A STUDY TO INVESTIGATE THE ASSOCIATION OF INTERLEUKIN-2 RECEPTOR BETA (IL2RB) GENE POLYMORPHISM IN ENDOMETRIOSIS

Research Project Submitted

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in

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By

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FACULTY OF BIOMEDICAL SCIENCES & TECHNOLOGY

DEPARTMENT OF HUMAN GENETICS

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This is to certify that, the dissertation/research project titled **A Study to Investigate The Association of Interleukin-2 Receptor beta (IL-2RB) Gene Polymorphism in Endometriosis** is based on the bonafide work carried out under my supervision by **Nandhini. S and Akshaya. J** (Registration No: B0118022 and B0118024) during the academic year 2021-2022 as a part of his/her B.Sc., Biomedical Sciences [Human Genetics] course. This dissertation/research project has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or similar title.

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LIST OF ABBREVIATIONS

S. No.	ABBREVIATIONS	EXPANSION
1.	IL-2	Interleukin- 2
2.	IL-2Rb	Interleukin- 2 Receptor beta
3.	°C	Degree Celsius
4.	mM	Millimolar
5.	ml	Millilitre
6.	mg	Milligram
7.	M	Molar
8.	µl	Microliter
9.	G	Gram
10.	Bp	Base Pair
11.	SNP	Single Nucleotide Polymorphism
12.	AGE	Agarose Gel Electrophoresis
13.	TE	Tris-EDTA
14.	rpm	Revolutions per minute
15.	pM	Picomolar
16.	KDa	Kilo Daltons
17.	µM	Micromolar

18.	PCR	Polymerase Chain Reaction
19.	EtBr	Ethidium Bromide
20.	RCLB	Red Cell Lysis Buffer
21.	NLB	Nucleated Cell Lysis Buffer
22.	SDS	Sodium Dodecyl Sulphate
23.	Taq	Thermophilus aquatics
24.	TAE	Tris-Acetic Acid EDTA
25.	Rpm	Revolution per minute
26.	TE	Tris- EDTA
27.	KCL	Potassium Chloride
28.	$MgCl_2$	Magnesium Chloride
29.	NaCl	Sodium Chloride
30.	HWE	Hardy Weinberg Equilibrium
31.	ESR	Estrogen Receptor
32.	C	Cytosine
33.	T	Thymine
34.	A	Adenine
35.	G	Guanine
36.	Ng	Nanogram
37.	Dntp	Deoxy Nucleotide Tris Phosphate

38.	Edta	Ethylene Diamine Tris Acetic Acid
39.	Hcg	Human Chronic Gonadotropin
40.	HCl	Hydrochloric acid
41.	NSAIDs	Non-Steroidal Anti Inflammatory Drugs
42.	NS	Non Significance
43.	OR	Odds Ratio
44.	CI	Confidence Interval
45.	CPP	Chronic Pelvic Pain
46.	LH	Luteinizing Hormone
47.	DIE	Deeply Infiltrating Endometriosis
48.	IFN-g	Interferon-gamma

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1.ABSTRACT

Introduction: Endometriosis is a benign gynecological disease characterized by ectopic endometrium that causes endometrium-like inflammatory lesions outside the uterine cavity. It causes a chronic inflammatory reaction that may result in the formation of scar tissue (adhesions, fibrosis) within the pelvis and other parts of the body. It occurs in 10% of women in reproductive age globally. The present study aimed to determine the association of interleukin-2 receptor beta (IL-2R b) gene C627T polymorphism with endometriosis.

Materials and Methods: 30 women with surgically or histologically diagnosed endometriosis were recruited for this study, and 30 patients with no evidence of endometriosis served as controls. The C627T polymorphism of the IL-2R b was assessed using the DNA sequencing to examine any differences in genotype distributions and allele frequencies of the IL-2R b C627T polymorphism between the endometriosis cases and the controls.

Results: There was no statistically significant difference in the frequency of the IL-2R b C627T polymorphism between the endometriosis patients and the controls (67% C/C, 26.6% C/T and 6.6% T/T versus 67%, 23%and 10%, respectively, P-value 0.23). No statistically significant in genotype distributions were observed among these groups.

Conclusions: Contrary to the recent data reported in a Taiwanese population, our results suggest that the C627T polymorphism of the IL-2R b gene may not be associated with the risk of endometriosis in the Indian population.

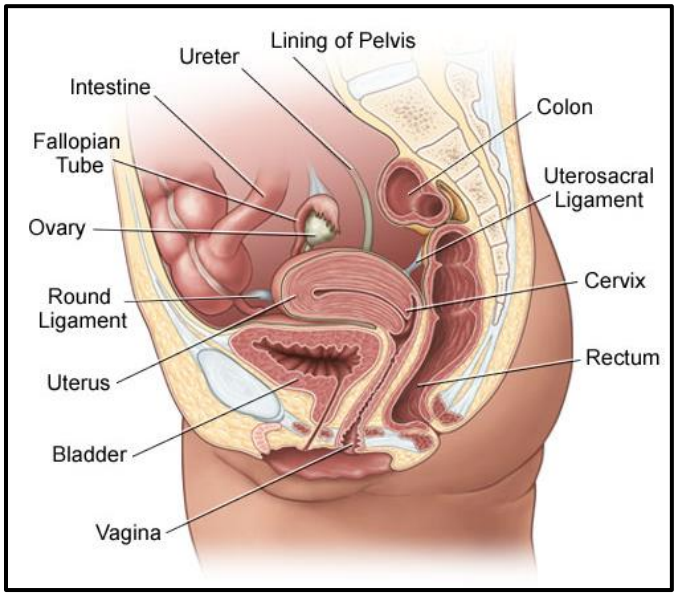
2.INTRODUCTION

2.1 Endometriosis

Endometriosis is a common gynecological condition affects women of childbearing age. This condition is named after the tissue endometrium, that lines the inner wall of the uterus. Endometriosis happens when tissue similar to the lining of the uterus grows outside of the uterus [1]. During regular menstrual cycle, the endometrium lines up and sheds if the women does not get pregnant. But for women with this condition, the tissue grows outside the uterus and reacts to the hormonal changes of the menstrual cycle and acts exactly like the endometrium and results in bleeding, inflammation, swelling, and scaring of the normal tissue surrounding the endometriosis implants. The incidence of this disease is around 10% among women but several more cases are being reported on account of performing diagnostic laparoscopy. The incidence is noticed to be 20% in infertile women according to studies and endometriosis is seen in 15% of women with Chronic Pelvic Pain (CPP) [2]. Endometriosis is estimated to affect 10-15% of women of reproductive age. However, it is difficult the prevalence because of diversity of symptoms and is found almost exclusively in women of reproductive age. Traditionally the diagnosis has not been commonly applied in adolescence but should be considered, as early recognition and treatment may be beneficial. Adenomyosis is the invasion of the endometrium by endometrial tissue. Extra uterine endometrial tissues cause inflammation, pain, dyspareunia and female infertility. Furthermore, nearly 50% of endometriosis patients are persecuted by fertility problems, including infertility and endometriosis could be explained by several etiopathogenesis, including implantation theory,

defective immune system, and Genetic factors. Endometriosis is usually confined to the peritoneal or serosal surfaces of pelvic organs, commonly the ovaries, broad ligaments, posterior and utero sacral ligaments, less common sites include fallopian tubes, serosal surfaces of the small and large intestines, ureters, bladder, cervix, and more rarely the pleura

Figure 1: Common locations of endometriosis



Source: Common locations of endometriosis within the pelvis and abdomen. (Reprinted after permission from the New England Journal of Medicine 2001. Olive DL, Pritts EA. Treatment of Endometriosis. Vol. 345:267)

As endometriosis has relatively high inheritability ratio of 51%, some researchers regarding genetic risk factors, such as estrogen receptor-1 (ESR1), estrogen receptor-2 (ESR2) and luteinizing hormone beta-subunit (LHB) genes, for endometriosis related infertility have been analysed. The exact pathophysiology of endometriosis is still unclear, but both environmental and genetic factors have been in the occurrence and progression of the disease[3]. Family

studies regarding endometriosis have indicated increased risk for close- relatives of patients with endometriosis, thus suggesting that genetics might have a contribution. Among the possible cause of endometriosis, it has been demonstrated that the deficiency in the immune system might act as an impediment to clear endometrial cells from the pelvic cavity. There are also evidences that B lymphocytes produce specific anti-endometrial autoantibodies and that Tregs, known as regulatory T-cells are key regulators to guarantee a specific immune response against ectopic endometrial fractions.

2.2 TYPES OF ENDOMETRIOSIS

Endometriosis is also grouped with the area of the pelvis or abdomen it affects[4]. There are four main types:

2.2.1 Superficial peritoneal endometriosis

The peritoneum is a thin membrane that lines your abdomen and pelvis. It also covers most of the organs in these cavities. In this type, the endometrial tissue attaches to the peritoneum. This is the least severe form of endometriosis

2.2.2 Endometriomas

These are dark, fluid-filled cysts. They are also called chocolate cysts. They vary in size and can appear in different parts of your pelvis or abdomen, but they’re most common in the ovaries.

2.2.3 Deeply infiltrating endometriosis (DIE)

In this type, the endometrial tissue has invaded the organs either within or outside your pelvic cavity. This can include your ovaries, rectum, bladder, and bowels. It's rare, but sometimes a lot of scar tissue can bond organs so they become stuck in place. This condition is called frozen pelvis. But this only happens to 1-5% of people with endometriosis[5].

2.2.4 Abdominal wall endometriosis.

In some cases, endometrial tissue can grow on the abdominal wall. The cells may attach to a surgical incision, like one from a C-section.

2.3 ENDOMETRIC LESSION

Three types of endometriotic lesions: superficial peritoneal endometriosis, ovarian endometriomas and deep infiltrating endometriosis. Typical endometriotic lesions show histological features similar to the endometrium, with stromal and glandular epithelium that respond to hormonal stimuli and exhibit some characteristics of growth[6].Lesion type appearance characteristics location peritoneal endometriotic lesions clear papules that resembles normal endometrium around the peritoneum, fallopian tubes and considered the most "active", highly vascularized; presence of pigmented hemosiderin deposits from "old blood" more advanced than red flare and considered less active ovarian endometriomas Brown tar like filled cysts contain shed menstrual debris, arise after endometrial ectopic implantation on ovaries Deep-infiltrating endometriosis Nodular in appearance Firm, solid tumor more than

5 mm from peritoneal surface into adjacent structures posterior sac, uterosacral ligaments, bladder and rectovaginal septum.

2.4 HISTORY OF ENDOMETRIOSIS

Although endometriosis is considered as 20 or 21th century disease, In 1922, Sampson described a series of cases of superficial and deep chocolate cyst, the life history of ovarian hematomas of endometrial type. The reactions of the lining of ovarian hematoma of endometrial type were similar to those of uterine mucosa mimicking menstruation in terms of bleeding and ageing process[7]. After discarding the term "perforating hemorrhagic cyst" referring hematomas of endometrial type. The first reference to endometriosis is found in which a treatment of painful disorder of menstruation is described. Endometriosis was first discovered microscopically by Karl von Rokitansky provided first identification and detailed description of endometriosis. But more detailed description of endometriosis was made in Daniel shroen‟s 1960 book titled as "Disputatio Inaugualis Medica de Ulceri" in which referred as endometriomas and adhesions associated with disease. Sampson published another key work on surgical treatment of endometrial intestinal adenoma. In 1927, he formulated a new concept in article titled peritoneal endometriosis due to menstrual Dissemination of endometrial tissue into the peritoneal cavity", which dominated hypotheses for the origin of endometriosis for the next 80 years. The term "chocolate cyst" was used for the first time in 1894, a critical reference for endometriosis[8]. This document is important because it relates to pelvic adenomas of endometrial type ("adenomyoma" of the uterus, sigmoid, etc). the first reference to endometriosis associated symptoms are found in the Ebers Papyrus, in which a treatment for a "painful disorder of menstruation" is described.

2.5 THEORIES OF ENDOMETRIOSIS

There are three theories that explain the etiology of endometriosis: Sampson's theory, Meyer's theory, and Halban's theory. Sampson's theory of transplantation and implantation is the most prevalent theory and the one supported by the most evidence to date. This theory emerged from observations made during surgeries in the 1920s that many women shed endometrial debris into the peritoneum through their fallopian tubes during menstruation [9] Not only has viable endometrial tissue been observed in women's fallopian tubes and peritoneal fluid, but endometrial tissue will grow if placed ectopically in humans and other animals. Endometriosis appears to be most common in the gravitationally dependent parts of the pelvis, lending credence to this theory [10]. Finally, endometriosis is more common in patients with mullerian anomalies or genital tract obstructions, both of which increase the likelihood of retrograde flow [11]. One flaw in this theory is that retrograde menstruation has been demonstrated in 76-90 percent of menstruating women, which is significantly higher than the prevalence of endometriosis [12,13].This disparity suggests that, in addition to the presence of ectopic tissue, other factors, such as the amount of endometrial debris that reaches the peritoneal cavity, the woman's immunocompetence to clear the debris, and the molecular abnormalities/properties inherent in the ectopic tissue, are required to establish the disease. Meyer's theory [14] proposes that endometriosis is caused by metaplasia of the coelomic epithelium. This theory is logical, because cells from both the peritoneum and the endometrium derive from the same embryological precursor: the coelomic cell. However, scientific support for this theory has been difficult to come by. If this hypothesis were correct, pleural endometriosis would be much more common than it is. Halban's theory proposes that distant lesions are formed through

hematogenous or lymphogenous spread of viable endometrial cells. This metastatic theory explains rare endometriotic lesions in the brain or lung, but it does not explain the gravitationally-dependent location of most endometriotic foci.

