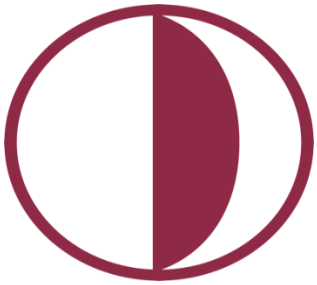

NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

THE EXPRESSION PROFILE OF *WNT3A*, *WNT4*, and *WNT5A* GENES IN SPONTANEOUS ABORTION MATERIALS

ARTHUR BODURIE CALVIN GARBER

MASTER THESIS MOLECULAR MEDICINE PROGRAM

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ASSOC. PROF. MAHMUT CERKEZ ERGOREN

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ACCEPTANCE/APPROVAL

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DECLARATION

I hereby declare that all information, documents, analysis, and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of the Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

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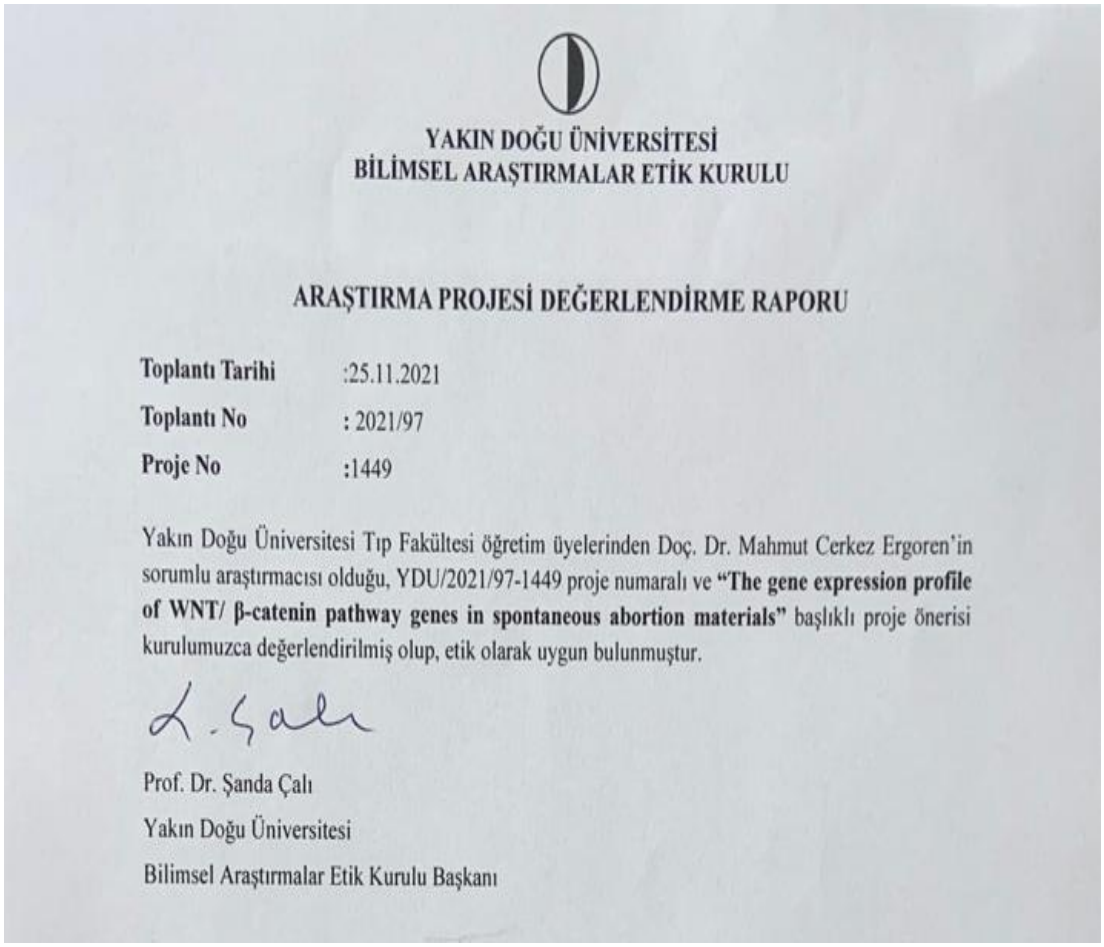
COMPLIANCE AND APPROVAL



YAKIN DOĞU ÜNİVERSİTESİ
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Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Mahmut Cerkez Ergoren'in sorumlu araştırmacısı olduğu, YDU/2021/97-1449 proje numaralı ve **"The gene expression profile of WNT/ β-catenin pathway genes in spontaneous abortion materials"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Prof. Dr. Şanda Çalı
Yakın Doğu Üniversitesi
Bilimsel Araştırmalar Etik Kurulu Başkanı

DEDICATION

This project is been dedicated to:

My parents Late Dr. Jimmy Melville Garber, Ms. Mariatu Kuyea Sesay, and late Ms. Olive Edith Ina Williams

My Aunty's Dr. Princess Dougan, Mrs. Annie Pratt, and Uncle Prof (Dr) Jack Nicholas Garber

My Son Alimamy Modupeh Garber.

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To him all glory be given, I want to say a very big thanks to God almighty for seeing me through this journey. It has always been God at the center of it all.

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To all my friends both at home and abroad I want to say I am grateful for your lives.

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LIST OF ABBREVIATION

Wnt-Wingless+Int-1

Fz-Frizzled

Dsh-disheveled

DNA-Deoxyribonucleic acid

RNA-Ribonucleic acid

Cdk3-Cyclin-dependent kinase 3

IVF-Invitro fertilization

ZGA-Zygotic genome activation

β-Beta

ACOG-American College of Obstetrics and Gynaecology

HLA-Human Leukocyte Antigen

APC-Adenomatous Polyposis Coli

GSK3-Glycogen synthase kinase 3

CK1α-Casein Kinase 1 Alpha

LRP5-Low Density Lipoprotein Receptor Related Protein 5

PCP- Polarity Cell Pathway

DAAM1- Dishevelled Associated Activator of Morphogenesis 1

CGM- Cyclic Guanosine Monophosphate

JNK-Jun N-Terminal Kinase

Wnt 3-Wingless 3 family member

Wnt 10B- Wingless 10B family member

Wnt 1- Wingless 1 family member

Wnt 2- Wingless 2 family member

Wnt 4 - Wingless 4 family member

Wnt 5A- Wingless 5A family member

Wnt 5B - Wingless 5B family member

Wnt 6 - Wingless 6 family member

Wnt 7- Wingless 7 family member

RSA- Recurrent spontaneous abortion

URSA- Unexpected Recurrent Spontaneous Abortion

cDNA-complimentary Deoxyribonucleic acid

bp-Base pair

EDTA-Ethylene Diamine Tetra-acetic Acid

TAE- Tris Acetic Acid EDTA

ml-Milliliter

mg-Milligram

µl- Microliter

PCR-Polymerase Chain Reaction

µm-Micro Molar

qRT-Quantittive Real Time

DH_2O- Deionized Water

Ct-Cycle threshold

ABSTRACT

THE EXPRESSION PROFILE OF WNT SIGNALLING PATHWAY GENES (*WNT3A*, *WNT4*, and *WNT5A*) IN SPONTANEOUS ABORTION MATERIAL

ARTHUR BODURIE CALVIN GARBER

SUPERVISOR:

ASSOC. PROF. MAHMUT CERKEZ ERGOREN

AIM OF THE STUDY

To investigate the expression levels of Wnt-signalling pathway genes (*WNT3A*, *WNT4*, and *WNT5A*) in spontaneous abortion materials.

BACKGROUND

The most common complication of pregnancy is a miscarriage, which is the involuntary loss of a fetus before reaching the phase of maturity. It includes all forms of pregnancy loss and the stage of conception to the 24th week of gestation according to the World Health Organisation. The two types of miscarriage are recurrent and sporadic. The current effect of recurrent miscarriage can affect just 1% of couples. About 25-50% of pregnant women experienced sporadic miscarriage which can be a result of some random fetal abnormalities at the chromosomal level. The Wnt proteins originate from a family of conserved glycoproteins, whose responsibilities are to act as ligand receptor-mediated signaling pathways. And some of these activities regulate various biological processes such as embryonic development and in adults. The Wnt signal pathways are carried out by the transmission of signals, and it is aided by proteins that facilitate the transfers of molecules from one cell to the other cell surface receptors. The name Wnt comes from the term wingless and Int-1. The pathways of Wnt can be achieved through cell-cell communication or the same cell-cell communication. The portion of Wnt genes can be found across animals with ranges from fruit flies to humans. The Wnt is composed of approximately 400 amino acids and comprises a varying family of secreted lipid-modified signaling glycoproteins.

The Wnt signaling pathways are categorized based on their characteristics such as Canonical, Noncanonical planar cell polarity, and Noncanonical Wnt/calcium pathways.

The different pathways are activated when a Wnt protein-ligand binds to a receptor family called frizzled family, thus passing the signals biologically to a Dishevelled protein that is inside a cell.

METHOD AND MATERIALS

The aborted materials were obtained from Near East University Hospital Medical Genetics Laboratory. A total of 24 samples were collected from the storage point. They were categorized into normal and abnormal karyotypes. This research study was undertaken at Near East University DESAM Institute Molecular Medicine Laboratory, Nicosia, Northern Cyprus.

The RNAs were extracted from the samples, and cDNA synthesis was performed followed by gene expression analysis by RT-qPCR.

RESULTS

Although statistically, they were all insignificant ($P<0.05$), a decrease in *WNT3A* and *WNT4* gene expressions were observed in samples with abnormal karyotype compared to gene expression in samples with normal karyotype ($P=0.170$ and $P=0.176$, respectively). In *WNT5A*, on the other hand, gene expression was slightly increased in samples with abnormal karyotype compared to samples with normal karyotype but there was no statistical significance ($P=0.592$). Correlation analysis revealed a positive correlation between *WNT3A* and *WNT4*, but a negative correlation between these two genes' expression and *WNT5A* expression.

CONCLUSION

Overall, changes in gene expression levels in *WNT3A*, *WNT4*, and *WNT5A* genes, which are involved in the Wnt-Beta catenin signaling pathway, in spontaneous abortion material with abnormal karyotype compared to materials with normal karyotype may be associated with the abnormalities in chromosome content or structure. Further analysis should be conducted to include protein expression levels of the studied genes to confirm that the changes detected in gene expression are reflected at the protein level with a larger cohort if possible.

KEYWORDS: spontaneous abortion, WNT signaling pathways, *WNT3A*, *WNT4*, *WNT5A*

CHAPTER 1: INTRODUCTION

1.1 Introduction

The most common complication of pregnancy is a miscarriage, which is the involuntary loss of a fetus before reaching the phase of maturity. It includes all forms of pregnancy loss and the stage of conception to the 24th week of gestation according to the World Health Organisation **("Definitions of Infertility and Recurrent Pregnancy Loss," 2008**). There are two types of miscarriages, recurrent and sporadic. The current effect of recurrent miscarriage can affect just 1% of couples **(Sonderegger et al., 2007**). About 25-50% of pregnant women experienced a sporadic miscarriage as a result of some random fetal abnormalities at the chromosomal level **(Pollheimer et al., 2006).** However, recurrent miscarriage is the simultaneous loss of pregnancy and is part of a range of reproductive disorders with similar but common causes (**El Hachem et al., 2017**). Furthermore, recurrent miscarriage is can be tied down to several features (**L. Qiao et al., 2019**). Recurrent miscarriage can occur even when there seem to be normal chromosomes and tends to affect women with underlying reproductive characteristics (**van der Horst et al., 2012)**.

The placenta is a unique organ that functions in the exchange of materials between the mother and her fetus. It is important for the success of pregnancy and the health of the fetus (**Brett et al., 2014**). There is an increased rate in maternal age which is associated with a range of causes such as embryo or oocyte aneuploidy, parental chromosomal abnormalities, maternal thrombophilias, obesity, and dysregulation **(Cimadomo et al., 2018)**.

However, abnormalities in trophoblast cell function are linked to complications such as intrauterine growth retardation, and pre-eclampsia **(Woods et al., 2018)**. But the underlying cause remains to be vastly unknown. Previous studies indicated that the wingless (Wnt) signal is essential in the physiological processes of the human trophoblast and tends to have a relationship with the pathogenesis of unexplained recurrent spontaneous abortion **(Knöfler & Pollheimer, 2013)**.

The trophoblast is vital in fetal and placenta development **(Pollheimer et al., 2006).** Although several proteins have been identified and their role in the Wnt signal pathway and the sole responsibility of these molecules remained to be unclear **(S. H. Bao et al., 2013)**.

