



THE ROLE OF PROSTAGLANDINS AND POLYMORPHISMS OF A2 PHOSPHOLIPASE GENES IN THE OCCURRENCE OF EARLY UTERINE CONTRACTIONS DURING PREGNANCY

Blanka Borowiec^{1,2}, Tomasz Hadada¹, Magdalena Kosińska¹

Abstract

This study focused on the role of polymorphisms in prostaglandin expression regulating genes in the occurrence of early uterine contractions during pregnancy. The analyzed genes were: PLA2G4C, encoding calcium independent phospholipase A2, and PLA2G4D, encoding IVD phospholipase A2. It was examined if known reference polymorphisms in these genes (rs1366442, [A/C/T] for PLA2G4C; and rs4924618, [A/T] for PLA2G4D), have any influence on preterm birth. Additionally, other biological, genetic and socio-economic factors were taken into account and analyzed, based on their role in induction of early resolve of pregnancy in the study group. Blood samples were taken from 20 patients. 15 of them gave birth preterm, 5 gave birth at the predicted date of pregnancy resolve. DNA was isolated from the samples, and subjected to PCR, with obtained amplified samples separated using electrophoresis on 1,5% agarose gel. Resulting material was subjected to high-throughput sequencing. Statistical analysis was performed using Statistica 13 software. Analyses have shown that the discussed biological, genetic and societal-economic factors have statistically significant influence on preterm birth. Sequencing results presented the suspected presence of the analyzed SNPs in most women from the studied groups, while not showing their presence in any of the controls. The biological, genetic, and socio-economic factors analyzed have a significant influence on pre-term birth. Presence of SNPs in PLA2G4C and PLA2G4D genes may increase the risk of early resolve of pregnancy. However, as the control and study groups were relatively small, it is suggested to repeat the studies on bigger samples to validate the results.

Running title: The role of SNPs in PLA2G4C and PLA2G4D genes in preterm labour

Keywords: preterm birth, preterm delivery, stillbirth, uterus, preterm labor, prostaglandin, phospholipase, phospholipase A2, pla2g4c, pla2g4d, polymorphism, allele, SNP, single nucleotide polymorphism, mutation, uterine contraction, metabolism, parturition

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Introduction

Premature births have long been the object of much research and scientific deliberations. Due to advanced perinatal clinical care and constantly evolving medical technology, the survival rate of newborns born prematurely, i.e. below 37 weeks of gestation, has improved significantly. However, a high percentage of pre-term pregnancies and, as a consequence, new-onset complications are still observed [1]. Maternal factors influencing the length of prenatal development include the health conditions and the environment of parents' life. However, genetic predispositions have recently been the most popular factor taken into consideration in relation to this topic [2,3]

So far, six most common risk factors for pre-term labor have been identified. These are infectious agents, placental, fetal, socio-economic, health and genetic factors (Bręborowicz and Banaszewska, 2007). According to the risk factors identified above, the idiopathic premature uterine contractile function may also be genetically determined. This function usually results in a delivery before the estimated date. Among the genes whose polymorphisms may be responsible for premature uterine contractions, there are those encoding oxytocin and relaxin receptors [4]. The genes coding for phospholipase A2 are particularly remarkable in that context.

This enzyme takes part in an important metabolic pathway, releasing arachidonic acid from phosphatidylinositol found in phospholipid cells. Arachidonic acid becomes a substrate for two important enzymes: cyclooxygenase (COX) and 5'-lipoxygenase. One of the products of cyclooxygenase is the PGG – prostaglandin, converted to PGH₂. It is a chemical compound from which other prostaglandins, thromboxanes and prostacyclins are formed [6].

Prostaglandins seem to be important mediators of uterine activity. Their structure consists of unsaturated carboxylic acids, containing a twenty-carbon backbone and a five-membered ring. They affect blood pressure, metabolism, body temperature, inflammatory reactions and a number of other significant processes taking place in the human organism. They are produced by almost every tissue in the body and serve as important relays or effectors in many different functions [7]. While the first hints of prostaglandin contribution to premature labor appeared, attempts were made to suppress their production to reduce myometrial contractions. However, they play an important role in maintaining the fetal and renal blood flow, so inhibiting their production would have destructive effects on certain body functions. Increase prostaglandin level to induce labor has been tried. However, the dosage was neutralized by prostaglandin centers located, among others, in the intestines and brain [8].

