Association of MTHFR rs1801133, PEMT rs4244593, CHKA rs7928739, VNTRs IL-1RN intron 2 and NOS3 intron 4 Polymorphisms with Unexplained Intrauterine Fetal Death in Bangladeshi Population

By

Deera Mahasin
20136037

Luthfa Akter Bushra
20136017

Shifat E Nusrat
20136038

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
B.Sc. in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is my/our original work while completing a degree at BRAC

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through complete and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all primary sources of help.

Student’s Full Name & Signature:

____

Deera Mahasin (20136037)

____

Luthfa Akter Bushra (20136017)

____

Shifat E Nusrat (20136038)

Approval

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1. Deera Mahasin (20136037)
2. Luthfa Akter Bushra (20136017)
3. Shifat E Nusrat (20136038)

of Spring, 2020 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of B.Sc. in Biotechnology in April, 2024.

Examining Committee:

Supervisor:
(Member)

____
Dr. Mohammed Mahboob Hossain
Professor, Department of Mathematics and Natural Sciences
BRAC University

Co-Supervisor:
(Member)

____
Dr. Yearul Kabir
Professor, Department of Biochemistry and Molecular Biology
University of Dhaka

Program Director:
(Member)

____
Dr. Munima Haque
Associate Professor, Biotechnology Program, Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

____
Dr. A.F.M.Yusuf Haider
Professor, Department of Mathematics and Natural Sciences
BRAC University

Ethics Statement

The study was conducted following the Helsinki Declaration and subsequent revisions. The Ethical Review Committee of the Department of Biochemistry and Molecular Biology, University of Dhaka, approved the study.

Dedication

Dedicated to the advancement of maternal and child health in Bangladesh

Acknowledgment

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List of Acronyms

SNP	Single Nucleotide Polymorphism
PCR	Polymerase Chain Reaction
MTHFR	Methylenetetrahydrofolate Reductase
PEMT	Phosphatidylethanolamine Methyltransferase
CHKA	choline kinase alpha
IL-1RN	Interleukin 1 receptor antagonist
IL-1 Family	Interleukin-1 Family
NOS3	Nitric Oxide Synthase 3
RFLP	Restriction Fragment Length Polymorphism
IMR	Infant Mortality Rate
CpG	Cytosine-Guanine Pairs
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
VLDL	Very low-density lipoproteins
SAM	S-adenosylmethionine
CTP	Cytidine Triphosphate
CDP	Cytidine 5'-diphosphocholine
DAG	Diacylglycerol
AFP	Alpha fetoproteins
TNF	Tumor Necrosis Factor
NLRP3	Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3
TRAPS	TNF-alpha associated periodic syndrome
CAPS	Cryopyrin associated inflammatory syndrome
VNTR	Variable Number of Tandem Repeats
UV	Ultraviolet
FTP	Full-term Pregnancy
OR	Odd Ratio

Abstract

This case-control study aimed to analyze interaction between increased risk of Intrauterine Fetal Death in Bangladeshi pregnant women and Single Nucleotide Polymorphism (SNP) of MTHFR (rs1801133), PEMT (rs4244593), CHKA (rs7928739), IL-1RN (intron 2 VNTR), and NOS3 (intron 4 VNTR). This study comprised 62 cases of IUFD and 64 controls, employed PCR/RFLP technique for MTHFR, PEMT, and CHKA gene, and only real-time PCR for IL1RN and NOS3 to observe repeat numbers. Compared to homozygous wild type (C/C) variant, heterozygous (C/A) and homozygous mutant (A/A) genotypes have shown increased risk (OR=3.18; 95% CI–1.02-9.91; p=0.04; OR=3.30; 95% Cl=0.98–11.07; p=0.05) of IUFD in PEMT analysis. However, the PEMT C/A and A/A genotypes were found to be associated with IUFD risk, in cases with no previous use of birth control (OR=0.27; 95% CI=0.08-0.92; p=0.04 and OR=0.02; 95% CI=0.06–0.79; p=0.02 respectively). IL1RN (Allele b/b) exhibited increased risk for IUFD when interacted with PEMT (A/A) genotype by 2 folds (OR=2; 95% CI=0.15–26.73; p=0.05). The genotypes of CHKA did not show any significant increase in IUFD risk. Interestingly, MTHFR and NOS3 manifested uniform distribution among cases and controls suggesting no association with IUFD risk in the Bangladeshi population.

Keywords: DNA repair genes; Polymorphism; PEMT; CHKA; MTHFR; Il-1RN; NOS3; Intrauterine Fetal Death, Biomarker

Chapter 1 Introduction

1.1 Intrauterine Fetal Death (IUFD) and Its Etiology

One of the most serious obstetrical consequences, intrauterine fetal death (IUFD) is a complex condition that contributes significantly to perinatal mortality (Seremak-Mrozikiewicz et al., 2018). However, this crucial and widespread issue has not received much attention in research (Gerber et al., 2005). The World Health Organization (WHO) defines Intrauterine Fetal Death (IUFD) as death that occurs before a human fetus is extracted or expelled from its mother; this is not an artificially caused pregnancy termination (Chowdhury et al., 2023). It is classified as an intrauterine fetal death (IUFD) or stillbirth when a fetus dies at 500 g at birth, or, in the absence of birth weight, at 22 weeks gestational age or 25 cm from crown to heel. The higher limit of 1000 g/28 weeks/35 cm for third-trimester IUFDs is also advised for reporting and international comparisons. ICD divides fetal fatalities into two categories: early (500–1000 g or 22–28 weeks) and late (those exceeding 1000 g or past 28 weeks) (Yeasmin et al., 2021).

Multiple processes of maternal, placental, or fetal origin may contribute to IUFD. The major causes of birth malformations, chromosomal abnormalities, multiple pregnancies, intrauterine growth restriction, and fetal hydrops of different etiologies are considered to be fetal reasons (Wolski et al., 2019). An intrinsic fetal cause is thought to be responsible for between 30% and 50% of stillbirths (Gerber et al., 2005). Placental diseases, preterm placental abruption, preterm premature rupture of membranes, feto-maternal hemorrhage, and placental insufficiency are among the causes of placental defects (Wolski et al., 2019). Of stillbirth occurrences, 10% to 15% have been linked to placental abnormalities and umbilical cord accidents (Gerber et al., 2005). Lastly, post-term pregnancy (defined as pregnancy lasting longer than 42 weeks), poorly managed diabetes, and other chronic illnesses like systemic lupus erythematosus, antiphospholipid syndrome, infections, hypertension, and preeclampsia/eclampsia are some of the major maternal causes of intrauterine fetal death. This category also includes abnormalities of the choline and folate cycles, as well as hereditary and acquired thrombophilia. In addition to considerably raising the chance of another miscarriage, hyperhomocysteinemia in pregnancy can cause

preeclampsia, fetal hypotrophy, preterm placental abruption, preterm delivery, neural tube defects, cleft palate, and intrauterine fetal death (Wolski et al., 2019). A significant maternal risk factor may be connected to 10% to 15% of stillbirths, whereas infections account for 15% of cases.

A particular cause of fetal mortality may be identified with the use of detailed laboratory tests, pathologic inspection of the placenta and fetus, and a careful assessment of the pregnancy and the mother's medical history (Gerber et al., 2005). However, identifying the precise cause and avoiding it in the future is frequently a difficult diagnostic and treatment task. Even though there are several established risk factors for pregnancy course failure, many instances remain undetected (Seremak-Mrozikiewicz et al., 2018). In as many as 15% to 40% of cases, despite thorough procedures, detailed autopsy, and all clinical investigations, the precise etiology of IUFDs remains unknown. These fall under the category of unexplained IUFD (Gerber et al., 2005).

1.2 Epidemiology of IUFD Worldwide

In developed nations, the prevalence of IUFD has decreased to a minimal incidence that cannot be avoided. In undeveloped and underdeveloped countries, however, it is still relatively high. (Chowdhury et al., 2023). Every year, 3.3 million stillbirths are reported worldwide, with 97% of those cases taking place in underdeveloped nations (Yeasmin et al., 2021). According to Liu et al. (2014), the incidence of IUFD varies between 5 and 32/1000 in different countries. Over the past three decades, the US has seen a substantial decrease in the rate of IUFD, from 14 to 6.7 per 1000 live births, owing to improved management of high-risk pregnancies (Gerber et al., 2005). Compared to previous times, a higher percentage of cases have had an explanation of death identified due to improved diagnostic methods and a deeper comprehension of the pathophysiology of IUFD (Yeasmin et al., 2021).

Despite the fact that antepartum fetal death has become less prevalent due to advancements in medicine and obstetrics, IUFD still accounts for over 50% of all perinatal deaths in developed countries (Gerber et al., 2005). Merely 4% of cases are officially reported in developing countries, where underreporting is frequent. An additional 1-2 million stillbirths

are thought to occur in these developing nations that are not reported (Yeasmin et al., 2021). Together, the developing nations of Asia and sub-Saharan Africa account for 70% of stillbirths worldwide. The leading causes of the high rate of perinatal fatalities in these areas include inadequate prenatal care, a lack of knowledge, women's lack of empowerment, and the accessibility or scarcity of healthcare facilities (Chowdhury et al., 2023).

1.3 Epidemiology of IUFD in Bangladesh

A society's perinatal death rate is a reliable predictor of the standard of antenatal care provided there (Chowdhury et al., 2023). The World Health Organization (2015) reported that the infant mortality rate (IMR) in Bangladesh and other ASEAN (Association of South East Asian Nations) countries is 27/1000 live births. From 2000-2015, Bangladesh had the quickest annual decline in both IUFD and stillbirth rate compared to all other South Asian nations (Yeasmin et al., 2021). Bangladesh's total IMR was recorded to be 22.23/1000 live births in the 2015 Inter-censal Population Survey (SUPAS) figures, surpassing the 2015 MDGs (Millennium Development Goals) target of 23/1000 live births. Despite this significant decline, Bangladesh remains seventh in the world in terms of the total number of stillbirths, with roughly 83,000 per year (Yeasmin et al., 2021).

1.4 MTHFR and IUFD Risk

1.4.1 Methylation Cycle

DNA methylation enables a cell’s genetic material to change covalently. DNA methylation means adding a methyl group to the 5-position of cytosine to create 5-methylcytosine (5mC), a critical DNA modification process observed in many eukaryotic organisms.

Mammals largely experience uniform pattern formation of DNA methylation on both strands when CpGdinucleotides are present. In the mouse genome, 60–80% of the CpG dinucleotides are methylated. The genome has a bidirectional distribution of 5mC. The two genomes that

combine to form a new person during reproduction are complimentary nonetheless distinct from one another. Because of this, healthy progeny cannot be produced by full parthenogenesis, in which oocytes split to create embryos without male fertilization, or androgenesis, in which embryos grow without an oocyte's genetic contribution. The features of the different regulatory mechanisms in male and female genomes are determined by methylation processes that control epigenesis and imprinting. Genes are silenced or activated by imprinting, which "tags" a chemical mark and is often parent-specific. Genes can be turned "on" or "off" within various organs or at specific times (e.g., during pregnancy). DNA methylation is vital for mammalian development and is also involved in many other biological processes, including transcriptional regulation, transposon silencing, genome-wide imprinting, and X chromosome inactivation. Erroneous DNA methylation patterns and polymorphisms in genes encoding DNA methylation regulators or enzymes have been associated with cancer and developmental problems (Liew & Gupta, 2015).

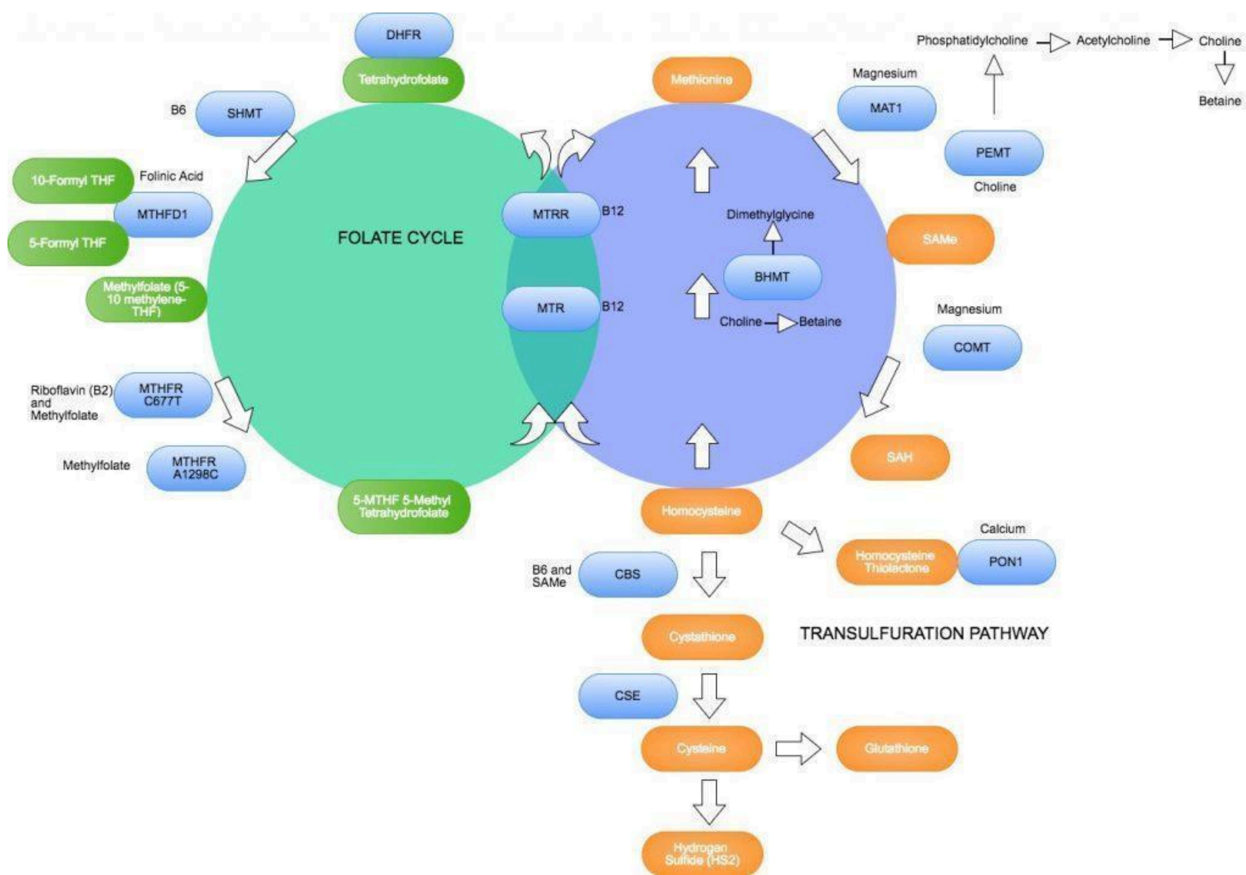

Figure 1: Methylation Cycle (Swanson, 2015)

1.4.2 Role of MTHFR in the Methylation Cycle

MTHFR stands for methylenetetrahydrofolate reductase, and the gene that encodes it is located on chromosome 1. The C677T polymorphism in the MTHFR gene decreases this enzyme activity, because of a mutation at exon 4, causing valine to be converted to alanine at codon 222 (Frosst et al., 1995). This enzyme reduces 5-MTHFR to 5-Methylenetetrahydrofolate. Moreover, MTHFR influences methyl group generation, neurotransmitter production, and other methylation reactions in the body (Jones, 2012). Again, MTHFR metabolizes sulfur-containing amino acid homocysteine in the body, which can potentially cause cardiovascular disease and certain deficiencies when found in excessive amounts (Clarke et al., 2012).

1.4.3 Polymorphisms in MTHFR Gene

C677T polymorphism: According to (Frosst et al.,1995), this polymorphism switches cytosine (C) to thymine (T) at position 677 of the MTHFR gene. This variation hinders the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, enzyme activity, and the DNA methylation process.

A1298C Polymorphism: Adenine (A) is switched to cytosine (C) at position 1298 of the MTHFR gene in this polymorphism. This polymorphism is also responsible for reduced enzyme activity, although the intensity is lesser than that of the C677T polymorphism. It can still harm folate metabolism and methylation reactions (Weisberg et al., 1998).

1.4.4 MTHFR rs1801133 Polymorphism and Fetal Development and IUFD

The MTHFR C677T polymorphism (rs1801133) is associated with an increased risk of Intrauterine Fetal Death (IUFD) that happens inside the uterus of pregnant women after 22 weeks of gestation. The MTHFR C677T genetic variant has been involved with increased homocysteine levels, damaged folate metabolism, certain placental imparity, thrombosis, and

cardiovascular issues linked to fetal death (Afaq et al., 2023). Moreover, the genetic variant and maternal folate status interplay, maternal age, and other lifestyle factors can play a role on an epigenetic level and elevate the chances of fetal demise.