The Wnt proteins originate from a family of conserved glycoproteins, whose responsibilities are to act as ligand receptor-mediated signaling pathways **(Komiya & Habas, 2008)**. And some of these activities regulate various biological processes such as embryonic development and in adults (**Komiya & Habas, 2008**). The Wnt signal pathways are carried out by the transmission of signals, and it is aided by proteins that facilitate the transfers of molecules from one cell to the other cell surface receptors **(Kleinert et al., 2010)**.

The name Wnt comes from the term wingless and Int-1 **(Komiya & Habas, 2008)**. The pathways of Wnt can be achieved through cell-cell communication or the same cell-cell communication **(Steinhart & Angers, 2018)**. The portion of Wnt genes can be found across animals with ranges from fruit flies to humans (**Lento et al., 2012**). The Wnt is composed of approximately 400 amino acids and comprises a varying family of secreted lipid-modified signaling glycoproteins **(Azbazdar et al., 2021**).

However, the modification of Wnt can occur through a process known as palmitoylation, and it is maintained in a serine residue **(Janda et al., 2015)**, which aids the binding of Wnt to the Wnt carrier proteins called Wntless. However, this can allow the transportation of Wnt to the plasma membrane for secretion and also the binding of Wnt protein binding to the frizzled (**Janda et al., 2015)**.

To enhance the proper secretion of Wnt it has to undergo glycosylation by attaching to carbohydrates **(Komekado et al., 2007)**. The Wnt signal proteins can aid the binding of other molecules in activating the various Wnt pathways through hormonal signals **(Chiang et al., 2012).** The Wnt signal plays a pivotal role in carcinogenesis and the development of embryos, and in the process of embryonic development, it controls the

patterning of the body axis, cells' final type, proliferation, and migration of cells. These are essential processes that aid the formation of tissues such as bones, heart, and muscles **(Lustig & Behrens, 2003).** Moreover, the role of the Wnt signal pathway in embryonic development came to the limelight when mutations were discovered in the Wnt pathway protein that was produced by the embryo of a fruit fly **(Bejsovec, 2014)**.

The Wnt signaling pathways are categorized based on their characteristics such as Canonical, Noncanonical planar cell polarity, and Noncanonical Wnt/calcium pathways **(Lhomond et al., 2012)**. The different pathways are activated when a Wnt protein-ligand binds to a receptor family called frizzled family, thus passing the signals biologically to a Dishevelled protein that is inside a cell **(Chong & Maiese, 2004)**. Furthermore, the canonical pathway brings about the regulation of gene transcription **(Komiya & Habas, 2008**).

The noncanonical planar cell polarity pathways play a vital role in regulating the cytoskeleton, by giving the cell a shape, however, the role of the noncanonical Wnt/calcium pathways plays a key role in calcium regulation inside cells (**De, 2011**). Furthermore, it was realized that these genes can have a role to play in breast cancer development in mice. The Wnt signal pathways function in controlling adult bone marrow tissue regeneration, skin, and the intestine **(Grigoryan et al., 2008)**. The Wnt pathways have shown to be of clinical importance as a result of mutations discovered in different health conditions such as type 2 diabetes, breast, and prostate cancer, and glioblastoma **(García-Jiménez et al., 2013) ((Xu et al., 2020)**.

The first successful information on the usefulness of the Wnt pathway was gathered as a result of the display of the inhibitory effect in mouse models having diseased proteins quite recently **(Clevers & Nusse, 2012)**.

1.2. Cell Divisions

All living organisms are made up of numerous numbers of cells, the number of cells in an organism can vary based on the complexity of the species ranging from tens to billions of cells **(Bianconi et al., 2013)**. These cells are made up of hundreds of intracellular molecules, and they are undergoing constant synthesis and degradation (**Sato et al., 2017)**.

Cell division is one of the most complex processes, and it is in high demand for energy. It is a process that requires a series of cellular events known as the cell cycle (**Mcintosh, n.d**.).

The outcome of the cell cycle is duplication of DNA and separation of newly duplicated genomes into daughter cells. In the G1 phase of cell division, enzyme synthesis takes place specifically for those used in genome duplication. However, in the G2 phase, the duplicated centromeres divide, and this separation can aid in organizing the mitotic spindle (**Mcintosh,n.d**.).

The chromosome is then condensed, nuclear envelope then breaks which will make way for mitosis (**Schooley et al., 2012**). There is a change structurally in the endoplasmic reticulum, Golgi apparatus, and mitochondria, ensuring the neat packaging between daughter cells **(Frohlick & Staehelin, 2000**).

Based on tradition cell division is divided into steps which are prometaphase, prophase, anaphase, and telophase and this classification was made according to the arrangement of chromosomes and cytoskeleton **(Frohlick & Staehelin, 2000**). However, having these steps in place help the regulation and execution of high spatial and temporal control in ensuring proper segregation of nucleic material in daughter cells **(Weiss, 2012**).

The newly formed daughter cells should be functioning just after the completion of cell division and can synthesize proteins, DNA, and other cellular makeup from nutrients

processing and metabolism (**Xiang et al., 2019)**. The newly formed cells are charged with the function of performing signal pathways in response to both internal and external pressures for their survival and growth (**Hotamisligil & Davis, 2016**). These activities normally take place in the membrane-bound organelles which are necessary for ensuring correct inheritance **(D. S. Schwarz & Blower, 2016**).

Almost all the cellular organelles undergo remodeling during the cell division stage by a mechanism that is not fully understood, other than those regulating cytoskeleton and chromosomes during cell division (**Ouellet & Barral, 2012**) (**Spichal & Fabre, 2017**). The already divided cells are faced with challenges in terms of organelle proportioning. These cells might vary in size, some do exist as single or with high copy numbers, with unique features in shape and location (**Chan & Marshall, 2010**).

In a review **(McCullough & Lucocq, 2005**) they compared the outcome of various organelles and the membrane structure during cell division and also highlighted so many questions relating to partitioning and its occurrence **(McCullough & Lucocq, 2005**). The mitochondrion is an abundant organelle that can change its morphology but does not rearrange itself **(Misgeld & Schwarz, 2017)**. Their morphological change can result in abnormal partitioning, and the accuracy varies between organelles. In cells with an abnormal Golgi, the chances of survival in the long term in uneven compared to other cells with shared mitochondria that may recover and grow well and divide during the cell cycle **(Misgeld & Schwarz, 2017) (Roger et al., 2017**).

For the cell to undergo cell division, an additional component needs to be added. So therefore cells need an extra mechanism to help them segregate during cell division (**Prosser & Pelletier, 2017**). However, in-depth knowledge of how cells talk between cell division and organelles occurrence is highly recommended, in as much as there are varieties of proteins that take part in the cell division process. A vivid example is RNA interference being used to screen for mitotic abnormalities, and analysis of proteomes of midbody proteins residing in membrane-bound organelles (**Eggert et al., 2006**).

According to **(Carlton et al., 2020**) they created a list of proteins that takes part in cell division and their role in membrane-bound structures. With many of these proteins little or no detail of their molecular functions are available.

The progression of the cell cycle can be achieved when cyclin-Cdk specific which controls many structural changes are activated **(Carlton et al., 2020)**. It also affects the change in the shape of mitochondria, which are charged with the responsibility of segregating daughter cells **(Salazar-Roa & Malumbres, 2017).**

The study of embryos by Aristotle in 384BC-322BC in comparing the development of aquatic animals with humans and chickens without the help of a microscope shed so much light on embryonic development. He later observed the development that is been taken place from the development of the embryo of cell patterns from holoblast to meroblastic phase **(Burggren, 2013)**.

There is more to learn about the regulation of the cell cycle during embryonic development, homeostasis, and also the study of the aquatic model will continue to pave the way for further research on embryonic development. Furthermore, there is an early sign of embryonic development in frogs, giving a clear indication of the cell cycle mechanism going on in the somatic cells **(Kane & Kimmel, 1993)**.

However, there is a cultivating knowledge and interest in how cells divide. But on the other hand, why do cells divide. According **(Lasota & Mackey, 1999 and Roeder, 2012)()**, the article concluded that cells divide to enable the stability, functions, and structure of an organism. Complications that may affect embryogenesis can occur as a result of the shift in cell division and migration (**Pakula, 2019)**.

1.2.1 Mitosis

The cellular cycles and division of cells are termed interphase, replication of DNA and other components of the cells are available sufficiently for the two cells **(Norbury &**

Nurse, 1992). The rearrangement of these materials takes place during mitosis, this will enhance growth and division among daughter cells **(Boettcher & Barral, 2013)**. The DNA by length is long and can only be separated by the mitotic spindle, and carrying out this task is key for the survival of an organism **(Mcintosh & Mcdonald, 1989).** The DNA duplex attaches to each sister chromatids through cohesion during the replication process **(Makrantoni & Marston, 2018)**.

In mitosis, the DNA duplexes are arranged into smaller objects having the same size as the cell **(Makrantoni & Marston, 2018)**. The Prophase is formed during the mitotic period as a result of chromosomal condensation which is vital for successful cell division **(Zoller et al., 2004) (Tiang et al., 2012)**. The spindle attaches to the chromosome during the prometaphase and then will organize them into metaphase. The chromosome segregates successfully during anaphase, with the help of two-fold symmetry at metaphase **(D. S. Schwarz & Blower, 2016)**. In a distance separation of the chromatids during metaphase, two functioning nuclei are formed distant from each other during telophase **(Rieder & Cole, 1999).** The cytokines will produce two cells, which can repeat the whole cycle at interphase **(Wagner & Glotzer, 2016)**

1.2.2 Meiosis

Meiosis is a biological process that involved the reduction of the chromosome number of an organism to half its normal count **(Tiang et al., 2012)**. The meiotic process is best achieved as a result of single DNA replication followed by the segregation of two chromosomes in a germ cell of a sexually active organism **(Solari, 2002)**. It has been observed that meiosis might be originating from mitosis but having different or more vital steps **(Wilkins & Holliday, 2009)**.

However, these steps include the pairing and synapsis of homologous chromosomes, non-sister chromatids recombination, and restricting sister chromatids from separating during prophase, DNA replication bypass in the two meiotic divisions **(Wilkins & Holliday, 2009)**. The germ cells then enter meiotic prophase 1 due to premeiotic DNA replication **(Baltus et al., 2006)**.

The meiotic prophase 1 divides itself cytologically into substages based on chromosomal behaviors and a scaffold of proteins known as the synaptonemal complex **(Subramanian & Hochwagen, 2014)**.

The protein backbone is formed among sister chromatids and this is recognized as the axial element in the early stage. The axial element is there to keep the sister chromatids intact until the second meiotic division. Subsequently, the chromosomes become condensed and long by length **(Klein et al., 1999)**.

The length of the axial element changes from a shorter to a longer band due to the progression of cells from the leptonema to the zygonema stage. Furthermore, during the zygonema stage, there is pairing taking place at the chromosomal level between axial elements which attach by a mechanism called the central element. The axial element becomes the lateral element at this very stage **(Yoon et al., 2018)**.

Also, the tripartite is formed surrounding the length of the chromosomes, taking us to the next substage which is called the pachynem. The homologs become fully synapsed followed by shortening and condensation of chromosomes at the end stage of pachytene **(Yoon et al., 2018)**. The synaptonemal complex dissociates at the end of prophase 1 giving way to diplonema **(Yoon et al., 2018)**. The chiasmata keep the homologs firmly attached. However, the chiasmata are formed during the process of homologous recombination which is aligned with chromosome synapsis **(H. Qiao et al., 2012)**.

1.2.2.1 Meiotic Recombination

Meiotic recombination is then initiated during the initial prophase 1 stage as a result of breaks in the double-helical structure of DNA. The breaks in the helical structure of DNA can be fixed either by crossover or noncrossover repair pathways resulting in chiasmata **(Hirose et al., 2011)**. Moreover, the role of chiasmata is to correctly align and separate homologous chromosomes during metaphase 1.

When the chromosomes fail to establish crossover, the chiasmata will not join and can lead to aneuploidy **(Martinez-Perez et al., 2008)**. Furthermore, the first mitotic division can cause a reduction, especially in the maternal and paternal chromosomal segregation into daughter cells **(Nasmyth, 2002)**. Meiotic recombination is vital on so many occasions, it is capable of forming genetic diversity, and the recombination of alleles can form from a new and unique allelic combination which is obliged to natural selection (**Ergoren, 2018**).

On the other hand, it forms strong bonds between homologous chromosomes, which play a role in ensuring chromosomal segregation in the first meiotic division as well as fixing the damaged ploidy of a gamete **(Theos et al., 2005)**.

1.2.2.2 Errors During Meiosis

Meiosis is a process of cells dividing to give rise to four cells, each half with the full complement of DNA. The sole purpose of meiosis is to produce gametes, these gametes need to have half the amount of DNA during fertilization **(Mercier et al., 2015)**. However, during meiosis, there is a tendency for error to take place. These errors can lead to the production of an extra or missing chromosome **(Hall et al., 2006)**. The aftermath of this phase depends on which particular chromosome is affected. Often they may not be viable, in another scenario these errors can lead to trisomy conditions or sex chromosomal disorders **(Kuliev et al., 2011)**.