Genes discussed in this paper are PLA2G4C and PLA2G4D. PLA2G4C encodes a phospholipase A2 from the IV C group (calcium-independent group), alternatively called cPLA2-gamma [9]. PLA2G4D encodes

phospholipase A2 from the IV D group, also known as cPLA-2delta [10]. Single nucleotide polymorphisms in these genes could potentially be used as markers of preterm birth.

To confirm the truth of the assumption that single nucleotide polymorphisms in the examined genes are classified as a risk factor for premature delivery, the occurrence of SNPs will be determined in patients from the test sample and control sample. The incidence of SNPs will be estimated in the group of women giving pre-term compared to the group of term birth givers. The SNP's ability to affect preterm delivery will also be assessed.

Materials and methods

Patients and collection of material

Two groups of women were used for the study - test and control. A qualification of pregnant women was carried out based on a gynaecological-obstetric interview conducted by the staff of the maternity ward of the Gynaecological and Obstetrics Clinical Hospital of Poznan University of Medical Sciences. The patients were given full information about the purpose and scope of the tests. They expressed their informed written consent in the presence of a witness - a department nurse or midwife.

During the qualification of patients to the study group, the following criteria were followed: time of termination of pregnancy in the range from 24 to 37 weeks of its duration, single pregnancy, belonging to the European population, Polish citizenship. During the qualification of patients to the control group, the following criteria were followed: time of termination of pregnancy after the 37th week of pregnancy, single pregnancy, belonging to the European population, Polish citizenship.

The study group included 15 women aged between 19 and 34 years ($\bar{x} = 28.6 \pm 5$, Med = 28), who gave birth prematurely ($\bar{x} = 33.5 \pm 4$, Med = 33.5); termination of pregnancy: 28-36 weeks. The control group consisted of 5 women between the ages of 18 and 40 ($\bar{x} = 29 \pm 4$, Me = 30) who gave birth at the time between 37 and 39 weeks of gestation ($\bar{x} = 38 \pm 1$, Me = 38).

Methods

The occurrences of SNPs in the PLA2G4C gene (rs1366442, [A / C / T]) (Fig. 1) and in the PLA2G4D gene (rs4924618, [A / T]) were assessed (Fig. 2). For this purpose, 2 ml of peripheral blood was collected from the women tested, from which DNA was subsequently isolated.

Blood collection

Patients underwent peripheral blood donation. It has been collected by qualified medical personnel. One sample was 2ml of blood and an anticoagulant - EDTA. Not exceeding 24 hours after collecting the material, it was transported in accordance with the requirements for the transport of biological material

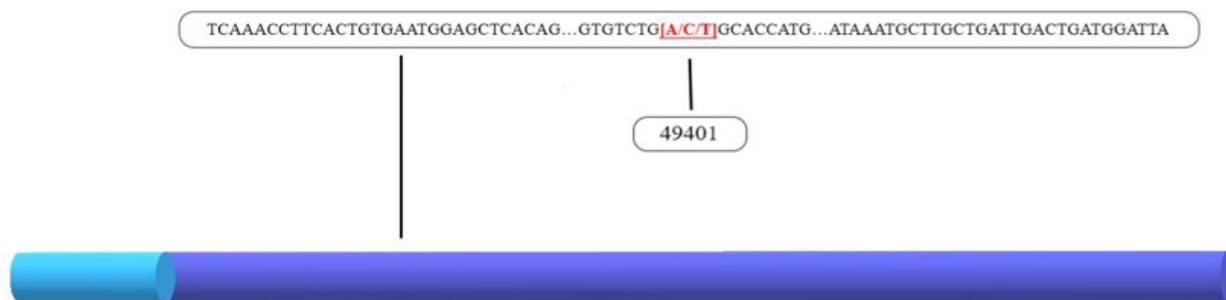


Figure 1 The considered SNPs in the 49401 position of the PLA2G4C gene. Light blue – promoter-regulator region, dark blue- the actual gene fragment

