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Genetic, Epigenetic and Inflammation Influences on Pregnancy Maintenance

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ABSTRACT

Genetic, Epigenetic and Inflammation Influences on Pregnancy Maintenance

Background: Early pregnancy loss (EPL) is defined as a nonviable intrauterine pregnancy diagnosed up to the first trimester (12 weeks) of gestation. There are about 23 million miscarriages every year worldwide, and 12% happen in the first trimester. In 50% to 82% of cases, EPL is due to genetic alteration, mostly abnormal foetal karyotypes (e.g., aneuploidies). Other risk factors such as female and male age, smoking, alcohol, stress, and previous miscarriages have been also reported as underlying causes of EPL. Pregnancy problems may share similar aetiologic processes overlapping with genetic variations. Understanding pathogenetic processes and finding potential genetic variants that result in miscarriages is important to identify possible common molecular pathways that lead to this outcome. Besides that, in the last years, DNA methylation and immune system misbalancing gained attention as prominent causes of EPL. Altered DNA methylation of placenta-specific genes, and immune-related genes, can affect directly or indirectly, embryo implantation, leading to the occurrence of EPL. Additionally, the immune system plays an important role in the crosstalk between mother and foetus, establishing a fine-tuning between the inflammatory response and immune tolerance in the endometrium. When this is disrupted, it may result in implantation failure affecting pregnancy progression. **Aims:** To identify risk factors to investigate several potential methods of exposure assessment during pregnancy and to better understand the causes of spontaneous abortion and recurrent spontaneous abortions. **Methods:** A total of 108 EPL patients were compared to 107 matched healthy females. DNA from cases and controls was assessed for the following genes: *MTHFR* (rs1801131; rs1801133), *APOE* (rs7412; rs429358), *F13A1* (rs5985; rs5982; rs3024477), *F13B* (rs6003), *CRP* (rs876538; rs2808635), *CFH* (rs1061170), *ACE* (rs1799752), *ACE2* (rs2285666), *FGA* (rs6050), *FGB* (rs1800790), *MMP12* (rs2276109), *TP53* (rs1042522) by multiple techniques; DNA methylation by pyrosequencing (Pyromark ID). The plasma of cases and controls was isolated to measure the levels of selected cytokines (IL-17a, IL-6, IL-23, IL-10) by multiplex immunoassay (Luminex, MAGPIX). **Results:** cases were in general older than controls (P=0.0001), with lower DNA methylation (Mean: 82.49%, SD± 3.46) (P=0.0001) and higher systemic inflammatory profile for all the cytokines studied IL-10 (Mean: 19.67 pg/mL vs 14.63 pg/mL; P=0.038), IL-17a (Mean: 12.69 pg/mL vs 9.77 pg/mL; P=0. 025), IL-23 (Mean: 506.3 pg/mL vs 397.7 pg/mL; P=0.018), and IL-6 (Mean: 3.93 pg/mL vs 3.36 pg/mL; P=0.018). Our results showed that the wild-type variants rs5985 (G/G genotype), rs6003 (C/C genotype), and rs2285666 (G/G genotype) in the *F13A1*, *F13B* and *ACE2* genes, respectively, were associated with a crude OR ranging from 2.72 to 3.94-fold risk increase for early pregnancy risk. We also found that the polymorphic allele for the variant rs1042522 in the *TP53* gene had a significant 1.3-fold (38% vs 50%) (OR: 0.57, CI: 0.33 to 0.98) (P=0.032) decrease among our cases compared to controls, representing a protective factor against EPL. Our findings showed an increased frequency of ApoE4 compared non- ApoE4 carriers among cases and controls (19% vs 16%) representing a 3.17-fold increase in risk for early pregnancy loss (OR: 3.17, CI: 1.03 to 8.78) (P=0.029). In our secondary analysis, we stratified cases by EPL and Recurrent Pregnancy Loss (RPL), we observed a very interesting fact in which no significant variants reported before followed cases stratification showed significant differences. For instance, a 3-34-fold increased risk for RPL women with C/C genotype for the *CFH* gene variant compared to EPL. Interestingly, both major alleles (C and T, respectively) for the CRP gene rs876538 and rs2808630 variants showed a similar protective role (OR: 0.13, CI: 0.01 to 0.98) (P=0.018; P=0.015); Simultaneously, an 8-fold risk increase was associated with the minor allele for

both variants (P=0.010; P=0.010). *FGA* and *FGB* genes variants' minor alleles (C and T alleles, respectively) were less represented among RPL cases than in EPL cases (P=0.028; P=0.019). Our fibrin bio-polymerization *in vitro* model demonstrated important architectural differences across cases and controls. Regarding the scaffold fibrin gel, our results suggested that controls had finer meshwork fibres with larger pores, this can be explained by the more regulated inflammatory process among controls. **Conclusions:** This thesis provided evidence of distinct genetic, epigenetic and systemic variables that can interact with each other and be a risk factor for early pregnancy loss and recurrent pregnancy loss. The genetic diversity observed in cases group versus intracase is a major highlight of how the clinical history of patients/individuals is essential to understand the differences observed, most importantly to use them as a prognostic tool.

Keywords: DNA-Methylation; Cytokines; Genomics, Epigenetics; Miscarriages; Molecular-biomarkers; Fibrin Scaffold.

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Abbreviations

ACE	Angiotensin Converting Enzyme
ACE2	Angiotensin Converting Enzyme 2
APOE	Apolipoprotein E
APS	Adenosine 5' Phosphosulfate
APTT	Activate Partial Thromboplastin Time
ART	Artificial Reproductive Therapies
BMI	Body Mass Index
CBC	Complete Blood Count
CCD	Charge-Coupled Device
CFH	Complement Factor H
CFI	Complement Factor I
CRP	C-Reactive Protein
dATPαS	Deoxyadenosine Alfa-Thio Triphosphate
DNMTS	DNA Methyltransferases
ECM	Extracellular Matrix
EPL	Early Pregnancy Loss
ESHRE	European Society Of Human Reproduction And Embryology
FGA	Fibrinogen Glycoprotein A
FGB	Fibrinogen Glycoprotein B
FGG	Fibrinogen Glycoprotein G
FSH	Follicle-Stimulating Hormone
HCG	Human Chorionic Gonadotropin
HCY	Homocysteine
HPLC	High-Performance Liquid Chromatography
HTC	Hematocrit
LDL	Low-Density-Lipoprotein
LH	Luteinising Hormone
LINE-1	Long Interspersed Transposable Element 1
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MMPS	Matrix Metalloproteinases

MTHFR	Methylenetetrahydrofolate Reductase Enzyme
PCA	Principal Component Analysis
PPI	Pyrophosphate
PPP	Platelet-Poor Plasma
PT	Prothrombin Time
RAS	Renin- Angiotensin System
RAS	Renin Angiotensin System
ROS	Reactive Oxygen Species
RPL	Recurrent Pregnancy Loss
SA	Spontaneous Abortion
SAM	S-Adenyl Methionine
SCRS	Short Consensus Repeats
SNPS	Single Nucleotide Polymorphisms
TH	Thyroid Hormones
UNICEF	United Nations Children's Fund
WBC	White Blood Cell Count
WHO	World Health Organization

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Chapter 1

Introduction

1.0. Introduction

According to a report published in 2020 by United Nations Children's Fund (UNICEF), there is one stillbirth every 16 seconds[1]. This indicates that approximately 2 million babies are stillborn annually[1]. Further in-depth investigations show that globally 23 million miscarriages occur every year and that mostly, this represents 44 pregnancies losses each minute [2]. From this total, 80% of miscarriages happen in the first trimester, and they are also known as early pregnancy loss (EPL)[1], [3].

The underlying cause for EPL is heterogeneous [2]. Chromosomal abnormalities are the most typical cause (e.g. aneuploidies) of EPL[4]. Most of the time, it is still too early to pinpoint the precise reason for the aberration. In a foetus with a genetically normal genome, the chance of early pregnancy loss diminishes with increasing gestational age and is quite low after 15 weeks gestation[2], [4]. Other risk factors such as female and male age, smoking, alcohol, stress, Body Mass Index (BMI), endometrial defects, infections, and previous miscarriages have been also reported as underlying causes of EPL[2], [4]–[8].

Despite the current knowledge and research, it seems we are far from understanding why some women undergo EPL, especially those with recurrent pregnancy losses. Therefore, the research described in this thesis represents an ongoing effort to clarify some of the early pregnancy-loss-related mysteries.

1.1. Background

In this chapter the following subjects will be covered: physiological pregnancy and its main changes in the human body during it; definitions, occurrences, and the aetiology of spontaneous abortion (SA), including recurrent spontaneous abortion. As well as recognised risk factors for spontaneous abortion and recurrent spontaneous abortion, with a focus on the key risk variables that were significant in the research aimed with this thesis.

1.2. Development of Pregnancy

To support and accommodate the growing foetus, the pregnant woman goes through considerable morphological and physiological changes[9]. In the early stages of the pregnancy, the ovum released by a matured follicle contains 23 pairs of chromosomes, and it is named the primary oocyte[10]. The ovum undergoes meiotic division just before ovulation. Upon division, the primary oocyte produces a secondary oocyte and a first polar body. The first polar body is expelled out within 23 chromosomes; hence the secondary oocyte remains with only 23 chromosomes[10]. The advent of fertilization starts when the ovum (secondary oocyte) released in the abdominal cavity fuses to the sperm, resulting in new offspring [9], [10].

For this to happen, during sexual intercourse at ovulation time the semen ejaculated in the vagina needs to reach the fallopian tube[9]. To the ovum fertilisation happen, the sperm needs to penetrate several layers of granulosa cells (corona radiata) that surround the ovum[9], [11]. This process is mediated and facilitated by the hyaluronidase and other proteolytic enzymes found in sperm's acrosome[9]. The proteolytic enzymes diffuse through the zona pellucida structures and inactivate the other sperms entering the ovum[9], [11], [12].

After the fertilization stage, the secondary oocyte stage ovum immediately divides into a matured ovum and a second polar body[9], [11]. The polar body is eventually expelled; and this mature ovum's nucleus develops into a female pronucleus with 23 chromosomes, including 22 autosomes and one X chromosome (also known as the sex chromosome). Simultaneously the sperm head swells and becomes the male pronucleus[10]. The combination of both in a single nucleus will then originate the 23 pairs of chromosomes, forming the diploid zygote[9].

A zygote in the fallopian tube takes around 3-5 days to reach the uterine cavity and undergoes a process called cleavage, in which multiple cell division happens until reaches the morula stage, a solid lump of very small cells[9], [11], [13]. From this stage onwards visible morphological differences appear, and different cell types can be distinguished[10]. The outer cell layer differentiates into a thin epithelial layer, the trophectoderm, whilst the cells inside the embryo form a compact unit, the inner cell mass (Figure 1)[9], [13].

The blastocoel, a cavity filled with fluid, forms in the centre of the embryo, forming the blastocyst [9], [14]. The blastocyst has the appearance of a balloon, it is covered by trophectoderm cells, and the inner cell mass is squashed to one side [10], [15]. Around the time of the blastocyst stage, the embryo implants in the uterus[15]. This is also the time when the zona pellucida begins to disappear, a process known as “hatching” and begins to interact with maternal tissues. The trophectoderm will differentiate into trophoblasts[9], [11], [16]. The trophoblast releases proteolytic enzymes onto the endometrium's surface[9]. These enzymes break down endometrial cells, which can then stick and invade the uterus, allowing the embryo to nestle in the uterine wall[11].

The interaction of the blastocysts with the endometrium during implantation can fail. If this is the case, the blastocyst and endometrium will both shed during the menstrual cycle[17].

However, if successful, the continuous supply of progesterone from the corpus luteum makes the endometrial cells increase in thickness even more and store more nutrients[17]. At this stage, the endometrial cells are called decidual and the area of implantation of the decidua[17], [18]. As trophoblastic cells invade the decidua, they bury themselves deeply among the uterine stromal cells that form the structural components of the uterus[9], [11].

The trophoblastic cells differentiate into cords attached to the decidual portion of the endometrium. These cords develop blood capillaries and around the 16th day after fertilization, the heart of the embryo starts pumping blood into the trophoblastic cords[11], [13]. Trophoblastic cells form vascular projections into which foetal capillaries grow[9]. At the same time, blood sinusoids develop around the trophoblastic cords. These sinusoids receive blood from the mother, further these projections become the placental villi[9], [10], [19]. Thus, the placenta has a foetal and a maternal part.

The two umbilical arteries that bring foetal blood to the placental villi through the capillaries are located in the foetal part of the placenta[9], [10], [13], [20]. The umbilical vein is where the blood returns to the foetus[9], [11]. The blood enters the sinusoids around the villi in the maternal placenta part by the uterine artery, and returns by the uterine vein[9]–[11]. Usually foetal and maternal blood do not mix, but because of the link between the two circulatory systems, nutrients and oxygen can be transferred from the mother to the foetus or embryo, and carbon dioxide and urea from the foetus to the mother[9], [10], [13].

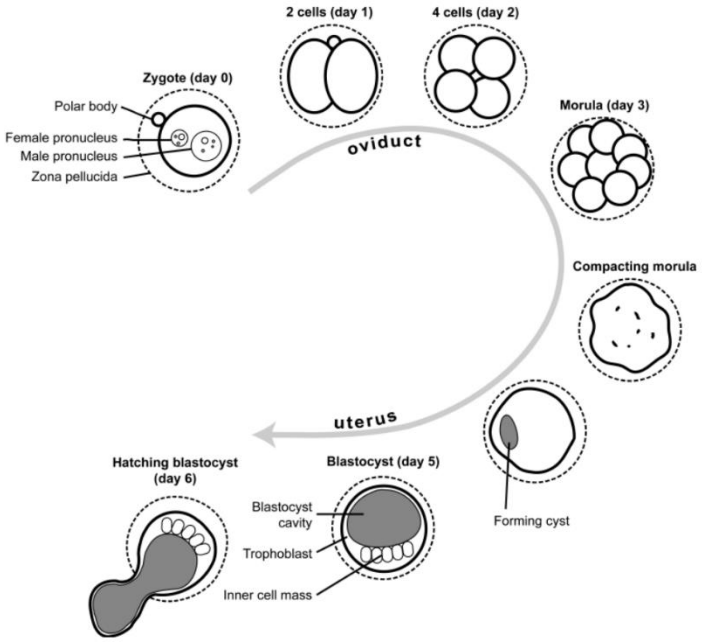


Figure 1. Fertilization through the implantation in the uterus[11]**.**

1.3. Physiological Changes During the Pregnancy

During pregnancy, the body undergoes significant changes to adapt the mother's body to the organic requirements of the mother-foetal and birthing complex[10], [20]. These changes first result from the effects of hormones from the placenta and corpus luteum, as well as from the second trimester and uterine growth[20]. The cardiovascular, pulmonary, and gastrointestinal systems of the mother experience the most significant physiological changes[20].

1.3.1. Reproductive System

The uterus grows to accommodate the foetus through gestation. It reaches 5 to 7 litres at the end of the pregnancy, half of this volume is occupied by the foetus and the other half is a combination of the placenta and amniotic fluid[20]. The shape goes from a pyriform (non-pregnant) to a globular (pregnant), returning to its original shape gradually post-partition.

The weight increases about 40 times, from 30 to 50g to 1000 to 1200g. After 3 months of pregnancy, the decidua has 3 different layers, the decidua basalis (maternal part), capsularis (surrounds the foetus) and parietalis (separates the embryo from the uterine lumen)[20]. Significant changes are seen in the vagina which acquires a purple colour due to the increased blood flow. The cervix meanwhile becomes soft and recovered by a mucus plug[20].

1.3.2. Cardiovascular System

The pregnancy increases cardiac output by 30% until the first trimester, decreasing gradually to normal by the end of the pregnancy[21], [22]. Mostly the increased output is delivered to the uterus, kidney and skin to provide nourishment to the foetus, eliminate maternal and foetal waste materials, and help in the mother's body temperature regulation[22]. Blood pressure changes very little during pregnancy. Systolic pressure usually is decreased until the 20th week of pregnancy and after rises again reaching pre-pregnancy levels[21]. Diastolic pressure is significantly reduced in early pregnancy but returns to pre-pregnancy in the last months[22].

Physiological changes are observed during the pregnancy to support maternal-foetal development. The plasma volume increases by 40-50%, due to wide pulse pressure and reduced mean arterial pressure, which leads to the retention of sodium and water through renin- the angiotensin system (RAS)[23]. Resulting in decreased haemoglobin concentration, haematocrit and red blood cell count, but with no alteration in the mean corpuscular volume (MCV) or mean corpuscular haemoglobin concentration (MCHC)[20], [24]. This haemodilution leads to the so-called "anaemia physiologic of pregnancy" or "pseudo-anaemia of pregnancy"[24].

Usually, the platelet count can drop progressively, but it resolves in the postpartum. Changes in the coagulation system during pregnancy produce a physiological hypercoagulable state. Fibrinogen levels rise significantly by up to 50% and fibrinolytic activity is decreased[22]–[24]. The concentrations of certain clotting factors, particularly VIII, IX and X, are increased, meanwhile factors II, V, XI and XIII maintain normal levels[24]. Endogenous anticoagulants such as protein S and antithrombin have lower concentrations[22], [24]. Thus, pregnancy affects the coagulation system's balance in favour of clotting, making pregnant and postpartum women more susceptible to venous thrombosis, from the first trimester (10 times risk) and continuing for at least 12 weeks after delivery (25 times risk)[22].

1.3.3. Respiratory System

There is an increased demand for oxygen during pregnancy[20]. In the first trimester of pregnancy, there is a significant increase of 40 to 50% in minute ventilation, due to higher tidal volume with an unchanged respiratory rate[25]. With foetus growth and uterus distension in late pregnancy, the diaphragm elevates alternating thoracic configuration[24]. As a result of end-expiratory gastric pressure the diaphragm is displaced upwards, generating a negative pleural pressure and the chest height becomes shorter with an increase of other thoracic dimensions to copy with total lung capacity, however functional activity remains unaltered[24], [25].

1.3.4. Gastrointestinal System

Decreased cardiac function, accompanied by the increase in the secretion of gastric juice observed in pregnancy, favours the occurrence of gastroesophageal reflux that leads to heartburn and even esophagitis[26]. Progesterone leads to the relaxation of the lower

oesophageal sphincter; As a result, the motility of the gastrointestinal system is affected and constipation, indigestion, and nausea [24], [27]. Nausea specifically happens in 50 to 80% of pregnancies, starting in the 4th week and ceasing by the end of the 20th week[28]. Most nausea and vomiting are linked to hormonal changes, and it is linked to the beta human chorionic gonadotropin (β-HCG), oestrogen, progesterone, and thyroid-stimulating hormones[26]. Combined with this mechanical changes also happen, the stomach is displaced upwards due to the higher volume in the uterus, generating increased intra-gastric pressure and this as well predisposes to nausea and vomiting[24].

1.3.5. Renal System

Renal blood flow and glomerular filtration rate rise rapidly during the first trimester of gestation, on the fourth month, values of up to 50% of those observed in non-pregnant women, however, no change is observed in histology or the number of nephrons[29]. In the last months, these values slowly decrease towards normal values[29], [30]. The creatinine clearance is usually increased, and therefore, the expected limit of urea and creatinine is lower in pregnant women[30]. In the last months, there is an expansion of renal calyces, pelvis and ureters, due to the action of progesterone, however, all the changes in the renal system return to the pre-pregnancy state by 4–6 weeks postpartum[22].

1.3.6. Endocrine and Metabolic System

During pregnancy, women experience great changes in their endocrine system to support the foetus's development. Thyroid hormones (TH) are crucial for the foetus' brain development and foetal growth in addition to many other aspects of pregnancy[31]. Thyroid stimulation starts as early as the first trimester by the β-HCG hormone. In the first stages of the pregnancy, the TH are mainly supplied by the mother[20], [24]. Preeclampsia, early labour,

foetal mortality, low birth weight, and developmental disabilities in the offspring have all been linked to evident maternal thyroid insufficiency during the first half of pregnancy[31].

The adrenal gland increases 3 times the secretion of aldosterone in the first trimester, and 10 times more in the third trimester[32]. Consequently, the renin-angiotensin-aldosterone system is overstimulated, and increased levels of angiotensin II are observed as well as renin activity compared to non-pregnant women[24], [32].

The pituitary gland during pregnancy increases by 50% mainly due to the proliferation of prolactin-producing cells in the anterior lobe[20], [33]. Continuous secretion of oestrogen and progesterone, from the corpus luteum, produces negative feedback in the levels of follicle-stimulating hormone (FSH) and luteinising hormone (LH) which are undetectable during pregnancy[33].

Alongside endocrine changes, several changes happen in the metabolism to maintain a healthy balance between the foetus and the mother. For instance, glucose initially in the first trimester of pregnancy drops and keeps constant levels until the last trimester which reduces again. This happens due to haemodilution and increased usage by the foetal-placental unit[34], [35]. The secretion of placental lactogen, growth hormone, progesterone, cortisol, and prolactin increases during the second reaching maximum in the third trimester, leading to a decrease in insulin sensitivity, therefore, maternal insulin resistance[24], [34].

Besides that, calcium serum concentrations are decreased because of haemodilution, which affects serum albumin binding fraction to calcium[24], [36]. Increased intestinal absorption is observed during the first months doubling at the end of the first trimester. This happens so the mother skeleton can store calcium to transfer to the foetus[36]. Foetal skeletal

development starts in mid-pregnancy and onward with maximum calcium accretion in the third trimester[36].

1.4. Definitions and Terminology

Gestation includes chemical and clinical pregnancy. The initial stage of pregnancy is named chemical pregnancy or unrecognised pregnancy, which occurs a few days after the embryo's implantation[37], [38]. Subsequently, the placental trophoblast cells of the conceptus increase the production of the circulating β-HCG [37], [39]. Clinical pregnancy or recognised pregnancy is characterised by the detection of an embryonic unit by ultrasound a few days to weeks after the embryo's implantation[37]. The termination of a clinical pregnancy without a medical reason is known as a miscarriage or a spontaneous abortion[37].

In 1970 the World Health Organization (WHO), described abortion as the termination of pregnancy 28 weeks before the foetus reached a gestational age compatible with extrauterine viability[40]. Gestational age (pregnancy duration measured from the last menstrual period), foetus weight, and foetus length can all be used to indicate viability[40]. Nowadays, spontaneous abortion is typically defined as the unintentional termination of an intrauterine pregnancy before foetal viability, with a maximum gestational age of 24 weeks[37], [41].

More specifically, when a spontaneous abortion happens between 0 to less than 14 weeks, it is known as early pregnancy loss[42], [43]. Meanwhile, the losses which happen between 14 weeks to 21 weeks, in the second trimester, are known as late pregnancy loss[44].

A total of 1-2% of the cases of EPL are from women with two or more cases of abortions, identified as Recurrent Pregnancy Loss (RPL)[41], [45]. There is no consensus if the pregnancy losses should be consecutive or not. For instance, while in the UK RPL is defined

as three or more consecutive pregnancy losses, in the USA defines recurrent miscarriage is as two or more failed clinical pregnancies[46]–[48]. The European Society of Human Reproduction and Embryology (ESHRE) describe cases where all pregnancy losses have been confirmed as intrauterine miscarriages without the stipulation that these losses need to be consecutive[49]. Therefore, even if there had been a livebirth between miscarriages, this definition would still be applicable. Due to these variations in the definition of RPL, the prevalence and the outcome of any subsequent pregnancies are significantly impacted[50].

1.5. Epidemiology and Incidence of Pregnancy Loss

In the world, approximately 242 million women find out they are pregnant each year (https://news.un.org/en/story/2022/03/1115062)[51], [52]. Quantifying the full burden of miscarriage is challenging because rates of pregnancy loss are high around the time that pregnancies are clinically recognised. Due to pregnancies ending in the first few weeks of gestation, an unknown number of women become pregnant without realising it[52]. The pregnancy's outcome can range from a live birth to a stillbirth or abortion (either spontaneous or induced)[46], [52].

According to the Italian Health Ministry (Ministero Della Salute) between 2019 and 2020 Italy registered an average rate of 140 spontaneous abortion cases per 1000 habitants aged between 15 to 49 years old[53], [54]. Most cases happened among women 33-35 years old and the overall region with the largest number of cases in 2019 and 2020 was the South of Italy with a rate of 195 and 163/1000 inhabitants (Table 1) [54].

Table 1. Number of spontaneous abortions in Italy by region between 2019 and 2020

Data type	2019			2020		
	Discharges for Miscarriage	Total Miscarriage Rate	Mean Age	Discharges for Miscarriage	Total Miscarriage Rate	Mean Age
Regions		1000 hab			1000 hab	
Italy	48932	150	34.55	41493	130	34.65
North	18557	127	34.73	16358	111	34.79
Northwest	10762	127	34.83	9528	115	34.78
Northeast	7795	130	34.59	6830	112	34.81
Centre	9121	146	35.13	7463	126	35.34
South	12298	177	34.25	9988	150	34.35
Islands	6572	167	34.26	5750	149	34.22

(http://dati.istat.it/Index.aspx)

Figure 2 shows the rate of spontaneous abortion per county in the years 2019 and 2020 in the Italian territory. In general, from 2019 (Mean: 137.7, SD ± 45.33) to 2020 (Mean: 113.8, SD± 46.16) a decrease in the number of spontaneous abortions was observed. The county of Marche had a reduction of 100% in 2020 compared to 2019. The counties with the lowest rate differences observed between the years were Valle d'Aosta (difference rate between 2019 and 2020: 1.06) and Umbria (difference rate between 2019 and 2020: 1.01). No data was registered to Veneto, probably due to COVID-19 which severely affected the region from 2019 to 2021 [54] (http://dati.istat.it/Index.aspx).

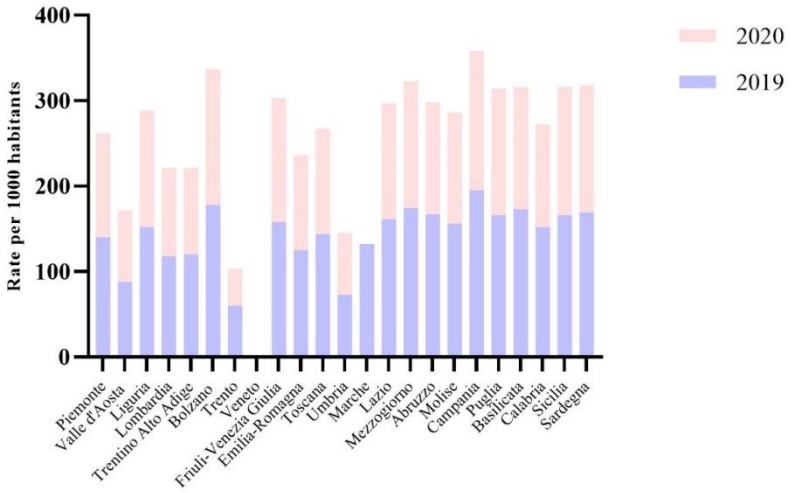


Figure 2. Number per 1000 habitants of spontaneous abortion between 2019 and 2020.

1.6. Risk Factors

Pregnancy loss has been associated with different risk factors. Maternal age and previous cases of spontaneous abortion are the most reported risk factors. Figure 3 summarizes some of the risk factors which were addressed in spontaneous abortion[55]. Further, this thesis will focus on epigenetics changes, more specifically methylation, genetic information for key genes during pregnancy, such as Apolipoprotein E, Methylenetetrahydrofolate Reductase, Fibrinogen beta chain genes; it will also be discussed the role of cytokines and its correlation with spontaneous abortion more thoroughly.

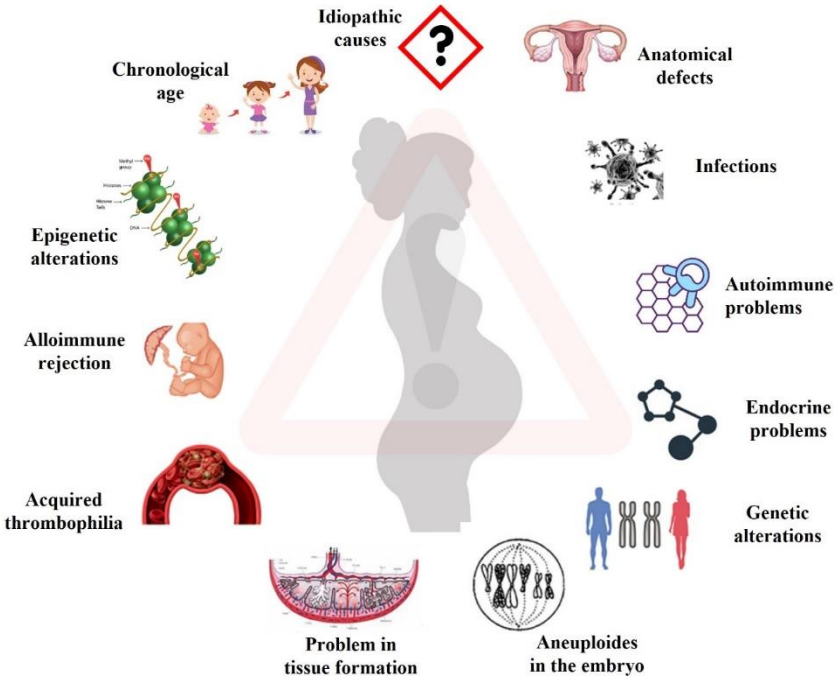


Figure 3. Risk factors associated with pregnancy loss and recurrent pregnancy. Genetic factors (chromosomal abnormalities, mutant genes), environmental toxins (drugs, lead, ionising radiation), infectious agents (viruses, bacteria), uterine abnormalities (malformations, fibroids, cervical insufficiency, post-operative changes), as well as other maternal or paternal factors, can all contribute to spontaneous abortion (chronic disease). Chromosome abnormalities are more likely to be the cause of early spontaneous abortions, but cervical incompetence is more often the cause of losses in the second trimester[55].

1.6.1. Maternal Age

The maternal age rose significantly. Birth rates increased for women in their early 30s, late 30s, and early 40s from 2007 to 2016 by 2%, 11%, and 19%, respectively[56]. This is also supported by the fact that the mean average age for childbearing increased from 27.5 between 1990-1995 to 30.6 in 2020 in the European territory[56]. Physiologically, fertility declines for women with advanced age, due to a normal age-related decrease in the number of eggs in the ovaries[56], [57].

Besides that, women aged 35 years old or more, can accumulate a series of risk factors which can contribute to spontaneous abortion and ectopic pregnancies, such as multiple partners, pelvic infections, prolonged smoking, tubal pathology, decreased tubal function, and delay of oocyte transportation[57], [58]. Quenby et al (2021) study showed that miscarriages risk increases to about 20% in women with 35 years old, increasing drastically to 65% in women aged 45 years old[46]. They also showed that women with no previous abortion history have the lowest miscarriage risk and women with previous abortion history have about a 10% increase in each additional miscarriage (Figure 4)[46].

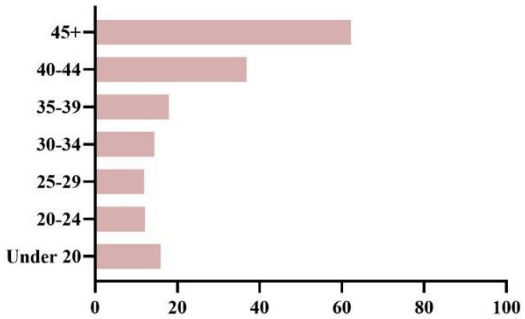


Figure 4. Risk of miscarriages by age group. Age is represented in years old and risk by percentual values up to 100%[46].

1.6.2. Genes

Most frequently, pregnancy problems have an inherited component. Pregnancy problems may share similar aetiologic processes overlapping with genetic variations[59]. Understanding pathogenetic processes and finding potential genetic variants that result in miscarriages is important to identify possible common molecular pathways that lead to this outcome[59].

A- *ACE* and *ACE-2* genes

The angiotensin-I-converting enzyme (ACE) is a peptidyldipeptidase A and contains 1277 amino acids[60]. ACE has the function of converting angiotensin-I into angiotensin-II in the RAS and degrading kinins in the kallikrein-kinin system[60], [61]. The gene has 25 introns and 26 exons that code the angiotensin-converting enzyme (*ACE*)[61]. It is found in the long arm of chromosome 17 (locus 17q23.3), is 21 kb long, and codes for a gene product with 1306 amino acids[60]. More than 160 SNPs are addressed to the *ACE* gene, only 34 are in coding regions, and 18 of them are missense mutations[61]. Among the many gene variations, insertion/deletion (I/D, rs1799752) of 287 bp in the *Alu*-elements in the intron 16 affect ACE expression and has received the most attention[61], [62].

