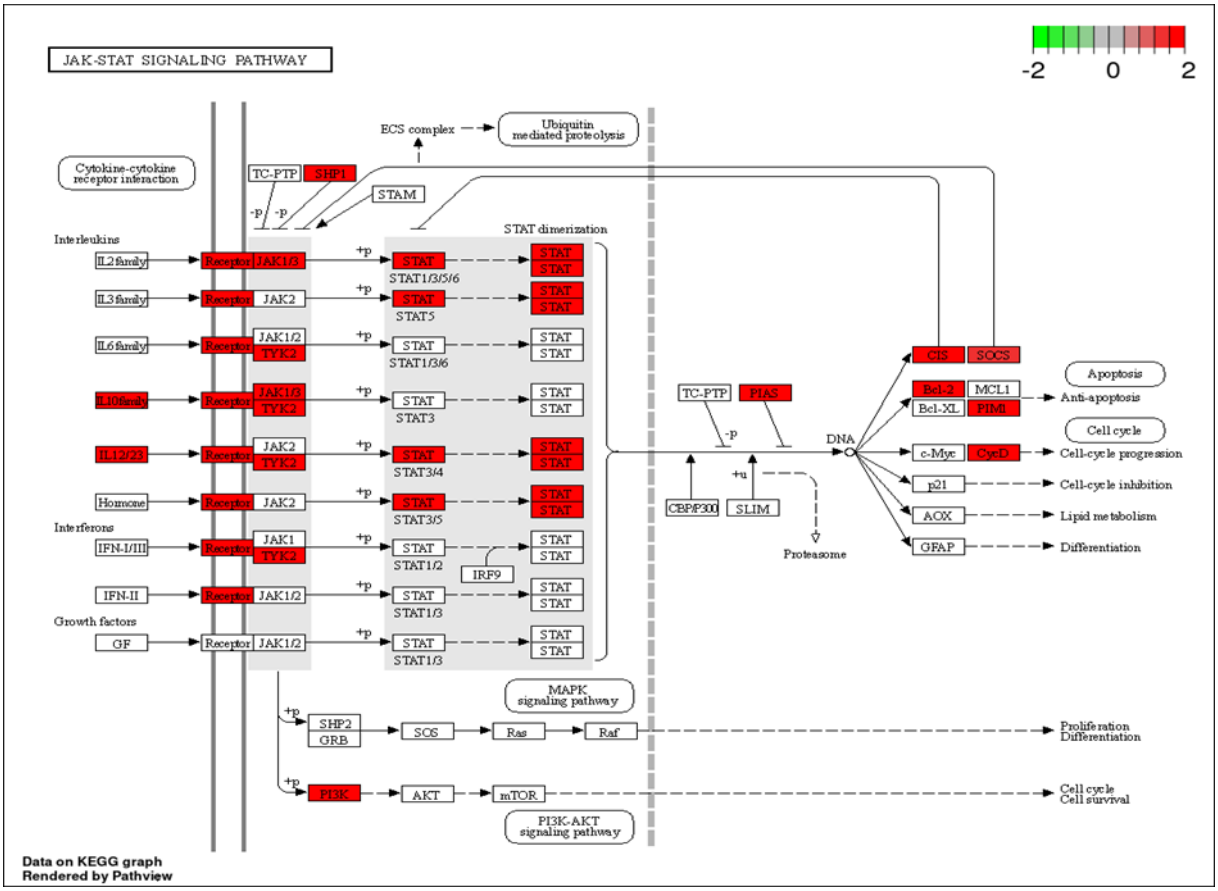



**Figure 7:** This networking plot also shows the relationship between enriched pathways. Two pathways (nodes) are connected if they share 20% (default) or more genes. The pathway enrichment analysis of top disease-related genes shows JAK-STAT and PI3K-Akt signaling pathways as the most enriched.