Furthermore, *de novo* autosomal monosomies and trisomies become invalid, these products are therefore eliminated during pregnancy and are been seen in spontaneous abortions **(Egozcue et al., 2000)**. The Trisomy remains to be the only fully autosomal anomaly, surviving to the postnatal stage. Trisomy 13 and 18 are the surviving anomalies to birth, trisomy 21 can survive until puberty and even adulthood. An error in meiosis may affect male de-novo sex chromosomal anomalies which include Klinefelter syndrome 47, XXY and 47, XYY aneuploidy. They originate from the paternal end and this error is produced in meiosis II (**Egozcue et al., 2000)**.

1.3 Embryogenesis

In human, the embryo is an indication of polarization which present itself around the eight-cell stage. In such a scenario the E-cadherins are located in the basolateral region and as well the gap junctions. In this manner, apical microvilli are seen in the eight human cells of the morula stage of the embryo (**Nikas et al 1996**). The tight and adherence junctions are maintained among phyla and they play a role in cell polarization and also the establishment of cell-cell communication **(Smith & Reese, 2016)**. However, there is a likelihood that the activity of cytoskeleton interactions and cell-cell communication cannot be conserved in the human embryo, but this remains to be looked into further **(Ajduk & Zernicka-Goetz, 2016**).

Furthermore, there is an expression of proteins such as ZO-1 and occludin which have been expressed in the human blastocyst and they are been linked with the formation of tight junction and desmosome **(Chiba et al., 2008)**. For implantation to occur, there has to be an expansion of the blastocyst by hatching outside the ZP and then cleaving to the uterine wall around 7-10 dpf **(S. J. Liu et al., 2020)**.

When the blastocyst fails to hatches the resulting outcome is a decrease in the chances of Invitro fertilization of the embryo **(Simon & Laufer, 2012)**. This outcome can be further understood with the help of molecular mechanisms, and can also improve IVF outcomes **(Koot et al., 2012)**. During embryogenesis in humans, the embryo progresses further even when there is no active transcription **(Jukam et al., 2017)**. But relies on maternal messenger RNAs (mRNAs) as well as protein deposits in the cytoplasm of an oocyte **(Deng et al., 2020)**. Furthermore, transcription control can be passed on to the embryo through the maternal to zygote transition **(Lee et al., 2014).**

The degradation of maternal products has facilitated a mechanism known as zygotic genome activation (embryonic genome activation) **(Schulz & Harrison, 2019)**. During the fourth and eighth cell stages, ZGA takes place in humans **(Rossant & Tam, 2017)**. Therefore there is a spontaneous upregulation that coincides with ZGA, other genes, and transposable elements **(Grow et al., 2015**). So far data collected indicating that the

formation of an amniotic cavity within the EPI, is formed after implantation, therefore forming a polar-like structure **(Bedzhov et al., 2014)**.

At the time of implantation, the EPI forms a pseudostratified columnar epithelium, giving rise to the bilaminar disc during implantation **(Weberling & Zernicka-Goetz, 2021)**. Conversely, multinucleated syncytiotrophoblast which has the responsibility of nutrient and gas exchange are formed at this stage. The differentiation of cytotrophoblasts into mononucleated extravillous cytotrophoblasts in mediating the immunological response of conception **(Turco & Moffett, 2019)**.

1.3.1 Stages of Human Embryo Development

The most crucial stage in embryonic development happens to be during primary body axes formation which gears toward whole body structure **(Fu et al., 2021)**. These body axes include the anteroposterior axis, dorsoventral axis, and right-left axis. However, there is an implication of Wnt signaling in the formation of anteroposterior and dorsoventral (DV) axes **(Bejoy et al., 2020).** In this regard, the formation of the following axis is seen in mammals **(Bejoy et al., 2020**). However, there is a production of a morphogenic compound that occurs in the primitive streaks and other surrounding tissues **(John & Rauzi, 2021)**.

In Mammals such as fish and frogs, the β-catenin molecule is been produced by canonical Wnt signals. This may lead to the formation of organizing centers. There is a mechanism behind the activation of gene complement that can alter the formation of the organizers, as well as hinder the result of canonical Wnt signal β-catenin production **(Kozmikova & Kozmik, 2020)**. Conversely, the expression of different mesodermal marker genes from avian gastrulation allows the movement of differential cell movement in the case of primitive streak formation **(Stankova et al., 2015)**. Wnt signaling is essential in the formation of specific body parts as well as organ systems during the late developmental phase. An increase in Wnt signaling can give rise to the dorsal and ventral axis **(Komiya & Habas, 2008)**.

The Wnt can participate in the formation of the central nervous system, and in guiding the axons of the spinal cord which is been done by Wnt protein **(Komiya & Habas, 2008).**

1.3.2 Molecular Basis For Embryonic Development

Understanding the mechanism behind normal and abnormal embryonic development using new molecular techniques keeps changing. Embryonic development cannot be further studied without incorporating the basic molecular and morphological aspects of the embryo **(Barkovich et al., 2012)**. The most reasonable and delicate aspect remains to be the conservation of genes, which have the role of guiding the development of an embryo **(Stern, 2007)**. From reviewed literature on sequencing studies, data has been collected on the changes of nucleotide bases.

However several regulatory genes of development are found in a range of species from Drosophila to humans **(Venter et al., 2001)**. The phylogenetic conservation has helped in identifying mammalian counterpart genes that are contributing to the development of other species functionally. And also these genes can participate in the development of the species in varying organs **(Pauli et al., 2012)**. In as much as so many developmental genes have been discovered and characterized, the ideal approach to understanding the molecular basis of embryonic development still rests on studies of developmental genetics in Drosophila **(He & Hannon, 2004)(Pauli et al., 2012).**

Given this, the early stages of human embryonic development can take place under less stringent genetic mechanisms when compared to Drosophila **(2017, 2017)**. The study of Drosophila embryonic development gave a better understanding of the molecular basis of human embryogenesis **(Penaloza et al., 2006)**.

In Drosophila, the embryonic development remains to be under stringent control, and in the early stages of development, there is an establishment of dorsoventral and anteroposterior axes of the embryo by the maternal effect genes **(Drosophila et al., 1986)**.

The oval embryo undergoes a series of steps sequentially resulting in the segmentation of the whole embryo along the anterior-posterior axis **(Meier & Reichert, 1990**). The segment that is formed then subdivides the embryo into a regional domain under the influence of the gap gene **(Jaeger, 2011)**.

The resulting loss of function of the gap mutants can result in the loss of structure or gap in the body **(Eldon & Pirrotta, 1991)**. The pair-rule genes are involved in forming seven pairs of stripes along the craniocaudal axis of the embryo during the second step **(Brand-saberi & Christ, 2000)**. The final stage is controlled by the segment polarity genes and works at different individual segments as well as during the anterioposterior organization **(Damen, 2007)**.

A Series of genes have shown to be key players in understanding the molecular basis of embryonic development, genes such as *Hox, Pax, Pit1, Oct1, Oct2, Unc86, Dlx, Msx, Tbx, Sox, WT1* **(Turgeon, 1986)**.

1.3.3 Developmental Disorders

Developmental disorders are called neurodevelopmental disorders, they are neurological conditions that can interrupt acquisition, retention, and skills application **(Morris-Rosendahl & Crocq, 2020)**. These disorders can cause dysfunction in attention, memory, perception, language, problem-solving or social interaction **(Morris-Rosendahl & Crocq, 2020)**. Developmental disorders are categorized into mild and severe.

The mild developmental disorders are manageable, whereas the affected child would be needing different kinds of support **(Salvador-Carulla et al., 2011)**. However, development disorders are caused by different conditions, such as genetic or chromosomal abnormalities, prenatal exposure to toxic substances, infection during pregnancy, and preterm birth **(Vorstman & Ophoff, 2013)**. There has been no cure for treating developmental disorders but the patients can be managed by treating them based

on their symptoms. The most common functional psychotic condition is schizophrenia, and it is affecting 1% of the world's population **(Patel et al., 2014)**. According to **(McCutcheon et al., 2020)** they used different therapy in managing a group of patients but later concluded that pharmacological treatment can help increase adaptive function. However, bipolar disorders become the sixth cause of developmental disorders, and individuals with these conditions can suffer developmental and progressive neurophysiological alterations (**Soiza et al., 2018)**.

1.4 Spontaneous Abortion

Spontaneous miscarriage is a loss of a pregnancy that is less than 20 weeks old in the gestation period **(Stephenson, 1996).** It is estimated by the American College of Obstetrics and Gynecologists as the most common loss of pregnancy with an estimated report of as many as above 20% resulting in miscarriage **(Bricker & Farquharson, 2002)**. Spontaneous abortion has been classified into primary and secondary spontaneous abortion. The primary spontaneous abortion is the loss of all subsequent pregnancies with no live birth. And the secondary spontaneous abortion in which there is a report of a surviving pregnancy **(Christiansen, 2021)**.

However, from epidemiological studies, there is a 24% risk of subsequent loss of pregnancy after two losses, 30% occurrence after three, and 40% after the fourth spontaneous abortion **(El Hachem et al., 2017)**. **Pandey et al., (2005)** mentioned earlier a host of etiological factors that can cause spontaneous abortion such as chromosomal alterations, genetic, anatomical, endocrine defects, anomalies relating to the placenta, infection, smoking, and alcohol consumption, exposure to certain chemicals and stressed induced factors.

1.4.1 Etiology of Spontaneous Abortion

In spontaneous abortion the loss of a pregnancy before the 20^{th} week of gestation can occur as a result of so many factors, the underlying causes often go undetected. Few causes have been highlighted which include chromosomal abnormalities, uterine anomalies, and immunologic factors **(Garrido-Gimenez & Alijotas-Reig, 2015)**. The

etiology of spontaneous abortion is still unclear, but few facts are pointing toward genetic susceptibility coupled with environmental factors **(Muyayalo et al., 2018)**. From subsequent research spontaneous abortion has been linked to genetic, structural, immune, infection, endocrine, or unexplained cause.

However, if the mother's immune system fails in recognizing the fetus, then an immune response will be raised which will affect the fetus causing spontaneous abortion (**Lin & Qiu, 2010**).

The association of thrombophilic disorder to spontaneous abortion has left so many questions on investigation and management plan. There has been a relationship between spontaneous abortion and other syndromes which include endocrine abnormalities, genetic and chromosomal abnormalities, anatomic anomalies, and blood coagulation defects **(Rull et al., 2012)**. There is a link between environmental factors such as cigarette smoking, and alcohol consumption, and how they affect the functionality of the trophoblast which might increase the risk of spontaneous abortion.

Furthermore, obesity has an association with spontaneous abortion **(Rasch, 2003**)According to **(Mohammed et al., 2014)** researched the etiology of spontaneous abortion using a questionnaire, and test procedures that tend to be necessary. In their conclusion, no direct cause of spontaneous abortion was identified with 76% remaining unexplained. It is sure to leave a gateway for further research to be undertaken and a better understanding of the molecular basis of spontaneous abortion.

1.4.2 Molecular Basis of Spontaneous Abortion

There is a prevalent increase in mitotic chromosomal error during implantation, both in young and old fertile women **(Mantikou et al., 2012) (Macklon et al., 2002).** The human embryo can allow the placenta to attach firmly to the walls of the uterus. However the synchrony during implantation and embryo development, thus the receptivity of the endometrium can prevent evasion of the mother by the embryo.

This was achieved from the molecular aspect when compared with a cancer cell **(Ledbetter, 2009).** However, when there is no available information on histology, researchers have turned to gene expressions and proteomic profiling in determining the molecular fingerprints of a receptive endometrium **(Brosens et al., 2009)**. It was reported that 14bp HLA-G gene variation might have an association with an increased risk of spontaneous pregnancy according to results gathered from this study **(Levkova et al., 2020)**.

1.5 WnT/β-Catenin Signaling Pathway

In this era of great development in molecular medicine, so much improvement has been realized in the past couple of decades. It has helped in the investigative process by using molecular mechanisms easier. A groundbreaking mechanism that is representing the fine art of scientists and diagnostic medicine is Wnt signal pathways. The Wnt is known for regulating cell proliferation, migration, determining the fate of cells, and tissues coupled with embryonic development. The name Wnt comes from the term wingless and Int-1 **(Komiya & Habas, 2008).** The Wnt signal can be activated when the Wnt proteins bind to a domain called the N-terminal cysteine-rich from the frizzled receptor **(Pei & Grishin, 2012).** The receptors can revolve around the plasma membrane several times to incorporate the G-protein coupled receptors (**Rothbächer et al., 2000**) **(Huang & Klein, 2004)**.