In individuals with the I/I genotype, decreased levels of ACE in the plasma are observed compared to those with D/D and I/D genotypes (rs1799752) [62]. This happens because the *ACE* D/D genotype is associated with the enhanced conversion of angiotensin-I to angiotensin-II[63]. In the microenvironment between mother and foetus RAS is associated with proliferation, placental angiogenesis, trophoblast invasion and cell growth[63], [64]. Pregnant women's D/D genotype is associated with higher uterine artery resistance compared to the I/I genotype[65], [66].

In a study by Kurzawinska et al (2016) 180 women with a history of two or more miscarriages in the first trimester were analysed for ACE I/D polymorphism (rs1799752)[63]. The D/D genotype was more frequent among cases (32%) than controls (23%)[63]. In a meta-analysis comprised of 11 research with 3357 individuals included[67]. Both the D/D and I/D genotypes were linked to a higher risk of recurrent miscarriage when compared to the ACE I/I genotype (OR D/D against I/I = 1.81, 95% CI 1.23-2.66, P = 0.003; OR I/D versus I/I = 1.50, 95% CI 1.25-1.80, P=0.001)[67].

The renin-angiotensin system (RAS) is negatively regulated by the carboxypeptidase ACE2, which can cause vasodilation by cleaving the vasoconstrictor angiotensin II to Ang (1-7)[68], [69]. Additionally, studies show that ACE2 functions as a protective element of the RAS to counteract the excessive activity of angiotensin-II, which is accountable for vasoconstriction, the production of reactive oxygen species (ROS), and inflammation, and thus plays a protective role in end-organ damage[68], [70]–[73]. The *ACE2* gene is located on chromosome Xp22 and consists of 18 exons, has a complete length of 41.04 kb, and codes for a gene product with 805 amino acids [68], [69], [74]. A total of 428 SNPs are attributed to the *ACE2* gene, 38 are in coding regions, and out of these 31 are missense polymorphisms[69], [74].

The *ACE2* rs2285666 in the intron 3 (c.439+4) (G>A), and is most frequently studied in association studies regarding cardiovascular diseases[69], [70], [74]. The polymorphism is located in a splice site and causes the impairment of mRNA translation efficiency[74]. The A/A genotype is responsible for a lower expression of the ACE2 receptor, compared to G/G genotype[69], [74].

Women with normal pregnancies have an increased level of ACE2 level through all gestation, compared to non-pregnant, and it is believed to play a role in the maintenance of blood pressure[68], [75]. *ACE* and *ACE2* gene polymorphisms may affect the balance in the RAS axis during pregnancy. This may happen because the polymorphisms can alter the signalling pathways by increasing/decreasing the production of angiotensin-II and Ang-(1-7), and this may result in preeclampsia, foetal growth restriction, preterm and we hypothesized miscarriages (Figure 5)[61], [65], [75], [76].

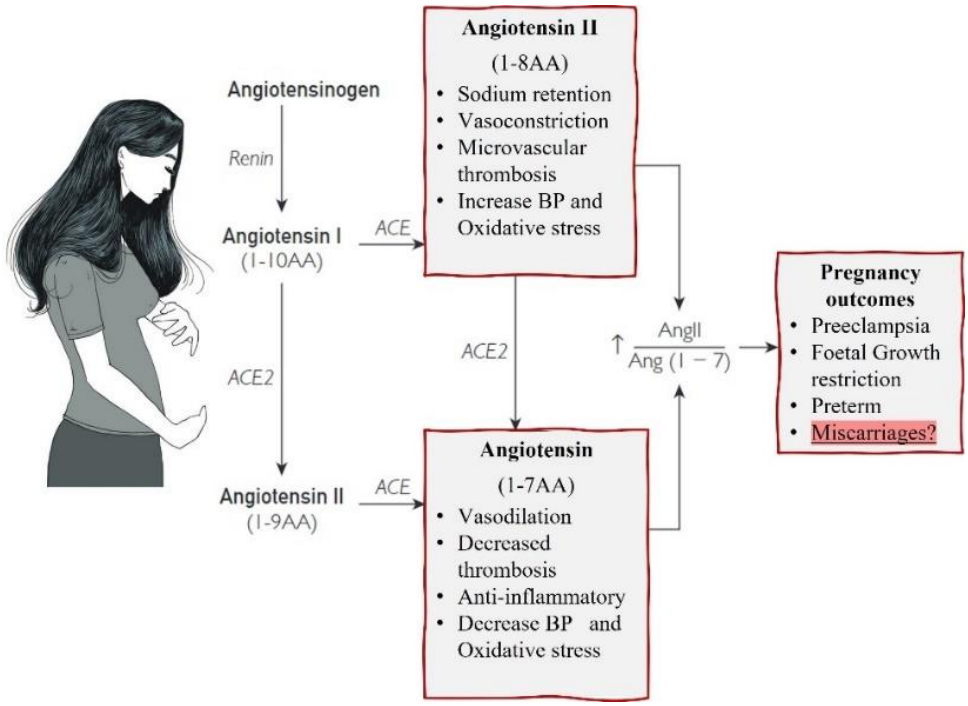


Figure 5. A representative image of the RAS axis system affecting pregnancy. ACE: angiotensin-converting enzyme; ACE-2: angiotensin-converting enzyme 2; BP: blood pressure[65], [75], [76]

B- APOE haplotype

The *APOE* gene is located in the long arm of chromosome 19 (locus 19q13.32), and consists of 4 exons and 3 introns with a total of 3597 bases pair, encoding a 34kDa glycoprotein with 299 amino acids[77], [78]. Apolipoprotein E (ApoE) glycoprotein has two domains, each of them with different structures and functions[79]. The receptor-binding region (136–150

amino acid) and the heparan sulphate proteoglycans binding region are both located in the N-terminal domain, which also includes a four antiparallel helix bundle[78], [79]. While, the C-terminal domain, is formed by amphipathic-helices in which are located the high-affinity lipid-binding region (244–272 amino acids), and the ApoE self-association region (267–299 amino acid)[78], [79].

ApoE has multiple functions throughout the human body, some of them include mediation in the metabolism of amyloid β, cholesterol haemostasis, antioxidant and anti-inflammatory effects, modulation of cell growth, differentiation and death, regulation of neuronal development, gonadal functions, lipid transport between and within tissues, participation in signal transduction pathways, and regulation of inflammatory response[78], [79].

The *APOE* gene is very polymorphic. A single polymorphic allele at amino acid 112 (rs429358) (T>C) and 158 (rs7412) (C>T) originate the Apo isoforms E2 (cys112, cys158), E3 (cys112, arg158), and E4 (Arg112, Arg158), which result in six different variants (E2/E2, E2/E3, E2/E4, E3/E3, E3/E4 and E4/E4)[77], [78]. In general, ApoE3 is the most common haplotype occurring in 77% of the general population, followed by ApoE4 (about 15%) and at least ApoE2 (around 8%) with the lowest frequency[78], [79].

The different isoforms and genotypes are associated with different physiological results. Higher plasma low-density lipoprotein (LDL) concentrations are seen in individuals with the E4 allele compared to individuals with the E2 and E3 alleles[80]. The E4 allele during the first trimester of pregnancy may contribute to the collection of plasma lipids in the intima of blood arteries, aiding the formation of thrombus, and promoting inflammatory endothelium cytokines[80]–[83]. As a consequence, the decreased placental blood flow and oxygen delivery caused by the development of thrombus in placenta vessels will result in trophoblast death and miscarriage. It is important to remember that cholesterol, as a precursor to

steroidogenesis, is essential for the survival of pregnancy[80], [84]. The presence of the E4 allele is associated with lower Apo affinity for LDL receptors, resulting in decreased absorption of bloodstream cholesterol, causing abnormalities in the production of steroid hormones and spontaneous abortion[80]–[83].

Li et al (2013) in a meta-analysis study comprising 975 women with recurrent pregnancy loss and 1533 normal controls estimated that cases with an E4 allele had about two times more risk of suffering RPL (OR: 1.919, CI: 1.016 to 3.625) compared to controls[77]. Another study in RPL also demonstrated that the E4 allele was relatively more frequent among cases (21.7%) than controls (5.4%)[85].

C- *CFH* gene

The gene that encodes human Complement Factor H (CFH) is found on chromosome 1 (locus 1q31.3), comprises 94kb of genomic DNA, and 1231 amino acids consisting of 20 short consensus repeats (SCRs), each with about 60 residues[86], [87]. SCR1-4 in the N-terminus mediate the cofactor/decomposition of C3 convertase and the degradation of complement component 3b (C3b), meanwhile, SCR19-20, in the C-terminus are crucial for cell surface mediation of CFH[87]. CFH is a fluid-phase protein that acts as a regulator of the complement system and can limit complement system-mediated activation by targeting C3 convertase in the alternative pathway[86]. It can also limit pathway amplification by acting as a cofactor for complement factor I (CFI) to inactive C3b to iC3b, through the inactivation of deposited C3b on self-cells after complement activation via any of the three initiation pathways, lectin, classical or alternative[86], [88].

A total of 550 SNPs were addressed to the *CFH* gene, mostly with an amino acid substitution[86]. The most investigate SNP of *CFH* is rs1061170, a missense variant located

in the coding region of the gene, that is found in the SCR7, which regulates the binding of CFH to C-reactive protein (CRP) and glycosaminoglycans[87], [89]. The polymorphism (T>C) results in amino acids change to p.Ile248Met, p.Glu936Asp, and p.His402Tyr, which may cause changes in amino acid characteristics and effect functions of those proteins [87], [90].

D- *CRP* gene

The human CRP gene is located on the long arm of chromosome 1 at locus 1q23.2 and has 2 exons, 1 intron, and a long 3' untranslated region[91]. Fundamentally, CRP gene expression transcription is stimulated by interleukin (IL)-6 as the primary inducer and IL-1 acts synergistically to increase the efficacy[91], [92]. CRP is a homopentameric acute-phase inflammatory protein, formed by 5 noncovalently bound subunits of 206 amino acids [92]. CRP increases drastically, from 1 µg/mL to 500µg/mL in space between 24 to 72 hours, during the inflammatory process, tissue injury, and progressive cancer[91].

Some patient traits and environmental variables such as smoking, infection, age, gender, cholesterol levels, and blood pressure, genetic polymorphisms might affect the baseline CRP level[91], [93]. Obesity has a significant role in determining CRP levels in humans, and high CRP levels indicate the onset of metabolic syndrome and type 2 diabetes, and insulin resistance[94]–[96]. Meanwhile, polymorphisms such as rs876538 (C>T) and rs2808635 (T>G) have been associated to influence CRP plasma levels in cardiovascular diseases, Crohn's disease, and age-related macular degeneration[97]–[101]. The polymorphism rs876538 is in the 3' flanking region (+7598). It has been demonstrated that carriers of the minor alleles in both polymorphisms (rs876538 and 2808635) (T and G alleles, respectively) had a better response to treatment for age-related macular degeneration than non-carriers[99], [102].

A rise in maternal CRP during pregnancy is expected after six-week pregnancy, and more evidently in the second and third trimesters, reaching a peak during labour[103], [104]. CRP level alteration has been linked to several pregnancy issues, such as early miscarriage, preterm labour, preeclampsia, foetal growth restriction, premature membrane rupture, and chorioamnionitis[103], [105]–[107].

E- *F13A1* and *F13B* genes

Factor XIII (FXIII) is a zymogen of the transglutaminase family essential to the coagulation cascade. In the plasma, FXIII circulates as a heterotetramer (FXIII-A_2B_2), the two subunits A have a catalytic function, and two B subunits inhibit the A subunit activation[108]. The activation peptide from the A subunit is removed by thrombin during the coagulation cascade, the B subunit dissociates in a Ca^{2+} dependent manner, and FXIII-A turns into an active transglutaminase that crosslinks fibrin strands, which explains its function in stabilising fibrin clots in the last stage of blood coagulation[108], [109].

The levels of the coagulation factors V, VII, VIII, IX, X, and XII, plasma fibrinogen, and von Willebrand factor rise dramatically during pregnancy[108], [110]. However, fibrin-stabilizing FXIII generally increases in the first weeks and then decreases throughout pregnancy, reaching 50% of the normal non-pregnant value at term[110]. During the pregnancy FXIII has an essential role in the early stages, FXIII-A accumulates in the placenta at the decidua, contributing to the formation of the cytotrophoblastic shell and stabilization of the fibrinoid layer[110]–[112]. Deficiency of FXIII-A is associated with failure in the formation of the cytotrophoblastic shell and layers, leading to detachment of the placenta, lower decidua angiogenesis, and subsequent spontaneous miscarriages (Figure 6) [110]–[115].

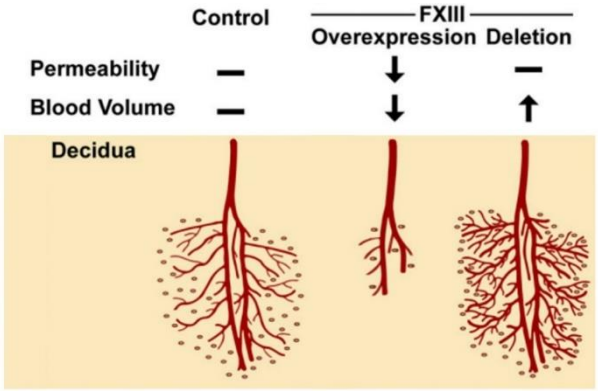


Figure 6. Trophoblast cells expressing FXIII alteration and decidua vascularization function effects. Embryo TC infected by lentivirus overexpressing FXIII display a decrease in decidual blood volume and a significant decrease in decidual blood vessel permeability compared to depletion of FXIII[115].

Mature FXIII-A has 731 amino acids, it is encoded in the *F13A1* gene covers 160 kb of chromosome 6 (locus 6p24–25) and comprises 15 exons and 14 introns[116]. Around 153 polymorphisms have been addressed to impact FXIII-A functions such as rs5985, rs5982, and rs3024477[112], [113]. Meanwhile, the FXIII-B subunit is encoded by the *F13B* gene located on chromosome 1 (1q31–32.1), consists of 12 exons, and 11 introns and covers a genomic region of 28 kb, a total of 16 mutations have been addressed to the gene, one of the most investigated is the rs6003 (Table 2) [112], [116], [117].

Table 2. Polymorphisms in *F13A1* and *F13B* gene summary

Gene	Amino Acid	cDNA	dbSNP	Exon	Domain	Effect	Associated Disease	Ref.
F13A1	V34L	c.103 G>T	rs5985	2	Activation peptide	Increases FXIII activation Alter fibrin cloth structure	MI, Ischemic stroke, ICH, DVT	[112], [116]–[121]
	T204P	c.614 A>T	rs3024477	5	Catalytic domain	Decrease FXIII plasma level and activity	Ischemic stroke	[112], [116], [122]
	P564L	c.1694 C>T	rs5982	12	Beta-barrel-1	Lower FXIII plasma levels but increased activity	Haemorrhagic and Ischemic stroke	[112], [116], [123]
F13B	H95R	c.344 G>A	rs6003	3	Second sushi	Increase FXIII subunit dissociation	Ischemic stroke, VTE	[112], [124]

MI: Myocardial Infarct; ICH: Intracranial haemorrhage; DVT: Deep Vein Thrombosis; VTE: Venous Thromboembolism

Jeddi-Tehrani et al (2010) reported that rs3024477 and rs5982 polymorphisms were significantly correlated with recurrent pregnancy loss in Iranian women. For both polymorphisms, rs3024477 and rs5985, it was observed a significant frequency of the minor allele (T allele for both) among cases, 84% and 68%, respectively, compared to controls, 46% and 31%, respectively; Representing 4-fold risk for recurrent pregnancy loss[125].

F- *FGA* and *FGB* genes

The fibrinogen is a glycoprotein of 340-kDa molecular mass, and it is composed of Aα, Bβ, and γ polypeptide chains linked by 29 disulfide bridges[126]. The Aα, Bβ, and γ chains are encoded by *FGA*, *FGB* and *FGG* genes, respectively, on chromosome 4 (locus 4q28-31) by grouped clusters of 50kb of extension[127], [128]. The *FGA* gene has a 7.6-kb size and consists of 6 exons, the *FGB* gene has an 8-kb size, and presents 8 exons, and *FGG* includes an 8.5-kb region and presents 10 exons [128].

Fibrinogen has a fundamental role in blood clotting since it is the precursor of fibrin [128]. The thrombin-mediated proteolytic cleavage and removal of N-terminal fibrinopeptide's from the Aα and Bβ chains is a necessary step in the synthesis of fibrin[127], [128]. This permits the half-staggered association of the C-terminal portion to the D-region of fibrin monomers into protofibrils[127]. Alongside, their fundamental role in fibrinolysis, fibrinogen and fibrin are essential in clot retraction, infection, wound healing, angiogenesis, cellular and matrix interaction, and inflammation[129].

Physiological levels of fibrinogen are 200-400 mg/dl, however, during pregnancy, the hypercoagulable state reaches up to 600 mg/dl[130]. Previous studies demonstrated that unbalanced levels (higher or lower) of fibrinogen can lead to miscarriages and pregnancy complications [126], [130]–[133]. Genetic variants such as rs6050 and rs1800790 can affect encoded proteins and eventually lead to undesired thrombosis within the placental vessels, detachment of the placenta and then abortion[132].

The polymorphism rs6050 in exon 5 occurs when the substitution of adenine to a guanine (A>G), originates an alanine instead of a threonine (p.Thr312Ala) in the *FGA* gene[132], [134], [135]. This region comprises FXIII-dependent cross-linking processes and may,

therefore, influence clot structure or rigidity[134]. The rs1800790 is located in the promoter region (-455) of the *FGB* gene, and substitution of guanine to an adenine (G>A) in the 5' flanking region, it is responsible for 7-10% higher plasma fibrinogen levels, potentially leading to enhanced platelet dependent coagulation processes[132], [136], [137].

G- *MMP12* gene

The matrix metallopeptidase (MMPs) are zinc-dependent endopeptidases that can cleave one or more components of the extracellular matrix (ECM) as well as non-matrix proteins, therefore with an essential role in remodelling[138]–[140]. They are a large family of 23 proteases with similar structural and functional characteristics, as well as shared end products[139].

During the pregnancy the success of trophoblast implantation and placentation depends on the function of proteases to mediate the degradation and remodelling of the ECM components such as various collagens, laminin, fibronectin, fibrillin and vitronectin, making MMP crucial for trophoblast invasion in the decidua; In fact, MMPs are secreted by both endometrial and trophoblast cells, in special MMP-12[141]–[143].

MMP-12 is commonly known as macrophage metalloelastase, it can break down a variety of ECM components such as elastin, including collagen type IV, fibronectin, laminin, vitronectin, proteoglycans, and plasminogen[139]. The MMP-12 is a 54-kDa elastolytic protease encoded on the *MMP-12* gene, it has 10 exons and 9 introns, and is located on chromosome 11q22.2-22.317, *MMP-12*'s expression level is mostly regulated at the transcriptional level[139], [144].

A common polymorphism (rs2276109) in the promoter region (-82 A>G) of the *MMP-12* gene can influence gene expression, the A allele enhances the binding of the transcription factor activator protein 1, increasing promoter activity[143]. It was previously associated with ischemic stroke, coronary artery disease, rheumatoid and inflammatory arthritis, skin diseases, and abdominal aortic aneurysms, [138], [140], [144]–[147].

A study demonstrated that trophoblasts had higher levels of expression of MMP-12 compared to other 12 different MMPs[143]. More precisely trophoblasts in the first trimester compared to trophoblasts isolated from term pregnancy had higher levels of MMP-12, demonstrating its relevant role in mediating invasion in the early stages of pregnancy[143].

H- *MTHFR* gene

The methylenetetrahydrofolate reductase enzyme (MTHFR) is encoded by the *MTHFR* gene located on chromosome 1 at locus 1p36. 3 and comprises 12 exons[148]. MTHFR is an enzyme that catalyzes the reduction of 5,10- methylenetetrahydrofolate to the active folate isoform, 5-methyltetrahydrofolate, the carbon donor for the remethylation of homocysteine (Hcy) to methionine, DNA, participates of S-adenosylmethionine synthases, proteins, neurotransmitters and phospholipids [148]–[150].

A total of 35 different mutations have been identified in the *MTHFR* gene[151]. The most commonly studied are *MTHFR* 677 (C>T) (rs1801133) and *MTHFR* 1298 (A>C) (rs1801131)[148]. In the population worldwide about 60% will have at least one of the variants (677T or 1298C) and 10% both of them[148]. The polymorphism C677T, results in a replacement of the exon 4 of alanine to valine (A223V), leading to high thermolability, and reduced enzyme activity that is particularly evident in folate-deficient states[152], [153].

Individuals with TT and CT genotypes have an enzymatic reduction of 70% and 35% compared to CC genotypes [152], [154].

Reduced MTHFR enzyme activity increases homocysteine concentrations associated with inflammation of the vascular endothelium [149]. The decrease of the active isoform 5-methyltetrahydrofolate increased uracil misincorporation into DNA, disturbance of nucleic acid integrity, slower DNA replication, and a higher risk of chromosome instability[151], [155]. It can also affect negatively impact pregnancy on several processes crucial to oocyte development, acquiring endometrial receptivity, embryo implantation, and maintaining a pregnancy (Figure 7)[151], [156]–[159].

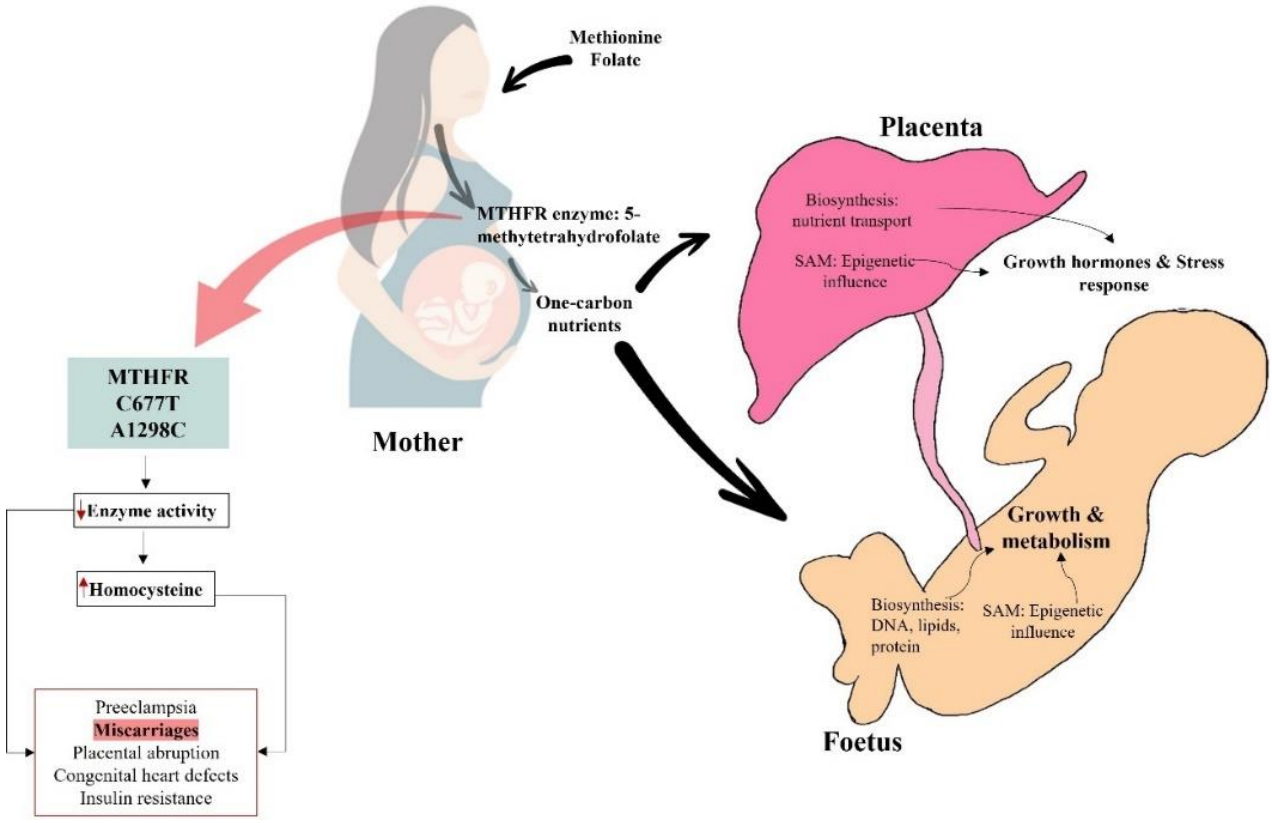


Figure 7. The role of one-carbon nutrients in foetal development. Maternal one-carbon nutrients intaken are essential to the placenta manutention and foetus development. Impacting the production of nucleic acids, proteins, and lipids as well as epigenetic control, ultimately affecting cellular growth and metabolism in the foetus. However, maternal polymorphisms such as *MTHFR* (C677T and A1298C) decrease significantly MTHFR enzyme activity, leading to a potential increase of homocysteine that eventually can lead to a series of pregnancy complications and miscarriages; the reduced enzymatic activity can

also contribute to chromosomal instability by affecting epigenetic markers, resulting in abnormalities[148], [158], [159].

Pregnant women with the 677TT genotype are more prone to undergo spontaneous abortion according to studies, including a meta-analysis which investigated 16 different studies in women with recurrent pregnancy loss[154], [160]. The A1298C also affect MTHFR enzyme activity, however, to a less extent. Individuals homozygous for the 1298CC have about 40% reduction; Meanwhile compound heterozygous for 677CT and the 1298AC have a reduction of 40 to 50% in enzymatic activity[148], [155].

MTHFR A1298C polymorphism is located in exon 7 and originates a substitution of glutamic acid by alanine at codon 429[148]. A large study with 18.000 women enrolled, identified that 1298CC could be associated with foetal growth restriction[161]. Another study by Mo et al (2018) identified that the 1298CC genotype was associated with adverse pregnancy outcomes in women from different regions of China[162]. Further, Enciso et al (2016) demonstrated 1298C allele was more frequent among multiple unsuccessful assisted reproductive treatments in the preimplantation of embryos, and women with at least one 677T allele had a mean embryo aneuploidy rate 20% higher than patients with homozygous 1298A and 677C[155].

I- *TP53* gene

The *TP53* gene encodes for tumour suppressor protein p53, a nuclear phosphoprotein, in the short arm of human chromosome 17p13.1 and consists of 11 exons [163], [164]. The p53 is an important apoptotic protein that is involved in most critical cellular processes such as cell cycle arrest, senescence, genomic stability, gene expression regulation, cell growth and DNA repair[164], [165].

Female germinative cells and embryos are protected from teratogenic agents by p53, which also induces the expression of the leukaemia inhibitor factor protein, an important mediator of the blastocyst implantation process[166]. Infertility, repeated implantation failure, and abortions are only a few of the negative pregnancy outcomes that have been linked to several polymorphisms in the *TP53* gene[165]–[169].

One of the most studied polymorphisms (rs1042522) in the *TP53* gene results in a change of guanine to cytosine in position 215 (c.215 C<G) in the exon 4; as a consequence a substitution from proline to arginine amino acid at codon 72 (Pro72Arg) [168]. Usually, arginine (G allele) is more effective than the proline (C allele) variant in stimulating leukaemia inhibitor factor protein expression, hence is a potent apoptosis inducer; Meanwhile, proline (C allele) is more operative in inducing cell arrest than arginine (G allele) [170].

1.6.3. DNA Methylation

DNA methylation plays a pivotal role in epigenetics in regulating gene expression and activities. The process happens throughout the human body and consists of the transference of a methyl group from the S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form a methylcytosine (5mC) catalyzed by a family of DNA methyltransferases (Dnmts)[171]. DNA methylation is mainly done by *de novo* Dnmt3a and Dnmt3b, which a new methylation pattern to unmodified DNA[172]; during DNA replication the Dnmt1 is responsible for copying the DNA methylation pattern onto the newly synthesized strand[55], [171], [172].

During early embryonic development and gametogenesis, DNA methylation has an essential role[173]. The whole genome goes into a reprogramming event of demethylation-remethylation to give continuity of the germline and the propagation of genetic material[171]. There are two waves of demethylation and remethylation during the life cycle[55], [173], [174]. The first one occurs during the migration of proliferating primordial germ cells and before the formation of mature gametes, respectively[171], [174], [175]. The second wave happens after fertilization, and the parental genomes undergo genome-wide demethylation when the epigenetic memory of the gametes is erased on a large scale, and the lowest DNA methylation has been reached in the blastocys[55], [173], [176]. Upon implementation, a wave of *de novo* methylation gradually occurs until it returns to the original high level, creating the initial embryonic pattern (Figure 8)[55], [171], [173], [174].

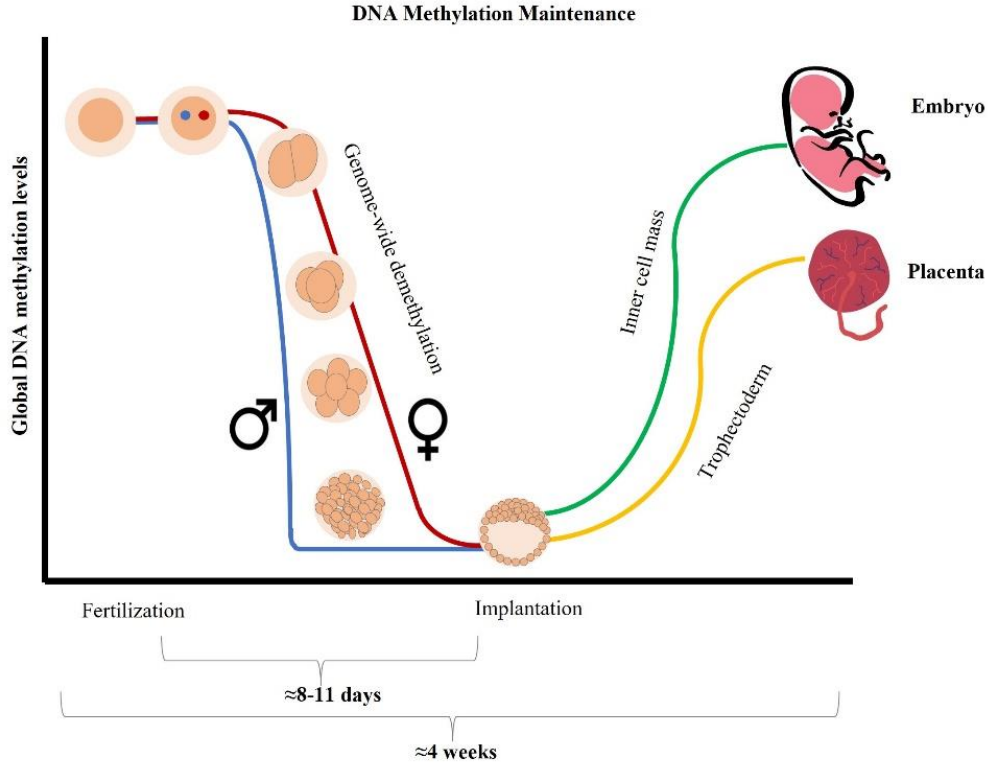


Figure 8. Changes in DNA methylation during embryonic development. The early human embryo genome's methylation pattern can be seen as follows: Before fertilisation, it is highly methylated. Then, during the cleavage stage, demethylation takes place, and after blastocyst development, the methylation level reaches a minimum level. Following implantation high methylation level can be seen through interaction with the uterus[174].

As the main controller of genetic information, DNA methylation only takes place at CpG dinucleotides[173]. They are widely and nonuniformly distributed across the human genome. Around 1-2% of these CpG-rich islands are frequently found in gene promoter regions, and their hypermethylation causes transcriptional suppression, resulting in gene silencing expression[177]. Meanwhile, genome-wide DNA hypomethylation of long interspersed transposable element 1 (LINE-1), which constitutes 17% of the human genome, predisposes cells to chromosomal defects and rearrangements that lead to genetic instability[173], [177], [178].