The most widely accepted theory is Sampson's transplantation and implantation theory, but most researchers agree that it grossly simplifies the disease process. While retrograde menstruation can transplant tissue fragments into the peritoneal cavity, the cells themselves must survive by avoiding apoptosis[15], adhering to the underlying peritoneum[16,17] degrading the underlying extracellular matrix[18],generating a new vascular supply[19], and evading the immune surveillance system[20].Endometriotic lesions and eutopic endometrium have distinct molecular differences. Increased levels of prostaglandins, chemokines, and cytokines, such as interleukin-1ß, intlerleukins-1, 6, 8, and tumor necrosis factor (TNF), are thought to enhance the adhesion of endometriotic implants to the peritoneal surface as a result of endometriosis inflammation.

These are linked to an increase in the production of prostaglandins, cytokines, and chemokines. Proteolytic membrane metalloproteinases, which are also elevated in endometriosis, aid in implantation. Endometriosis also alters angiogenesis and apoptosis in favor of implant survival. Increased monocyte chemoattractant protein 1, interleukin-8 in endometriosis attract granulocytes, macrophages, and natural killer cells. Autoregulatory positive feedback loops cause these inflammatory mediators to accumulate in endometriotic tissue[21]. Endometriotic lesions cause erythrocyte breakdown, and the non-protein-bound catalytic iron increases the production of reactive oxygen species, perpetuating peritoneal damage and inflammation.

2.6 GENETIC FACTORS

Endometriosis is thought to have a genetic predisposition, which may make some people more likely to get it than others. Multiple studies have examined familial patterns and endometriosis. Several clinical studies indicate that there is a relation between genetic factors and endometriosis. The prevalence of this disease is predominantly visible in certain families and it is also noted that a woman with an affected immediate relative is seven times more susceptible in developing endometriosis[22]. The most notable evidence linking genetic factor to endometriosis arises from studies conducted on monozygotic twins show greater concurrence for endometriosis than dizygotic twins. The same studies conclude that genetic factors contribute to half the variation related to the risk of endometriosis and a heritability of 51% in population [23].

2.7 CAUSES OF ENDOMETRIOSIS

The cause of endometriosis is unknown. Some experts believe that pieces of endometrium travel back through the fallopian tubes and pass out into the pelvic cavity (space inside the pelvis that holds the reproductive organs). Tiny pieces of tissue may lodge on surfaces of the reproductive organs[24]. During menstruation, the tissue bleeds, just like the endometrium inside the uterus. Surrounding tissue may become inflamed. Over time, scar tissue and cysts can form.

2.8 SIGNS AND SYMPTOMS

- Pain, especially excessive menstrual cramps that may be felt in the abdomen or lower back
- Pain during intercourse

- Abnormal or heavy menstrual flow
- Infertility
- Painful urination during menstrual periods
- Painful bowel movements during menstrual periods
- Gastrointestinal problems, such as diarrhea, constipation and nausea.

Any symptoms mentioned previously, surgical procedure like laparoscopy has to be performed to confirm the diagnosis. In laparoscopy a small scope is inserted through a small incision in the abdomen so the endometrial implants can be viewed. Biopsy of these implants confirm the diagnosis. Then treatment can be planned according to the severity of the endometriosis.

2.9 STAGES OF ENDOMETRIOSIS

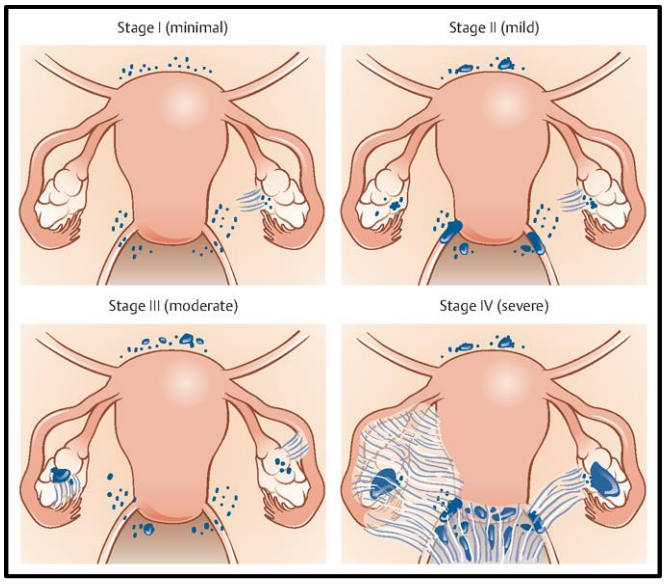
Endometriosis appears in many different forms with a wide variety of clinical presentations depending on the extension of the disease, organs affected and individual circumstances. The most used and best-known system was developed by the American Society for Reproductive Medicine (ASRM – formerly ASF)[25].

The ASRM classification system is divided into four stages or grades according to the number of lesions and depth of infiltration:

Table 1: Stages of endometriosis

STAGE	DISEASE	DESCRIPTION
I	Minimal	The implants are small, few in number, and shallow and no significant adhesions
II	Mild	Characterized by superficial implants and no significant adhesion
III	Moderate	Involves multiple deep endometrial implants, small cysts on at least one ovary.
IV	Severe	Multiple deep endometriosis implants, large cysts on one or both ovaries and many dense adhesions throughout the pelvic region

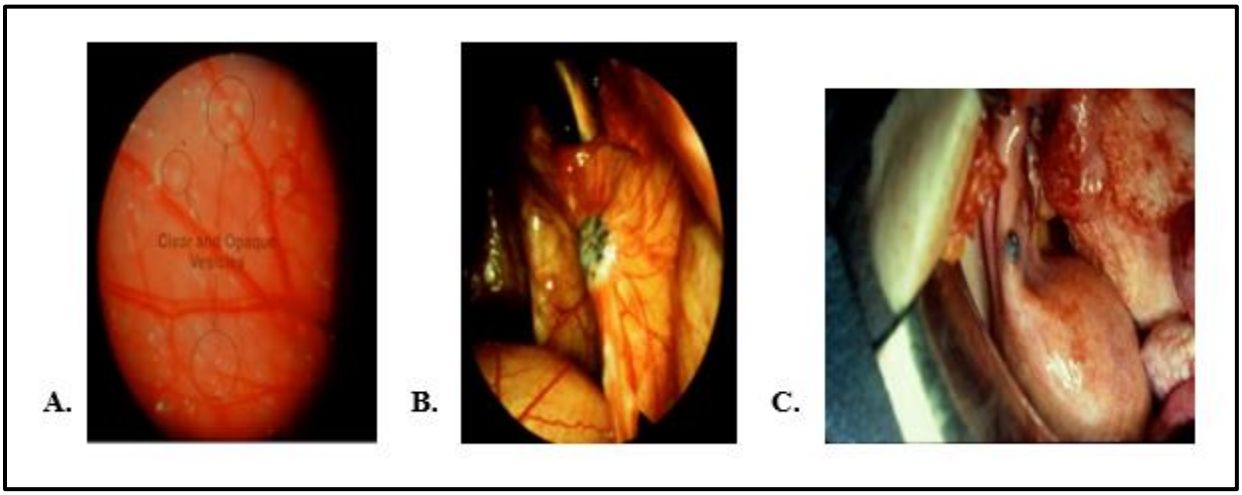
Figure 2: Stages of endometriosis



2.10 CLINICAL APPEARANCE

Peritoneal endometriosis can appear in a variety of ways. Endometriosis implants were traditionally thought to be blue-black "powder-burns" or "mulberry lesions" of the peritoneum. More recently, several stages of implant development have been appreciated, each with a corresponding appearance. Early, active lesions can appear as papular excrescences or vesicles that range in color from clear to pink or bright red [26]. A third of these lesions are in phase with the eutopic endometrium and have a tendency to grow and regress spontaneously, implying a fluctuation in proliferation associated with cyclic hormone production during the menstrual cycle [27]. Advanced, active lesions are characterized by inflammation, fibrosis, and hemorrhage, and have a more recognizable appearance at surgery. These lesions can be a variety of colors, ranging from black to brown, purple, red, or green. These are caused by the presence of heme degradation products as the foci bleed and fibrose. Dormant and healed lesions appear white or calcified and are the remnants of glands embedded in fibrous tissue[28]. The peritoneum's surface may also be puckered. Endometrioma, also known as a chocolate cyst, is a specific manifestation. These ovarian cysts were named after the characteristic chocolate syrup appearance of their contents when ruptured. Microscopic lesions have been found in the normal appearing peritoneum of women with and without endometriosis using electron microscopy [29]. Although the clinical significance of these findings is unknown at this time, the presence and potential tissue activity in these occult lesions may contribute to the occurrence of endometriosis or the recurrence of symptoms in women even after successful ablation or excision of visible lesions.

Figure 3: Intraoperative appearance of endometriosis. (a) The vesicular appearance of early, active lesions. (b) Peritoneal windows. (c) The characteristic blue-black appearance of more advanced, active implants.



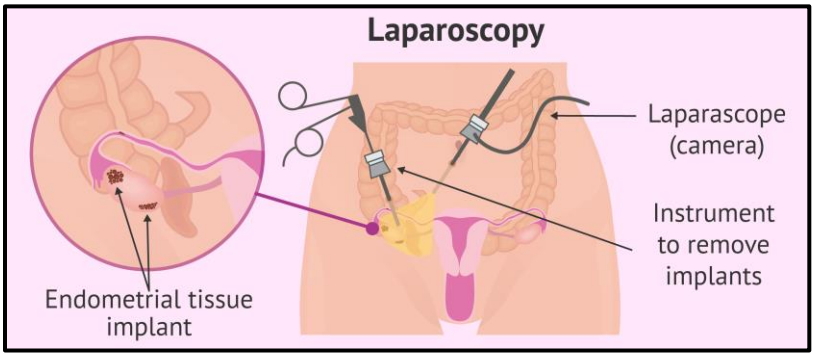
Source : Carpinello OJ, Sundheimer LW, Alford CE, Taylor RN, DeCherney AH. Endometriosis.[Updated 2017 Oct. 22]. South Dartmouth (MA): MDText. com, Inc.; 2000.

2.11 DIAGNOSIS

A complete history and physical examination, including speculum and bimanual examination, may aid in diagnosis. As an estrogen-dependent disease occurring in women with heavy menses, endometriosis has been suspected most commonly in women with menstrual-associated cyclic pain. Such cyclic pain is not pathognomonic for endometriosis, as women with fibroids and adenomyosis may also have dysmenorrhea. Furthermore, many patients with endometriosis have non-menstrual chronic pelvic pain, complaining of pain at other predictable times of their menstrual cycle, such as at ovulation. Patients may also have dyspareunia, bowel or bladder pain, or chronic fatigue.

2.11.1 Laparoscopy

Figure 4: Laparoscopy to diagnose Endometriosis



Source: Bafort C *et al*, Laparoscopic surgery for endometriosis. Cochrane Database of Systematic Reviews. 2020(10).

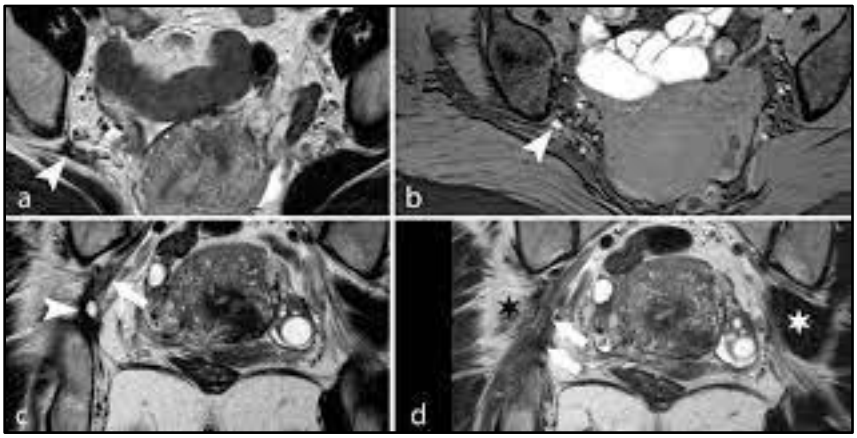
At laparoscopy, endometriosis may be visualized as peritoneal implants, peritoneal windows, endometriomas, and deep infiltrating nodules of endometriosis which may each be associated with adhesions. The color, size, and morphology of endometriotic lesions are highly variable from person to person. Endometriotic implants in the pelvis occur more often on the left side, although the reason for this asymmetry is not known. On histopathology, the diagnosis of endometriosis requires the presence of two or more of these histologic features: endometrial epithelium, endometrial glands, endometrial stroma, and hemosiderin-laden macrophages.