1.5 PEMT and IUFD Risk

1.5.1 Kennedy Pathway

To preserve the structural integrity and selective permeability of cells, the phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) form a fluid lipid bilayer, which is an essential part of biological membranes. Phospholipids are involved in cell division, autophagy, and apoptosis. They also act as chaperones for proteins, second messengers, and receptors that bind to membrane-bound proteins (Gibellini & Smith, 2010).

Ethanolamine and choline are nutrients gained through diet (Wiedeman et al., 2018). The primary destination of these nutrients is the phospholipid Kennedy pathway (Figure 2), which is in charge of the de novo synthesis of PC and PE (Blunsom & Cockcroft, 2020). The Kennedy pathway's PC and PE branches are called the CDP-choline and CDP-ethanolamine pathways.. These routes involve a sequence of analogous enzymatic processes that convert choline to PC and ethanolamine to PE. The first step in the CDP-choline branch of the Kennedy pathway is the phosphorylation of choline by Choline Kinase (CK). CCT/PCYT1 then combines phosphocholine with CTP to create CDP-choline. To create PC, this product is then combined with DAG via CPT or CEPT. By corresponding reactions catalyzed successively by ETNK, ECT/PCYT2, and EPT/SELENO1, the CDP-ethanolamine branch becomes PE. The primary regulating enzymes of the Kennedy pathway are PCYT1 and PCYT2 (Bakovic, 2022). PS synthase 1 (PTDSS1) and PS synthase 2 (PTDSS2), respectively, catalyze "base-exchange" processes in the endoplasmic reticulum (ER) to generate PS, the third bilayer phospholipid, from PC and PE. Moreover, PE methyltransferase (PEMT) converts PE to PC while mitochondrial PS decarboxylase (PISD)

converts PS to PE. These processes support the preservation of the membrane's phospholipid ratio and homeostasis (McMaster, 2017).

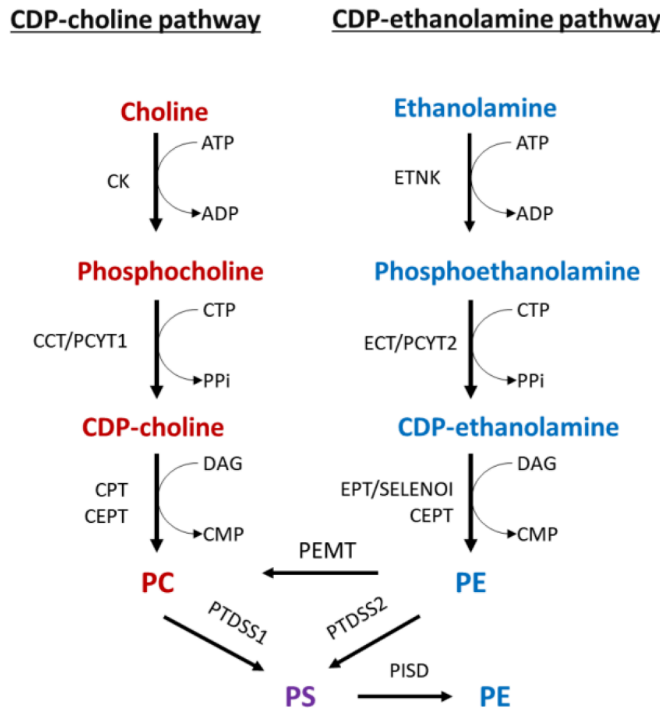

Figure 2: Kennedy Pathway for the formation of bilayer phospholipids by de novo pathways, methylation, and base-exchange reactions (Bakovic, 2022)

1.5.2 Role of PEMT in Kennedy Pathway

In the Kennedy route, phosphatidylethanolamine (PE) is converted to phosphatidylcholine (PC) by the transferase enzyme phosphatidylethanolamine N-methyltransferase (PEMT) (EC 2.1.1.17) (Vance, 2013). The PEMT gene located in the Smith-Magenis syndrome region of chromosome 17 encodes it in humans (Walkey et al., 1999).

About 70% of PC biosynthesis in the liver occurs via the CDP-choline pathway, which converts choline from diet or the metabolism of lipids containing choline to PC. Nevertheless, the PEMT pathway has been demonstrated to be crucial in the evolution of PC production during periods of starvation. Moreover, PC produced by PEMT is involved in

several physiological processes, including choline production, hepatocyte membrane construction, bile secretion, and the secretion of very low-density lipoproteins (VLDL).

The PEMT enzyme transforms phosphatidylethanolamine (PE) to phosphatidylcholine (PC) through three sequential methylations by S-adenosylmethionine (SAM). The enzyme is present in membranes connecting to the mitochondria and the endoplasmic reticulum. It makes up around 30% of PC biosynthesis, with the Kennedy or CDP-choline pathways accounting for roughly 70% (Vance, 2014). More than half of phospholipids in cell membranes and about 30% of the total lipid content of cells are made up of PC, which is usually the most prevalent phospholipid in plants and animals. Thus, preserving membrane integrity requires the PEMT pathway (Christie, 2012).

Phospholipases C/D can break down PC produced by the PEMT pathway, which leads to the de novo synthesis of choline. As a result, the PEMT pathway supports the body's larger-scale energy metabolism and the proper functioning of the liver and brain (Vance, 2014).

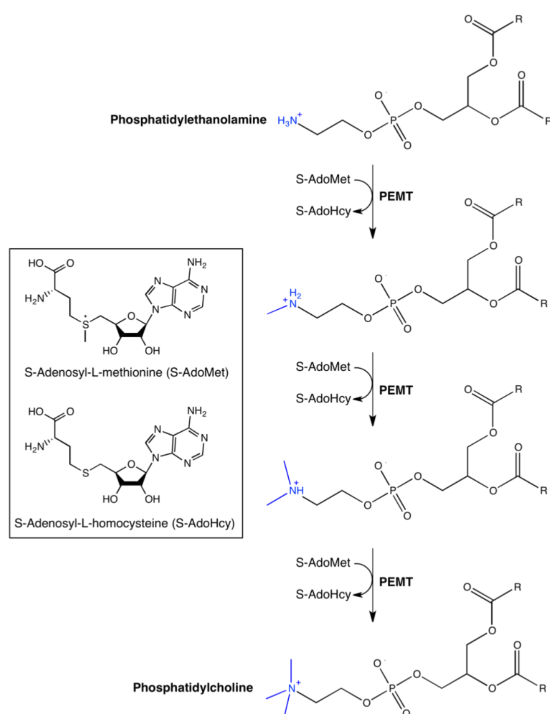

Figure 3: PEMT enzyme converting phosphatidylethanolamine (PE) to phosphatidylcholine (PC) by three sequential methylations by S-adenosylmethionine (SAM)

1.5.3 Polymorphisms in PEMT Gene

The PEMT gene is located on chromosome 17 with seven exons and six introns. It is important to note that PEMT is highly polymorphic, implying that different stimuli can independently alter its activity and cause different disruptions (Seremak-Mrozikiewicz et al., 2018). This gene has 98 single nucleotide polymorphisms (SNPs) found so far. Two of those have been identified as functional SNPs: PEMT rs7946 (5465G→A) and PEMT rs12325817 (–744G→C). These SNPs impact protein activity and may be related to choline needs and health effects. At position 175, the PEMT rs7946 polymorphism substitutes methionine for valine, leading to a loss of function and an elevated risk of nonalcoholic fatty liver disease. The PEMT rs12325817 is located around 50 base pairs inside the estrogen response element in the promoter region of the gene. The rs12325817 mutation may impact gene expression and is linked to a higher risk of choline shortage in women, potentially due to altered estrogen-mediated PEMT gene induction (Ivanov et al., 2009). Their effect is most significant in women who have gone through menopause.

1.5.4 PEMT Polymorphism and Fetal Development

Choline is one of the most critical components for proper fetal and placental development. Research has previously demonstrated a beneficial association between the fetal nervous system's adequate development and the availability of choline during pregnancy (Shaw et al., 2009). Furthermore, choline seemed necessary for placental development and the healthy operation of the feto-placental unit (Jiang et al., 2012). Neural tube defects and other abnormalities are linked to the poor choline status of expecting mothers (Zeisel, 2006). Choline for developing fetuses can come from two possible sources: the diet of pregnant women and the liver's de novo production. PEMT has some control over the synthesis of choline in the liver. It's also important to note that choline synthesis is the primary function of PC produced by the PEMT pathway. Serum homocysteine, which can be changed into cysteine or methionine, is another important component of PEMT (Vance, 2014).

During pregnancy, elevated estrogen concentrations promote the production of phosphatidylcholine and are a crucial positive regulator of hepatic PEMT transcription. The PEMT gene's promoter region's loss of estrogen binding sites may cause a severe choline deficit and related problems. Furthermore, PEMT is the sole enzyme in humans that produces de novo choline (Resseguie et al., 2011). It is well known that specific PEMT polymorphisms can lower the protein's sensitivity to rising serum estrogen levels, while other variants may impair choline synthesis and its proper utilization. Additionally, it might adjust the demand for choline in the diet, which is crucial during pregnancy (Yan et al., 2011).

1.6 CHKA and IUFD Risk

1.6.1 Role of CHKA in Kennedy Pathway

CHKA gene encodes for choline kinase alpha, an enzyme that catalyzes the initial phospholipid synthesis stage in the Kennedy pathway's CDP-choline branch.The enzyme phosphorylates choline using ATP, converting it into phosphocholine and adenosine diphosphate (ADP). Phosphocholine reacts with cytidine triphosphate (CTP) to form CDP (cytidine diphosphate)-choline. This reaction is catalyzed by the enzyme CTP: phosphocholinecytidylyltransferase. CDP (cytidine diphosphate)-choline serves as a precursor for the synthesis of phosphatidylcholine. It combines with diacylglycerol (DAG), phosphatidylcholine with the help of enzyme CDP:cholinephosphotransferase.

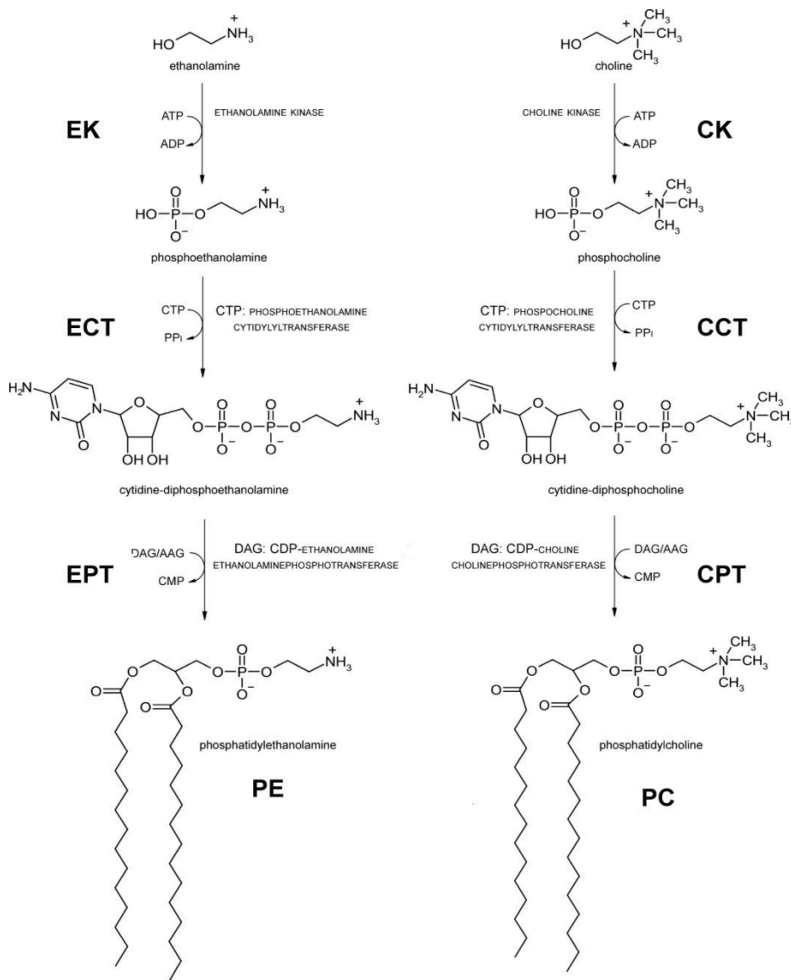

Figure 4: CHKA participation in Kennedy pathway (Gibellini, 2010)

1.6.2 Polymorphism in CHKA gene

CHKA (Choline Kinase Alpha) gene maps to chromosome 11q13.2. It comprises 17 exons and encodes 457 amino acids with a molecular mass of 52,249 Da. One of the CHKA SNP's rs10791957 found within the first intron is a potential enhancer, and it correlates with a low prevalence of type 2 diabetes as well as organ dysfunction in women who are choline-deficient (Da Costa, 2014). The CHKA rs10791957 variant has been identified as a potential modulator of phosphatidylcholine (PC) homeostasis, with a specific role in decreasing the use of dietary choline for PEMT-PC synthesis relative to CDP-PC synthesis (Ganz, 2017). Another CHKA SNP rs792873 consisting of a minimum of one C allele has

found a correlation with a lower prevalence of spina bifida in the California population (Enaw et al., 2006). Two SNPs (rs3794186 and rs11481) on the 3′-UTR of CHKA genes have significantly impacted the expression of Alpha-fetoprotein. It is a biomarker used for hepatocellular carcinoma (HCC). Still, these two SNPs are not correlated with the progression of the HCC.T allele of the exonic CHKA SNP rs3794186, in particular, was associated with lower serum AFP levels in hepatocellular carcinoma (HCC) patients, suggesting a potential protective effect (Park et al., 2012).

1.6.3 CHKA Polymorphism and Fetal Development

Choline is an essential vitamin that modulates stem cell proliferation and apoptosis, as well as the formation and function of the brain and spinal cord. It ultimately plays a crucial role in fetal development (Zeisel, 2006). Alteration in the amounts of choline negatively affects choline metabolism in expecting mothers by disrupting the formation of cell membranes, elevating homocysteine levels, and/or hindering methylation processes and acetylcholine production, which eventually affects fetal development as well (Hogeveen, 2013). The choline kinase alpha (CHKA) gene plays a key role in the biosynthesis of phosphatidylcholine, a major phospholipid contributing significantly to the structural integrity and functionality of eukaryotic cell membranes (Gruber, 2012). Choline kinase alpha, which is involved in phospholipid metabolism and cell cycle regulation also affects cell proliferation and transformation (De Molina, 2008). Lecithin, a vital component of lung surfactant in developing fetal rat lungs, is synthesized by the choline kinase alpha gene, which further proves the gene’s contribution to fetal development (Farrell, 1974).

1.7 IL-1RN and IUFD Risk

1.7.1 IL-1 Family Signaling Pathways

The Interleukin-1(IL-1) cytokine family is a group of 11 different cytokines. 7 of them ligands with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) and 4 of them have antagonistic activities [IL-1 receptor antagonist (IL-1Ra), IL-36Ra, IL-37, IL-38.

Though there are many types of interleukin 1 cytokine, the well-known is Interleukin 1 B, which plays an important role in inflammation. Interleukin 1 B is a potent pro-inflammatory cytokine produced by the immune cells notably, the innate immune system as well as epithelial cells during a stimulus such as an inflammation or infection. Interleukin 1 B, once secreted, acts on different types of cells in the body; for example, it acts on the cells of the vessel increasing the expression of adhesion molecules and when it does this, it allows migration of other immune cells to the site of inflammation circulating neutrophils and circulating monocytes can get recruited to the sites of the tissue in inflammation: again, promoting the inflammatory response. Interleukin B can also directly activate mature immune cells signaling them to produce more pro-inflammatory cytokines such as -Interleukin 1 B as well as TNF-alpha. Interleukin 1 B has other functions, including acting as a pyrogen and increasing body temperature- a hallmark of inflammation. Interleukin 1 B promotes T-cell activity, a part of the adaptive immune response. Interleukin 1 B also stimulates fibroblast proliferation and collagen production, increasing scarring and chronic inflammatory changes.

Immune cells have many receptors on the cell surface that respond to different inflammatory stimuli such as cytokines component of pathogens or damaged associated molecular patterns from injured cells. These receptors get stimulated, which initiates a cascade of intracellular events, ultimately activating transcription factors, which are essential in the inflammatory process, such as NF kappa B. If Kappa B reads genes, it will eventually make proteins TNF alpha and pro interleukin 1 and Pro Interleukin 1 B. TNF alpha is a proinflammatory cytokine, and so are the interleukins, but these interleukins are not active yet. Intracellular proteins called node-like receptor 3 (nlrp 3) are important in interleukin 1 B activation as well as interleukin 1 A activation. Essentially, they will activate Pro caspase-1 to caspase-1,

converting Pro Interleukin 1 A and Pro Interleukin 1 B to Interleukin 1 A and Interleukin 1 B. Interleukin-1 B is released from the cell and will target these other cells around the area. For example, it will target other immune cells here. This target cell will have Interleukin 1 B receptors on its cell surface and so when interleukin 1 B binds to interleukin 1 B receptors, it will again trigger a cascade of intracellular events, within that cell which will essentially result in the things, we described earlier depending on the cell target. In summary, interleukin 1 B is a potent pro-inflammatory cytokine.