Altered DNA methylation results in faulty embryonic development, birth defects, and other illnesses, like human cancer. In a study by Burris et al (2012) women who developed preeclampsia in the first trimester had lower genome-wide (LINE-1) methylation levels (84.1%) compared to women who did not (84.3%)[179]. The methylation profile can be affected by environmental factors such as stress, hormones, drugs, and diet. Vasilyev et al (2020) analysed 141 chronic villi miscarriages and identified that regardless of karyotype LINE-1 methylation profile increased with gestational age. However, decreased with maternal age in miscarriages with a normal karyotype (P=0.029)[180].

1.6.4. Cytokines

Cytokines are soluble proteins or glycoproteins produced by the leucocytes and diverse other cell types (e.g., endothelial cells, fibroblasts)[181]. They also can have autocrine (act on the cells that secreted the cytokine), paracrine (act in the cells nearby) and endocrine (act in distant cells) actions[182]. It is common for the same cytokine to act in multiple cells, and that different cytokines stimulate similar functions[181]. Some cytokines can act synergistically or antagonistically, as a mechanism of cellular control[181].

In pregnancy, the maternal immune system adjusts to prevent the rejection of foetal (paternal) tissues in a way that preserves immunological competence to combat pathogens[182]. The immune system and cytokines also play additional roles, such as supporting foetal-maternal communication and settling appropriate physiological changes, such as in the clotting system and vascular endothelium, certain cytokines promote foetal growth locally by acting as growth factors, and Th1 cells cytokines stimulate vasculogenesis (Figure 9) [183]–[186].

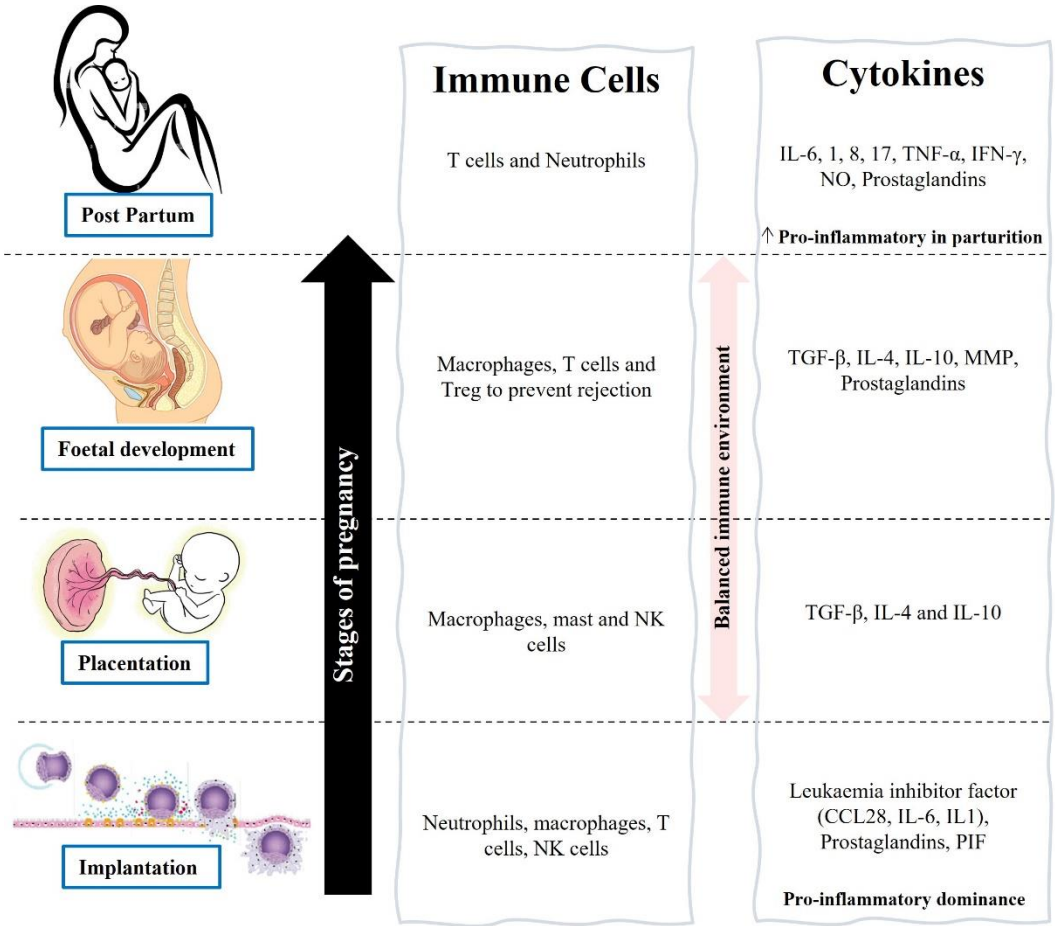


Figure 9. Cytokine's roles during pregnancy. In the implantation of the blastocyst in the uterine endometrium cytokines such as IL-6, IL-1, and PIF secreted by neutrophils, macrophages M1, $CD4^+$ Th1 cells, neutrophils will be slightly increased in response to local inflammation. NK cells will surround the trophoblast after implantation. In the third trimester, the development of the placenta depends upon Macrophages M2, NK and mast cells for uterine spiral and artery remodelling. The immune system controls strictly immune responses and cytokines production by favouring T regulatory cells and anti-inflammatory cytokines and TGF-β to stimulate foetal development. During parturition pro-inflammatory cytokines such as IL-6 and IL-1 are released by immune cells and endothelium to induce muscle contraction. IL: Interleukin; NK cells: Natural Killers cells; CCL: C-C motif ligand 28; PIF: Preimplantation Factor; TGF-β: Transforming Growth Factor beta: Th: T helper;

MMP: Matrix Metalloproteinases; TNF-α: Tumour Necrosis Factor-alpha; IFN-γ: Interferon-gamma; NO: Nitric Oxid[186].

In the course of the pregnancy, protracted exposure to Th1 cytokines from a cell-mediated immune response can harm the foetus and lead to pregnancy losses[187]–[189]. As a result, it's normal to see a change from proinflammatory Th1 immunity to anti-inflammatory Th2 immunity throughout the stages of successful pregnancies[189].

A study showed that women who suffered spontaneous abortion in the first trimester had considerably higher levels of Th1 cytokines (IL-2, IFN- γ and TNF- α) than pregnant women with no further complications in their gestational period [185]. Maciej et al (2021) showed that women with recurrent miscarriages in the first trimester had a strong positive correlation between IL-4 and IFN-γ[183]. This finding can be explained by the fact that in the group of women who had miscarriages, IL-4 concentration did not alter the proinflammatory polarisation of cytokines since it was accompanied by a large increase in INF- γ concentration, and the INF-/IL-4 ratio remained twice as high as in the control group[183].

Daher et al (2004) analysed 29 women with at least 3 consecutive pregnancy losses and a mean age of 35 years old, and no previous successful pregnancy[189]. Interestingly at the time, these women were not pregnant, however, displayed considerably higher levels of Th1 cytokine profile (IFN- γ: 355.8 pg/mL) to more than 3 compared to the controls (IFN- γ: 98.0 pg/mL), supporting the role of Th1 in the pathogenesis of miscarriages[189].

1.7. Purpose of the Study

Early pregnancy loss aetiology is varied, and several factors may interact to contribute to foetal loss. This heterogeneous origin makes it extremely difficult to truly identify causative factors and prognostic factors. More importantly, early pregnancy loss and recurrent

pregnancy loss risk factors may vary greatly to disentangle aetiologic screening biomarkers. Therefore, more thorough knowledge may discover prenatal and perinatal interventions that could lower the risk of future miscarriages.

1.8. Hypotheses

1- Age is a major risk factor for early and recurrent pregnancy loss.

2- Routine blood exams itself are not able to identify pregnancy loss risk.

3- The genetic environment is mediated in part by genetic factors differences between cases compared to controls, and intracase, which can further highlight the risk population.

4- DNA methylation displays large and point scale differences in cases and intracase.

5- Pro and anti-inflammatory levels increase in response to abortion.

6- Risk genes for miscarriages can be associated with epigenetic changes.

7- Genetic, epigenetic, and systemic factors affect scaffold-gel polymerization.

1.9. Thesis Aims

The main aim of this research was to identify and access potential risk factors exposure during pregnancy and to better understand the causes of spontaneous abortion and recurrent spontaneous abortions. The specific aims were:

- To identify demographic characteristics in the risk population.

- To characterise cases and controls, as well as intracases, genes with haemostatic functions (e.g., coagulation, metabolic, and folate processing) for pregnancy success.

- To explore the DNA methylation profile in cases and controls, as well as intracases. Furthermore, to assess the potential functional significance of methylation differences using other bioinformatics tools.

- To study the association of genetic, epigenetic, and systemic influences and risk for spontaneous abortion.

- To *in vitro* characterize genetic, epigenetic, and systemic influences in the scaffold gel fibrin of cases and controls. Further, systematically analyse these differences with an image analyser to evaluate microenvironment risk factors for implementation.

- To investigate the best prognostic biomarker in the early identification of pro one miscarriage women.

Chapter 2

Research Materials and Methods

2.0. Introduction

A retrospective cohort study was conducted, composed of five independent investigations, that later were combined. Firstly, an extensive database was constructed to identify specific features relevant to the studied population. Cases and controls were characterized according to ethnicity, demographical information, and pre-surgery (curettage) laboratory blood test results.

After, in the second step, DNA extracted from whole blood was used to perform genetic investigations. A total of 11 distinct genes, accounting for 17 different single nucleotide polymorphisms and 2 mutations were selected based on previous studies (vide Chapter 1.6.2.). This resulted in the identification of risk and protective genetic factors and their implications during EPL.

Thirdly, bisulfite conversion was applied to the same DNA used for the genetic investigation. This process was performed to distinguish methylated from unmethylated cytosines in genomic DNA at a single base resolution. This allowed the establishment of a global DNA methylation profile for both groups, and further evaluation of the profile combined with secondary elements, such as metabolic pathways, and genetic information.

As part of the fourth step, a total of 4 cytokines were measured, three of them being pro-inflammatory and one anti-inflammatory. Throughout the pregnancy, it's important to maintain a healthy balance of pro- and anti-inflammatory cytokines, and dysregulated cytokine expression is associated with a poor pregnancy environment. Therefore, within this crucial step, circulating biomarkers were identified, as well as their potential to affect early miscarriages, when also associated with epigenetic, genetic, and metabolic components.

Finally, an in vitro model of a fibrin gel scaffold was performed. During the initial stage of pregnancy, the blastocyst must complete two processes after fertilisation to ensure continued pregnancy development, the attachment, and the invasion of the endometrium's luminal epithelium, in which fibrin mesh plays an important role in the stabilization. This step contributed to the visualization of the fibrin gel scaffold microenvironment differences according to specific information gathered from genetics and circulating biomarkers.

2.1. Case-Control Study Design

A case-control study was undertaken on pregnant females who had spontaneous miscarriages (cases) and on females who chose pregnancy termination (controls). The study was carried out at the University of Ferrara between January 2016 and January 2019. Ethical approval number: NCT03585023. All participants gave informed consent.

2.2. Feasibility of Recruitment

A significant increase in spontaneous miscarriages worldwide was observed from 1985 (12.5%) to 2016 (15%). One out of seven pregnant females will suffer from early pregnancy loss, and 85% of the cases will be from an undetermined cause. Due to this, females who had spontaneous pregnancy loss in their first trimester from idiopathic causes identified at the Department of Obstetrics and Gynaecology of Cona Hospital, Ferrara, Italy were recruited to be part of the research from 2016 to 2019, with a total of 108 females enrolled in the study as cases, and 107 as controls.

2.3. Recruitment Criteria

To minimise substantial confounding variables, that would lead to pregnancy loss, only females who satisfied the following requirements were included as cases in the study:

- Aged 18 to 55 years
- Be between 6 days to 12 weeks of completed pregnancy
- A diagnosis of undetermined spontaneous abortion based on clinical history, examination, and transvaginal ultrasound scanning
- Natural conception
- Singleton pregnancy
- Single or multiple spontaneous abortions from natural conception
- Signature in the informed consent form accepting to participate and agreeing with blood collection

For the control group selected females who opted for the pregnancy interruption were included according to the following specific requirements:

- Aged 18 to 55 years
- Be between 6 days to 12 weeks of completed pregnancy
- Natural conception
- Singleton pregnancy
- A diagnosis confirming pregnancy normality based on clinical history, examination, and transvaginal ultrasound scanning
- Signature in the informed consent form accepting to participate and agreeing with blood collection

2.4. Exclusion Criteria

Any spontaneous pregnancy loss that was likely to be affected by any confounding variables was excluded from the cases group. Thus, the following exclusion criteria were applied:

- Known maternal drug or excess alcohol intake
- Pregnancies where the mother is affected by a significant medical condition, such as cardiac, renal, or haematological disease
- Known foetal congenital infections such as CMV or toxoplasmosis
- Known congenital chromosomal abnormalities
- Known congenital structural malformations
- Pregnancies conceived via artificial reproductive therapies (ART)
- Maternal gestational diabetes

Regarding the control group, the following exclusion requirements were stipulated:

- Known spontaneous abortion
- Known maternal drug or excess alcohol intake
- Pregnancies where the mother is affected by a significant medical condition, such as cardiac, renal, or haematological disease
- Known foetal congenital infections such as CMV or toxoplasmosis
- Known congenital chromosomal abnormalities
- Known congenital structural malformations
- Pregnancies conceived via artificial reproductive therapies (ART)
- Maternal gestational diabetes

2.5. Study Protocol

The phenotypic measurements of the participants (i.e., case and controls) and whole blood collection were performed in Cona Hospital using identical equipment. All the case groups had an incomplete spontaneous abortion, therefore surgical curettage intervention had to be performed. Whole blood was collected for all cases and controls, as standard procedure to evaluate their health status before the intervention, to assess Erythrogram, Leukogram, Coagulogram, and Coagulation function. Females who accepted to participate in the study were asked to answer a questionnaire and to donate an extra vacutainer blood tube, for DNA extraction performed in the Translational Medicine Department at the University of Ferrara (Figure 1).

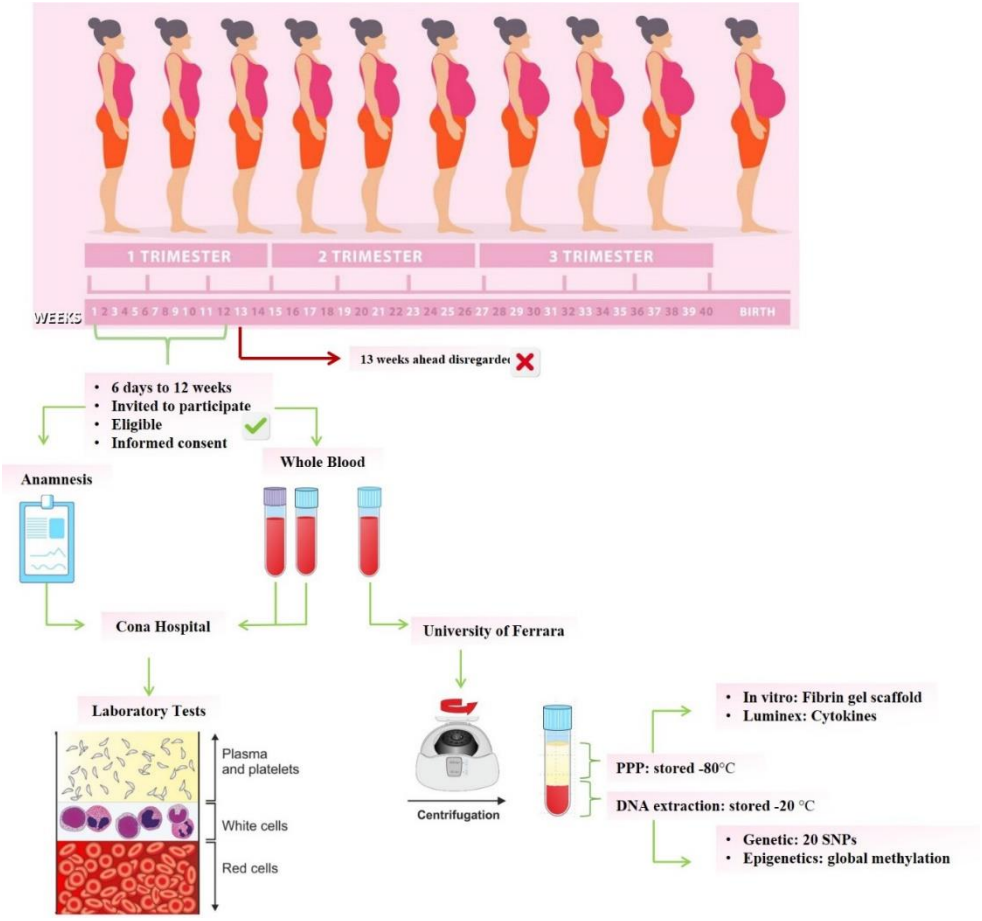


Figure 1. The summary approach of the steps taken during project development

2.6. Cases and Control's Anamnesis

A well-discriminated questionnaire was constructed to characterize the cases and controls recruited for the study and reduce any confounding factors. The questionnaire was applied in a mixed style of closed and open-ended questions referred to the patients.

The questionnaire was completed by a nurse on the day after the intervention for those patients that accepted to participate in the study formally and had donated blood for DNA extraction. Patients were asked about their birth date, weight, height, ethnicity, menarche age, the date of their last period before pregnancy, the date of the surgical curettage, in the case of the volunteer group, the date of their pregnancy termination, the number of cigarettes per day in case of a smoker, diagnosed comorbidities, regular use of medications, any previous birth, and spontaneous abortions up to four precedent pregnancies. Patients were also requested to authorize access to the whole blood results performed on the day before the intervention by the hospital.

2.7. Blood Sample Collection, Processing, and Storage

A total of 3 vacutainers, 10-12 mL of whole blood final volume, were taken from all participants before the surgical procedure, two of them were collected in sodium citrate and one in EDTA anticoagulant. The blood tubes collected in EDTA and one in the citrate were sent to the hospital laboratory, for haematological and coagulation analysis. This is a standard procedure to guarantee the patients' well-being and avoid any further complications before the surgical intervention in all females who had a spontaneous abortion and volunteer pregnancy termination. The other sodium citrate blood was delivered at room temperature to the Translational Medicine department of the University of Ferrara for DNA extraction and plasma storage within 2 hours post-collection.

The two blood vacutainers that remained at the Cona Hospital were analysed and processed at the haematology department in the hospital laboratory. The procedures and protocols followed the standards and quality control as prescribed by the hospital, with results revised and ensured by the responsible for the sector.

Upon arrival at the Translational Medicine Department, the citrate sodium blood was centrifuged at 2002g for 10 minutes at RT for platelet-poor plasma (PPP) collection. For each participant, 3 properly identified samples containing 500µL of plasma were prepared and stored at -80°C for further investigation.

Subsequently plasma separation, and blood was homogenized, ensuring that peripheral human mononuclear cells were incorporated into the red blood cells, and stored at -20°C until use. Before DNA extraction, the frozen blood was placed at RT until complete thawing. Genomic DNA was extracted with the automatic EZ1 Advanced and BioRobot system (Qiagen, Hilden, Germany) instrument and its respective kit EZ 1&2^{TM} DNA Blood 350µL (Qiagen, Hilden, Germany), following the manufacturer's instructions. For all the participants 350µL of blood was used to isolate and purify the DNA in a final dilution elution volume of 100µL and stored at -20°C after proper DNA quantification. The automatic steps for DNA extraction and purification included (Figure 2):

1- DNA lyses due to DNA binding to the silica surface of the particles in the presence of a chaotropic salt
2- DNA binds to magnetic particles
3- Magnetic separation of the DNA
4- The DNA is then efficiently washed
5- Elution step in either water or TE buffer

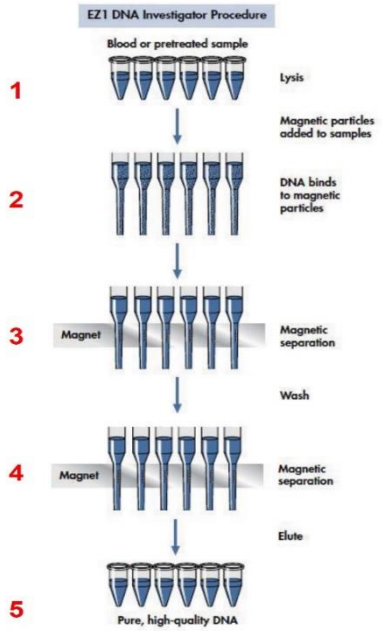


Figure 2. DNA extraction and purification flowchart.

Following extraction, DNA concentration and purity were assessed by the Agilent Cary60 spectrophotometer (Agilent Technologies, Mulgrave, Australia) according to the manufacturer's protocol. A volume of 2µl of DNA was employed to measure the 260/280 and 260/230 ratios using Cary60. Samples with a 260/280 ratio <1.75 and a 260/230 ratio > 1.8 were reextracted, and in case of the same results were discarded from the analysis. A 1% Agarose gel was run to assess DNA integrity. Agarose gels were prepared by weighing out the appropriate amount of agarose in a conical flask and adding the correct volume of 1x TBE. The flask contents were mixed by gentle swirling and heated in a microwave to dissolve the agarose. The dissolved agarose was left to cool to approximately 60°C before the addition of 1x GelRed® Nucleic Acid Dye (Biotium, California, USA).

The melted agarose was then poured into a pre-sealed gel cast containing a well-comb. Agarose gels were left to set for 20-30 minutes at room temperature. The agarose gel was placed in an electrophoresis tank (E844, Consort, Turnhout, Belgium) containing a sufficient 1x TBE buffer. Electrophoresis was carried out at 80 volts for 1 hour or until the required resolution was achieved. DNA fragments were visualised in Axygen® Gel Documentation

Systems (Corning, New York, USA) with a UV light and then photographed on a trans illuminator. Stock DNA was normalised to a concentration of 50ng/µl by dilution with nuclease-free water (Qiagen, Hilden, Germany).

2.8. Genomic Studies and Early Spontaneous Pregnancy Loss

About 50% of sporadic miscarriages are caused by genetic abnormalities, which can be divided into chromosomal or monogenic. Chromosomal abnormalities are the most frequent cause of miscarriage and can concern both the number of chromosomes (aneuploidies) and their structure (structural abnormalities).

The genetic studies were performed on 11 different genes and 17 polymorphisms and 2 mutations. Overall, they are associated at different levels with important metabolic functions essential to haemostasis and physiological stability during pregnancy. For each variation, the best suitable gold-standard methodologies were used to characterize genotypically cases and controls (Table 1). Two genes were included, *F2* and *F5*, as a screening method to identify risk factors for venous thrombophilia as a possible cause of pregnancy loss. The frequency of the polymorphism (mutated allele) in the cases was comparable to normal frequencies expected in the worldwide population. No homozygote polymorphic cases were identified, out of 108, 4 heterozygotes were identified for *F2* and 5 for *F5* genes.

Table 1. Genes and the reference sequence.

Genes	Name	Chr	Ref. Sequence	Location	Nucleotide Variation	Change	Method
ACE1	Angiotensin I Converting Enzyme	17	rs1799752	Intron 16	I/D	287 *Alu*	Agarose Gel Check
APOE	Apolipoprotein E	19	rs7412	Exon 4	C>T	R158C	Pyrosequencing
		19	rs429358	Exon 4	T>C	C112R	
CFH	Complement Factor H	1	rs1061170	Exon 9	T>C	H402Y	
CRP	Protein C Reactive	1	rs876538	3'Flanking	C>T	+7598	
		1	rs2808635	Intragenic	T>G	NA	
F13A1	Coagulation Factor XIII A chain	6	rs5985	Exon 2	G>T	V34L	
		6	rs5982	Exon 12	C>T	P564L	
		6	rs3024477	Exon 5	A>T	Y204F	
F13B	Coagulation Factor XIII B chain	1	rs6003	Exon 3	G>A	H95R	
FGA	Fibrinogen alpha chain	4	rs6050	Exon 5	A>G	T312A	
FGB	Fibrinogen beta chain	4	rs1800790	5'Flanking	G>A	-455	
MTHFR	Methylenetetrahydrofolate Reductase	1	rs1801131	Exon 8	A>C	E429A	
		1	rs1801133	Exon 5	C>T	A223V	
ACE2	Angiotensin Converting Enzyme 2	X	rs2285666	Intron 3	C>T	c.439+4	Real-Time PCR
F2	Coagulation Factor II	11	rs1799963	3'UTR	G>A	c.20210	
F5	Coagulation Factor V	1	rs6025	Exon 10	T>C	A534G	
MMP12	Matrix Metallopeptidase 12	11	rs2276109	Promoter	A>G	-82	
TP53	Tumour Protein p53	17	rs1042522	Exon 4	C>G	P72R	

2.8.1. PCR and Agarose Gel Check

In essence, the PCR method depends on the fact that heat denatures the DNA strand into single strands, which will then anneal with primers and nucleotide bases to reform a double strand at lower temperatures. Therefore, the first step is to denature the DNA for a brief period at a high temperature. Lower temperatures are used after this initial phase in the presence of complementary primers to the DNA on each side of the sequence being investigated. When these primers anneal, a DNA polymerase adds bases by bases of nucleotides, replicating the targeted area of the DNA (Figure 3).

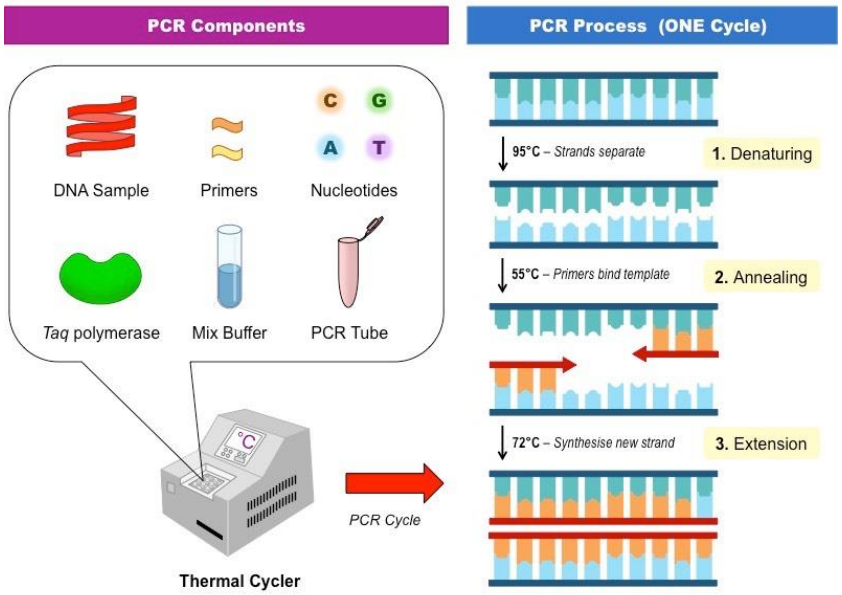


Figure 3. Polymerase Chain Reaction components and steps involved in the amplification of a DNA fragment. https://microbenotes.com/polymerase-chain-reaction-pcr-principle-steps-applications/

PCR for ACE1 and before pyrosequencing analysis (vide table) was performed in a final volume of 40µl/well. Each reaction contained, 2µl of 50ng/µl DNA, lx buffer (Roche, Mannheim, Germany), 0.2 mM of each dNTP (dATP, dGTP, dTTP, and dCTP) (Promega, Madison, USA), 25 pmol of each oligonucleotide (IDT, Coralville, USA), and 0.06U/µl Taq DNA polymerase (Roche, Mannheim, Germany). The annealing temperature was optimised for each oligonucleotide pair. Reactions were performed in a 96-Well reaction plate (Applied

Biosystems™ MicroAmp™, Waltham, USA). The cycling conditions (SureCycler 8800, Agilent Technologies, Mulgrave, Australia) were variable according to the investigated polymorphisms. For each set of PCR reactions, a negative control was carried out (by using sterile distilled water in place of DNA) to test for DNA contamination, as well as internal controls for each of the polymorphisms.

A total of 20μl/well of PCR product for *ACE1* was checked in a 2.5% Agarose gel placed in the electrophoresis tank (E844, Consort, Turnhout, Belgium) containing a sufficient 1x TBE buffer and 1x GelRed® Nucleic Acid Dye (Biotium, California, USA), and PCR marker 1x (BioLabs, Texas, USA). Electrophoresis was carried out at 100 volts for 30' or until the required resolution was achieved. DNA fragments were visualised in Axygen® Gel Documentation Systems (Corning, New York, USA) with a UV light and then photographed on a trans illuminator (Figure 4).

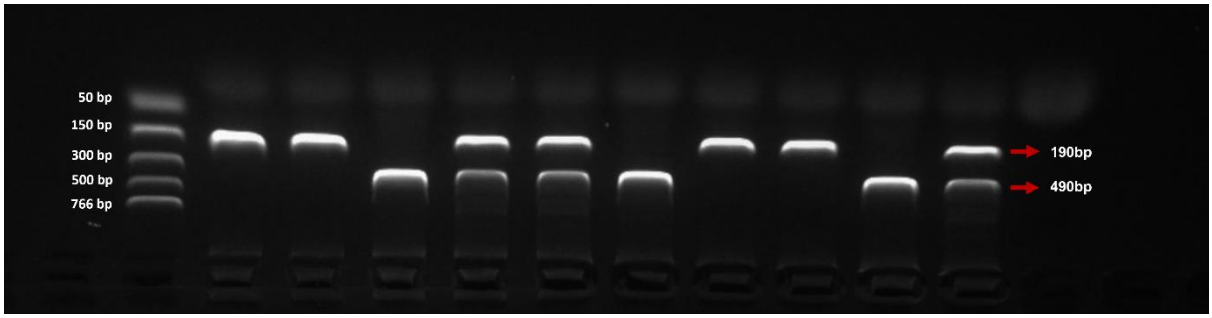


Figure 4. *ACE1* 2.5% agarose gel check and genotype information was acquired for cases and controls. Insertion: 490bp, Deletion: 190bp.

Before pyrosequencing, the PCR products were also checked on a 2.5% agarose gel. To ensure standard results, a total of 10μl/well of final PCR product was used to confirm the absence of any contaminants and product quality (Figure 5). The steps regarding the gel production and check were the same as the ones for *ACE1*.

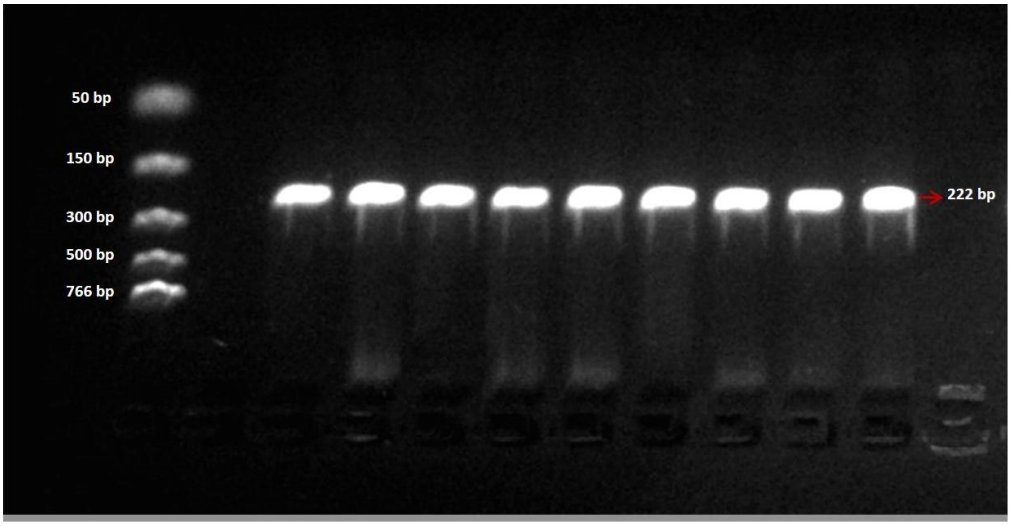


Figure 5. MTHFR A1298C (rs1801131) 2.5% agarose gel check. All PCRs before proceeding to pyrosequence were checked as part of standard quality control of the PCR products. PCR fragment 222bp. The first lane represents PCR markers from 50 bp to 766 bp, the second negative control, followed by amplification of cases and control samples.

