Investigating the functional impact of *WNT-4*, *VEGF-A* and *MMP-2* genetic polymorphism in Pakistani patients with endometriosis and fibroids

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By

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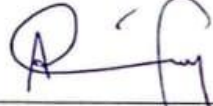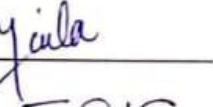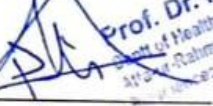
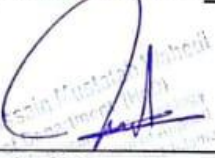**H-12, Islamabad, Pakistan**

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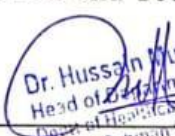
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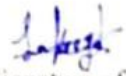
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Declaration

I certify that this research work titled "*Investigating the functional impact of WNT-4, VEGF-A and MMP-2 genetic polymorphism in Pakistani patients with endometriosis and fibroids*" is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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Pakeezah Shahid

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Dedicated to my amazing parents and beloved siblings, whose unwavering cooperation and support helped me achieve this amazing achievement.

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List of Acronyms

VEGF	Vascular Endothelial Growth Factor
MMP-2	Matrix metalloproteinase-2
MRI	Magnetic Resonance Imaging
CT	Computed Tomography
SNP	Single Nucleotide Polymorphisms
WNT4	Wingless type MMTV integration site family member 4
ECM	Extracellular Matrix
GnRH	Gonadotropin-Releasing Hormone
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
IUDs	Intrauterine Devices
GWAS	Genome-Wide Association Study
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
MED12	Mediator complex subunit 12
HMGA2	Mammalian High-Mobility-Group Protein AT-Hook 2
ART	Assisted Reproductive Technology
FH	Familial Hypercholesterolemia
IVF	*In vitro* Fertilization
NCBI	National Centre for Biotechnology Information
UniProt	Universal Protein Resource
SIFT	Sorting Intolerant from Tolerant
PolyPhen-2	Polymorphism Phenotyping version 2
PANTHER	Protein Analysis Through Evolutionary Relationships
P-Value	Probability Value
pH	Potential Hydrogen
TM Score	Template Modeling Score
RMSD	Root Mean Square Deviation
PDB	Protein Data Bank
PTMs	Post Translation Modifications
IRB	Institutional Review Board
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium Dodecyle Sulphate

DNA   Deoxyribonucleic Acid

Rpm   Revolutions per minute

TAE   Tris Acetate Ethylene-diamine-tetra-acetic acid

UV-Vis   Ultraviolet-Visual

ARMS-PCR   Amplification Refractory Mutation System Polymerase Chain Reaction

Tm Melting   Temperature

MgCl2   Magnesium Chloride

PPIs   Protein-Protein Interactions

Abstract

Uterine fibroids and endometriosis are common gynecological disorders that significantly impact women's well-being as well as complex aetiologies. This study uses an in silico examination of three important genes WNT-4, VEGF-A, and MMP-2 to investigate their genetic foundation. By using sophisticated computational methods to find single nucleotide polymorphisms (SNPs), we were able to distinguish harmful variations using SNP predictors like SIFT, Polyphen2, and PROVEAN. Three significant SNPs in VEGF-A, twelve in MMP-2, and seven in WNT-4 were found by our research. The majority of these SNPs have a deleterious effect on protein stability, according to structural and stability analyses. SUMOylation, N-glycosylation, and phosphorylation alterations were further highlighted by post-translational modification studies. Notably, the relationship between endometriosis and fibroids and SNP rs121908651 in WNT-4 was studied. There was a significant correlation with uterine fibroids ($p = 0.0001$) and with endometriosis ($p = 0.0026$). There is the need for more studies using larger cohorts to validate these SNPs' involvement in disease susceptibility and to pinpoint possible targets for treatment.

Keywords: Endometriosis, Uterine Fibroids, SNP Analysis, WNT-4, VEGF-A, MMP-2, In Silico Methods

Chapter 1. Introduction

1.1 Uterine fibroid and Endometriosis

A persistent gynecological disease that affects women who are fertile, endometriosis is inflammatory and hormone-driven. When Uterine-like tissue proliferates ectopically beyond the uterus, it becomes apparent. The pelvic cavity, fallopian tubes, ovaries, appendix, colon (P. R. de C. França et al., 2022), cervix, rectum, small intestine, vagina, abdominal walls, and bladder are examples of pelvic structures to which this tissue typically extends and impairs physiological activities (Rasheed & Hamid, n.d.). Rarely, it can spread to extra-pelvic locations like the brain, lungs, and eyes and show symptoms similar to tumor metastasis (Saunders & Horne, 2021). Increased vulnerability to comorbidities such as diabetes, hepatic and pelvic problems, cardiovascular diseases, and several autoimmune syndromes has been linked to endometriosis (Chauhan et al., 2022). Furthermore, it is associated with higher rates of severe psychological distress, such as worry and depression, which can be attributed to the incapacitating symptoms, the need for numerous surgical procedures, and the elevated risks of infertility (Horne & Missmer, 2022).
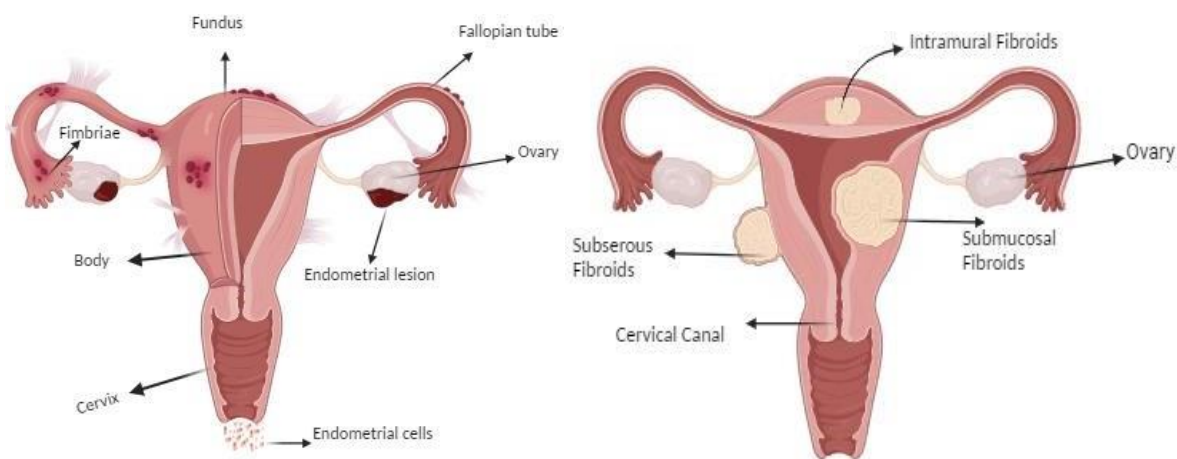
Figure 1.1. Endometrial lesions and uterine fibroids' locations within the pelvic cavity, showing how they could affect adjacent tissues

The benign tumors known as uterine fibroids, or uterine leiomyomas, develop from the smooth muscle cells in the uterus (Yang et al., n.d.). Even while these growths are frequently asymptomatic, they can potentially lead to serious health problems such irregular uterine flow,

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pelvic pain, and difficulties with reproduction (Wise & Laughlin-Tommaso, 2012). The size and location of uterine fibroids can vary; some may remain intramural or subserosal, while others may protrude into the uterine cavity (Segars, 2014). Remarkably, chromosomal anomalies may account for about half of uterine fibroids, indicating a genetic predilection (Sefah et al., 2023). Moreover, studies are conducted to clarify the molecular mechanisms that underlie the genesis and development of fibroid tumors (Segars, 2014). The locations of uterine fibroids and endometrial lesions within the pelvic cavity are shown in Figure 1.1

There exists a proposed correlation between endometriosis and uterine fibroids, suggesting possible etiological parallels between the two conditions. Both disorders respond to oestrogen similarly and are impacted by steroid hormones. Prior studies have demonstrated a greater incidence of endometriosis in females with uterine fibroids relative to those without fibroids (Uimari et al., 2011).

1.2. Epidemiology of endometriosis and uterine fibroids

Endometriosis strikes women at menarche and peaks between the ages of 25 and 45. Seldom does it continue after menopause. Uterine fibroids mainly afflict women who are of child-bearing age, which may continue after menopause and peak in prevalence between the ages of 35 and 49. Both disorders require specialized management techniques due to their substantial financial, social, and medical costs (Smolarz et al., n.d.)(Uimari et al., 2021a).

1.2.1 Global Prevalence

Approximately 10% of all women globally suffer from endometriosis, which impacts 190 million women (Rasheed & Hamid, n.d.). Its frequency in women without symptoms is between 2% and 11%, while in those who have pelvic pain, it is between 5% and 21% (Shafrir et al., 2018). It affects 0.1% of women between the ages of 15 and 49 annually (Vercellini et al., n.d.). About The risk of infertility is doubled for fifty percent of women who have endometriosis when compared to women without the disease (Králíčková et al., 2020). Women undergoing gynecological operations have diagnosis rates ranging from 0.1% to 53% during laparoscopy and laparotomy (Smolarz et al., n.d.).

Globally, uterine fibroids impact a substantial proportion of the female populace, with approximate prevalence rates varying from 4.5% to 68.6% based on study methodology, geographic region, and demographic characteristics (Giuliani et al., 2020). According to recent research, the global age-standardized incidence rate of uterine fibroids is trending upward, indicating a rising prevalence of this disorder (Li et al., 2023). Variations in prevalence rates

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between different locations can be attributed to various factors, including age, ethnicity, and hormonal impacts (Dai et al., 2024).

1.2.2 Prevalence in Pakistan

An estimated 6% of people in Pakistan have endometriosis (Khan et al., n.d.). In a different study, endometriosis was identified in 44 patients, or 27.5%, of 160 patients who had laparoscopies for different gynecological complications (Tahira et al., n.d.). Several studies have found that the prevalence of fibroids in Pakistan ranges from 20% to 40% (Zehra et al., 2022). Literature review on the clinical appearance and the incidence of fibroids have revealed that Pakistani women are more likely than Indian, urban, and Nigerian women to experience uterine fibroids (Munusamy et al., n.d.).

The precise prevalence of endometriosis and uterine fibroids is unknown despite being chronic diseases and the main causes of infertility in women. This is because the conditions are often diagnosed too late and receive insufficient care. In order to better comprehend the epidemiology of these medical conditions and its many characteristics, more thorough investigations are required.

1.3. Risk Factors

Genetic and epigenetic variables, including as exposure to diethylstilbestrol, early menarche, reduced body weight, irregular menstrual cycles, decreased waist to hip ratio, and nulliparity, are linked to an high risk of endometriosis (Zondervan et al., 2020). The risk is further increased by lifestyle choices including alcohol and tobacco use, sedentary behavior, and diets high in fat, hormones, and dioxins. Age also matters a lot; women between the ages of 25 and 29 are more vulnerable (Smolarz et al., n.d.), and current research has connected obesity with an increased risk of endometriosis (Tang et al., 2020).

In a similar vein, age, race, family history, obesity, and high blood pressure are risk factors for uterine fibroids (Pavone et al., 2018). The growth of fibroid tumors is influenced by hormones like progesterone and oestrogen levels (Yang et al., n.d.). The risk of fibroid tumors is also influenced by lifestyle factors such as food, stress, physical activity, smoking, and caffeine intake(Vafaei et al., 2024). Furthermore, uterine fibroids have been linked to vitamin D insufficiency and specific dietary variables in later life as triggers (Vafaei et al., 2024). These risk factors emphasize how crucial it is to comprehend how hereditary and environmental variables interact to form gynecological problems.

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1.4 Pathogenesis aetiology

1.4.1 Uterine Fibroids Etiopathogenesis

The smooth muscle tissue of the uterus gives rise to benign tumors called leiomyomas, or uterine fibroids. Their aetiology has been explained by a number of theories, but the precise mechanisms are still unknown. The following are the main mechanisms and factors:

1.4.1.1. Hereditary Factors

Research has shown that fibroids have a hereditary predisposition to develop. Individuals diagnosed with fibroids have been found to have specific gene abnormalities and familial patterns. According to research, the formation and development of fibroid tumors may be influenced by particular genetic pathways (Uimari et al., 2021b).
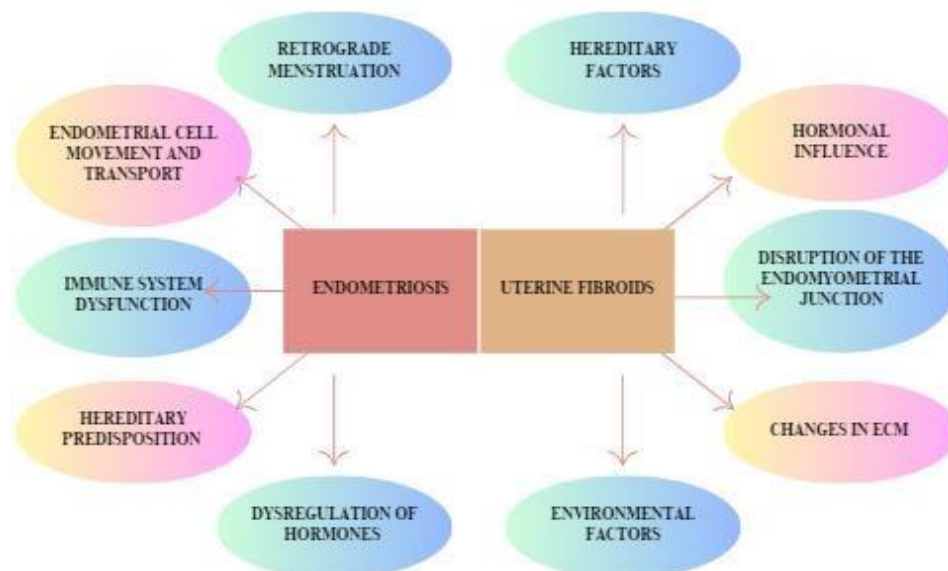
Figure 1.2.Mechanisms implicated in Endometriosis Etiopathogenesis

1.4.1.2. Hormonal Influence

Progesterone and oestrogen are essential for the development of fibroids. Compared to a normal myometrium, fibroid tissues contain greater concentrations of these hormones. The size and severity of fibroid symptoms might be influenced by hormonal fluctuations that occur during the menstrual cycle (Ciavattini et al., 2013).

1.4.1.3. Disruption of the Endomyometrial Junction

Infertility, uterine hemorrhage, and the development of submucosal and intramural myomas are among symptoms associated with fibroids that may result from disruption of the

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endomyometrial junction, which is where the endometrium joins the myometrium (Ciavattini et al., 2013).

1.4.1.4. Changes in the Extracellular Matrix (ECM)

Fibroids have a different ECM composition, which contributes to their development and stiffness. Fibroid tissue characterized through elevated collagen deposition and modifications in extracellular matrix proteins (Ciavattini et al., 2013).

1.4.1.5. Environmental Factors

Diet, obesity, and exposure to specific chemicals are examples of lifestyle and environmental factors that may have an impact on the development of fibroids. These elements may have an impact on uterine health generally and hormonal balance (Uimari et al., 2011).

1.4.2. Endometriosis Etiopathogenesis

The development of tissue that resembles endometrium beyond the uterus is a hallmark of endometriosis, which results in discomfort and infertility. The elements involved are depicted in figure, however other theories have been put out regarding the precise methods of its growth.

1.4.2.1. Retrograde Menstruation

According to this idea, menstrual blood flows backward via the fallopian tubes and into the abdominal cavity, where endometrial cells settle exterior to the uterus. It is believed that this procedure is the main way that endometriosis develops (Tanbo et al., 2017).

1.4.2.2. Endometrial Cell Movement and Transport

According to a different notion, endometrial cells can pass through uterine lymph nodes and capillaries to reach the bloodstream and lymphatic system. These cells can move to far-off places including the brain, lungs, and abdominal cavity once they enter the circulatory and lymphatic systems. There, they can become endometrial lesions that are regulated by different reproductive hormones (Jerman et al., n.d.)(P. R. de C. França et al., 2022).

1.4.2.3. Immune System Dysfunction

Immune system abnormalities may impede the removal of ectopic endometrial cells, enabling them to proliferate and implant outside of the uterus. This malfunction may include changed cytokine production and decreased natural killer cell activity (Smolarz et al., 2021).

1.4.2.4. Hereditary Predisposition

The heritable genetic components linked to endometriosis have been the subject of much investigation for many years. Endometriosis has complicated genetics that are altered by a

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number of variables. Research suggests that endometriosis is impacted by both epigenetic and genetic variables, both in its onset and progression. According to twin studies, endometriosis has a heritability of between 47 to 51%, with genetic variants responsible for about 26% of this heritability (Saha et al., n.d.).

1.4.2.5. Dysregulation of Hormones

The pathophysiology of endometriosis involves steroid hormones in a significant way. The overproduction of the oestrogen synthesizing enzyme aromatase, which is produced from the ovary in addition to cholesterol, is present in endometrial lesions and encourages the formation of these ectopic lesions. Elevated levels of oestrogen receptor β are noted in cases of endometriosis, which is thought to result from methylation of the gene's promoter. This increased level encourages the formation and inflammation of endometrial lesions by lowering the level of oestrogen receptor α and creating progesterone resistance in them (Vercellini et al., 2013).

1.4.3. Combining Perspectives on Pathogenesis

The aetiologies of endometriosis and uterine fibroids are complicated and involve overlapping genetic, hormonal, and environmental factors. They frequently coexist, pointing to potential shared routes or risk factors.

1.4.3.1. Genetic Foundations

There are strong genetic foundations for both diseases. Certain genes may play a function in the formation of fibroids, as evidenced by the identification of genetic variants and familial predispositions. Certain genetic variations correlated with a higher risk of endometriosis and fibroids in recent investigations, indicating that both conditions are related by common genetic pathways (Uimari et al., 2021a). Genome-wide association studies that have found similar genetic origins between the two conditions provide evidence that genetic vulnerability can play a significant role in the development of these gynecological issues (Gallagher et al., 2019a).

1.4.3.2. Hormonal Environment

The pathophysiology of endometriosis and uterine fibroids is significantly influenced by oestrogen and progesterone. These hormones, which support uterine fibroids' growth and maintenance, are present in uterine fibroids' fibroid tissues at greater concentrations than they are in the normal myometrium (Ciavattini et al., 2013). Progesterone resistance and oestrogen both contribute to the duration and severity of lesions in endometriosis, which is also highly

susceptible to these hormones. Oestrogen drives the development of ectopic endometrial tissue. The symptoms and development of fibroids and endometrial lesions are made worse by hormonal variations that occur during the menstrual cycle (Gruber & Mechsner, 2021).