Interleukin 1 inhibitors include ANAKINRA (IL1B receptor antagonist) and CANAKINUMAB (IL1B cytokine Antagonist).

ANAKINRA is an interleukin 1 B receptor antagonist. It prevents interleukin 1 B from binding to its receptor. ANAKINRA does so by essentially binding to the interleukin 1 B receptor site where interleukin 1 cytokines would otherwise bind.

CANAKINUMAB is a monoclonal antibody that binds to interleukin 1 B cytokines, preventing their effects on the target cells. Anakinra is used for rheumatoid arthritis and acute gouty arthritis, as well as TNF-alpha-associated periodic syndrome (TRAPS) and cryopyrin-associated inflammatory syndrome (CAPS). In CAPS, one has a mutation in nlrp3, which is known as cryopyrin. NLRP3 is an enzyme important in the activation of interleukin 1. Mutation in NLRP3 results in overactivation of interleukin 1, so people get febrile, joint aches, and other inflammatory phenomena.

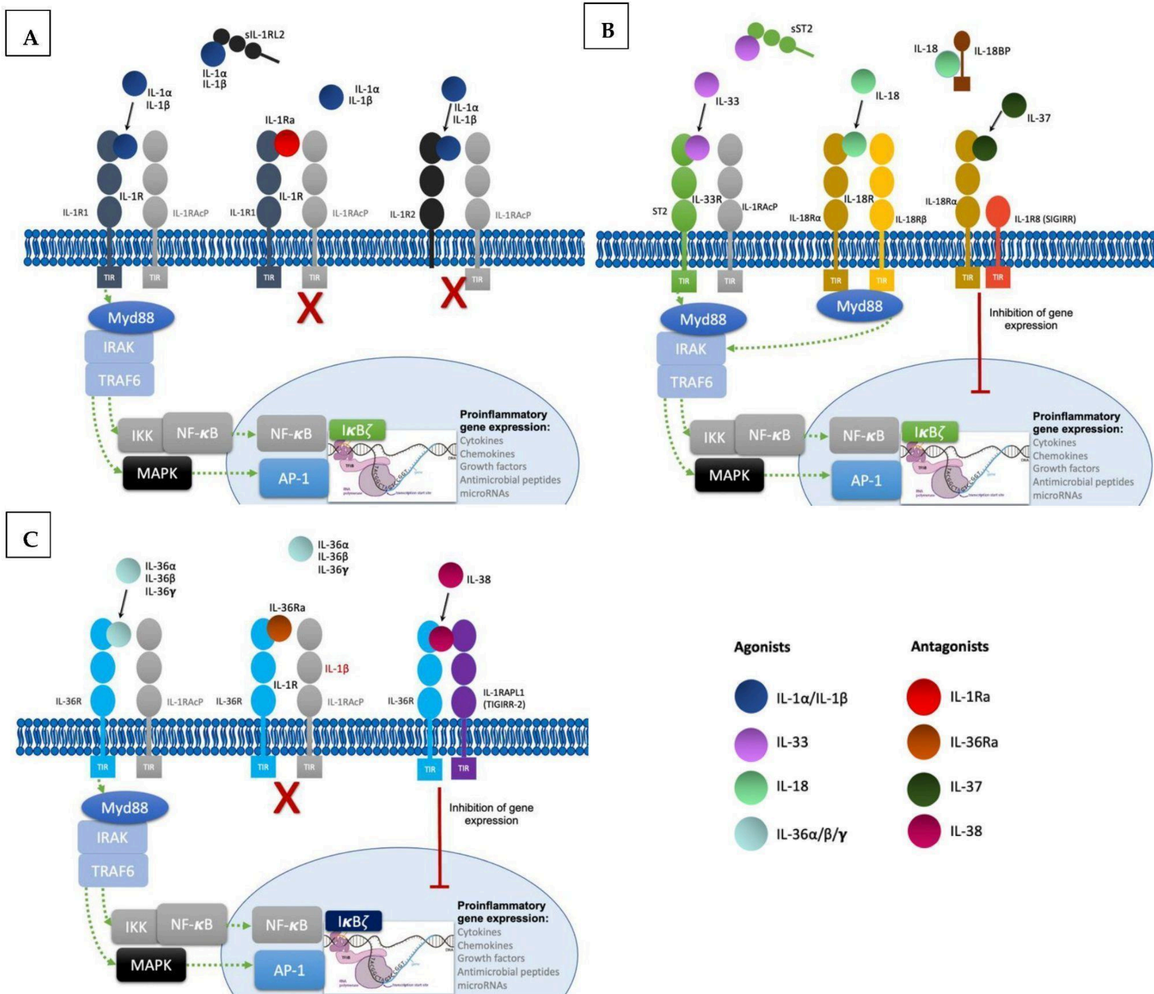

Figure 5: IL-1 Family Signaling Pathways (Iznardo&Puig, 2022)

1.7.2 Role of IL-1RN in IL-1 Family Signaling Pathways

According to (Redlich et al., 2003), IL-1RN (Interleukin-1 receptor antagonist) works as an inhibitor in the IL-1 family signaling pathways. IL-1α and IL-1β are pro-inflammatory cytokines within the IL-1 family, and IL-1RN is a natural antagonist of them. IL-1RN acts as an opponent with IL-1α and IL-1β to bind with the IL-1 receptor (IL-1R). When it successfully binds to IL-1R, it prevents the binding of IL-1α and IL-1β and their signaling cascades from being activated. IL-1RN maintains pro-inflammatory and anti-inflammatory signals in the body by downregulating IL-1 signaling(Dinarello, 2009). In addition, IL-1RN

helps to maintain homeostasis and modulate immune responses by suppressing elevated IL-1 activity (Mantovani et al., 2019).

1.7.3 Polymorphisms in IL-1RN Gene

IL-1RN gene encodes the interleukin-1 receptor antagonist (IL-1ra), which functions as a natural inhibitor of the pro-inflammatory cytokines interleukin-1 alpha (IL-1α) and interleukin-1 beta (IL-1β). Variation in this gene can hinder the production or function of IL-1ra, potentially causing inflammatory diseases. One of the most crucial polymorphisms in the IL-1RN gene is the variable number tandem repeat (VNTR) polymorphism located in intron 2. It consists of perfect repeats of an 86-bp sequence. Five allelic variants from two to six have been established, where 2 and 4 are most prominent.

IL-1RN polymorphisms are associated with autoimmune disorders, infectious diseases, and inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, periodontal disease, and cardiovascular diseases. Yet, the findings have led to ambiguous conclusions, and the impact of IL-1RN polymorphisms on disease predisposition depends on genetic background, environmental factors, and interactions with other genes.

1.7.4 IL-1RN Allele 2 Polymorphism and Fetal Development

IL-1RN is a polymorphic gene. The IL-1RN intron 2 is linked with elevated proinflammatory responses in the body. Gerber et al. (2005) found that fetuses that carry two copies of the IL-1RN intron 2 may be at a higher risk of intrauterine fetal death. Though it did not conclude causality, there was an association between unexplained stillbirth and fetal homozygous IL-1RN intron 2 carriage. This implies that the IL-1RN intron 2 may be a genetic risk factor for stillbirth, possibly through its influence on the inflammatory response.

1.8 NOS3 and IUFD Risk

1.8.1 Nitric Oxide Synthase (NOS) Pathway

The synthesis of free radicals, which are necessary for some physiological activities but can also lead to oxidative stress and cell damage, is elevated in several disorders. Enzymes mainly mediated the changes linked to free radicals.. Nitric oxide synthase (NOS), which catalyzes the production of nitric oxide (NO), is one of them. There are three different variants of this enzyme (NOS1, NOS2, and NOS3), and each is encoded by their respective gene (Król Kępińska, 2020). NOS1, NOS2, and NOS3 are alternatively referred to as neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), respectively. The iNOS isoform is expressed in a variety of nonvascular cell types, including macrophages, hepatocytes, and fibroblasts, in contrast to the nNOS and eNOS isoforms, which are primarily found in neuronal and endothelial cells, respectively (Nathan, 1992). One of the primary functional distinctions between iNOS and the other NOS isoforms is that, while nNOS and eNOS produce NO as a signaling molecule to control physiological processes like vasodilatation, iNOS produces NO in response to inflammatory mediators (Klinger & Kadowitz, 2017). In the presence of molecular oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), and L-arginine, all three isoforms catalyze the same reaction that generates NO from the amino acid's nitrogen residue. N-hydroxy-L-arginine, an intermediate molecule produced in the first stage of catalysis, is oxidized to L-citrulline, resulting in NO (Förstermann& Li, 2011). Additionally, NOS binds to heme, tetrahydrobiopterin (BH4), calmodulin, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (Król & Kepinska, 2020).

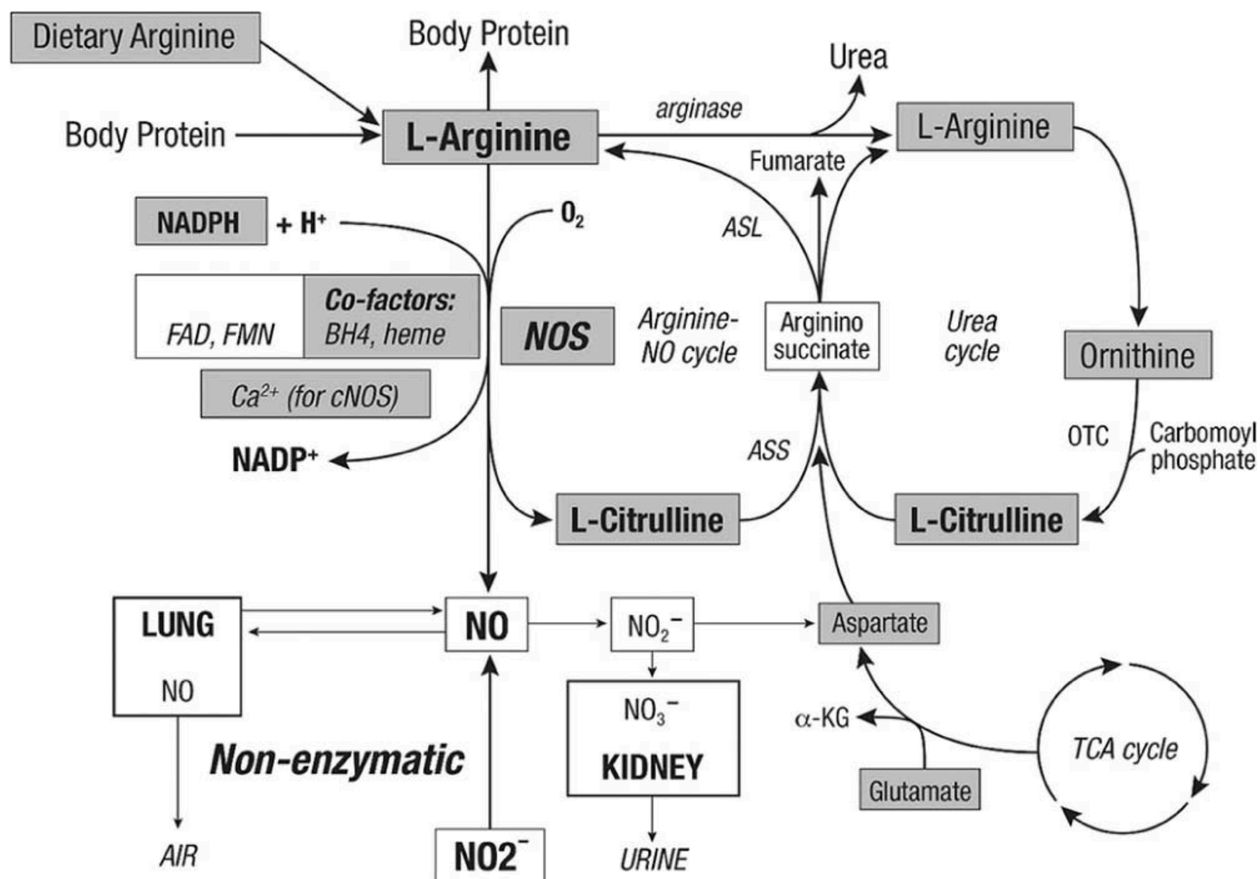

Figure 6: NOS Synthase Pathway (Klinger & Kadowitz, 2017)

1.8.2 Role of NOS3 in NOS Pathway

Nitric oxide (NO) generated from the endothelium is an essential protective molecule of the vasculature. It is produced by the NOS3-producing enzyme endothelial NO synthase (eNOS) (Förstermann& Li, 2011). The gene responsible for encoding the enzyme NOS3 can be found at location 36.1 on the longer arm of chromosome 7. The gene produces a protein of 1153 amino acids. NOS3 contributes to the generation of NO in the vascular endothelium (Fish & Marsden, 2006), which is a single layer of flat cells surrounding the inner surface of the blood vessels (Sumpio et al., 2002). According to Förstermann and Münzel (2006), NO generated by NOS3 in the vascular endothelium is essential for controlling leukocyte

adherence, platelet aggregation, vascular tone, and cellular proliferation. Therefore, a healthy cardiovascular system depends on a functional NOS3.

Like other NOS isoforms, functioning NOS3 transports electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to the heme in the amino-terminal oxygenase domain via the flavins, flavin mononucleotide, and flavin adenine dinucleotide in the carboxy-terminal reductase domain. Here, NO and L-citrulline are produced through the oxidation of the substrate L-arginine. Bioactive NO is decreased by cardiovascular risk factors such as hypertension, diabetes mellitus, high cholesterol, and cigarette smoking. Reactive oxygen species (ROS) in the vessel wall are produced more frequently as a result of these risk factors (Förstermann & Li, 2011).

1.8.3 Polymorphisms in NOS3 Gene

The gene NOS3 has 26 exons (Tran et al., 2022) and is highly prone to polymorphisms. Numerous diseases and medical conditions have been studied in relation to its polymorphisms. While NOS3 polymorphisms have not been related to the timing of menopause (Hefler et al., 2002) or ovarian cancer (Hefler et al., 2002), they have been associated with brain infarction (Elbaz et al., 2000), pre-eclampsia (Tempfer et al., 2000), idiopathic recurrent miscarriage (Tempfer, 2001), and hypertension (Miyamoto et al., 1998).

Reduced eNOS expression results from the C allele for the NOS3 786T > C polymorphism of the eNOS gene, which in turn results in less NO being produced (Seremak-Mrozikiewicz et al., 2010). Those with this condition will be more susceptible to developing preeclampsia (Dai et al., 2013), diabetic nephropathy (Dai & Qi, 2011), retinopathy (Shoukry et al., 2012), migraine (Eroz et al., 2014), and hypertension (Niu & Qi, 2011). Similarly, higher episodes of coronary vasospasm are also linked to the 786T > C (Nakayama et al., 1999). There is a significant correlation between the C-allele of the 786T > C single-nucleotide polymorphism variant and the incidence of aneurysm rupture, which is followed by subarachnoid hemorrhage and vasospasm. Patients with a four-repeat allele of the variable number of

tandem repeats in this genetic variant have also been shown to have an increased risk of aneurysm ruptures (Hendrix et al., 2017).

1.8.4 NOS3 Polymorphism and Fetal Development

It has been demonstrated that NO produced by the endothelium, eNOS, or NOS3 regulates vascular tone by diffusing into nearby vascular smooth muscle cells from endothelial cells. There, it induces relaxation through a guanylate cyclase route (Vane et al., 1990). Numerous studies indicate that NO plays a significant function as a modulator of uterine quiescence and utero-placental blood flow during pregnancy (Al-Hijji et al., 2003). Numerous research in mouse models has demonstrated that blocking NO production during pregnancy results in reduced fetal and placental perfusion, elevated fetal growth restriction, and an increased rate of hypertension (Hefler et al., 2001). A higher frequency of abnormal prenatal development, such as fetal growth restriction, limb deformities, and late intrauterine fetal death, was observed in mice lacking eNOS (Hefler et al., 2001). In the case of humans, NO concentrations in the amniotic fluid of women who went on to have IUFD during pregnancy were considerably lower than in controls (Huber et al., 2005). According to specific theories, a premature decrease in NO may cause poor placental vascular development and endothelial control, which could result in IUFD (Tranquilli et al., 2004).

1.9 Objectives

1.9.1 General objective

In this study, we expect to uncover significant evidence of the genotype and allele frequencies of PEMT rs4244593, CHKA rs7928739, MTHFR rs1801133, NOS3 intron 4, and IL-1RN intron 2 gene polymorphisms in mothers with IUFD occurrences.