2.11.2 IMAGING

Imaging has limited utility in the diagnosis of endometriosis, as it lacks adequate resolution to identify adhesions or superficial peritoneal implants. Ultrasound is cheap and easy to

perform, but user-dependent; MRI is more accurate but considerably more expensive. As CT of the pelvis does not visualize pelvic organs well, it is not useful in the diagnosis of endometriosis. An important role for the CT scan with contrast is to detect ureteral involvement and possible renal insufficiency.

Figure 5: Imaging of Endometriosis



Ultrasound is a readily available and inexpensive tool for the diagnosis of large endometriosis lesions. Transvaginal ultrasound can help diagnose endometriomas, bladder lesions, and deep nodules such as those in the rectovaginal septum. Lesions identifiable on ultrasound include hypoechogenic linear thickening or nodules/masses with or without regular contours. With an experienced sonographer, transvaginal ultrasound has high specificity and sensitivity in the diagnosis of ovarian endometriosis. While an adnexal mass may be suspicious for an endometrioma, the differential diagnosis includes dermoid cyst, hemorrhagic cyst, neoplasm, ovarian abscess, and ectopic pregnancy. Transrectal ultrasound may also be used to demonstrate rectal involvement in endometriosis, the depth

of infiltration by endometriosis, and to detect lesions on the posterior bladder wall, but it has not been shown to be superior to transvaginal ultrasound.

2.11.3 SERUM MARKERS

Serum markers for endometriosis have been eagerly sought for their use in diagnosis, to measure disease activity, and to monitor improvement. Serum cytokines, matrix metalloproteinases, adhesion molecules, and markers of angiogenesis or inflammation have been investigated. While peritoneal markers have also been investigated, the cyclic variation in hormonal influences and the amount of peritoneal fluid makes this impractical and difficult to standardize.

2.11.4 ENDOMETRIAL NERVE FIBRE

Endometrial biopsy is being explored for the diagnosis of endometriosis. Recent studies have shown an increased number of nerve fibers in the endometrium of women with endometriosis compared to women without endometriosis. These nerve fibers are reported to be primarily small unmyelinated sensory C fibers in the functional layer of endometrium, which are identified by their staining with PGP9. VIP, and substance P, but not with neurofilament. Some evidence suggests that endometriosis patients on hormonal treatment also have fewer nerve fibers compared to endometriosis patients who are not on hormones. A significant limitation in these studies is the lack of information about pain symptoms in these mixed cohorts of patients with infertility and chronic pain.

2.12 TREATMENT

2.12.1 Pain medication

NSAIDs (Non-steroidal anti-inflammatory drugs)- eg: ibuprofen that can be obtained OTC for relieving pain. Although it is not always successful in pain reduction.

2.12.2 Hormone medication therapy

Hormone therapy can help the body to regulate hormonal alterations that induce endometrial tissue growth outside of the uterus. Supplemental hormones can help in relieving pain and also is known to slow down the progression of endometriosis in some cases [30]. Varieties include hormonal birth control medication, Gonadotropin releasing hormone (gnRH0 agonists and antagonists, Medroxyprogesterone and danazol. Medroxyprogesterone is administered through injection. It halts menstruation and also known to stop the occurrence of endometrial tissue. Gonadotropin-releasing hormone (GnRH) agonists and antagonists block the production of estrogen and thereby prevent the stimulation of ovaries. As a result, artificial menopause is induced. Danazol is taken orally and helps in the halting of menstruation. GnRH analogues have been largely replaced by danazol for their lesser side effects. Hormonal and physical contraceptive method. Hormonal contraceptives are primarily used in the treatment of endometriosis to hinder monthly accumulation of endometrial tissues. In less serious cases birth control pills, patches, IUD and vaginal rings have the potential to alleviate or even prevent pain.

2.12.3 Surgery

Removal of endometrial tissues outside the uterus. It is an invasive treatment procedure and is performed as a last resort if the other treatments are ineffective. Conservative surgery: Surgery performed on prospective pregnant women or women with constant severe pain. This surgery aims to eliminate endometrial tissues present outside the uterus while conserving the structure and function of the reproductive organs.

2.12.4 Laparoscopy:

Endometrial deposits can be viewed and removed using laparoscopy which is minimally invasive. The removal of endometrial tissue is performed by vaporization by making small incisions in the abdomen.

2.12.5 Hysterectomy:

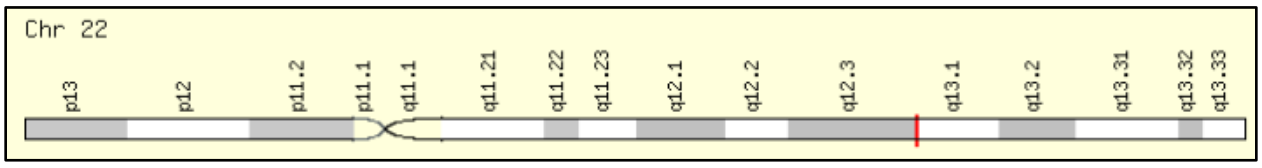
It is recommended as a last resort as it involves in the permanent removal of ovaries, cervix and the uterus. Rigorous counselling sessions are recommended prior to surgery as the woman would be rendered sterile after the procedure and is irreversible.

2.13 ROLE OF INTERLEUKIN -2

Interleukins, also known as IL are a type of cytokine that were initially thought to be produced only by leukocytes but have been later found to be expressed by several other cells of the body. Interleukins have the vital role in the activation and differentiation, proliferation, maturation,

migration and adhesion of cells related to immunity. ILs additionally have pro and anti-inflammatory properties and hence controls development, differentiation and activation of cells during an immune response. A main characteristic of ILs is its ability to elicit chemical reactions in cells and tissues through high-affinity binding to cell surface receptors[20]. The pro-inflammatory cytokines such as interferon-gamma (IFN-g) and interleukin-2 have a potential effect in the advanced stage of endometriosis Interleukin-2 (IL-2), binding with the specific interleukin-2 receptor (IL-2R), is one of the well-known cytokines due to the stimulating action on the proliferation and differentiation of B cells, T cells and non-specific cytotoxic cells including natural killer and lymphokine-activated killer cells [21].

Figure 6: Structure of Gene



Source: https://www.genecards.org/cgi-bin/carddisp.pl?gene=IL2RB

3.REVIEW OF LITERATURE

Jenkins S*, et al*. endometriosis is a chronic gynecologic disease characterized by the development and presence of histological elements such as endometrial glands and stroma outside of the uterine cavity. Chronic pelvic pain and infertility are the two most common clinical manifestations of the disease. Endometriosis lesions can be found in a variety of locations, with the ovaries being the most commonly involved focus of the disease, followed by the posterior broad ligament, anterior cul-de-sac, posterior cul-de-sac, and uterosacral ligament.

Akdis M, *et al*. Interleukins, A main characteristic of ILs is its ability to elicit chemical reactions in cells and tissues through high-affinity binding to cell surface receptors[31] .The pro-inflammatory cytokines such as interferon-gamma (IFN-g) and interleukin-2 have a potential effect in the advanced stage of endometriosis Interleukin-2 (IL-2), binding with the specific interleukin-2 receptor (IL-2R), is one of the well-known cytokines due to the stimulating action on the proliferation and differentiation of B cells, T cells and non-specific cytotoxic cells including natural killer and lymphokine-activated killer cells.

Gyoung Hoon Lee*, et al*. performed a study to investigate the potential association of the C627T polymorphism in the interleukin-2 receptor b gene (IL-2R b) with the risk of endometriosis in Korean women. In this study they found that there was no statistically significant difference in the frequency of the IL-2R b C627T polymorphism between the endometriosis patients and the controls or in the T allele Frequencies. Even when the endometriosis cases were subdivided into stages III and IV, no statistically significant

differences in genotype distributions or allele frequencies were observed among the three groups. They concluded in contrary to the recent data reported in a Taiwanese population, and results suggest that the C627T polymorphism of the IL-2R b gene may not be associated with the risk of endometriosis in the Korean population [32].

X.-Q. WANG, *et al*. performed a study investigate the relationship between single nucleotide polymorphism (SNP), as well as serum levels of interleukin 2 (IL-2) and interleukin 6 (IL-6) in patients with endometriosis (EMT) and disease susceptibility the difficulty in the detection and diagnosis of EMT may result in a considerable probability of delayed diagnosis. Meanwhile, no reliable laboratory bio marker has been identified to help diagnose this disease. As a result, a special observation on highly susceptible population is the best prevention and treatment method for EMT. He concluded that IL-2 rs11575812 (T>C) TT genotype, rs2069772 (A>G) AG genotype, and rs2069762 (T>G) GG genotype increase the risk of EMT, which are correlated with the serum levels of IL-2 and IL-6 [33].

Yao-Yuan Hsieh, *et al.* the study was about Interleukin-2 receptor (IL-2R)-627*C homozygote but not IL-12R1 codon 378 or IL-18 105 polymorphism is associated with higher susceptibility to endometriosis. The effects of IL-12 and IL-8 on endometrial cell survival varied according to the disease state and the concentration of the cytokines. He concluded that, IL-2R-627*C homozygote and C allele are associated with higher susceptibility to endometriosis. In contrast, the IL-12R1 codon 378 C/G and IL-18 105 A/C gene polymorphisms are not related to the susceptibility to endometriosis. This suggested that IL-2R gene polymorphisms may be the candidate genetic markers in the susceptibility to endometriosis and provided valuable insight into the pathogenesis of endometriosis [34].

4.AIM AND OBJECTIVES

4.1 AIM

To investigate the association of interleukin 2 (IL-2) gene polymorphism related to risk of endometriosis.

4..2 OBJECTIVES

1. Identify the role of interleukin -2 (IL-2) gene polymorphism in endometriosis.

2. To compare the frequencies of interleukin -2 (IL-2) polymorphism between the cases and control.

3. To estimate a relative risk in the study population.

The present case-control study was approved by the Institutional Ethics Committee of Sri Ramachandra Institute of Higher Education and Research (Deemed to be University) (CSP/22/MAR/106/95) (Annexure 1)

5. SAMPLES AND SUBJECTS

5.1 STUDY POPULATION

The present case-control study is the comparison of endometriosis and the control group of fertile women. The variations between the case and control group have been used to calculate the relative risk. About 2-3ml of peripheral blood was collected after explaining the details of the study and obtaining the informed consent. The samples were collected in EDTA vacutainer and stored at $4^{o}C$

5.2 CASE SAMPLES

The study comprised of 30 clinically confirmed endometriosis patients. The patients were recruited from the Department of Obstetrics and Gynecology, Sri Ramachandra Institute of Higher Education and Research (Deemed to be University), Chennai. Relevant clinical data were collected from all the patients. All the samples were collected after explaining the processes that is going to be performed with their sample and getting their sign in the Patient Consent form (Annexure 2).

INCLUSION CRITERIA:

Dysmenorrhea, pelvic pain, ectopic pregnancy and diagnosis of endometriosis was made on the basis of laparoscopic findings. Medical record of the patients will be referred to rule out abnormalities, infections and uterine abnormalities.

EXCLUSION CRITERIA:

Patients with inflammatory bowel disease, pelvic inflammatory disease, urinary tract infection was excluded from study. Patients who are unwilling to participate in the study were not included as well.

5.3 CONTROL SAMPLES

The study comprised of 30 female participants with a minimum of at least one child born with no history of endometriosis or endometriotic lesions.

INCLUSION CRITERIA:

Fertile women with regular menstrual cycle, women had normal vaginal deliveries, as well as no family history, clinical symptoms or diagnostic evidence of endometriosis

EXCLUSION CRITERIA:

Women with past or present endometriotic lesions, cysts, PCOS, inflammatory disorders or gynecological disorders

6. MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 GENOMIC DNA ISOLATION FROM PERIPHERAL BLOOD:

6.1.1.1 Stock solutions

TRIS-HCL – 1M (PH 7.5):

12.11 g of Tris-base was dissolved in 80ml of double distilled water and the pH was adjusted to 7.5/8.0 with concentrated HCl. The final volume was made up to100ml with distilled water and sterilized by autoclaving.

POTASSIUM CHLORIDE (KCl) – 1M:

7.45g of Potassium Chloride was added to 80 ml of double distilled water, after complete dissolution of the salt, the final volume was made up to 100ml and sterilized by autoclaving.

MAGNESIUM CHLORIDE ($MgCl_2$) – 1M:

40.6g of Magnesium Chloride was dissolved in 80 ml of double distilled water, after complete dissolution of the salt, the final volume was made up to 100ml and sterilized by autoclaving.

EDTA DISODIUM SALT – 0.5M (PH 8.0):

18.6g of di-Sodium EDTA was dissolved in 80ml of double distilled water and Sodium Hydroxide pellets were added to dissolve the salt completely.1N HCl was added to bring pH to 8.0. The final volume was made up to 100ml with double distilled water and sterilized by autoclaving.