However, to get the full potential of Wnt signaling, there must be co-receptors interacting between the Wnt protein and frizzled receptors. As a result of the activation of the receptors, signals are been sent to the cytoplasm having the phosphoprotein disheveled **(Sheets, 2015),** and signal transduction is taken place between frizzled and disheveled. Moreover, all organisms are having the Dsh proteins and there is a significant resemblance in terms of its functions in all organisms **(Dillman et al., 2013)** The Wnt pathway is characterized into three different categories such as canonical, non-canonical planar polar pathway, and non-canonical wnt/ calcium pathway **(Komiya & Habas, 2008)**

Canonical Pathway

As the name implies the canonical pathway falls in the category of canonical pathways which has a link with the beta-catenin (β-catenin) **(Komiya & Habas, 2008**). It can result in the accumulation of β-catenin in the cytoplasm. Furthermore results in the translocation in the nucleus as well as a transcriptional coactivator of transcriptional factors. Conversely, with the absence of Wnt, there would be no accumulation of β-catenin and would be degraded by a destruction complex **(Komiya & Habas, 2008)**. Furthermore, the function of the destruction complex is being carried out by a series of other proteins, which are responsible for the destruction of the β-catenin complex. These proteins are made up of a series of proteins such as Adenomatous Polyposis Coli (*APC*), Glycogen Synthase Kinase 3 (*GSK3*), Casein Kinase 1α (*CK1α*) **(Dillman et al., 2013)**.

These proteins degrade β-catenin by a mechanism known as ubiquitination, which can automatically translocate it for digestion by the proteasome **(Aberle et al., 1997)**. Furthermore, after the digestion process, Wnt then binds to Fz and LRP5 and also inactivates the function of the destruction complex **(MacDonald et al., 2009)**. The inactivation of the destruction complex is due to translocation of negative Wnt regulator employing translocation of axin and destruction complex into the plasma membrane **(van Kappel & Maurice, 2017)**. Protein phosphorylation in the destruction complex can lead to the binding of Axin to LRP5 via the cytoplasmic tail.

The axin then becomes de-phosphorylated resulting in decreasing its stability level **(van Kappel & Maurice, 2017)**. The Dsh is then activated through the process of phosphorylation, as well as inhibiting the *GSK3* through the DIX and ODZ domain. It then allows β-catenin accumulation which is then contained in the nucleus. However, impeding the cellular response through gene transduction along with the transcription factor **(Rothbächer et al., 2000)**. There have been no stated facts about how β-catenin can activate target gene expression, moreover, tissue-specific players show the prospect of assisting β-catenin in defining genes target **(Söderholm & Cantù, 2020**). So many controversies concerning β-catenin protein interaction, and whether it is been

phosphorylated by Akt at Ser552, which in turn is causing dissociation from cell-cell contact and accumulation in the cytosol **(Fang et al., 2007)**.

Noncanonical Pathway

Noncanonical planar cell polarity (PCP) is a pathway lacking β-catenin, however, this pathway does not make use of LRP-5/6 as a co-receptor (**Sun**, **2011**) (**Ren et al., 2021**). The PCP pathway can be activated when Wnt binds to Fz and its co-receptor. A complex is formed with Dishevelled-associated activator morphogenesis, which comes about as a result of Dsh recruitments, Dsh uses its PDZ and DIX domains in the activation process **(Katoh, 2017)**. The G-protein Rho is then activated by DAAM1 through a guanine exchange factor, and Rho activates Rho-associated kinase (ROCK) **(Habas et al., 2001)**.

The ROCK happens to be one major regulatory mechanism of the cytoskeleton **(Amano et al., 2010)**. There is a complex formation between the Dsh and *RACL* gene as well as mediating the binding profile of actin. The activation of *JNK* is favored by *RACL* and thus may lead to the polymerization of actin. The restructuring of cytoskeleton and gastrulation is favored when there is a binding between profiling and actin **(Van Aelst & D'Souza-Schorey, 1997).**

Noncanonical Wnt/calcium Pathway

In this pathway, β-catenin does not have a direct impact, but instead, aids the regulation of calcium and calcium release from the endoplasmic reticulum (ER) in controlling calcium at the intracellular level. The same mechanism that applies to other Wnts pathways is applicable here, wherein the ligand-binding will foster the activation of the Fz receptor directly with then interact with Dsh in turn activating Dsh-protein domains **(De, 2011)**. The PDZ and DEP domains are both involved in Wnt/calcium signaling (**De, 2011)**.

Furthermore, there are varying characteristics of the Fz receptor which is quite different from other Wnt pathways, characteristically is indirectly related to being interfacing with trimeric G-protein **(De, 2011)**.

However, the co-stimulation of Dsh and G-protein can then trigger PLC and or cGMP-specific PDE activation. The activation of PLC can cause the cleavage of plasma membrane components into the DAG and IP3 **(Francis et al., 2010)**. The binding of IP3 on ER receptors can cause the release of calcium, and thus the increased concentration of DAG and calcium can lead to the activation of Cdc42 via PKC **(Kania et al., 2017)**. Cdc42 plays an integral role in ventral patterning. Calcium increased will activate both calcineurin and CaMKII, CaMKII impedes the activation of transcription factor which is a regulatory mechanism of cell adhesion, migration, and separation of tissues **(Y. Z. Liu et al., 2014).**

However, the coupling of both canonical and non-canonical pathways has been looked into keenly proposing a convergent Wnt pathway (**Nelson & Nusse, 2004**). Likewise, the convergent evidence of the Wnt signaling pathway displays integration and activation of the Wnt/$Ca2^{+}$ and also Wnt/β-catenin signaling for several other Wnt ligands **(Nelson & Nusse, 2004)**.

1.5.1 The Role of WnT/β-Catenin Signaling in Embryonic Development

There has been so much evidence stating the role of Wnt/β-catenin signaling and its regulating effect on the embryo in the mouse model and also regulating the formation of luminal epithelial evagination. Over decades ago, a study was carried out on Wnt signal, using mutated embryos in Wnt genes. So far genes coding for other Wnt family members such as *Wnt3* and *Wnt10B* were found to be carriers of MT insertions (**Tekmal & Keshava, 1997)**. There came about the demonstration of the Wnt in blood vessel development **(Scholz et al., 2016)**.

In context, the Wnt signal plays a more specific and integral role, and also its effect on cell physiology **(Sethi & Vidal-puig, 2015)**. The Wnt signal pathways play an integral

role in the development of an embryo, it thus functions in different species such as vertebrates and invertebrates which include humans, frogs, zebrafish, and drosophila **(Holstein, 2012)** The Wnt genes are notably found in the genomes of Drosophila, Xenopus, and other complex organisms, with 19 essential Wnt genes having different functions **(Miller, 2002)**. However, it points noting down that these genes remained to be vital in the multicellular development of animals (**Holstein, 2012**).

According to the review article, data demonstrated that Wls is essential in the development of the embryo **(Biechele et al., 2011)**. Wnt pathways were discovered in the polarity of the Drosophila segment, promoting the establishment of both anterior and posterior regions **(Clevers & Nusse, 2012)**. It has been shown to play a critical role in the formation of the axis body, as seen in Drosophila, especially in the anterior, posterior, dorsal, and ventral axes. It is known for initiating the differentiation of cells which will trigger the formation of vital organs such as lungs and ovaries **(Aros et al., 2021)**. The development of vital organs can be achieved when regulation of cell proliferation and migration are accomplished **(Aros et al., 2021)**.

A better understanding of the transduction mechanism of Wnt/β-catenin can be achieved with in-depth knowledge of how Wnt operates in the homeostatic range, also in cell fate-proliferation and stem–cell renewal **(Clevers & Nusse, 2012)**.

Furthermore, the Wnt signaling is been divided into segments such as axis patterning, cell fate specification, cell proliferation, and migration **(Barbato et al., 2017)**.

The *Wnt 1* molecule was firstly identified in the growth and development of the breast and is induced in mouse mammary oncogenesis **(Feng et al., 2018).** It was after the *Wnt3* gene got discovered, and provided the insertion mechanism in mammary tumors in mice (**Feng et al., 2018**). However neither *Wnt 1* nor *Wnt 3* are been expressed in the development of an adult mammary gland, but other members of Wnt such as *Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6,* and *Wnt7* in different stages of development are being expressed **(Feng et al., 2018)**. The GSK and Wnt signaling was identified in 1980 which is greatly conserved by mammals but originally from yeast. In mammals, there are two *GSK3* isoforms and they code for target genes sharing a 97% amino-acid sequence.

From research, the isoforms are of *GSK3* which are dysregulated, they are key players in tumorigenesis **(Chong & Maiese, 2004).** From previous research, the studies demonstrated the importance of the Wnt signal in neural development and differentiation **(Kriska et al., 2021)**. Furthermore little is known about Wnt/β-catenin independent signal in terms of neural development.

1.5.2 WnT/β-Catenin Signaling In Early Pregnancy

The reproductive tract of female mammals is made up of strong tissues such as oviducts, uterus, cervix, and vagina **(Tung & Suarez, 2021)**. The mammalian embryo has two pairs of ducts, which arise before sexual differentiation. This duct known as the Mullerian duct can give rise to the female reproductive tract and differentiate into the oviduct, uterus, cervix, and the upper end of the vagina **(J. M. Schwarz, 2015).**

The formation of Mullerian duct is formed during the embryonic day, which incorporates itself into the oviduct, uterus, and cervix. However, the epithelial cells from the coelomic wall facilitate the formation process of the Mullerian duct (**Mullen & Behringer, 2014**). The research was carried out to study the relation between Wnt/βeta-catenin signal and recurrent spontaneous abortion during the first trimester using fresh tissue villous and decidual tissues. They concluded that β-catenin may be used as biomarkers for unexplained recurrent spontaneous abortion (RSA) but ruled out the relationship between Wnt and Early pregnancy **(Li et al., 2015)**.

The *Wnt 4* is expressed after the duct has been formed, and it is highly expressed when surrounding the mesenchymal cells **(Deutscher & Hung-Chang Yao, 2007).** Both the male and female sexes of *Wnt4* mutant mice were lacking the Mullerian duct and as a result, the females lack the reproductive tract. However, data suggested that *Wnt 4* plays a critical role in the development of ducts before sexual differentiation occurs. According to the review, a lot of evidence were gathered which states *Wnt 4* plays an integral role in the differentiation of cells and also aid the process of growth and development of human and should be explored more (Q. Zhang et al., 2021).

The *Wnt 5a* is also an essential gene for its sole responsibility in the development of the reproductive tract **(Yu et al., 2015)**. From the genetic data analysis, it was revealed that

Wnt5a works concomitantly with *Wn7a* **(Mericskay et al., 2004)**. It has shown that the role of *Wnt5a* is critical in the formation of the uterus, cervix, and vagina **(Roly et al., 2018)**. The *Wnt2a* is important for the development of the placenta and also the vascularization of the placenta. Its role in the trophoblast cells during the first trimester remained to be unknown.

In a study, they compared the expression level of *Wnt2* in the villous obtained from tissues of patients who had suffered from URSA to that of a healthy woman using different techniques. In conclusion, they concluded that *Wnt2* is an important tool in the function of the trophoblast. However, insufficiency in *Wnt2* may lead to the impairment of cell proliferation (**Befani, Christina; Liakos, 2017)**.

1.6 The Aim Of This Study

To investigate the expression level of Wnt-signaling pathway-related genes such as *WNT3A*, *WNT4*, and *WNT5A*, which are suspected to cause an impact on embryonic development leading to spontaneous abortion.

1.7 Significance of the study

Studies conducted by different researchers point toward the significance of Wnt signal pathways genes and their contribution to miscarriage. Therefore investigating these genes will give a better understanding of their role during implantation and miscarriage which can be implored and also rectifying errors using IVF techniques.

CHAPTER II: MATERIALS AND METHODS

2.1 Materials

2.1.1 Sample Collection (Abortion Materials)

The abortion materials were obtained from Near East University Hospital Medical Genetics Laboratory after it was approved by the Near East University Scientific Review Board. A total of 24 samples were collected from the storage point and 23/24 were used in carrying out this study. They were categorized into normal and abnormal karyotypes. This research study was undertaken at Near East University DESAM Institute Molecular Medicine Laboratory, Nicosia Northern Cyprus.

2.1.2 Manufacturing Company

cDNA synthesis kit (Canada), Thermo-scientific marker (USA), Nanodrop (Thermo-scientific 2000 spectrophotometer), Applied Bio-systems (USA), Eppendorf Scientific (Germany), Bio-Rad Instrument (Cleverer Scientific Limited), Ultraviolet Trans Illuminator (Bio-imaging system), Rotar Gene Real-Time PCR (Qiagen, Germany), Weighing balance ((Balance Adam Equipment), RNA isolation Kit (Hibrigen, Turkey), Bio-Rad Electrophoresis (UK).