1.4.3.3. Inflammatory and Immune Factors

Both diseases include persistent inflammation and dysregulated immune responses as major contributing factors. Immune system malfunction in endometriosis may lead to an inability to eliminate ectopic endometrial cells, which permits them to proliferate and implant outside of the uterus (Gruber & Mechsner, 2021). The environment that chronic inflammation produces is ideal for the growth of these cells. Likewise, alterations in immunological responses and inflammation of the uterine tissue are linked to uterine fibroids. Fibroid cell development and maintenance are facilitated by growth hormones and inflammatory cytokines (Ciavattini et al., 2013).

1.4.3.4. Factors related to Lifestyle and Environment

The likelihood and severity of uterine fibroids and endometriosis can be influenced by factors like food, obesity, and exposure to environmental contaminants, which can also impair immune system function and hormonal balance(Vallée et al., 2023);(Wise, 2015);(Vafaei et al., 2024) .

1.5 Clinical Manifestations of Fibroids and Endometriosis

The symptoms of endometriosis and fibroids frequently overlap, which makes diagnosis and treatment extremely difficult. Menorrhagia, or excessive menstrual bleeding, and dyspareunia, or pain during sexual activity, are symptoms that can be caused by either disorder (Uimari et al., 2021a). Infertility and severe dysmenorrhea, or painful periods, are notably linked to endometriosis. Many affected women also experience devastating cyclical or non-cyclical pelvic discomfort (Uimari et al., 2021a). Other typical digestive symptoms include bloating, nausea, and dyschezia, or difficulty passing gas. However, symptoms associated to mass may arise from fibroids, depending on their location and size such constipation, pelvic discomfort, and frequent urination (Farris et al., 2019). Fibroids can also cause irregular uterine bleeding, complicate pregnancies, and cause repeated miscarriages (Mukhopadhaya et al., 2007). The combination of these symptoms from both diseases can negatively impact the quality of life, so management and treatment must be comprehensive and tailored to each patient.

1.6 Diagnosis

1.6.1. Diagnosing Endometriosis

A multimodal method is used to diagnose endometriosis.

1.6.1.1. Clinical Assessment

Medical professionals evaluate symptoms such non-menstrual pelvic discomfort, dyspareunia (pain during sexual activity), and dysmenorrhea (difficult periods). These signs and symptoms direct the initial endometriosis suspicion (Allaire et al., 2023)(Health & 2024, n.d.).

1.6.1.2. Imaging Studies

With a sensitivity of roughly 93% and specificity of 95%, endometrial lesions in the ovaries, urinary tract, and recto-sigmoid area can be found using magnetic resonance imaging (MRI) and transvaginal ultrasound (TV-USG). However, because peritoneal lesions are deep enough to be detected beyond the limits of these procedures, they cannot be diagnosed using these methods (H. S. Taylor et al., 2021).

1.6.1.3. Laparoscopy

One of the best ways to diagnose endometrial abnormalities is via small-scale surgical examination of the pelvic and abdominal cavity, or laparoscopy. It aids in the treatment of illness and draws attention to the development, size, and appearance of lesions (Rolla, 2019).

1.6.2. Diagnosing Uterine Fibroids

There are various techniques for diagnosing leiomyomas, another name for uterine fibroids

1.6.2.1. Clinical Examination

Doctors examine the pelvis to feel for any abnormalities, such as lumps or noticeable masses in the uterus. This examination facilitates the evaluation of the fibroids' location, size, and shape (Cruz et al., 2017).

1.6.2.2. Imaging Techniques

Due to affordability and ease of use, ultrasound is initially the main imaging modality. It enables the visual representation of fibroids and the evaluation of their attributes, including size, quantity, and location, using transvaginal or transabdominal techniques (George, 2023). However, MRI or CT scans may be used for additional assessment in complex instances that call for thorough visualization. Particularly MRI provides better soft tissue contrast, which helps differentiate fibroids from other pelvic tumors. These imaging technologies work in

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concert to diagnose and evaluate uterine fibroids, allowing medical professionals to efficiently customize treatment regimens depending on the specific needs of each patient (Shahzad et al., 2023).

1.6.2.3. Hysteroscopy and Sonohysterography

Hysteroscopy is a method that allows direct imaging of the uterine chamber by inserting a narrow, illuminated telescope-like equipment through the cervix into the uterus. This technique is particularly useful for assessing submucosal fibroids that penetrate the uterus, enabling precise diagnosis and possible therapy (Martín-Merino et al., 2016). On the other side, sonohysterography uses ultrasound imaging after expanding the uterus with saline injection. This method helps detect anomalies such as intracavitary fibroids by providing fine-grained pictures of the uterine lining (Martín-Merino et al., 2016).

1.7. Treatment

1.7.1. Endometriosis Treatment

It's critical to take into account various aspects while choosing an endometriosis treatment plan, including symptoms, age, the course of the disease, and possible adverse effects. Endometriosis does not yet have a treatment, and it frequently returns. Thus, a balanced diet and lifestyle are essential, in addition to pharmaceuticals and hormonal therapy targeted at symptom relief and hormone balance. Removing endometrial lesions surgically is another popular method.

1.7.1.1. Hormonal Therapies and Medications

Due to their affordability and pain-relieving properties, progestins and oral contraceptives are the recommended 1st therapy for endometriosis. But not all patients should use them, and they might have negative effects. Antibodies that release gonadotropin-releasing hormone (GnRH) are regarded as secondary choices; however, oral administration of these agents is not feasible. The novel GnRH antagonist taken orally, Elagolix, decreases oestrogen levels but may be harmful to bone health. Other drugs that have demonstrated efficacy in treating endometriosis include danazol, gestrinone, and other progestogens.

Recently, endometriosis treatment has included aromatase inhibitors, which target and limit the production of oestrogen. These medications are frequently used in combination with nonsteroidal anti-inflammatory drugs (NSAIDs) to treat pain (Ferrero et al., 2018). Furthermore, studies have demonstrated the efficacy of natural compounds like curcumin and puerarin in treating endometriosis symptoms (França et al., 2022.).

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1.7.1.2. Surgical Procedures

Surgery is the last alternative for women with endometriosis whose painkillers are unable to ease their symptoms. The goal of surgical operations is to eradicate endometrial lesions from different regions of the body completely. Laparoscopy, laparotomy, and hysterectomy are among the techniques. Nevertheless, there is a significant chance of pain recurrence, and these operations are not totally curative (Tanbo et al., 2017). Research indicates that the likelihood of requiring a second operation increases to 50% after five to seven years, with a 15-20% risk after two years (Vercellini et al., 2009). With the development of robotic-assisted laparoscopy, patients with obesity and deep lesions can now benefit from increased precision. Although this method is more expensive, it is safe and efficient (Restaino et al., 2020).

1.7.1.3. Non-medical treatments for management of symptoms

Endometriosis symptoms are frequently managed with a variety of non-medical therapies. These include antioxidants to lower oxidative stress, homoeopathic medicines, acupuncture, reflexology, traditional Chinese herbal treatments, and stress-relieving therapies. Additionally, helpful in reducing discomfort and regaining normal pelvic function is post-surgical physical therapy (Wójcik et al., n.d.). Although these alternative therapies can reduce symptoms, the endometrial lesions are not cured by them.

1.7.2. Uterine fibroid Treatment

Like endometriosis it is critical to take age, disease progression, side effect likelihood, and symptoms into account while choosing a treatment plan for uterine fibroids. It is crucial to understand that there is no known treatment for uterine fibroids, and there is a considerable risk of recurrence. Thus, in addition to drugs and hormonal therapy to control symptoms and restore hormonal balance, as well as operations to remove fibroids if necessary, a healthy diet and lifestyle are essential.

1.7.2.1. Hormonal Therapies and Medications

The mainstay of treating uterine fibroids is hormonal therapy. Hormonal IUDs and birth control pills are the first-line therapy for symptoms including excessive bleeding. Although GnRH agonists are quite successful at shrinking fibroids, their substantial adverse effects usually limit their use (Ciebiera et al., 2023). Another pharmacological option for treating fibroid symptoms and size is to use anti-hormonal medications such ulipristal acetate and mifepristone, which have demonstrated efficacy in doing so (D. Taylor et al., n.d.).

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1.7.2.2. Surgical Procedures

Surgical alternatives are required for women whose fibroids are big or unresponsive. A myomectomy is the best option for people who want to maintain their fertility since it removes fibroids without damaging the uterus. Conversely, although a hysterectomy is a definite treatment for fibroids, it causes infertility. An alternate strategy is provided by minimally invasive techniques such as uterine fibroids embolization, which reduce fibroids by obstructing their blood supply (Laughlin-Tommaso, 2018) .

1.7.2.3. Non-medical Treatments

Symptom control is the main goal of non-medical therapy. Lifestyle modifications, such as keeping a healthy weight and balanced food, can help manage symptoms. Although there is little scientific support for alternative medicines like acupuncture and herbal remedies, they are nevertheless utilized (Stener-Victorin et al., 1996).

1.8. Pakistani Research Priorities: Genetic Association in Endometriosis and Fibroids

Uterine fibroids and endometriosis are common disorders that cause a great deal of morbidity, such as infertility and persistent discomfort. Despite their impact, there is currently no proven cure, and the majority of current therapies are symptomatic and include surgery or medication (Uimari et al., 2021). Genetic investigations conducted by researchers in different nations have expanded our understanding of these diseases by identifying many gene associations. On the other hand, little study has been done in Pakistan specifically on the genetic associations of endometriosis and uterine fibroids. The existing body of knowledge frequently overlooks Pakistan in favor of larger populations or certain genetic variations (Kabodmehri et al., 2022);(Liaqat et al., 2013);(Latif et al., n.d.). This points out how important it is to do more local research in order to understand the genetic factors influencing these medical conditions in Pakistani women. Local studies are necessary to close this knowledge gap.

Three genes—VEGFA, WNT4, and MMP2—have been chosen for the current study based on their reported links to endometriosis and uterine fibroids in worldwide studies. Due to its function in blood vessel production and tissue growth, the VEGFA (Vascular Endothelial Growth Factor A) gene is essential for angiogenesis and has been connected to the emergence of both diseases (Prokofiev et al., 2020);(Rashidi et al., 2019). Due to its function in cell proliferation and differentiation, the WNT4 (Wnt Family Member 4) gene is critical for the development of the female reproductive system and has been linked to a number of

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reproductive diseases, including fibroids and endometriosis (Pitzer et al.,20121). Variations in the MMP2 (Matrix Metalloproteinase 2) gene have been linked to pathological alterations in the remodeling of tissues and healing processes. This gene is essential for the degradation of extracellular matrix (Korompelis et al., 2015);(Jana et al., 2012).

In order to fill the research gap and enhance patient outcomes in the area, this study intends to investigate these genetic connections within the Pakistani population. The results could lead to better diagnostic tools and targeted therapeutics.

1.9. Objectives of Study

The following are the study's primary objectives:

i. To use in-silico analysis to determine whether the SNPs of particular genes are damaging.

ii. To use in-vitro analysis to look into the relationship between uterine fibroids and endometriosis and specific SNPs.

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Chapter 2. Literature Review

2.1. Endometriosis and Uterine Fibroids

Two of the most common gynecological conditions affecting women of reproductive age are endometriosis and uterine fibroids, which can cause severe morbidity and negatively influence quality of life.

2.1.1. Endometriosis

The presence of endometrial-like tissue outside the uterine cavity is a characteristic of endometriosis, a long-term inflammatory disorder. The ovaries, fallopian tubes, the peritoneum and in rare instances, the area beyond the pelvis, can all contain this ectopic tissue. Around 10% of women worldwide who are of reproductive age are thought to be affected by the disorder. relying on where the lesions are located, symptoms might range from dysmenorrhea (painful periods), persistent pelvic pain, and infertility to gastrointestinal and urinary tract issues (Ciarmela et al., 2013.). Endometriosis is a multifaceted and intricate aetiology. Numerous explanations, such as immunological dysfunction, coelomic metaplasia, hereditary susceptibility, and retrograde menstruation, have been put forth. According to the theory of retrograde menstruation, endometrial cells implant and proliferate in the pelvic cavity as a result of menstrual blood flowing backward through the fallopian tubes. On the other hand, not every woman who experiences retrograde menstruation goes on to acquire endometriosis, suggesting that other elements, like compromised immune systems and genetic predispositions, are important (Ciarmela et al., 2013.).

Endometriosis is heritable, as evidenced by the identification of many susceptibility loci linked to the condition in recent genetic research. For example, the condition has been associated with mutations in the WNT4 gene, which is essential for the development of the female reproductive system. Environmental variables that alter hormone regulation and immunological responses, such as exposure to endocrine-disrupting chemicals, may also play a role in the onset and progression of endometriosis (Uimari et al., 2021a).

2.1.2. Uterine Fibroids

Bilateral smooth muscle tumors of the uterus are called uterine fibroids, also called leiomyomas or myomas. Their estimated occurrence by the age of 50 is as high as 70-80%, making them

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the most prevalent pelvic tumors in women. A variety of symptoms, including heavy menstrual bleeding (menorrhagia), pelvic pressure or pain, frequent urination, and reproductive problems like infertility and pregnancy complications, can be caused by fibroids, which can vary greatly in size, number, and location within the uterus (Ciarmela et al., 2013.). While the precise cause of uterine fibroids is still unknown, it is known that oestrogen and progesterone play a major role in their development. A major part of their development is also influenced by genetic factors. Numerous chromosomal abnormalities and genetic mutations have been found in fibroid tissues through research, indicating a significant hereditary component. For instance, mutations in the MED12 gene have been routinely found in fibroid cells, suggesting that this gene plays a role in the aetiology of fibroids (Ciavattini et al., 2013).

2.2. Genetic Insight

Uterine fibroids and endometriosis are common gynecological disorders that have a major negative influence on women's health. Genetic, hormonal, and environmental variables interact intricately to influence both diseases.

2.2.1. Genetic Basis of Endometriosis

Endometriosis is regarded as a polygenic/multifactorial disorder, indicating that various genes and environmental factors interact to cause it. Numerous susceptibility loci linked to endometriosis have been discovered by genome-wide association studies (GWAS). These loci contain genes related to inflammation, immunological response, and hormone control (Mcgrath et al., 2023);(Rahmioglu et al., 2023.).

2.2.1.1. Key Findings

The key genetic findings are as follows:

1. Chromosomal Loci: Numerous chromosomal regions, including 1p36, 7p15.2, 9p21, and 12q22, have been linked to endometriosis. Genes involved in cell adhesion, immunological response, and oestrogen signaling are located in these areas (Rahmioglu et al., n.d.).

2. ESR1 and ESR2 Genes: Because endometriosis is hormone-dependent, the oestrogen receptor genes ESR1 and ESR2 are particularly interesting. Differences in these genes could affect how oestrogen receptors are expressed and function, which could affect how the disease develops and progresses (V. S. Baranov et al., 2016).

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3. WNT4 Gene: Endometriosis has been linked to the WNT4 gene, which is involved in cell signaling pathways. It contributes to the regulation of endometrial cell proliferation and the development of the female reproductive tract (McGrath et al., 2023).

4. VEGFA Gene: Angiogenesis is aided by VEGFA, also known as vascular endothelial growth factor A. It plays a crucial part in endometriosis since the disorder causes new blood vessels to emerge in order to support the growth of ectopic endometrial tissue (Donnez et al., 1998).

5. MMP2 Gene: Angiogenesis and remodeling of tissues are two critical processes in the pathophysiology of endometriosis, and MMP2 is involved in the breakdown of extracellular matrix components (Barbe et al., 2020).

2.2.2. Genetic Basic of Uterine Fibroids

Leiomyomas, often known as uterine fibroids, are benign tumors of the uterine muscle. Both genetic and epigenetic changes are present in fibroids. Numerous genes and chromosomal anomalies have been related to the formation of fibroid lesions through genetic investigations.

2.2.2.1. Key Findings

The key genetic findings for uterine fibroids are as follows:

1. MED12 Mutations: The most common genetic changes associated with uterine fibroids are mutations in the mediator complex subunit 12 (MED12) gene. Because MED12 regulates transcription, mutations in the gene can cause aberrant cell growth (V. Baranov et al., n.d.).

2. HMGA2 Gene: Rearrangements in the High-mobility group AT-hook 2 (HMGA2) gene are also frequently observed in fibroids. Tumor growth can be attributed to the deregulation of HMGA2, which is involved in the regulation of gene expression and chromatin structure (V. Baranov et al., n.d.).

3. FH Gene: The formation of fibroid tumors has been linked to mutations in the fumarate hydratase (FH) gene, which is involved in the Krebs cycle. FH mutations may cause oncometabolites to build up and accelerate the initiation of tumors (V. Baranov et al., n.d.).

4. VEGFA Gene: Angiogenesis is aided by VEGFA, also known as vascular endothelial growth factor A. It is important for fibroid growth because the condition causes new blood vessels to emerge to support the growth of fibroid tissue (Kirschen et al., n.d.).

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5. WNT4 Gene: Uterine fibroids have been linked to the WNT4 gene, which is involved in cell signaling pathways. It contributes to the regulation of cell proliferation and the development of the female reproductive tract (Kirschen et al., n.d.).

6. MMP2 Gene: Essential to the pathophysiology of uterine fibroids, matrix metalloproteinase-2 (MMP2) breaks down extracellular matrix components to promote tissue remodeling and angiogenesis (Onishi et al., n.d.).

2.3. VEGFA's role in uterine fibroids and endometriosis

Due to its involvement in angiogenesis—the process of generating new blood vessels from pre-existing ones vascular endothelial growth factor A (VEGFA) plays a critical role in both endometriosis and uterine fibroids. This angiogenic factor plays a role in the development and progression of both diseases by being involved in their pathogenesis.

VEGFA is increased in endometriosis and stimulates the growth of ectopic endometrial tissue by forming new blood vessels. Studies show that compared to healthy endometrial tissue, VEGFA expression is higher in endometriotic lesions. The creation and maintenance of endometriotic lesions are aided by the enhanced angiogenesis that VEGFA facilitates. This enhances the survival and multiplication of endometrial cells outside the uterus (Chung & Han, 2022).
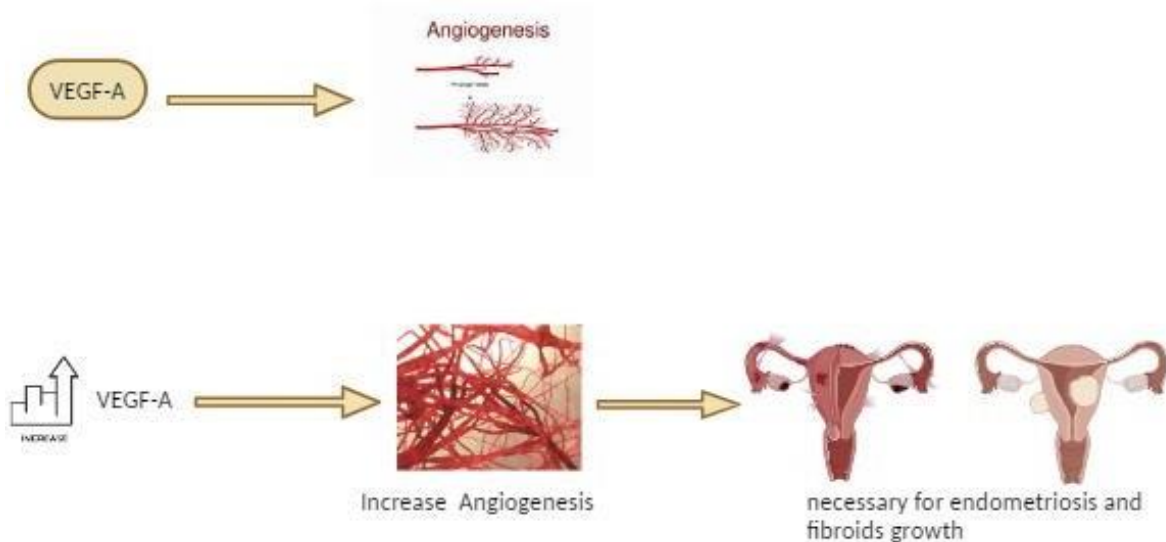
Figure 2.1. A higher expression of VEGF-A stimulates angiogenesis, which helps in the growth of fibroids and endometriosis.