1.9.2 Specific purpose

The purpose of this study is to:

i) To determine the frequency distribution of the MTHFR rs1801133, PEMT rs4244593, CHKA rs7928739, IL-1RN intron 2 and NOS3 intron 4 polymorphisms in intrauterine fetal death patients and healthy controls among the Bangladeshi population.

ii) To investigate the association of the SNPs with susceptibility and risk of intrauterine fetal death.

iii) To estimate the odds ratios of IUFD based on genetic polymorphism

iv) To study the risk of IUFD associated with SNPs based on maternal and fetal age and obstetric history of the mother.

v) To explore the association of the SNPs with clinical and biochemical parameters of IUFD patients.

Chapter 2 Materials and Methods

2.1 Study Design

The study was designed as a hospital-based case-control study (Figure 7).

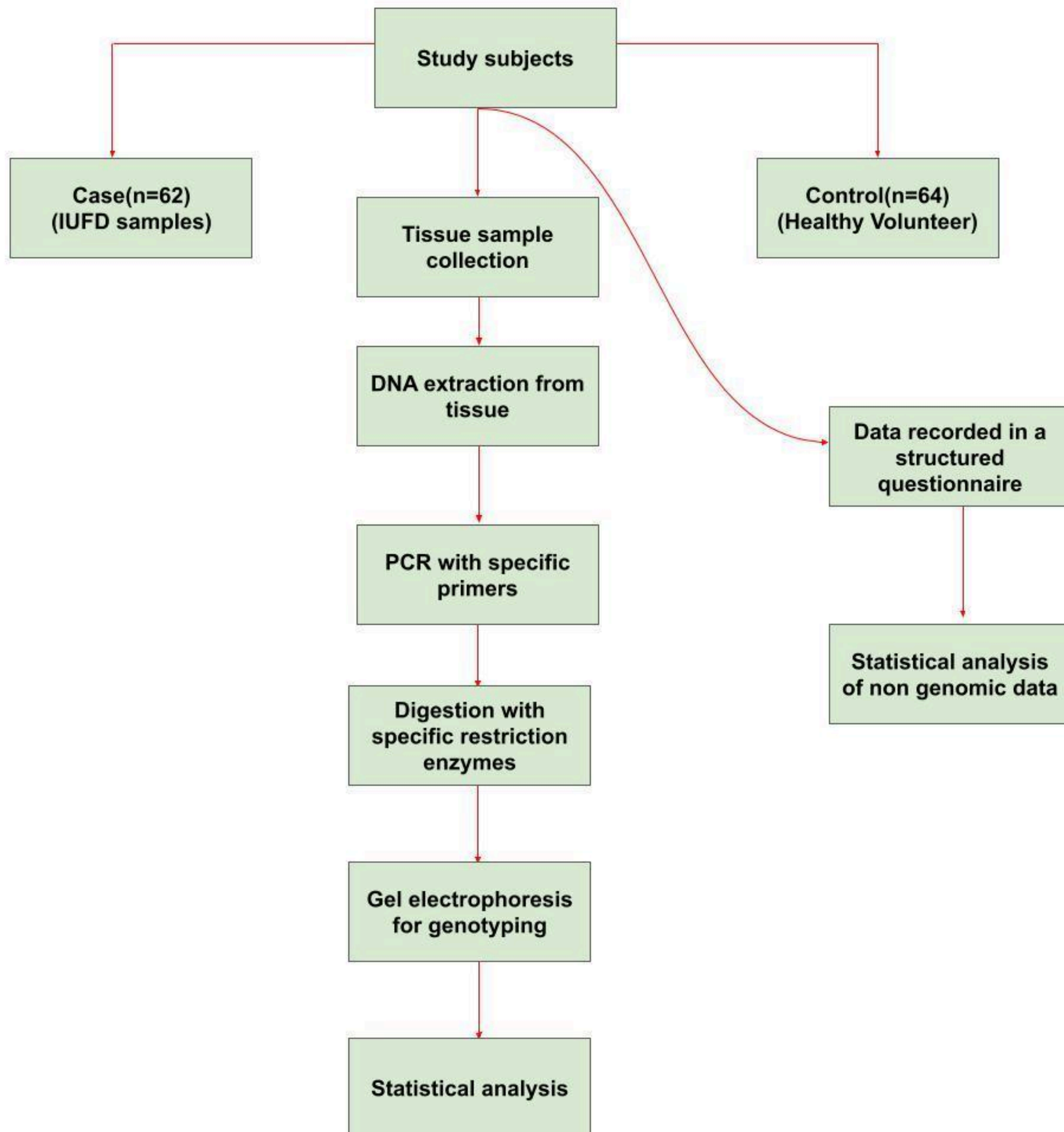

Figure 7: Schematic diagram of study design

2.2 Selection of the Patients and Controls

The study was performed on collected data among 62 women with a diagnosis of intrauterine fetal death (IUFD). Placental tissue samples were collected from the Department of Gynecology and Obstetrics at Dhaka Medical College and Ad-Din Medical College Hospital. The control group comprised 64 women with a history of giving healthy birth. The subjects were selected using cluster random sampling technique and their demographic data were collected from the hospital through interviews.

Inclusion Criteria

Case:

i. Patients with singleton pregnancy

ii. Fetal death cases with gestational age equal to or greater than 22 weeks.

iii. Unexplained cases where the mother and fetus had no visible risk factors.

Control:

i) Healthy individuals who gave birth to healthy babies.

Exclusion Criteria

i. Having a history of comorbidity, e.g. Diabetes, high blood pressure.

ii. Having a history of any autoimmune diseases, e.g., rheumatoid arthritis

iii. Having any chromosomal abnormalities in pregnancy.

2.3 Data Collection

The demographic and clinical data of the patients were collected using a structured questionnaire. The questionnaire had details of each study subject's age, occupation, blood group, blood pressure, and duration of marriage. The questionnaire also included details of obstetric history, including full-term pregnancy, surgery or Cesarean section, birth control, and pregnancy loss such as spontaneous abortion, intrauterine death, stillbirth, preterm labor, preeclampsia, eclampsia, and intrauterine growth restriction. Besides, it also had a section that contained information about the fetus, including gestational age in weeks, weight of the fetus (in kg), and gender of the fetus.

2.4 Consent and Ethical Issues

All participants were provided with extensive information about the purpose of the study and the experimental design of the procedure. Each participant gave their informed consent as required. A structured questionnaire was used to record details of the patients. The study followed the guidelines of the Helsinki Declaration and its further amendments and revisions ("World Medical Association Declaration of Helsinki, 2013," n.d.). The Ethical Review Committees of the Department of Biochemistry and Molecular Biology, University of Dhaka approved the project.

2.5 Sample Collection and Storage

A standardized amount of placental tissue was collected from each participant, adhering to all medical and surgical precautions. The collected tissues were immediately transferred to RNA later solution-containing tubes. The placental tissues were collected from four placental sites, and they were named M1 and M2 (positioned in the direction of the mother's body). The other two were named F1 and F2 (positioned in the direction of the fetus). The RNA later solution-containing tubes were kept in an icebox for transportation to the laboratory and stored at −20°C until DNA extraction.

2.6 DNA Extraction and Quantification

2.6.1 DNA Extraction from Tissue

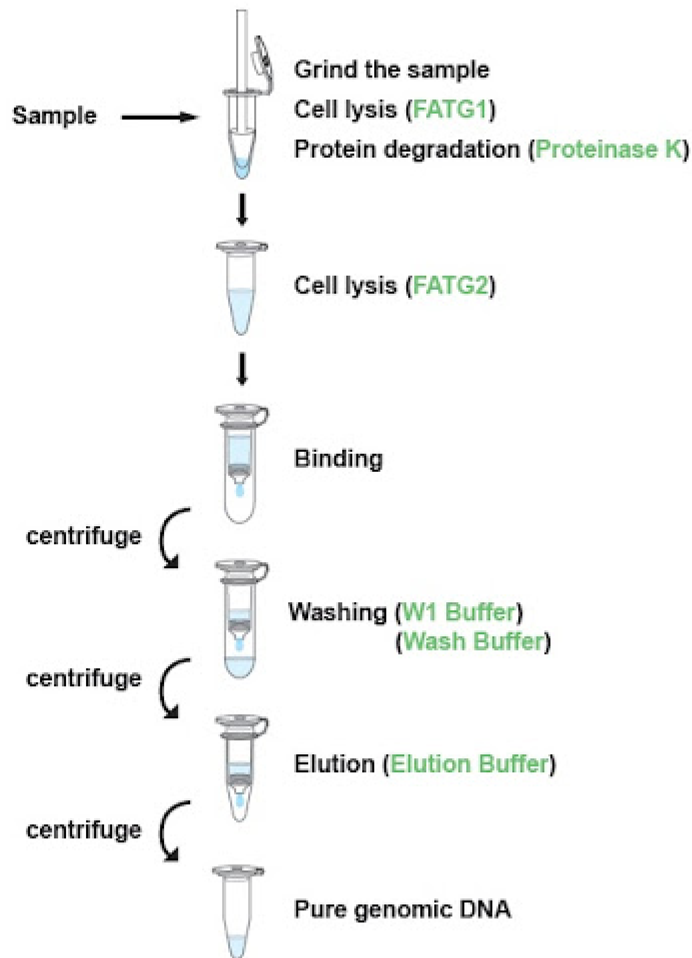

Figure 8: Steps of DNA extraction from animal tissue

Genomic DNA was extracted from the tissue samples using (FavorPrep™ Tissue Genomic DNA Extraction Kit) by Favorgen Biotech Corporation.

Preparation: Water baths were set dried at 60 °C for step 4 and 70 °C for step 6.

- Ethanol was added into the W1 Buffer and wash buffer when they were first opened.

i. Cell lysis

Up to 25 mg tissue samples were cut and then retained in microcentrifuge tubes. Mortar, scalpel, and forceps were used to grind the tissue sample. 200 µl FATG1 Buffer was mixed well by pipette tip. 20 µl Proteinase K was added (10mg/ml) to the sample mixture. The sample was mixed thoroughly by vortexing. The disintegrated tissue sample was incubated at 60 °C until the tissue was lysed completely. The tubes were vortexed occasionally during Incubation. The samples were incubated overnight for complete lysis.

ii. Soluble DNA separation from cellular components

A 200 µl of FATG2 buffer was added to the sample mixture, it was mixed thoroughly by pulse-vortexing, and incubated at 70 °C for 10 min. Then, 200 µl ethanol (96-100%) was added to the sample mixture. It was mixed thoroughly by pulse-vortexing. The tube was briefly spun (for 15 seconds) to remove drops from the inside of the lid.

iii. Binding DNA to a purification system

A FATG mini-column was placed in a collection tube. Then the mixture was transferred (Including any precipitate) carefully to the FATG Mini Column Centrifuge at maximum speed (-16,100 x g) for 1 min and then placed the FATG Mini Column to a new collection tube.

iv. Washing

For the washing step, 400 µl W1 Buffer was added to the FATG Mini Column, centrifuged at full speed for 1 min, and then discarded the flow-through. 750 µl Wash Buffer was added to the FATG Mini Column, centrifuged at maximum speed (-16,100 x g) for 1 min, then discarded flow-through. It was centrifuged at full speed for an additional 3 min to dry the column. In this step, the residual liquid was removed.

v. Elution

To elute the DNA, a 100 µl of preheated Elution Buffer was added to the membrane of the FATG Mini Column. The column was kept standing for 3 minutes. For effective elution, it was made sure that the elution solution was dispensed onto the membrane center and was

absorbed completely. In the last step, it was centrifuged at a maximum (-16,100×g) speed for 2 minutes to elute DNA. The extracted DNA was stored at -20 ºC.

2.6.2 Quantification of DNA

The quantity and purity of the genomic DNA were assessed by taking absorbance values at 260 nm (A260) and at 280 nm (A280 using Thermo Scientific™ NanoDrop™. The quality of DNA elutes (4 µl) was also assessed by agarose gel electrophoresis. Gel electrophoresis was performed using 2% and 3% agarose gel run at a low voltage (100V for 90 minutes) and (100V for 120 minutes), respectively, for RFLP and (120V for 90 minutes) for VNTR. Then, the gels were stained in the Ethidium bromide solution for 20 minutes, maintaining safety precautions. Finally, the gels were visualized after staining using a gel documentation system (AlphaImager mini, USA).

2.7 PCR-RFLP

The PEMT rs4244593, CHKA rs7928739, and MTHFR rs1801133 genotypes were determined using conventional Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The Variable Number of Tandem Repeats (VNTRs), NOS3 Intron 4, and IL-1RN Intron 2 genotypes were determined using conventional Polymerase Chain Reaction (PCR).

2.7.1 PCR

2.7.1.1 Primer Selection

The primer sequences of PEMT rs4244593, CHKA rs7928739, and NOS3 Intron 4 and IL-1RN Intron 2 PCR conditions were selected from previously published work and used after validated through NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and UCSC

In-Silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) (Seremak-Mrozikiewicz et al., 2018; Drews et al., 2017; Nasr et al., 2016; Rafiei et al., 2013). The primer sequences of MTHFR rs1801133 were designed using Primer3web version 4.1.0. The primer sequences and the resulting digested products of each amplicon are listed in Table 1.

Table 1: Primer sequences for allele determination, with amplicon size and the resultant digested product size

Gene	dbSNP ID	Primer Sequence	Amplicon size	Band Size of Restriction Digestion Products/ PCR Products (in cases of VNTRs)
MTHFR	rs1801133	F:5'-TGGGAAGAACT CAGCGAACT-3' R:5'-TTACTACCCCAG ATGCTGCC-3'	672 bp	G/G: 672 bp G/A: 672 bp, 447 bp and 225 bp A/A: 447 bp and 225 bp
PEMT	rs4244593	F:5’-CTGCCTCCTCAC GACCTGTA-3’ R:5’-GCGTGGTCCTC CACTCTTTC-3’	637 bp	C/C: 637 bp C/A: 637 bp, 401 bp and 237 bp A/A:401 bp and 237 bp
CHKA	rs7928739	F:5'-TGATTTCCAATG TCGAATCC-3' R:5'-TAAGTCAAAATG CCGCTCTG-3'	474 bp	C/C: 474 bp C/A: 474 bp, 272 bp and 202 bp A/A: 272 bp and 202 bp

IL-1RN	Intron 2	F:5'-CTCAGCAACACT CCTAT-3' R:5'-TCCTGGTCTGCA GGTAA-3'	410 bp (4 repeats)	Allele a: 410 bp (4 repeats) Allele b:240 bp (2 repeats) Allele c: 326 bp (3 repeats) Allele d: 498 bp (5 repeats) Allele e: 595 bp (6 repeats)
NOS3	Intron 4	F:5'-ACCTCAGCCCAG TAGTG-3' R:5'-GCAAGTGTCAG ATAGGATT-3'	600 bp (5 repeats)	Allele a: 600 bp (5 repeats) Allele b: 573 bp (4 repeats)

2.7.1.2 PCR Reagents

The reagents used for PCR-RFLP are listed in Table 2.

Table 2: Reagents used for PCR-RFLP

	Reagents	**Company**
PCR	DreamTaq Green PCR Master Mix	Thermo Fisher, USA
	Primers	Macrogen, South Korea
	10% Dimethyl Sulfoxide	Sigma-Aldrich, Germany
	Nuclease Free Water	New England Biolabs, USA
Restriction Digestion	Restriction Enzymes (*HinfI, TaqI*)	New England Biolabs, USA
Gel Electrophoresis	Agarose Powder	Invitrogen, USA
	Midori Green Advance-Nucleic acid Stain	NIPPON Genetics, JAPAN
	GeneRuler 100 bp DNA Ladder, ready-to use	Thermo Fisher, USA

2.7.1.3 Composition of PCR mix

Table 3: Composition of the PCR reaction mixture

Component	Volume (µL)
Green PCR Master Mix	7.0
Nuclease-free water	3.0
10% Dimethyl sulfoxide	1.0
Forward Primer	0.5
Reverse Primer	0.5
Genomic DNA	3.0
Total Volume	**15.0**

2.7.1.4 PCR Conditions

The PCR thermal conditions of the MTHFR rs1801133, PEMT rs4244593, CHKA rs7928739, IL-1RN Intron 2, and NOS3 Intron 4 have been illustrated in Figures 8 to 12, respectively.