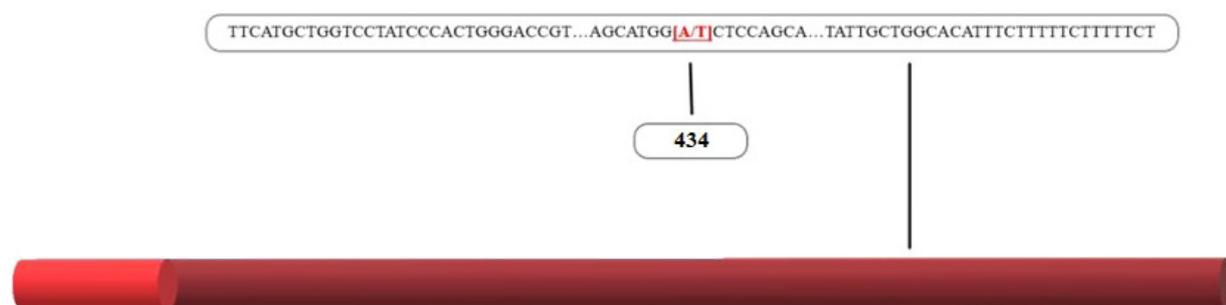


Figure 2 The considered SNPs in the 434 position of the PLA2G4D gene. The location is an example of a missense mutation. Light red – promoter-regulator region, dark red- the actual gene fragment

(transport in an anti-contaminated vessel filled with ice for the transport of biological material, where the temperature did not exceed 4°C).

DNA isolation

For DNA isolation from peripheral blood of the studied women a set of QIAGEN Columns and reagents (DNeasy® Blood & Tissue Kit) was used.

The isolation procedure was carried out according to the original protocol attached to the above set of reagents. The Eppendorf MiniSpin® laboratory centrifuge was used to centrifuge all samples. The entire procedure was carried out according to the original, unchanged content of the protocol attached to the kit. After the DNA isolation process, the amount obtained was determined using a Nan-

Table 1 The sequences of starters used in the PCR

Starters	Forward starter (5'→3')	Reverse starter (5'→3')	Product length
PLA2G4C	CTGGGGTGCTTCCTACAAGG	AAGCGACTTCATCCAACCCC	172
PLA2G4D	GGCAGCATTCCAAAGTGAGG	CCCTCGTTCTTCCAGGACAA	384

Table 2 The contents of reaction mix

Contents	Volume (µl)
NZYTaq II 2x Green Master Mix	5
Water	3,5
Forward starter	0,25
Reverse starter	0,25
DNA	1

Table 3 Specific stages of the PCR

Stages	Temperature (°C)	The length of the stage (min.)	Number of repetitions (cycles)
Initial denaturation	94	5	
Denaturation	94	1	5
Annealing	60	1	35
Elongation	72	1	35
Ultimate elongation	72	10	
Storage	4		

oDrop spectrophotometer 230nm absorbance ratio. The samples were stored at -20°C until the PCR reaction was performed.

Polymerase chain reaction (PCR)

The following table (**Tab. 1**) shows a summary of the primer sequences used in the PCR reaction. The synthesis of the primers was commissioned to Sigma-Aldrich Sp. z o.o. (Poznan, Poland). In both primers, the PCR reactions were carried out in a volume of 10 µL. The composition of the reaction mixture is presented in the table below (**Tab. 2**). A ProFlex™ PCR System thermocycler was used to carry out the PCR. The program used included 35 cycles that comprised DNA denaturation, primer attachment and DNA elongation. A description of all reaction stages is presented in a table below (**Tab. 3**). The PCR products were stored in 20µL Eppendorf tubes at -20 ° C until re-used.

DNA electrophoresis on 1.5% agarose gel

Separation of amplified DNA samples was performed on 1.5% agarose gel in TAE buffer. The GelRed® fluorescent dye, in a volume of 4µL, was added to the gel prior to congelation. 5 µL of the PCR reaction product and 5 µL of the Thermo Scientific GeneRuler 100 bp DNA Ladder reagent, diluted in accordance with the protocol attached, were applied to the gel.