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The growth and development of fibroid tissue in uterine fibroids is facilitated by VEGFA, which also stimulates angiogenesis. According to studies, fibroid tissue has higher levels of VEGFA expression than normal myometrium does. Because VEGFA increases vascularity, fibroid cells' metabolic requirements are met, allowing for their survival and multiplication in the uterine wall (Navarro et al., 2021).

VEGFA promotes angiogenesis, which is a key factor in the pathophysiology of uterine fibroids and endometriosis alike. Ectopic endometrial cells and fibroid cells can proliferate and survive more easily when this gene is overexpressed because it increases vascularity in endometriotic lesions and fibroid tissue, respectively. In order to manage the symptoms and evolution of these diseases, it is helpful to comprehend the role of VEGFA in them.

2.4. WNT4 Gene's Role in Endometriosis and Uterine Fibroids: Consequences for Reproductive Health

The WNT4 gene profoundly affects reproductive health through its complex involvement in endometriosis and uterine fibroids. WNT4 plays a role in cell signaling pathways essential for the development of the female reproductive tract in endometriosis (Pavličev et al., 2024). The pathophysiology of endometriosis may be aided by WNT4 signalling dysregulation, which encourages aberrant cell proliferation and tissue invasion (Pitzer et al., n.d.). Furthermore, research indicates that abnormal WNT4 expression could interfere with immunological responses and hormone regulation, aggravating symptoms of endometriosis (Kiewisz et al., n.d.).
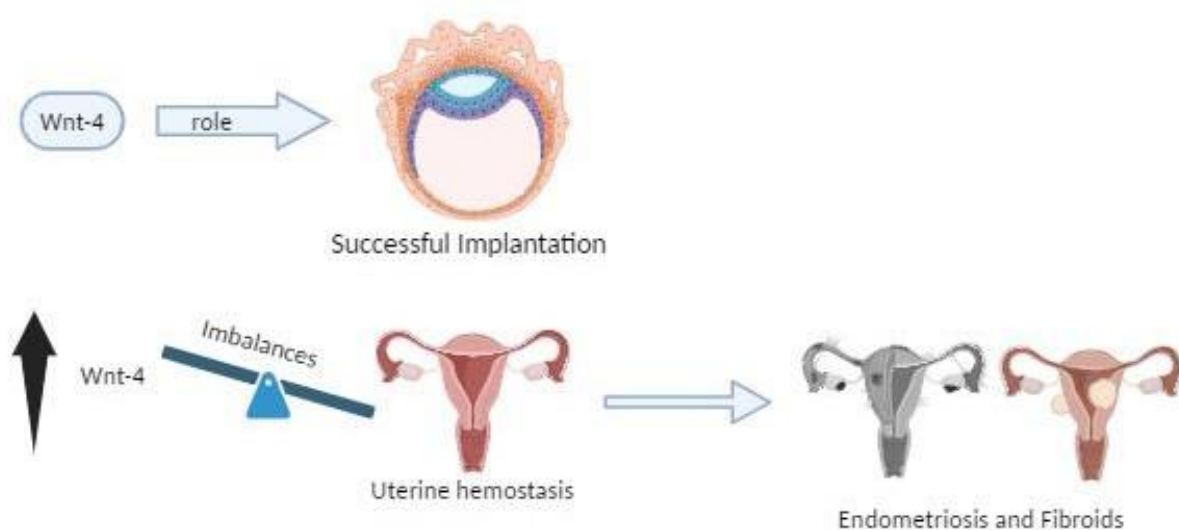
Figure 2.2 Increased WNT4 contributes to endometriosis and fibroids by interrupting uterine homeostasis.

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WNT4 is also connected to the pathophysiology of uterine fibroids. The growth of fibroid may be aided by dysregulated WNT4 signalling, which may encourage aberrant cell proliferation and extracellular matrix remodelling (Kho et al., 2021). Additionally, WNT4-mediated signalling pathways contribute to angiogenesis, an essential process for the growth and vascularization of fibroid tissue. In addition to affecting tissue homeostasis and hormone responsiveness, WNT4 dysregulation in uterine fibroids may also have an impact on disease progression and symptom intensity (Ali et al., 2023).

Comprehending WNT4's complex involvement in endometriosis and uterine fibroids carries noteworthy consequences for reproductive health. Modulating WNT4 signalling pathways through targeted medicines may provide new approaches to treating various gynaecological problems and enhancing the success of reproductive treatments (Uimari et al., 2021a)

2.5. MMP2 in the Pathophysiology of Uterine Fibroids and Endometriosis

The matrix Metalloproteinase 2 (MMP2) plays a critical role in tissue remodeling, especially in the extracellular matrix (ECM), and disorders of this enzyme have been linked to endometriosis and uterine fibroids, among other gynecological problems. MMP2 promotes inflammation, angiogenesis, cell migration, and ECM breakdown. Aberrant MMP2 activity in endometriosis enhances ectopic endometrial tissue invasion, which is correlated with the severity of the disease (Barbe et al., 2020).

In similar ways, MMP2 promotes the growth and breakdown of extracellular matrix in uterine fibroids. Fibrotic alterations and fibroid progression are caused by dysregulated MMP2 activity, which modifies collagen turnover (Akram et al., 2022). Fibrid angiogenesis is further supported by MMP2-mediated ECM remodelling (Governini et al., n.d.).

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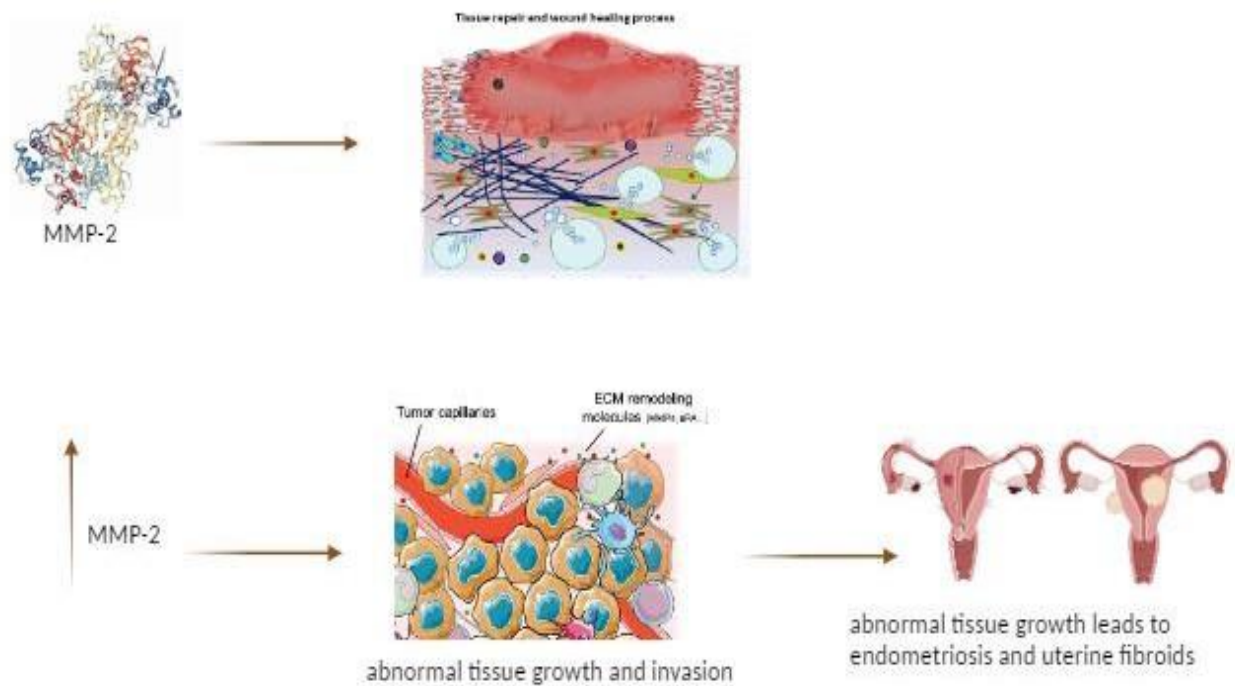
Figure 2.3. higher MMP-2 levels result in aberrant invasion and development of tissue, promotes to fibroids and endometriosis.

The involvement of MMP2 in the pathogenesis of endometriosis and its dysregulated expression in gynecological disorders are highlighted in studies by Barbe et al., and Akram et al., offering insights into the mechanisms behind the disease's development (Barbe et al., 2020);(Akram et al., 2022).

Determining MMP2's significance in endometriosis and uterine fibroids requires an understanding of its role in tissue remodeling. Modulating MMP2 activity through targeted therapies shows potential for treating various disorders and enhancing patient outcomes (Onishi et al., n.d.).

2.6. Patients with endometriosis and fibroids: Reproductive Outcomes and Fertility Preservation

Common gynecological disorders that can have a major impact on reproductive outcomes and fertility preservation in those who are affected are endometriosis and uterine fibroids. Giving patients complete care requires an understanding of how these disorders affect fertility.

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2.6.1. Reproductive Outcomes and Endometriosis

Numerous reproductive difficulties, such as infertility, miscarriage, and unfavourable pregnancy outcomes, are linked to endometriosis (Macer & Taylor, 2012). Endometriotic diseases can cause pelvic anatomy to be distorted, which can impede the fallopian tubes mechanically and lower the quality of the eggs. Furthermore, sperm function and embryo implantation may be adversely affected by endometriosis-related chronic inflammation and oxidative stress (Bonavina & Taylor, 2022). Consequently, in order to become pregnant, endometriosis patients frequently need assisted reproductive technologies (ART), such as in vitro fertilisation (IVF) (Al Shukri et al., 2023).

2.6.2. Strategies for Preserving Fertility

For patients with endometriosis and fibroids, especially those undergoing surgical procedures, fertility preservation is a major concern. The removal of endometriotic lesions or possibly a hysterectomy may be necessary for the surgical treatment of endometriosis, which could jeopardise ovarian reserve and fertility (Rangi et al., 2023). Preoperative counselling is also crucial, as is thinking through possibilities for fertility preservation including cryopreservation of eggs or embryos (Rangi et al., 2023). Individualised treatment regimens are crucial because fibroid therapy techniques, including myomectomy, can also affect fertility (Supermaniam & Thye, 2019);(Rezk et al., 2021).

2.6.3. Consequences of Hormonal Treatments

Gonadotropin-releasing hormone (GnRH) agonists and aromatase inhibitors are two typical hormonal medications used to treat endometriosis and fibroids; however, they can also decrease ovarian function and cause hypoestrogenic conditions (Rousseau, 1999). These therapies may reduce symptoms, but they may also short-term reduce fertility. Therefore, it is crucial to carefully evaluate the time of treatment and the aims of reproduction.

2.6.4. Multidisciplinary Method

Specialists in reproductive endocrinology, gynecology, and fertility management must collaborate to manage endometriosis and fibroids (Sänger et al., 2023). When medical professionals work together, patients receive comprehensive care that includes preconception counselling, appropriate ART interventions, and fertility preservation counselling (Tsonis et al., 2023). Holistic care also includes treating psychological issues and supporting patients as they navigate infertility obstacles (Vitale et al., 2017).

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In order for best reproductive outcomes and maintain fertility in those who are impacted, it is crucial to comprehend the intricate interactions that exist between endometriosis, fibroids, and fertility.

2.7. Diagnostic Challenges

Globally, diagnosing endometriosis and uterine fibroids is extremely difficult due to the lack of conclusive diagnostic indicators and the generic character of symptoms. The overlap of symptoms between endometriosis and fibroids, which might include pelvic pain, irregular uterine bleeding, and infertility, is one of the main challenges in making this distinction. Due to the need for clinicians to sort through differential diagnoses in order to identify the underlying ailment, these common symptoms frequently result in delays in diagnosis. The process is further complicated by the lack of trustworthy biomarkers for endometriosis diagnosis, which forces medical professionals to rely on invasive procedures, imaging tests, and clinical evaluation as means of confirmation (Parasar et al., 2017).

Similar diagnostic difficulties arise in Pakistan as a result of restricted access to resources and specialised healthcare services. Ultrasonography is frequently used to assess female infertility caused by endometriosis as well as uterine fibroids. Despite its usefulness, ultrasound is not always able to make a definitive diagnosis, especially when it is difficult to differentiate between fibroids and endometriosis lesions. Accurate identification is further hampered by the nonspecificity of symptoms and endometriosis's propensity to resemble other medical conditions. These elements lead to delays in diagnosis and highlight the need for better diagnostic techniques and patient and healthcare provider knowledge (Noor et al., 2022).

The diagnosis procedure is further complicated by the presence of adenomyosis, a disorder that is strongly related to endometriosis. It might be difficult to distinguish between adenomyosis and fibroids based alone on clinical presentation because these illnesses can coexist and have similar symptoms. Because many reproductive health diseases are complex, a multidisciplinary approach to diagnosis is required. For a clear diagnosis, this approach should include clinical evaluation, imaging modalities, and, in certain circumstances, histological analysis (Habiba & Benagiano, 2021).

It is critical to improve healthcare infrastructure, raise professional understanding, and fund research to find new diagnostic markers and techniques in order to overcome these diagnostic problems. People with endometriosis and uterine fibroids can benefit from prompt treatments

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and appropriate management catered to their individual needs by increasing diagnosis accuracy and decreasing delays.

2.8. New Therapeutic Targets and Innovative Treatment Strategies

Given the intricacy of endometriosis and fibroids and the variable responses to conventional treatments, addressing new therapeutic targets and innovative treatment modalities for these disorders poses a major global issue. On the other hand, new discoveries about the molecular mechanisms of fibroids and endometriosis have opened the door to the investigation of novel therapeutic approaches. Researchers from all around the world are concentrating on finding novel therapeutic targets in an effort to create medicines that target certain pathways implicated in the development of various diseases.

In recent years, a number of intriguing therapeutic targets have surfaced, providing novel possibilities for the development of tailored treatments for fibroids and endometriosis. various targets include angiogenic factors, growth factors, hormone receptors, and inflammatory cytokines that are involved in the pathophysiology of various disorders. Novel therapy techniques seek to enhance long-term patient outcomes, reduce side effects, and achieve more precise and effective symptom control by focusing on specific molecular processes implicated in disease progression.

Meeting the treatment needs of people with endometriosis and fibroids is still a critical issue in Pakistan, where access to specialised healthcare treatments may be restricted. Researcher and healthcare professional collaboration is essential for determining therapeutic targets pertinent to Pakistani population and customising innovative treatment techniques for regional settings. Furthermore, programmes targeted at expanding knowledge about new treatment choices and expanding access to cutting-edge therapies are crucial for raising the standard of care for people with these diseases.

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Chapter 3. Methodology

3.1. In-Silico Investigation of Missense SNPs

The genes VEGFA, WNT4, and MMP2 were first subjected to in-silico analysis in this study in order to forecast the functional effects of missense SNPs. Researchers were able to quickly identify potentially harmful variations and comprehend how they affected the structure and function of proteins attributable to the computational methods.

Because missense single nucleotide polymorphisms (SNPs) can accurately and economically anticipate the possible effects of genetic differences on protein structure and function, in-silico investigation of these SNPs is essential. Single amino acid changes in proteins, known as missense SNPs, can have a major impact on the development and susceptibility to disease. Through the use of computational methods, scientists can prioritize SNPs for additional experimental validation by screening and identifying those that are most likely to be harmful (Ali et al., 2022).
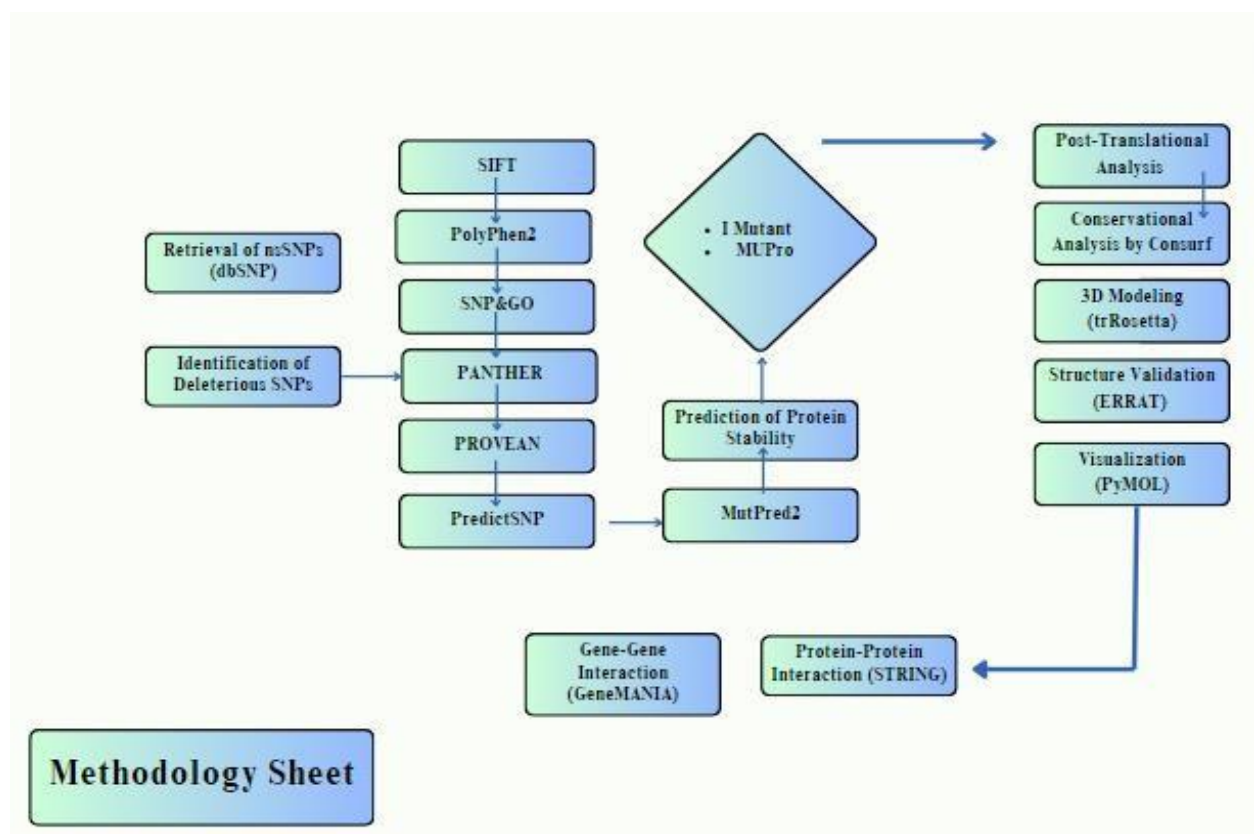
Figure 3.1. An illustration of the in-silico methods used in computational analysis to select missense SNPs.