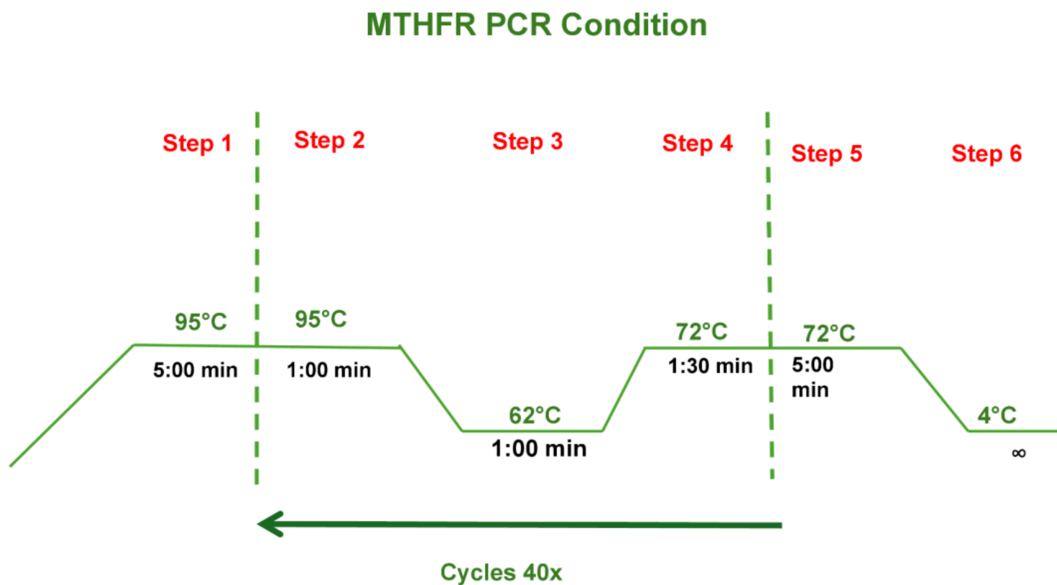

Figure 9: Thermal conditions for amplification of MTHFR rs1801133

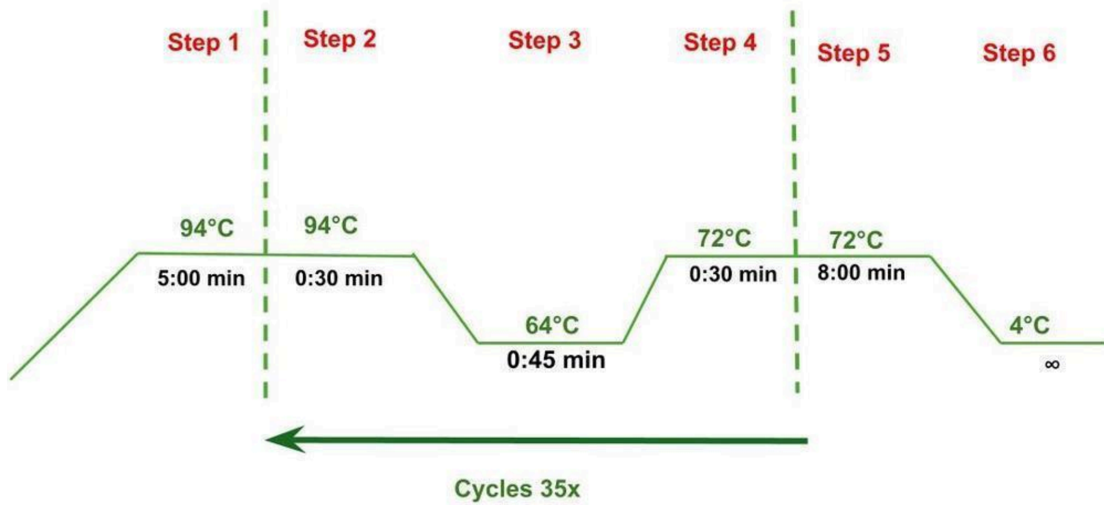

Figure 10: Thermal conditions for amplification of PEMT rs4244593

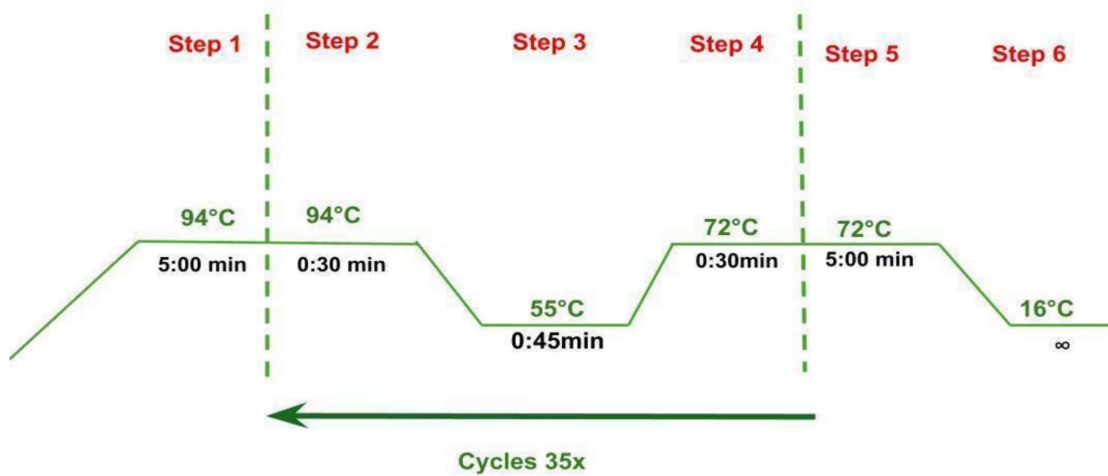

Figure 11: Thermal conditions for amplification of CHKArs7928739

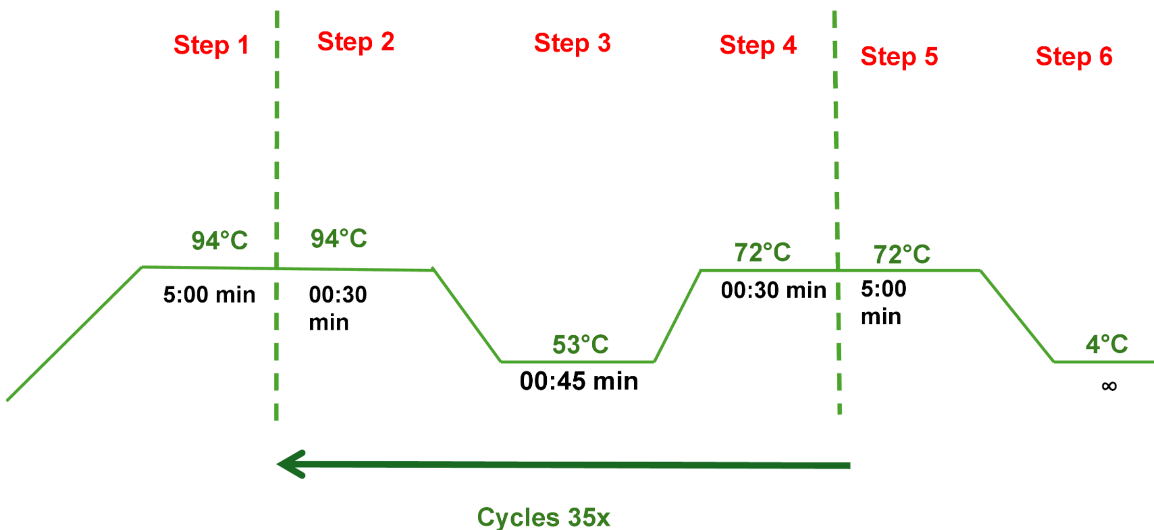

Figure 12: Thermal conditions for amplification of IL-1RN intron 2

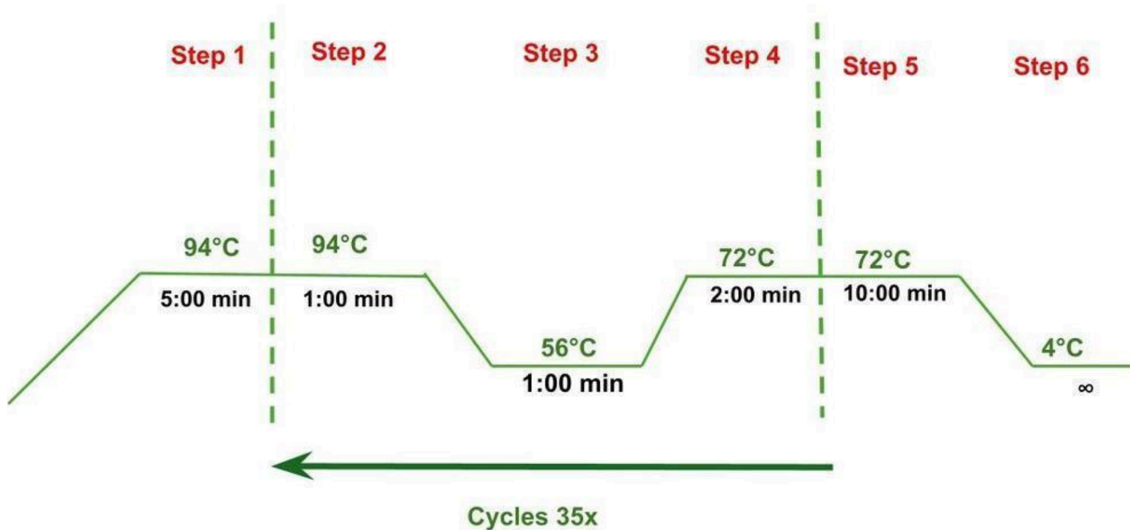

Figure 13: Thermal conditions for amplification of NOS3 Intron 4

2.7.1.5 Evaluation of PCR

The amplified PCR products of the MTHFR, PEMT, CHKA, IL-1RN, and NOS3 were checked for amplification by gel electrophoresis in 2% agarose gel stained with Midori Green stain. The amplified DNA was then visualized under UV light (Figures 13 and 17).

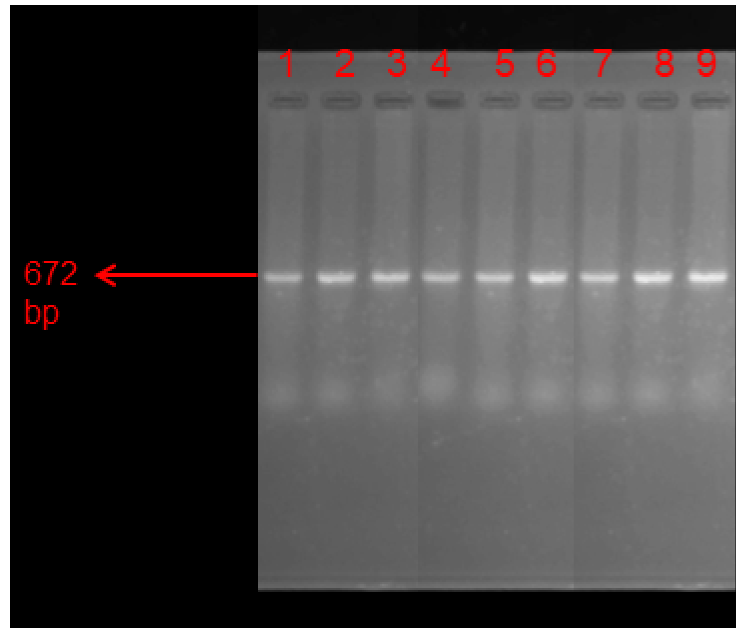

Figure 14: PCR products of the MTHFR gene in a 2% agarose gel. *The MTHFR PCR product (672 bp) is in lanes 1 to 9.*

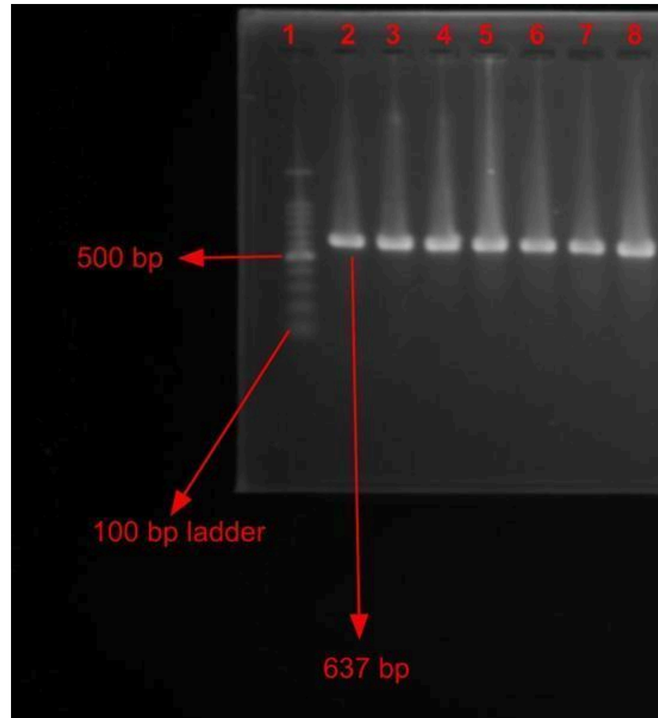

Figure 15: PCR products of the PEMT gene in 2% Agarose gel. *PCR product of PEMT (637 bp) is in lanes 2 to 8. Lane 1 contains a DNA ladder*

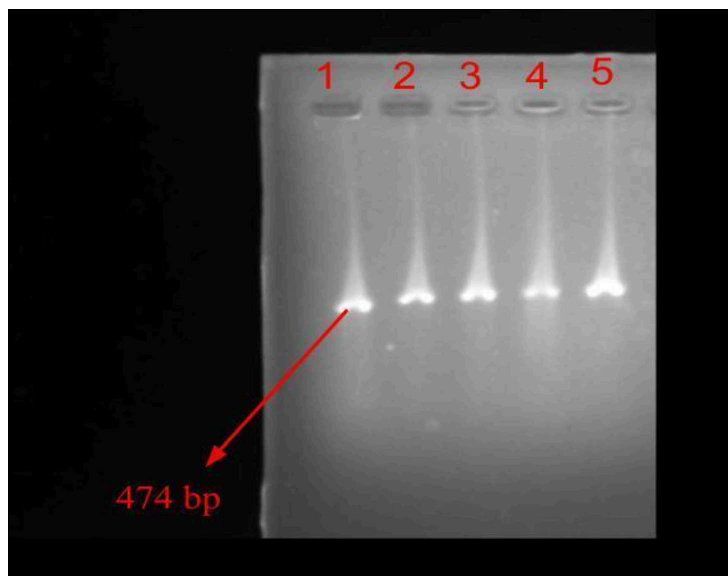

Figure 16: PCR products of CHKA gene in 2% Agarose gel. *PCR products of CHKA (474 bp) are in lanes 1 to 5.*

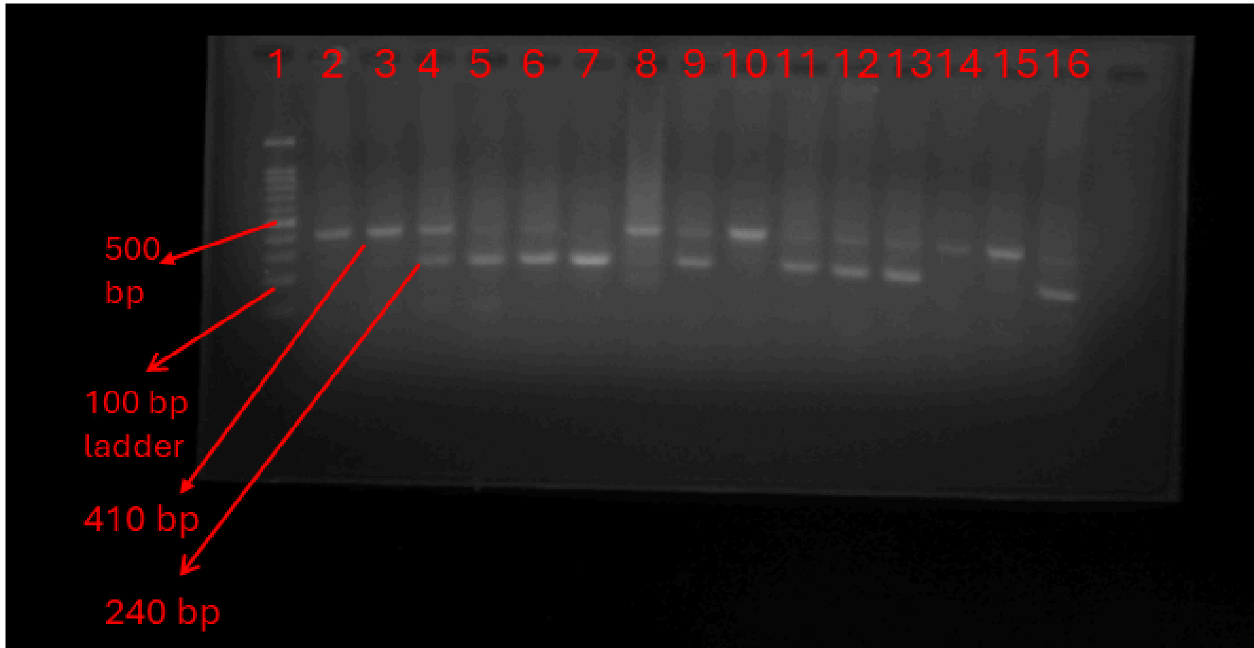

Figure 17: PCR products of the IL-1RN gene in a 2% agarose gel. *Lane 1 contains a DNA ladder. Lanes 2, 3, 7, 8, 10.14, and 15 are homozygous alleles, and Lanes 4, 6, 9, 11, 12, 13, and 16 are heterozygous alleles. Lane 1 contains a DNA ladder.*

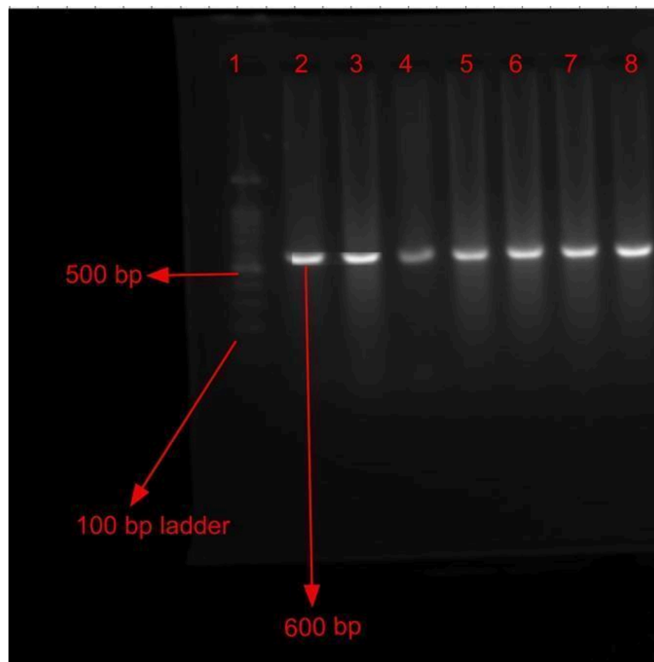

Figure 18: PCR products of NOS3 gene in 2% Agarose gel. *PCR products of NOS3 (600 bp) are in lanes 2 to 8. Lane 1 contains DNA ladder.*

2.7.2 RFLP Analysis

2.7.2.1 Restriction Digestion of MTHFR PCR Products

The restriction enzyme *HinfI* was used to digest the 672 bp MTHFR rs1801133 PCR products following the manufacturer's instructions (incubated at 37°C for 2.5 hours). The 672 bp PCR products of the homozygous mutant genotype (G/A) were fragmented into 447 bp and 225 bp fragments. The two bands appeared under UV light. The homozygous mutant genotype (G/A) produces two bands since it has both alleles (447 bp and 225 bp).