2.1.3 Chemical Reagents

2.1.3.1 Molecular Weight Marker

GelPilot 50 bp DNA ladder (QIAGEN, Hilden, Germany) catalogue no. 239025) and GeneRuler 50 bp DNA ladder (Thermo Scientific™, Pittsburg, USA, catalogue no. SM0371) were used as a molecular weight marker.

2.1.3.2 Oligonucleotides

The primer pairs which were designed for *WNT3A*, *WNT4*, and *WNT5A* primers were obtained from (Turkey)

2.1.3.3 Standard Solutions

50X Tris Acetic Acid EDTA (TAE) electrophoresis buffer was prepared as marked out by **Sambrook et al 1989**. It was later diluted with 1X (80ml from 10X TAE +980 ml distilled water). The dilution was done to reduce the concentration of TAE which may cause delays in-band movements**.**

2.1.3.4 Chemical Agent

Agarose biomax 100mg, Ethidium bromide (Serva, Heidelberg, Germany)

2.1.3.5 Softwares

GelCapture Software packages were used to view and analyze the gel images and store the imaging data. Statistical analysis of data has been done using Statistical Package for the Social Sciences (SPSS).

2.2 Methods

2.2.1 RNA Extraction from Abortion Materials

The RNA was extracted from samples(abortion materials) using RNA extraction kit (Hibrigen, Turkey) following manufacturer guidelines (Hibrigen, Turkey).

2.2.2 RNA Concentration Measurement (Nanodrop 260/280)

The concentration of RNA and purity was measured using an optical density at 260/280 nm wavelength with the aid of a Nanodrop (Thermo-scientific Pittsburg USA).

2.2.3 Complementary DNA (cDNA) Synthesis

The synthesis of cDNA was carried out using the OneScript Plus cDNA synthesis kit (abmGood, Canada). The kit contained five components it was recorded and presented in **Table 2.1**

Kit Composition for Mix	**1X**
Buffer	4 µl
dNTPs	1 µl
Primer	1 µl
RNAse H_20	5 µl
Enzyme	1 µl
RNA sample	8 µl
Total	**20 µl**

Table 2.1 Calculations for cDNA synthesis using the cDNA synthesis kit protocol

2.2.4 Primer Optimization for Gradient PCR

In optimizing the primers oligomer stock was prepared for three genes. A certain quantity of deionizing water was added to each primer specific giving a total of (100µM). It was further diluted to a 10 µM working solution by taking 10 µl of stock primer added to 90µl deionized water

The primer pairs which were designed for *WNT3A*, *WNT4*, and *WNT5A* were presented in **Table 2.2**

Oligo Name	Base Sequence 5'-3'
WNT3A-Forward	GAGCAGGACTCCCACCTAAAC
WNT3A-Reverse	AGACACTAGCTCCAGGGAGGA
WNT4-Forward	CATGAGTCCCCGCTCGTG
WNT4-Reverse	CCAGGTACAGCCAGTTGCTC
WNT5A-Forward	TCGCTGATGGACGTTGGAAA
WNT5A-Reverse	CCAATGGACTTCTTCATGGCG

Table 2.2 The sequence of primers forward and reverse primers obtained from oligomer stock for three genes **(*WNT3A*, *WNT4*, and *WNT5A*)**

Gradient PCR was performed using the bio-systems 96 well thermal cycler PCR which was used in differentiating the different optimum temperature conditions for the qRT-PCR. The gradient PCR was done for the 3 Wnt genes at a selected temperature range between 56℃ to 61℃. Calculations were done for 23 samples and 1 negative control which was presented in **Table 2.3**.

The analysis of gradient PCR lasted for 1hr 40 minutes.

Component	**1X**	**24X**
TaqMix	5 µl	120 µl
Forward Primer	0.5µl	12 µl
Reverse Primer	0.5 µl	12 µl
Glycerol	0.6 µl	14.4 µl
DH_2O	1.4 µl	33.6 µl

Table 2.3 The calculation for gradient PCR mixture

In carrying out the procedure 8µl from the final mixture + 1µl of cDNA giving a total of 9µl were put into the Eppendorf PCR tubes. The total was for 23 samples and 1 negative control. These steps were repeated for all three genes using the primer specific to these genes.

Annealing temperatures ranging from 56℃-61℃ were used for the gradient PCR during this experiment which was presented in **Table 2.4**.

Stage	Temperature	Time	Time
Initial Denaturation	95℃	5 mins	1 cycle
Denaturation	95℃	15mins	35 cycles
Annealing	56℃-61℃	30mins	
Extension	72℃	45mins	
Termination	72℃	5mins	1 cycle

Table 2.4 Optimum conditions used for gradient PCR

Agarose Gel Electrophoresis

The product from gradient PCR was later taken for gel electrophoresis using Agarose (Sigma-Aldrich, catalogue no. 11388983001). The gel concentration was prepared at 2%, in which 4 grams of agarose were weighed and dispensed into a transparent glass holding 200 ml TAE buffer. The solution was then taken into the microwave at high voltage to ensure there was clarity seen in the glass every 30 seconds. The glass was taken to a cool dry place to ensure the cooling down of the mixture. The tray (20cm x 20cm) was wiped to remove debris, Ethidium Bromide (Ethidium bromide (EtBr) (Sigma-Aldrich, catalogue no. E1385) 5 µl was added to the solution before it was poured into the tray for it to get solidified. Both loading dye and sample were mixed (2µl to 8µl respectively), and were loaded into the wells. The ladder wells were later loaded with 2µl and the tank was covered. The samples ran for 1hr 30mins at 100volts. The bands were later viewed using an ultraviolet trans-illuminator (DNR Bioimaging system, Neve Yamin, Israel).

2.2.6 Quantitative-PCR (RT-qPCR)

The reaction for PCR thermal cycler and RT-qPCR were undertaken in a sterilized environment using a category II laminar flow hood. The plastic wares and reagents were sterilized and assigned for this procedure. The RT-qPCR master mix was done for all 23 samples which were presented in **Table 2.5**.

Component	**1X**	**24X**
SYBR GREEN	10 µl	240 µl
Forward Primers	2 µl	48 µl
Reverse Primers	2 µl	48 µl
DH_20	5 µl	120 µl

Table 2.5 RT-qPCR calculation mixture for *WNT3A*, *WNT4*, and *WNT5A*

The optimum condition for qRT-PCR at different stages was recorded and presented in **Table 2.6**

Stages	**Temperature**	**Time**	**Cycles**
Initial Denaturation	**95 ℃**	**2 minutes**	**1 cycle**
Denaturation	**95 ℃**	**30 seconds**	**30 cycles**
Annealing	**57 ℃**	**30 seconds**	
Extension	**72 ℃**	**45 seconds**	
Termination	**72 ℃**	**10 minutes**	**1 cycle**

Table 2.6 The optimum conditions for qRT-PCR

CHAPTER III: RESULTS

3.1 Introduction

To investigate the expression profile of WNT signaling pathway genes (*WNT3A*, *WNT4*, and *WNT5A*) in spontaneous abortion materials a total of 24 abortion samples were analyzed. These materials were categorized into two: Normal and Abnormal karyotypes (**Table 3.1** and **Table 3.2**). Since the Wnt/β-catenin is known for its role in the development of the embryo, it has been mapped in the drosophila model (**MacDonald et al., 2009**). The expression level of these genes was determined using different methods and procedures which include, RNA isolation, cDNA synthesis, Gradient PCR, and later qRT-PCR to get the expression profile of these genes.

Group one contains eight samples that were of abnormal karyotype and were presented in **Table 3.1**

Sample Identity	**Abnormal Karyotype (Abnormal Chromosomes)**	**Year**
001	(92,XX)	2020
002	(45,X)	2019
003	(47,XX)	2022
004	(46,XY)	2018
009	(69,XXY)	2018
016	(46,XY)	2017
021	(45,X)	2019
022	(45,XX)	2017

Table 3.1 Samples with abnormal karyotype

Group two contains eight samples that were of normal karyotype and were presented in **Table 3.2**

Sample Identity	**Normal Karyotype (Normal Chromosomes)**	**Year**
005	(46,XX)	2021
006	(46,XX)	2021
007	(46,XX)	2021
008	(46,XX)	2021
010	(46,XX)	2021
011	(46,XX)	2020
012	(46,XX)	2020
013	(46,XX)	2019
014	(46,XX)	2020
015	(46,XX)	2021
017	(46,XX)	2018
018	(46,XX)	2019
019	(46,XX)	2019
020	(46,XX)	2019
023	(46,XX)	2017

Table 3.2 Samples with normal karyotype

3.3 RNA Synthesis and Concentration Measurement

The RNA synthesis and concentration measurement for 24 samples was performed using the cDNA synthesis kit and the concentration was measured using the nanodrop technique which is presented in **Table 3.3**

Sample Identity	**RNA Concentration (ng/ µl)**	**A260/280**
001	19.5	1.7
002	6.4	2.2
003	112.0	2.0
004	16.2	1.9
005	17.3	1.9
006	24.2	1.6
007	19.2	1.6
008	17.3	1.7
009	27.7	1.5
010	28.8	1.9
011	7.2	1.8
012	1.6	2.4
013	6.7	2.0
014	56.0	2.0
015	47.8	2.0
016	6.5	1.6
017	21.5	1.6
018	26.3	1.7
019	37.2	1.6
020	2.7	1.7
021	2.7	1.9
022	4.8	1.7
024	314.7	2.0

Table 3.3 RNA concentration and purity ratio of the abortion materials using Nanodrop

3.4. Gradient PCR and Gel Electrophoresis (Agarose)

Gradient PCR has been carried out to get the optimum annealing temperature for the genes (*WNT3A*, *WNT4*, *WNT5A*, and *β-actin*). The bands displayed were recorded at varying base pairs (50bp- 95bp) they are presented in **Figure 3.**1, **Figure 3.2**, **Figure 3.3**, and **Figure 3.4**.

The *WNT3A* gene displayed bands at 60 bp at a temperature ranging from 56 ℃-61℃ and was observed as primer dimers (**Figure 3.1**).

Figure 3.1 *WNT3A* gene at different optimizing temperatures ranging from 56℃-61℃ **Line 1**. The ladder show *WNT3A* display bands at 60bp, **Lines** 56-NTC is a display of varying temperatures

The *WNT4* gene displayed bands at 61bp at a temperature ranging from 56 ℃-61℃ and was observed as primer dimers (**Figure 3.2**).

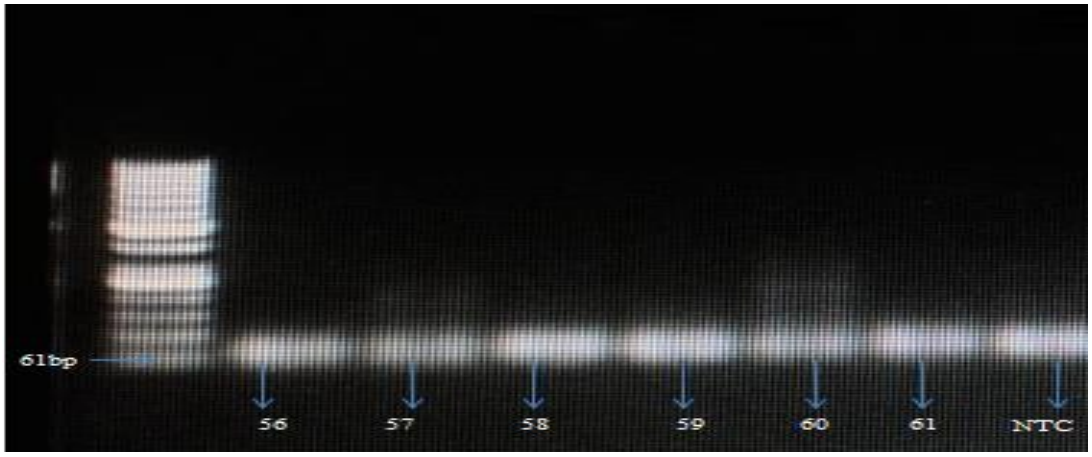

Figure 3.2 *WNT4* gene at different optimizing temperatures ranging from 56℃-61℃ **Line 1**. The ladder show *WNT4* display bands at 61bp, **Lines** 56-NTC is a display of varying temperatures

The *WNT5A* gene displayed bands at 55 bp at a temperature ranging from 56℃-61℃ and was observed as primer dimers (**Figure 3.3**).