2.8.2. Pyrosequencing

Pyrosequencing is a sequence-based detection technology that enables rapid and accurate quantification of sequence variation. The septs associated with the principle behind the methodology are:

1- A DNA segment is amplified with a biotinylated primer which will serve as a pyrosequencing template. After the denaturation, step the biotinylated single strand is isolated and hybridized with a single sequencing primer-template.

2- The hybridized primer and single-stranded template are incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase, as well as the substrates adenosine 5' phosphosulfate (APS) and luciferin.

3- DNA polymerase catalyses the addition of the dNTP to the sequencing primer if the nucleotide is complementary to the base in the template strand. This event will generate the release of pyrophosphate (PPi) at a proportional level to the amount of incorporated nucleotide (Figure 6 steps 1-3).

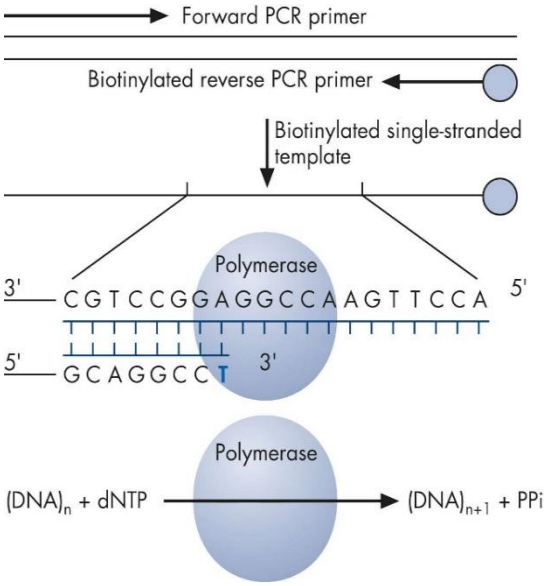


Figure 6. Pyrosequencing steps 1-3.

4- When adenosine 5' phosphosulfate is present, ATP sulfurylase transforms the PPi into ATP. The amount of visible light produced is proportional to the amount of ATP that is used in the reaction to mediate luciferase conversion of luciferin to oxyluciferin. A charge-coupled device (CCD) camera detects the light generated in the luciferase-catalyzed process and records it as a peak in the output of raw data (Pyrogram). The height of each peak (light signal) is proportional to the number of nucleotides incorporated (Figure 7).

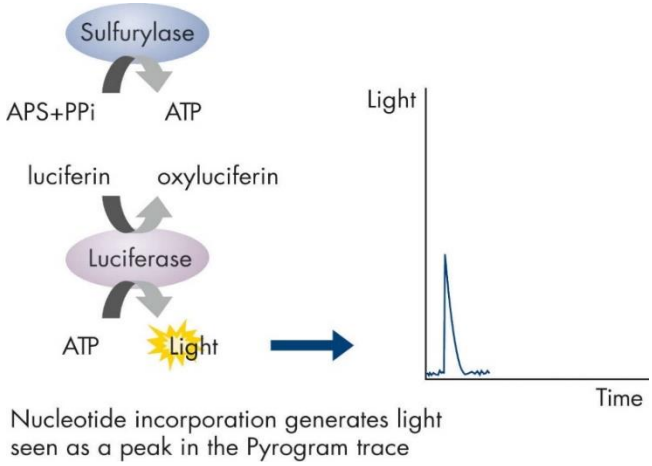


Figure 7. Pyrogram generation principles behind pyrosequencing

5- Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added (Figure 8).

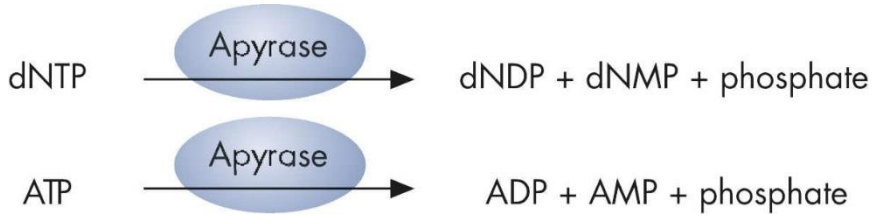


Figure 8. Degradation of unincorporated nucleotides. dNDP, deoxynucleoside diphosphate; dNMP, deoxynucleoside monophosphate; AMP: Adenosine monophosphate; ADP: Adenosine diphosphate.

6- Sequential addition of dNTPs is carried out. It should be mentioned that deoxyadenosine alfa-thio triphosphate (dATPαS) is effectively utilised by the DNA polymerase but is not recognised by the luciferase. The complementary DNA strand is constructed while the process progresses, and the nucleotide sequence is identified from the signal peaks in the Pyrogram trace (Figure 9).

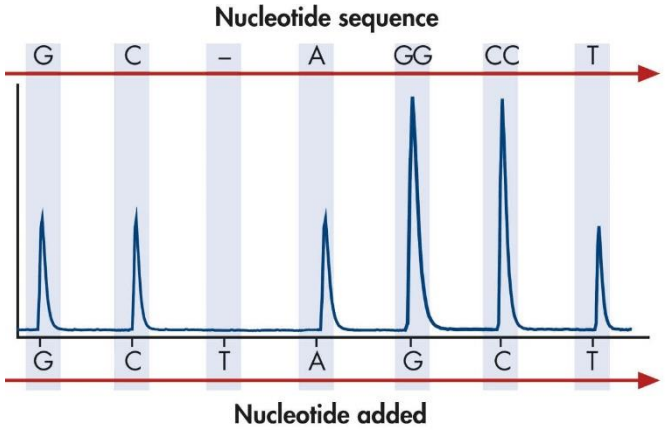


Figure 9. The final visualization of a pyrosequencing pyrogram.

All the genotype analyses were performed in the PyroMark ID Q96 instrument (Biotage, Uppsala, Sweeden), with a total volume of 30µl/well of PCR product. The binding and annealing solutions were prepared as suggested by the manufacturers. The binding solution's final volume was 80µl, which consisted of 30µl of PCR product, 10µl of distilled water, 3µl of streptavidin Sepharose™ high performance (Cytiva, Marlborough, USA), and 37µl of

binding buffer (Qiagen, Hilden, Germany) for each well analysed. The solution was dispensed in the wells containing the PCR product and placed in a thermal mixer at RT for 10 minutes. The aneling solution's final volume was 40µl, comprised of 2.25µM high-performance liquid chromatography (HPLC) sequence primer (IDT, Coralville, USA) in 39.1µl of the aneling buffer. The vacuum prep workstation (Biotage, Uppsala, Sweeden) for each 96-well plate was composed of 100ml of each: 70% ethanol, denaturation solution (Qiagen, Hilden, Germany), 1x wash buffer (Qiagen, Hilden, Germany), and nuclease-free water (Qiagen, Hilden, Germany). Using the vacuum tool, the PCR processed in the workstation was dispensed into the aneling solution and placed at 80°C for 2 minutes. The volumes for dATP, dGTP, dTTP, dCTP, substrate, and enzyme (PyroMark Gold Q96 Reagents, Qiagen, Hilden, Germany) were pipetted according to manufactures recommendation, and their respective volume was based on the plate template and target interest sequence inserted in the pyrosequencing software 1.00 (Qiagen, Hilden, Germany) (Figure 10).

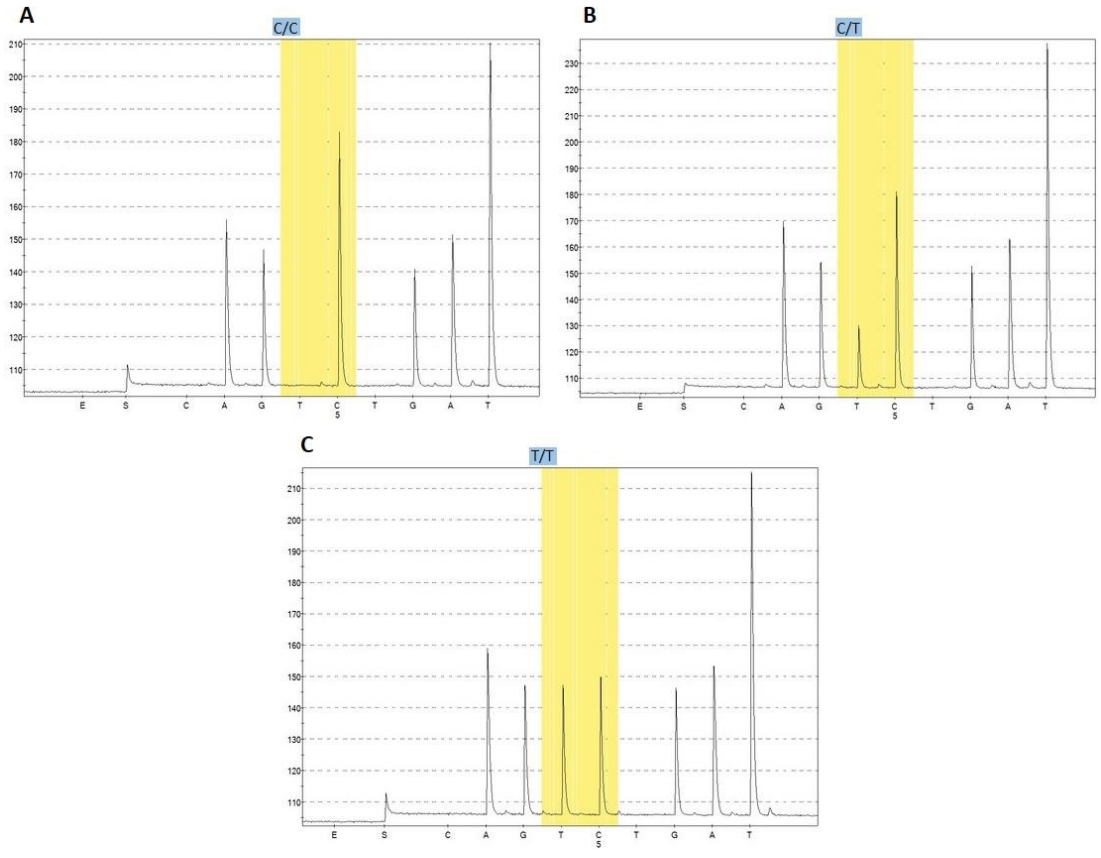


Figure 10. Pyrogram analysis of MTHFR C677T (rs1801133). The genotypes referred to in the pyrogram are wild type (A), heterozygote (B), and polymorphic (C). The same approach was performed for all polymorphisms performed with pyrosequencing.

2.8.3. Real-Time PCR

The Real-Time PCR was performed with rhAmp SNP Genotyping technology. In sum, this technology uses a unique two-enzyme system coupled with RNA-DNA hybrid primers to precisely interrogate target SNPs sequences. The steps in general are:

1- The rhAmp master and reporter mix, and SNP assay are combined in one vial and dispensed into the samples, and placed in the thermocycler (Figure 11).

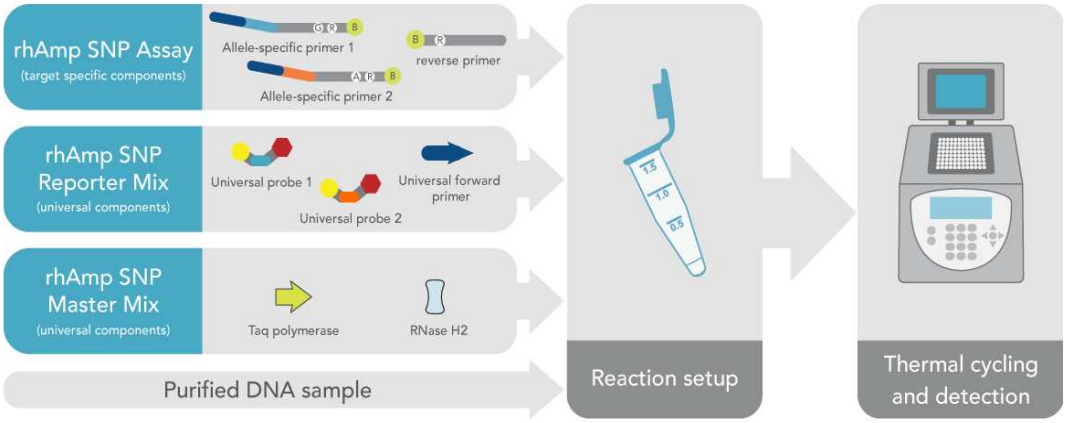


Figure 11. General reagents and process required to perform rhAmp genotyping assay.

2- The first stages of the Real-Time PCR in the thermocycler are identified as allelic discrimination. Initially, both allele-specific primers query the SNP locus, in which allele 1 is read on a FAM dye and refers to the reference Allele, and allele 2 is read in a VIC dye and refers to the altered allele. Only the primers that are precisely annealed to the target sequence are cleaved by the RNase H2 enzyme, hence removing the RNA base and 3' blocking modification, allowing extension by the Hawkeye™ Taq Polymerase. A tail sequence is then integrated into the amplicon during the first two cycles of

amplification and is subsequently detected by a universal reporter system based on probes (Figure 12).

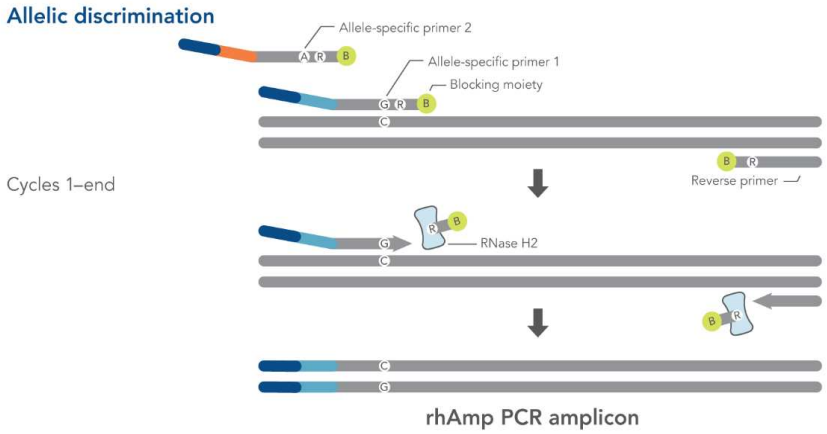


Figure 12. Schematic representation of allelic discrimination on QuantStudio Real-Time PCR.

3- The polymerase extension leads to the degradation of the probe and signal generation, which then is represented in a dot plot graph based on the respective genotyping analysis (Figure 13).

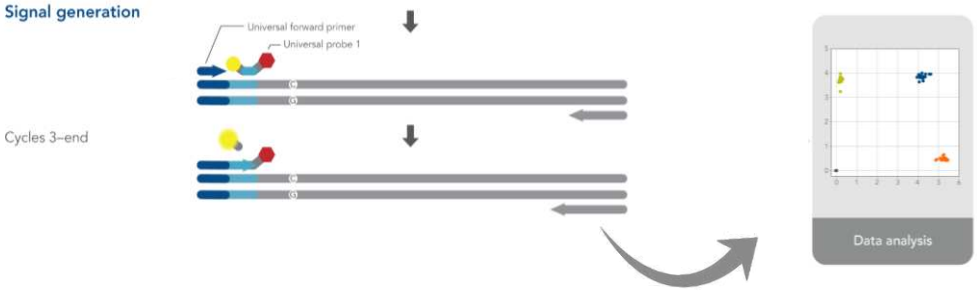


Figure 13. General reagents and process required to perform rhAmp genotyping assay.

All Real-Time PCRs were performed in QuantStudio™ 3 (ThermoFisher Scientific, Waltham, USA) with a final volume of 10µl, constituted of 1x rhAmp® Genotyping master mix (IDT, Coralville, USA) 1x rhAmp® Reporter mix (IDT, Coralville, USA), 1x of SNP assay (IDT, Coralville, USA), distilled water (IDT, Coralville, USA), and 5ng of DNA, following the manufacturer's recommendations. For each set of Real-Time PCR reactions,

a negative control was carried out (by using sterile distilled water in place of DNA) to test for DNA contamination and internal controls for each of the polymorphisms (wild-type, heterozygotes and polymorphic homozygotes). The cycling conditions were 60°C for 30 seconds for the pre-read stage, hold-stage at 95°C for 10 minutes, the PCR stage of 40 cycles at 95°C for 10 minutes, 60°C for 30 seconds, 68°C for 20 seconds, and post-read stage at 60°C for 30 seconds. The analyses of the genotyping were performed in the Thermo Fisher Connect™ software (ThermoFisher Scientific, Waltham, USA), which provided visuals and integrated traces of allelic discrimination plots to allow thorough QC of SNP assays to accurately reflect the true signals versus background noise (Figure 14).

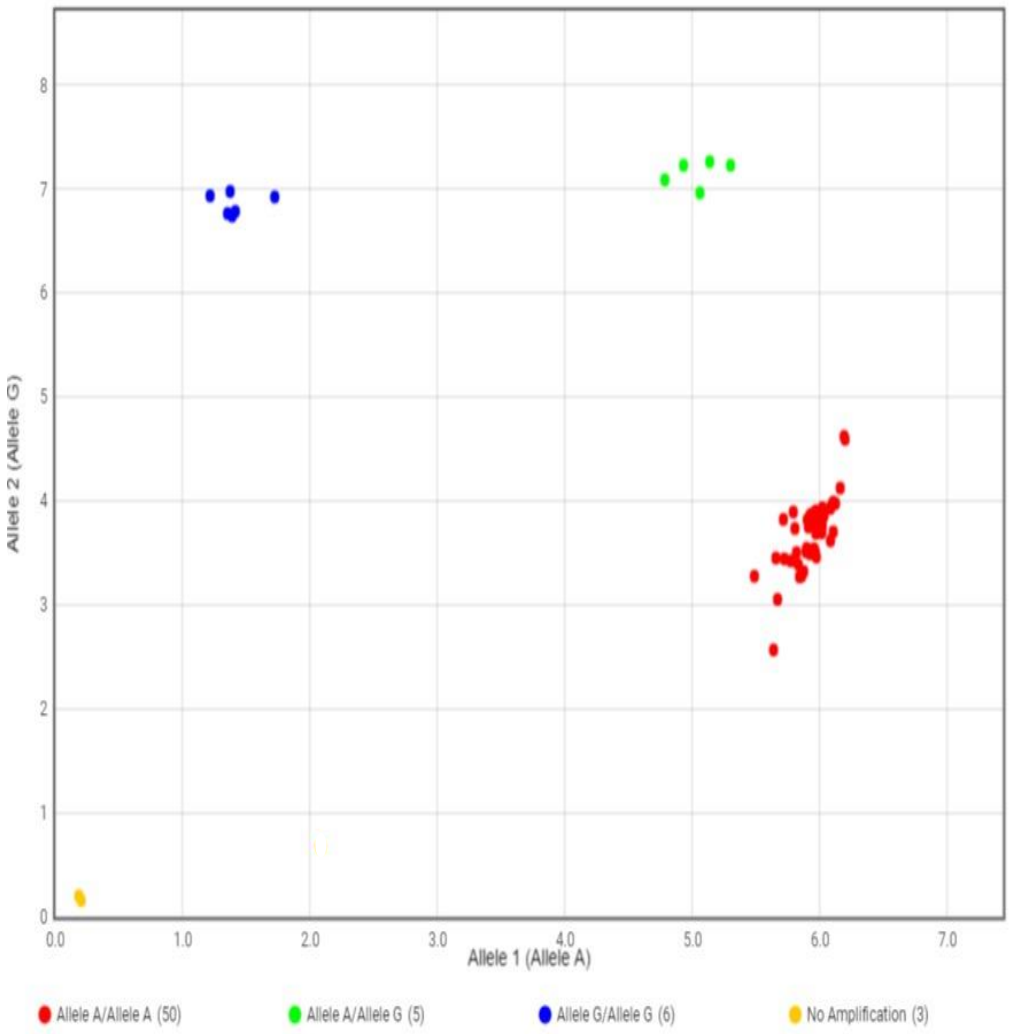


Figure 14. Genotyping analyses of *ACE2*. Dot plot graph representation for cases and controls analysis of rs2285666. Allele 1 represents the reference allele (read on FAM dye) seen as red dots; Allele 2 represents the altered allele (read on VIC dye) seen as blue dots;

Individuals represented by green dots are carriers of both, allele 1 and allele 2, therefore heterozygotes.

2.9. Bisulfite Conversion of Blood-derived DNA samples and DNA Methylation Analysis in Blood

Histone modifications and DNA methylation are two examples of changeable structural DNA alterations known as epigenetic modifications that affect gene expression and phenotype without changing the nucleotide sequence. Numerous studies have been conducted on DNA methylation which occurs as a result of the covalent attachment of a methyl group to cytosine residues in cytosine/guanine-rich regions of DNA (CpG islands). There are several ways to assess methylation ranging from locus-specific to genome-wide, one of them is pyrosequencing which was used in this work. The best method for evaluating DNA methylation is bisulfite conversion. This method allows unmethylated cytosine residues in the target DNA to undergo conversion to uracil after being exposed to bisulfite, whereas the methylated cytosines remain unaltered. As a result, methylated and unmethylated DNA have distinct DNA sequences post-bisulfite treatment (Figure 15).

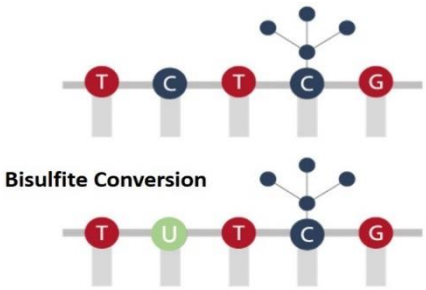


Figure 15. DNA bisulfite conversion.

An initial volume of 500ng of DNA was used for all bisulfite conversions. The bisulfite conversion was performed according to the recommendations of the manufacturers (Qiagen,

Hilden, Germany) and a final volume of 50 µl bisulfite-converted DNA was stored at -20°C (Figure 16).

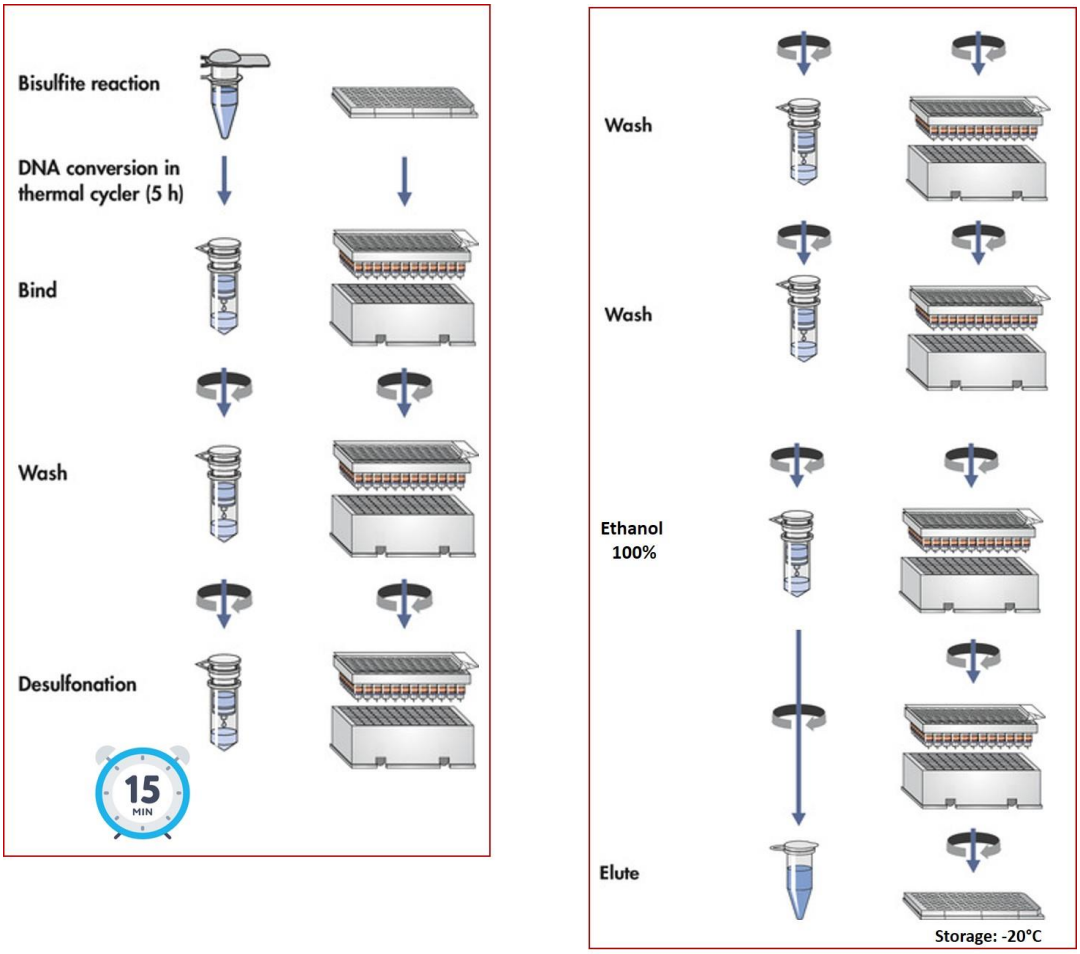


Figure 16. Bisulfite conversion workflow. The steps regarding the bisulfite conversion procedure.

After bisulfite conversion, the long interspersed nucleotide element 1 (LINE-1) was analysed to investigate genome-wide DNA methylation. In total 5 CpGs (positions +306 to +364) sites were amplified by PCR and then analysed by pyrosequencing with PyroMarkQ96 ID (Qiagen, Hilden, Germany), however due to lack of precise information, probably because adjacent CT dinucleotide, the CpG site at +364 position was disregarded. The PCR was performed using the PyroMark PCR kit (Qiagen, Hilden, Germany), following the recommendations of the fabricant. The final volume of the PCR was 25µl, containing 2µl of bisulfite converted DNA, 1x Master Mix, 1x CoralLoad Concentrate, 1x Q-Solution, and

0.5µM of each oligonucleotide (IDT, Coralville, USA), RNAse-free water. For each set of PCR reactions, a negative control was carried out (by using sterile distilled water in place of DNA) to test for DNA contamination. The cycling profile was composed 3 stages, the first one was the initial activation for 15 minutes at 95°C, the second stage comprised 38 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes to improve full-length replication (SureCycler 8800, Agilent Technologies, Mulgrave, Australia).

The amplicon was analysed by loading 5µl of PCR product in an 8.5% polyacrylamide gel placed in the vertical electrophoresis tank (E844, Consort, Turnhout, Belgium) containing sufficient 1x TBE buffer, PCR marker 1x (BioLabs, Texas, USA). Electrophoresis was carried out at 100 volts for 90 minutes. The gel was post-stained with 3x GelRed® Nucleic Acid Dye (Biotium, California, USA) with an incubation period of 30 minutes. Fragments were visualised in Axygen® Gel Documentation Systems (Corning, New York, USA) with a UV light and then photographed on a trans illuminator (Figure 17).

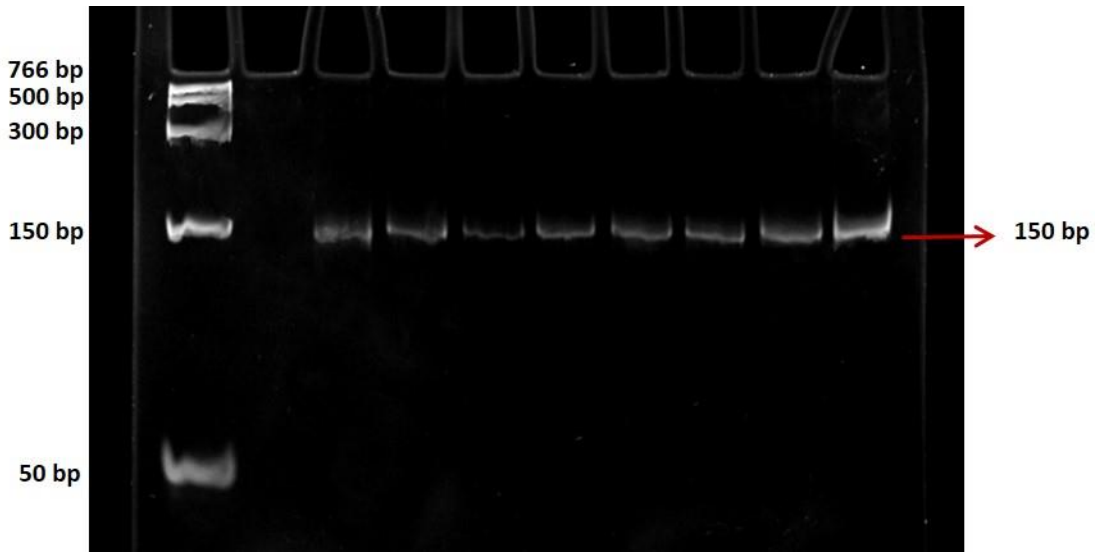


Figure 17. LINE-1 check on 8.5% polyacrylamide gel. First, the band lane represents the PCR marker from 50bp to 766bp, the second lane is negative control; the other lanes represent cases and controls investigated, and the amplicon size is a fragment of 150bp.

The remaining volume of 20µl of PCR product was used in pyrosequencing. The binding and aneling solutions were prepared as suggested by the manufacturers. The binding solution's final volume was 80µl, which consisted of 20µl of PCR product, 20µl of distilled water, 3µl of streptavidin Sepharose™ high performance (Cytiva, Marlborough, USA), and 37µl of binding buffer (Qiagen, Hilden, Germany) for each well analysed. The solution was dispensed in the wells containing the PCR product and placed in a thermal mixer at RT for 10 minutes. The aneling solution's final volume was 40µl, comprised of 2.25µM High-performance liquid chromatography (HPLC) sequence primer (IDT, Coralville, USA) in 39.1µl of the aneling buffer. The vacuum prep workstation (Biotage, Uppsala, Sweeden) for each 96-well plate was composed of 100ml of 70% ethanol, denaturation solution (Qiagen, Hilden, Germany), 1x wash buffer (Qiagen, Hilden, Germany), and nuclease-free water (Qiagen, Hilden, Germany). Using the vacuum tool, the PCR processed in the workstation was dispensed into the aneling solution and placed at 80°C for 2 minutes. The volumes for dATP, dGTP, dTTP, dCTP, substrate, and enzyme (PyroMark Gold Q96 Reagents, Qiagen, Hilden, Germany) were pipetted according to manufactures recommendation, and their respective volume was based on the plate template and target interest sequence inserted in the CpG islands software 1.01 (Qiagen, Hilden, Germany). Using the combined average data, the overall LINE-1 DNA methylation values were calculated as the mean of the proportions of C (%) at the 4 CpG sites analysed, (positions +306, +318, +321, and +328) and this indicated the level of methylation of LINE-1 elements (Figure 18).

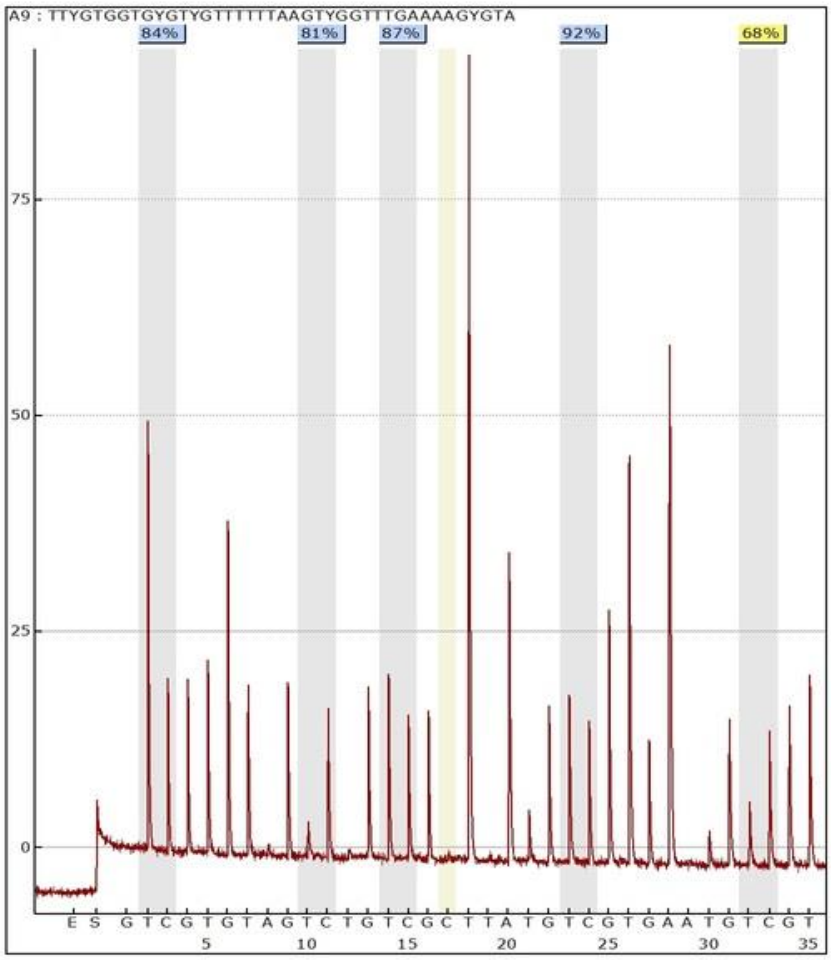


Figure 18. Pyrogram of 5 CpG islands. Nucleotide dispensation is indicated above of pyrogram. After the 3rd CpG island site, a bisulfite nucleotide control was inserted to ensure standard quality for all samples. Due to a lack of information, the last CpG island percentual value was excluded and the mean percentual was recalculated for 4 CpG islands.