The electrophoresis process was carried out for 90 minutes in an electric field of 100V voltage and 100A current. The result of the above reaction was recorded and visualized using the transilluminator G: BOX (Syn-gene, Cambridge, UK).

DNA sequencing

The rest of the PCR product was accurately described and delivered to the Laboratory of Molecular Biology at the Faculty of Biology of the Adam Mickiewicz University in Poznan, for the purpose of sequencing.

Analysis of sequencing results

Sequencing results were obtained in the form of AB1 extension files. The analysis performed involved

identification of the location of single nucleotide polymorphisms:

1. In the PLA2G4C gene, encoding phospholipase A2 IVC, conversion of adenine to cytosine or thymine (A / C / T).
2. In the PLA2G4D gene encoding phospholipase A2 IVD, conversion of adenine to thymine (A / T).

Two variants were considered in each gene:

1. Homozygous variant - identical nucleotides occur in the SNP site on homologous chromosomes.
2. Heterozygous variant - different nucleotides occur in the SNP site on homologous chromosomes.

The analysis and visualization of the results of sample sequencing were compiled in the SnapGene Viewer 4.2.4 program.

Ethical approval

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

The consent for testing was provided by the Poznan University of Medical Sciences Bioethical Commission (No. 538/14). The patients were given full information about the purpose and scope of the tests. They expressed their informed written consent in the presence of a witness - a department nurse or midwife.

Results

A group of 20 unrelated and unpaired women was tested for the polymorphisms of genes encoding phospholipase A2 IVC and IVD. One of the SNPs was located in the PLA2G4C gene at position 49401 and consisted of the conversion of adenine to cytosine or thymine (A / C / T). The second was located in the PLA2G4D gene at position 434 and was defined as the change of adenine to thymine (A / T).

The study group consisted of 15 women in whom preterm delivery was observed. The control group

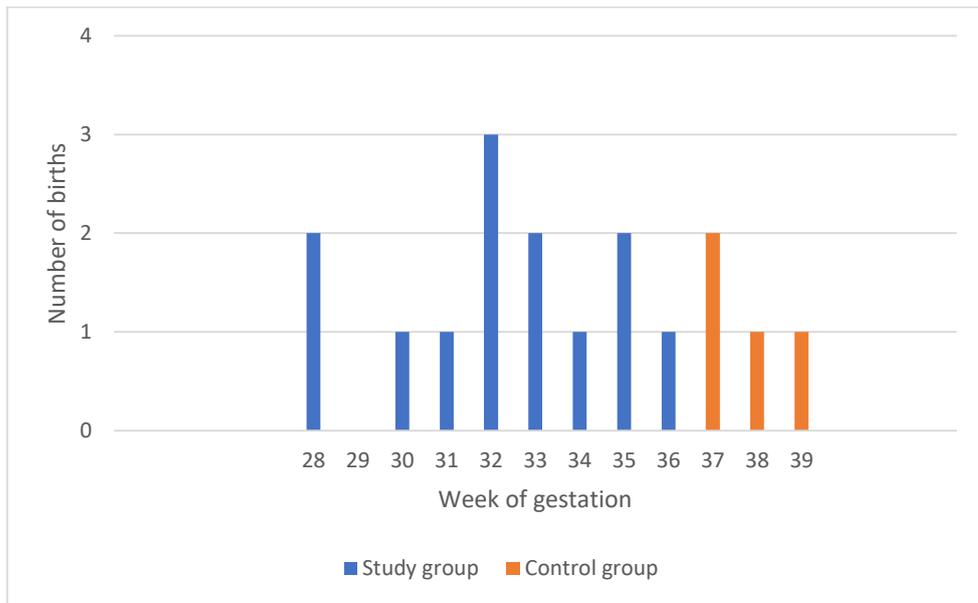


Figure 3 Distribution of the rate of births in the consecutive weeks of gestation



Figure 4 Exemplary results of genomic DNA electrophoresis on 1,5% agarose gel. 1kb marker – standard mass distribution. 011-018 – genomic DNA samples, isolated from peripheral blood of the patients from the study group. The picture was taken using the G:Box transilluminator [16]