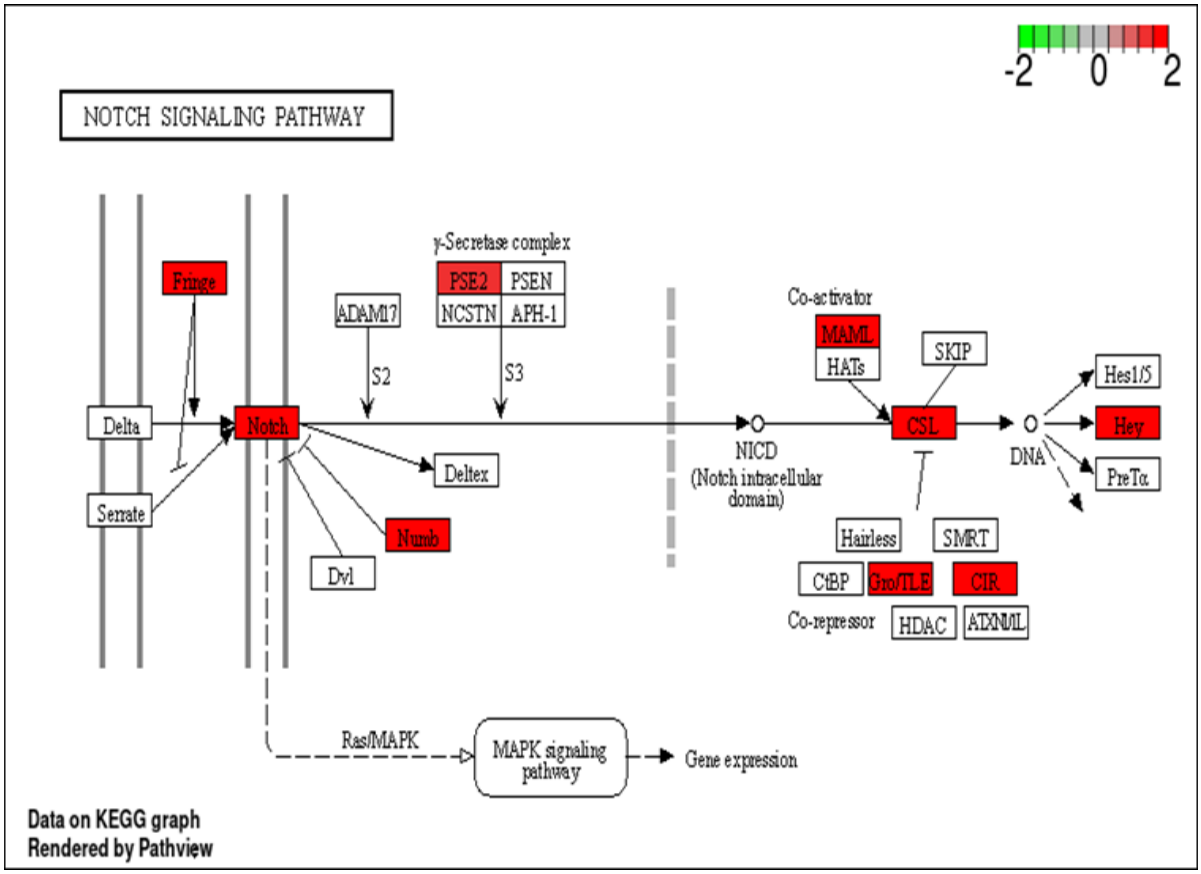




**Figure 8:** The differential expressed genes in enrichment analysis of top diseases related genes shows JAK--STAT pathway as most enriched pathway