2.10. Inflammatory Systemic Profile Measurement

Multiple biomarkers can be detected at once thanks to Luminex multiplex technology. This technique utilizes colour-coded superparamagnetic beads covered with analyte-specific antibodies. Various target analytes are mixed with the beads and incubated with the sample. A combination of biotinylated detection antibodies and a streptavidin-phycoerythrin conjugate are then used to detect the captured analytes. The steps are:

1- The sample is mixed with coloured beads, which are internally dyed with red and infrared fluorophores of differing intensities, and that have already been pre-coated with capture antibodies specific to the analyte. The target analytes are bound by the antibodies (Figure 19).

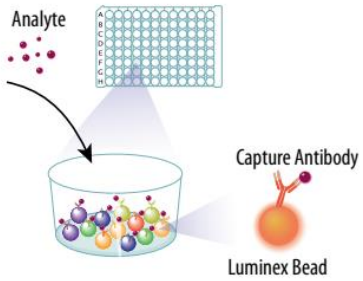


Figure 19. Stage 1 Luminex multiplex immunoassay.

2- After an analyte from a test, the sample is captured by the bead, and a biotinylated detection antibody is introduced, forming an antibody-antigen sandwich. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere (Figure 20).

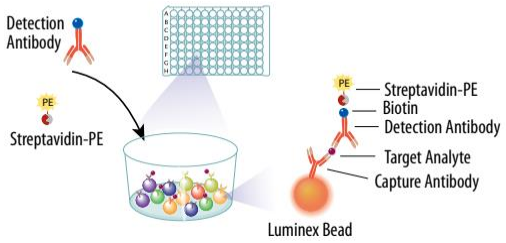


Figure 20. Stage 2 Luminex multiplex immunoassay.

3- Dual-laser flow-based detection equipment is used to read the beads. The classification of the bead by one laser identifies the analyte being detected. The magnitude of the PE-derived signal, which is directly proportional to the volume of the analyte bound, is determined by the second laser. The Luminex MAGPIX® analyser can scan magnetic beads in addition to flow-based analysers. The magnetic beads are captured and held in a monolayer by a magnet in the MAGPIX analyser while being illuminated by two spectrally separate light-emitting diodes. The analyte being detected is identified by one

LED, and the strength of the PE-derived signal is determined by the second LED (Figure 21).

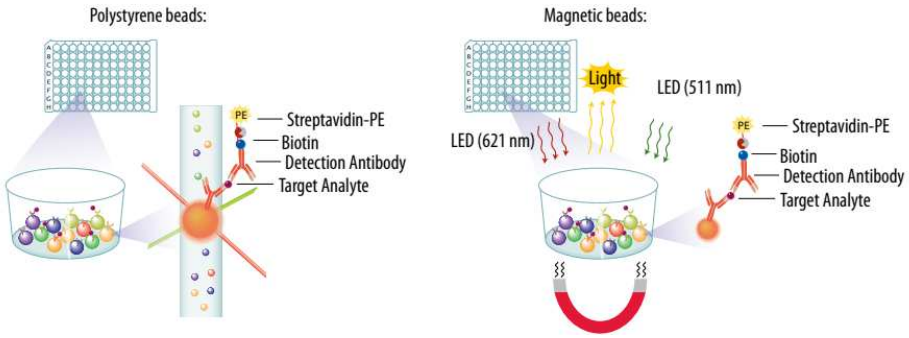


Figure 21. Stage 3 Luminex multiplex immunoassay.

A volume of 25µl/well of plasma was analysed with a Milliplex® Human High Sensitivity T Cell Magnetic Bead Panel, #catHSTCMAG-28SK (Merck, Darmstadt, Germany) for IL-6 (bead 57), IL-23 (bead 54), IL-17A (bead 39) and IL-10 (bead 27), using a multiplex MAGPIX™ (Luminex XMAP Technology, Austin, USA) analyser. The four analytes were simultaneously detected in a 96-well plate. According to the manufacturer's recommendations, 25 µL/well of the bead mixture containing the four analytes was added to the plate. Sample and antibody-coated capture beads were mixed and incubated at 4 °C, overnight (protected from light). The next day, the plate was incubated at RT for 30 minutes with shaking at 500 rpm. The plate was washed three times using a hand-held magnetic separation block (Merck, Darmstadt, Germany) to retain the beads. Washed beads were further incubated with biotin-labelled anti-human cytokine antibodies for 60 minutes at RT with shaking at 500 rpm. Followed by streptavidin-phycoerythrin incubation for 30 minutes at RT with shaking at 500 rpm. The plate was washed three times using a hand-held magnetic separation block (Merck, Darmstadt, Germany) to retain the beads. In the end, 150µl/well of drive fluid PLUS (Merck, Darmstadt, Germany) was added, and in sequence, the plate was read on the MAGPIX instrument. Samples were assayed in duplicate to ensure quality control, and reproducibility of the results, the final value was set as the mean of the two

values found, and samples with discrepancies were repeated or excluded in case of equivalent results.

2.11. Fibrin Bio-Polymerization *in vitro* Model

Due to Fibrinogen's important role in adhesion between the placenta and the uterus, and it is significance for maintaining pregnancy, fibrin gels scaffolds were performed. Cases and controls of fibrin gel scaffold were investigated based on the combination of circulating levels of fibrinogen and genotype for XIIIa V34L (rs5985). In total, 4 cases and 4 controls were selected, two of them were wild type (G/G genotype) and two polymorphic (T/T genotype) (Table 2).

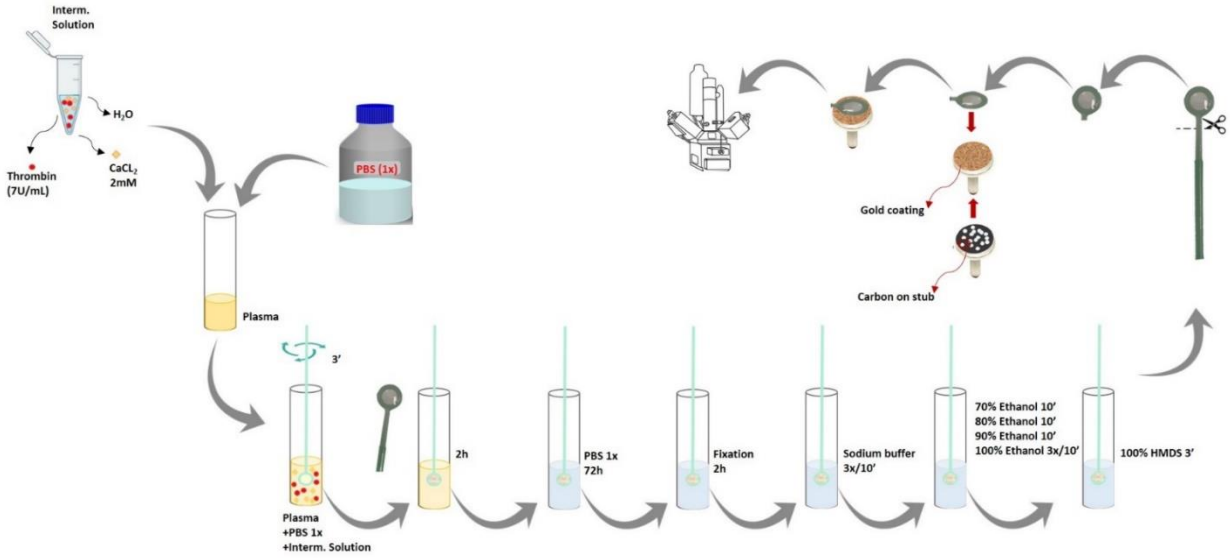
Table 2. Summary of selected cases and controls

ID		V34L	IL-6 (pg/mL)	Fibrinogen (mg/dl)
Cases	1	GG	0.77	331
	2	GG	2.32	362
	3	TT	2.21	314
	4	TT	1.7	309
Controls	5	GG	2.33	365
	6	GG	1.96	292
	7	TT	2.1	386
	8	TT	1.37	296

The fibrin gel scaffold was prepared in a final volume of 500µl, in which 116.1µl of plasma, 348.2 of PBS pH 7.4 (1x) (Gibco, ThermoFisher Scientific, Waltham, USA), and 35.7µl of intermediate solution (prepared in a volume of 750µl): containing 2mM of calcium (ThermoFisher Scientific, Waltham, USA) and 7U/mL of bovine thrombin (Werfen, Milan, Italy). The reagents were combined with the diluted plasma in an open tube and roughly mixed with sterile plastic inoculation loops of 2mm (Looplast, Milan, Italy) for 3 minutes, followed by 2 hours of incubation at RT. After, the inoculation loops were washed with 5mL

of PBS pH 7.4 (1x) (Gibco, ThermoFisher Scientific, Waltham, USA) with daily change for up to 72 hours. The fibrin gel scaffolds were fixed by permeating them with a 2% (v/v) glutaraldehyde solution for 2 hours at RT, followed by 3 washes in 0.1M sodium cacodylate buffer, pH 7.4, each of them with 10 minutes of incubation at RT. Further, they were processed by dehydration using a stepwise ethanol gradient, in which the samples were dehydrated in 70%, 80%, 90%, and free times in 100% ethanol solutions, each of them for 10 minutes. For the critical point in drying, the samples were immersed for 3 min in 100% HMDS (Sigma-Aldrich, St. Louis, USA) after the last 100% ethanol step. After HMDS treatment, the samples were removed from the culture wells and excess HMDS was blotted away by filter paper, and sputter coating with gold-palladium. The fibrin gel scaffold was observed and photographed digitally in at least 6 different areas using a scanning electron microscope (EVO® 40, Zeiss, Oberkochen, Germany) (Figure 22).

Figure 22. Schematic representation of workflow in fibrin gel scaffold preparation



In order, to avoid bias an operator who was unaware of the genotype measured 50 nanofiber diameters and pores size from each micrograph. The images were acquired on 8-bit grayscale, a working distance of 15mm, and in a magnification of 8k, 14k, and 25k for the same targeted region. The images were analysed with ImageJ 1.53q (National Institutes of Health, Maryland, USA), and the scale of the images was set to 1μm. Filter lookup tables (LUTs) to highlight features of interest in greyscale images were applied, and 50 nanofibers were measured for the diameter, while properly identifying the areas counted to avoid miscounting. To identify the pores, a threshold of a minimum of 84 pixels and a maximum of 255 pixels were applied for all the grayscale images analysed, pores were then processed to find edges, and a composition image was created to confirm the pore's location compatible with the original grey image (Figure 23).

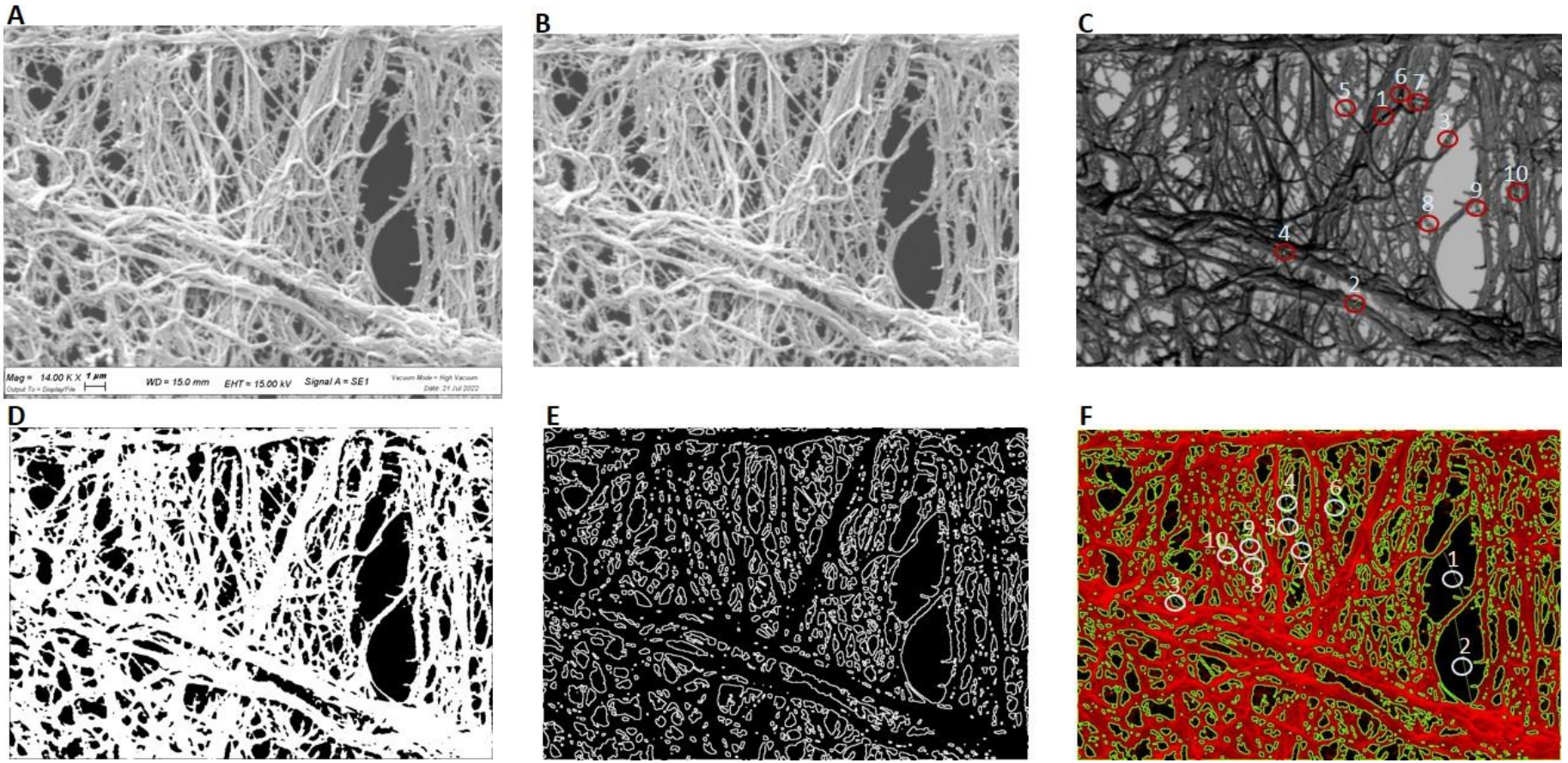


Figure 23. Representation of stepwise to evaluate fibrin gel scaffold. A: an original image was taken with 14k magnification. B: selection of analysed area. C: LUT application and nanofiber diameter count. D: threshold application. E: image processed to find the edges referent to pores. F: Images B and E are combined to overlap pores in the original image, only clearly identified pores were counted, average per image 50 pores.

2.12. Statistics Analysis

Using straightforward descriptive statistics, the comparability of cases and controls was evaluated. Means and standard deviations are used to summarize the baseline characteristics of the cases and controls (or medians and interquartile ranges, if appropriate). For continuous data, such as age, 95% confidence intervals were used provided. Frequency counts and percentages are provided for categorical variables, such as ethnicity. Version 8 of GraphPad Prism was used for the statistical analysis (SPSS Inc., Chicago, USA). Visual assessment of normality curves for variables was undertaken to assess for normality.

Using the chi-square test, deviations from the Hardy-Weinberg equilibrium were taken into account. Hardy-Weinberg equilibrium calculates the expected genotype distribution as $p2+2pq+q2$, where $p2$ is the predicted frequency for homozygosity of the common allele, q the predicted frequency for homozygosity of the rare allele, and $2pq$ is the heterozygotes. This genotype distribution is based on the observed frequency of the rare allele (q) and common allele (p). These frequencies are expected provided the sample is drawn from a population with random mating and no strong selection.

The statistics details were included in the relevant chapter, however, in general analysis of variance (ANOVA) was used to assess the association between genotypes and baseline characteristics on normally distributed data, a 2x2 table was utilized for categorical data and an unpaired t-test for continuous variables for comparing the means of two variables. The correlation analysis was performed with Pearson and linear regression. P-values <0.05 were considered statistically significant.

Chapter 3

Research Results

3.0. Research Results Introduction

The first part of the results covers the demographic and medical clinical characterization findings of cases and controls. These results were crucial to highlight the main general demographic differences within the case group. To achieve this, important information such as age, ethnicity, previous abortion experience for the cases, parity going from 0 to up to 1 or more, body mass index (BMI) and smoking as health habit indicators were analysed. The clinical medical characterization findings were used to express the differences expected in the routine blood tests during an event of abortion (**Section 3.1.**).

In the second part of the results, cases and controls were genetically ascribed based on the individual findings and grouped accordingly. The genes were allocated in sections based on their main role. Thus, all genes and their respective polymorphisms related to coagulation (*F13A1* and *F13B*), folate metabolism (*MTHFR*) and general body metabolism were separated into sections 3.2, 3.3 and 3.4, respectively. The genetic results initially helped to identify the main differences and important polymorphism between cases and controls. Most importantly it targets specific genes associated with abortion risk and protective genetic factors(**Sections 3.2. to 3.4.**).

In the third part of the results, cases and control DNA methylation profiles were shown. The findings were used to investigate the global DNA methylation variation levels between cases and controls. After, demographic data (chronological age) was used to demonstrate the correlation variation between age and methylation, since age is known to be associated with DNA methylation across several sites in the human genome (**Section 3.5.**).

After, the results regarding the systemic levels of cytokines in the advent of abortion and also for healthy controls were investigated. Primarily statistical findings were obtained

between cases and controls, allowing the identification of the circulating inflammatory process during the abortion (**Section 3.6.**).

Further, all the previous information was combined in different approaches. Firstly cases were genetically separated by abortion status, cases with the absence of previous abortion were allocated to the non-previous abortion group ($Cases^1$), and then by previous abortions (women with recurrent episodes of abortion) ($Cases^2$). The results helped to identify which polymorphisms may offer a significant risk or protective role effect according to specific demographic characteristics and how those characteristics can affect genomic studies. Secondly, alongside the previous approach epigenetic and cytokine results were gathered together to identify possible pre-screening variables (**Section 3.7.**).

Finally, an elegant in vitro model demonstrated the fibrin gel scaffold environment in the plasma of cases and controls. This result explored how the coagulation polymorphisms, circulating fibrinogen, the IL-6, and IL-10 cytokines levels differences are prone to interfere with the success of the pregnancy (**Section 3.8.**).

3.1. Exploring the Impact of Health Characterization on Early Pregnancy Loss

Compared to the controls, the cases were older (P=0.0001, Mean: 35.19, SD± 5.55), more often nulliparous (73%, P=0.0001), and more than half of the cases had experienced at least one previous case of spontaneous abortion (56%, P=0.0001). No significant difference was observed between Body Mass Index (BMI), ethnicity, and smokers between cases and controls (Table 1).

Table 1. Selected demographic and clinical characteristics for cases and controls

Characteristics	Cases (n=108)	Control (n=107)
Age*		
≤ 24	6 (5%)	22 (21%)
25-34	34 (31%)	55 (51%)
≥35	68 (63%)	30 (28%)
Ethnicity		
Caucasian	102 (94%)	85 (80%)
Asian	2 (2%)	11 (10%)
African	4 (4%)	11 (10%)
BMI		
Underweight	6 (6%)	5 (4%)
Normal weight	89 (82%)	79 (74%)
Overweight	7 (6%)	17 (16%)
Obesity Class I	6 (6%)	6 (6%)
Smoker		
Yes	29 (27%)	33 (31%)
No	79 (73%)	74 (69%)
Parity*		
0	79 (73%)	6 (6%)
≥1	29 (27%)	101 (94%)
Previous Abortion*		
0	48 (44%)	107 (100%)
≥ 1	60 (56%)	0 (0%)

*significant differences P<0.05

In the whole blood analysis compared to the control, EPL cases had lower levels of circulating lymphocytes (P=0.043) and fibrinogen (P= 0.041); Meanwhile higher levels of the hematocrit test (HTC) (P=0.010) and activated partial thromboplastin time (P=0.0004). No significant differences were observed in total white blood cell count (WBC), neutrophils, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) (Table 2).

Table 2. Selected clinical laboratory analysis for cases and controls

Laboratory Clinical Analysis	**Cases (n=108)** (Mean ± SD)	**Controls (n=107)** (Mean ± SD)	**P value**
WBC (4.00 - 11.00x10^3/µl)	8.13 ± 2.41	7.94 ± 2.23	0.402
Lymphocytes (1.5-5x10^3/µl)	1.98 ± 0.49	2.10 ± 0.51	0.043*
Neutrophiles (2.00 - 7.50x10^3/µl)	5.43 ± 2.28	5.21 ±± 2.05	0.300
RBC (3.8-5.8x10^6/µl)	4.33 ± 0.45	4.43 ± 0.47	0.070
HTC (40-54%)	36.27 ± 3.09	38.59 ± 2.70	0.010*
MCV (76-96fl)	86.67 ± 9.41	87.21 ± 7.41	0.403
MCH (27-32pg)	29.05 ± 2.94	29.06 ± 3.13	0.431
Platelets (150 - 450 x10^3/µl)	264.4 ± 57.78	258.9 ± 56.33	0.246
Fibrinogen (150-400 mg/dl)	332.3 ± 70.78	352.1 ± 76.68	0.041*
PT (Ratio)	1.05 ± 0.088	1.05 ± 0.07	0.364
aPTT (Ratio)	1.01 ± 0.07	0.98 ± 0.08	0.0004*

*significant differences P<0.05

3.2. The Influence of Coagulation Factors Polymorphisms on Miscarriages

The allele frequencies of polymorphisms in the genes *F13A1*, *F13B*, *FGA,* and *FGB* were compared between the cases and controls, and the results are shown in Table 3. A significant difference was observed in the frequency of alleles for SNP rs6003 and rs5985 in the *F13A1* and *F13B* genes, respectively.

For both SNPs, the risk allele seems to be in the major allele instead of the minor. A lower frequency of the minor allele (T allele) was observed in cases for the SNP rs6003 compared to the controls (15% vs 22%), and consequently higher frequency of the major allele (C allele) among cases than in controls (85% vs 78%) (P= 0.037). In the allele frequency of

polymorphism rs5985, it was observed that the major allele (G allele) was more predominant in the cases than in the controls (90% vs 84%); While the minor allele (T allele) was more frequent in controls than in cases (10% vs 16%) (P= 0.020). The remaining SNPs investigated did not show a significant result.

Table 3. Allele frequencies of genes associated with coagulation pathways

Genes and SNPs	**Cases (n=108)**	**Controls (n=107/%)**	**P**
F13A1	**n / %**	**n / %**	
rs5985			
G	183 (85%)	167 (78%)	0.037*
T	33 (15%)	47 (22%)	
rs5982			
G	179 (83%)	165 (77%)	0.067
A	37 (17%)	49 (23%)	
rs3024477			
A	212 (98%)	210 (98%)	0.494
T	4 (2%)	4 (2%)	
F13B	**n/%**	**n/%**	
rs6003			
C	195 (90%)	179 (84%)	0.020*
T	21 (10%)	35 (16%)	
FGA	**n/%**	**n/%**	
rs6050			
T	168 (62%)	159 (74%)	0.199
C	48 (38%)	55 (26%)	
FGB	**n/%**	**n/%**	
rs1800790			
C	175 (81%)	179 (84%)	0.237
T	41 (19%)	35 (16%)	

*significant differences P<0.05

When analysing the genotype distribution of the previous genes and respective SNPs, the results did not show a significant difference (Table 4).

Table 4. Genotype distribution of different polymorphisms between cases and controls

Genes and SNPs	Cases (n=108)	Controls (n=107)	P
F13A1	**n / %**	**n / %**	
rs5985			
GG	78 (72%)	70 (65%)	0.116
GT	27 (25%)	27(24%)	
TT	3 (3%)	10 (11%)	
rs5982			
GG	75 (69%)	64 (61%)	0.32
GA	29 (27%)	37 (34%)	
AA	4 (4%)	6 (5%)	
rs3024477			
AA	104 (96%)	104 (97%)	0.435
AT	4 (4%)	2 (2%)	
TT	0	1 (1%)	
F13B	**n/%**	**n/%**	
rs6003			
CC	89 (82%)	79 (76%)	0.150
CT	17 (16%)	21 (18%)	
TT	2 (2%)	7 (6%)	
FGA	**n/%**	**n/%**	
rs6050			
TT	67 (62%)	62 (58%)	0.693
TC	34 (31%)	35 (32%)	
CC	7 (7%)	10 (10%)	
FGB	**n/%**	**n/%**	
rs1800790			
CC	70 (65%)	76 (71%)	0.492
CT	35 (32%)	27 (25%)	
TT	3 (3%)	4 (4%)	

*significant differences P<0.05

Further genotypes were investigated and combined in the dominant, recessive, and homozygote models summarized in table 5. The first row represents the homozygote model (- - vs - +), the second the dominant model (++/ - + vs - -), and the third the recessive model (+ + vs - - / - +).

According to the results, rs5985 polymorphism on the homozygote model, the wild type G/G genotype was associated with 3 times increased risk for EPL (OR: 3.71, CI: 1.01 to 12.90)

(P= 0.020). Meanwhile, for the same polymorphism, T/T and G/T genotypes, analysed by the recessive model, were associated with a 73% protective role in EPL (OR: 0.27, CI: 0.08 to 0.99) (P=0.039). A 4-fold increased risk among C/C genotype for the rs6003 variant (OR: 3.94, CI: 0.83 to 19.10) (P= 0.036) was observed. The different genetic models did not show a significant difference for the other polymorphisms. Due to the lack of homozygote polymorphic for the polymorphism rs3024477 odds ratio could be analysed, with an exception of the dominant model.

Table 5. Genetic models applied in cases and controls in essential genes for the coagulation cascade

Genes and SNPs	P	OR (CI -95%)
F13A1		
rs5985		
GG vs TT	0.020*	3.71 (1.01 to 12.9)
TT + GT vs GG	0.176	0.72 (0.40 to 1.27)
TT vs GG + GT	0.039*	0.27 (0.08 to 0.99)
rs5982		
GG vs AA	0.196	1.75 (0.44 to 5.69)
AA + GA vs GG	0.091	0.65 (0.37 to 1.16)
AA vs GA + GG	0.368	0.64 (0.20 to 2.51)
rs3024477		
AA vs TT	0.159	N/A
TT + AT vs AA	0.505	1.33 (0.35 to 5.38)
TT vs AA + AT	0.497	N/A
F13B		
rs6003		
CC vs TT	0.036*	3.94 (0.83 to 19.1)
TT + CT vs CC	0.087	0.60 (0.31 to 1.16)
TT vs CC + CT	0.083	0.26 (0.05 to 1.26)
FGA		
rs6050		
TT vs CC	0.202	1.54 (0.59 to 4.00)
CC + TC vs TT	0.318	0.84 (0.48 to 1.45)
CC vs TT + TC	0.300	0.67 (0.25 to 1.79)
FGB		
rs1800790		
CC vs TT	0.396	1.22 (0.32 to 5.00)
TT + CT vs CC	0.203	1.33 (0.76 to 2.35)
TT vs CC + CT	0.494	0.73 (0.18 to 2.80)

The first model represents the homozygote model; The second represents the dominant model; The third represents the recessive model; N/A: not applicable; +: major allele; -: minor allele; *significant differences P<0.05.

3.3. The Association of Maternal One-Carbon Genes and Pregnancy

The allele frequencies of polymorphisms in the *MTHFR* gene were compared between the case and control groups (Table 6). A significant difference was observed in the frequency of alleles in the rs1801133 polymorphism. The minor allele frequency (T allele) was 2 times more represented in cases than in the controls (57% vs 32%), and consequently lower frequency of the major allele among cases than in controls (C allele) (43% vs 68%) (P= 0.0001).

Table 6. Allele frequencies of genes associated with one-carbon gene pathways in cases and controls

Genes and SNPs	**Cases (n=108)**	**Controls (n=107)**	**P**
MTHFR	**n / %**	**n / %**	
rs1801133			
C	92 (43%)	145 (68%)	
T	124 (57%)	69 (32%)	0.0001*
rs1801131			
A	151 (70%)	151 (71%)	
C	65 (30%)	63 (29%)	0.441

*significant differences P<0.05

When analysing the genotype distribution of the previous genes and respective SNPs, a significant difference was observed in the genotype distribution of the polymorphism rs1801133. Overall cases had an increased frequency of C/T (48% vs 35%) and T/T (18% vs 15%) genotypes. This led to 1.5 times less representation of the C/C genotype among

cases (33% vs 50%) (P=0.05) (Table 7). While no significant difference was seen in the rs1801131 polymorphism.

Table 7. Genotype distribution in cases and control of the one-carbon gene pathway

Genes and SNPs	Cases (n=108)	Controls (n=107)	P
MTHFR	**n / %**	**n / %**	
rs1801133			
CC	36 (33%)	53 (50%)	0.05*
CT	52 (48%)	39 (35%)	
TT	20 (18%)	15 (15%)	
rs1801131			
AA	55 (51%)	52 (47%)	0.525
AC	41 (38%)	47 (44%)	
CC	12 (11%)	8 (9%)	

*significant differences P<0.05

As the next step, the genotypes were combined in the same genetic models previously described (section 3.2). The C/C genotype for rs1801133 polymorphism had a 50% protective role (OR: 0.50; CI: 0.23 to 1.14) (P= 0.046). While for the same polymorphism, the C/T and T/T genotypes analysed in the dominant model had 1.3 times more risk for EPL (OR: 1.96; CI: 1.13 to 3.46) (P=0.011) (Table 8). No significant difference was seen in the rs1801131 polymorphism.

Table 8. Genetic models in one-carbon gene pathway approach for cases and controls

Genes and SNPs	P	OR (CI -95%)
MTHFR		
rs1801133		
CC vs TT	0.046*	0.50 (0.23 to 1.14)
TT + CT vs CC	0.011*	1.96 (1.13 to 3.46)
TT vs CC + CT	0.239	1.39 (0.65 to 2.93)
rs1801131		
AA vs CC	0.205	0.66 (0.25 to 1.73)
CC + AC vs AA	0.526	1.01 (0.60 to 1.71)
CC vs AC + AA	0.248	1.54 (0.63 to 3.86)

The first model represents the homozygote model; The second represents the dominant model; The third represents the recessive model; *significant differences P<0.05

3.4. Metabolic Genes Traits Effect on Risk of EPL

Table 9 is composed of key genes associated with metabolic functions, such as inflammation, oxidative stress, lipid transportation, blood pressure regulation, and electrolyte balance. The genes studied were *CRP*, *CFH*, *ACE1*, *ACE2*, *MMP12*, *TP53,* and *APOE*, and 9 polymorphisms were selected.

There was a statistically significant difference in the distribution of minor (A allele) (35% vs 51%) and major (G allele) (65% vs 49%) alleles between cases and controls for the polymorphism rs2285666 in the gene *ACE2* (P=0.0003). The same significant difference was seen for the polymorphisms rs1042522 and rs7412, in the *TP53* and *APOE* genes, respectively. The major allele (C allele) frequency for the polymorphism rs1042522 showed an increased proportion among cases than in controls (77% vs 70%) (P= 0.05), and a lower frequency for the minor allele (G allele) (23% vs 30%). For rs7412 cases had 2 times less frequency of the minor allele (T allele) compared to the controls (5% vs 10%) (P=0.021), and a 1-fold increase of the major allele (C allele) (95% vs 90%).