SODIUM CHLORIDE (NaCl) – 5M:

29.2g of Sodium Chloride was added to 80ml of double distilled water and warmed to assist dissolution. The final volume was made up to 100ml with doubledistilled water and the solution was sterilized by autoclaving.

SODIUM DODECYL SULPHATE (SDS) – 10% (PH 7.0):

10g of SDS was dissolved in 80 ml of sterile double distilled water gently by slowmixing to avoid frothing. The solution was kept in a water bath at 65% to assist complete dissolution.The final volume was made up to 100ml and the solution was filter sterilized.

TRITON X-100:

Commercially available Triton X-100 was used.

ABSOLUTE ETHANOL:

Absolute Ethanol was commercially produced and stored in the refrigerator.

ETHANOL – 70%:

70ml of absolute ethanol was made up to 100ml with distilled water and stored in4°C.

6.1.1.2 Working Solution

RED CELL LYSIS BUFFER (RCLB) (Low salt buffer)

For 100 ml of RCLB - 1 ml of Tris buffer (1M). 1ml of Mgcl2 (1M), 1ml of KCl (1M), 0.4ml of Di-sodium EDTA (0.5 M), was used and made up to 100ml with sterile double distilled water and stored at room temperature (25°C). From the stock of the reagents, the working Solutions were prepared freshly prior to use.

Table 2: Red cell lysis buffer (RCLB)

REAGENT	STOCK CONCENTRATION	WORKING CONCENTRATION
Tris buffer	1M	10mM
KCl	1M	10mM
MgCl2	1M	10mM
Di-sodium EDTA	0.5M	2mM

Nucleated cell lysis buffer (NLB)

This buffer is also termed as White Blood Cell Lysis Buffer or high salt buffer.

For 100ml of NLB- 1ml of Tris buffer (1M), 1ml of MgCl2 (1M). 1ml of KCl (1M), 8ml of Nacl (5M), 0.4ml of Di-Sodium EDTA (0.5M), was used and made up to 100ml with Sterile double distilled water and stored at room temperature (25°C). from the stocks of the Reagents working solutions were freshly prior to use to reach the working

concentrations As listed below.

Table 3: Nucleated cell lysis buffer (NLB)

REAGENT	STOCK CONCENTRATION	WORKING CONCENTRATION
Tris buffer	1M	10mM
KCl	1M	10mM
MgCl2	1M	10mM
NaCl	5M	400mM
Di-sodium EDTA	0.5M	2mM

6.1.2 Qualitative analysis of DNA

6.1.2.1 Agarose Gel Electrophoresis

Reagents and Buffer preparation

AGAROSE

Commercially available Agarose was used.

TAE Buffer (Tris-acetate EDTA Buffer) – 50X (pH 7.2):

242 gm of tris base and 14.6 gm of disodium EDTA were dissolved in sterile double distilled water.Using glacial acetic acid, the pH was adjusted to 7.2. The final volume was made up to 1000 ml and sterilized by autoclaving. The solution was stored in a clean sterile reagent

bottle at room temperature (25°C). From this, 20ml was taken and Made up to 1000ml using sterile distilled water to make 1x TAE buffer.

Ethidium Bromide (10mg/ml):

To 1ml of sterile double distilled water, 10 mg of Ethidium Bromide was added and mixed well for complete dissolution of the dye. the stock solution was storedin in air tight containers wrapped with aluminum foil.

DNA Sample Loading Dye (6X):

Table 4: Components of Loading dye

Glycerol	18.72ml
EDTA 0.5M	0.6ml
Xylene cyanol (0.12%)	60mg
Bromophenol blue (0.12%)	24mg

All components were mixed in 30.68ml of sterile double distilled water and stored at room temperature.

DNA Ladder (100bp):

1. **Stock ladder:** Commercially available from GeNei (100 µg)

2. Working ladder:

Table 5: DNA Ladder

Stock ladder	10µl
Loading dye	20 µl
Sterile water	30 µl

6.1.3 POLYMERASE CHAIN REACTION (PCR)

All the reagents for PCR reaction were commercially procured. The reagents required forPCR are as follows:

- 1x PCR Buffer
- dNTP mix
- Taq DNA polymerase
- Sterile water
- Template DNA
- Primers (forward and reverse)

The primers were obtained as lyophilized powder and reconstituted in 100 µl Nuclease- free sterile double distilled water. Further dilutions were performed to obtain working concentrationsof the primers. The forward and reverse primer Sequences are given in table.6

Table 6: Primer Sequence

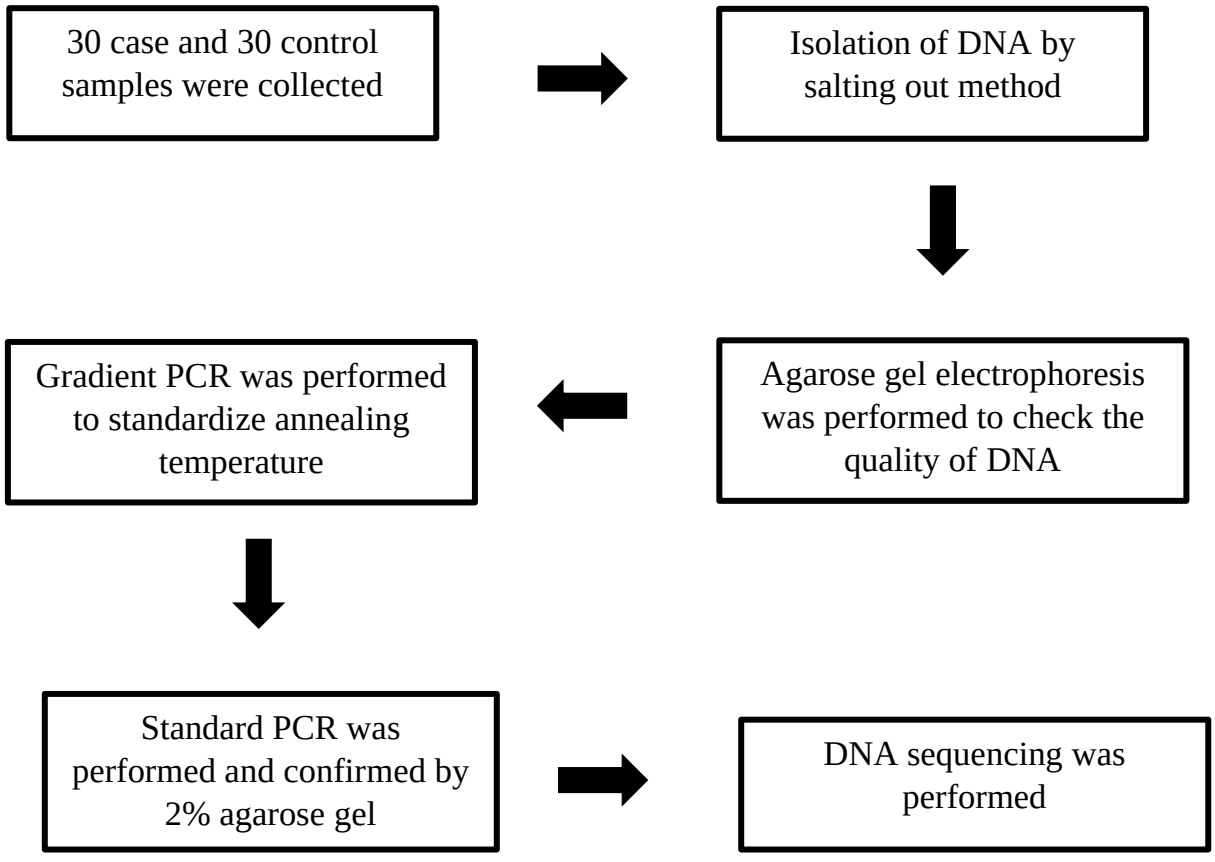
GENE	PRIMER	SEQUENCE (5' – 3')	LENGTH
Interleukin-2	Forward	AAGGACACCATTCCGTGGCT	20
	Reverse	CCGGTGTTCCTGCAGTTGAT	20

6.1.4 DNA SEQUENCING

Followed by cycle sequencing, the microtiter plate is subjected to DNA sequencing.

- RR Mix (big dye termination v3.1)
- Hi-Di formamide
- EDTA
- Sodium acetate 3M

EXPERIMENTAL DESIGN



6.2 METHODOLOGY

6.2.1 Isolation of high molecular weight genomic DNA from peripheral blood by salting out method

Blood sample collected from endometriosis patients and controls in EDTA vacutainers was used for isolation of High molecular weight genomic DNA by Salting Out Method.

Red blood cell lysis (Non-Nucleated cell lysis)

1. To 2ml of blood sample, double the volume of RCLB and 0.1% of triton X-100 (5-6 drops) was added and gently mixed
2. The content was then incubated at 37°C in water bath for 10 minutes
3. Following incubation, the samples were centrifuged at 2500 rpm for 15 minutes at 4°C
4. The supernatant was discarded and the pellet was resuspended in 10ml of RCLB and mixed well
5. The sample was then centrifuged at 2500 rpm for 15 minutes at 4°C
6. The wash was repeated until white pellet was obtained

White blood cell lysis (Nucleated cell lysis)

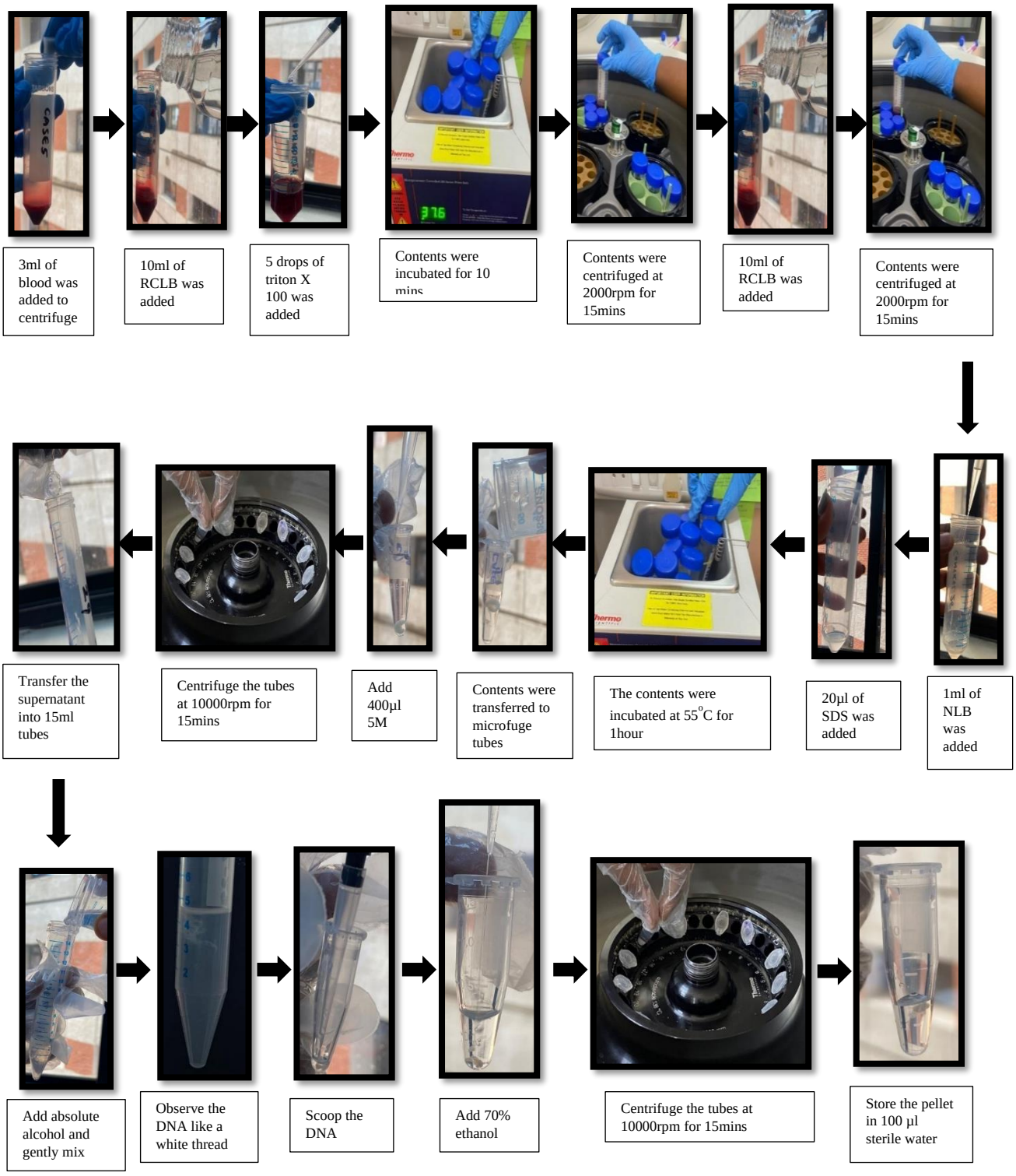
7. To the white pellet, 1ml of NLB was added and suspended well
8. 20µl of 10% SDS was added and incubated in water bath at 55°C for 60 minutes

Purification and precipitation of DNA

9. After incubation, the contents were then transferred to 2ml sterile microfuge tube
10. To the content, 400 µl of NaCl was added and mixed well
11. It was then centrifuged at 10000 rpm for 15mins at 4°C

12. The supernatant was then transferred to 15ml centrifuge tube and double the volume of absolute ethanol was added. The contents were then gently mixed by inverting the tube to precipitate the DNA.
13. The precipitated DNA was scooped out into 1.5ml sterile microfuge tube and 200µl of 70% ethanol was added. The mixture was centrifuged at 2000rpm for 5 minutes
14. The supernatant was decanted and the DNA pellet was air dried.
15. The pellet was then dissolved in 100µl of sterile water and stored at 4°C for further experimental use.