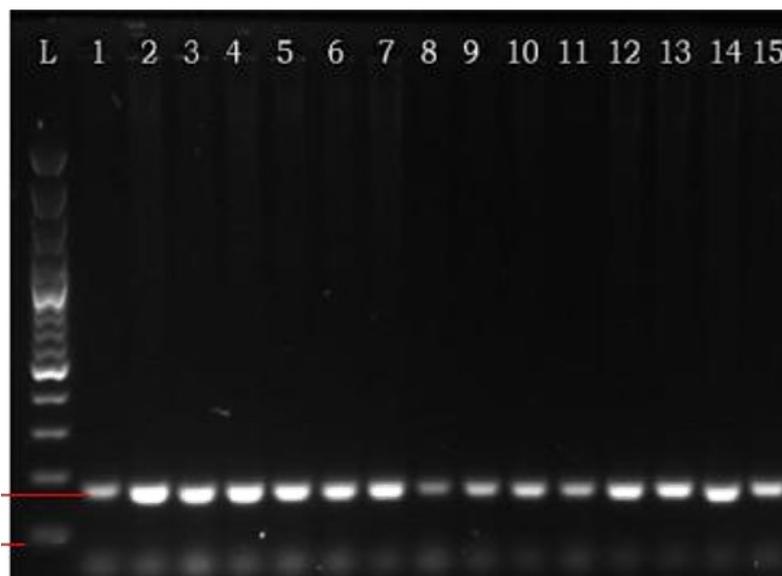


Figure 5 Electrophoretic separation of PCR samples of 174bp predicted length, amplified with the use of primers allowing for detection of SNP in PLA2G4C gene, on 1,5% agarose gel. Study group. The picture was taken using the G:Box transilluminator. L – sample mass template, 1-5 – numbers of PCR samples from the study group

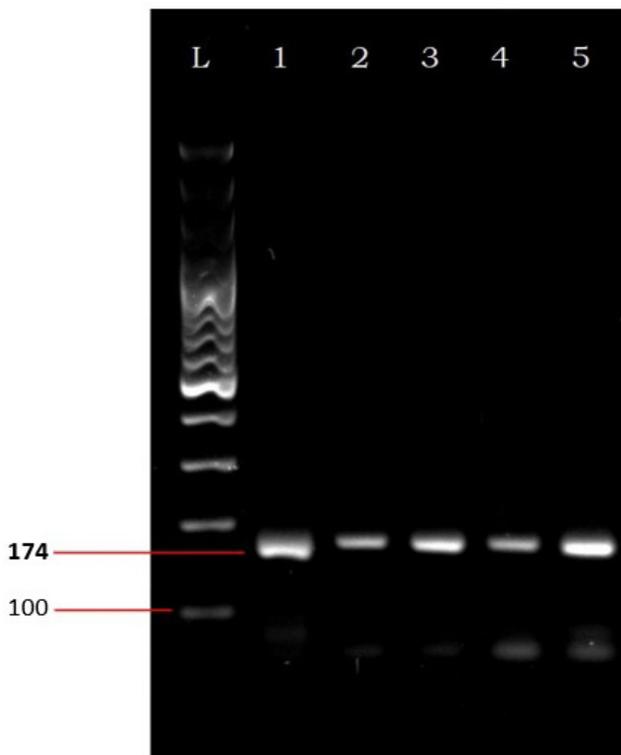


Figure 6 Electrophoretic separation of PCR samples of 174bp predicted length, amplified with the use of primers allowing for detection of SNP in PLA2G4C gene, on 1,5% agarose gel. Control group. The picture was taken using the G:Box transilluminator. L – sample mass template, 1-5 – numbers of PCR samples from the control group