Table 9. Allele frequencies distribution in genes associated with systemic metabolism in cases and controls

Genes and SNPs	**Cases (n=108)**	**Controls (n=107)**	**P**
CRP	**n / %**	**n / %**	
rs876538			
C	163 (75%)	167 (78%)	0.263
T	53 (25%)	47 (22%)	
rs2808635			
T	152 (70%)	147 (69%)	0.352
G	64 (30%)	67 (31%)	
CFH			
rs1061170			
T	133 (62%)	136 (64%)	0.335
C	83 (38%)	78 (36%)	
ACE1			
rs1799752			

D	131 (61%)	132 (62%)	0.22
I	85 (39%)	82 (38%)	
ACE2			
rs2285666			
G	141 (65%)	105 (49%)	0.0003*
A	75 (35%)	109 (51%)	
MMP12			
rs2276109			
T	180 (83%)	185 (86%)	0.183
C	36 (17%)	29 (14%)	
TP53			
rs1042522			
C	166 (77%)	150 (70%)	0.05*
G	50 (23%)	64 (30%)	
APOE			
rs429358			
T	195 (90%)	197 (92%)	0.258
C	21 (10%)	17 (8%)	
rs7412			
C	205 (95%)	192 (90%)	0.021*
T	11 (5%)	22 (10%)	

*significant differences P<0.05

The information regarding the genotype findings is shown in table 10. A significant difference was observed in the polymorphisms rs2285666 and rs7412 in the *ACE2* and *APOE* genes, respectively. When analysing *ACE2* gene polymorphism cases had 50% of G/G genotype and controls 31%, this represented 1.6 times more frequency of major homozygote allele genotype in cases; Meanwhile a decrease of 1.6 in the frequency of A/A genotype (P=0.010). Statistically, a significant difference was also seen for the rs7412 in the *APOE* gene (P=0.034), this result was further explored in this chapter. No significant difference was observed in the other polymorphisms.

Table 10. Genotype distribution in cases and control of the systemic metabolism-associated genes

Genes and SNPs	Cases (n=108)	Controls (n=107)	P
CRP	**n / %**	**n / %**	
rs876538			
CC	65 (60%)	66 (64%)	0.506
CT	33 (30%)	35 (30%)	
TT	10 (10%)	6 (6%)	
rs2808635			
TT	54 (50%)	55 (52%)	0.447
TG	44(40%)	37 (35%)	
GG	10 (10%)	15 (13%)	
CFH			
rs1061170			
TT	47 (43%)	47 (44%)	0.776
TC	39 (37%)	42 (39%)	
CC	22 (20%)	18 (17%)	
ACE1			
rs1799752			
DD	40 (37%)	38 (34%)	0.665
DI	51 (47%)	56 (53%)	
II	17 (16%)	13 (13%)	
ACE2			
rs2285666			
GG	54 (50%)	33 (31%)	0.010*
GA	33 (30%)	39 (36%)	
AA	21 (20%)	35 (33%)	
MMP12			
rs2276109			
TT	76 (70%)	80 (74%)	0.626
TC	28 (26%)	25 (24%)	
CC	4 (4%)	2 (2%)	
TP53			
rs1042522			
CC	67(62%)	52 (50%)	0.110
CG	32 (30%)	46 (42%)	
GG	9 (8%)	9 (8%)	
APOE			
rs429358			
TT	87 (80%)	90 (84%)	0.837
TC	21 (20%)	17 (16%)	
CC	0	0	
rs7412			
CC	97 (90%)	85 (79%)	0.034*
CT	11 (10%)	22 (21%)	
TT	0	0	

*significant differences P<0.05

All the genetic models performed were statistically significant for *ACE2* gene polymorphism (rs2285666). The results show that in the homozygote model, the G/G genotype represents a risk for EPL (OR: 2.72; CI: 1.39 to 5.36) (P=0.0020). While G/A and A/A genotypes have a protective role (OR: 0.44; CI: 0.25 to 0.32) (P=0.003) in the dominant and recessive models (OR: 0.49; CI: 0.25 to 0.94) (P=0.019). It was also seen in the dominant model a significant difference for the polymorphism rs1042522 in the *TP53* gene, the G/G and G/C genotypes represented a protection of 43% for EPL (OR: 0.5786; CI: 0.3347 to 0.9834) (P=0.0324). No significant difference was observed in the other polymorphisms.

Table 11. Genetic models applied in cases and control of the systemic metabolism genes

Genes and SNPs	**P**	**OR (CI -95%)**
CRP		
rs876538		
CC vs TT	0.165	0.59 (0.20 to 1.60)
TT + CT vs CC	0.466	1.05 (0.61 to 1.84)
TT vs CC + CT	0.224	1.71 (0.64 to 4.89)
rs2808635		
TT vs GG	0.194	1.47 (0.60 to 3.60)
GG + GT vs TT	0.472	1.08 (0.62 to 1.78)
GG vs TT + GT	0.190	0.62 (0.27 to 1.42)
CFH		
rs1061170		
TT vs CC	0.232	1.32 (0.64 to 2.83)
CC + CT vs TT	0.475	1.05 (0.62 to 1.79)
CC vs TT + CT	0.249	0.73 (0.82 to 1.07)
ACE1		
rs1799752		
DD vs II	0.126	1.60 (0.69 to 3.73)
II + DI vs DD	0.464	0.93 (0.53 to 1.64)
II vs DD + DI	0.287	1.35 (0.61 to 2.88)
ACE2		
rs2285666		
GG vs AA	0.002*	2.72 (1.39 to 5.36)
AA + GA vs GG	0.003*	0.44 (0.25 to 0.32)
AA vs GG + GA	0.019*	0.49 (0.25 to 0.94)
MMP12		
rs2276109		
TT vs CC	0.194	0.47 (0.08 to 2.09)

CC + TC vs TT	0.284	1.24 (0.67 to 2.21)
CC vs TT + TC	0.345	2.01 (0.46 to 10.7)
TP53		
rs1042522		
CC vs GG	0.308	1.28 (0.50 to 3.30)
GG + GC vs CC	0.032*	0.57 (0.33 to 0.98)
GG vs CC + GC	0.588	0.98 (0.40 to 2.42)

The first model represents the homozygote model; The second represents the dominant model; The third represents the recessive model; *significant differences P<0.05

Regarding the *APOE* gene, the two polymorphisms (rs429358 and rs7412) were further combined as previously described (1.6.2. B) (Figure 1). The combination of both SNPs allowed haplotype identification. The results showed that 17% (n=18) of the controls had at least one allele for apopoliprotein E2, while cases had 7% (n=7). Meanwhile, the number of females who had EPL carrying at least one apopoliprotein E4 allele was 19% (n=21) and the controls 16% (n=17). When analysed statistically, carriers of haplotype E2 had 69% of protection for early pregnancy loss (OR: 0.31; CI: 0.11 to 0.96); Meanwhile, carriers of the E4 haplotype had a 3-fold increased risk for EPL (OR: 3.17, CI: 1.03 to 8.78) (P=0.029).

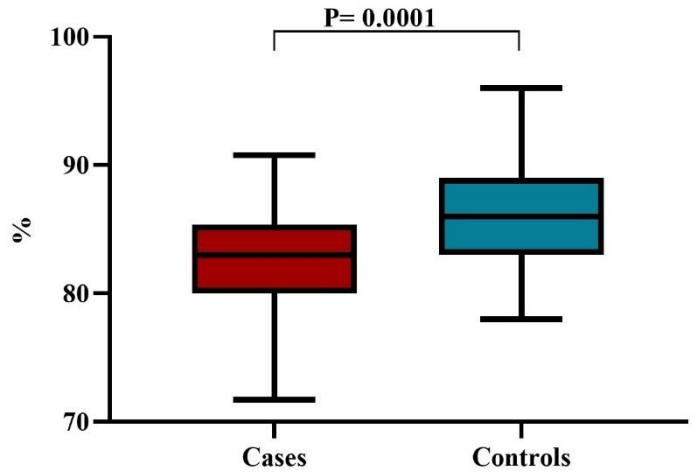
	ApoE 112	ApoE 158
Call	rs429358	rs7412
E2, E2	**TT**	**TT**
E3, E3	**TT**	**CC**
E4, E4	**CC**	**CC**
E2, E3	**TT**	**TC**
E2, E4	**TC**	**TC**
E3, E4	**TC**	**CC**

Figure 1. *APOE* haplotypes call based on the combination of the genotypes information of the polymorphisms rs429358 and rs7412

3.5. The DNA Methylation Profile in EPL

Figure 2 shows a significant statistical difference between the global DNA methylation in cases and controls. The minimum percentage found among controls (n=107) was 78% and in cases (n=108) was 71.70%, the maximum percentage value was 90.8% and 96% for cases

and controls, respectively. Very similar findings were observed for the 25th (80% vs 83%), 75th (85.35% vs 89%) percentile, and median (83% vs 86%) when comparing cases and controls. Despite the slight difference in the mean, overall cases (Mean: 82.49%, SD± 3.46)



had lower percentage levels of methylation than controls (Mean: 85.82%, SD± 3.65) (P=0.0001).

Figure 2. Global DNA methylation analysis between cases and controls. Mean values are represented by the percentage expression for the methylation profile. *Significant differences P<0.05

Since ageing is strongly correlated with changes in DNA methylation, the correlation was performed to evaluate the association between age and methylation profile in cases and controls. As previously mentioned cases were older than controls. While the cases median was 36 years old (Min=23 and Max= 45), controls were 30 years old (Min=18 and Max= 55), hence a higher mean value among cases (Mean: 35.19 SD± 5.55) than in controls (Mean: 30.47 SD± 6.67). When comparing age versus global DNA methylation the same findings were observed between cases (P=0.001; r= -0.27, CI: -0.44 to -0.08) (Figure 3. A) and controls (P=0.0001; r= -0.38, CI: -0.53 to -0.20) (Figure 3. B) in which an increased age is followed by lower percentage levels of methylation, highlighted more evidentially in cases (Figure 3. C).

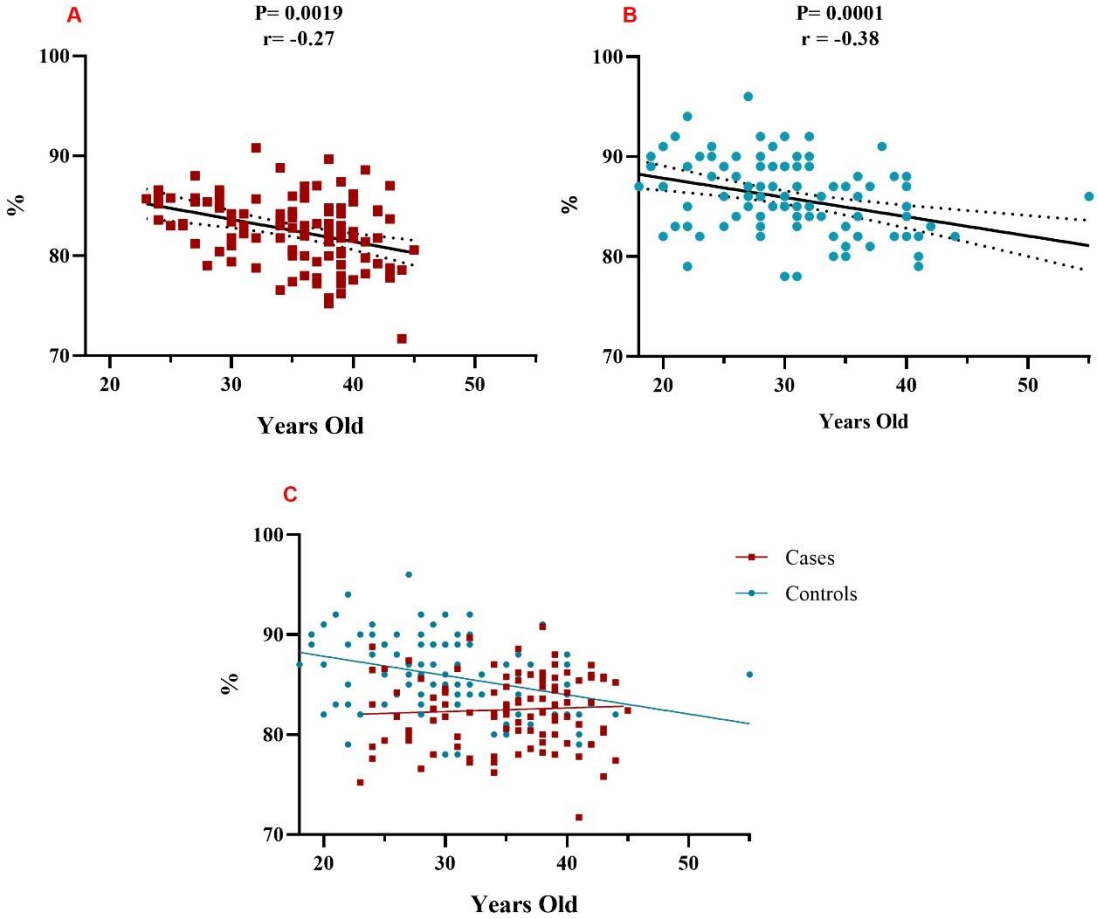


Figure 3. Correlation status of age and global DNA methylation between cases and controls. A: cases (n=108); B: controls (n=107); C: cases and controls combined. Significant differences P<0.05

3.6. Roles of Pro and Anti-Inflammatory Cytokines in the First Trimester

Cytokines have important roles during pregnancy and aberrant expression of pro and anti-inflammatory cytokines contributes toward preterm labour, pre-eclampsia, and gestational diabetes mellitus. Therefore, we analysed the following cytokines IL-10, IL-6, IL-23 and IL-17a. Due to the lack of material for some cases and controls, we were unable to perform cytokines investigation, thus they were not included in the analysis. Table 12 summarises the information regarding the findings for cases (n=92) and controls (n=89).

Table 12. Cytokines analyses for cases and controls main findings.

Statistical Information		IL-10 (pg/mL)	IL-23 (pg/mL)	IL-17a (pg/mL)	IL-6(pg/mL)
Cases (n=92)	Min-Max	0.81-90.55	40.75-2053	1.28-39.71	0.11-15.44
	25th-75th Percentile	10.25-32.70	281.1-809.6	8.83-15.96	2.38-7.13
	Median	19.67	506.3	12.69	3.93
	Mean ±SD	24.43 ± 18.59	595.6 ± 411.5	13.34 ± 6.47	4.97 ± 3.33
Controls (n=89)	Min-Max	0.60-76.38	24.06-1708	1.25- 30.33	0.1-15.28
	25th-75th Percentile	7.65-26.69	216-605.5	7.18-15.80	1.46-4.74
	Median	14.63	397.7	9.77	3.36
	Mean ±SD	19.46 ± 15.99	455.8 ± 307.7	11.23 ± 6.42	3.83 ± 3.02
P value		0.038*	0.018*	0.025*	0.018*

*significant differences P<0.05

As shown in the previous table (Table 12), cases had higher overall levels of pro and anti-inflammatory cytokines when compared to controls (Figure 4). We observed significantly higher levels of anti-inflammatory cytokine IL-10 (Mean: 19.67 pg/mL vs 14.63 pg/mL; P=0.038) (Figure 4. A), as well as pro-inflammatory cytokines IL-17a (Mean: 12.69 pg/mL vs 9.77 pg/mL; P=0. 025) (Figure 4. B), IL-23 (Mean: 506.3 pg/mL vs 397.7 pg/mL; P=0.018) (Figure 4. C), and IL-6 (Mean: 3.93 pg/mL vs 3.36 pg/mL; P=0.018) (Figure 4. D) in EPL women compared to controls.

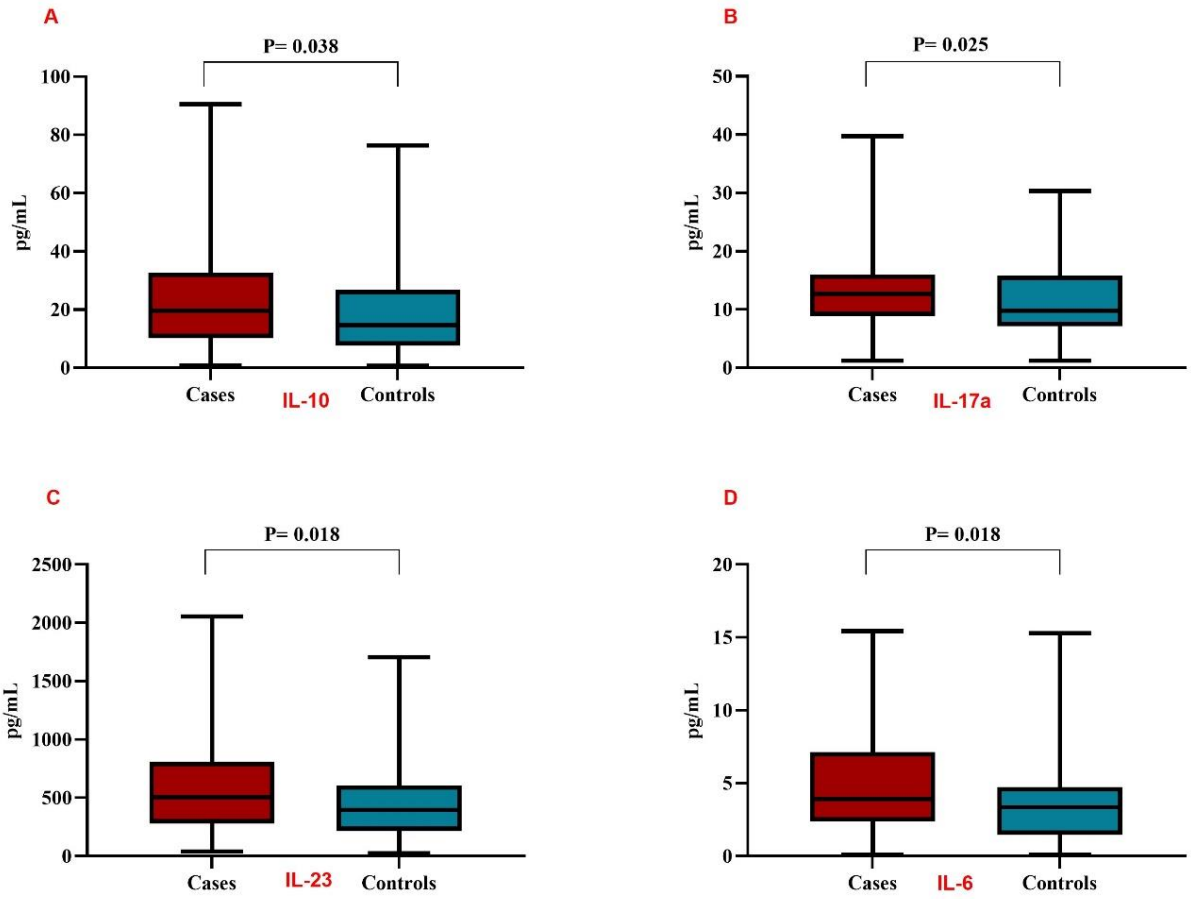


Figure 4. Cytokines expression in cases and controls. A: IL-10. B: IL-17a. C: IL-23. D: IL-6. *significant differences P<0.05

Further cytokines, age, and methylation were combined to evaluate multivariable correlation. Cases are represented in figure 5. A, as demonstrated in the graph cytokines are strongly positively correlated to each other, especially IL-23 and IL-10 (r= 0.90, P=0.0001), IL-6 and IL-23 (r= 0.85, P=0.0001), IL-6 and IL-10 (r= 0.83, P=0.0001), IL-6 and IL-17a (r= 0.79, P=0.0001), IL-23 and IL-17a (r= 0.71, P=0.0001), IL-17a and IL-10 (r= 0.71, P=0.0001). A slightly lower correlation was also seen between IL-6 and age (r= 0.17, P=0.05), and a negative correlation between age and methylation (r= -0.26, P=0.007). Figure 5. B shows the findings for controls, as well as in cases, had a strong positive correlation within cytokines, IL-23 and IL-10 (r= 0.90, P=0.0001), IL-6 and IL-10 (r= 0.90, P=0.0001), IL-6 and IL-23 (r= 0.87, P=0.0001), IL-6 and IL-17a (r= 0.86, P=0.0001), IL-17a and IL-10

(r= 0.81, P=0.0001), IL-23 and IL-17a (r= 0.79, P=0.0001). Controls cytokines IL-10 and IL-17a were also slightly negatively correlated with methylation (r= -0.17, P=0.05, for each), and with age (r=-0.24, P= 0.012) (Figure 5).

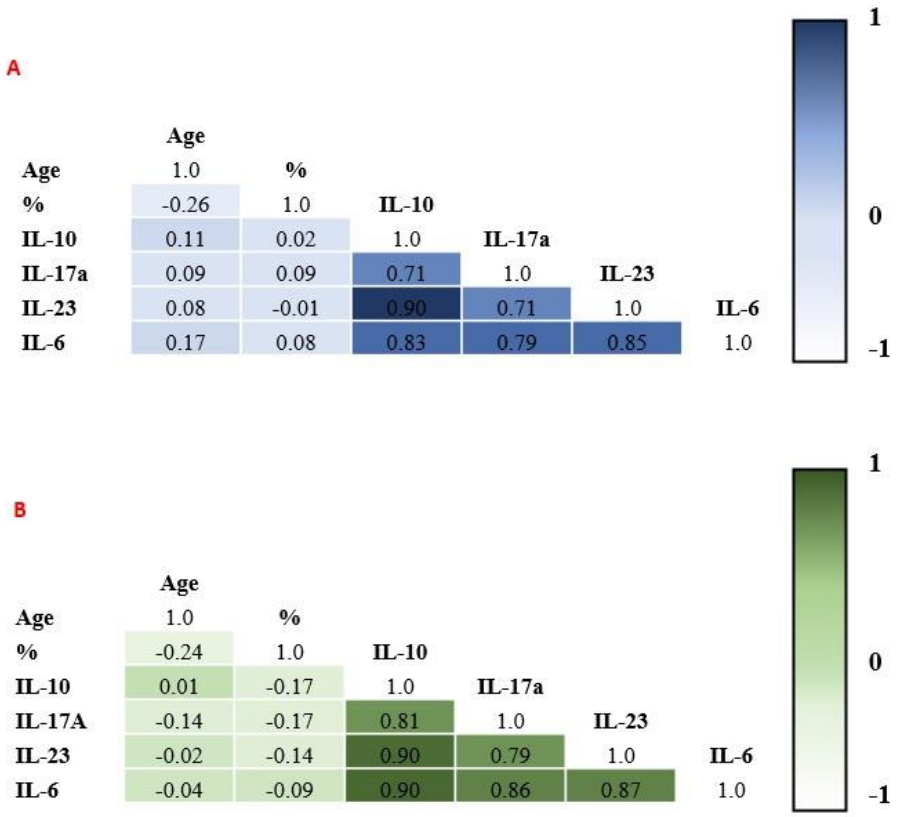


Figure 5. Correlation profile of multivariable. A: represent the r value for cases age, methylation (%), IL-6, IL-10, IL-23 and IL-17a variables. B: represent the r value for controls age, methylation (%), IL-6, IL-10, IL-23 and IL-17a variables.

3.7. Genetic, Epigenetic, and Systemic Holistic Analysis in EPL

Cases were further separated into two groups, Cases[1] representing cases with non-previous abortions and recurrent abortions (one or more previous abortion episodes) were identified as Cases[2]. This was performed to identify subcases related differences for demographic, clinical laboratory analysis, genetic, epigenetic, and inflammatory profiles. For instance, cases[2] were, in general, older (Mean: 35.19, SD± 5.55) (P=0.017) than cases[1] (Mean: 33.76, SD± 4.92), and controls (Mean: 30.47, SD± 6.67) (P=0.0001). Also, cases [1] were older than the controls (P=0.0009) (Table 13).

Table 13. Intracases analysis of demographic characteristics

Characteristics	Cases[1] (n=48)	Cases[2] (n=60)	Control (n=107)	Cases[1] vs Controls	Cases[2] vs Controls	Cases[1] vs Cases[2]
Age						
≤ 24	1 (2%)	5 (8%)	22 (21%)	0.0009*	0.0001*	0.017*
25-30	20 (42%)	14 (24%)	27 (25%)			
≥35	27 (56%)	41 (68%)	58 (54%)			
Ethnicity						
Caucasian	47 (98%)	55 (92%)	85 (80%)	0.01*	0.113	0.312
Asian	0 (0%)	2 (3%)	11 (10%)			
African	1 (2%)	3 (5%)	11 (10%)			
BMI						
underweight	4 (8%)	2 (3%)	5 (5%)	0.49	0.198	0.558
Normal weight	38 (80%)	51 (85%)	79 (73%)			
Overweight	4 (8%)	3 (5%)	17 (16%)			
Obesity Class I	2 (4%)	4 (7%)	6 (6%)			
Smoker						
Yes	12 (25%)	17 (28%)	33 (31%)	0.458	0.734	0.697
No	36 (75%)	43 (72%)	74 (69%)			
Parity						
0	29 (60%)	50 (83%)	6 (6%)	0.0001*	0.0001*	0.0038*
≥1	19 (40%)	10 (17%)	101 (94%)			

*significant differences P<0.05

Forward in the re-analysis of clinical laboratory findings, it was observed that cases[1] (Mean: 37.98, SD± 3.48) (P=0.013) and cases[2] (Mean: 36.27, SD± 2.74) (P=0.05) had lower levels

of HTC compared to controls (Mean: 38.59, SD± 2.70). Cases[2] platelets were lower (Mean: 255.1, SD± 56.80) compared to cases[1] (Mean: 276.0 ± 57.03) (P=0.003). Controls fibrinogen levels (Mean: 352.1, SD± 76.6) were increased compared to cases[2] (Mean: 322.3, SD ± 61.21) (P=0.021). Meanwhile, activated partial thromboplastin time was higher among cases[2] (Mean: 1.01, SD± 0.07) than in controls (Mean: 0.98, SD± 0.08) (P=0.001).

Table 14. Differences between laboratory clinical findings

Laboratory Clinical Analysis	Cases[1] (n=48)	Cases[2] (n=60)	Control (n=107)	Cases[1] vs Controls	Cases[2] vs Controls	Cases[1] vs Cases[2]
CBC (4.00 - 11.00x10^3/µl)	7.97 ± 2.04	8.27 ± 2.67	7.94 ± 2.23	0.49	0.341	0.331
Lymphocytes (1.5-5x10^3/µ)	1.97 ± 0.50	1.99 ± 0.49	2.10 ± 0.51	0.063	0.073	0.477
Neutrophiles (2.00 - 7.50x10^3/µl)	5.27 ± 1.92	5.55 ± 2.55	5.21 ±± 2.05	0.368	0.277	0.363
RBC (3.8-5.8x10^6/µl)	4.46 ± 0.47	4.33 ± 0.44	4.43 ± 0.47	0.069	0.121	0.353
HTC (40-54%)	37.98 ± 3.48	36.27 ± 2.74	38.59 ± 2.70	0.013*	0.05*	0.204
MCV (76-96fl)	86.31 ± 11.07	86.97 ± 7.97	87.21 ± 7.41	0.408	0.45	0.465
MCH (27-32pg)	29.21 ± 2.90	28.92 ± 2.99	29.06 ± 3.13	0.344	0.437	0.305
Platelets (150 - 450 x10^3/µl	276.0 ± 57.03	255.1 ± 56.80	258.9 ± 56.33	0.05*	0.344	0.033*
Fibrinogen (150-400 mg/dl)	344 ± 80.60	333 ± 61.21	352.1 ± 76.68	0.304	0.021*	0.092
PT (Ratio)	1.03 ± 0.07	1.06 ± 0.09	1.05 ± 0.07	0.206	0.352	0.136
aPTT (Ratio)	1.01 ± 0.07	1.01 ± 0.07	0.98 ± 0.08	0.008	0.001*	0.347

Cases[1]: stands for women in the cases group with non-previous abortion experience (n=48); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes (n=60); Controls: health women with no previous abortion advent (n=107); *significant differences P<0.05.

*significant differences P<0.05

Table 15 summarizes the allelic frequency of the genes studied stratified by intracase. When analysing cases[1] and controls the major allele had a protective role for the polymorphisms rs1800790 (C allele) (P=0.013) and rs1801133 (C allele) (P=0.021); While a risk factor for the variants rs6003 (P=0.015), rs2285666 (P=0.000), and rs10442522 (P=0.039). It was observed a 1.61-fold risk increase between cases[2] and controls (P=0.038) for rs10442522 polymorphism, and 1.70-fold for rs5985 (P=0.041). Interestingly, after analysing cases [1] and cases[2] for rs1800790 polymorphisms in the *FGB* gene, it was observed that the major allele played a 2-fold risk for EPL (P=0.018).

Table 15. Major and Minor allele re-analyses based on the abortion status

	Cases[1]		Cases[2]		Controls		Cases[1] vs Controls	Cases[2] vs Controls	Cases[2] vs Cases[1]
Ref. Seq.	**Major Allele**	**Minor Allele**	**Major Allele**	**Minor Allele**	**Major Allele**	**Minor Allele**	**P**	**P**	**P**
rs5985	80 (83%)	16 (17%)	103 (86%)	17 (14%)	167 (78%)	47 (22%)	0.142	0.041*	0.507
rs5982	78 (81%)	18 (19%)	101 (84%)	19 (16%)	165 (77%)	49 (23%)	0.206	0.062	0.285
rs3024477	95 (99%)	1 (1%)	117 (98%)	3 (2%)	210 (98%)	4 (2%)	0.296	0.349	0.214
rs6003	89 (93%)	7 (7%)	106 (88%)	14 (12%)	179 (84%)	35 (16%)	0.015*	0.122	0.140
rs6050	70 (73%)	26 (27%)	98 (82%)	22 (18%)	159 (74%)	55 (26%)	0.797	0.074	0.062
rs1800790	71 (74%)	25 (26%)	104 (87%)	16 (13%)	179 (84%)	35 (16%)	0.045*	0.062	0.018*
rs1801133	52 (54%)	44 (46%)	72 (60%)	48 (40%)	145 (68%)	69 (32%)	0.021*	0.153	0.389
rs1801131	73 (76%)	23 (24%)	78 (65%)	42 (35%)	151 (71%)	63 (29%)	0.319	0.293	0.078
rs876538	76 (79%)	20 (21%)	87 (73%)	33 (27%)	167 (78%)	47 (22%)	0.362	0.255	0.257
rs2808635	72 (75%)	24 (25%)	80 (67%)	40 (33%)	147 (69%)	67 (31%)	0.259	0.703	0.182
rs1061170	64 (67%)	32 (33%)	69 (58%)	51 (42%)	136 (64%)	78 (36%)	0.596	0.275	0.168
rs1799752	64 (67%)	32 (33%)	67 (56%)	53 (44%)	132 (62%)	82 (38%)	0.400	0.296	0.105
rs2285666	68 (71%)	28 (29%)	73 (61%)	47 (39%)	105 (49%)	109 (51%)	0.000*	0.038*	0.125
rs2276109	79 (82%)	17 (18%)	101 (84%)	19 (16%)	185 (86%)	29 (14%)	0.341	0.568	0.713
rs1042522	78 (81%)	18 (19%)	88 (73%)	32 (27%)	150 (70%)	64 (30%)	0.039*	0.530	0.175

Cases[1]: stands for women in the cases group with non-previous abortion experience (n=48); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes (n=60); Controls: health women with no previous abortion advent (n=107); *significant differences P<0.05.

When analysing Cases[1] vs controls a statistical difference was observed in the polymorphism rs1801133 (P=0.013), rs2808635 (P=0.050), rs2285666 (P=0.006). When investigating cases[2] vs controls a significant difference was observed in the polymorphism rs876538 (P=0.045). Interestingly, cases[1] compared cases[2] for the polymorphism in the *FGB* gene (rs1800790), cases[1] had considerably more frequency (46%) of the minor allele (T allele) than cases[2] (27%) (P=0.036) (Table 16).