Figure 7: Isolation of genomic DNA by salting out method



6.2.2 QUALITATIVE AND QUANTITATIVE ANALYSIS OF DNA

6.2.2.1 Agarose gel electrophoresis

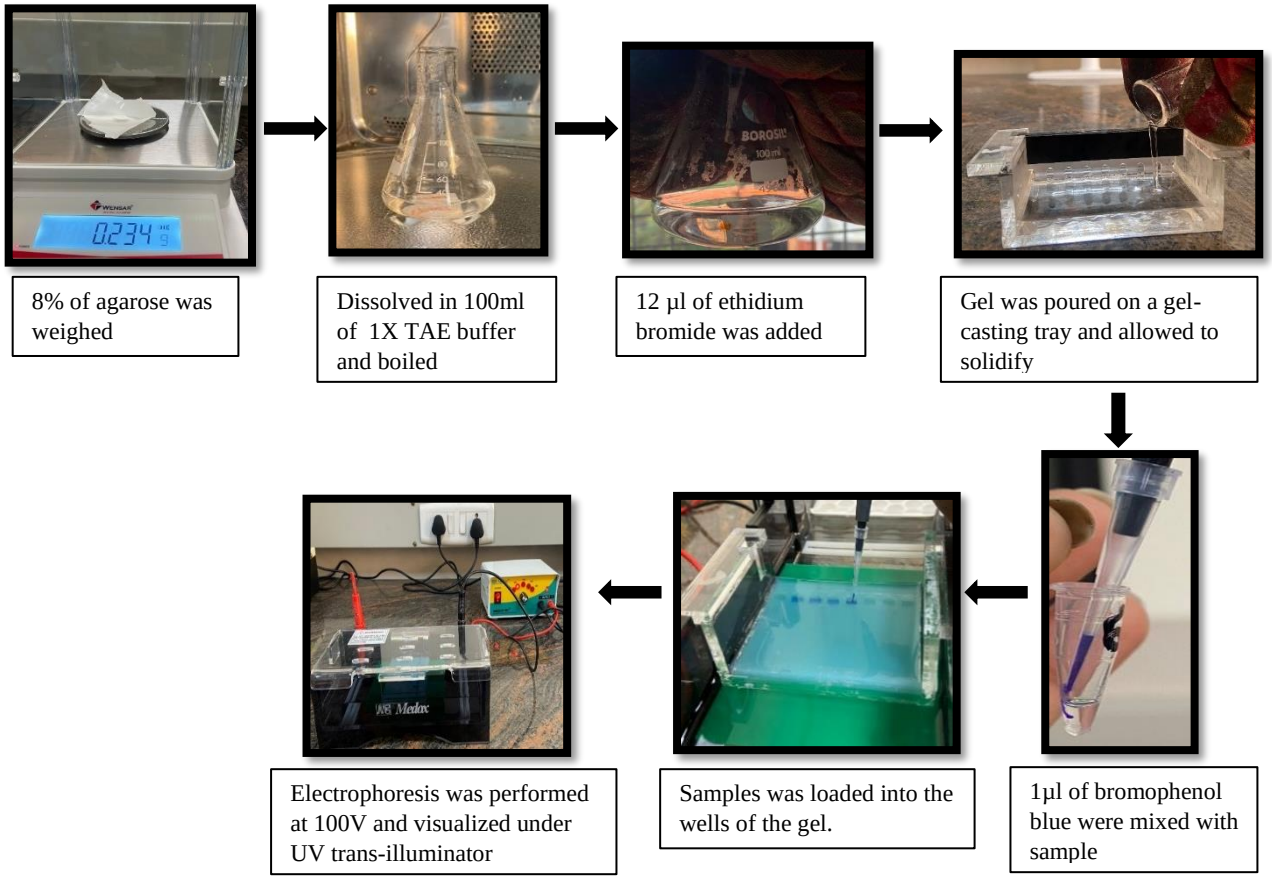
The quality of the DNA was checked using 0.8% agarose gel

1. 0.8g of agarose was weighed and dissolved in 100ml 1X TAE buffer by boiling.
2. 12 µl of ethidium bromide was added to the molten agarose for visualization of the genomic DNA under UV light.
3. The gel was poured on a gel-casting tray and allowed to solidify.
4. After solidification, the gel was placed in an electrophoresis tank with 1X TAE buffer.
5. The samples (5µl each) were mixed with 1µl of bromophenol blue (loading dye) and loaded into the wells of the gel.
6. The electrophoresis was performed at 100V till the dye reaches near the end of the gel and visualized under UV trans-illuminator.

6.2.2.2 Spectrophotometric Analysis

The quality of the DNA was checked by spectrophotometric analysis. 1µl of the DNA sample was taken and placed on the NANODROP spectrophotometer. TheOD was read at 260nm and 280nm. The concentration of the sample wasdetermined based on the OD value at 260nm.

Figure 8: Qualitative analysis of DNA



6.2.3 POLYMERASE CHAIN REACTION (PCR)

6.2.3.1 Standardizing annealing temperature by Gradient PCR

1. The annealing temperature was standardized by performing agradient PCR.
2. A master mix was prepared for 12 samples.
3. It was aliquoted into 12 PCR tubes with the final volume of 20 µl each.
4. Each lane was attributed a different temperatures as per the gradient of 55±65°C.
5. On completion of PCR reaction, the products were run on 2% agarose gel

to Determine the optimum annealing temperature.

6. The annealing temperature was found to be 57°C for both gene polymorphism primers.

5.2.3.2 In-vitro amplification of IL-2Rb (C627T) gene by PCR

A PCR Master mix was prepared for the samples in a microfuge tube as shown in table

1. Equal volume of the master mix was aliquoted into all the PCR tubes.
2. To this, 2 µl of the template DNA was added to all the PCR tubes.
3. The tubes were placed in a polymerase chain reactor and the reaction was set For a span of 1 hour 40 minutes for 35 cycles as per program.
4. The obtained PCR product was validated by performing agarose gel electrophoresis by using a 2% agarose gel and visualized by using UV trans-illuminator.

Table 7: PCR Reaction Mixture

CONTENTS	STOCK CONCENTRATION	WORKING CONCENTRATION	VOLUME PER TUBE (µL)
Nuclease freewater			14.7
Taq polymerase	3U/µl	10U/µl	0.5
10x PCR Buffer	10x	1x	2
dNTP mix	10mM	0.2mM	0.4
Forward primer	10nM	30-50pM	0.2
Reverse primer	10Mn	30-50pM	0.2
Template DNA		50-100ng	2
Final volume			20

Table 8: PCR program

S.NO	STEPS	TEMPERATURE	DURATION
1	Initial denaturation	94°C	5 minutes
2	Denaturation	94°C	60 Seconds
3	Annealing	55°C	45 Seconds
4	Extension	72°C	45 Seconds
Steps 2-4 are repeated for 30 cycles			
5	Final extension	72°C	7 minutes
6	Hold	4°C	

6.2.4 DNA SEQUENCING

The master mix of Big Dye, primer and PCR water were prepared and then dispensed equally into MicroAmp96 well plate. The pcr products were then added to the wells and subjected to sequencing PCR reaction. Sequencing PCR was carried out in the Gene Amp for 9600 thermal cycler. The reaction conditions were repeated for 30 cycles and subjected to sequencing by an ABI 3730 automated DNA sequencer (Applied Biosystem).

Table 9: Sequencing PCR Reaction

REAGENTS	VOLUME(µL)
Big dye	0.25
Forward or Reverse primer	1.0
PCR water	6.0
PCR product (10ng/µl)	1.0
Sequencing buffer	1.75

6.2.5 Purification of cycle sequencing product

1. Master mix I: 10µl of distilled water +2µl 125 Mm EDTA per sample
2. Master mix II: 2µl of 3m sodium acetate (PH 4.6) +50µl of ethanolper sample.
3. 12µl and 52µl of master mix 1 and 11 was added, mixed welland incubated at room temperature for 10 minutes.

4. It was centrifuged at 4000rpm for 20 minutes at 30°C.
5. The supernatant was discarded and to it 70% ethanol was added.
6. The plate was then sealed with septa and loaded into thesequencer.

6.2.6 STATISTICAL ANALYSIS:

The expected genotypes and allele frequencies were calculated for both the cases and controls. These frequencies were used to test if the population followed Hardy - Weinberg equilibrium (A chi-square x^2 test was used to examine the difference in genotype distribution between case and control). The interaction between interleukin 2 receptor beta genotypes was evaluated by calculating the odds ratio for mutant genotypes as compared to the wild type. 95% confidence intervals (CI) were calculated to estimate the risk of different genotypes. All statistical analyses were done by using SPSS (statistical package for the social sciences)

7. RESULTS

This study population consisted of 30 cases with confirmed diagnosis of endometriosis and 30 age matched control. High molecular weight genomic DNA was isolated by Salting out method for 30 case and 30 control samples. (Figure 10 A.). The quality of the DNA was checked using nanodrop (Figure 10 B.) The quality of the DNA was checked in 0.8% agarose gel electrophoresis (Figure 10 C.) The annealing temperature for the primers was optimized using gradient PCR and the optimum temperature was found to be 60°C for both the genes. The standard PCR product was obtained and was checked for amplifications by using 2% agarose gel electrophoresis (Figure 11).

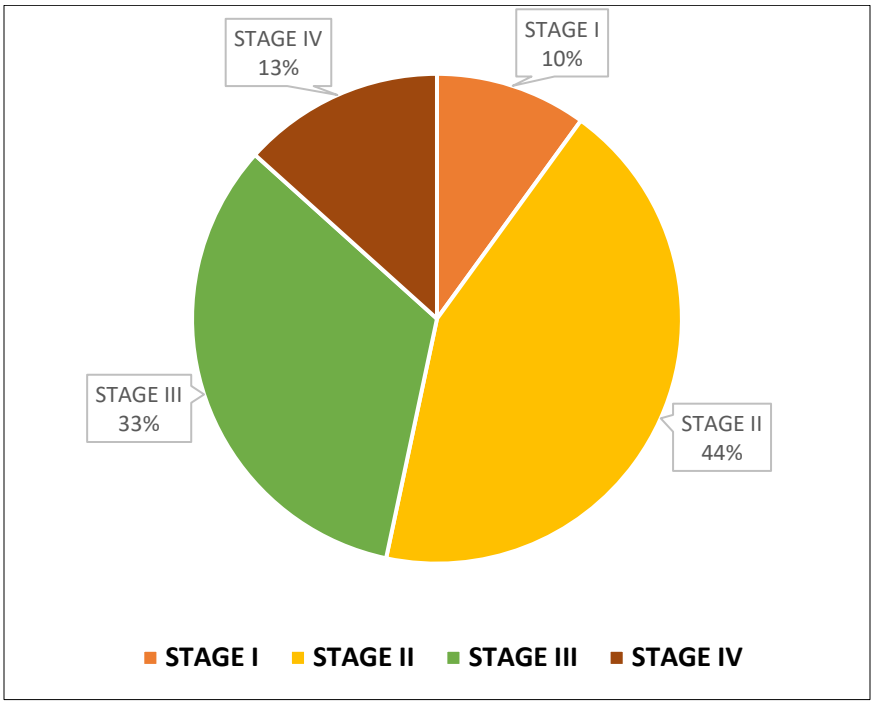


Figure 9: Stages of Endometriosis

DNA Sequence Analysis

The products obtained after the standard PCR were sequenced in order to identify the genotypes. All the samples were analyzed for base calling and the quality index. The genotypes were assigned by using the FINCH TV software. The genotypes were observed. The three possible genotypes which were observed in the present study are given in table

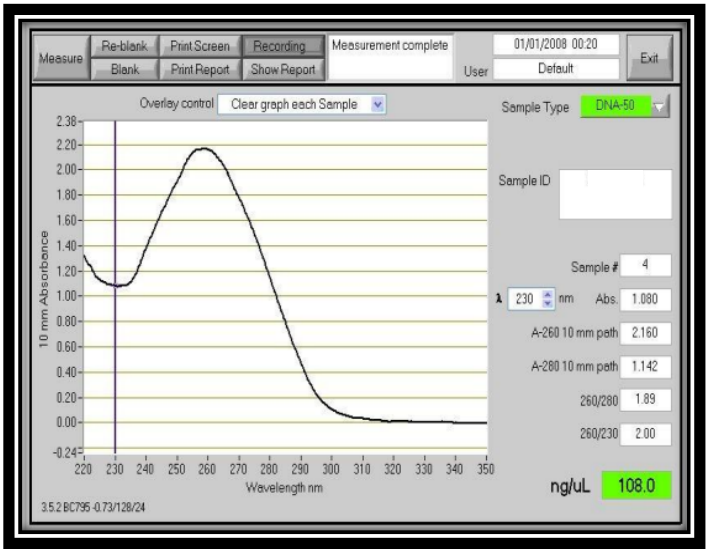
Table 10: Genotypes observed in present study

CC	Wild type
TT	Mutant
CT	Heterozygous

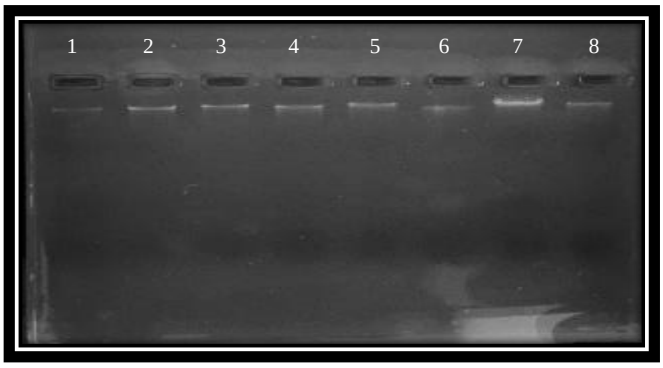
Figure 10: Isolation, Quantitative and Qualitative analysis of the DNA

A.