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3.1.1. SNP retrieval

The dbSNP database of the National Centre for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov/snp/ was the source of the SNP data for the genes VEGFA, WNT4, and MMP2 (accessed on 03-10-2023). This dataset included information on SNP allele variants, chromosomal locations, and SNP IDs.

3.1.2. Retrieval of Protein Sequence

WNT4, MMP2, and VEGFA canonical protein sequences were retrieved in FASTA format from the UniProt database (https://www.uniprot.org/). The total amino acid count of the WNT4 protein sequence is 351, the VEGFA protein sequence is 232, and the MMP2 protein sequence is 660.

3.1.3. Identification of missense variants with detrimental functions

Employing the Sorting Intolerant from Tolerant (SIFT) tool https://sift.bii.a-star.edu.sg/, which assesses the effect of amino acid alterations on protein function, the missense single nucleotide polymorphisms (SNPs) from the National Centre for Biotechnology Information (NCBI) were examined. deleterious SNPs were defined as those with a SIFT score of less than 0.05 (Kumar et al., n.d.). Five further online tools (PolyPhen-2, SNP&GO, PANTHER, PROVEAN, and PredictSNP) were then used to evaluate these damaging SNPs. While PolyPhen-2 (Polymorphism Phenotyping v2) http://genetics.bwh.harvard.edu/pph2/ evaluates the structural or functional implications of amino acid substitutions (Adzhubei et al., n.d.), SNP&GO https://snps-and-go.biocomp.unibo.it/snps-and-go/ uses functional protein annotations to classify amino acid alterations as either neutral or pathogenic (Capriotti et al., 2013). PANTHER (Protein Analysis Through Evolutionary Relationships) http://www.pantherdb.org/tools/csnpScoreForm.jsp uses the wild-type amino acid's conservation time to forecast how harmful amino acid alterations will be (Thomas et al., n.d.). PROVEAN http://provean.jcvi.org/index.php assesses the functional effects of changes in protein sequence, such as indels and substitutions of amino acids (Choi et al., 2012). PredictSNP http://loschmidt.chemi.muni.cz/predictsnp evaluates the impact of SNPs on protein function by combining several prediction methods (Bendl et al., 2014). SNPs identified by all six tools to be damaging underwent a further investigation.

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3.1.4. Evaluation of detrimental Missense SNPs' Structural and Functional Impact

Using the MutPred2 web server (http://mutpred.mutdb.org/), the effects of harmful missense single nucleotide polymorphisms (SNPs) on protein structure and function were assessed. MutPred2 is a computational tool that predicts the structural and functional changes brought about by amino acid substitutions in protein sequences, hence evaluating the possible harmful implications of these substitutions (Pejaver et al., 2020). The MutPred2 server received the protein sequences in FASTA format along with a list of substitutions for amino acids. The resulting P-values were used to establish the level of confidence in the predictions; values below 0.05 were regarded as confident, and values below 0.01 as highly confident.

3.1.5. Identifying the Impact of Deleterious Missense SNPs on the Stability of Proteins

For proteins to function normally, protein stability is vital. The I-Mutant2.0 https://folding.biofold.org/i-mutant/i-mutant2.0.html online tool was utilized to evaluate the effect of SNPs on protein stability (Capriotti et al., 2005). I-Mutant 2.0 forecasts if a change in an amino acid would increase or decrease protein stability. The protein sequence was entered into I-Mutant2.0 in FASTA format, along with information about amino acid changes, under default settings (pH: 7 and temperature: 25°C). The tool then produced reliability indices (RI) and ΔΔG values. Increased protein stability is indicated by a positive ΔΔG value (>0), while decreased stability is indicated by a negative value (<0) (Capriotti et al., 2005).

To evaluate the impact of missense SNPs on protein stability, a similar technique known as MUpro was also employed. Mupro http://mupro.proteomics.ics.uci.edu/ is an ensemble of machine learning algorithms created to anticipate the effects of single-site mutations in amino acids on the stability of proteins. For this, it provides two machine learning techniques. To determine how a mutation would affect the stability of a protein, users can enter information about the mutation, such as its name and position (Cheng et al., n.d.).

3.1.6. Analysis of Post Translation Modification (PTM)

Protein interactions and functionality depend on Post-Translation Modifications (PTMs), which can be altered by the presence of deleterious missense SNPs. The gain or loss of PTM sites as a result of the amino acid alterations brought on by these SNPs was predicted using prediction algorithms. Different computational techniques were used to evaluate PTMs such as

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SUMOylation, Glycosylation, Ubiquitination, Methylation, and Phosphorylation. Protein kinases catalyze phosphorylation, which primarily targets serine, threonine, and tyrosine residues. Arginine and lysine residues are the main parts affected by methylation. Some particular spots on proteins are the focus of ubiquitination. A solitary platform was employed to conduct an analysis of these post-translational alterations available at https://www.musite.net/ (Wang et al., n.d.). The GPS-SUMO 2.0 platform, accessible at https://sumo.biocuckoo.cn/online.php, was utilized to perform SUMOylation predictions (Zhao et al., n.d.). NetNGlyc 1.0 was used for N-linked glycosylation analysis; it may be accessed at https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/(Gupta & Brunak, 2002). Additionally, NetOGlyc 4.0 available at https://services.healthtech.dtu.dk/services/NetOGlyc-4.0/ was used to perform O-linked glycosylation predictions (Steentoft et al., 2013).

3.1.7. Analysis of Evolutionary Conservation

ConSurf https://consurf.tau.ac.il/consurf_index.php was used to evaluate the effect of missense SNPs on evolutionarily conserved amino acids inside a protein sequence. ConSurf uses homologous sequence phylogenetic analysis to determine the rates of amino acids evolution. ConSurf delivers conservation ratings for each amino acid, with 9 denoting the greatest degree of conservation and 1 indicating the least, upon submission of the protein sequence in FASTA format. It also provides information on whether an amino acid residue is accessible or buried, as well as its structural or functional importance (Ashkenazy et al., n.d.).

3.1.8. 3D Model Prediction

Using the trRosetta (https://yanglab.nankai.edu.cn/trRosetta), protein modelling was performed on the missense SNPs found in earlier steps. By using deep learning techniques, trRosetta is highly proficient at accurately predicting protein structures. Using a restricted Rosetta architecture and direct energy minimization, trRosetta builds three-dimensional models based on inputted amino acid sequences. In order to improve accuracy, this method incorporates inter-residue distance and orientation distributions that are predicted using a deep neural network. Homologous templates also improve the accuracy of predictions. TrRosetta outperformed other approaches in benchmark evaluations using the CASP13 and CAMEO datasets, highlighting its effectiveness in protein structure prediction (Du et al., n.d.).

3.1.9. 3D Protein Structure Validation

ERRAT was used to do extra quality validation on protein structures produced by trRosetta. Employing atomic interactions, ERRAT https://saves.mbi.ucla.edu/ is a tool that evaluates the

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quality of protein structures and may be accessed at https://saves.mbi.ucla.edu. Values higher than 50 denote acceptable quality. It calculates an overall quality factor. By carefully examining their atomic connections and overall structural integrity, our procedure guarantees the accuracy of projected protein structures (Colovos & Yeates, 1993).

3.1.10. Comparison of the Wild and Mutant 3D Protein Structures

TM-align was used to superimpose and compare the wild-type and mutant protein structures in order to evaluate the differences between them. The programme TM-align, which can be accessed at https://zhanggroup.org/TM-align/, makes it easier to accurately align protein structures and provides values for Root Mean Square Deviation (RMSD) and Template Modelling scores (TM scores). TM scores indicate the degree of alignment and range from 0 to 1, with 1 denoting perfect alignment. On the other hand, greater dissimilarity between the wild-type and mutant proteins is indicated by larger RMSD values, which measure structural differences between them. In order to determine TM scores and RMSD values (Zhang & Skolnick, 2005), TM-align examined the protein models produced by trRosetta in PDB format. This allowed for the clarification of structural differences between the wild-type and mutant proteins.

3.1.11. Visualization of the Wild and Mutant Protein Models

PyMOL version 2.5.5 was used to display and evaluate structural changes in wild-type and mutant amino acid residues. PyMOL is highly regarded because to its strong features, which enable the investigation of different chemical aspects of the structures and visual representation of protein models. Researchers can use PyMOL to evaluate the fine features of protein structures, including conformational changes brought on by mutations, and to learn more about how these changes affect the activity of the protein (Jubb et al., 2017).

3.1.12. Analysis of Protein-Protein Interactions

Proteins participate in complex interactions that are essential for controlling basic biological functions; perturbations in these interactions can hasten the onset of disease. The Protein-Protein Interaction Prediction Tool STRING was used in this study to forecast protein-protein interactions (PPIs). By providing information in the form of interaction pathways, STRING https://string-db.org/ makes it easier to investigate the functional and structural relationships between different proteins. Researchers could access customized interaction pathways by entering the name of the protein and the organism. This allowed them to gain insights into the

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network of protein relationships that are essential for both cellular processes and disease mechanisms (Szklarczyk et al., 2011).

3.1.13. Gene-Gene Interaction Analysis

Deciphering gene-gene interactions is essential to understanding biological processes that are intricate. To analyze these interactions, a useful tool is GeneMANIA https://genemania.org/. It offers insights into the links between genes, including physical interactions, co-expression, and pathway associations, and it makes the prediction of gene function easier. GeneMANIA provides a thorough investigation of the interconnectivity of the WNT4, VEGFA, and MMP2 genes in the context of evaluating their interactions. Through the use of GeneMANIA, it is possible to identify shared pathways, regulatory mechanisms, and possible functional relationships between these genes. Understanding the molecular mechanisms behind a variety of physiological and pathological processes, including development, the course of disease, is made easier with the help of this study (Akhtar et al., 2019).

3.2. *In-vitro* Analysis

3.2.1. Study Participants and Sample Collection

Comparing patients with endometriosis and uterine fibroids to normal controls, the case-control study sought to understand the significance of WNT gene polymorphisms. Every participant provided written, informed consent. 30 normal controls, 30 people with uterine fibroids, and 30 people with endometriosis by clinical diagnosis made up the study's 90 human subjects. Nine months were spent gathering samples from the Research and Diagnostic Laboratory at ASAB, Holy Family Hospital Rawalpindi, Benazir Bhutto Hospital Rawalpindi, and the District Headquarter Hospital Rawalpindi.

3.2.2. The inclusion and exclusion criteria

The inclusion and exclusion criteria listed below in table 3.1. were achieved.

Table 3.1. Inclusion and Exclusion Criteria.

Criteria Type	Description
Inclusion Criteria	
Age Range	Participants aged 18-55 years
Gender	Female participants

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Health Status	Diagnosed with specific conditions (e.g., endometriosis, uterine fibroids)
Consent	Provided informed written consent
Location	Residing within the study's geographical area
Exclusion Criteria	
Age Range	Participants younger than 18 or older than 55 years
Health Status	Presence of other chronic diseases or conditions that may confound the study results
Pregnancy	Pregnant women
Recent Treatments	Participants who have undergone major surgery or treatments within the last 6 months
Incomplete Data	Incomplete medical records or missing essential health information
Non-Consent	Failure to provide informed written consent
Geographic Limitation	Residing outside the study's geographical area

3.3.3. Blood Sample Collection and Storage

The individuals' blood was drawn by hospital personnel into 3 ml EDTA tubes. The name, age, and collection date of each EDTA tube were appropriately labelled. The tubes holding the blood samples were brought to the lab in an icebox and kept in a refrigerator at 4°C.

3.3.4. Genomic DNA Extraction Using the Phenol-Chloroform Method

Whole blood samples were treated with phenol-chloroform extraction to obtain DNA. This two-day, low-cost technique is renowned for its dependability and good outcomes. Before being used, all plastic and glassware used in the DNA extraction procedure were carefully cleaned, autoclaved, and rinsed.

3.3.4.1. Preparation of Solutions for DNA Extraction

All of the solutions employed in the phenol-chloroform method of DNA extraction have the following compositions and purposes:

Table 3. 1. The solutions needed to extract DNA and their purposes.

Solution	Component	Molarity	Quantity	Function

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A	Sucrose	0.32 M	109.55 g	Facilitates the release of cell components, including DNA, by causing cell lysis and breakage of cell membrane
	Tris (pH 7.5)	10 mM	12.114 g	
	Magnesium Chloride	5 mM	0.476 g	
	Triton X-100 (1% V/V)	-	10 ml	
	Autoclaved Distilled Water	-	Up to 1000 ml	
B	Tris (pH 7.5)	10 mM	12.114 g	Results in the DNA precipitation and separation of the proteins
	Sodium Chloride	400 mM	23.37 g	
	Ethylene Diamine Tetra Acetic Acid (EDTA)	2 mM	0.58 g	
	Autoclaved Distilled Water	-	Up to 1000 ml	
C	Phenol	-	250 µl	Helps in the separation of DNA from protein and other cell debris by forming an aqueous layer
D	Chloroform	-	48 mL	Involved in DNA purification by stabilizing the coagulated proteins and reducing foaming
	Iso-amyl Alcohol	-	2 mL	
20% SDS	Sodium Dodecyle Sulphate (SDS)	20% w/v	20 g	Involved in denaturation of proteins and lipids, separating them from DNA
	Autoclaved Distilled Water	-	Up to 100 ml	
3M Sodium Acetate	Sodium Acetate	3 M	12.3 g	Involved in precipitation of DNA by neutralizing the negative charges on phosphate backbone

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Autoclaved Distilled Water	-	Up to 50 ml	

3.3.4.2. Reagents for DNA Extraction

The following are the additional chemicals needed for the phenol-chloroform DNA extraction procedure, along with an explanation of their purposes:

Table 2.3. Chemicals needed to extract DNA and how they work.

Reagent	Function
Proteinase K	Releases DNA by digesting proteins.
Isopropanol	Dissolves the solvation shell of DNA to precipitate it.
98% Ethanol	Helps to precipitate the DNA.
PCR Water	Stops the deterioration of DNA through storing.

3.3.4.3. The DNA Extraction Protocol

Day 1:

1. The blood in the EDTA tube should be allowed to come to room temperature and properly mixed by repeatedly flipping the tube. After transferring 750 µl of the blood into a 1.5 ml microcentrifuge tube, add 750 µl of Solution A in equal measure.
2. After four to six inversions, leave the microcentrifuge tube at ambient temperature for ten minutes.
3. The mixture should be centrifuged for ten minutes at 13,000 rpm.
4. After disposing of the supernatant with caution, gently resuspend the nuclear pellet in 400 µl of Solution B.
5. Use constant tapping or gentle vortexing to fully dissolve the nuclear pellet in Solution B.
6. Using 13,000 rpm for 10 minutes, centrifuge the mixture once more.
7. Remove the supernatant and proceed to add 400 µl of Solution B, 15 µl of 20% SDS solution, and 8 µl of Proteinase K to the microcentrifuge tube.
8. Mixture should be incubated for one night at 37°C.

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Day 2:

1. Make a fresh mixture in a microcentrifuge tube with 250 µl of each of the solutions C and D, then add it to the tube that has been incubated for the entire night.

2. The resultant mixture should be centrifuged for ten minutes at 13,000 rpm.

3. Move the DNA-containing aqueous layer to a fresh microcentrifuge tube.

4. After the aqueous layer has been separated, add 500 µl of Solution D and centrifuge for 10 minutes at 13,000 rpm.

5. Once again, transfer the aqueous layer to a fresh microcentrifuge tube and fill it with 500 µl of isopropanol and 55 µl of 3M sodium acetate solution.

6. After repeatedly flipping the tube to precipitate the DNA, centrifuge at 13,000 rpm for 10 minutes.

7. After discarding the supernatant, fill the tube holding the DNA pellet with 200 µl of chilled 98% ethanol.

8. Centrifuge at 13,000 rpm for 8 minutes.

9. After letting the ethanol air dry, mix the DNA pellet with 100 µl of PCR water, and keep it chilled at -20°C.

3.3.5. DNA Gel Electrophoresis

The isolated DNA was subjected to a 2% (w/v) agarose gel electrophoresis to evaluate its purity. The following describes how to prepare the buffer solutions and reagents needed for the gel electrophoresis:

3.3.5.1. EDTA Solution, 0.5 M

One can make a 0.5 M EDTA solution by dissolving 186.12 grams of EDTA in 1000 ml of distilled water. When making TAE buffer, which is used in gel electrophoresis, this solution is necessary. A pH of 8 should be maintained in the 0.5 M EDTA solution.

3.3.5.2. 50X Tris Acetate Ethylene-diamine-tetra-acetic acid (TAE) Buffer

The following steps were taken to prepare a 50X stock solution of Tris Acetate Ethylene-di-amine-tetra-acetic Acid (TAE) Buffer:

Table 3.3. Ingredients needed to prepare the TAE buffer.

Component	Quantity
Tris Base	242 g

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0.5 M EDTA Solution	100 ml
Glacial Acetic Acid	57.1 ml
Deionized Water	Up to 1000 ml

The 50X TAE buffer was made to have a pH between 8.2 and 8.4. The buffer was autoclaved after preparation and kept at room temperature.

3.3.5.3. 1X Tris Acetate Ethylene-diamine-tetra-acetic acid (TAE) Buffer

In order to create a 1X TAE buffer solution, 980 ml of distilled water was mixed with 20 ml of the 50X TAE stock solution.