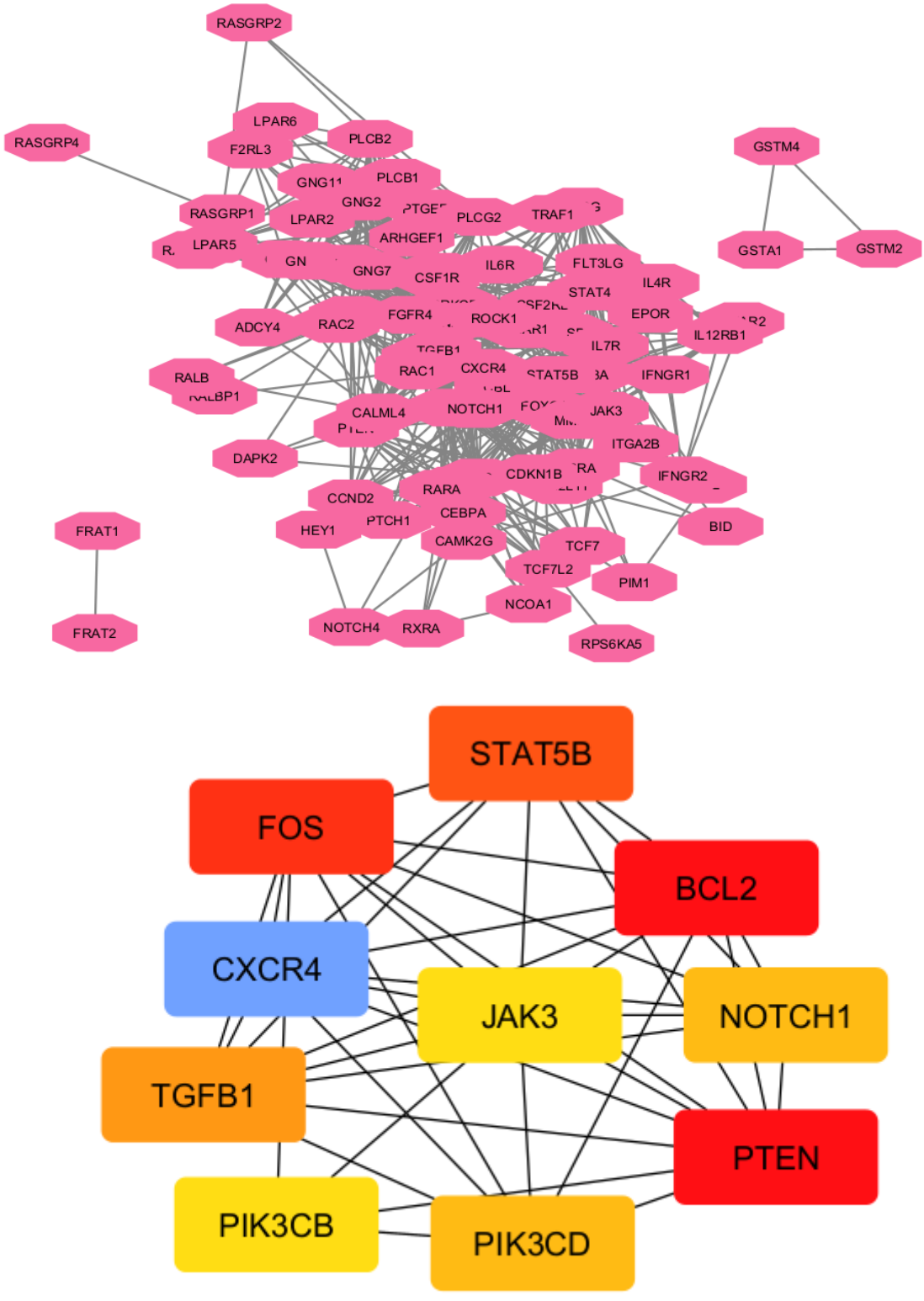



**Figure 9:** Visualization of the protein–protein interaction (PPI) network and the candidate hub genes of 92diseases related genes. (A) PPI network of 132 genes. (B) Hub genes of 92 diseases-related genes. The darker red of nodes presents a higher MCC score.

## DISCUSSION

Assisted reproductive technology (ART) treatments involve the systemic administration of high doses of Gonadotropin to induce the stimulation of multiple ovarian follicles, followed by the retrieval of mature oocytes through ovarian puncture (Huang JY et al., 2014). Due to the elevation of endogenous estrogen levels and enhanced ovarian cell proliferation associated with these treatments, there has been speculation about the potential impact of ART on the risk of ovarian cancer and other reproductive cancers. Ovarian

cancer, a relatively uncommon yet life-threatening disease, constitutes 3.6% of incident cancers and contributes to 4.3% of cancer-related mortality in women globally (Liu et al., 2024; Lundberg et al., 2019). Identified risk factors for ovarian cancer encompass factors such as nulliparity, early onset of menstruation, late onset of menopause, a family history of ovarian cancer in first-degree relatives, and a negative association with increasing age at first childbirth (Ahmed et al., 2019). Additionally, certain conditions, including endometriosis, irregular menstruation, and infertility evaluation, have been linked to an elevated risk of ovarian cancer (Králíčková et al., 2020).

The impact of assisted reproductive technology (ART) on the risk of ovarian cancer has been a subject of investigation, with varying and inconclusive outcomes in several studies (Sandvei et al., 2024; Del Pup et al.,2018). Most studies conducted thus far have compared the risk of ovarian cancer post-ART to that of the general population. While three studies indicated an increased risk among women undergoing ART, discerning whether these associations are attributed to the treatments or the infertility itself remains unclear. Studies comparing the risk of ovarian cancer after ART to that in other infertile women have also yielded inconclusive results. In a study conducted by Frida E.Lundberg et al.,2019, which encompassed all women who experienced their initial live birth from 1982 to 2012, as documented in the population-based Swedish Multi-Generation Register(MGR), with a total sample size of 1,535,678, showed that there is an increased likelihood of ovarian cancer and borderline ovarian tumors (BOT) in women who have undergone assisted reproductive technology (ART).