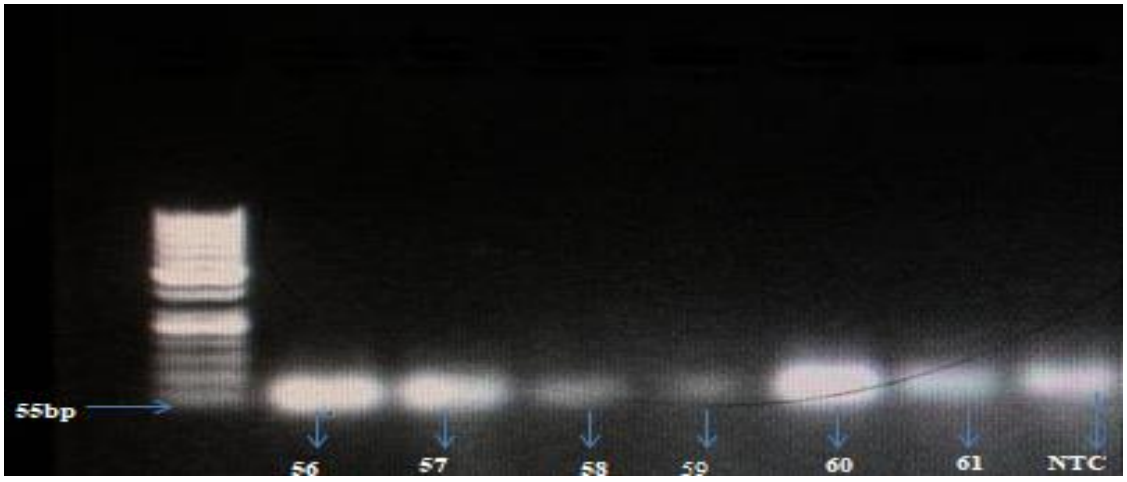

Figure 3.3 *WNT5A* gene at different optimizing temperatures ranging from 56℃-61℃ **Line 1**. The ladder shows *WNT5A* display bands at 55bp, and **Lines** 56-NTC is a display of varying temperatures.

The housekeeping gene (*β-actin*) was supposed to display bands at 112 bp, unfortunately, the band was visualized at 95 bp at 56℃-61℃ and was observed as primer dimers (**Figure 3.4**).

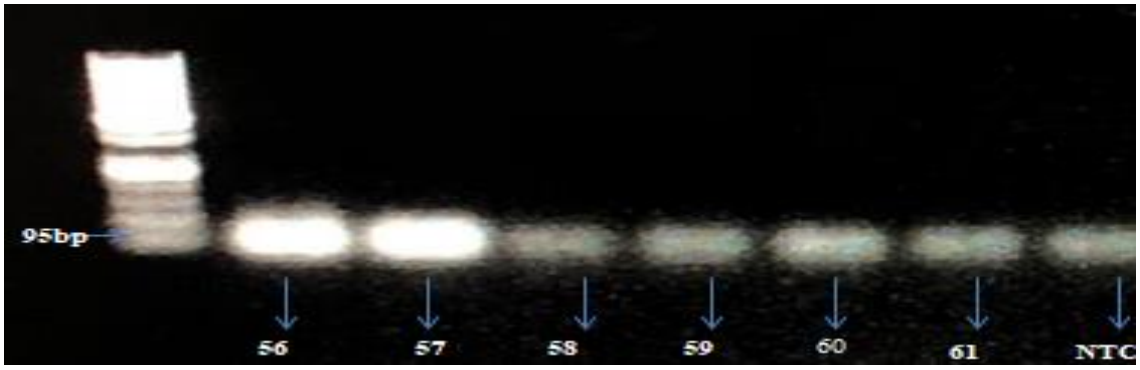

Figure 3.4 *β-actin (*Housekeeping gene)gene at different optimizing temperatures ranging from 56℃-61℃
(Line 1. The ladder shows ***β-actin*** display bands at 95bp, **Lines** 56-NTC is a display of varying temperatures)

3.5 Gene Expression Analysis of *WNT3A, WNT4,* and *WNT5A* genes

The expression profiles of these genes were carried out using cDNA synthesized samples for all three Wnt signal genes (*WNT3A*, *WNT4*, and *WNT5A,* **).** The samples were subjected to qRT-PCR for analysis. Based on observation the reaction becomes positive when there is an accumulation of signals carried out by fluorescent dyes. A keen interest was paid on the cycle threshold which is the exact cycle value required for a fluorescent signal to go above the threshold. The Ct values are a reversible scale that can be used to compare the nucleic acid quantity in a sample. As such it is interpreted as the lower the Ct value, the greater the nucleic acid quantity in a sample.

The expression profile of the three genes (*WNT3A*, *WNT4*, and *WNT5A*) including the housekeeping gene (***β-Actin)*** in all 23 samples and a negative template control was done using qRT-PCR and the values were recorded which is presented in **Table 3.4**

Sample Identity	***WNT3A***	***WNT4***	***WNT5A***	***β-Actin*** **(Housekeeping gene)**
001	19.30	21.81	22.80	17.90
002	18.13	19.27	23.09	19.09
003	20.40	19.61	22.54	17.72
004	17.44	21.75	22.74	20.02
005	17.84	17.58	22.90	18.03
006	17.37	16.51	22.71	20.37
007	19.31	18.54	22.59	16.54
008	16.76	22.26	21.92	22.65
009	16.21	23.37	22.66	23.59
010	16.72	18.00	21.68	19.68
011	16.71	26.75	23.06	18.85
012	16.60	25.63	22.10	18.39
013	15.62	27.08	21.67	17.31
014	15.86	24.32	22.37	18.05
015	15.29	24.95	21.45	13.86
016	15.52	24.42	21.19	18.03
017	15.57	25.51	21.16	20.07
018	16.38	26.89	21.15	23.08
019	15.41	21.56	20.80	17.80
020	15.08	27.39	20.75	24.33
021	17.68	25.76	21.11	18.54
022	17.10	20.37	21.13	22.15
024	15.60	23.50	22.31	19.23
NTC	20.63	29.35	24.99	21.23

Table 3.4 Cycle threshold results of WNT signal genes expression using qRT-PCR

The expression profile of *WNT3A* for all 23 samples and a negative template control was done using qRT-PCR which is presented in **Figure 3.5**

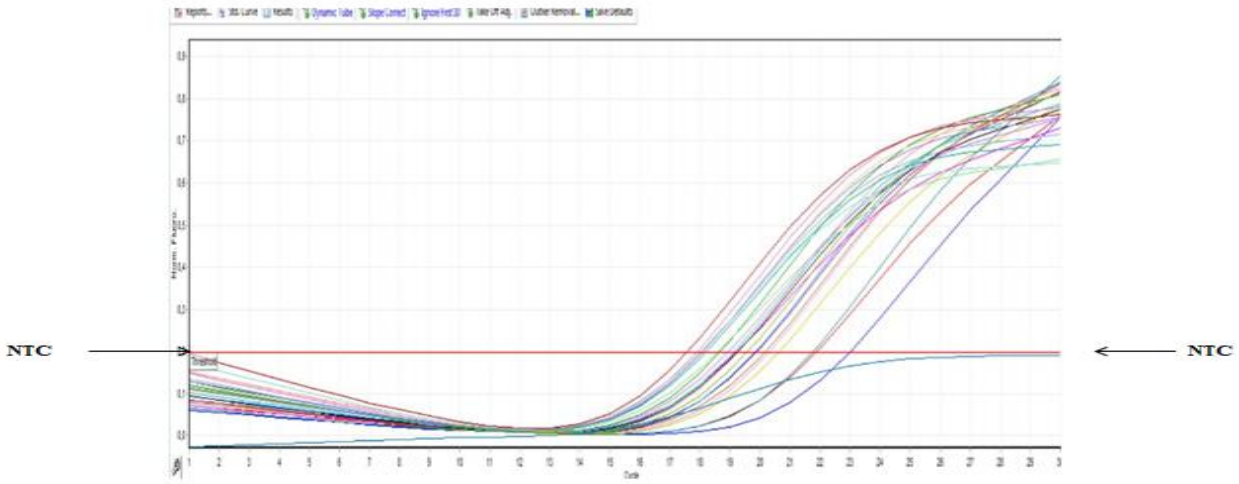

Figure 3.5 Gene expression level for *WNT3A* from the qRT-PCR with curves

The expression profile of *WNT4* for all 23 samples and a negative template control was done using qRT-PCR which is presented in **Figure 3.6**

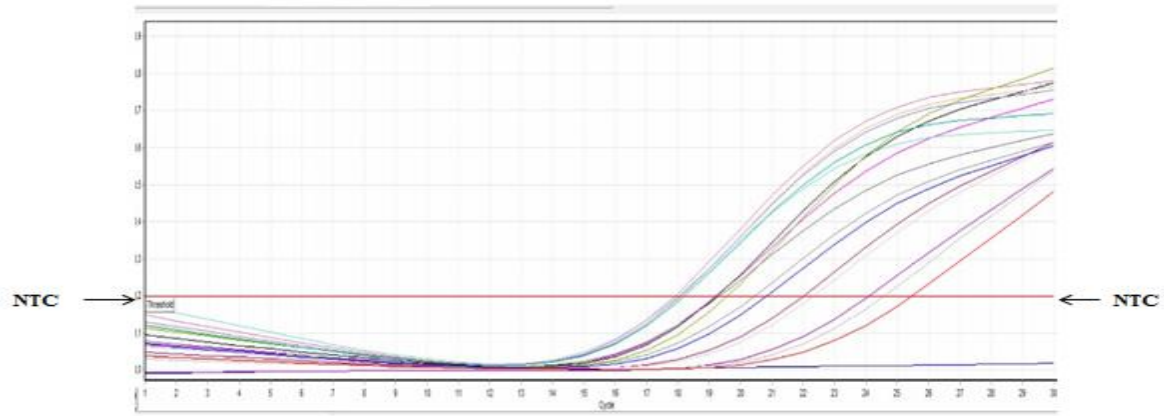

Figure 3.6 Gene expression level for *WNT4* from the qRT-PCR with curves

The expression profile of *WNT5A* for all 23 samples and a negative template control was done using qRT-PCR which is presented in **Figure 3.7**

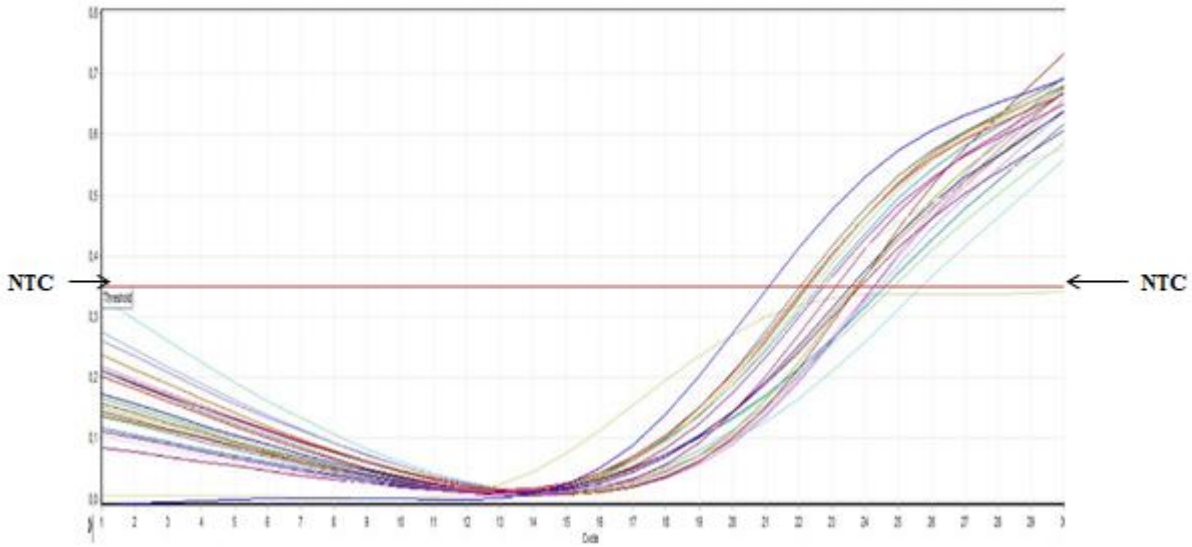

Figure 3.7 Gene expression level for *WNT5A* from the qRT-PCR with curves

The expression profile of ***β-actin*** for all 23 samples and a negative template control was done using qRT-PCR which is presented in **Figure 3.8**

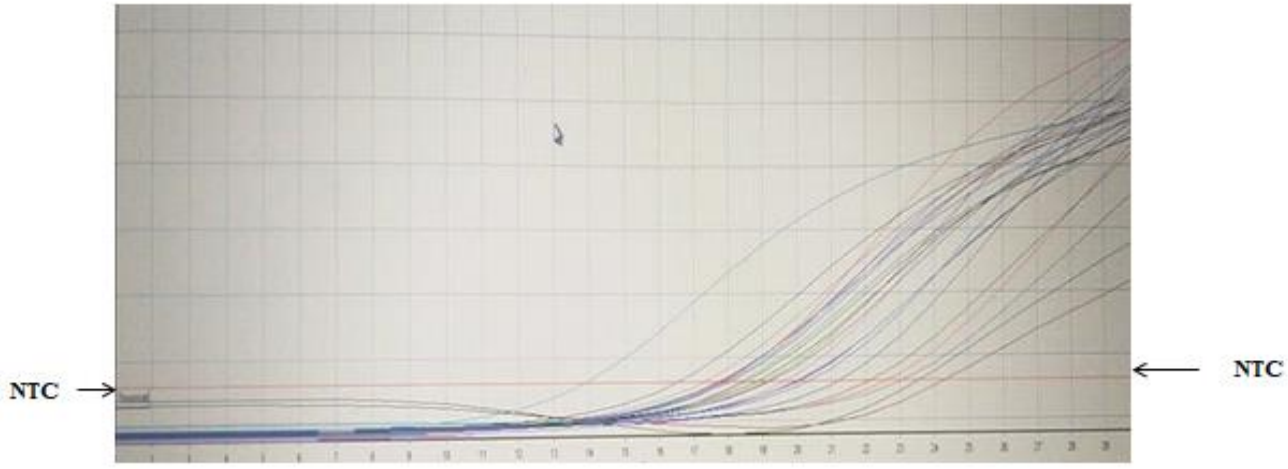

Figure 3.8 Gene expression level for *β-actin* from the qRT-PCR with curves

3.6 Statistical Analysis

The statistical analysis of *WNT3A, WNT4,* and *WNT5A* for the two groups (normal and abnormal karyotypes) for all 23 samples are presented in **Table 3.5** with P values <0.05=Significant and P values >0.05=Not significant.