3.3.5.4. Protocol

1. Two grams of agarose were measured and added to 100 ml of 1X TAE buffer using an electronic balance.
2. After that, the agarose mixture was cooked for around two minutes in a microwave oven in order to dissolve it.
3. To stain the DNA, 4 µl of ethidium bromide was added when the mixture had somewhat cooled.
4. After the gel solution was added to a gel casting tray, it was left to set at room temperature and combs were used to create wells.
5. After the gel formed, it was put in an electrophoresis tank with 1X TAE buffer in it.
6. Each DNA sample was loaded onto the gel wells by combining 6 µl of extracted DNA with 2 µl of loading dye.
7. Additionally, a 1 Kbp DNA ladder was put into a well to be used as a guide when measuring the lengths of DNA samples.
8. For thirty minutes, gel electrophoresis was run at 80 volts.
9. The gel was subjected to DNA band analysis and comparison with a DNA ladder using a UV trans-illuminator and ChemiDoc system after electrophoresis was finished.

3.3.6. DNA Quantification

Using the ThermoScientific the NanoDrop 2000 UV-Vis Spectrophotometer and the NanoDrop 2000™ software, the extracted DNA samples were quantified. First, as a blank, 1 µl of PCR water was put on the pedestal. Next, 1 µl of the DNA sample was used to measure the standard

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absorbance for DNA quantification at 260 nm wavelength. The absorbance ratio at 260/280 nm was used to measure the purity of DNA; an ideal ratio of roughly 1.8 indicated pure DNA.

3.3.7. Designing of Primer

To enable allele-specific Amplification Refractory Mutation System PCR (ARMS-PCR), primers were specifically created for WNT-4 gene variant for *In-vitro* analysis. SNP sequence was obtained from NCBI, and SNP was given a set of three primers that were created in accordance with predefined standards, as detailed in the experimental technique.

Table 3.4. ARMS PCR-specific primers.

Gene	SNP	Variation	Primer Sequence (5' to 3')	Tm	GC Content	Product Size
WNT 4	rs121908651	G>A	FORWARD PRIMER WILD: **CACACCTGCCGAAGAGATGG** FORWARD PRIMER MUTANT: **CACACCTGCCGAAGAGATGA** REVERSE PRIMER: **TCTGCCTGTCTTGCTCCCTC**	55.9	60	173

The primer properties, such as GC concentration, melting temperatures (Tm), hairpin formation, and self-complementarity, were examined using the software OligoCalc. UCSC in-silico PCR software and Primer-BLAST were used to determine primer specificity, making it easier to identify amplicon sizes and exact primer binding positions.

3.3.8. Preparation of Working Dilutions of Primers

The primers that were supplied were lyophilized, which meant that each primer required a 100 µM stock solution. The nmol of the primer multiplied by 10 was used to determine the volume of nuclease-free water added for reconstitution. After that, in order to reduce the chance of contamination and freeze-thaw cycles, working dilutions of the primer stock were made. In a sterile microcentrifuge tube, 10 µl of the stock primer was diluted with 90 µl of nuclease-free water.

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3.3.9. Allele Specific Amplification Refractory Mutation System PCR (ARMS-PCR)

On extracted DNA samples, Allele-Specific Amplification Refractory Mutation System PCR (ARMS-PCR) was used to confirm the existence of SNPs. Two distinct PCR reactions were needed for each SNP, with a similar primer being used in each. A wild type primer was used in one reaction and a mutant primer in the other. The corresponding SNP was present, according to amplification. A 20 µl reaction mixture was included in each PCR tube, as shown in Table 3.6

Table 3.5. PCR reaction mixture components.

Component	Quantity
PCR Water	12 µl
Forward Primer	1 µl
Reverse Primer	1 µl
DNA Template	2 µl
PCR Master Mix	4 µl
Total Volume	**20 µl**

A biosafety cabinet was used to carefully prepare the reaction mixture in order to reduce the possibility of contamination. To prevent the DNA polymerase enzyme from activating too soon, it was kept on ice. After preparation, a quick centrifugation step was performed on the mixture to remove any trapped air bubbles and guarantee uniform homogeneity. Then, in order to aid in amplification, the PCR tubes holding the reaction mix were heated in a thermocycler. Gradient PCR was utilized for the first optimization of primers, guaranteeing accurate conditions for the subsequent amplification of sample DNA to identify single nucleotide polymorphisms (SNPs).

The optimized conditions for PCR reaction for Wnt-4 (rs121908651) are as follow:

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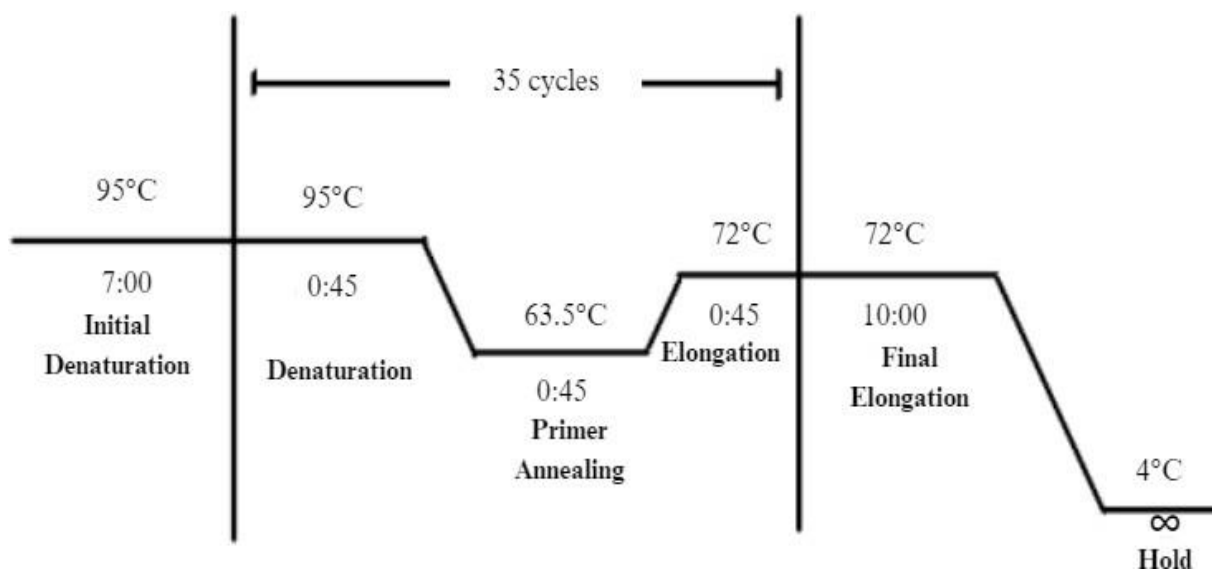
Figure 3.2. Schematic representation of PCR Profile of WNT-4 wild (rs121908651)
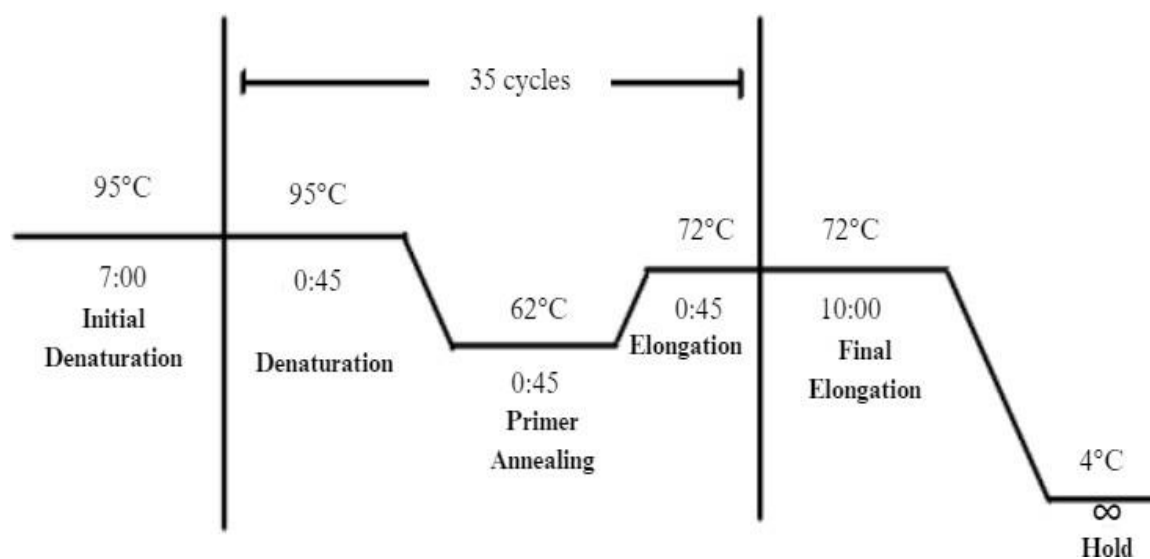
Figure 3.3. Schematic representation of PCR Profile of WNT-4 mutant (rs121908651)

3.3.10. Gel Electrophoresis for Analysis of PCR Products

Using 2% gel electrophoresis and the previously described methodology, the ARMS PCR products were subjected to further examination. The gel was electrophoresed for 45 minutes at 80 volts this time, and a 50 bp ladder was used. After that, a UV trans-illuminator and

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ChemiDoc system were used to visualize the gel. A thorough examination and comparison with the ladder were performed on the resultant PCR products to ensure accuracy.

3.3.11. Statistical Analysis

The statistical study was conducted using version 10 of GraphPad Prism. The Chi-square test ($\chi 2$) was used to evaluate the relationship between single nucleotide polymorphisms (SNPs) and endometriosis risk after allele and genotype frequencies were calculated. The following significance thresholds were applied: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

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Chapter 4. Results

4.1. Retrieving nsSNPs out of the NCBI-SNP database

A precise genetic investigation revealed that the dbSNP database contained 10,754 SNPs in the WNT4 gene, 7,726 SNPs in the VEGF A gene, and 12,140 SNPs in the MMP2 gene. Of these, there were synonymous coding differences for 214 SNPs in WNT4, 262 in VEGF A, and 324 in MMP2. On the other hand, 563 missense mutations were found in the VEGF A gene, 591 in the MMP2 gene, and 360 in the WNT4 gene. Additionally, it was found that intronic and other areas contained 10,180 SNPs from the WNT4 gene, 6,901 from the VEGF A gene, and 11,225 from the MMP2 gene. Given their possible impact on protein function, missense non-synonymous SNPs in all genes were given particular attention for the investigations that followed. The goal of the subsequent functional and clinical relevance analysis of missense SNPs was to clarify their functions in different genetic and disease situations.
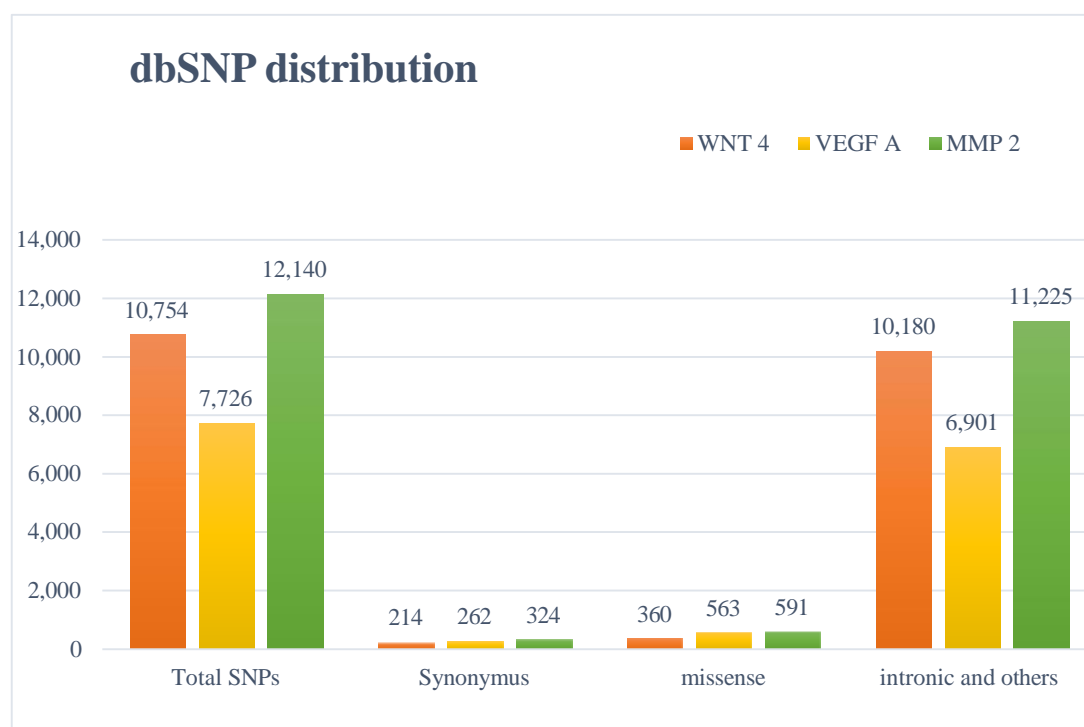
Figure 4.1. Distribution of different types of SNPs of genes WNT-4, VEGF-A, and MMP-2.

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4.2. Deleterious SNPs

We employed the SIFT method to identify potentially harmful single nucleotide polymorphisms as the initial stage of our thorough investigation. Out of the 563 missense SNPs for the VEGF-A gene, 135 have been identified as harmful after using the SIFT method. Of the 360 missense SNPs found for the WNT4 gene, 36 were shown to be harmful. Similar findings were seen for the MMP2 gene, where 112 of the 591 missense SNPs were identified as possibly harmful. By combining the usage of SIFT and PolyPhen tools, this initial choice was substantially improved. When both methods were used in tandem, 11 harmful SNPs for the VEGF-A gene, 13 for the WNT4 gene, and 30 for the MMP2 gene were filtered out for further analysis. The selection procedure was improved by additional research employing the SNP-GO tool, which identified variants in the VEGF-A, WNT4, and MMP2 genes that may be pathogenic. This method identified functional annotations indicating disease causation for 5 SNPs in VEGF-A, 7 SNPs in WNT4, and 15 SNPs in MMP2. Additionally, PROVEAN analysis showed 3 significant SNPs in the VEGF-A gene, 14 significant SNPs in the MMP2 gene, and 7 significant SNPs in the WNT4 gene. Panther analysis further validated the detrimental nature of all SNPs previously discovered. Ultimately, 3 SNPs in VEGF-A, 7 SNPs in WNT4, and 12 SNPs in MMP2 were further confirmed to be harmful by Predict-SNP. This provided more evidence of their possible influence on protein function and significance in the context of genetics and pathology.

These missense SNPs that were filtered out of six in silico approaches and then sent to Structural and Functional Impact methods for additional validation.
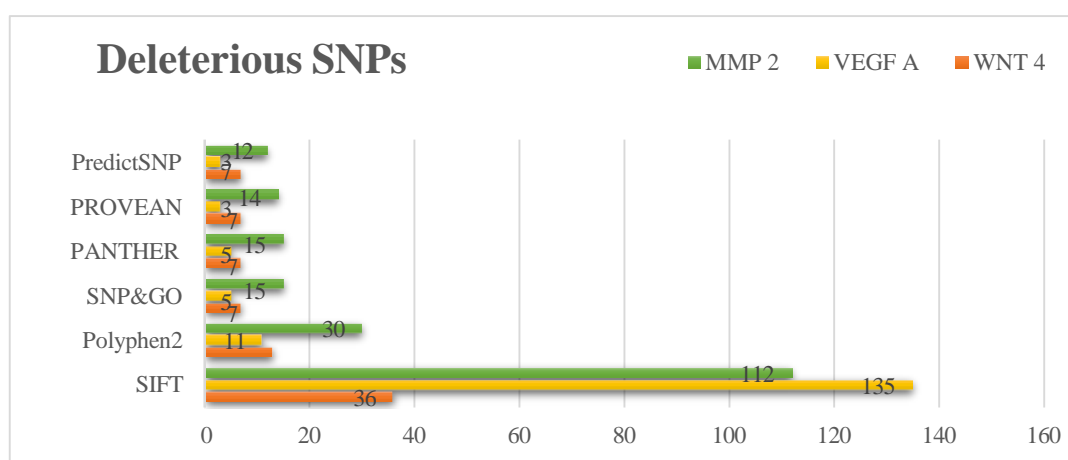
Figure 4.2. Results of analysis of number of missense SNPs of WNT-4, VEGF-A, and MMP-2 by six different computational tools.

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4.3. Analysis of the Structural and Functional Effects of deleterious Missense SNPs

The VEGF-A gene's MutPred2 study indicates that the G84R change has a score of 0.727, indicating that it is probably detrimental to mechanisms such as disulfide linkage and metal binding. The C86Y mutation affects disulfide linkage, metal binding, transmembrane protein structure, and has a higher score (0.929) that strongly supports pathogenicity. The R108W alteration largely affects intrinsic disorder and protein stability, and even with a lesser score of 0.531, it still suggests a possible negative impact.

Numerous high-impact missense substitutions with high pathogenicity scores which are suggestive of significant potential negative impacts on protein function are included in the MutPred2 analysis for the WNT4 gene. Interestingly, missense R86H (0.923) and G194S (0.946) are implicated in disulfide linkage formation, transmembrane protein structure, and metal binding. R83W (0.881) and A114V (0.917), for example, alter structural components and protein interactions. Furthermore, several ELM motifs and protein structures, such as disordered and ordered interfaces, catalytic and allosteric sites, and glycosylation, are impacted by R83Q (0.836), P225L (0.842), and G293S (0.897). These mutations raise the possibility of disruptions in WNT4-related pathways, which could affect the stability, structure, and function of proteins as well as cause dysregulation and pathogenic phenotypes.

The MMP-2 MutPred analysis indicate that different substitutions have different effects on protein function. For example, substitution R101H has a score of 0.886 for MutPred2, affecting motifs ELME000270, ELME000276, and PS00546. This has an effect on metal binding and catalytic sites in the molecular mechanisms. Substitution R146H has a score of 0.508 for MutPred2, affecting the motif ELME000328, which has an effect on ordered interfaces and DNA binding. Substitution V154M, with a score of 0.585, affects motifs ELME000063, ELME000136, ELME000153, and ELME000159, resulting in an effect on different protein interactions. Substitution G330D has a score of 0.923, affecting disordered interfaces, metal binding, and transmembrane protein motifs, among other things. With a score of 0.904, substitution C332F affects the transmembrane protein, metal binding, and disulfide linkage motifs. With an inherent disorder score of 0.832, substitution W374S affects the transmembrane protein and ordered interface. The transmembrane protein, metal-binding motifs, and ordered interface are all impacted by the S396R mutation, which has a score of 0.881. With a score of 0.9, the E404K alteration had an impact on catalytic sites and metal

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binding. A score of 0.916 was assigned to substitution G406D, which had an impact on helix formation and metal binding. The R482C mutation changed metal binding, ordered interface motifs, and relative solvent accessibility, with a score of 0.829. Transmembrane proteins and metal-binding motifs were among the areas that were impacted by the R495W alteration, which received a score of 0.888. MutPred2 scored 0.82 for substitution Y543C in MMP2. It influenced metal binding, changed ordered and disordered interfaces, and reduced relative solvent accessibility. These modifications are significant because they raise the possibility that SNPs may impact MMP2 protein activity and influence the development or susceptibility of disease.