Table 16. Cases and controls genetic analyses after clinical stratification

Genes and SNPs	Cases[1] (n=48)	Cases[2] (n=60)	Controls (n=107)	Cases[1] vs Control	Cases[2] vs Controls	Cases[1] vs Cases[2]
F13A1	**n/%**	**n/%**	**n/%**	**P**	**P**	**P**
rs5985						
GG	33 (69%)	45 (75%)	70 (65%)	0.257	0.265	0.639
GT	14 (29%)	13 (22%)	27 (25%)			
TT	1 (2%)	2 (3%)	10 (10%)			
rs5982						
GG	33 (69%)	42 (70%)	64 (61%)	0.494	0.284	0.442
GA	12 (25%)	17 (28%)	37 (34%)			
AA	3 (6%)	1 (2%)	6 (5%)			
rs3024477						
AA	47 (98%)	57 (95%)	104 (97%)	0.798	0.398	N/A
AT	1 (2%)	3 (5%)	2 (2%)			
TT	0 (0%)	0 (0%)	1 (1%)			
F13B	**n/%**	**n/%**	**n/%**			
rs6003						
CC	42 (88%)	47 (78%)	79 (74%)	0.153	0.365	0.396
CT	5 (10%)	12 (20%)	21 (20%)			
TT	1 (2%)	1 (2%)	7 (6%)			
FGA	**n/%**	**n/%**	**n/%**			
rs6050						
TT	25 (52%)	42 (70%)	62 (58%)	0.514	0.304	0.120
TC	20 (42%)	14 (23%)	35 (32%)			
CC	3 (6%)	4 (7%)	10 (10%)			
FGB	**n/%**	**n/%**	**n/%**			
rs1800790						
CC	26 (54%)	44 (73%)	76 (71%)	0.122	0.316	0.036*
CT	19 (40%)	16 (27%)	27 (25%)			
TT	3 (6%)	0 (0%)	4 (4%)			
MTHFR	**n/%**	**n/%**	**n/%**			
rs1801133						
CC	12 (25%)	24 (40%)	53 (50%)	0.013*	0.419	0.148
CT	28 (58%)	24 (40%)	39 (35%)			
TT	8 (17%)	12 (20%)	15 (15%)			
rs1801131						
AA	28 (58%)	27 (45%)	52 (47%)	0.716	0.232	0.241
AC	17 (36%)	24 (40%)	47 (44%)			
CC	3 (6%)	9 (15%)	8 (9%)			
CRP	**n/%**	**n/%**	**n/%**			
rs876538						
CC	29 (60%)	36 (60%)	66 (64%)			

CT	18 (38%)	15 (25%)	35 (31%)	0.564	0.045*	0.101
TT	1 (2%)	9 (15%)	6 (6%)			
rs2808635						
TT	25 (52%)	29 (48%)	55 (52%)	0.050*	0.061	0.931
TG	22 (46%)	22 (37%)	37 (35%)			
GG	1 (2%)	9 (15%)	15 (13%)			
CFH	**n/%**	**n/%**	**n/%**			
rs1061170						
TT	23 (48%)	24 (40%)	47 (44%)	0.936	0.396	0.356
TC	18 (38%)	21 (35%)	42 (39%)			
CC	7 (16%)	15 (25%)	18 (17%)			
ACE1	**n/%**	**n/%**	**n/%**			
rs1799752						
DD	21 (44%)	19 (32%)	38 (34%)	0.62	0.392	0.266
DI	22 (46%)	29 (48%)	56 (53%)			
II	5 (10%)	12 (20%)	13 (13%)			
ACE2	**n/%**	**n/%**	**n/%**			
rs2285666						
GG	27 (56%)	27 (45%)	33 (31%)	0.006*	0.169	0.412
GA	14 (29%)	19 (32%)	39 (36%)			
AA	7 (16%)	14 (23%)	35 (33%)			
MMP12	**n/%**	**n/%**	**n/%**			
rs2276109						
TT	32 (67%)	44 (73%)	80 (74%)	0.574	0.517	0.421
TC	15 (31%)	13 (22%)	25 (24%)			
CC	1 (2%)	3 (5%)	2 (2%)			
TP53	**n/%**	**n/%**	**n/%**			
rs1042522						
CC	32 (67%)	35 (58%)	52 (49%)	0.106	0.244	0.349
CG	14 (29%)	18 (30%)	46 (43%)			
GG	2 (4%)	7 (12%)	9 (8%)			

Cases[1]: stands for women in the cases group with non-previous abortion experience (n=48); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes (n=60); Controls: health women with no previous abortion advent (n=107); *significant differences P<0.05

Table 17 contains the findings for the genetic model's analysis for cases[1] vs controls. The results showed that the G/G genotype for the rs5985 variant in the *F13A1* gene represents a 4.71-fold (P=0.05) and T/T genotype 80% of protection for EPL; The same average protection was observed for the T/T genotype for the polymorphism rs6003 located in *F13B* gene (P=0.028). In the *MTHFR* gene variant rs1801133 the wild-type genotype, C/C, plays a protective role (P=0.05) and the polymorphic genotype, T/T and C/T a risk factor (P=0.002). While the opposite was seen in the *CRP* gene variant rs2808635, the wild-type T/T genotype represented a 6-fold increase in risk (P=0.020), meanwhile, the polymorphic G/G genotype an 87% of protection (P=0.012). The G/G genotype for the variant rs2285666 in the *ACE2* gene is a risk factor (P=0.001) and the A/A is protective (P=0.009). The *TP53* gene polymorphisms in the G/G genotype had a protective role (P=0.018). Cases [2] vs controls analysis demonstrated that the rs876538 C/C genotype played a protective role (P=0.033) and the polymorphic allele T/T a risk factor (P=0.020). The contrary was seen for *ACE2* polymorphism, in which the wild-type genotype (G/G) was a risk factor (P=0.033) and the polymorphic genotype (A/A) as protective (P=0.033).

Intriguing findings were observed when comparing cases[2] vs cases[1]. For instance, the polymorphic allele for rs6050 (C/C genotype) and rs1800790 (T/T genotype) added a protective factor among cases [2] (P=0.028; P=0.019, respectively). Both variants in the *CRP* gene had similar results, the wild-type genotype for rs876538 (C/C) and rs2808635 (T/T) (P=0.018; 0.015, respectively) as protective, and the polymorphic (T/T and G/G, respectively) as risk (P=0.010 for both) for EPL. Likewise, a variant not reported as significant previously, following case stratification appears as a risk factor for EPL rs1061170 (C/C genotype) (P=0.001) for early pregnancy loss.

Table 17. Intracases genetic model analysis

Genes and SNPs	Cases[1] vs Control		Cases[2] vs Controls		Cases[2] vs Cases[1]	
F13A1	**OR (CI)**	**P**	**OR (CI)**	**P**	**OR (CI)**	**P**
rs5985						
GG vs TT	4.71 (0.73 to 52.6)	0.05*	3.21 (0.80 to 15.1)	0.062	1.46 (0.16 to 21.8)	0.378
TT + GT vs GG	0.86 (0.40 to 1.77)	0.342	0.63 (0.30 to 1.24)	0.099	1.16 (0.56 to 2.47)	0.342
TT vs GG + GT	0.20 (0.01 to 1.28)	0.05*	0.33 (0.07 to 1.54)	0.074	0.61 (0.04 to 5.45)	0.347
rs5982						
GG vs AA	1.03 (0.27 to 3.95)	0.478	3.93 (0.59 to 46.1)	0.09	3.82 (0.54 to 50.5)	0.112
AA + GA vs GG	0.67 (0.32 to 1.37)	0.147	0.63 (0.31 to 1.25)	0.094	0.94 (0.43 to 2.14)	0.443
AA vs GA + GG	1.21 (0.29 to 4.20)	0.437	0.28 (0.02 to 1.82)	0.111	0.25 (0.01 to 51.8)	0.105
rs3024477						
AA vs TT	NA	NA	NA	NA	NA	NA
TT + AT vs AA	0.73 (0.05 to 5.06)	0.396	1.82 (0.41 to 7.98)	0.232	2.47 (0.36 to 32.7)	0.212
TT vs AA + AT	NA	NA	NA	NA	NA	NA
F13B						
rs6003						
CC vs TT	3.72 (0.62 to 42.8)	0.098	4.16 (0.70 to 47.8)	0.078	1.12 (0.06 to 21.7)	0.468
TT + CT vs CC	0.40 (0.16 to 0.99)	0.028*	0.78 (0.37 to 1.66)	0.258	1.94 (0.67 to 5.61)	0.106
TT vs CT + CC	0.30 (0.02 to 1.70)	0.123	0.24 (0.02 to 1.43)	0.078	0.79 (0.04 to 15.4)	0.436
FGA						
rs6050						
TT vs CC	1.34 (0.33 to 4.84)	0.335	1.69 (0.51 to 5.14)	0.197	1.26 (0.30 to 5.00)	0.386
CC + TC vs TT	1.26 (0.64 to 2.48)	0.248	0.59 (0.29 to 1.61)	0.061	0.47 (0.22 to 1.04)	0.028*
CC vs TT + TC	0.64 (0.18 to 2.40)	0.260	0.69 (0.23 to 2.20)	0.275	1.07 (0.22 to 3.62)	0.465
FGB						
rs1800790						

CC vs TT	0.45 (0.11 to 1.92)	0.152	NA	NA	NA	NA
TT + CT vs CC	2.16 (1.07 to 4.09)	0.014*	0.89 (0.44 to 1.770	0.375	0.43 (0.20 to 0.98)	0.019*
TT vs CC + CT	1.71 (0.41 to 2.39)	0.243	NA	NA	NA	NA
MTHFR						
rs1801133						
CC vs TT	0.42 (0.15 to 1.32)	0.05*	0.56 (0.24 to 1.36)	0.106	1.33 (0.47 to 4.30)	0.308
TT + CT vs CC	2.94 (1.39 to 6.28)	0.002*	1.41 (0.75 to 2.75)	0.142	0.50 (0.23 to 1.18)	0.051
TT vs CC + CT	1.22 (0.48 to 3.21)	0.334	1.53 (0.65 to 3.47)	0.156	0.80 (0.29 to 2.16)	0.328
rs1801131						
AA vs CC	1.43 (0.38 to 5.30)	0.306	0.46 (0.15 to 1.32)	0.073	0.32 (0.09 to 1.20)	0.051
CC + AC vs AA	0.90 (0.45 to 1.82)	0.391	1.55 (0.80 to 2.90)	0.093	1.71 (0.81 to 3.77)	0.084
CC vs AC + AA	0.82 (0.22 to 2.89)	0.397	2.18 (0.83 to 6.06)	0.061	0.37 (0.10 to 1.32)	0.075
CRP						
rs876538						
CC vs TT	2.63 (0.39 to 31.2)	0.181	0.36 (0.11 to 1.02)	0.033*	0.13 (0.01 to 0.98)	0.018*
TT + CT vs CC	1.48 (0.74 to 2.92)	0.130	1.05 (0.50 to 2.09)	0.441	1.01 (0.46 to 2.65)	0.482
TT vs CC + CT	0.35 (0.03 to 2.31)	0.164	2.97 (1.02 to 8.91)	0.020*	8.29 (1.25 to 92.7)	0.010*
rs2808635						
TT vs GG	6.81 (1.47 to 74.8)	0.020*	0.87 (0.36 to 2.14)	0.393	0.13 (0.01 to 0.96)	0.015*
GG + GT vs TT	0.97 (0.49 to 1.89)	0.468	0.98 (0.52 to 1.85)	0.486	1.16 (0.54 to2.48)	0.349
GG vs TT + GT	0.13 (0.01 to 0.83)	0.012*	1.08 (0.45 to 2.66)	0.431	8.29 (0.01 to 0.80)	0.010*
CFH						
rs1061170						
CC vs TT	1.16 (0.43 to 2.98)	0.384	3.88 (1.42 to 10.94)	0.393	3.34 (1.46 to 7.60)	0.001*
TT + CT vs CC	0.88 (0.42 to 1.74)	0.362	1.22 (0.64 to 2.37)	0.271	1.38 (0.64 to 3.00)	0.204
TT vs CC + CT	0.90 (0.35 to 2.36)	0.417	1.76 (0.84 to 3.94)	0.075	1.95 (0.20 to 1.32)	0.098
ACE1						
rs1799752						

DD vs II	1.43 (0.47 to 4.07)	0.269	0.54 (0.20 to 1.44)	0.103	0.38 (0.12 to 1.28)	0.057
II + DI vs DD	0.70 (0.35 to 1.43)	0.164	0.78 (0.38 to 1.54)	0.248	1.11 (0.51 to 2.44)	0.405
II vs DD + DI	0.84 (0.31 to 2.49)	0.371	1.80 (0.79 to 4.39)	0.086	0.46 (0.17 to 1.33)	0.087
ACE2						
rs2285666						
GG vs AA	4.09 91.50 to 10.1)	0.001*	2.04 (0.91 to 4.56)	0.033*	0.50 (0.19 to 1.48)	0.096
AA + GA vs GG	0.34 (0.17 to 0.69)	0.003*	0.54 (0.27 to 1.06)	0.033*	1.57 (0.74 to 3.43)	0.122
AA vs GG + GA	0.35 (0.13 to 0.82)	0.009*	0.62 (0.29 to 1.26)	0.108	0.56 (0.22 to 1.48)	0.126
MMP12						
rs2276109						
TT vs CC	0.80 (0.09 to 11.92)	0.428	0.36 (0.06 to 1.88)	0.132	0.46 (0.03 to 3.22)	0.241
CC + TC vs TT	1.41 (0.69 to 3.01)	0.148	1.07 (0.52 to 2.21)	0.419	0.73 (0.32 to 1.65)	0.225
CC vs TT + TC	1.11 (0.07 to 9.78)	0.464	2.76 (0.54 to 15.8)	0.127	0.40 (0.03 to 2.80)	0.212
TP53						
rs1042522						
CC vs GG	2.76 (0.63 to 13.3)	0.097	0.88 (0.29 to 2.32)	0.396	0.31 (0.06 to 1.65)	0.074
GG + GC vs CC	0.47 (0.23 to 0.94)	0.018*	0.67 (0.35 to 1.26)	0.113	1.43 (0.65 to 3.05)	0.187
GG vs CC + GC	0.47 (0.09 to 1.94)	0.170	1.43 (0.54 to 3.91)	0.246	0.32 (0.06 to 1.63)	0.08

Cases[1]: stands for women in the cases group with non-previous abortion experience (n=48); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes (n=60); Controls: health women with no previous abortion advent (n=107); *significant differences P<0.05

To investigate the differences in the methylation profile among women with recurrent abortion, intracase analyses were performed. Table 18 shows the main statistical findings between intracases and stratified cases vs controls.

Table 18. DNA methylation profile among intracase

Summary	Cases[1] (n=48) %	Cases[2] (n=60) %	Control (n=107) %	Cases[1] vs Controls	Cases[2] vs Controls	Cases[1] vs Cases[2]
Min-Max	79 - 90.8	71.7 – 86.6	78 - 96	0.0005	0.0001*	0.0005*
25th-75th Percentile	81.8 - 85.93	78.05 - 84.55	83 – 89			
Median	83.40	81.80	86			
Mean ±SD	83.86 ± 2.86	81 ± 3.54	85.82 ± 3.65			

Cases[1]: stands for women in the cases group with non-previous abortion experience (n=48); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes (n=60); *significant differences P<0.05.

The graphs below show the information shown in table 18. It was observed that cases[1] and cases[2] had a lower percentage of DNA methylation than controls (P=0.0005; P=0.0001, respectively) (Figure 6. A and B); and case[2] than cases[1] (P=0.0005) (Figure 6. C).

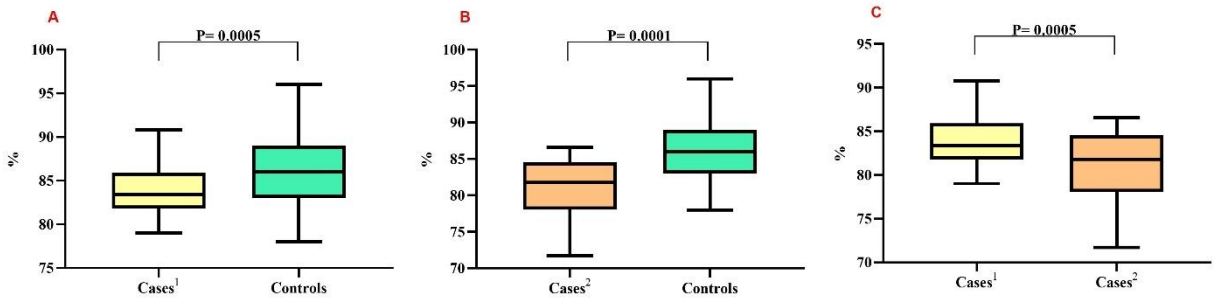


Figure 6. DNA methylation profile among intracase. A: comparison of levels of DNA methylation between cases[1] and controls. B: comparison of levels of DNA methylation between cases [2] and controls. C: comparison of levels of DNA methylation between cases [1] and cases[2]. Cases[1]: stands for women in the cases group with non-previous abortion experience (n=48); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes (n=60); *significant differences P<0.05.

Methylation is well known to impact major functions of genes, such as the interactions with DNA of both chromatin proteins and specific transcription factors. Therefore, the cases[1] and cases[2] methylation profiles were analysed according to specific polymorphisms that were previously demonstrated to have an impact on the risk and protective role of EPL (Table 19).

Table 19. The global DNA methylation according to rs5985 polymorphism in the *F13A1* gene

rs5985 - Major Allele G/G genotype	**Cases[1] (n=33) %**	**Cases[2] (n=45) %**	**Control (n=70) %**	**Cases[1] vs Controls**	**Cases[2] vs Controls**	**Cases[1] vs Cases[2]**
Min-Max	79 - 90.8	71.7 - 86.6	78 - 96	0.012*	0.0001*	0.002*
25th-75th Percentile	81.8 – 86	78.4 - 84.7	83 – 89			
Median	84.2	81.8	86			
Mean ±SD	84.14 ± 2.91	81.53 ± 3.63	85.83 ± 3.68			
rs5985 - Minor Allele T/T+G/T genotype	**(n=15) %**	**(n=15) %**	**(n=37) %**			
Min-Max	80.2 - 88.8	77.2 – 85.8	78 – 94	0.015*	0.0001*	0.003*
25th-75th Percentile	82.2 - 85.8	78 – 83.6	83.5 – 88			
Median	83.6	79.8	86			
Mean ±SD	83.69 ± 3.33	80.5 ± 3.08	85.81 ± 3.64			

Cases[1]: stands for women in the cases group with non-previous abortion experience, a total of 33 were wild-type (G/G genotype) and 15 polymorphic homozygotes and carriers (T/T + G/T genotypes); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes, a total of 45 were wild-type (G/G genotype) and 15 polymorphic homozygotes and carriers (T/T + G/T genotypes); *significant difference $P<0.05$.

When the methylation profile was analysed by the major and minor allele of the rs5985 polymorphism in the *F13A* gene; cases[1] for the G/G genotype had lower levels of methylation than controls ($P = 0.012$) (Figure 7. A). However, the lowest levels were observed among cases[2], for the same genotype, compared to controls ($P=0.0001$) (Figure 7. B) and cases[1] ($P=0.002$) (Figure 7. C). The same was seen for the T/T + G/T genotype, where cases[1] had lower levels of methylation profile than controls ($P=0.015$) (Figure 7. D), but lowest within the case[2] group compared to controls ($P=0.0001$) (Figure 7. E) and cases[1] ($P=0.002$) (Figure 7. F).

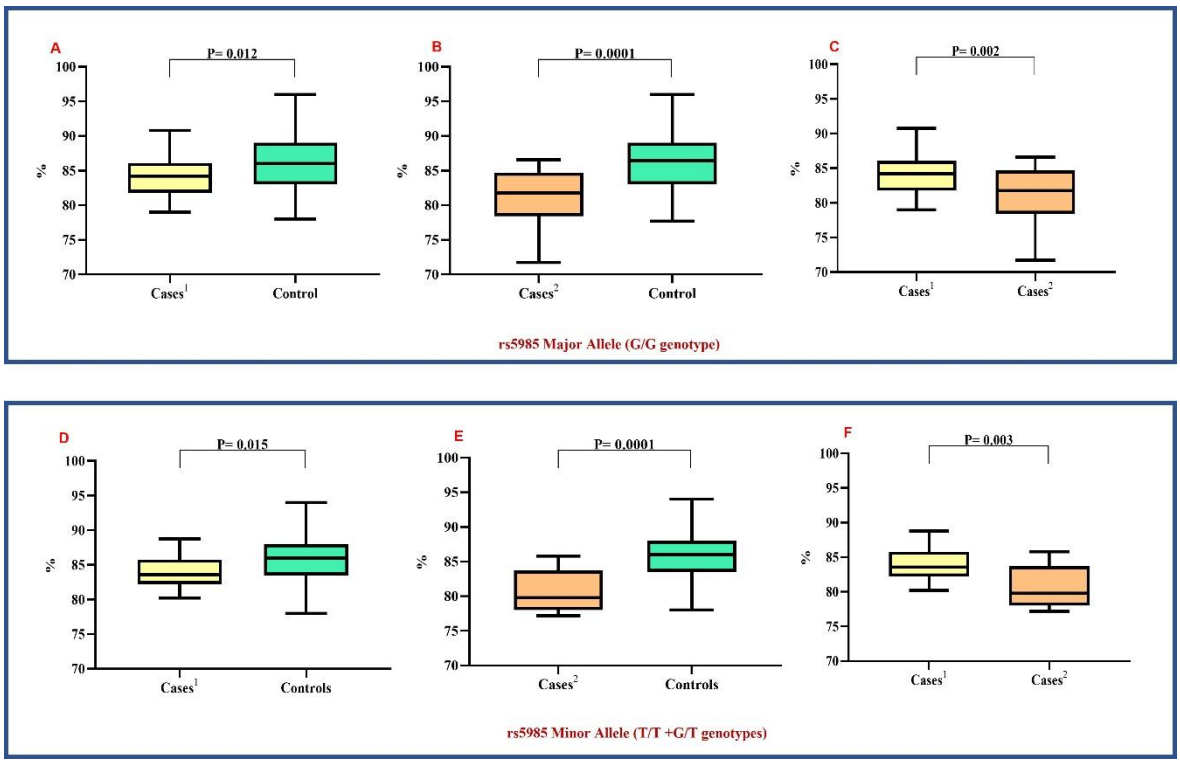


Figure 7. Methylation profile via rs5985 major and minor alleles. Global DNA methylation of rs5985 SNP, A: major allele of cases[1] and controls. B: major allele of cases[2] and controls. C: major allele of cases[1] and cases[2]. D: minor allele of cases[1] and controls. E: minor allele of cases[2] and controls. F: minor allele of cases[1] and cases[2]; a total of 33 were wild-type (G/G genotype) and 15 carriers and polymorphic homozygotes (G/T+T/T genotypes); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes, a total of 45 were wild-type (G/G genotype) and 15 carriers and polymorphic homozygotes (G/T+T/T genotypes); *significant differences $P<0.05$.

Cytokines analysis was performed to identify differences according to case stratification (Table 20). Despite no significance found, overall cases[2] had higher levels for all the cytokines than cases[1]. As expected controls had lower levels of IL-10 (P=0.05), IL-17a (P=0.033), IL-23 (P=0.049), and IL-6 (P=0.032) than cases[1]. The same was seen when comparing controls to cases[2] IL-10 (P=0.028), IL-17a (P=0.024), IL-23 (P=0.011), and IL-6 (P=0.016).

Table 20. Cytokines analyses after cases stratification

Cytokines	Stat. Analysis	Cases[1] (n=38) (pg/mL)	Cases[2] (n=54) (pg/mL)	Controls (n=89) (pg/mL)	Cases[1] vs Controls	Cases[2] vs Controls	Cases[1] vs Cases[2]
IL-10	Min-Max	2.38 - 54.7	0.81 - 90.55	0.60-76.38	0.05*	0.028*	0.461
	25th-75th Percentile	8.38 - 37.58	12.06 - 31.17	7.65-26.69			
	Median	21.43	18.38	14.63			
	Mean ±SD	23.62 ± 16.33	25.01 ± 20.16	19.46 ± 15.99			
IL-17a	Min-Max	3.89 - 30.33	1.28 - 16.79	1.25- 30.33	0.033*	0.024*	0.358
	25th-75th Percentile	9.58 - 15.67	8.52 - 16.79	7.18-15.80			
	Median	12.21	13.58	9.77			
	Mean ±SD	12.96 ± 5.26	13.61 ± 7.25	11.23 ± 6.42			
IL-23	Min-Max	81.91 - 1413	40.75 - 2053	24.06-1708	0.049*	0.011*	0.328
	25th-75th Percentile	253.4 - 8181.8	287.4 - 779.9	216-605.5			
	Median	499	519.2	397.7			
	Mean ±SD	548.4 ± 331.2	628.9 ± 459.7	455.8 ± 307.7			
IL-6	Min-Max	0.78 - 10.10	0.11 - 15.44	0.1-15.28	0.032*	0.016*	0.359
	25th-75th Percentile	2.3 - 6.66	2.48 - 7.97	1.46-4.74			
	Median	3.96	3.64	3.36			
	Mean ±SD	4.12 ± 2.72	4.42 ± 6.24	3.83 ± 3.02			

Cases[1]: stands for women in the cases group with non-previous abortion experience (n=38); Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes (n=54); *significant differences $P<0.05$.

Epigenetic mechanisms regulate many inflammatory factors, including DNA methylation and histone modifications. In particular, DNA and histone methylation are crucial forms of transcriptional regulation, therefore DNA methylation profile and cytokines correlation were evaluated. After analysis of cases[1], a negative correlation was observed between methylation profile (%) and age (r=-0.22, P=0.05); IL-17a (r= -43; P=0.006), followed by and IL-6 (r= -0.35; P=0.003); a strong positive correlation between IL-10 and IL-17a (r= 0.71; P=0.0006), IL-23 (r= 0.91; P=0.0001), IL-6 (r= 0.91; P=0.0001); forward by a strong positive correlation between IL-17a and IL-23 (r=0.69; P=0.007) and IL-6 (r=0.84; P=0.004); and among IL-6 and IL-23 (r=0.82; P=0.006) (Figure 8).

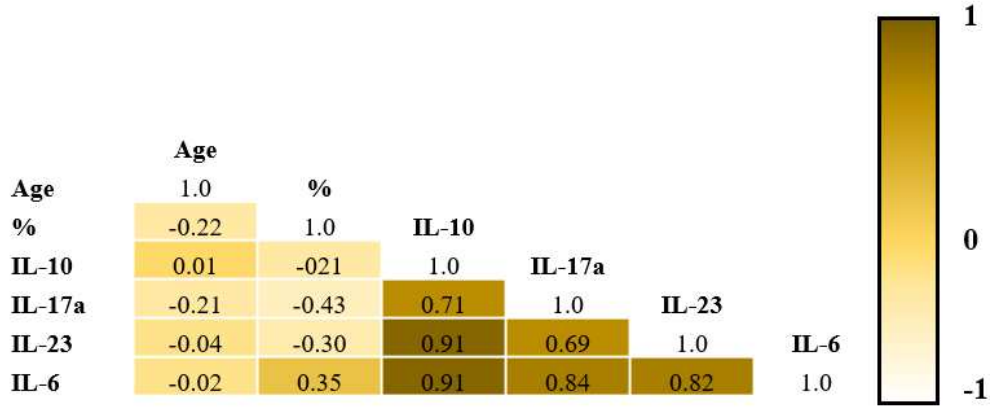


Figure 8. Cases[1] multivariable analysis. Cases[1]: stands for women in the cases group with non-previous abortion experience (n=38) and cytokine information; r values displayed in the inner of squares, r values were colour-coded according to its relevance, for reference use the bar on the right side; *significant differences P<0.05.

Furthermore, the same analysis was performed on case [2]. A positive correlation was observed between IL-10 and IL-17a (P=0.007), IL-23 (P=0.001), and IL-6 (P=0.002). It was also seen a positive correlation between IL-17a, IL-23 (P=0.007), and IL-6 (P=0.002); IL-6 was positively correlated to IL-23 (P=0.002). A negative correlation was seen between (Figure 9).

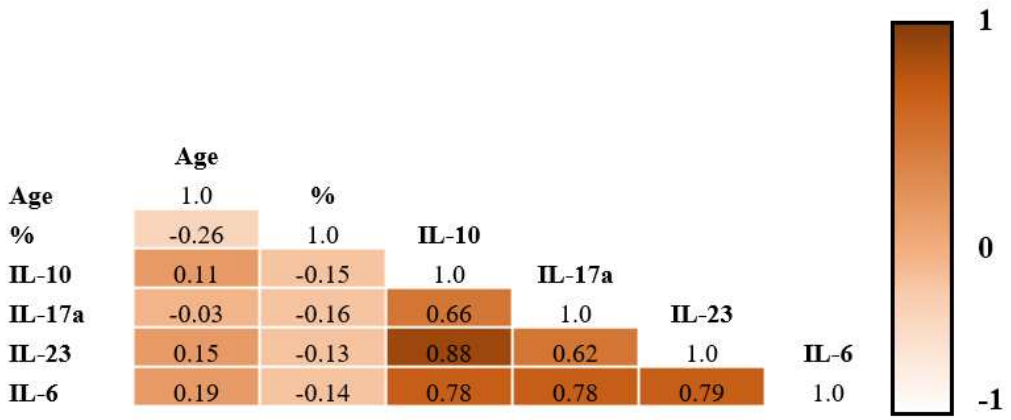


Figure 9. Cases [2] multivariable analysis. Cases[2]: stands for women in the cases group with one or multiple previous abortion episodes (n=54) and cytokine information; r values displayed in the inner of squares, r values were colour-coded according to their relevance, for reference use the bar on the right side; *significant differences P<0.05. r values displayed in the inner of squares, r values were colour-coded according to their relevance, for reference use the bar on the right side; *significant differences P<0.05.

After the multivariable analysis to determine which variable may be the best predictor for future analysis the Principal Component Analysis (PCA) was applied to investigate the multi-colinearity existent between the features/variables. In figure 10. It is possible to see how the variables interact with each other in our cases. For instance, the cytokines are strongly correlated to each other concerning principal component 1 (PC1), being the most impactful in the composition of PC1. While, principal component 2 (PC2) is mainly composed of age and methylation, with negative effects on each other. PC1 and PC2 had eigenvalues greater than 1 (3.52 and 1.15, respectively) they had a cumulative proportion of variance of 77.86% (Figure 10. B). Meanwhile, PC3, PC4, PC5 and PC6 (0.82, 0.28, 0.12, and 0.09, respectively) were below this threshold, therefore contributing the least to the cumulative proportion of variance (less than 23%) (Figure 10. B). Principal component 1 had largest contributions from IL-10 (coeficient: 0.941), IL-17a (coeficient: 0.873), IL-23 (coeficient: 0.946), IL-6 (coeficient: 0.949). Meanwhile, PC2 is mainly composed of age (coefficient: -0.80). Through this analysis and the results obtained,t is possible to confirm the importance of the study of cytokines for abortion. It helps to identify that in the future

the best cytokines to be studied are IL-6 and IL-23 since they had the highest coefficients to PC1 (Figure 10. C).