B.

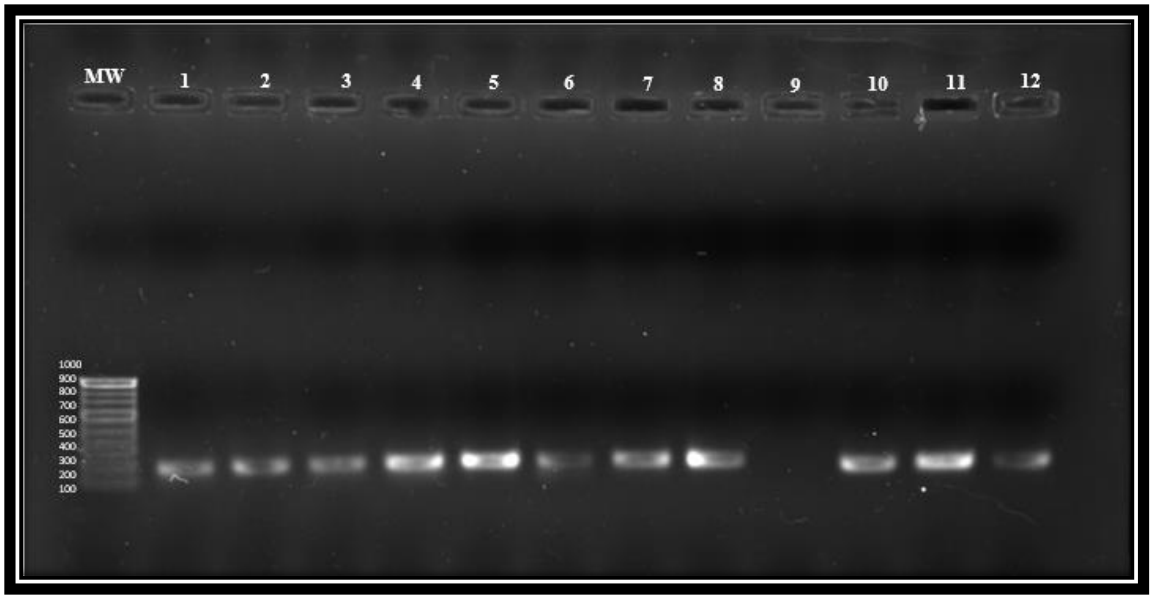


C.



A: Isolated DNA The genomic DNA was extracted from peripheral blood using the Standard Salting out Method. **B: DNA concentration**: Checked with nanodrop spectrophotometry (ND1000 spectrophotometer) and the resultant 260/280 ratio, 260/230 ratio and the concentration of the given DNA sample was found to be 1.89, 2.00 and 108 ng/µL respectively. **C: DNA quality:** The quality of DNA extracted from peripheral blood lymphocytes (lane 1 to 8) was determined using 0.8% agarose gel electrophoresis and was observed to be good. The intensity of the band depends on the concentration of DNA.

Figure 11: PCR Amplification of the DNA samples confirmed by 2% agarose gel electrophoresis



Lanes MW – DNA ladder 1-12 showing 167 bp amplicon in all samples

Figure 12: CC Genotype observed in the study

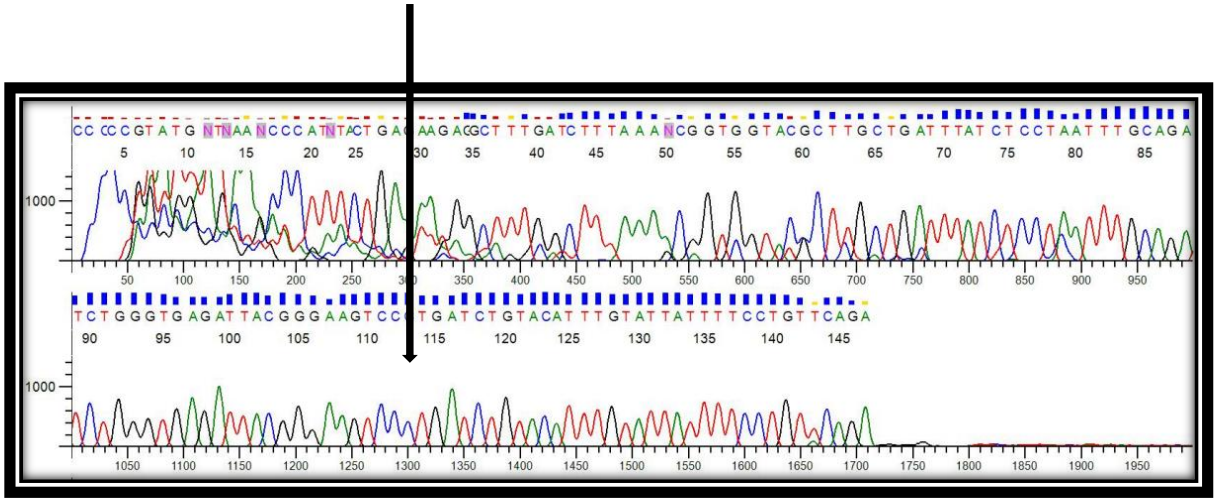


Figure 13: CT Genotype observed in the study

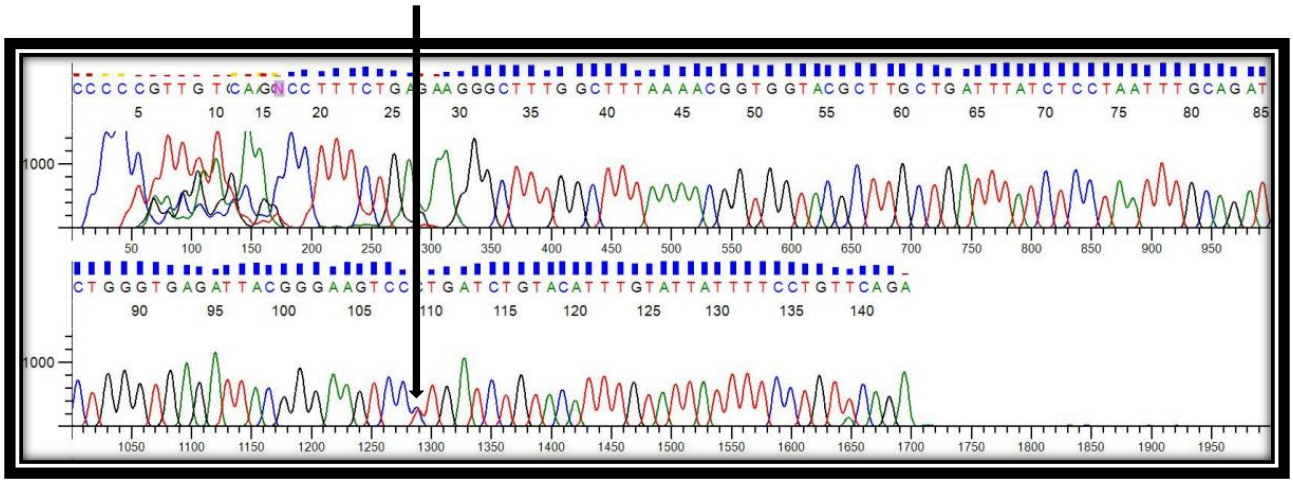


Figure 14: TT Genotype observed in the study

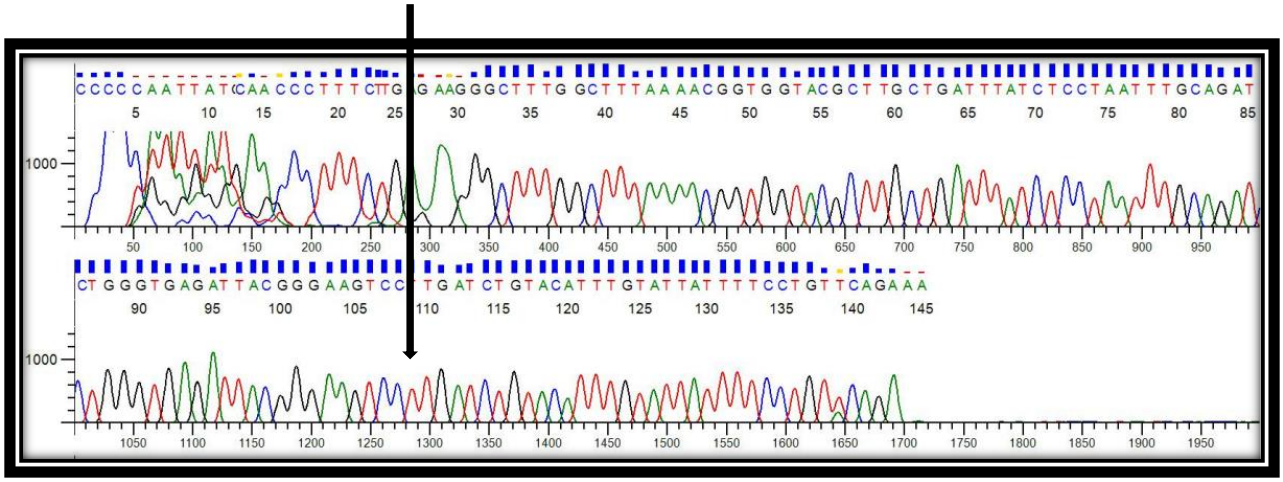


Table 11: Genotype distribution of interleukin- 2 receptor beta gene (C627T) polymorphism in cases and controls

Interleukin- 2 Receptor beta gene(C627T) polymorphism		GENOTYPES			ALLELE FREQUENCY		*P* VALUE
		CC	CT	TT	C	T	
CONTROLS (n= 30)	Observed	20	7	3			0.2308 (Non-significant)
	Expected	18.40	10.18	1.40	0.783	0.216	
CASES (n= 30)	Observed	20	8	2			0.6592 (Non-significant)
	Expected	19.2	9.6	1.2	0.8	0.2	

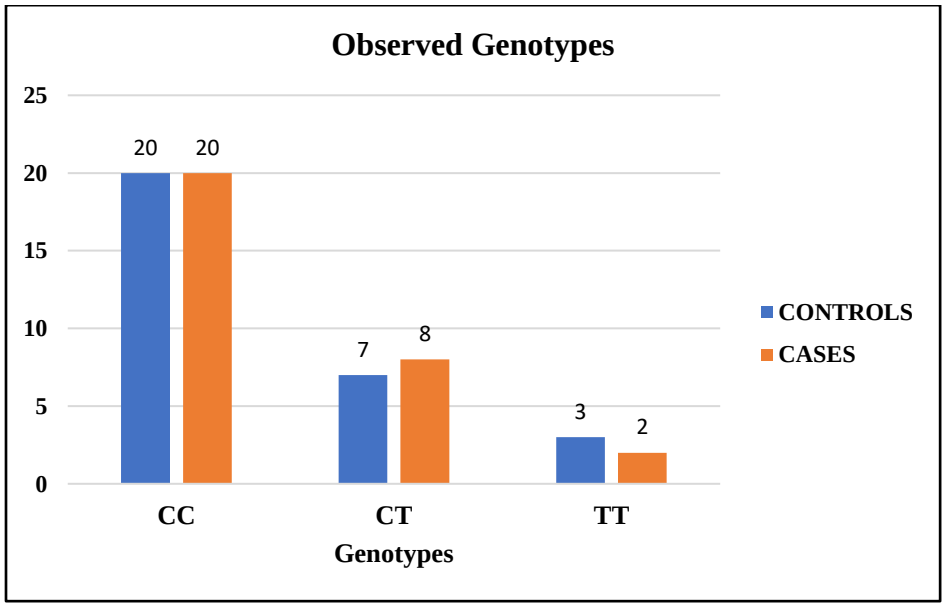


Figure 15: Graphical Representation of Observed Genotypes

The allele and genotype frequencies of the Interleukin 2 Receptor Beta gene (C627T) polymorphism were calculated. The expected genotype frequencies were calculated and were found to follow Hardy Weinberg equilibrium among cases and controls. The analysis of sample revealed that TT (homozygous variant) was found to be 6.6% in Cases and 10% in Controls. But the CT (heterozygous variant) was found to be higher in cases (26.6%) than in controls (23%). The wild type CC was found to be equal in both controls (67%) and cases (67%). The frequency of T allele was 0.152 in controls and 0.182 in cases. The frequency of C allele was 0.7 in controls and 0.8 in cases. The p value of cases and controls shows there is no significance between cases and controls in present study.