4.4. Protein Stability Prediction

I-Mutant and MuPro, two bioinformatic tools, were employed in this study to examine the impact of certain SNPs on protein stability. Based on both techniques, we were able to derive a cohesive result from this study that indicated that the three SNPs in the VEGF-A gene that were found in the previous step all had a destabilizing effect on protein stability.
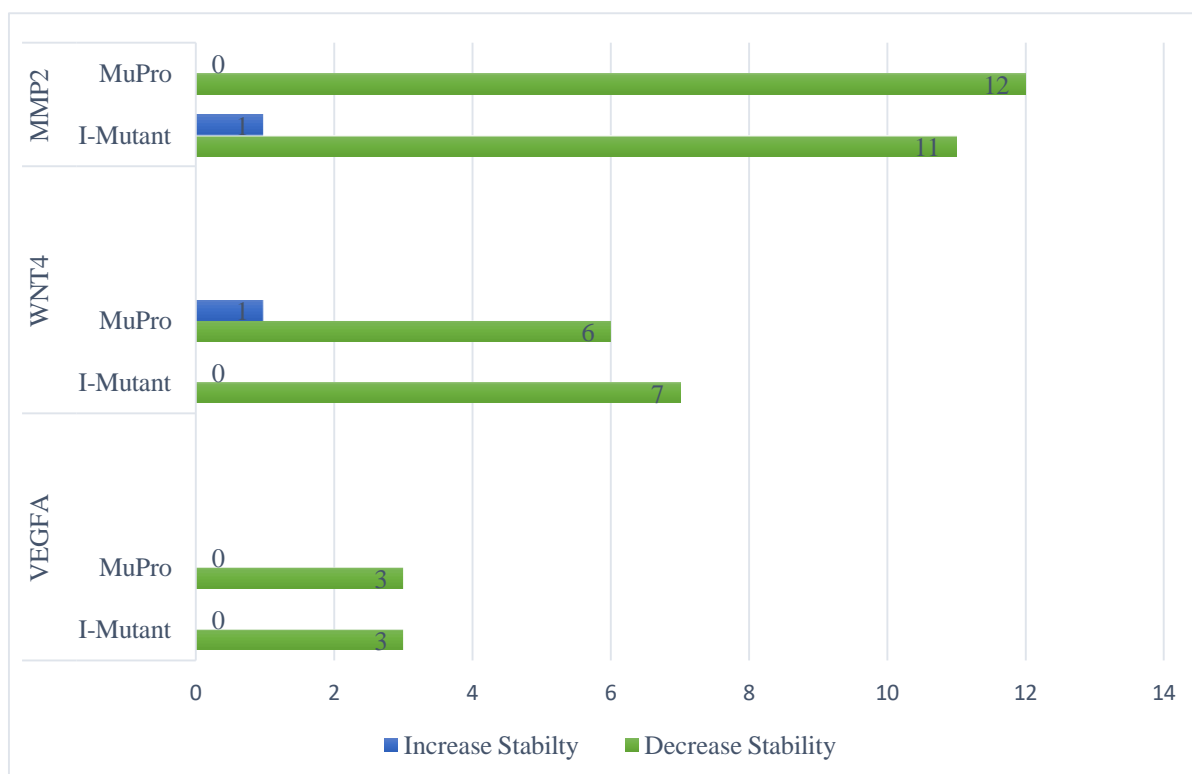
Figure 4.3. Number of SNPs by I-mutant, and MuPro.

Similarly, for the SNPs in the WNT-4 gene, a reduction in protein stability was computed by all platforms, yielding 6, SNPs with similar destabilizing effect. Moreover, the similar pattern is shown when SNPs within the MMP-2 gene are examined; 11, SNPs consistently show

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causing instability effects on protein stability based on both computational methods. Consequently, a thorough use of I-Mutant and MuPro highlights the disrupting impact of SNPs in the VEGF-A, WNT-4, and MMP-2 genes on protein stability, as demonstrated by the consistent prediction of destabilization across many computational studies. Robust evidence is provided by this comprehensive approach about the impact of genetic variants in destabilizing protein structure and function.

4.5. PTM Analysis

We found multiple post-translational modification (PTM) sites in the wild-type WNT4 protein through our careful in-silico study. The locations 284–288 and the N–glycosylation sites at 28 and 237, as well as the phosphorylation sites at 129, 251, and 276, were particularly noteworthy. Positions 235, 237, and 263 were also identified as ubiquitination sites. It appears that the mutations did not cause any appreciable changes in PTM patterns because comparisons between wild-type and mutant WNT4 proteins did not reveal any notable alterations in these PTM positions.

Table 4.1. PTM Analysis of WNT-4 selected SNPs.

SNP	AA CHANGE	SUMO	N-GLY	O-GLY	MET	PHO	UBI
RS202139199	G293S	-	-	-	-	-	-
RS369386711	G194S	-	-	-	-	-	-
RS121908651	A114V	-	-	-	-	-	-
RS34644882	R86H	-	-	-	-	-	-
RS368808392	R83Q	-	-	-	-	-	-
RS121908652	R83W	-	-	-	-	-	-
WILD	284-288		28, 287	No site	313	129, 251, 276	235, 237, 263

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Comparably, several PTM sites were identified by VEGF-A study. There was an N-glycosylation site at position 41 and SUMOylation at sites 14 and 50. There were methylation sites at position 145 and phosphorylation sites at locations 143, 162, and 164. It's interesting that the VEGF-A sequence did not have any ubiquitination sites according to our expectations. The SUMOylation site at position 14 was absent from the G84R mutant version, and no further alterations to PTM sites were seen in relation to the other mutant forms.

Table 4.2. PTM Analysis of VEGF-A SNPs selected in the previous step.

SNP	AA CHANGE	SUMO	N-GLY	O-GLY	MET	PHO	UBI
RS368256497	G84R	14 site removed	-	-	-	-	-
RS374420337	C86Y	-	-	-	-	-	-
RS114262569	R108W	-	-	-	-	-	-
WILD TYPE		14, 50	41	No site	145	143, 162, 164	No site

Several important PTM sites were found during our analysis of MMP2. Positions 160–164, 579, 299, 594, 210, 164, 127, and 379 were specifically identified as having SUMOylation. Positions 132, 155, 434, 448, 458, 465, and 652 were shown to have phosphorylation sites. Positions 62, 187, 439, 470, and 639 were shown to have ubiquitination sites, and position 62 had a methylation site. Interestingly, wild-type MMP2 was expected to have neither O-glycosylation nor N-glycosylation sites. Mutant study revealed distinct changes in PTMs: The R482C mutant lacked the ubiquitination site at position 439, while the R146H mutant lacked phosphorylation at sites 132 and 155. The W374S, G406D, and G330D mutants all showed evidence of O-glycosylation at position 401.

These findings provide a comprehensive overview of PTMs in the WNT4, VEGF-A, and MMP2 proteins. They also clearly demonstrate the significance of these alterations in protein activities and the impact of specific mutations on PTM patterns

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Table 4.3. PTM Analysis of MMP-2 SNPs.

SNP	AA CHANGE	SUMO	N-GLY	O-GLY	MET	PHO	UBI
RS121912953	R101H	-	-	-	-	-	-
RS148810689	V154M	-	-	-	-	-	-
RS368282133	R146H	-	-	-	-	132, 155 site removed	-
RS111609606	C332F	-	-	-	-	-	-
RS150524555	W374S	-	-	41 site added	-	-	-
RS121912955	E404K	-	-	-	-	-	-
RS121908741	G406D	-	-	41 site added	-	-	-
RS121908741	G330D	-	-	41 site added	-	-	-
RS151265434	R482C	-	-	-	-	-	439 site removed
RS141033596	R495W	-	-	-	-	-	-
RS138709475	Y543C	-	-	-	-	-	-
WILD		160-164, 579, 299, 594, 210, 164, 127, 379	No site	No site	62	132, 155, 34, 448, 458, 465, 652	187, 439, 470, 639

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4.6. Conservational Analysis

ConSurf was used to analyze the amino acid sequences of VEGFA, WNT4, and MMP2 in order to determine their evolutionary conservation. For every amino acid site, ConSurf assigns a score
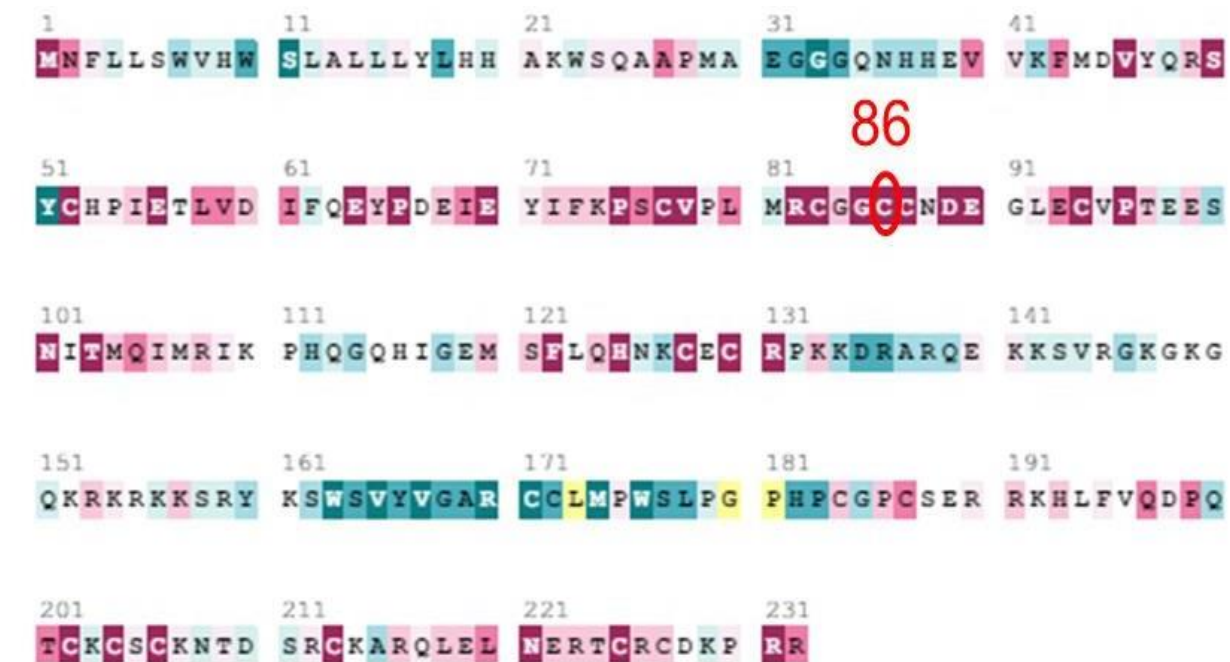
Figure 4.4. ConSurf prediction for deleterious missense SNPs of VEGF-A.

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between 1 and 9, with 1 denoting less conservation and 9 denoting strong conservation. One of the three SNPs in VEGFA, C86Y, is located within the highly conserved region (9). Similarly, three of the six SNPs in WNT4, A114V, G194S, and R86H were found to be located in areas that are extremely conserved. Comparably, high conservation areas are home to seven of the eleven SNPs in MMP2: C332F, E404K, G330D, G406D, R101H, R146H, and W374S.

When SNPs are found in areas that are highly conserved, there is a greater chance that these variants will be harmful than when they are found in areas that are less conserved. This is because of the fact that changes made to regions with high conservation are more likely to change the stability and function of proteins since they are more likely to be structurally and functionally significant. Our results highlight the significance of using evolutionary conservation analysis as a prediction method when assessing the functional implications of SNPs.

Figure 4.5. ConSurf prediction for deleterious missense SNPs of WNT-4.

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Figure 4.6. ConSurf prediction for deleterious missense SNPs of MMP-2.

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4.7. Analysis of 3D Protein Structure

With the use of the ERRAT tool, the quality of the wild-type and mutant protein models created by trRosetta was confirmed. An acceptable score is one that is greater than 50 in the overall quality factor calculated by ERRAT. After that, the wild-type and mutant structures were compared using TM-align, which produced the Root Mean Square Deviation (RMSD) counts. From 0 to 5, the RMSD values are used to indicate how far the mutant structure deviates from the wild-type structure. Table 4 displays all model ERRAT scores and RMSD values. A score of ERRAT more than 80 was obtained by both the wild-type and mutant structures that were developed, indicating protein structures of higher quality.

Table 4.4. ERRAT score, TM-score and RMSD values of wild and mutant structures of VEGF-A, WNT-4, and MMP-2.

Genes	Model	ERRAT Confidence Score	TM-Score	RMSD Value
VEGF-A	Wild	89.4118	-	-
	C86Y	82.8402	0.4672	5
WNT-4	Wild	93.5484	-	-
	A114V	90	0.99154	0.66
	G194S	93.4426	0.98374	1.02
	R86H	94.4262	0.99107	0.69
MMP2	Wild	90.2362	-	-
	C332F	84.9765	0.73832	4.53
	E404K	84.7154	0.67483	2.64
	G406D	85.3618	0.6634	1.78
	R101H	87.0662	0.67201	1.57
	R146H	86.3142	0.74731	4.32
	G330D	88.6115	0.72008	4.2
	W374S	87.5	0.7046	2.43

4.8. Structure Visualization

PyMOL was used to superimpose the models in order to better clarify the structural variations between the wild-type and mutant proteins. Through this procedure, particular alterations in amino acids and the ensuing structural differences between the two forms could be seen. All three genes have overlaid structures that clearly show the changes in amino acid residues, making it easy to compare the mutant and wild-type configurations. The resulting stacked structures are shown in the figure, which clearly illustrates how the mutant and wild-type proteins differ from one another.
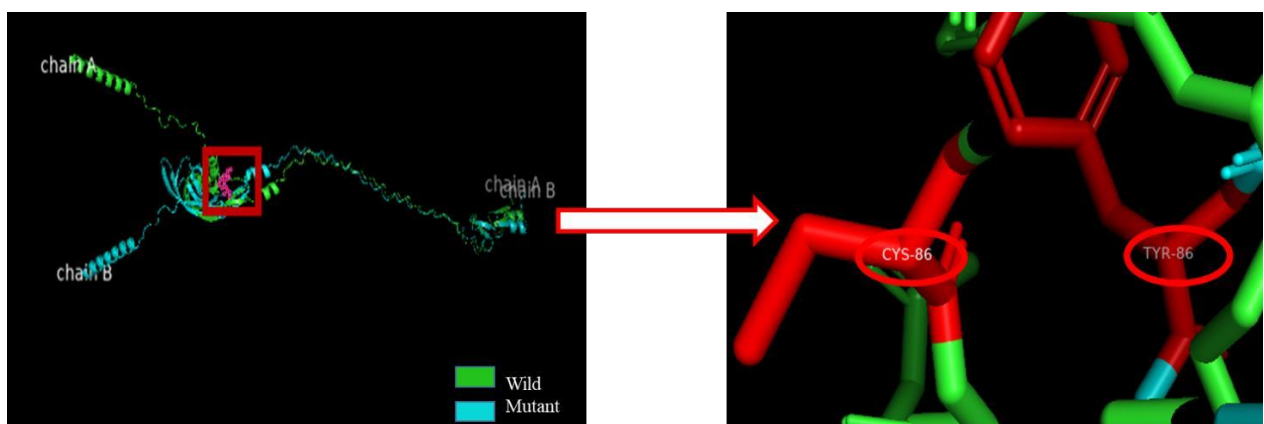
Figure 4.7. Superimposition of VEGF-A wild and mutant C86Y and Highlighted amino acid change from cysteine (wild) to Tyrosine (mutant).
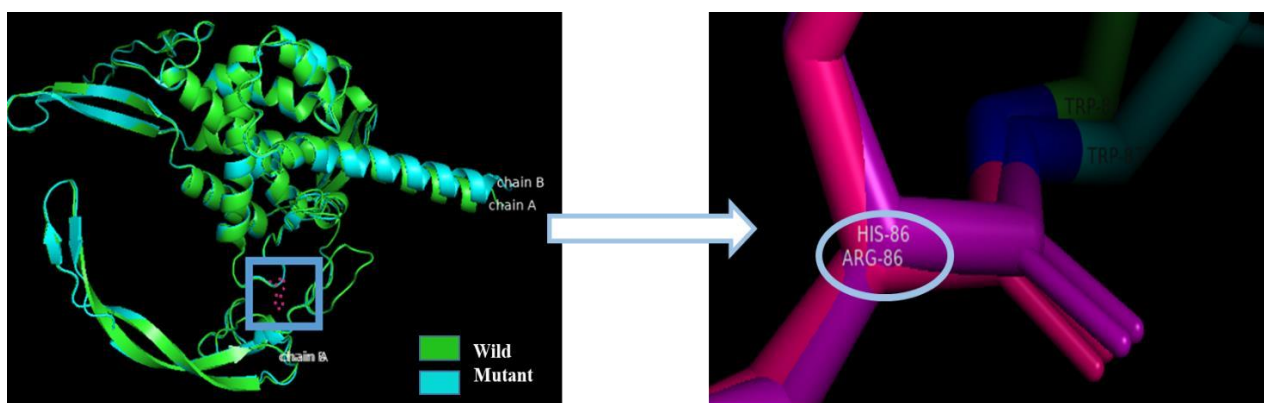
Figure 4.8. Superimposition of WNT-4 wild and mutant R86H and Highlighted amino acid change from arginine(wild) to histidine (mutant).