2.7.2.2 Restriction Digestion of PEMT PCR Products

The restriction enzyme *TaqI* was used to digest the PEMT rs4244593 637 bp PCR products following the manufacturer's instructions (incubated at 65°C for 5 hours). The 637 bp PCR products of the homozygous mutant genotype (C/C) were fragmented into 401 bp and 237 bp fragments. The wild-type allele (C) was not cleaved and appeared as one band (637 bp) under UV light. The heterozygous mutant genotype (C/A) produces all three bands since it has both alleles (637 bp, 401 bp, and 237 bp).

2.7.2.3 Restriction Digestion of CHKA PCR Products

The restriction enzyme *HinfI* was used to digest the CHKA rs7928739 474 bp PCR products following the manufacturer's instructions (incubated at 37°C for 2.5 hours). The 474 bp PCR products of the heterozygous genotype (C/A) were fragmented into 272 bp and 202 bp fragments. The homozygous mutant genotype (A/A) produces two bands since it has both alleles (272 bp and 202 bp).

2.7.2.4 Composition of Restriction Digestion Mix

Table 4: Composition of the reaction mixture for HinfI restriction enzyme digestion

Component	Volume (µL)
PCR grade H_2O	7.9
rCutSmart™ Buffer	1.5
HinfI enzyme	0.6
PCR product	5.0
Total Volume	**15.0**

Table 5: Composition of the reaction mixture for TaqI restriction enzyme digestion

Component	Volume (µL)
PCR grade H_2O	5.7
10X buffer	1.0
TaqI enzyme	0.3
PCR product	9.0
Total Volume	**16.0**

2.7.2.5 RFLP Analysis of Digested Products

The enzyme digestion product was run on a Midori Green stained 2% agarose gel for MTHFR and PEMT and 3% agarose gel for the CHKA gene. The gel was then visualized using gel documentation under ultraviolet (UV) light (Figure 18-20). The product size was determined by matching against a 100-base pair DNA ladder.

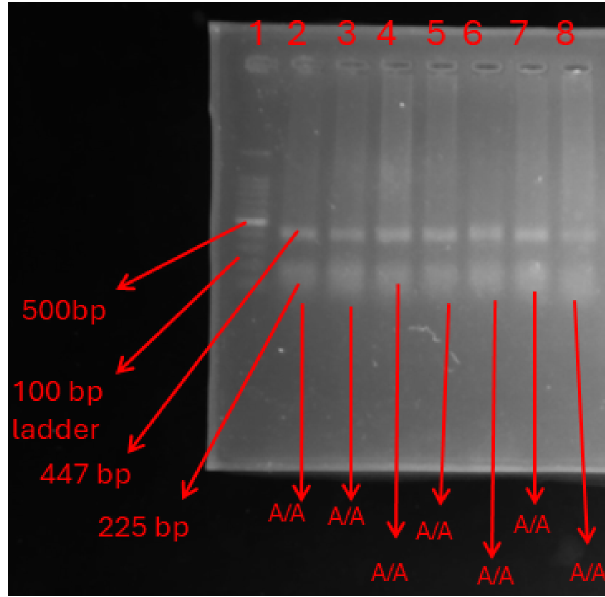

Figure 19: Restriction digested products of MTHFR in 2% agarose gel. *Restriction digestion fragments of MTHFR (Lanes- 2 to 8) resolved in a 2% agarose gel. Lanes 2 to 8 are mutant homozygotes (447 bp and 225 bp). Lane 1 contains a DNA ladder.*

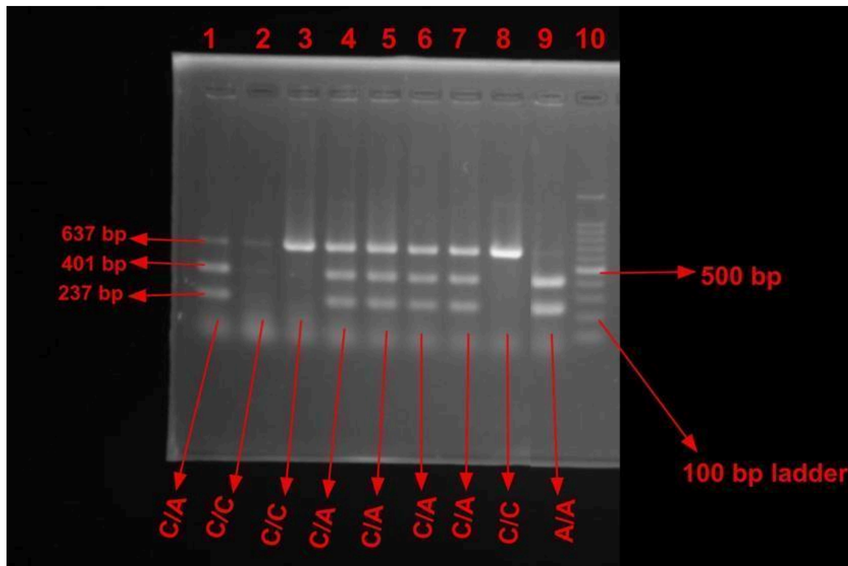

Figure 20: Restriction digested products of PEMT in 2% agarose Gel. *Restriction digestion fragments of PEMT (Lanes 1 to 9) resolved in 2% agarose gel. Lanes 2, 3, and 8, wild-type homozygote (637 bp); Lanes 1, 4 to 7, heterozygote (637, 401, 237 bp) and Lane 9, mutant homozygote (401bp and 237 bp). Lane 10 contains a DNA ladder.*

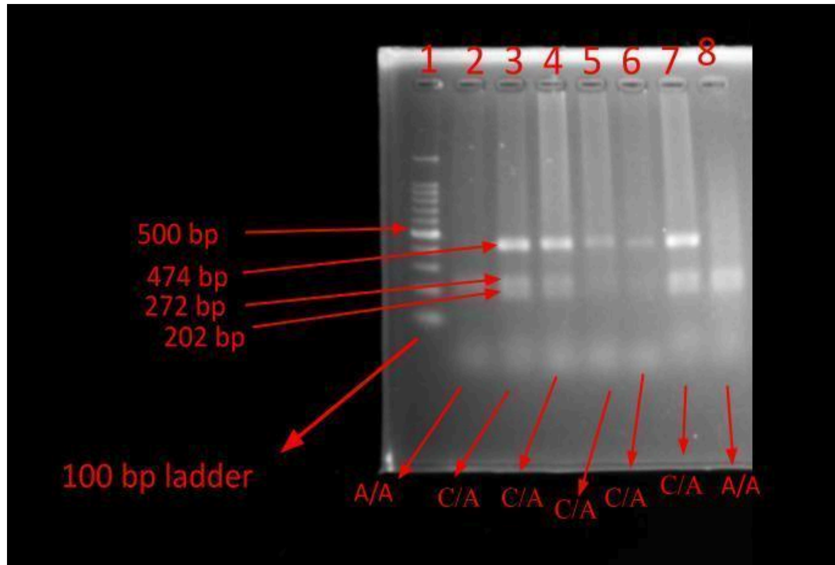

Figure 21: Restriction digested products of CHKA in 3% agarose Gel. *Restriction digestion fragments of CHKA (Lanes 2 to 8) resolved in 3% agarose gel. Lanes 3, 4,5,6,7 heterozygote (474 bp, 272 bp,202 bp); Lanes 2 and 8 mutant homozygote (272 bp and202 bp). Lane 1 contains a DNA ladder.*

2.8 Statistical Analysis

Binomial logistic regression, t-test, and Pearson chi-square were performed through SPSS software, version 29 (SPSS 29.0, SPSS Inc., Chicago, IL). The relative associations were determined by calculating the odds ratio (OR) with 95% confidence intervals (CIs) and level of significance (p). All statistical tests were two sided; a $p<0.05$ was taken as the level of significance.

Chapter 3 Results

This chapter presents the results of the study on the association of PEMT rs4244593, CHKA rs7928739, MTHFR rs1801133, VNTRs NOS3, and IL-1RN gene polymorphisms with the incidence of unexplained intrauterine fetal death is presented in this chapter. The study was conducted with 62 intrauterine fetal death placentas of subjects and 64 controls.

3.1 Baseline Characteristics of the Study Subject

This study was conducted with 62 IUFD subjects and 64 controls, whose age, blood pressure, history of pregnancy and pregnancy loss, occupation, marital years, birth control history, fetus age, and history of surgery are listed in Table 6. Patients were 17–37 years old, with a mean age of 24.66±0.69, while controls were 17–40 years old, with a mean age of 24.52±0.68. Half of the IUFD subjects belonged to the 20-30 age group. Since controls were similar in age to patients, age differences between cases and controls were not statistically significant.

Compared with control subjects, fetuses from IUFD were more likely to be aged between less than 37 weeks (82.3% in IUFD cases vs. 56.2% in control), and the difference was statistically significant (P<0.002), which is also the reason why the cases were statistically (p<0.001) more likely to weight less than 2.5 kgs (67.7% of the cases compared to 34.4% of controls).

While analyzing Full Term Pregnancies (FTP), previously full-term pregnant patients manifested a higher possibility of fetal death than the control subjects with a statistically significant p-value of 0.004. Moreover, 9.7% of the total case subjects were found to have been using contraceptive pills as a method of birth control compared to control, which resulted in a statistically significant p-value of 0.03.

Table 6: Baseline characteristics of the study subjects

Variable		Control (n=64) n (%)	Case (n=62) n (%)	p-value
Age of the Mother (Year)[a]:		24.52 ± 0.68	24.66 ± 0.69	0.59
<20		19 (29.7)	20 (32.3)	0.60
20-30		37 (57.8)	31 (50.0)	
>30		8 (12.5)	11 (17.7)	
Blood pressure (mm of Hg):				
Systolic	≤120	52 (81.2)	51 (82.3)	0.88
	>120	12 (18.8)	11 (17.7)	
Diastolic	≤80	53 (82.8)	55 (88.7)	0.34
	>80	11 (17.2)	7 (11.3)	
Married for (Years)[a]:		6.95 ± 0.80	6.30 ± 0.75	0.59
<5		35 (54.7)	38 (61.3)	0.69
5-10		15 (23.4)	11 (17.7)	
>10		14 (21.9)	13 (21.0)	
Obstetric History				
History of FTP[c]:				
Yes		36 (56.2)	19 (30.6)	**0.004**
No		28 (43.8)	43 (69.4)	
History of surgery/ C-section:				
C-section		7 (10.9)	6 (9.7)	0.58
Other surgeries		0 (0.0)	1 (1.6)	
No record of surgery		57 (89.1)	55 (88.7)	
History of pregnancy loss:				
Yes		5 (7.8)	1 (1.6)	0.10
No		59 (92.2)	61 (98.4)	

Continued table 6

History of birth control:			
Contraceptive pill	2 (3.1)	6 (9.7)	**0.03**
Injectable depo	11 (17.2)	3 (4.8)	
No birth control used	51 (79.7)	53 (85.5)	
Fetal Information			
Age of the Fetus (Week)[a]:	37.31 ± 0.29	34.92 ± 0.41	0.007
≤37	36 (56.2)	51 (82.3)	**0.002**
>37	28 (43.8)	11 (17.7)	
Gender of the Fetus:			
Male	33 (51.6)	38 (61.3)	0.27
Female	31 (48.4)	24 (38.7)	
Weight of the fetus (kg):			
≤2.5	22 (34.4)	42 (67.7)	**<0.001**
>2.5	42 (65.6)	20 (32.3)	

[a]Mean±SEM; [b]Numbers in parentheses show percentages; [c]FTP=Full-term pregnancy; $p<0.05$ was taken as the level of significance

3.2 Frequency distribution of MTHFR rs1801133, PEMT rs4244593, CHKA rs7928739, IL-1RN intron 2 and NOS3 introns 4 genotype and IUFD risk

Genotypic and allelic distributions of MTHFR rs1801133, PEMT rs4244593, CHKA rs7928739, IL-1RN intron 2 and NOS3 intron 4 polymorphisms in control and patients with their association with IUFD are outlined in Table 7.

The MTHFR gene showed only homozygous mutant allele (A/A) in all subjects. No other variants of MTHFR were detected in this study.

In 62 IUFD case samples, 8.1% were homozygous wild type (C/C) of PEMT, 58.1% were heterozygous mutant variants (C/A), and 33.9% were homozygous mutant variants (A/A). Controls had 21.9% homozygous wild type (C/C), 50.0% heterozygous mutant variant (C/A), and 28.1% homozygous mutant variant (A/A). There is a statistically significant association between C/A (OR=3.18; 95% CI=1.02–9.91; p=0.04) and A/A (OR=3.30; 95% Cl=0.98–11.07; p=0.05) genotype with IUFD.

The homozygous wild type (C/C), heterozygous mutant variants (C/A), and homozygous mutant variants (A/A) of the CHKA gene were 9.7%, 62.9%, and 27.4% respectively. Controls had 10.9% homozygous wild type (C/C), 57.8% heterozygous mutant variant (C/A), and 31.3% homozygous mutant variant (A/A).

The most frequent allele of the IL-1RN gene in IUFD cases was allele a/a, with an estimated frequency of 45.2%, followed by allele a/b, with a frequency of 35.5%. The alleles e/e in controls and a/d in cases were not detected. The most frequent genotype was allele a/a, followed by allele a/b. No allele has been found to be statistically significant here.

The VNTR, NOS3 showed only one allele (allele a) containing 5 repeats in intron 4 among all subjects. No other variants of repeats of NOS3 were detected in this study.

Table 7: Genotypic frequency of MTHFR, PEMT, CHKA, IL-1RN and NOS3 in the study subject and risk of IUFD

Gene	**Genotype**	**Control (n=64) n (%)**	**Case (n=62) n (%)**	**OR* (95%CI)**	**p-value**
MTHFR					
	A/A	64 (100)	62 (100)	-	-
PEMT					
	C/C	14 (21.9)	5 (8.1)	1 (Ref)	
	C/A	32 (50.0)	36 (58.1)	3.18 (1.02-9.91)	**0.04**
	A/A	18 (28.1)	21 (33.9)	3.30 (0.98-11.07)	**0.05**
CHKA					
	C/C	7 (10.9)	6 (9.7)	1 (Ref)	

Continued table 7

C/A	37 (57.8)	39 (62.9)	1.25 (0.37-4.15)	0.71
A/A	20 (31.3)	17 (27.4)	1.02 (0.27-3.71)	0.98
IL-1RN				
Allele a/a	26 (40.6)	28 (45.2)	1 (Ref)	
Allele b/b	3 (4.7)	10 (16.1)	3.09 (0.76-12.51)	0.11
Allele e/e	0 (0.0)	1 (1.6)	14E+8⁎	1.00
Allele a/b	33 (51.6)	22 (35.5)	0.62 (0.29-1.32)	0.22
Allele a/c	0 (0.0)	1 (1.6)	15E+8⁎	1.00
Allele a/d	2 (3.1)	0 (0.0)	0.0 (0.0)	0.99
NOS3				
Allele a/a	64 (100)	62 (100)	-	-

*Adjusted by age; Odds ratios (OR) and 95% confidence interval (95%CI); Logistic regression was done to evaluate significance; p < 0.05 was considered as the level of significance; ⁎High odds ratio due to missing values and outliers of the model

3.3 Frequency distribution of MTHFR rs1801133, PEMT rs4244593, CHKA rs7928739, IL-1RN intron 2 and NOS3 intron 4 alleles and IUFD risk

In the allele frequency analysis of PEMT, the wild-type C and mutant A alleles were present in 37.1% and 62.9% among cases, whereas 46.9% and 53.1% were in controls, respectively. However, neither allele significantly increased IUFD risk (OR=0.67; 95% CI-0.40-1.11; p=0.12).