Table 12: Analysis of odds ratio and confidence interval of interleukin 2 receptor beta (C627T) gene polymorphism in cases and controls

Genotypes IL-2Rb gene (C627T) polymorphism	**Control (n= 30)**	**Case (n= 30)**	**Odds Ratio (OR)**	**Confidence Interval (CI)**	***P* Value**
CC	**20**	**20**			
CT	**7**	**8**	**0.96**	**0.34to 2.68**	**0.9445 (NS)**
TT	**3**	**2**	**0.66**	**0.10 to 4.42**	**0.6747 (NS)**

With CC as reference genotype, the OR (95% CI) for heterozygosity and homozygosity of C allele was determined. The results revealed no significant increase in the frequency of CT allele among the cases and controls (OR- 0.96; 95% CI: 0.34 -2.68). Similarly, the TT allele frequency among the cases and controls was also non-significant (OR-0.66; 95% CI: 0.1 – 4.42). the results suggestthat study groups were statistically non-significant.

8.DISCUSSION

The purpose of this study was to evaluate whether the C627T polymorphism of the IL-2R b gene may be associated with the risk of endometriosis in the Indian population. SNPs in several members and regulatory components of the interleukin family, including IFN-g, IL-1 b, IL-4, IL-10, IL-1 receptor 1, IL-1 receptor antagonist, and IL-2R, have recently been investigated. Although the genetic contribution of the interleukin family to endometriosis has been suggested, several IL-4, IL-6 and IL-1 b polymorphisms were reported to show no association with endometriosis [35]. However, a study on 2005 showed an association between an IL-2R b polymorphism and endometriosis in Taiwanese women [36].

Among the various polymorphisms of IL2, C627T may be one of the least researched SNPs, hence selected in this Indian population-based association study. In this case –control study, we have shown that the C627T polymorphism in the IL-2R b gene was not associated with the risk of endometriosis. These findings contradict previous findings by Hsieh YY, who found that the non-CC genotype may have some protective function against severe endometriosis (OR 0.32; 95% CI 0.18 –0.60) [36].This inconsistency may be explained in part by the fact that the genotype frequencies observed in Hsieh's study differed from the assumption of random mating (C/C of 18, C/T of 111, and T/T of 30). Non-random mating, mutations, limited population size, selection, and other factors could all contribute to the HWE violation in the controls [37]. In genetic studies, the assessment of HWE is used as an initial quality control step. In general, genotyping errors are known to cause distortions in genotype distributions and deviations from HWE. When disease prevalence is low, it is crucial that control groups exhibit the HWE state in order to represent the general population [38].

Furthermore, discrepancies in the data reported by these two studies could be due to ethnic differences. Endometriosis pathogenesis may be influenced by subtle genetic differences between ethnicities.

Recruiting controls from the older population seems to make the genotype of our controls more homogenously distributed by reducing the number of patients likely to develop endometriosis as much as clinically possible [39].

It is also evident that the lack of reproducibility is related to sample size. According to Salanti et al, the most evident OR (1.1–1.5) between SNPs and a complex trait disease can be obtained with a sample size of at least 1000 subjects. Given that our study did not have such an ideal sample size, future meta-analysis with multiple studies will be required to show the true effect magnitudes.

9.CONCLUSION

In conclusion, we found that the IL-2R b gene C627T polymorphism is not associated with advanced stage endometriosis in an Indian population. To the best of our knowledge, no study has looked into the relationship between the IL-2R b polymorphic loci and other complex diseases. More research is necessary to determine the role of IL-2R b in the pathogenesis of endometriosis, as well as to confirm the possible association of the IL-2R b gene polymorphism with endometriosis susceptibility.

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ANNEXURE I: IEC CLEARANCE LETTER

SRI RAMACHANDRA
INSTITUTE OF HIGHER EDUCATION AND RESEARCH
(Deemed to be University)

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ETHICS COMMITTEE FOR STUDENTS PROJECTS

Chairperson:
Dr. Radha Madhavan
Member Secretary:
Dr.S.Aruna
Members:

Dr.Ganesh Venkataraman	Dr.Arathi Ganesh
Dr. Linga Devi	Dr.M.Haripriya
Dr. Suresh K. Rayala	Mr.S.Rethinakumar - Legal Consultant
Dr.Krishnendu Mukhopadhyay	Ms.Anitha Jayaraj - Legal Consultant
Dr. Thilakavathy	Mrs.Manjula. R – Lay Person

31.03.2022

To
Ms. Akshaya. J
Ms. Nandhini. S
B.Sc (Hons) Biomedical Sciences (IV Year)
Department of Human Genetics
Sri Ramachandra Institute of Higher Education & Research (DU)

REF: CSP/22/MAR /106/95
SUB: A study to investigate the potential association of Interleukin-2 receptor B gene (IL-2R b) with risk of Endometriosis in Indian women.

.....

Thank you for submitting the clarifications. The Institutional Ethics Committee (for UG & Non-Med. PG Students), SRIHER (DU) approves the project.

You are advised to be familiar with ICMR guidelines on Biomedical Research in human beings and also to adhere to the Principles of good clinical practice.

- **You are required to inform the IEC when the study initiated and**
- **Submit the final report on the completion of study to the Committee for Students Proposals, SRIHER (DU).**

Dr.S.Aruna
Member Secretary
Note: Please quote CSP Reference number in all future communications

ANNEXURE II: INFORMED CONSENT

Title of the project: A study to investigate the potential association of the interleukin-2 receptor B gene (IL-2Rb) with risk of endometriosis in Indian women

Name of the Principle Investigator:

Nandhini.S and Akshaya.J

B.Sc. Biomedical Science IV year,

Department of Biomedical Sciences,

Sri Ramachandra Institute of Higher Education and Research,

Porur, Chennai

Description of the study:

This study deals with the association of C627T gene in endometriosis women. Identification of such Polymorphism as cause for endometriosis will help in better management and counselling of the patient. 3-5mL human peripheral blood sample will be collected in a sterile heparin vacutainer for the study. The collected blood sample will be used to study the gene polymorphism. The participation in this study for sample collection will require approximately 5-10 minutes of the volunteer's time.

Possible Risks to the participants:

There is no major risk for participants, because the study is based on blood samples which will be collected by the well-trained phlebotomist.

Possible Benefits to the participant:

There are no direct benefits to the participants.

Cost and Payments to the participant:

There is no cost for participation in this study. Participation is completely voluntary and no payment will be provided.

Confidentiality:

Information obtained in this study is strictly confidential. Your name will not be used in reporting of information in publications or conference presentations.

Participant's right to withdraw from the study:

You have the right to refuse to participate in this study, the right to withdraw from the study and the right to have your data destroyed at any point during or after the study, without penalty.

Voluntary consent by the participant: PARTICIPATION IN THIS STUDY IS COMPLETELY VOLUNTARY, AND YOUR CONSENT IS REQUIRED BEFORE YOU CAN PARTICIPATE IN THIS STUDY.

I have read this consent form (or it has been read to me) and I fully understand the contents of this document and voluntarily consent to participate in the study. All of my questions concerning this study have been answered. If I have any questions in the future about this study, they will be answered by the investigators listed below. I understand that this consent ends at the conclusion of this study.

Contact Address with phone number:

PI – from SRMC	Collaborator (if any) outside SRMC
Nandhini.S and Akshaya.J B.Sc. Biomedical Science IV year, Department of Biomedical Sciences, Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai Phone: 9551754305/9841413838	Not applicable

A copy of the participant/patient information sheet should be given to the participant for her/ his record.

[In case of illiterate participant, the information is explained and thumb impression is obtained, in the presence of an unrelated witness. Left hand thumb impression for male and right-hand thumb impression of female]

By signing this form, I agree to participate in this study. A copy of this form has been given to me.

Date:

Name:

Participant's signature:

Thumb impression:

Witness name:

Witness signature:

Certification of INFORMED CONSENT

I certify that I have explained the nature and purpose of this study to the above-named individual, and I have discussed the potential benefits of this study participation. The questions the individual had about this study have been answered, and we will always be available to address future questions.

Date: Signature of person obtaining consent

Name: Nandhini.S

Signature of PI

Name: Akshaya.J

Signature of PI

ஸ்ரீ இராமச்சந்திரா உயர் கல்வி மற்றும் ஆராய்ச்சி நிறுவனம்

விவரம் அறிந்து ஒப்புதல் கோரும் படிவம்

ஆராய்ச்சியின் தலைப்பு:

இந்தியப் பெண்களில் எண்டோமெட்ரியோசிஸ் அபாயத்துடன் இண்டர்லூகின்-2 ஏற்பி B மரபணுவின் (IL-2Rb) சாத்தியமான தொடர்பை ஆராய்வதற்கான ஒரு ஆய்வு

ஆராய்ச்சியாளரின் விலாசம்:

நந்தினி.சு மற்றும் அக்ஷயா.ஜெ

நான்காம் ஆண்டு, உயிர் மருத்துவ அறிவியல்

மனித மரபியல் துறை

ஸ்ரீ இராமச்சந்திரா மருத்துவ கல்லூரி மற்றும் ஆராய்ச்சி நிறுவனம்

சென்னை - 600116.

ஆராய்ச்சியின் விளக்கம்:

இந்த ஆய்வு எண்டோமெட்ரியோசிஸ் பெண்களில் C627T மரபணுவின் தொடர்பைக் கையாள்கிறது. எண்டோமெட்ரியோசிஸின் காரணமான பாலிமார்பிஸத்தை கண்டறிவது, நோயாளியின் சிறந்த மேலாண்மை மற்றும் ஆலோசனைக்கு உதவும். 3-5 மில்லிலிட்டர்

மனித புற இரத்த மாதிரி ஆய்வுக்காக ஒரு மலட்டு ஹெப்பரின் வெற்றிடத்தில் சேகரிக்கப்படும். சேகரிக்கப்பட்ட இரத்த மாதிரி மரபணு பாலிமார்பிஸத்தை ஆய்வு செய்ய பயன்படுத்தப்படும். மாதிரி சேகரிப்புக்கான இந்த ஆய்வில் பங்கேற்பதற்கு

தன்னார்வலரின் நேரத்தின் தோராயமாக 5-10 நிமிடங்கள் தேவைப்படும்.

பங்கேற்பாளருக்கு இடையூறு:

பங்கேற்பாளருக்கு இரத்தம் சேகரிக்கும் போது சிறு வலி தோன்றி சில நொடிகளில் மறைந்துவிடும்.

பங்கேற்பாளருக்கு நன்மைகள்:

இந்த ஆராய்ச்சியின் மூலம் பங்கேற்பாளருக்கு எவ்வித நேரிடை நன்மைகள் இல்லை. ஆனால் இந்த ஆய்வின் முடிவு சமுதாயத்திற்குப் பயன்படும்.

பங்கேற்பாளர் பணம் செலுத்துதல்:

இந்த ஆராய்ச்சிக்காக பங்கேற்பாளர் பணம் ஏதும் செலுத்த தேவையில்லை.

காப்பு தகவல்:

இந்த ஆராய்ச்சியில் பயன்படுத்தப்படும் பங்கேற்பாளரின் கோப்புகள் காப்புதகவலாய் பாதுகாக்கப்படும். மேலும் எதிர்கால பதிப்புகளிலோ அல்லது கருத்தரங்குகளிலோ உங்கள் பெயரைச் சேர்க்கமாட்டோம்.

பங்கேற்பாளரின் விலகல் உரிமை:

இந்த ஆராய்ச்சிக்குப் பங்கேற்பாளர்கள் விருப்பப்படவில்லை எனில் ஆராய்ச்சியில் இருந்து எந்தநேரத்திலும் விலகிக்கொள்ளலாம். இதற்கு பணம் ஏதும் செலுத்த தேவையில்லை.

சேமிப்பு தகவல்:

இந்த ஆய்விற்க்கு எடுக்கப்படும் தகவல்கள் பிற்காலத்தில் சேமிக்கப்படும்.

பங்கேற்பாறளரின் ஒப்புதல்:

இந்த ஆராய்ச்சியில் பங்கேற்கும் பங்கேற்பாளரின் முழு சம்மதத்துடன் ஒப்புதல் படிவம் பெறபடுகிறது.