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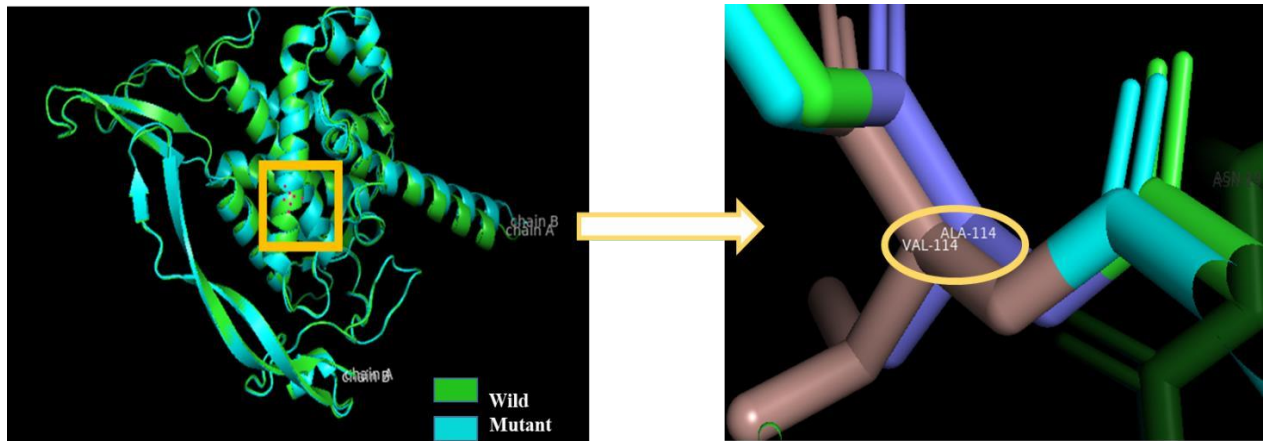
Figure 4.9. Superimposition of wnt-4 wild and mutant A114V and Highlighted amino acid change from Alanine (wild) to Valine (mutant).
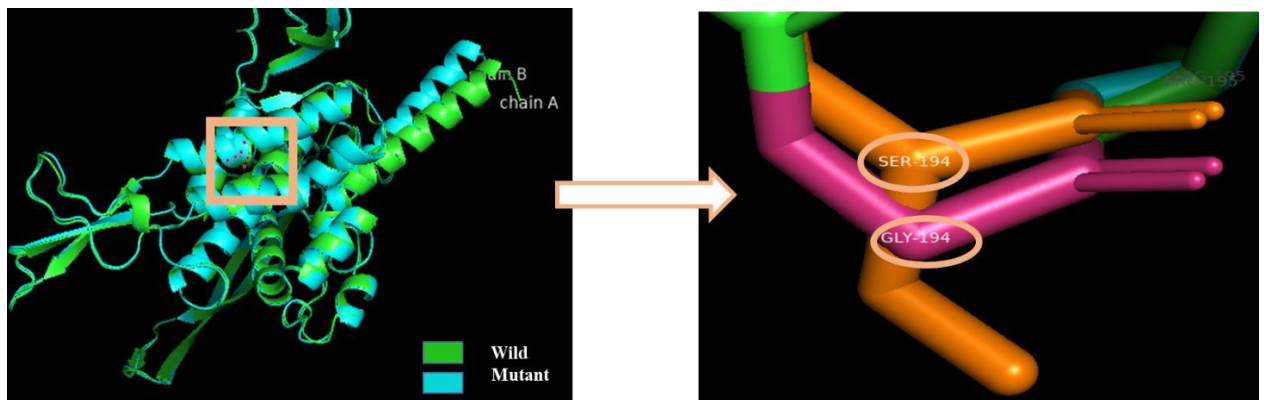
Figure 4.10. Superimposition of WNT-4 wild and mutant G194S and Highlighted amino acid change from Glycine (wild) to Serine (mutant).
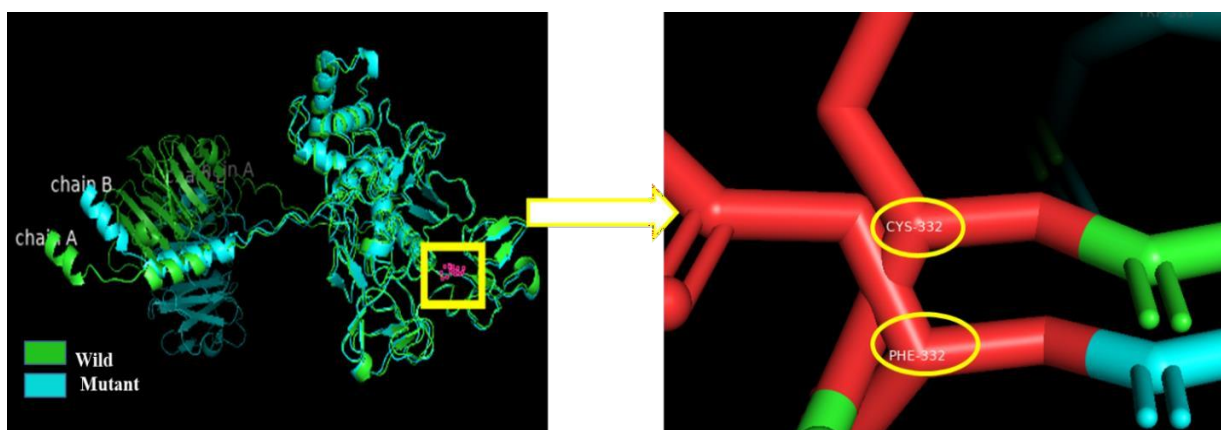
Figure 4.11. Superimposition of MMP-2 wild and mutant C332F and Highlighted amino acid change from Cysteine(wild) to Phenylalanine (mutant).

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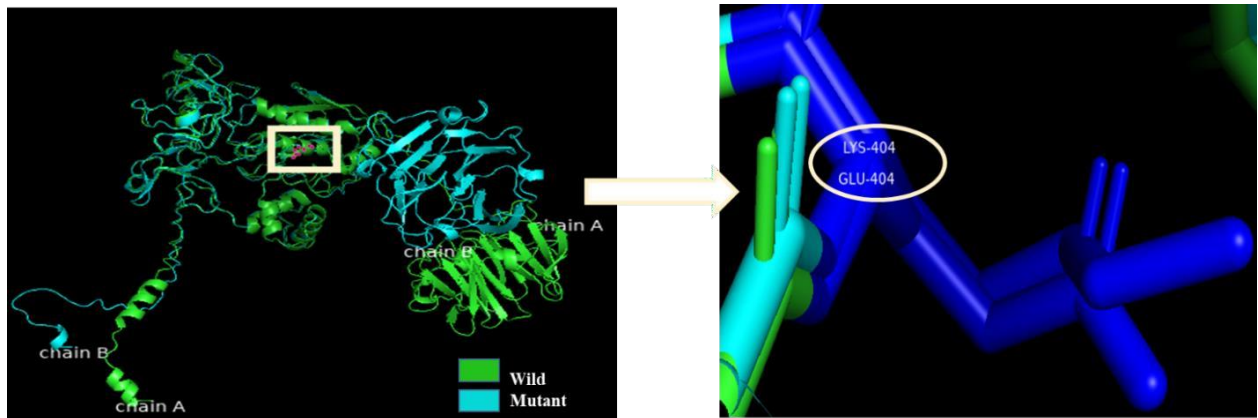
Figure 4.12. Superimposition of MMP-2 wild and mutant E404K and Highlighted amino acid change from Glutamic acid (wild) to Lysine (mutant).
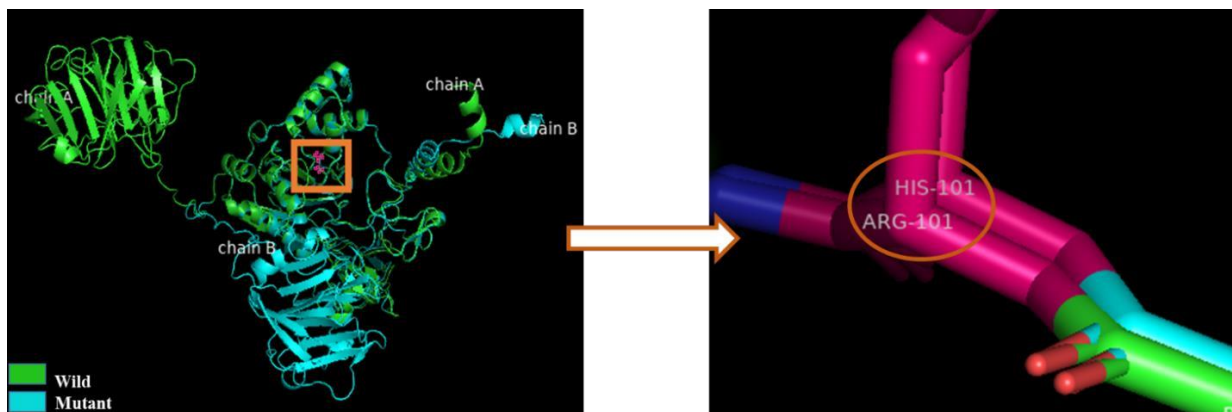
Figure 4.13. Superimposition of MMP-2 wild and mutant R101H and Highlighted amino acid change from Arginine (wild) to Histidine (mutant).
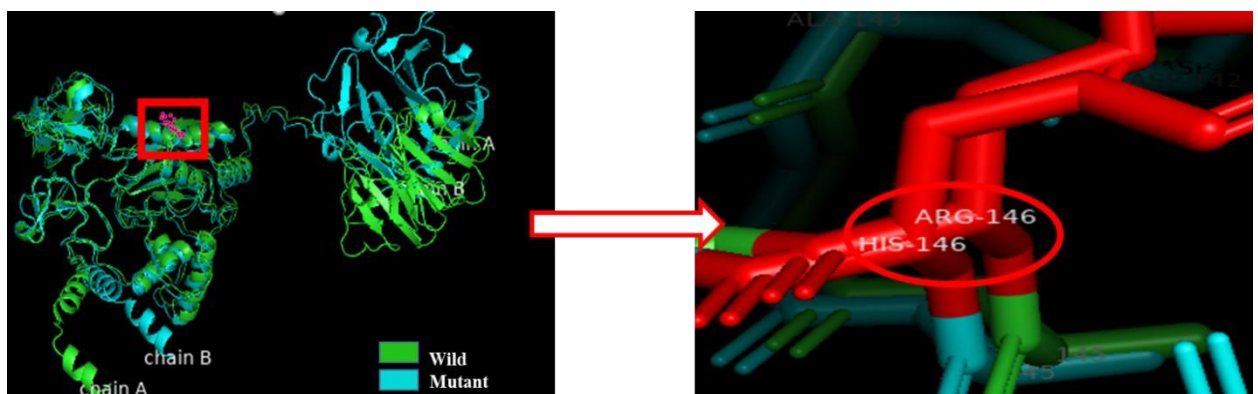
Figure 4.14. Superimposition of MMP-2 wild and mutant R146H and Highlighted amino acid change from Arginine (wild) to Histidine (mutant).

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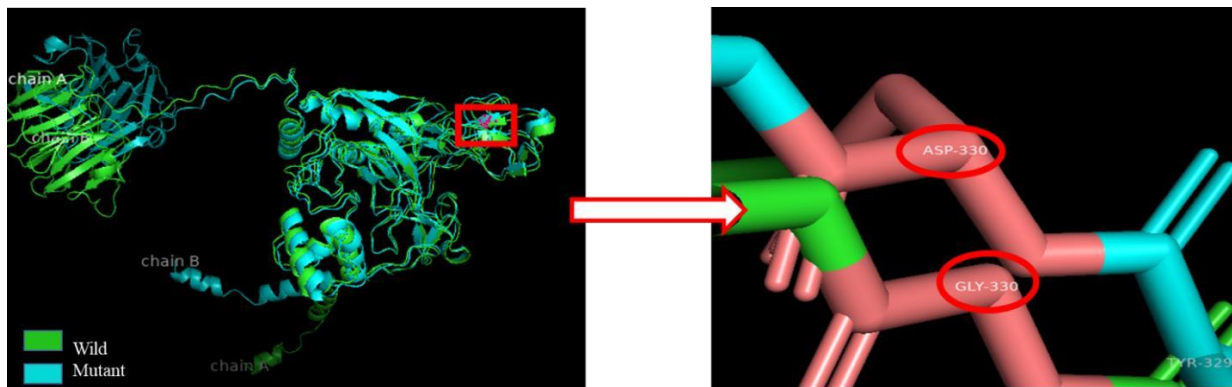
Figure 4.15. Superimposition of MMP-2 wild and mutant G330D and Highlighted amino acid change from Glycine (wild) to Aspartic acid (mutant).
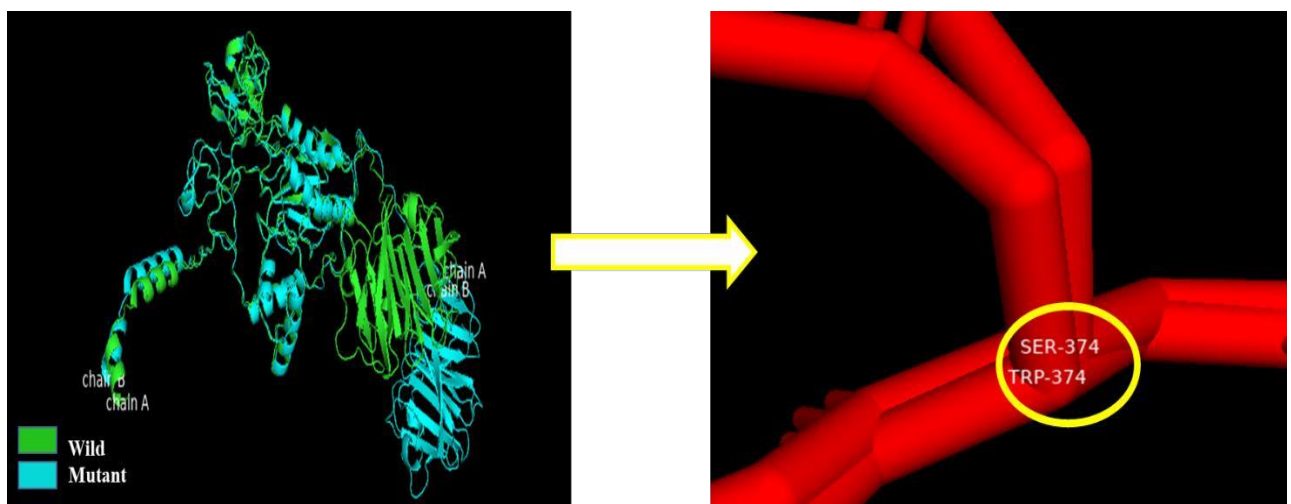
Figure 4.16. Superimposition of MMP-2 wild and mutant W374S and Highlighted amino acid change from Tryptophan (wild) to Serine (mutant).

4.9. Protein-Protein Interaction

The STRING database, a potent resource for forecasting protein-protein interactions, was used to study the interactions of the WNT-4 and MMP-2 proteins with numerous other proteins. Since they govern different pathways, protein-protein interactions are vital for many vital biological processes. Numerous diseases may result from these relationships being disrupted. As seen in Figure 4, STRING analysis predicted that WNT-4 interacts with ten more proteins. With an interaction score of 0.987, WNT-4 and FZD3 showed the strongest interaction of all of them. Further interactions with FZD6, FZD2, WIF1, FZD4, FZD5, FZD9, FZD8, PORCN, and SFRP1 were found, demonstrating the wide range of interactions in which WNT-4 is involved.

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Likewise, MMP-2 study showed interactions with 10 other proteins. With an interaction score of 0.999, which denotes a highly significant interaction, TIMP2 was found to have the strongest interaction.

It should be mentioned, though, that VEGF-A interaction data were not present in the STRING database, making a comparable analysis of this protein unfeasible. The significance of protein-protein interactions in preserving biological function and the possible consequences of their disturbance in disease settings are highlighted by these results.
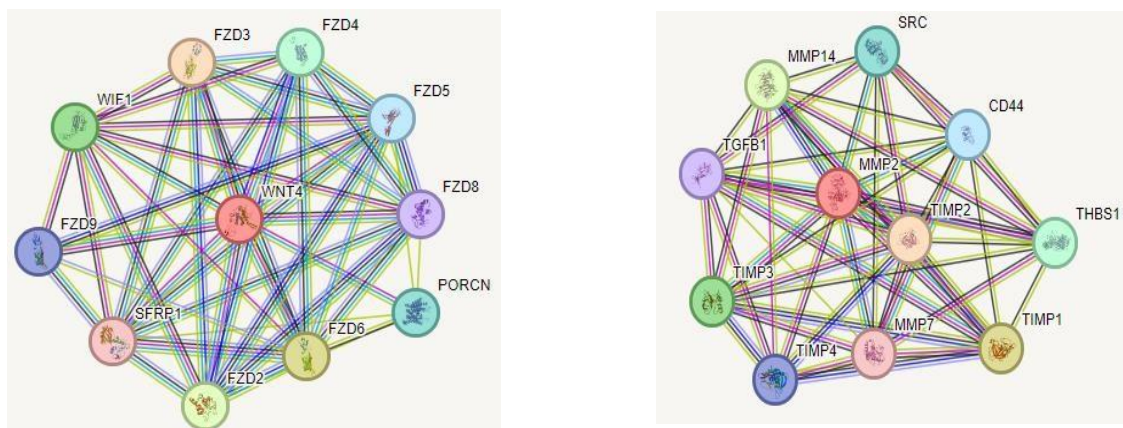
Figure 4.17. a). Protein-protein interaction of WNT-4 predicted by STRING, b) Protein-protein interaction of MMP-2 predicted by STRING.

4.10. Gene-Gene Interaction

To decipher the complex network of gene-gene interactions involving the essential triad of WNT4, VEGFA, and MMP2 genes, GeneMANIA was employed. With this methodology, we were able to identify a complex co-expression landscape among the genes in the network, which is clearly illustrated in Figure 4. The unique expression patterns of WNT9B, WNT8A, WNT9A, and PAK4 were noteworthy exceptions. On the other hand, a group of genes, which included NRP1, NRP2, KDR, PGF, MMP2, ARNT, and BACE1, clearly displayed consistent co-localization patterns, suggesting that they might be involved in functional connections.

Furthermore, the identification of genetic relationships between every gene in the network, except for TIMP2, VEGFB, SHC2, WNT8B, WNT8A, WNT9A, and WNT16, highlights the complex interactions and mutual dependency among the chosen genes. Because of their interdependence, genes may be affected by changes in another gene, which could have an effect on the physiological landscape. This suggests a delicate equilibrium.

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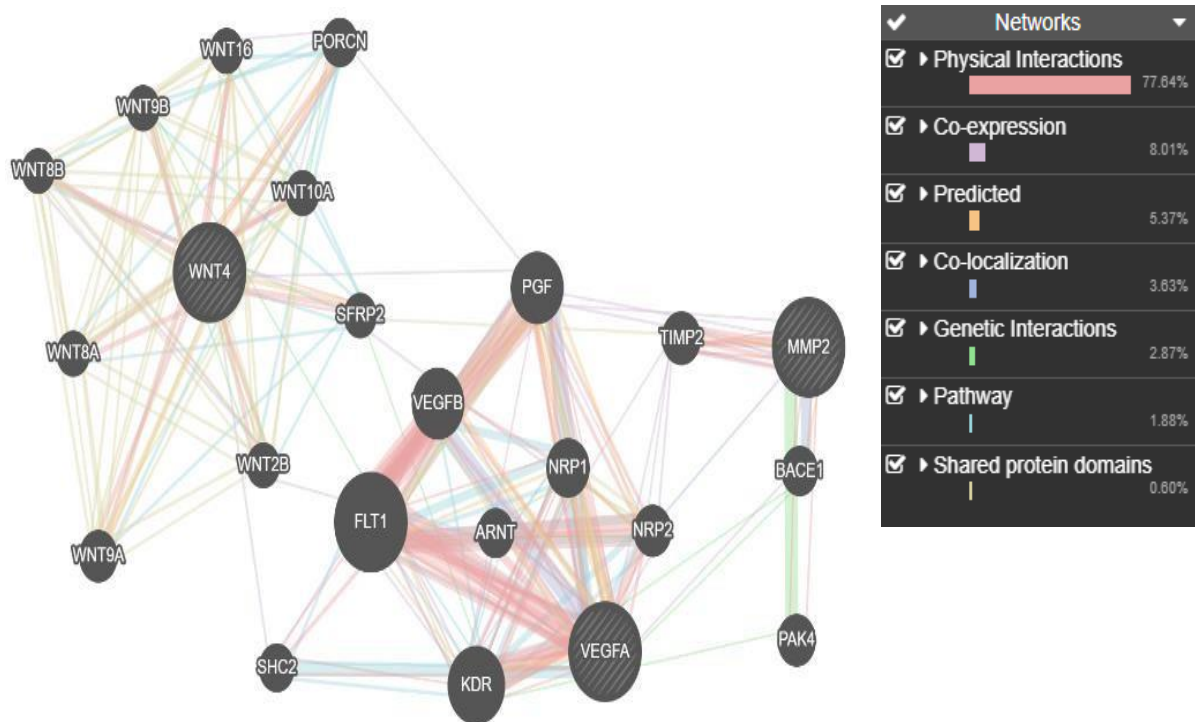
Figure 4.19. Interaction of VEGF-A, WNT-4, and MMP-2 with each other and other genes predicted by GeneMANIA.