In the case of CHKA, the wild-type C allele, and mutant A allele were present in 41.1% and 58.9% of cases, respectively, whereas 39.8% and 60.2% were in controls, respectively. However, neither allele significantly increased IUFD risk (OR=1.05; 95% CI=0.64–1.75; p=0.84). In the VNTR IL-1RN, alleles a and b had the maximum frequency, whereas, the rest

of the alleles had a very low and negligible percentage within both the cases and the controls. However, none of the alleles showed any significant association with IUFD.

Table 8: Allele frequency of MTHFR, PEMT, CHKA, IL-1RN, and NOS3 in the study subject and risk of IUFD

Gene	Allele	Control Alleles (n=128) n (%)	Case Alleles (n=124) n (%)	OR (95%CI)	p-value
MTHFR					
	A Allele	128 (100)	124 (100)	-	-
PEMT					
	C Allele	60 (46.9)	46 (37.1)	1 (Ref)	-
	A Allele	68 (53.1)	78 (62.9)	0.67 (0.40-1.11)	0.12
CHKA					
	C Allele	51 (39.8)	51 (41.1)	1 (Ref)	-
	A Allele	77 (60.2)	73 (58.9)	1.05 (0.64-1.75)	0.84
IL-1RN					
	Allele a	87 (68.0)	79 (63.7)	1 (Ref)	
	Allele b	39 (30.5)	42 (33.9)	0.84 (0.49-1.43)	0.53
	Allele c	0 (0.0)	1 (0.8)	0.0 (0.0)	1.00
	Allele d	2 (1.6)	0 (0.0)	14E+8*	0.99
	Allele e	0 (0.0)	2 (1.6)	0.0 (0.0)	0.99
NOS3					
	Allele a	128 (100)	124 (100)	-	-

Odds ratios (OR) and 95% confidence interval (95%CI); Logistic regression was done to evaluate significance; $p < 0.05$ was considered as the level of significance; *High odds ratio due to missing values and outliers of the model

3.4 Association between PEMT and CHKA with IUFD using Genetic Models

The effects of PEMT rs4244593 and CHKA rs7928739 genotypes on IUFD risk were evaluated using codominant, dominant, recessive, and over-dominant models (Table 9). In the overdominant model, the risk of IUFD was highest (0.72 folds) in individuals with the heterozygous genotype of PEMT (C/A), but it was statistically insignificant (OR=0.72; 95% CI=0.36–1.46; p=0.36). In CHKA, the heterozygous genotype (C/A) also showed the highest risk (0.8 folds) in the overdominant model, but that was too statistically insignificant (OR=0.80; 95% CI=0.39–1.65; p=0.56).

Table 9: Genotype frequencies of PEMT rs4244593 and CHKA rs7928739 using codominant, homozygous dominant, homozygous recessive, and over-dominant models

Gene	Genotype model	Genotype	Control (n=64) n (%)	Case (n=62) n (%)	OR (95%CI)	p-value
PEMT	Codominant model	C/C	14 (21.9)	5 (8.1)	1 (Ref)	-
		C/A	32 (50.0)	36 (58.1)	3.42 (1.00-11.69)	0.5
		A/A	18 (28.1)	21 (33.9)	3.06 (0.83-11.34)	0.93
	Dominant model	C/C	14 (21.9)	5 (8.1)	1 (Ref)	-
		C/A+A/A	50 (78.1)	57 (91.9)	3.06 (0.83-11.34)	0.93
	Recessive model	C/C+C/A	46 (71.9)	41 (66.1)	1 (Ref)	-
		A/A	18 (28.1)	21 (33.9)	0.76 (0.36-1.62)	0.48
	Over dominant	C/C+A/A	32 (50.0)	26 (41.9)	1 (Ref)	-
		C/A	32 (50.0)	36 (58.1)	0.72 (0.36-1.46)	0.36
CHKA	Codominant model	C/C	7 (10.9)	6 (9.7)	1 (Ref)	-
		C/A	37 (57.8)	39 (62.9)	1.18 (0.33-4.25)	0.79
		A/A	20 (31.3)	17 (27.4)	1.07 (0.27-4.22)	0.92
	Dominant model	C/C	7 (10.9)	6 (9.7)	1 (Ref)	-
		C/A+A/A	57 (89.1)	56 (90.3)	0.87 (0.27-2.76)	0.81

Continued table 9

	Recessive model	C/C+C/A	44 (68.8)	45 (72.6)	1 (Ref)	-
		A/A	20 (31.3)	17 (27.4)	1.20 (0.56-2.59)	0.64
	Over dominant	C/C +A/A	27 (42.2)	23 (37.1)	1 (Ref)	-
		C/A	37 (57.8)	39 (62.9)	0.80 (0.39-1.65)	0.56

Odds ratios (OR) and 95% confidence interval (95%CI); p < 0.05 was considered as the level of significance

3.5 Combined Genotypic effects of PEMT, CHKA, and IL-1RN polymorphisms on IUFD Risk

The analysis of pairwise joint associations of CHKA rs7928739 and PEMT rs4244593in genotypes with IUFD risk are shown in Table 10.

The risk of intrauterine fetal death was highest (1.29 folds) in individuals with CHKA A/A and PEMT C/A genotype, but it was statistically insignificant (OR=1.29; 95% CI=0.07–24.38; p=0.87).

Table 10: Interaction between CHKA rs7928739 and PEMT rs4244593 genotypes and IUFD risk

CHKA	PEMT	Controls (n=64) n (%)	Cases (n=62) n (%)	OR (95% CI)	p-value
C/C	C/C	1(1.60)	1(1.60)	1 (Ref)	-
C/C	C/A	4 (6.30)	3 (4.80)	0.75 (0.03-17.5)	0.86
C/C	A/A	2 (3.10)	2 (3.20)	1 (0.03-29.80)	1
C/A	C/C	6 (9.40)	2 (3.20)	0.33 (0.01-8.18)	0.50
C/A	C/A	21 (32.80)	24 (38.70)	1.14 (0.07-19.42)	0.93
C/A	A/A	10 (15.60)	13 (21.00)	1.3 (0.07-23.43)	0.86
A/A	C/C	7 (10.90)	2 (3.20)	0.28 (0.01-6.91)	0.44
A/A	C/A	7 (10.90)	9 (14.50)	1.29 (0.07-24.38)	0.87
A/A	A/A	6 (9.40)	6 (9.70)	1 (0.05-19.96)	1

Odds ratios (OR) and 95% confidence interval (95%CI); p < 0.05 was considered as the level of significance

The analysis of pairwise joint associations of CHKA rs7928739 and IL-1RN intron 2in genotypes with IUFD risk are shown in Table 11.

The risk of intrauterine fetal death was highest (9 folds) in individuals with IL-1RN (Allele b/b) CHKA (C/A) genotype, but it was statistically insignificant (OR=9; 95% CI=0.57–143.89; p=0.12).

Table 11: Interaction between IL-1RN Intron 2 and CHKA rs7928739 genotypes and IUFD risk

IL-1RN	CHKA	Controls (n=64) n (%)	Cases (n=62) n (%)	OR (95%CI)	p-value
Allele a/a	C/C	3 (4.70)	1 (1.60)	1 (Ref)	-
Allele a/a	C/A	16 (25)	17 (27.40)	3.19 (0.3-33.89)	0.33
Allele a/a	A/A	7 (10.90)	10 (16.10)	4.29 (0.37-50.19)	0.24
Allele b/b	C/C	1 (1.60)	3 (4.80)	9 (0.37-220.92)	0.18
Allele b/b	C/A	2 (3.10)	6 (9.70)	9 (0.57-143.89)	0.12
Allele b/b	A/A	0 (0.00)	1 (1.60)	4.80E+09*	1
Allele e/e	C/C	0	0	-	-
Allele e/e	C/A	0	0	-	-
Allele e/e	A/A	0 (0.00)	1 (1.60)	4.80E+09*	1
Allele a/b	C/C	3 (4.70)	2 (3.20)	2 (0.11-35.80)	0.63
Allele a/b	C/A	18 (28.10)	16 (25.80)	2.67 (0.25-28.28)	0.41
Allele a/b	A/A	12 (18.80)	4 (6.50)	1 (0.08-12.55)	1
Allele a/c	C/C	0	0	-	-
Allele a/c	C/A	0	0	-	-
Allele a/c	A/A	0 (0.00)	1 (1.60)	4.80E+09*	1
Allele a/d	C/C	0	0	-	-
Allele a/d	C/A	1(1.60)	0 (0.00)	0	1
Allele a/d	A/A	1(1.60)	0 (0.00)	0	1

Odds ratios (OR) and 95% confidence interval (95%CI); p < 0.05 was considered as the level of significance; *High odds ratio due to missing values and outliers of the model

Table 12 shows the analysis of pairwise joint associations of PEMT rs4244593 and IL-1RN intron 2 in genotypes with IUFD risk.

The incidence of IL-1RN (Allele b/b) PEMT (A/A) genotype was statistically significant, increasing the risk of IUFD twice (OR=2; 95% CI=0.15–26.73; p=0.05).

Table 12: Interaction between IL-1RN Intron 2 and PEMT rs4244593 genotypes and IUFD risk

IL-1RN	PEMT	Controls (n=64) n (%)	Cases (n=62) n (%)	OR (95%CI)	p-value
Allele a/a	C/C	4 (6.30)	2 (3.2)	1 (Ref)	-
Allele a/a	C/A	13 (20.30)	12 (19.40)	1.84 (0.285-11.978)	0.59
Allele a/a	A/A	9 (14.10)	14 (22.60)	3.11 (0.469-20.649)	0.52
Allele b/b	C/C	0 (0)	1 (1.60)	3.20E+09*	0.24
Allele b/b	C/A	1 (1.60)	7 (11.30)	14 (0.944-207.597)	1
Allele b/b	A/A	2 (3.10)	2 (3.20)	2 (0.15-26.734)	**0.05**
Allele e/e	C/C	0 (0)	1 (1.60)	3.20E+09	0.6
Allele e/e	C/A	0	0	-	-
Allele e/e	A/A	0	0	-	-
Allele a/b	C/C	8 (12.50)	1 (1.60)	3230949686*	1
Allele a/b	C/A	18 (28.10)	16 (25.80)	0.25 (0.017-3.66)	0.31
Allele a/b	A/A	7 (10.90)	5 (8.10)	1.78 (0.286-11.039)	0.53
Allele a/c	C/C	0	0	1.43 (0.184-11.085)	0.73
Allele a/c	C/A	1 (1.60)	0	3.20E+09*	1
Allele a/c	A/A	0	0	-	-
Allele a/d	C/C	2 (3.10)	0	-	0.99
Allele a/d	C/A	0	0	-	-
Allele a/d	A/A	0	0	-	-

Odds ratios (OR) and 95% confidence interval (95%CI); $p < 0.05$ was considered as the level of significance; *High odds ratio due to missing values and outliers of the model

The analysis of joint associations of IL-1RN Intron 2, CHKA rs7928739, and PEMT rs4244593 genotypes and IUFD risk is shown in Table 13.

No significant association was found among the genotypes IL-1RN Intron 2, CHKA rs7928739, and PEMT rs4244593.

Table 13: Interaction among IL-1RN Intron 2, CHKA rs7928739 and PEMT rs4244593 genotypes and IUFD risk

IL-1RN	PEMT	CHKA	Controls (n=64) n (%)	Cases (n=62) n (%)	OR (95%CI)	p-value
Allele a/a	C/C	C/C	0	-	-	-
Allele a/a	C/C	C/A	2 (3.10)	0 (0.0)	1 (Ref)	-
Allele a/a	C/C	A/A	1 (1.60)	1 (1.60)	1615471580*	0.99
Allele a/a	C/A	C/C	2 (3.10)	1 (1.60)	807735790.2 *	0.99
Allele a/a	C/A	C/A	8 (12.50)	7 (11.30)	1413537633*	0.99
Allele a/a	C/A	A/A	6 (9.40)	9 (14.50)	2423207370*	0.99
Allele a/a	A/A	C/C	2 (3.10)	1 (1.60)	807735790.2 *	0.99
Allele a/a	A/A	C/A	3 (4.70)	5 (8.10)	2692452634*	0.99
Allele a/a	A/A	A/A	2 (3.10)	4 (6.50)	3230943161*	0.99
Allele b/b	C/C	C/C	0	0	-	-
Allele b/b	C/C	C/A	0 (0.0)	2 (3.20)	2.60E+18*	0.99
Allele b/b	C/C	A/A	1 (1.60)	1 (1.60)	1615471580*	0.99
Allele b/b	C/A	C/C	0 (0.0)	1 (1.60)	2.60E+18*	0.99
Allele b/b	C/A	C/A	1 (1.60)	5 (8.10)	8077357902*	0.99
Allele b/b	C/A	A/A	1 (1.60)	0 (0.0)	1	0.99
Allele b/b	A/A	C/C	0	0	-	-
Allele b/b	A/A	C/A	0	0	-	-
Allele b/b	A/A	A/A	0 (0.0)	1 (1.60)	2.60E+18*	0.99
Allele e/e	C/C	C/C	0	0	-	-
Allele e/e	C/C	C/A	0	0	-	-
Allele e/e	C/C	A/A	0	0	-	-
Allele e/e	C/A	C/C	0	0	-	-
Allele e/e	C/A	C/A	0	0	-	-
Allele e/e	C/A	A/A	0	0	-	-
Allele e/e	A/A	C/C	1 (1.60)	0(0.00)	2.60E+18*	0.99
Allele e/e	A/A	C/A	0	0	-	-
Allele e/e	A/A	A/A	0	0	-	-
Allele a/b	C/C	C/C	1 (1.60)	1 (1.60)	1615471580*	0.99
Allele a/b	C/C	C/A	1 (1.60)	2 (3.10)	807735790.2 *	0.99
Allele a/b	C/C	A/A	0	0	-	-
Allele a/b	C/A	C/C	0 (0.00)	3 (4.70)	1	1

Continued table 13

Allele a/b	C/A	C/A	12 (19.40)	12 (18.80)	1615471580*	0.99
Allele a/b	C/A	A/A	4 (6.50)	3 (4.70)	2153962107*	0.99
Allele a/b	A/A	C/C	0 (0.00)	4 (6.30)	1	1
Allele a/b	A/A	C/A	3 (4.80)	4 (6.30)	1211603685*	0.99
Allele a/b	A/A	A/A	1 (1.60)	4 (6.30)	403867895.1*	0.99
Allele a/c	C/C	C/C	0	0	-	-
Allele a/c	C/C	C/A	0	0	-	-
Allele a/c	C/C	A/A	0	0	-	-
Allele a/c	C/A	C/C	0	0	-	-
Allele a/c	C/A	C/A	0	0	-	-
Allele a/c	C/A	A/A	0	0	-	-
Allele a/c	A/A	C/C	0	0	-	-
Allele a/c	A/A	C/A	0	1 (1.60)	2.61E+18*	0.99
Allele a/c	A/A	A/A	0	0	-	-
Allele a/d	C/C	C/C	0	0	-	-
Allele a/d	C/C	C/A	0	0	-	-
Allele a/d	C/C	A/A	0	0	-	-
Allele a/d	C/A	C/C	1 (1.60)	0	1	0
Allele a/d	C/A	C/A	0	0	-	-
Allele a/d	C/A	A/A	0	0	-	-
Allele a/d	A/A	C/C	1 (1.60)	0	1	0
Allele a/d	A/A	C/A	0	0	-	-
Allele a/d	A/A	A/A	0	0	-	-

Odds ratios (OR) and 95% confidence interval (95%CI); $p < 0.05$ was considered as the level of significance; *High odds ratio due to missing values and outliers of the model

3.6 PEMT, CHKA, and IL-1RN Genotype on the risk of IUFD according to History of a Full-Term Pregnancy and Birth Control

The association of PEMT, CHKA, and IL-1RN genotypes and IUFD according to the history of a full-term pregnancy is shown in Table 14. A 2.4-fold greater risk of IUFD was observed in cases with the CHKA C/A genotype who had previously had a successful pregnancy, but this was not statistically significant (OR=2.4; 95% CI=0.14–42.6; p=0.55). On the other hand, none of the genotypes of PEMT, CHKA, and IL-1RN showed significant association with individuals who had previous successful pregnancies. Surprisingly, there was a

statistically significant association between IUFD and the use of birth control in cases of C/A (OR=0.27; 95% CI=0.08–0.92; p=0.04) and A/A (OR=0.02; 95% CI=0.06–0.79; p=0.02) genotypes of the PEMT gene, whereas, in cases of both CHKA and IL-1RN, no genotypes showed any statistically significant difference.