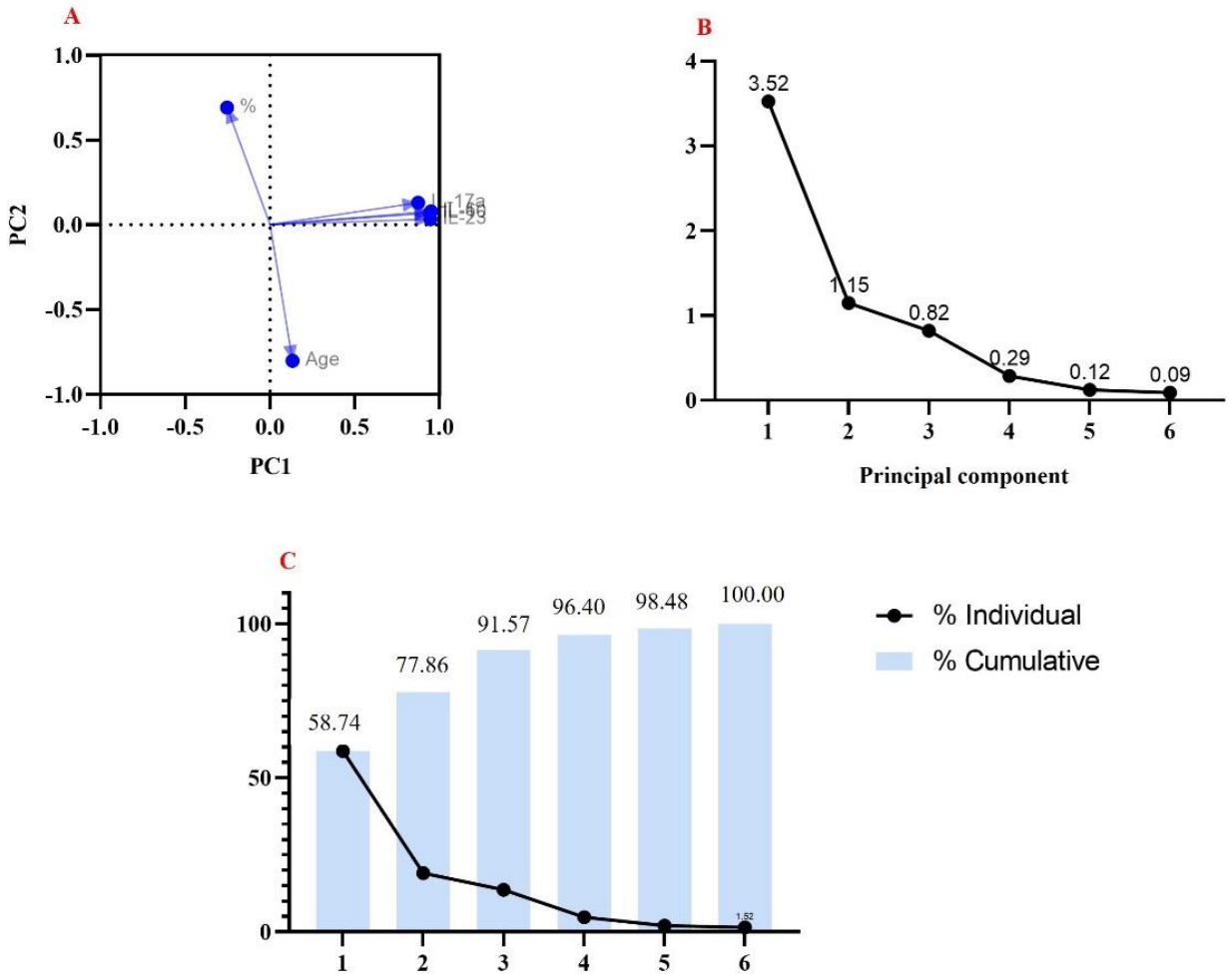


Figure 10. Principal component analysis among cases. A: loading graphic of IL-6, IL-10, IL-23 and IL-17a. B: Eigenvalue representation. C: cumulative proportion of variance.

3.8. Combination of Genetic and Systemic Biomarkers in a Fibrin Bio-Polymerization *in vitro* Model: A Perspective for the Future

Coagulation factor XIII (FXIII) may have a significant impact on placental formation since FXIII covalently cross-links fibrin and affects fibrinolysis. The minor allele (T/T genotype) of the polymorphism rs5985 in the *F13A1* gene is known to be associated with earlier cross-

linking, formation of a finer fibrin meshwork, and susceptibility to fibrinolysis. However, it has to be taken into account that the development of coagulation-based pregnancy complications is usually a multifactorial event involving gene-gene and gene-environment interactions. Therefore, to study and investigate the different architectural structures in the fibrin scaffold gel, 4 cases and 4 controls were selected. Figure 11. A and B represent cases with genotype wild-type for the rs5985 polymorphism, and Figure 11. C and D are controls for the same genotype.

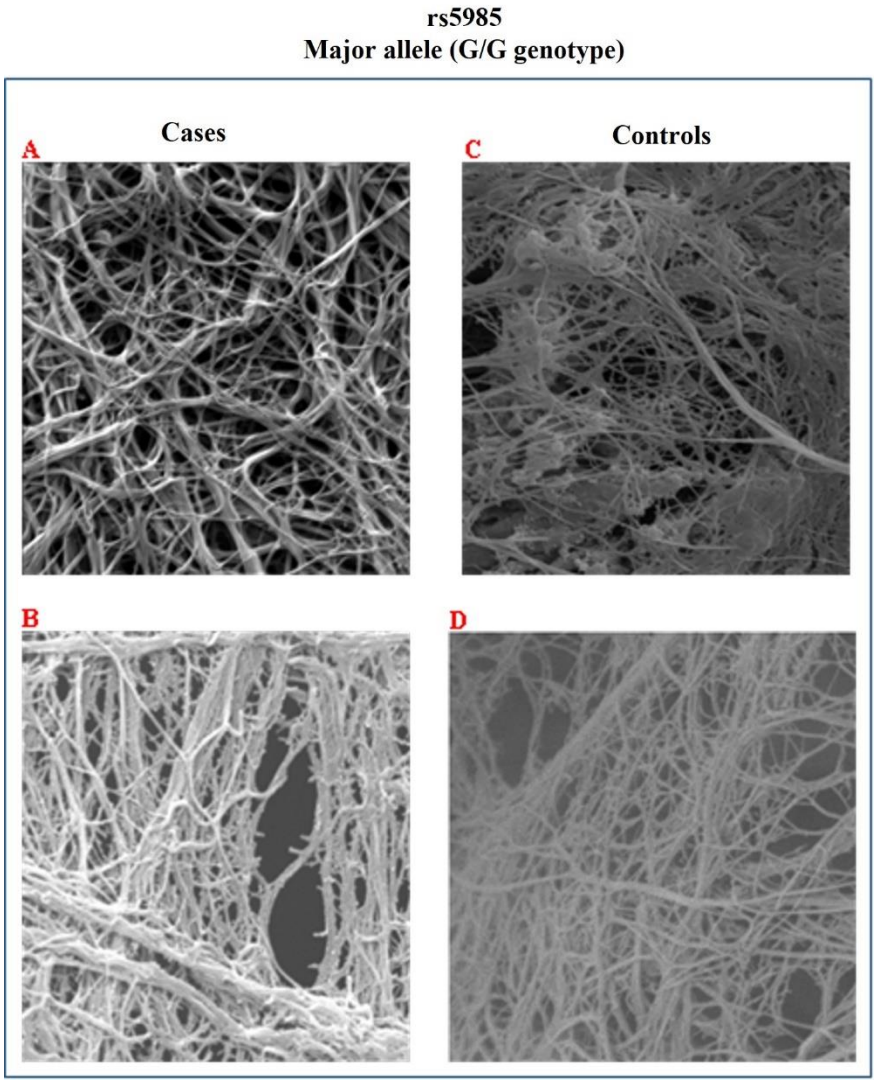


Figure 11. Fibrin scaffold gel in vitro structure for cases and controls with two major alleles for the rs5985 polymorphism in the *F13A1* gene. A and B: cases. C and D: controls. Image analysis in 14k magnification.

The minor allele for the polymorphism rs5985 was also analysed by structural differences in the scaffold fibrin gel (Figure 12). Figure 12. A and B represented scaffolds from two different cases with the minor allele. While Figure 12. C and D are two controls with a similar genotype.

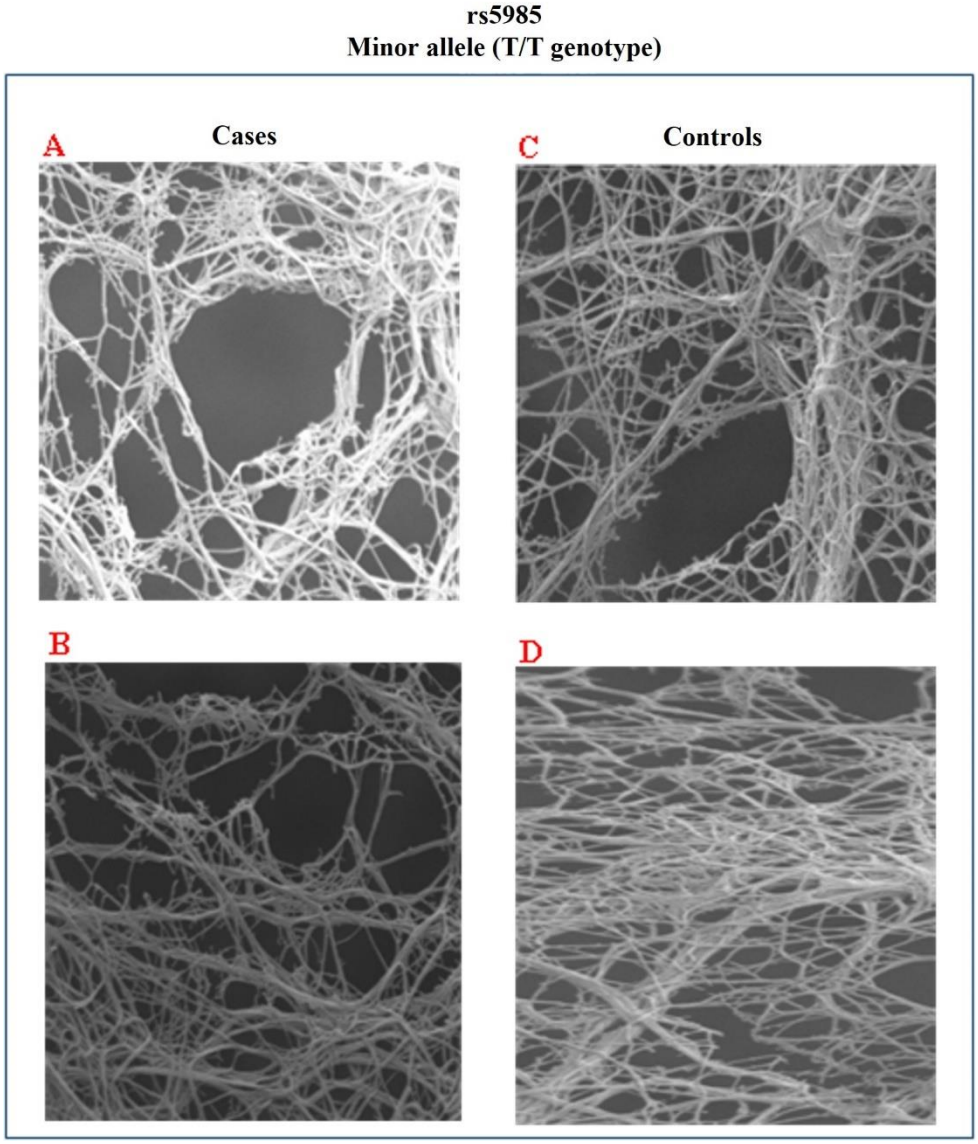


Figure 12. Fibrin scaffold gel in vitro structure for cases and controls with two minor alleles for the rs5985 polymorphism in the *F13A1* gene. A and B: cases. C and D: controls. Image analysis in 14k magnification.

Table 21 summarizes the information regarding the findings for the nanofibres measurement in the fibrin scaffold gel among the selected genotypes. When comparing the scaffold structure between cases and controls with two major alleles, it was seen that cases (Mean:

9.41, SD ± 3.10) had thicker fibres than controls (Mean: 7.77, SD ± 2.77) (P=0.0001); and also than cases with two minor alleles (Mean: 7.63, SD ± 2.48) (P=0.0001).

Table 21. Architectural differences in the nanofibres according to *F13A1* gene polymorphism

rs5985 - Major Allele	**Cases (n=2)**	**Controls (n=2)**	**P value**
Min-Max	4.48-20.22	2.01 - 19.14	0.0001*
25th-75th Percentile	7.07 - 11.15	5.83 - 9.29	
Median	8.91	7.66	
Mean ±SD	9.41 ± 3.10	7.77 ± 2.77	
rs5985 - Minor Allele	**(n=2)**	**(n=2)**	
Min-Max	3.00 - 13.60	2.50 - 10.77	0.0001*
25th-75th Percentile	5.83 - 9.01	4.12 - 6.05	
Median	7.07	4.92	
Mean ±SD	7.63 ± 2.48	5.28 ± 1.62	
P value	0.0001*	0.0001*	

The n=2 is referent to the number of cases and controls enrolled, however for each of them, 50 nanofibres were measured individually and grouped as part of their respective group, case or control*significant differences P<0.05.

Table 22 comprises information on the area of the pores found through the images. Controls (Mean: 69.04, SD± 46.39) with two major alleles had a larger area of pores than cases (Mean: 30.38, SD± 13.28) (P=0.0001). The same was seen for controls with two minor alleles (P=0.0001). No significant differences were observed between cases with two major and minor alleles (P=0.161), and controls with two major and minor alleles (P=0.405).

Table 22. Architectural differences in the pores according to *F13A1* gene polymorphism

rs5985 - Major Allele	**Cases (n=2)**	**Controls (n=2)**	**P value**
Min-Max	5.88 - 68.41	6.34 - 230.9	0.0001*
25th-75th Percentile	19.53 - 38.14	33.24 - 102.4	
Median	29.53	51.87	
Mean ±SD	30.38 ± 13.28	61.04 ± 46.39	
rs5985 - Minor Allele	**(n=2)**	**(n=2)**	
Min-Max	2.08 - 154.5	18.60 - 285.4	0.0001*
25th-75th Percentile	22.33 - 43.22	35.41 - 68.70	
Median	32.81	45.59	
Mean ±SD	35.03 ± 21.31	70.23 ± 43.22	
P value	0.161	0.405	

The n=2 is referent to the number of cases and controls enrolled, however for each of them, 50 pores were measured individually and grouped as part of their respective group, case or control*significant differences P<0.05.

Chapter 4

Discussion

&

Conclusions

4.0. Discussion

We investigated the effects of multiple variables' roles in early pregnancy loss in a cohort of 108 cases. The main findings of our survey are the impact of age, methylation and cytokines associated with genetic information in pregnancy loss, and how their influence may vary according to the background, i.e. recurrent miscarriages.

In total 63% of women with early pregnancy loss were more than 35 years old and 73% nulliparous (P=0.0001). In specific, in this group, 68% were women with recurrent pregnancy loss, and 83% of them were nulliparous (P=0.017). Maternal and neonatal pregnancy outcomes are typically associated with maternal age greater than 35 [56]. Underlying comorbidities, such as hypertension, diabetes, and obesity are also more common in older women leading to more complications during pregnancy [56]. An increased risk of pregnancy-specific problems such as preeclampsia, intrauterine growth restriction, premature birth, and stillbirth are also linked to advanced mother age [56], [190], [191]. As demonstrated previously, pregnant women with age ≥35 are 2 times more likely to develop gestational diabetes, and placenta praevia and undergo parturition complications, such as postpartum haemorrhage[56], [192]. This risk is likely to increase to 3 times in women aged ≥45 when considering clinical background as demonstrated by Fitzpatrick et al (2017) in a cohort of 233 pregnant women aged 45, in which 33% of them were overweight, 23% obese, 53% nulliparous, 44% had pre-existing medical conditions, and 78% conceived following assisted conception[58].

Age can also influence DNA methylation, for instance, our cases and controls were negatively correlated to age (r= -0.27, P=0.0019; r= -0.38, P=0.0001, respectively), therefore, with an increase in age a tendency of decrease in methylation, was observed. The same has been previously seen in other models, such as cancer and metabolic diseases [137].

Advanced chronological age is associated with more cell divisions of long-lived stem cell populations, leading to cumulative exposure to environmental risk factors, as well as changes that may develop with age in response to underlying genetic risk factors, resulting in age-associated DNA methylation alterations [193], [194].

In general, our cases (Mean: 82.49%, SD± 3.46) had lower levels of DNA methylation than controls (Mean: 85.82%, SD± 3.65) (P=0.0001), and cases with multiple abortions had even lower methylation than cases with single abortion (Mean: 81.39% and 83.86%, respectively). Other authors have found that altered DNA methylation results in faulty embryonic development and birth defects. In a study by Burris et al (2012) women who developed preeclampsia in the first trimester had lower genome-wide (LINE-1) methylation levels compared to women who did not [179]. The methylation profile can be affected by environmental factors such as stress, hormones, drugs, and diet. Vasilyev et al (2020) analysed 141 chronic villi miscarriages and identified that regardless of karyotype, LINE-1 methylation profile increased with gestational age. However, decreased with the advance in maternal age in miscarriages with a normal karyotype[180].

It has been also demonstrated that age-related methylation loss increases circulating cytokines[195], [196]. A moderate systemic inflammatory response occurs in a healthy pregnancy, and it increases as the pregnancy grows and peaks in the third trimester[184]. The mild increase in cytokine concentration in the bloodstream and the activation of white blood cells including neutrophils, lymphocytes, monocytes, and granulocytes are two characteristics of this physiological inflammatory response[183]. Interestingly, we found a lower number (1.98 vs $2.10x10^3/\mu l$), but within the normal range, of circulating lymphocytes in cases compared to controls (P=0.043). However, higher circulating levels of IL-10 (24.43 pg/mL), IL-6 (4.97 pg/mL), IL-23 (595.6 pg/mL), and IL-17a (13.34 pg/mL), cytokines in our cases compared to controls (19.46, 3.93, 455.8, and 11.23 pg/mL, respectively).

Indicating increased pro and anti-inflammatory cytokines in the maternal circulation, which has been shown before to be associated with miscarriages[181], [184], [188], [189], [197].

Despite the higher cytokines levels among cases than controls, both of them had the same tendencies in correlation as also seen in the principal component analysis (section 3.8, figure 10). However, correlations were mostly stronger among controls than in cases, this suggests that controls had a much more simultaneously balanced cytokine levels increase compared to cases. Our results showed that IL-6 was positively correlated with IL-10 (cases: r=0.83, P=0.0001; controls: r=0.90, P=0.0001), IL-23 (cases: r=0.85, P=0.0001; controls: 0.87, P=0.0001), IL-17a (cases r= 0.79; P=0.0001; controls: r= 0.86, P=0.0001). While IL-23 was positively correlated to IL-10 (cases: r= 0.90, P=0.0001; controls: r= 0.81, P=0.0001), IL-17a (cases: r= 0.71, P=0.0001; controls: r= 0.79, P=0.0001).

Alongside, IL-23 had a higher ratio difference among all cytokines compared to controls (1.3-fold). In fact, in cases and controls the strongest correlation was between IL-10 and IL23 (r=0.90, P=0.0001). Authors demonstrated that IL-23 up-regulates the IFN-γ production in activated cord blood naive T cells, as well as IL-10 and IL-17a [198]. IL-23 and IL-17a axis induces Th1/Th17- types with a pro-inflammatory cytokine profile that is unfavourable to pregnancy success and has been associated with pregnancy loss, preterm birth, recurrent pregnancy miscarriages, and preeclampsia [199]–[203]. Concurrently, IL-10 has the opposite effects of IL-23 and IL-17a, IL-10 maintains immunotolerance during pregnancy by inhibiting the NF-κB signalling pathway, and decreasing pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α, by inducing heme oxygenase-1, and by inhibiting antigen presentation via blocking major histocompatibility complex (MHC) class II expression[204]. Kaislasuo et al (2020) observed that IL-10 concentrations in normal pregnancies were significantly higher than in pregnancies ending in a loss starting at 6-8

weeks of gestation [205]. Other studies also demonstrated that IL-10 levels are lower in the peripheral blood mononuclear cells in women with recurrent pregnancy loss [206], [207].

Conversely, other studies have shown no difference or association between IL-10 levels and miscarriages or pregnancy complications [208]–[210]. While some authors demonstrated an increase in IL-10 levels in women with recurrent pregnancy loss[211]. An explanation for this difference across publications can be the time point of blood collection. For instance, blood drawn in women with a history of miscarriages is expected to have much lower levels of IL-10 than pregnant women, undergoing complex physiological changes to support maternal-foetus tolerance. In our case, this strong positive correlation observed between IL-10 and IL-23 is believed to be due to the pregnancy loss inflammatory niche and failure in the shift of immune response instead of being the major cause for miscarriages.

Cytokines are part of a regulatory network complex system to modulate foetal and maternal interactions. Genes expression will greatly not only affect cytokines production but also functional differences in the foetal-maternal environment supporting the physiological changes, however, polymorphisms in key genes for pregnancy success can affect/alter gene expression favouring miscarriages [212], [213]. For instance, our results showed that the wild-type variants rs5985 (G/G genotype), rs6003 (C/C genotype), rs2285666 (G/G genotype) in the *F13A1*, *F13B* and *ACE2* genes, respectively, were associated with a crude OR ranging from 2.72 to 3.94-fold risk increase for early pregnancy risk. While, rs5985, and rs2285666 polymorphic alleles (T and A alleles) were underrepresented, yielding significant protection of 73% and 56% for EPL (P=0.039; P=0.0003, respectively).

The *ACE2* variant G/G genotype was associated with a 2.72-fold increased risk for EPL (OR: 2.72, CI: 1.39 to 5.36) (P=0.002). This genotype was described as a risk factor for hypertension among women [214], [215]. Brosnihan et al (2004) demonstrated that ACE2

levels are increased among normal pregnancies and decreased in pregnancies with preeclampsia [75], [76]. The idea that Ang-(1-7) may play a significant role in blood pressure management in pregnancy is further supported by the enhanced Ang-(1-7) vasodilation in mesentery tissue[75].

The *F13A1* (rs5985) and *F13B* (rs6003) gene variants have divergent findings across the literature. While our findings demonstrated that G/G genotype for rs5985 had an increase of 3.71 (OR: 3.71, CI: 1.01 to 12.9) (P=0.20) in the risk for EPL, previous studies state that risk relays in the minor allele. For example, a study performed on women with recurrent abortion history showed that carriers of the minor allele for rs5985 polymorphism (T allele) had a 5-fold increased risk for early pregnancy loss[216]. However, another study demonstrated that either a minor (T allele) or major (G allele) allele can be a risk factor, and that is modulated depending on the levels of the circulating fibrinogen[217].

As per the rs6003, the minor allele (T allele) has a very low frequency among Caucasians as demonstrated in previous studies, supporting our results, since 94% of cases were Caucasians [124], [218]. Despite minor allele being associated with increased risk for venous thromboembolism and ischemia stroke, and with higher dissociation of the FXIII subunits in plasma, our study showed that the homozygote polymorphic (T/T genotype) had a protective effect among EPL, and instead, wild-type (C/C genotype) adds in as risk factor for EPL (OR: 3.94, CI: 0.83 to 19.1) (P=0.036), however to the date no information regarding miscarriages has been postulated in previous studies [218], [219].

We also found that the polymorphic allele for the variant rs1042522 in the *TP53* gene had a significant 1.3-fold decrease (38% vs 50%) (OR: 0.57, CI: 0.33 to 0.98) (P=0.032) among our cases compared to controls, representing a protective factor against EPL. Pietrowski et al (2005) demonstrated a statistically significant association between carriage of the proline

allele (C allele) and the occurrence of recurrent pregnancy loss [220]. Arturo et al (2021) and Kang et al (2018) demonstrated that having two proline alleles (C/C genotype) leads to a decrease in implantation and clinical pregnancy rates (38% vs 61%) compared to women with at least one arginine allele (G/G vs G/T) [164], [167].

Differently, the polymorphic allele (T allele) for the variant rs1801133 in the *MTHFR* gene was overrepresented among cases than in controls (66% vs 50%), representing a 2-fold risk increase for EPL (OR: 1.96, CI: 1.13 to 3.46) (P=0.011). This is in agreement with previous studies[149]–[151], [153], [155], [221]. In general, the T allele is associated with increased thermolability of MTHFR, causing impaired folate binding and reduced enzymatic activity[222]. Therefore, the T allele is associated with decreased concentrations of folate in serum, plasma, and red blood cells, and mildly increased plasma Hcy concentration[223], [224]. Mehandjiev et al (2019) and Merviel et al (2017) studies on pregnancy loss and recurrent pregnancy loss, respectively, showed an increased frequency of the T/T genotype among cases compared to controls (29.3% vs 9.8%) leading to 1.65-fold increased risk for EPL[225], [226]. *MTHFR* and ApoE variants, specifically ApoE4, have been also associated with venous thrombosis and cardiovascular risk, by stimulating a higher pro-inflammatory profile, additionally affecting oxidative stress, platelet function, and apoptosis [227]–[229]. Overall, the ApoE4 allele is linked to greater low-density lipoprotein cholesterol, and the ApoE2 allele is related to lower levels[230]. Our findings show an increased frequency of ApoE4 compared non- ApoE4 carriers among cases and controls (19% vs 16%) representing a 3.17-fold increase in risk for early pregnancy loss (OR: 3.17, CI: 1.03 to 8.78) (P=0.029). Our results are also supported by other authors who have described ApoE4 as a risk factor among early pregnancy miscarriages and also among recurrent pregnancy loss [229]–[231]. Even though, other studies, have reported ApoE4 to be associated with a lower risk for

endometriosis and an increase in fertility due to its role in endothelial and endocrine dysfunction[232].

In our secondary analysis, we observed a very interesting fact in which no significant variants reported before followed cases stratification showed significant differences. The variants rs6050 (*FGA* gene), rs1800790 (*FGB* gene), rs876538 (*CRP* gene), rs2808635 (*CRP* gene), and rs1061170 (*CFH* gene), results before and after stratification findings represent the importance of the clinical background of the patient's prior genetic studies.

We found a 3-fold increased risk for RPL women with C/C genotype for the *CFH* gene variant compared to EPL. The minor allele of the *CFH* polymorphism rs1061170 (T allele) was associated with a decreased risk of pregnancy loss in a study performed by Cho et al (2020) and the major allele (C allele) to a higher risk for developing preeclampsia in a study by Lenhart et al (2015)[87], [233]. Studies in other diseases models (lupus nephritis, age-related macular degeneration, systemic lupus erythematosus) showed that the C/C and C/T genotypes increased systemic inflammation due to reduction in the binding ability to CRP and glycosaminoglycan and inhibition of complement turnover. Currently, the models investigating the relationship between pregnancy outcome and the polymorphism point out that during the pregnancy genetic and clinical factors in combination with the CFH risk allele (C allele) may play a role in miscarriages[233], [234]. For instance, CFH has been shown to negatively correlate with insulin sensitivity and glucose tolerance, which may be especially important during pregnancy[233]–[236].

Interestingly, both major alleles (C and T, respectively) for the CRP gene rs876538 and rs2808630 variants showed a similar protective role (OR: 0.13, CI: 0.01 to 0.98) (P=0.018; P=0.015); Simultaneously, an 8-fold risk increase was associated with the minor allele for both variants (P=0.010; P=0.010). Similarly, a study showed that women with recurrent

miscarriages and minor allele carriers for the polymorphism rs2808630 (G allele) had an increase of 4-fold (OR: 4.88, CI: 1.7-54.1) than non-carriers (OR: 2.90, CI: 0.84-38.10) of CRP in their bloodstream[237]. Another study by Best et al (2013) demonstrated pregnant women in the first trimester with preeclampsia carriers of the minor allele (T allele) for the polymorphism rs876538 were more prone to develop severe preeclampsia[105].

FGA and *FGB* genes variants' minor alleles (C and T alleles, respectively) were less represented among RPL cases than in EPL cases (P=0.028; P=0.019). Our results did not agree with the previous finding by Kamimoto et al (2017) that demonstrated a higher frequency (86.7%) of the polymorphic allele (G allele) in the rs6050 variant among women with a history of early pregnancy loss compared to controls, cases also presented a hypofibrinogenemia status. They also have higher frequencies of coagulopathies comorbidities, such as deep vein thrombosis (83.3%) and prosthetic valves (81.6%)[135]. The fact that among Japanese the FGA rs6050 polymorphism is more frequent (46.5%) compared to Europeans (29%) could have contributed to the differences observed in our investigations. Meanwhile, Schwedler et al (2021) observed that the minor allele of polymorphism rs1800790 (A allele) under proinflammatory stimulation increased the levels of fibrinogen in pregnant women with a history of foetal loss, placental dysfunction, pregnancy complications or venous thromboembolism [137].

Considering the important impact of genes variant expression (rs5985 G/G and T/T genotypes) and circulating biomarkers (i.e. fibrinogen and IL-6), our fibrin bio-polymerization *in vitro* model highlighted architectural differences across cases and controls with the same genotype (rs5985) G/G vs G/G (Mean: 9.41 vs 7.77, P=0.0001) and T/T vs T/T (Mean: 7.63 vs 5.28; P=0.0001); As well as for different genotype G/G vs T/T among cases (Mean: 9.41 vs 7.63; P=0.0001) and controls (Mean: 7.77 vs 5.28; P=0.0001) in the thickness of nanofibres. Meanwhile considering pores findings, only within the same

genotypes were observed statistical differences G/G vs G/G (Mean: 30.38 vs 61.04; P=0.0001) and T/T vs T/T (Mean: 35.03 vs 70.23; P=0.0001). Our results suggest that controls had finer meshwork fibres with larger pores, this can be explained by the more regulated inflammatory process among controls. An interesting finding relies upon the fact that despite the transcription of fibrinogen being significantly upregulated by IL-6, our cases had lower levels of fibrinogen compared to controls (332.3 vs 352.1 mg/dl), however, higher levels of IL6 than controls (4.97 vs 3.83) (P=0.018); this may highlight an aberrant intercommunication downstream of our cases cytokines [238]. The fact that IL-10 can downregulate the synthesis of fibrinogen by inhibiting IL-6, explains the strong positive correlation seen between IL-6 and IL-10 among our controls (r=0.90), but less strongly among our cases (r=0.83). Besides that, the rs5985 variant can affect cross-linked fibrin structure, in which the polymorphic genotype (T/T genotype) cleaves thrombin rapidly compared to the wild-type genotype (G/G genotype) resulting in altered instability and clot architecture[239], [240]. However, rather than just varying according to genotype, the combination of genotype and fibrinogen levels will determine the fibrin scaffold gel. Authors demonstrated that a decrease in the permeability in the scaffold was observed with an increase in the fibrinogen levels among wildtype (G/G genotype) and carriers (G/T genotype for the rs5985 variant, and that the permeability had less variability as fibrinogen concentrations increased. While in individuals with polymorphic genotype (T/T genotype), the fibrinogen levels did not alter permeability, showing overall more stability [217]. Moreover, in contrast to G/G genotypes, the T/T genotype increases the fibrin cross-linking activity and results in a fibrin clot that is less sensitive to fibrinolysis at low levels of normal fibrinogen [217]. How this could affect the pregnancy is still unclear, we hypothesize that in the early stages of the pregnancy, during the implementation of the trophoblast in the decidual endometrium a stable cloth with resistance to fibrinolysis is essential to pregnancy success, especially within a normal range of fibrinogen expected in early pregnancy.

4.1. Conclusions and Limitations

In conclusion, this thesis provides evidence of distinct genetic, epigenetic and systemic variables that can interact with each other and be a risk factor for early pregnancy loss and recurrent miscarriages. The genetic diversity observed in cases group versus intracase is a major highlight of how the clinical history of patients/individuals is essential to understand the differences observed, most importantly to use them as a prognostic tool. More preeminently our principal component analysis demonstrated how cytokines analysis can significantly contribute as a major prognostic biomarker associated with early pregnancy loss. Age and genome-wide-methylation were negatively correlated indicating that advanced age, in our study ≥35 years old, is ascribed to miscarriages for factors such as chromosomal abnormalities and alternated genetic expression, which can be the results of altered DNA methylation profiles. Polymorphisms across different and within the same genes can result in risk or protective factors, and the interpretation of the findings can vary according to the clinical background of investigated cases, i.e. single abortion or multiple abortions. The *in vitro* model to current knowledge was the first model to be applied associated with pregnancy loss, most previous models were used to investigate cardiovascular diseases (e.g., thrombosis, myocardial infarction), despite a small number, it provides the first insights into how not only fibrinogen and *F13A1* variants can influence the architecture of fibrin gel but also IL-6. The studies of this thesis demonstrated a series of non-modifiable factors that play major roles in pregnancy loss outcomes.

Some of the limitations of this thesis included the small sample size, and the absence of 16 plasma samples for cases and 18 for controls. Another important limitating factor is the fact that from our 60 cases of recurrent abortion, only 13 had karyotypic information from their previous abortions indicating trisomy 13 (Patau syndrome) 18 (Edward's syndrome), and 21 (Down syndrome). Out of those cases, a total of 12 had at least two previous abortions with

trisomy (13,18 or 21, not precisely specified); one case had 3 spaced abortions all of them identified with trisomy 18. In the ideal scenario having blood, one-month post miscarriages would allow us to identify tendencies in a haemostatic environment related to DNA methylation and cytokines expression. Unfortunately, we did not have access to the maternal placenta to investigate the methylation profile and cytokines supernatant profile, which could provide a better understanding of the maternal-foetus environment.

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