Group Statistics					
Genes	Karyotype	Sample Numbers	Mean	p-Value	Std. Error Mean
WNT3A	Abnormal	8	0.7922	0.170	0.32046
	Normal	15	1.9469		0.74129
WNT4	Abnormal	8	1.4901	0.176	0.51976
	Normal	15	2.8678		0.83561
WNT5A	Abnormal	8	0.5011	0.592	0.12427
	Normal	15	0.4101		0.11080

Table 3.5 Statistical analysis of *WNT3A, WNT4,* and *WNT5A*

(P values <0.05)= Significant (P values >0.05)= Not significant

When we compared the three genes ***WNT3A, WNT4,* and *WNT5A***,
Out of 23 samples of *WNT3A,* we found 8 abnormal karyotypes and 15 normal karyotypes with a mean difference of 1,9469± 0,74129 and 0,7922±0,32046 respectively, According to the data obtained no statistically significant difference was found between the two groups analyzed (**P=0.170**), As shown in **Table 3.5.**
Out of 23 samples In *WNT4* we also found 8 abnormal karyotypes and 15 normal karyotypes with a mean difference of 1,4901±0,51976 and 2,88678±0,83561. According to the data obtained no significant difference was observed between groups (**p=0.176**). **As shown in Table 3.5**

Out of 23 samples in *WNT5A,* we found 8 abnormal karyotypes and 15 normal karyotypes with a mean difference of 0,5011±0,12427 and 0,4101±0,11080, According to the data obtained, no statistically significant difference was observed between the groups analyzed (**p=0.592**). As shown in **Table 3.5**

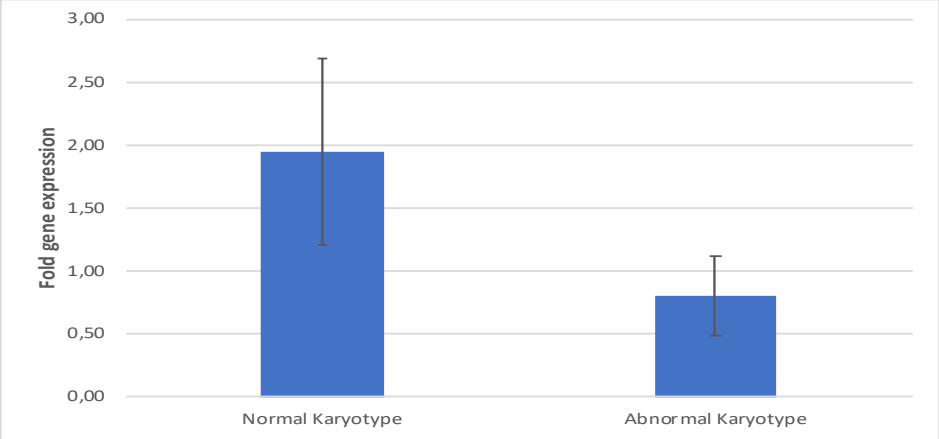

Figure 3.9a Graphical representation of *WNT3A* gene expression in normal and abnormal karyotypes

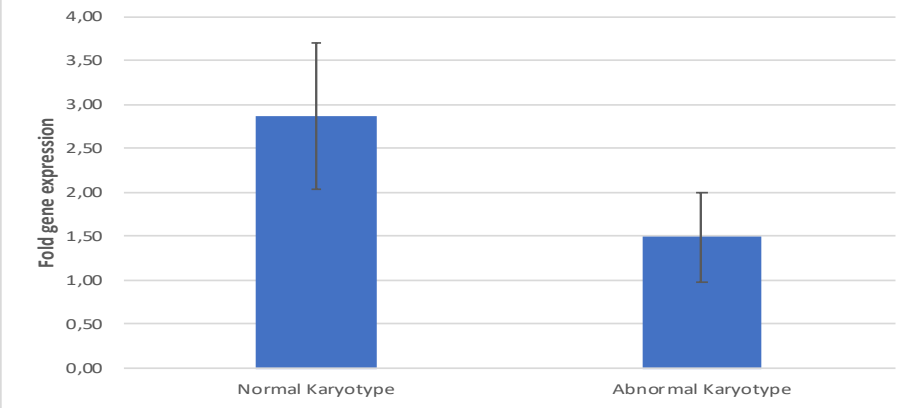

Figure 3.9b Graphical representation of *WNT4* gene expression in normal and abnormal karyotypes

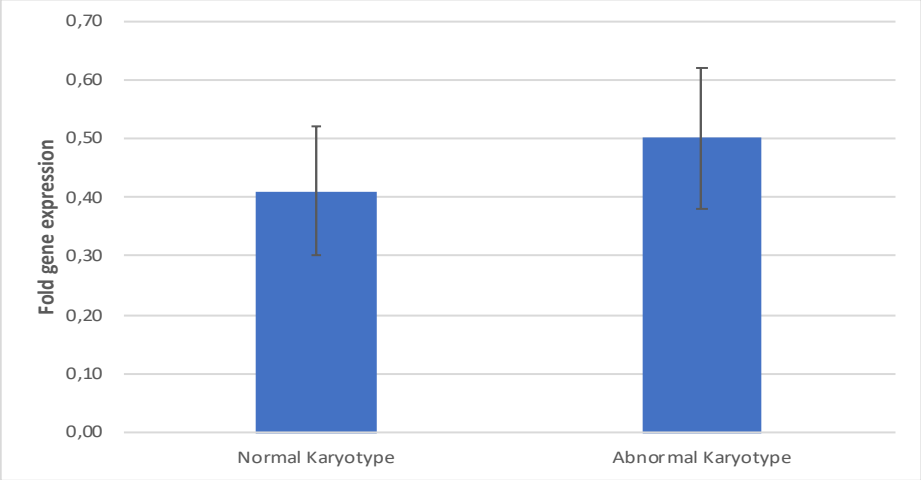

Figure 3.9c Graphical representation of *WNT5A* gene expression in normal and abnormal karyotypes

The Gene Correlation Analysis of *WNT3A*, *WNT4*, and *WNT5A* for the two groups (normal and abnormal karyotypes) for all 23 samples is presented in **Table 3.6** with Correlation significance = 0.05 level and Correlation significance = 0.01 level P values <0.05=Significant and P values >0.05=Not significant.

Gene Correlation Analysis				
Genes		***WNT3A***	***WNT4***	***WNT5A***
WNT3A	Correlation coefficient	1.000	0.522	-0.489
	Significance	.	0.011	0.018
	N	23	23	23
WNT4	Correlation coefficient	0.522	1.000	-0.958
	Significance	0.011	.	0.000
	N	23	23	23
WNT5A	Correlation coefficient	-0.489	-0.958	1.000
	Significance	0.018	0.000	.
	N	23	23	23

Table 3.6 Correlation significance between *WNT3A*, *WNT4*, and *WNT5A*

Correlation significant = 0.05 level Correlation significant = 0.01 level

When we compared the correlation between the three genes we found a statistically significant difference between *WNT3A*, *WNT4*, and *WNT5A* (**p=0,011,0,018**), Respectively.

WNT4 has a significant difference from *WNT3A* and *WNT5A* (**P=0,011,0,000**), respectively.

WNT5A has also a significant difference from *WNT3A* and *WNT4* (**P=0,018,0,000**), respectively. The correlation analysis shows the relationship between all three Wnt genes (*WNT3A, WNT4, and WNT5A***).** However *WNT3A* and *WNT4* are positively correlated, but no further correlation between *WNT3A and WNT4* compared to *WNT5A***.** This explains further as expressions were observed in *WNT3A* and *WNT4*, and no further expression of *WNT5A***.**

CHAPTER IV: DISCUSSION AND CONCLUSION

4.1 Discussion

Miscarriage is the most common form of complication during pregnancy, and it is characterized by the involuntary loss of a fetus before maturation **("Definitions of Infertility and Recurrent Pregnancy Loss," 2008).** Recurrent and sporadic are the two types of miscarriages (**Sonderegger et al., 2007**). However recurrent miscarriage can affect 1% of couples, and 25-50% of pregnant women from data can experience a sporadic miscarriage as a result of random fetal abnormalities taking place in the chromosome (**Pollheimer et al., 2006**). A protein called the Wnt which is formed from glycoproteins can act in the capacity of receptor-ligand mediating signal pathways**.** The Wnt proteins are there to regulate biological processes which include the development of an embryo. The name Wnt is an acronym for Wingless and Int-1**(Komiya & Habas, 2008).** The signal pathways can be carried out through the transmission of signals, promoting the transfer of molecules from one cell to another cell surface receptors (**Kleinert et al., 2010**). The Wnt genes are found in lower to complex animals ranging from fruit flies to humans. From research Wnt signals can regulate different types of activities such as pluripotency and proliferation of an embryo from a mouse model stem cells and as well somatic cells adjustment (**de Jaime-Soguero et al., 2018)**
There have been reported crosstalks between the uterus and hatched blastocyst during the receptive phase, considerably this promotes implantation and gives further adjustment to the uterine tissue (**Kaloğlu et al., 2003**).

4.2. Wnt Signalling in Fetal Development

The role of Wnt/β-catenin have being studied expressly using the mouse model on its effect on the embryo. The Wnt/ β-catenin can regulate certain mechanisms in the embryo such as the formation of the luminal epithelial evagination. Relatively studies have identified *WNT3* and *WNT10B* as sole carriers of MT insertions (**Tekmal & Keshava, 1997**). As a result of a controversial report, the Wnt signal was identified to be vital in developing blood vessels **(Benz et al., 2019**). The Wnt signal pathways have more specific and vital roles to play in the physiology of cells (**Ng et al., 2019**).

The Wnt signal was first identified in Drosophila, but later found to be present in a variety of other species such as humans, zebrafish, and frogs comprising 19 essential Wnt genes with varying functions (**Kozmikova & Kozmik, 2020)**. It was after this discovery that researchers did a thorough study and came up with a conclusion that these genes can contribute to the development of the embryo in Drosophilia (**Pond et al., 2020**). Furthermore, the Wnt can facilitate the development of the body axis by promoting and establishing regions (**Niehrs, 2022**). However, the Wnt signaling can also aid the development of organs and organ systems employing regulation and proliferation (**Ramirez et al., 2020**)

4.3 Literature Review on Wnt Signalling Genes (*WNT3A, WNT4, WNT5A*)

The *WNT3A* is a canonical ligand pathway that helps in promoting embryonic development, which includes regulating pluripotency, migration of cells during neurulation and gastrulation, and the formation of body axis (**Denicol et al., 2013**). From review literature, *WNT3A* has been shown to promote both β-catenin dependent and β-catenin independent YAP/TAZ responses (**Park et al., 2015**). The *WNT3A* plays an integral role in the maintenance of bovine trophoblast by regulating and activating CDX2 expression levels using the Wnt-YAP/TZ signal pathways (**Wang et al., 2019)**.

There has been evidence which was gathered using animal models, suggesting the key role of Wnt signaling in placental development and also impedes the regulation of trophoblast proliferation and invasion (**Chen et al., 2021**). It has been observed that a disruption in the Wnt signal component is largely associated with a gestational disease in mice **(Sonderegger et al., 2010**). The Wnt beta-catenin has been shown to inactivate the function of blastocyst implantation **(Z. Zhang et al., 2017**). According to **(H. Bao et al., 2020)**, the over-expression of Wnt-Beta signaling, when the Wnt inhibitors are silent can hinder trophoblast differentiation.