Table 14: PEMT rs4244593, CHKA rs7928739, and IL-1RN intron 2 genotypes on risk of IUFD according to the history of FTP and birth control

Baseline Characteristics	Gene	Genotype	Control (n=64) n (%)	Case (n=62) n (%)	OR (95%CI)	p-value
History of FTP[c]						
No previous pregnancy	PEMT	C/C	4 (6.3)	4 (6.5)	1 (Ref)	-
		C/A	14 (21.9)	25 (40.3)	0.46 (0.12-2.59)	0.56
		A/A	10 (15.6)	14 (22.6)	0.68 (0.14-3.56)	0.71
	CHKA	C/C	6 (9.4)	5 (8.1)	1 (Ref)	-
		C/A	13 (20.3)	29 (46.8)	0.37 (0.09-1.45)	0.15
		A/A	9 (14.1)	9 (14.5)	1.83 (0.18-3.75)	0.81
	IL1RN	Allele a/a	10 (15.6)	21 (33.9)	1 (Ref)	-
		Allele b/b	3 (4.7)	7 (11.3)	0.9 (0.19-4.23)	0.89
		Allele e/e	0 (0.0)	0 (0.0)	-	-
		Allele a/b	15 (23.4)	15 (24.2)	2.1 (0.74-5.94)	0.16
		Allele a/c	0 (0.0)	0 (0.0)	-	-
		Allele a/d	0 (0.0)	0 (0.0)	-	-
Previous successful pregnancy	PEMT	C/C	10 (15.6)	1 (1.6)	1 (Ref)	-
		C/A	18 (28.1)	11 (17.7)	0.16 (0.02-1.46)	0.11
		A/A	8 (12.5)	7 (11.3)	0.11 (0.01-1.13)	0.06
	CHKA	C/C	1 (1.6)	1 (1.6)	1 (Ref)	-

		C/A	24 (37.5)	10 (16.1)	2.4 (0.14-42.26)	0.55
		A/A	11 (17.2)	8 (12.9)	1.38 (0.74-25.43)	0.83
	IL1RN	Allele a/a	16 (25.0)	7 (11.3)	1 (Ref)	-
		Allele b/b	0 (0.0)	3 (4.8)	0	0.99
		Allele e/e	0 (0.0)	1 (1.6)	0	1
		Allele a/b	18 (28.1)	7 (11.3)	1.13 (0.32-3.91)	0.85
		Allele a/c	0 (0.0)	1 (1.6)	0	1
		Allele a/d	2 (3.1)	0 (0.0)	706770243.7*	0.99
History of birth control						
Contraceptive pill	PEMT	C/C	0 (0.0)	1 (1.6)	1 (Ref)	-
		C/A	1 (1.6)	4 (6.5)	403868950.2*	1
		A/A	13 (20.3)	4 (6.5)	5E+9*	1
	CHKA	C/C	1 (1.6)	2 (3.2)	1 (Ref)	-
		C/A	1 (1.6)	2 (3.2)	1.00 (0.34-29.81)	1
		A/A	0 (0.0)	2 (3.2)	0	0.99
	IL1RN	Allele a/a	1 (1.6)	3 (4.8)	1 (Ref)	-
		Allele b/b	0 (0.0)	1 (1.6)	0	1
		Allele e/e	0 (0.0)	0 (0.0)	-	-
		Allele a/b	1 (1.6)	2 (3.2)	1.50 (0.05-40.6)	0.81
		Allele a/c	0 (0.0)	0 (0.0)	-	-
		Allele a/d	0 (0.0)	0 (0.0)	-	-
Injectable depo	PEMT	C/C	1 (1.6)	0 (0.0)	1 (Ref)	-
		C/A	6 (9.4)	3 (4.8)	0	1
		A/A	4 (6.3)	0 (0.0)	1	1
	CHKA	C/C	1 (1.6)	0 (0.0)	1 (Ref)	-
		C/A	7 (10.9)	3 (4.8)	0	1
		A/A	3 (4.7)	0 (0.0)	1	1

	IL1RN	Allele a/a	2 (3.1)	1 (1.6)	1 (Ref)	-
		Allele b/b	0 (0.0)	0 (0.0)	-	-
		Allele e/e	0 (0.0)	0 (0.0)	-	-
		Allele a/b	9 (14.1)	2 (3.2)	2.25 (0.13-38.8)	0.58
		Allele a/c	0 (0.0)	0 (0.0)	-	-
		Allele a/d	0 (0.0)	0 (0.0)	-	-
No birth control used	PEMT	C/C	13 (20.3)	4 (6.5)	1 (Ref)	-
		C/A	25 (39.1)	29 (46.8)	0.27 (0.08-0.92)	**0.04**
		A/A	13 (20.3)	20 (32.3)	0.2 (0.06-0.79)	**0.02**
	CHKA	C/C	5 (7.8)	4 (6.5)	1 (Ref)	-
		C/A	29 (45.3)	34 (54.8)	0.68 (0.17-2.78)	0.59
		A/A	17 (26.6)	15 (24.2)	0.91 (0.21-4.01)	0.89
	IL1RN	Allele a/a	23 (35.9)	24 (38.7)	1 (Ref)	-
		Allele b/b	3 (4.7)	9 (14.5)	0.35 (0.08-1.45)	0.15
		Allele e/e	0 (0.0)	1 (1.6)	0	1
		Allele a/b	23 (35.9)	18 (29.0)	1.33 (0.58-3.09)	0.5
		Allele a/c	0 (0.0)	1 (1.6)	0	1
		Allele a/d	2 (3.1)	0 (0.0)	1E+9*	0.99

[c]FTP=Full-term pregnancy; Odds ratios (OR) and 95% confidence interval (95%CI); Logistic regression was done to evaluate significance; $p < 0.05$ was considered as the level of significance; *High odds ratio due to missing values and outliers of the model

Chapter 4 Discussion

4.1 Association of MTHFR rs1801133 Polymorphism with IUFD Risk

The MTHFR gene is essential for DNA synthesis and methylation processes and this gene is involved in folate metabolism (vitamin B9). Many of the identified genetic variations for the MTHFR gene are functional, and the gene is highly polymorphic. In our research, all the subjects revealed the same genotype, which is homozygous mutant allele (A/A). In a Polish study, researchers have shown a probability of single nucleotide polymorphism associated with an increased risk of IUFD and other reproductive complications. There was no significant association between a specific MTHFR polymorphism and IUFD etiology. In another multicenter study, no significant associations were found when combinations of different polymorphisms in MTHFR and other genes were analyzed (Hefler et al., 2004).

One of the most vital reasons for such an outcome can be that the SNP is monomorphic (fixed for one allele) or almost monomorphic for both cases and controls. In addition, population genetics might be associated with this outcome. Different populations and ethnic groups express contrasting allele frequencies because of evolutionary pressure or random genetic drift (Wolski et al., 2019).

4.2 Association of PEMT rs4244593 Polymorphism with IUFD Risk

It is vital to emphasize that the issue of IUFD is a syndrome with numerous probable causes, some of which have been identified and documented in scientific papers concerning a range of pregnancy issues (such as preeclampsia, fetal growth restriction, gestational diabetes mellitus, etc.). Various genetic markers, such as cytogenetic and molecular ones, are well recognized to be associated with intrauterine fetal death, particularly in cases of repeated failures.

According to our research, women with the PEMT rs4244593 C/A genotype had a 3.2 times and A/A genotype had 3.3 times greater chances of developing IUFD than those with the C/C

genotype after being adjusted by age (OR=3.18; 95% CI=1.02–9.91; p=0.04; OR=3.30; 95% Cl=0.98–11.07; p=0.05) (Table 7). In line with our findings, in the Polish population, 4 SNP sites of PEMT were studied for association with IUFD (rs4646406, rs4244593, rs897453 and rs12325817). Among them, the largest statistically significant differences were observed for rs4244593 C > A polymorphism. The frequency of the AA genotype was significantly higher (OR=0.43, 95% CI= 0.19–0.98; p=0.007) in IUFD women compared to the healthy subjects. (Seremak-Mrozikiewicz et al., 2018). The same study found the dominant model to be the best-fit model (CC vs. AC+AA) (OR=0.38, 95%CI=0.20–0.73; p = 0.002). Their discovery conflicts with our findings from the dominant model analysis, which showed a p-value of 0.93, hence, non-significant (Table 9). Differences in ethnic, regional, and environmental factors may be responsible for the contradiction in the result.

The statistically significant differences demonstrated the association between severe obstetrical problems and PEMT polymorphisms. PEMT's role in the choline cycle could be a partial explanation. Because impaired choline metabolism is correlated with vitamin B and methionine metabolism, it is critical for normal fetal and placental development. Those metabolic pathways have common ground in synthesizing methionine and homocysteine methylation. These enzymatic cycles appear essential for the proper availability of methyl groups, which is necessary to develop the fetal epigenome (Mehedint, 2010).

4.3 Association of CHKA rs7928739 Polymorphism with IUFD Risk

In our study, we found no statistically significant connection between the CHKA rs7928739 variants and the risk of intrauterine fetal death. The heterozygous genotype C/A was associated with a higher risk of developing intrauterine fetal death compared to the wild-type CC variant, although the correlation was not statistically significant (OR=1.25; 95% CI=0.37–4.15; p=0.79) (Table 7). In a study of the Polish population, the genotype A/A was more associated with intrauterine fetal death compared to other genotypes but it was statistically insignificant (OR=1.035; CI=0.494–2.168; p=0.92695) (Drews, 2017). Besides, the study also showed that the combined effect of CHKA rs7928739AC/CHDH rs2289205. AA genotypes were significantly more common in the study group than in the control group

(p=0.031). In addition, the study also found the dominant model to be the most suitable even though it was statistically insignificant (CC vs AC+AA) (OR=1.017; 95% CI=0.527–1.960; p=0.96063). But in our study the recessive model was the most suitable one for CHKA but it was insignificant (OR=1.20; 95% CI=0.56–2.59; p=0.64) (Table 9).

Thus, our study did not find a statistically significant connection between CHKA rs7928739 variants and intrauterine fetal death risk, and further research is needed to explore the specific role of the CHKA gene polymorphism in obstetrics.

4.4 Association of IL-1RN intron 2 Polymorphism with IUFD Risk

The Interleukin- 1 Receptor Antagonist (IL-1RN) gene affects the body's immune response and inflammation. Studies in divergent populations have found an association between IL-1RN gene intron 2 and IUFD risk. In our research, no allele of IL-1RN intron 2 expressed statistical significance. Then, while comparing genotypes of CHKA rs7928739, no significant association was found between interactions in any genotype of both genes. However, when the analysis was done on combined effect of IL-1RN intron 2 and PEMT rs4244593, there was a statistically significant outcome when the interaction between allele b/b of IL-1RN and A/A genotype of PEMT rs4244593. The p-value was 0.05. In an IL-1RN intron 2 polymorphism study done on the Brazilian population, the same allele, allele b/b, was linked with preterm delivery of pregnant women. Moreover, mothers who tested positive for two copies of IL-1RN intron 2 were more than twice as likely to have experienced a prior fetal death (Chaves et al., 2008). In another study on Hispanic descent, the IL-1RN intron 2 was at an increased risk for spontaneous preterm delivery (odds ratio, 3.8; 95% CI, 1.1–13.1) (Genç et al., 2002).

The statistically significant value of interaction between IL-1RN allele b/b and PEMT rs4244593 A/A genotype depicts an association between reproductive complications in those two genotypes.

The IL-1RN gene is responsible for higher production of an anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1Ra). This allele b/b can play a role in the immune and inflammatory response of numerous tissues, including the ones that participate in reproduction. On the other hand, PEMT is concerned with phospholipid metabolism and is essential for phosphatidylcholine synthesis. The combined effect of these two suggests that excessive anti-inflammatory response from IL-1RN allele b/b and PEMT rs4244593 altering placental regular function can potentially impair fetal growth. There is also the possibility that some of these results resulted from random chance. As there was no statistically significant value for allele b/b in the IL-1RN gene individually, the interaction between two different genotypes of PEMT and IL-1RN implies the necessity to consider multiple genetic factors and their interplay in the context of complex biological processes like pregnancy (Blue et al., 2018).

4.5 Association of NOS3 intron 4 Polymorphism with IUFD Risk

Clinical and experimental data indicate that thrombophilic and vascular genes play a critical role in the pathophysiology of pregnancy-related problems, including IUFD (Martinelli et al., 2000). Results from studies on vascular and thrombophilic genes in IUFD were inconsistent. Some authors mentioned a specific function of these genes. However, several researchers could not find any connection between thrombophilic and vascular gene SNPs and IUFD incidence (Morssink et al., 2004). Both in humans and animal models, the NOS3 gene is a strong regulator of vascular tone. The polymorphism under investigation has previously been connected to several "vascular" illnesses, including stroke and hypertension (Huber et al., 2005).

Our study found no statistically significant connection between the NOS3 intron 4 variants and the risk of IUFD (Table 7). Among the two expected alleles (a and b), only allele a was found among the study subjects and the controls. Our results contradict an earlier study on the relationship between the NOS3 intron 4 polymorphism and IUFD in the European population (Huber et al., 2005). Even though the study could not establish any statistically

significant association between NOS3 intron 4 polymorphism and the increased risk of IUFD, their investigation portrayed reasonable proof that it might modulate the timing of IUFD in affected pregnancies.

4.6 PEMT rs4244593, CHKA rs7928739, and IL-1RN intron 2 Polymorphisms on IUFD Risk Based on History of Full Term Pregnancy and Birth Control

Previous pregnancies and birth control attempts have been important factors to consider for a successful or unsuccessful pregnancy. In previous studies, it has been observed that healthy women had an increased number of previous full-term pregnancies than women with IUFD (Huber et al., 2005). In other studies, the use of oral contraceptives is examined for an effect on the increasing risk of fetal death. However, no significant association was found (Jellensen et al., 2008).

Our study examined the interaction of the history of previous FTP and birth control (classified by oral contraceptive pill, Injectable Depo, and no birth control history) with PEMT rs4244593, CHKA rs7928739, and IL-1RN intron 2 Polymorphisms on increased risk of IUFD. No statistically significant differences were observed in the history of previous FTP. However, there was a statistically significant association between IUFD and the use of birth control in cases of C/A (OR=0.27; 95% CI=0.08–0.92; p=0.04) and A/A (OR=0.02; 95% CI=0.06–0.79; p=0.02) genotypes of the PEMT gene. Both the results were inconsistent with the previous studies (Jellensen et al., 2008; Huber et al., 2005). The different distributions of genetic and environmental factors in the study cohort, which may affect the consequences of any specific genetic variant, could cause inconsistent results among the epidemiological studies.

4.7 Strengths and Limitations

This study is the first investigation on polymorphisms associated with IUFD in Bangladesh. However, owing to the rarity of the incidence of unexplained IUFD, the sample size was very small. We excluded tribal or other ethnic groups from participating in this study, ensuring all subjects had a uniform genetic background. Although due to financial constraints, we were unable to cross-check our results through sequencing. To ensure the reproducibility of our results, genotype assays were repeated on 10% of the randomly chosen samples, and the repetitions were 100% concordant.

Conclusion

The application of modern diagnostic techniques, along with an in-depth knowledge of the pathophysiology of intrauterine fetal death, has made it possible to determine the cause of death in a higher percentage of cases than in the past. However, the existence of unexplained IUFD proves that there are still many underlying factors of IUFD that are still unexplored. In this regard, identifying genetic polymorphisms can serve as an excellent biomarker.

Our findings indicate a potential link between genetic variations and increased IUFD risk. We discovered that the C/A and A/A variants of PEMT rs4244593 is associated with IUFD risk in Bangladeshi women. However, the MTHFR rs1801133, CHKA rs7928739, VNTRs IL-1RN intron 2, and NOS3 intron 4 polymorphisms were not significantly associated with an elevated risk of developing IUFD. However, when interacting with the PEMT A/A genotype, the IL1RN b/b genotype exhibited a statistically significant risk for IUFD. The PEMT C/A (heterozygous mutant) and A/A (homozygous mutant) genotypes also exhibited association with IUFD in cases of no use of birth control. A study with larger sample sizes is required to examine the link between these polymorphisms and IUFD further.

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