இந்த படிவத்தை நான் படிக்கபெற்றும் (அல்லது நான் படித்தும்) முழு விவரங்களை விளக்கப்பட்டேன். இதன் மூலம் எல்லா விதமான சந்தேகங்களும் நீக்கப்பட்டன. மேலும் எதிர்காலத்தில் ஏதேனும் சந்தேகம் ஏற்படின் கீழே கொடுக்கப்பட்டுள்ள ஆராச்சியாளரை அணுகி நிவர்த்தி செய்துகொள்வேன்.

ஆராய்ச்சியாளரின் விலாசம்

நந்தினி.சு மற்றும் அக்ஷயா.ஜெ

நான்காம் ஆண்டு, உயிர் மருத்துவ அறிவியல்

மனித மரபியல் துறை

ஸ்ரீ இராமச்சந்திரா மருத்துவ கல்லூரி மற்றும் ஆராய்ச்சி நிறுவனம்

சென்னை – 600116.

இந்த ஒப்புதலை நான் கீழே கொடுக்கப்பட்டுள்ள கையொப்பத்தின் மூலம் உறுதி செய்கிறேன்.

பங்கேற்பாளரின் பெயர்: கையொப்பம்/பெருவிரல் ரேகை அச்சு: தேதி:

சாட்சியின் பெயர்: கையொப்பம்: தேதி:

தெரியப்படுத்தும் அனுமதி சான்றிதழ்

நான் மேலே குறிப்பிட்டுள்ள நபருக்கு இந்த ஆய்வின் தன்மை மற்றும் முக்கியத்துவம் குறித்து விலகினேன். நான் இந்த ஆய்வின் சாத்தியமுள்ள பலன்களைப பற்றி விளக்கினேன். பங்கேற்பளாரின் கேள்விகளுக்கு பதில் அளிப்பேன்.

தேதி:

ஆராய்ச்சியாளரின் பெயர்: நந்தினி.சு

கையொப்பம்:

கைப்பேசி எண்:

ஆராய்ச்சியாளரின் பெயர்: அக்ஷயா.ஜெ

கையொப்பம்:

கைப்பேசி எண்:

ANNEXURE III: QUESTIONNAIRE

BASIC QUESTIONS	YOUR RESPONSES
1. Name:	
2. Age/Sex	
3. Marital status	
4. Contact address:	
5. Date of your first menstrual period ever:	
6. Date of your most recent period:	
7. Duration of your period (in days):	
8. Type of flow (heavy, medium, light):	
9. Do you menstruate every 28 to 30 days,or are your cycles irregular?	☐Yes ☐No
10. What medications, birth control pills, hormones, and/or supplements that you take on a regular basis?	
11. Do you have painful bowel movements?	☐Yes ☐No □Sometimes
12. Do you have pelvic pain	☐Yes ☐No ☐Sometimes
13. Type of pain?	
14. Severity of pain?	Pain on a scale of 1-10: ____
15. Do you have unusual vaginal bleeding anytime during your cycle?	☐Yes ☐No
16. Do you experience painful urination orblood in urine at any time during your cycle?	☐Yes ☐No

INFORMED CONSENT

Title of the project: A study to investigate the potential association of the interleukin-2 receptor B gene (IL-2Rb) with risk of endometriosis in Indian women

Name of the Principle Investigator:

Nandhini.S and Akshaya.J

B.Sc. Biomedical Science IV year,

Department of Biomedical Sciences,

Sri Ramachandra Institute of Higher Education and Research,

Porur, Chennai

Description of the study:

This study deals with the association of C627T gene in endometriosis women. Identification of such Polymorphism as cause for endometriosis will help in better management and counselling of the patient. 3-5mL human peripheral blood sample will be collected in a sterile heparin vacutainer for the study. The collected blood sample will be used to study the gene polymorphism. The participation in this study for sample collection will require approximately 5-10 minutes of the volunteer's time.

Possible Risks to the participants:

There is no major risk for participants, because the study is based on blood samples which will be collected by the well-trained phlebotomist.

Possible Benefits to the participant:

There are no direct benefits to the participants.

Cost and Payments to the participant:

There is no cost for participation in this study. Participation is completely voluntary and no payment will be provided.

Confidentiality:

Information obtained in this study is strictly confidential. Your name will not be used in reporting of information in publications or conference presentations.

Participant's right to withdraw from the study:

You have the right to refuse to participate in this study, the right to withdraw from the study and the right to have your data destroyed at any point during or after the study, without penalty.

Voluntary consent by the participant: PARTICIPATION IN THIS STUDY IS COMPLETELY VOLUNTARY, AND YOUR CONSENT IS REQUIRED BEFORE YOU CAN PARTICIPATE IN THIS STUDY.

I have read this consent form (or it has been read to me) and I fully understand the contents of this document and voluntarily consent to participate in the study. All of my questions concerning this study have been answered. If I have any questions in the future about this study, they will be answered by the investigators listed below. I understand that this consent ends at the conclusion of this study.

Contact Address with phone number:

PI – from SRMC	Collaborator (if any) outside SRMC
Nandhini.S and Akshaya.J B.Sc. Biomedical Science IV year, Department of Biomedical Sciences, Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai Phone: 9551754305/9841413838	Not applicable

A copy of the participant/patient information sheet should be given to the participant for her/ his record.

[In case of illiterate participant, the information is explained and thumb impression is obtained, in the presence of an unrelated witness. Left hand thumb impression for male and right-hand thumb impression of female]

By signing this form, I agree to participate in this study. A copy of this form has been given to me.

Date:

Name:

Participant's signature:

Thumb impression:

Witness name:

Witness signature:

Certification of INFORMED CONSENT

I certify that I have explained the nature and purpose of this study to the above-named individual, and I have discussed the potential benefits of this study participation. The questions the individual had about this study have been answered, and we will always be available to address future questions.

Date: Signature of person obtaining consent

Name: Nandhini.S

Signature of PI

Name: Akshaya.J

Signature of PI

ஸ்ரீ இராமச்சந்திரா உயர் கல்வி மற்றும் ஆராய்ச்சி நிறுவனம்

விவரம் அறிந்து ஒப்புதல் கோரும் படிவம்

ஆராய்ச்சியின் தலைப்பு:

இந்தியப் பெண்களில் எண்டோமெட்ரியோசிஸ் அபாயத்துடன் இண்டர்லூகின்-2 ஏற்பி B மரபணுவின் (IL-2Rb) சாத்தியமான தொடர்பை ஆராய்வதற்கான ஒரு ஆய்வு

ஆராய்ச்சியாளரின் விலாசம்:

நந்தினி.சு மற்றும் அக்ஷயா.ஜெ

நான்காம் ஆண்டு, உயிர் மருத்துவ அறிவியல்

மனித மரபியல் துறை

ஸ்ரீ இராமச்சந்திரா மருத்துவ கல்லூரி மற்றும் ஆராய்ச்சி நிறுவனம்

சென்னை – 600116.

ஆராய்ச்சியின் விளக்கம்:

இந்த ஆய்வு எண்டோமெட்ரியோசிஸ் பெண்களில் C627T மரபணுவின் தொடர்பைக் கையாள்கிறது. எண்டோமெட்ரியோசிஸின் காரணமான பாலிமார்பிஸத்தை கண்டறிவது, நோயாளியின் சிறந்த மேலாண்மை மற்றும் ஆலோசனைக்கு உதவும். 3-5 மில்லிலிட்டர்

மனித புற இரத்த மாதிரி ஆய்வுக்காக ஒரு மலட்டு ஹெப்பரின் வெற்றிடத்தில் சேகரிக்கப்படும். சேகரிக்கப்பட்ட இரத்த மாதிரி மரபணு பாலிமார்பிஸத்தை ஆய்வு செய்ய பயன்படுத்தப்படும். மாதிரி சேகரிப்புக்கான இந்த ஆய்வில் பங்கேற்பதற்கு

தன்னார்வலரின் நேரத்தின் தோராயமாக 5-10 நிமிடங்கள் தேவைப்படும்.

பங்கேற்பாளருக்கு இடையூறு:

பங்கேற்பாளருக்கு இரத்தம் சேகரிக்கும் போது சிறு வலி தோன்றி சில நொடிகளில் மறைந்துவிடும்.

பங்கேற்பாளருக்கு நன்மைகள்:

இந்த ஆராய்ச்சியின் மூலம் பங்கேற்பாளருக்கு எவ்வித நேரிடை நன்மைகள் இல்லை. ஆனால் இந்த ஆய்வின் முடிவு சமுதாயத்திற்குப் பயன்படும்.

பங்கேற்பாளர் பணம் செலுத்துதல்:

இந்த ஆராய்ச்சிக்காக பங்கேற்பாளர் பணம் ஏதும் செலுத்த தேவையில்லை.

காப்பு தகவல்:

இந்த ஆராய்ச்சியில் பயன்படுத்தப்படும் பங்கேற்பாளரின் கோப்புகள் காப்புதகவலாய் பாதுகாக்கப்படும். மேலும் எதிர்கால பதிப்புகளிலோ அல்லது கருத்தரங்குகளிலோ உங்கள் பெயரைச் சேர்க்கமாட்டோம்.

பங்கேற்பாளரின் விலகல் உரிமை:

இந்த ஆராய்ச்சிக்குப் பங்கேற்பாளர்கள் விருப்பப்படவில்லை எனில் ஆராய்ச்சியில் இருந்து எந்தநேரத்திலும் விலகிக்கொள்ளலாம். இதற்கு பணம் ஏதும் செலுத்த தேவையில்லை.

சேமிப்பு தகவல்:

இந்த ஆய்விற்க்கு எடுக்கப்படும் தகவல்கள் பிற்காலத்தில் சேமிக்கப்படும்.

பங்கேற்பாறளரின் ஒப்புதல்:

இந்த ஆராய்ச்சியில் பங்கேற்கும் பங்கேற்பாளரின் முழு சம்மதத்துடன் ஒப்புதல் படிவம் பெறபடுகிறது.

இந்த படிவத்தை நான் படிக்கபெற்றும் (அல்லது நான் படித்தும்) முழு விவரங்களை விளக்கப்பட்டேன். இதன் மூலம் எல்லா விதமான சந்தேகங்களும் நீக்கப்பட்டன. மேலும் எதிர்காலத்தில் ஏதேனும் சந்தேகம் ஏற்படின் கீழே கொடுக்கப்பட்டுள்ள ஆராச்சியாளரை அணுகி நிவர்த்தி செய்துகொள்வேன்.

ஆராய்ச்சியாளரின் விலாசம்

நந்தினி.சு மற்றும் அக்ஷயா.ஜெ

நான்காம் ஆண்டு, உயிர் மருத்துவ அறிவியல்

மனித மரபியல் துறை

ஸ்ரீ இராமச்சந்திரா மருத்துவ கல்லூரி மற்றும் ஆராய்ச்சி நிறுவனம்

சென்னை – 600116.

இந்த ஒப்புதலை நான் கீழே கொடுக்கப்பட்டுள்ள கையொப்பத்தின் மூலம் உறுதி செய்கிறேன்.

பங்கேற்பாளரின் பெயர்: கையொப்பம்/பெருவிரல் ரேகை அச்சு: தேதி:

சாட்சியின் பெயர்: கையொப்பம்: தேதி:

தெரியப்படுத்தும் அனுமதி சான்றிதழ்

நான் மேலே குறிப்பிட்டுள்ள நபருக்கு இந்த ஆய்வின் தன்மை மற்றும் முக்கியத்துவம் குறித்து விலகினேன். நான் இந்த ஆய்வின் சாத்தியமுள்ள பலன்களைப பற்றி விளக்கினேன். பங்கேற்பளாரின் கேள்விகளுக்கு பதில் அளிப்பேன்.

தேதி:

ஆராய்ச்சியாளரின் பெயர்: நந்தினி.சு

கையொப்பம்:

கைப்பேசி எண்:

ஆராய்ச்சியாளரின் பெயர்: அக்ஷயா.ஜெ

கையொப்பம்:

கைப்பேசி எண்:

QUESTIONNAIRE

BASIC QUESTIONS	YOUR RESPONSES
1. Name:	
2. Age/Sex	
3. Marital status	
4. Contact address:	
5. Date of your first menstrual period ever:	
6. Date of your most recent period:	
7. Duration of your period (in days):	
8. Type of flow (heavy, medium, light):	
9. Do you menstruate every 28 to 30 days,or are your cycles irregular?	☐Yes ☐No
10. What medications, birth control pills, hormones, and/or supplements that you take on a regular basis?	
11. Do you have painful bowel movements?	☐Yes ☐No □Sometimes
12. Do you have pelvic pain	☐Yes ☐No ☐Sometimes
13. Type of pain?	
14. Severity of pain?	Pain on a scale of 1-10: ____
15. Do you have unusual vaginal bleeding anytime during your cycle?	☐Yes ☐No
16. Do you experience painful urination orblood in urine at any time during your cycle?	☐Yes ☐No