A total of fourteen Wnt ligands and eight FZ receptors were identified in the placenta tissue during the first trimester (**Sonderegger et al., 2010)**. The *Wnt4* is mostly expressed in Wnt signal genes. However, studies on epigenetics have demonstrated

promoter methylation for four Wnt inhibitors in the placenta and trophoblast tissue of humans (**Apicella et al., 2019**). The ability of the human embryo can be compromised when there is a loss of function of beta-catenin which can affect blastulation and a decrease in trophectoderm cells (**Krivega et al., 2015**).

The *Wnt4* and Beta-catenin from animal model studies have shown to play a role in implantation and placental development **(S. H. Bao et al., 2013**). The downregulation of Dkk can affect the invasion of trophoblast negatively **(Zhu et al., 2016**). Therefore an increase in Dkk-1 and sFRP4 expression may result in a decrease in *Wnt4* and *β-catenin* expression, which might either decrease or increase *Wnt5A* expression (**Chronopoulou et al., 2022**). According to (**Chronopoulou et al., 2022**) aimed to study the expression of *Wnt4*, *Wnt6*, and *β-catenin* using human placenta tissue obtained from first-trimester miscarriage, to detect the relationship between alterations in signaling pathway and early pregnancy failure in humans. In their conclusion, their finding shows the significance of balanced Wnt signaling in an event surrounding early pregnancy. A study demonstrated that a total of fourteen Wnt ligands and eight Fzd receptors were expressed in the placenta of a human sample, indicating the role of the Wnt signaling pathway in placental development (**Sonderegger et al., 2007**). The β-catenin expression may lead to hyperplasia and subfertility, and the destruction of β-catenin can lead to infertility (**Jeong et al., 2009**).

In animals, a balanced state of equilibrium of Wnt-signaling is important for placentation in humans. In two different ways such as the hyper-activation and under-activation of Wnt signaling are associated with the pathology of the placenta and trophoblast abnormalities (**Zhu et al., 2016**).

4.4 Results Generated in this Study

A total of 23 samples were obtained from the IVF clinic at Near East Hospital, wherein they were placed into two categories namely Normal and Abnormal karyotype. The study was carried out to observe the expression level of Wnt-signalling pathway genes in (*WNT3A, WNT4*, and *WNT5A*) in all 23 abortion materials. The RNAs were isolated from these samples and further processed for cDNA synthesis. The gradient PCR was performed to obtain the optimum temperature ranging from 56℃ to 61℃ of the three genes along with the housekeeping gene (β-actin). The genes were further processed using Agarose gel electrophoresis, but each gene displayed no bands rather primer dimers were visualized.

There was a 1.1-fold decrease in abnormal karyotype was observed when compared to normal karyotype in *WNT3A*. However (**Ross et al., 2014**) conducted a study on rare human syndrome on genetic evidence of Wnt signal and reprogramming. They demonstrated that a balanced activation of the Wnt signal can yield a normal karyotype, whereas an abnormal stimulation of *WNT3A* could produce an abnormal karyotype.
A 1.5-fold decrease in abnormal karyotype was observed when compared to normal karyotype in *WNT4* and a negative fold difference in abnormal karyotype when compared to normal karyotype was observed in *WNT5A* in this study. In a study, "link in *WNT4, WNT6, β-catenin* expression in human placenta tissue during the first trimester". A 6.1, 5.1, and 7.6-fold increase was observed in the different subgroups of the *WNT4* gene. The differences in the subgroups were not statistically significant, as a result of a significant increase in *WNT4* placenta expression could be associated with early pregnancy loss (**Chronopoulou et al., 2022**). The correlation analysis shows the relationship between all three Wnt genes (*WNT3A, WNT4*, and *WNT5A***).** However *WNT3A* and *WNT4* are positively correlated as they were both upregulated, but no further correlation between *WNT3A and WNT4* compared to *WNT5A* which was downregulated**.** This explains further when there is an upregulation of *WNT3A* and *WNT4,* there will be the downregulation of *WNT5A* from the correlation analysis result.

A decreased expression in *WNT3A* and *WNT4* and an increased expression in *WNT5A* were observed in abnormal karyotypes when compared to normal karyotypes. The Wnt signaling genes have a significant role in ensuring implantation, migration of cells, promoting embryonic development, neuralation and gastrulation.
However, the *WNT3A* from a study has a function of promoting β-catenin-dependent and independent responses. Furthermore, *WNT4* remains to be the most expressed Wnt signaling gene (**Apicella et al., 2019**). So far there has been a lot of research carried out on *WNT4* in the adult uterus, crucial in implantation and formed parts of the decidua (**Hayashi et al., 2009)**.
Overall according to the gene expression analysis, the *WNT3A*, *WNT4*, and *WNT5A* were expressed in the abnormal karyotype when compared to the normal karyotype in the spontaneous Abortion materials. Furthermore, the gene expression analysis of *WNT3A*, *WNT4*, and *WNT5A* demonstrated to be associated with spontaneous abortion.

The canonical pathway can be activated by *WNT4* in the presence of LRP5/6, but it also forms part of the non-canonical pathway making it classified as a non-canonical Wnt signaling molecule (**Ring et al., 2014**). An increase in Dkk-1 and sFRP4 expression decreased the expression of *Wnt4* and *β-catenin*, subsequently, resulting in an increase or decrease in *WNT5A* expression, associated with pre-eclampsia development (**Z. Zhang et al., 2013)**.

The Wnt/*β-catenin* has been shown to contribute immensely toward the development of organ systems, which include the digestive system, respiratory system, skeletal system, nervous system, cardiovascular, hematopoietic, and reproductive systems **(van Amerongen & Nusse, 2009)**. Furthermore, the Wnt/*β-catenin* signaling plays a pivotal role in tissue homeostasis, and most importantly development and differentiation of trophoblast (**Gough, 2012**). An alteration in *β-catenin* can hinder the process of blastulation **(Krivega et al., 2015**).

4.5 CONCLUSION

Miscarriage is a common complication that can lead to the loss of a fetus involuntarily before maturity. It is been divided into two types, which are sporadic and recurrent miscarriage. A big gap of about 25-50% of pregnant women may experience a sporadic miscarriage. However, the simultaneous loss of a fetus is spontaneous abortion, it is been reported to be a reproductive disorder of concern.

The Wnt is a combination of Wingless and Int-1, it is known for regulating several other processes such as cell proliferation, migration, cell fate determination, and embryonic development. The main reasons for implantation failure can be originated from uterine or embryonic factors as it is pointed out in major papers, as well as from embryonic factors. Cross-talk between embryo and uterus is considered to be vital and these factors should be considered in ruling out implantations failure.

In conclusion, the result gathered in this study according to the gene expression analysis, the WNT signal genes (*WNT3A*, *WNT4*, and *WNT5A)* were expressed in the abnormal karyotype when compared to the normal karyotype in the spontaneous Abortion materials. However, a decreased expression of *WNT3A*, and *WNT4*, and an increased expression in *WNT5A* in abnormal karyotype when compared to the normal karyotype in the spontaneous Abortion materials was observed.

Overall, changes in gene expression levels in *WNT3A*, *WNT4*, and *WNT5A* genes, which are involved in the Wnt-Beta catenin signaling pathway, in spontaneous abortion material with abnormal karyotype compared to materials with normal karyotype may be associated with the abnormalities in chromosome content or structure. Further analysis should be conducted to include protein expression levels of the studied genes to confirm that the changes detected in gene expression are reflected at the protein level with a larger cohort if possible. However, in this study, there were limitations with the RNA concentration measurements, as the samples were not freshly collected. Further research must be carried out to investigate factors related to spontaneous abortion associated with Wnt signal genes in terms of implantation. Furthermore, a better understanding of the role of Wnt genes during implantation can be implored, as also rectifying errors using IVF techniques.

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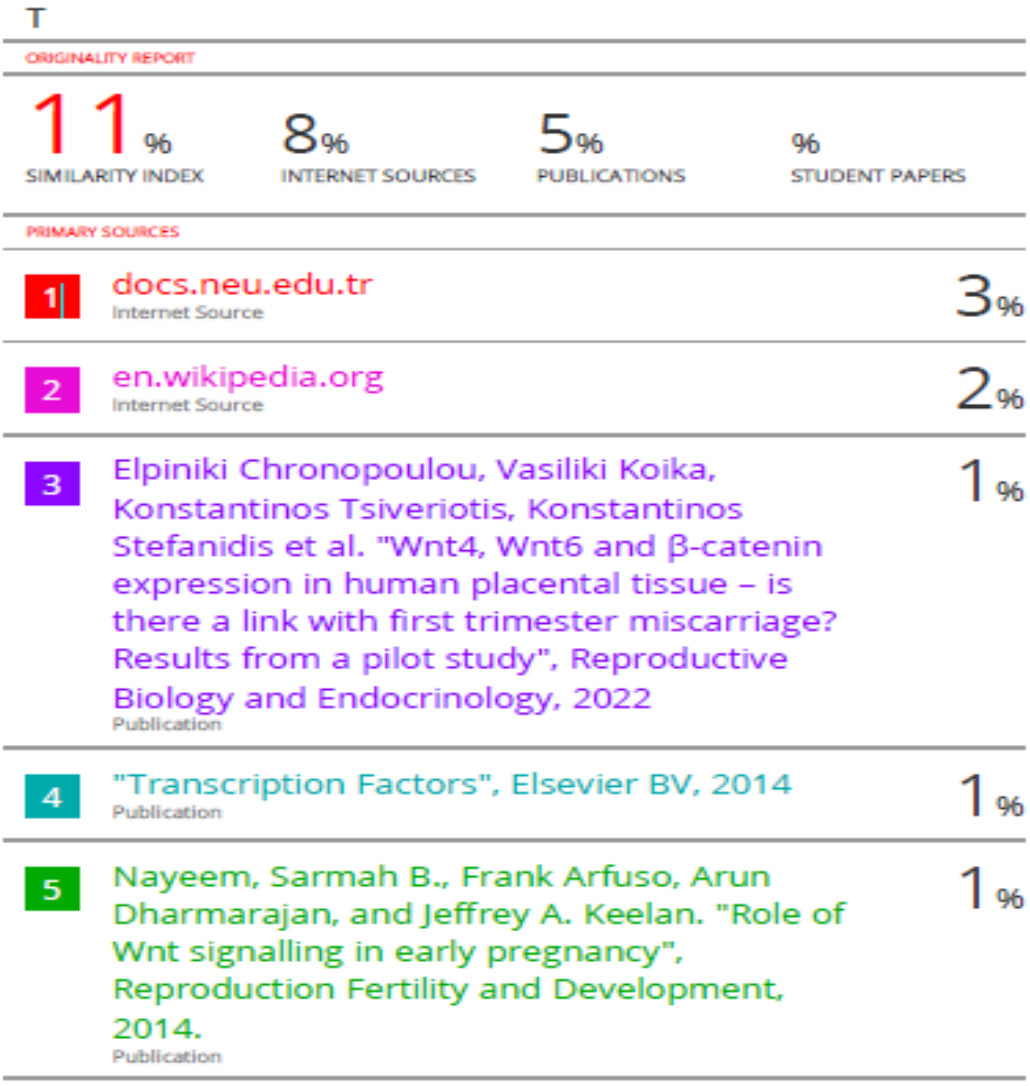
Appendix A: Turnitin Similarity Report



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Appendix B: Ethical Approval Document

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi :25.11.2021
Toplantı No : 2021/97
Proje No :1449

Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Mahmut Cerkez Ergoren'in sorumlu araştırmacısı olduğu, YDU/2021/97-1449 proje numaralı ve **"The gene expression profile of WNT/ β-catenin pathway genes in spontaneous abortion materials"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Prof. Dr. Şanda Çalı
Yakın Doğu Üniversitesi
Bilimsel Araştırmalar Etik Kurulu Başkanı

Kurul Üyesi	Toplantıya Katılım	Karar
	Katıldı(✓)/ Katılmadı(X)	*Onay(✓)/ Ret(X)*
Prof. Dr. Tamer Yılmaz	✓	✓
Prof. Dr. Şahan Saygı	✓	✓
Prof. Dr. Nurhan Bayraktar	✓	✓
Prof. Dr. Mehmet Özmenoğlu	✓	✓
Prof. Dr. İlker Etikan	✓	✓
Doç. Dr. Mehtap Tınazlı	✓	✓
Doç. Dr. Nilüfer Galip Çelik	✓	✓
Doç. Dr. Emil Mammadov	✓	✓
Doç. Dr. Ali Cenk Özay	✓	✓

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