In a study conducted by D. Vassard et al., 2021 which included women with no cancer before ART treatment were included (n=61 579). Women from the background population with similar age and no prior history of ART treatment were randomly selected as comparisons (n=579760) revealed that the relationship between assisted reproductive technology (ART) treatment and the risk of breast cancer exhibited variations based on the age at treatment initiation, showing a tendency towards an elevated risk with increased age at treatment initiation. Notably, among women aged 40 years and older at the onset of treatment, the risk of breast cancer was heightened compared to untreated women of a similar age. Specifically, 102 cases (2.1%) of breast cancer were diagnosed in ART-treated women, contrasted with 661 cases (1.4%) among untreated women (Hazard Ratio [HR] 1.37, 95%Confidence Interval [CI] 1.29–1.45) (Vassard et al.,2021). In a 30-year cohort study involving 2,431 women diagnosed with infertility, researchers found a notable increase in the risk of endometrial cancer among those treated with clomiphene citrate (CC) and human menopausal Gonadotropin (hMG) compared to the general population (Standardized Incidence Ratio [SIR] = 5.0, 95% Confidence Interval [CI] = 2.15-9.85, $p < 0.05$). However, it's important to mention that this connection was not supported by subsequent multivariable analysis (Diakosavvas et al., 2021). Genetic testing via Next Generation, RNA Sequencing has been proven to be a powerful tool for understanding the different pathways that might been activated to develop Cancer. Next-generation sequencing (NGS) is a technology that enables simultaneous multi-gene analysis, utilizing minimal amounts of nucleic acids and offering a rapid turnaround time.NGS data gives a comprehensive and unbiased profile of the cancer genome. NGS can easily find targetable, novel, and mutation sites to guide for precision with the help of bioinformatics tools (Li et al., 2015). Bioinformatics along with the help of cutting-edge technologies like Artificial intelligence and machine learning could land in potential precise and precision medicine. Performing NGS standards of care and precision is commonplace for many malignancies but for other cancers including ovarian cancer is still emerging (Boussios et al.,2020). As of now, there is no established and universally effective standard treatment for restraining the metastasis of reproductive cancers. Personalized approaches hold significant potential as an available option for enhanced patient management. Utilizing RNA sequencing through gene expression profiling, coupled with bioinformatics analysis, can offer valuable insights into malignancies, aiding in the identification of specific pathways for targeted interventions. In our study, a comprehensive bioinformatics analysis identified 1588 Differentially Expressed Genes (DEGs), shedding light on the underlying mechanisms of various cancers and enhancing our understanding of pathogenesis and prognosis.

Subsequent downstream analysis highlighted 10 hub genes, namely **STAT5B, FOS, BCL2, CXCR4, JAK3, NOTCH1, TGFB1, PIK3CB, PIK3CD, and PTEN,** which may play critical roles in early diagnosis, tumor staging, and predicting poor outcomes in ovarian cancer. This emphasizes the importance of considering these hub genes in the comprehensive assessment of ovarian cancer. It is worth noting that when determining the risk of cancer, it is essential to factor in the type of infertility (primary vs. secondary) and the underlying causes. Tailoring interventions based on this understanding can contribute to more effective and personalized strategies for managing reproductive cancers and their metastasis.

## LIMITATIONS:

A comprehensive review of medical and medication records is essential before drawing any conclusions. Sample size has to be increased to get more accurate results. Future studies could help delve deeper to understand the correlation between Cancer and Assisted Reproductive Technology (ART), shedding light on potential pathogenic mechanisms.

## CONCLUSION

Women undergoing infertility treatments, particularly in vitro fertilization (IVF), face a slightly elevated risk of severe maternal morbidity. There is a crucial need to pinpoint predictors specific to patients and treatments that influence severe maternal morbidity, potentially shaping the choice of treatment offered to a woman. Data on the association between fertility treatments and cancer risk are limited.

**In our study, RNA Sequencing data and pathway analysis of Ovarian, Breast and Endometrial Cancer patients previously treated with ART showed up-regulation of 10 hub genes** namely **STAT5B, FOS, BCL2, CXCR4, JAK3, NOTCH1, TGFB1, PIK3CB, PIK3CD, and PTEN and JAK-STAT and PI3K-Akt signaling pathways as the most enriched.** This is the **first**

**study** that involved molecular study of different reproductive cancer genes of ART-treated patients. However, we could not completely attribute the development of cancer to the ART Treatment, we could identify JAK-STAT and PI3K-Akt signaling pathways as the most enriched in these patients. This could help in providing personalized treatment to the patients. Further to prove this data more samples need to be studied and follow-up of ART-treated patients should be done.

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**Conflict of Interest**
The authors declare no conflict of interest.

**Ethical statement**
All procedures performed in studies involving human participants were by the ethical standards.

**Author contributions**
All authors contributed equally to this work; RR, AGA, VAA,SSA, AAK, SJ designed the study; AAK, SJ, WT conducted the laboratory work; RR, SSA, AAK, SJ, AA wrote the manuscript. All authors reviewed and approved the final manuscript.

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