4.11. In- Vitro Analysis

The relationship between the missense SNP rs121908651 of the WNT-4 gene and endometriosis risk was studied in a case-control study. 30 whole blood samples from patients with endometriosis,30 from patients with fibroid disease, and 30 from healthy controls were used in the study. These samples were taken from the Research and Diagnostic Laboratory at ASAB, the District Headquarter Hospital in Rawalpindi, the Benazir Bhutto Hospital in Rawalpindi, and the Holy Family Hospital in Rawalpindi.

The Phenol-Chloroform method was used to extract DNA, and a ThermoScientific Nanodrop 2000 UV-Vis Spectrophotometer and 2% agarose gel electrophoresis were used to evaluate the DNA's quality and amount. Allele-specific ARMS PCR was used to analyze DNA samples that met the purity threshold of around approximately 1.8 absorbance ratio. The purpose of this process was to detect the existence of polymorphisms. Following PCR, the results were examined using 2% agarose gel electrophoresis. To ascertain whether there was a significant correlation between the selected SNP and endometriosis, statistical analysis of the ARMS PCR data was carried out.

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4.12. Association Analysis of WNT-4 rs121908651 Polymorphism

The WNT-4 rs121908651 variant is a missense single nucleotide polymorphism (SNP) in which the risk allele A replaces the ancestral allele G at position 114 of the WNT4 protein, this SNP causes an amino acid substitution from alanine (A) to valine (V) in exon 3 of the WNT4 gene on chromosome 1p36.12. By employing 2% agarose gel electrophoresis to assess the presence of this SNP, 173 base pair PCR products for both disease samples and healthy controls are shown in figure 4.20 and 4.22.

 Table 4 provides specifics on chi-square test results, observed genotype and allele frequencies, and graphical representations in Figures 4.20 and 4.21. Based on statistical analysis, there was a significant difference between endometriosis patients and healthy controls in the frequency of the ancestral G allele compared to the risk A allele. In patients with fibroids, a notable significant was observed. There is a significant correlation between the rs121908651 SNP and the risk of endometriosis, according to the Chi-square test ($\chi^2 = 11.88$, p = 0.0026, df = 2), On the other hand, the rs121908651 SNP of the WNT-4 gene was shown to be highly significantly associated with the risk of uterine fibroids, according to the results of the Chi-square test for fibroids ($\chi^2 = 17.79$, p = 0.0001, df = 2). This suggests that the observed genotypes were in Hardy-Weinberg equilibrium.
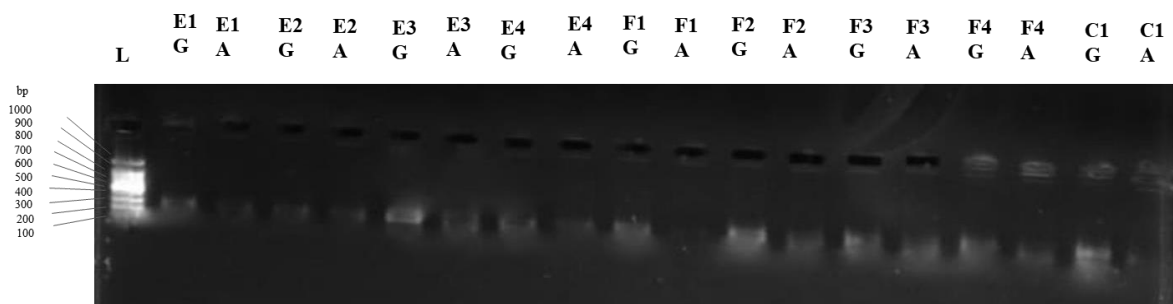
Figure 4.20. Agarose gel electrophoresis (2%) showing the WNT-4 rs121908651 polymorphism PCR results in different samples. The DNA ladder is shown in Lane L. Endometriosis samples are indicated by the letters E in the lanes, fibroids by the letters F, and control samples by the letters C. Whereas a single band denotes homozygosity for either the G or A allele, two bands in a lane show that the person is heterozygous, having both the G and A allele.

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Table 4.5. Genotype, allele frequencies, and chi square x2 test of the WNT-4 rs121908651

polymorphism with endometriosis.

Cases/Controls WNT-4	Genotype Frequency						Allele Frequency			
	GG		GA		AA		G		A	
	n	%	n	%	n	%	n	%	n	%
Cases n= 30 (Endometriosis Patients)	11	36.67%	13	43.33%	6	20.00%	35	58.33%	25	41.67%
Controls n=30 (Healthy Females)	22	81.48%	4	14.81%	1	3.70%	48	88.89%	6	11.11%
x^2	11.88						13.40			
p-value	0.0026						0.0003			
Df	2						1			
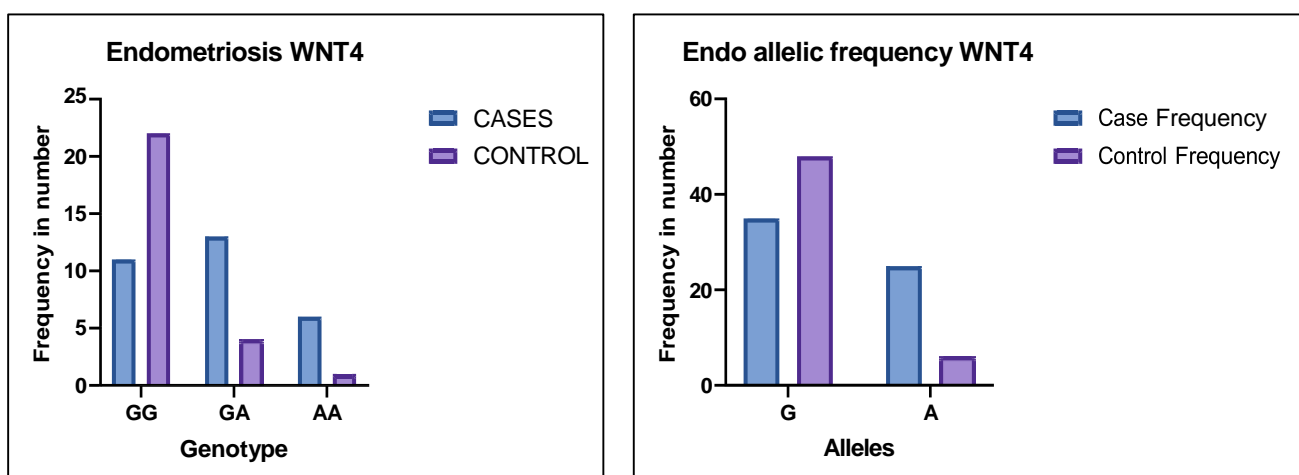
Figure 4.21. Genotype and allelic distribution of WNT-4 rs121908651 with Endometriosis

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Table 4.6. Genotype, allele frequencies, and chi square x2 test of the WNT-4 rs121908651 polymorphism with endometriosis.

Cases/Controls WNT-4	Genotype Frequency						Allele Frequency			
	GG		GA		AA		G		A	
	n	%	n	%	n	%	n	%	n	%
Cases n= 30 (Fibroids Patients)	8	26.67%	12	40.00%	10	33.33%	28	46.67%	32	53.33%
Controls n=30 (Healthy Females)	22	81.48%	4	14.81%	1	3.70%	48	88.89%	6	11.11%
x^2	17.79						22.80			
p-value	0.0001						<0.0001			
Df	2						1			
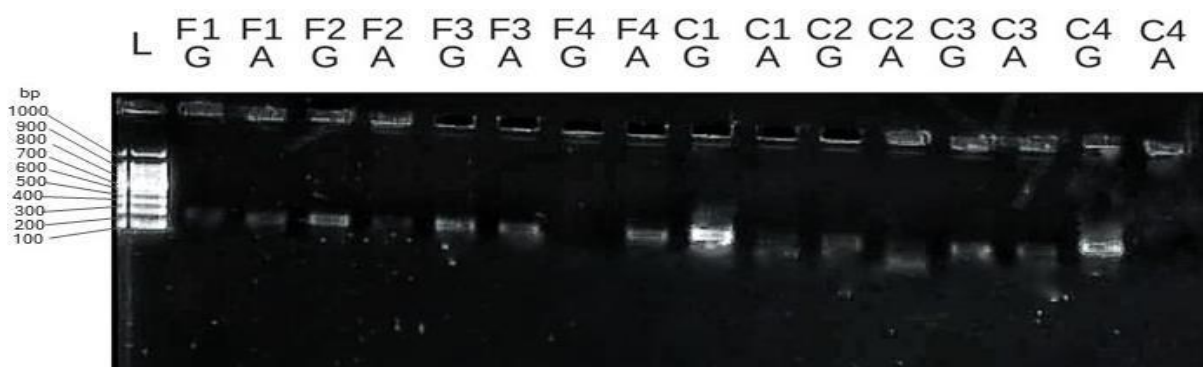
Figure. 4.22. Agarose gel electrophoresis (2%) showing the WNT-4 rs121908651 polymorphism PCR results in different samples. The DNA ladder is shown in Lane L. fibroids by the letters F, and control samples by the letters C. Whereas a single band denotes homozygosity for either the G or A allele, two bands in a lane show that the person is heterozygous, having both the G and A allele.

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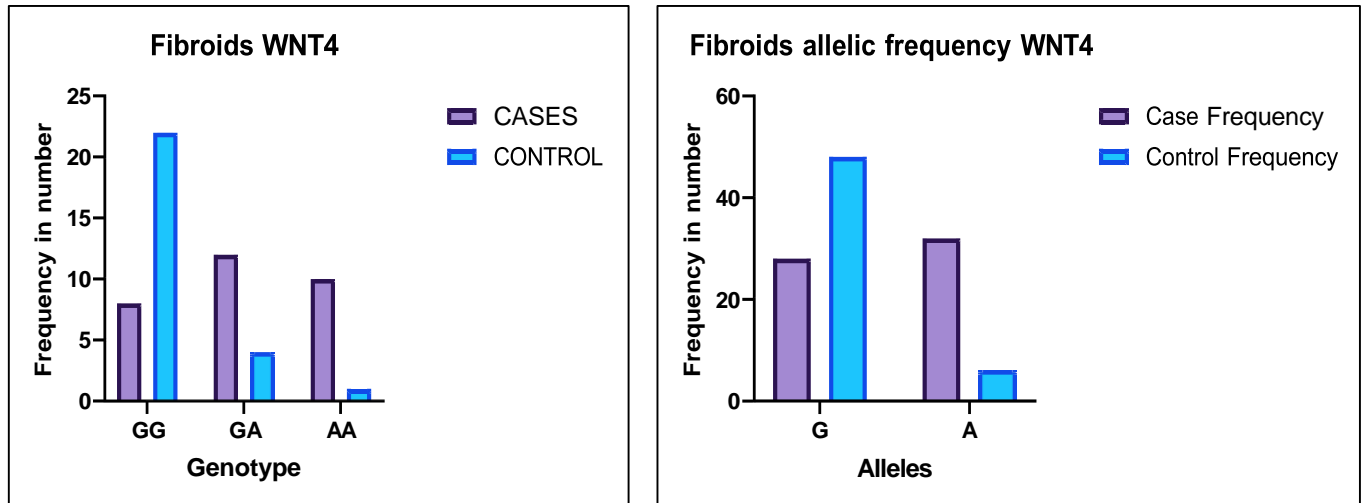
Figure 4.23. Genotype and allelic distribution of WNT-4 rs121908651 with Fibroids.

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Chapter 5. Discussion

Chronic reproductive disorders like endometriosis are typified by the growth and proliferation of endometrial tissue in areas of the body other than the uterus. This condition affects about 10% of women globally (Rasheed & Hamid, n.d.) and 6% of women in Pakistan (Khan et al., n.d.); it is most common in those between the ages of 25 and 45 (Smolarz et al., 2021). However, because to the disease's poor management and delayed diagnosis, the precise prevalence cannot be determined. It is understood that endometriosis is a disease with a complicated and uncertain aetiology that involves a number of genetic, epigenetic, hormonal, immunological, and retrograde menstrual variables in addition to endometrial cell movement. Infertility and pelvic pain are the two main symptoms that worsen as endometriosis progresses (Soliman et al., 2017).

It is thought that endometriosis has a strong hereditary foundation. Research reveals that single nucleotide polymorphisms play a role in the development of disease and that endometriosis has a polygenic inheritance pattern (Hansen & Eyster, 2010). Numerous genes have been implicated in the development of endometriosis, according to candidate gene analysis and GWAS research, and roughly 19 SNPs have been linked to the condition (Sapkota et al., 2017). Comparably, leiomyomas, another name for uterine fibroids, are non-cancerous growths in the uterus that can result in a great deal of morbidity, such as heavy menstrual flow, pelvic pain, and problems with reproduction. Given that endometriosis and uterine fibroids are both impacted by comparable hormonal and genetic factors, there is evidence to imply that the two disorders have similar genetic origins. The overlapping genetic foundation of both disorders has been highlighted by the identification of genetic variations through genome-wide association studies that increase the chance of developing both conditions (Gallagher et al., 2019b).

In this work, three genes WNT-4, VEGF-A, and MMP-2 were examined using in silico methods. The identification of single nucleotide polymorphisms (SNPs) and the evaluation of their possible effects on protein function have become more feasible with the development of computational techniques. Interestingly, many missense SNPs, such as MMP2 (591), VEGF-A (563), and WNT4 (360), have been found in these proteins by the NCBI database. This emphasizes how crucial it is to comprehend how these variations affect structure and function.

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We used a number of deleterious SNP predictors (web tools), including PolyPhen2, SIFT, SNP&GO, PANTHER, PROVEAN, and PredictSNP, to distinguish between neutral and harmful SNPs. 3 significant SNPs in VEGF-A, 12 in MMP-2, and 7 in WNT-4 were found by our research. Of these, it was discovered that one SNP in each of WNT4 and MMP2 was linked to improved stability, whereas six SNPs in WNT4 and eleven in MMP2 were shown to decrease the protein stability.

Post-translational modifications (PTMs) were investigated further, and it was found that the WNT4 protein is SUMOylated at locations 284–288; N-glycosylated at sites 28 and 237; and phosphorylated at positions 129, 251, and 263. These PTM patterns remained mostly unchanged by mutations. MMP2 and VEGF-A showed similar patterns, with major differences in the PTM locations of the mutant proteins. ERRAT and TM-Align, two programmes for 3D protein modelling, were used in structural analysis to identify discrepancies between wild-type and mutant proteins, which may have pathogenic implications. The connections and signaling networks of these proteins were further clarified using STRING studies.

SNP rs121908651 of the WNT-4 gene was carefully investigated for any possible connections to endometriosis and uterine fibroids during the study's in-vitro phase. A statistically significant relationship between this SNP and endometriosis was found, after a thorough statistical analysis using GraphPad Prism version 10 and a strong case-control design were used. For endometriosis, the data produced a p-value of 0.0026, which is less than the typical cutoff point of 0.05 for statistical significance. On the other hand, the study discovered a p-value of 0.0001 for the SNP rs121908651 and uterine fibroids relationship. As the p-value is less than 0.05, there is a significant association between the SNP with the chance of getting fibroids. There is a necessity of more studies using bigger cohorts to elucidate the function of the SNP in endometriosis and substantiate its correlation with fibroids.

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Conclusion

In conclusion, the thorough examination of VEGF-A, MMP-2, and SNP rs121908651 in the WNT-4 gene offers important insights into the genetic underpinnings of endometriosis and uterine fibroids. Three major SNPs in VEGF-A, twelve in MMP-2, and seven in WNT-4 were found to be harmful variations by the in silico investigation, which made use of cutting-edge computational technologies. Prominently, these SNPs impacted the post-translational modifications and structural stability of these proteins, exposing alterations in phosphorylation, ubiquitination, and SUMOylation patterns. The significance of these mutations in changing the interactions and activities of proteins and possibly influencing the pathophysiology of disease is highlighted by these studies. SNP rs121908651 was significantly associated with endometriosis (p-value = 0.0026) in the in-vitro phase, but it did show a little more significantly association with uterine fibroids (p-value = 0.0001), indicating that this SNP may be involved in the formation of fibroid growth. A bigger sample size may be able to demonstrate a more conclusive link. These findings emphasize the need for more research to confirm these conclusions and clarify the genetic basis of these disorders. In summary, this study highlights the possibility of combining in silico and in-vitro approaches to find significant genetic connections and their biological implications, and it furthers our understanding of how certain SNPs contribute to disease risk.

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Future Prospects

With multiple directions for future research to deepen our understanding of the genetic basis of endometriosis and uterine fibroids, the future possibilities of this study are highly intriguing. This research must be expanded to include the two other genes examined in the in silico phase, MMP-2 and VEGF-A, in light of the noteworthy discoveries surrounding SNP rs121908651 in the WNT-4 gene. The computational study suggested that both genes may be important, and that empirical research is necessary to confirm their links to endometriosis and fibroids. To boost the results' statistical power and dependability, future studies should concentrate on increasing the sample size. Furthermore, by using proteomics techniques to explore these SNPs' functional effects in more detail, we may be able to learn more about how these genetic variations affect protein function and contribute to disease pathways. These investigations may be able to confirm the present findings and even point to novel treatment targets or biomarkers for these diseases.

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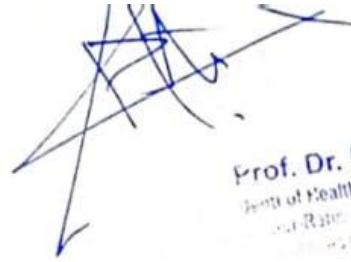Zhang, Y., & Skolnick, J. (2005). TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic Acids Research*, *33*(7), 2302–2309. https://doi.org/10.1093/NAR/GKI524

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