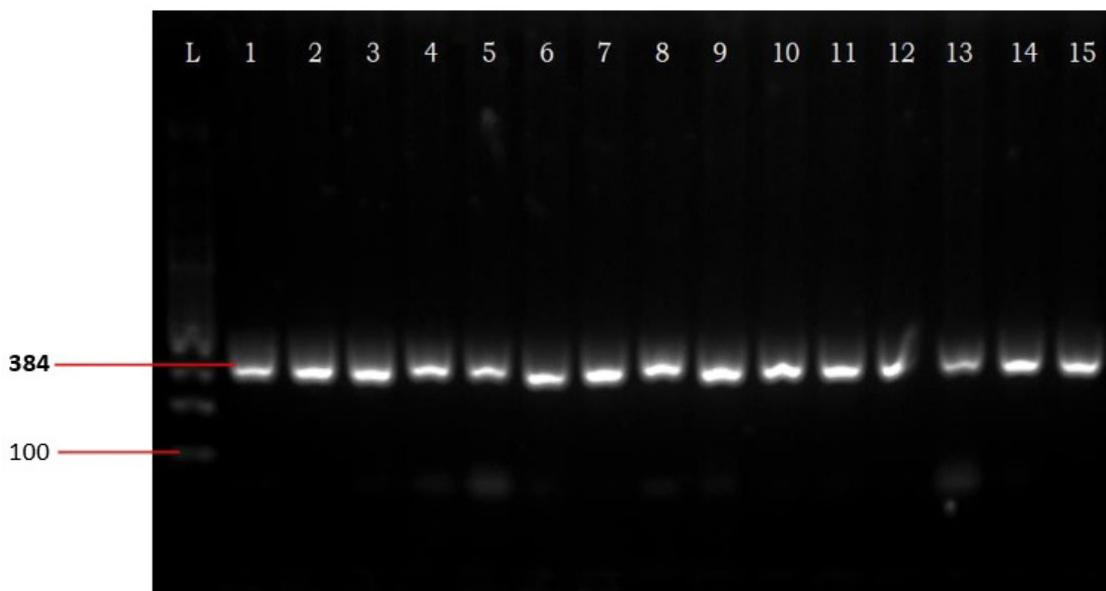


Figure 7 Electrophoretic separation of PCR samples of 174bp predicted length, amplified with the use of primers allowing for detection of SNP in PLA2G4D gene, on 1,5% agarose gel. Study group. The picture was taken using the G:Box transilluminator. L – sample mass template, 1-15 – numbers of PCR samples from the study group

consisted of 5 women who gave birth at predicted time, i.e. after the 37th week of pregnancy. The results of fetal age distribution at the time of birth in the study and control groups are presented in the graph below (**Fig. 4**).

In the study group, the majority of births occurred in the 32nd and 33th weeks of pregnancy. In

the control group, the majority of births were observed at 37 and 39 weeks of pregnancy.

The isolation of genomic DNA from the peripheral blood of the studied patients was the first stage of the genetic tests discussed. The research was carried out by the High-Technology Laboratory at the Faculty of Biology of the Adam Mickiewicz Uni-

versity in Poznan. After isolation, the material was stored at -20°C.

Figure 5 shows the results of the agarose gel electrophoretic separation of several genomic DNA samples obtained. The photo confirms the satisfactory quality of the isolated material.

The DNA preparations obtained as a result of the isolation were used to amplify the PLA2G4C and PLA2G4D gene fragments. Fragments that could contain single nucleotide polymorphisms were selected. The exemplary results of PCR reactions are presented in next four figures (**Fig. 6, 7, 8, 9**).

The obtained PCR products were sequenced. Exemplary sequencing results can be found in **figure 10** and **figure 11**. As part of the study, the occurrence of SNPs in the PLA2G4C and PLA2G4D genes for women from the test and control groups was

estimated. The estimated data is presented in the tables below (**Tab. 4, 5**) and on the charts (**Fig. 12, 13**).

In the PLA2G4C gene, the analyzed SNP did not occur in the control group. The highest number of heterozygous forms [A / C] was observed in the study group (9 out of 15 women). Heterozygous forms of AT and homozygous TT did not appear in any of the patients. There were two patients in the study group who gave birth prematurely without any polymorphism in the PLA2G4C gene.

In the PLA2G4D gene, the analyzed SNP did not occur in the control group. In the study group, the most heterozygous forms [A / T] were observed. There was one patient who gave premature birth despite the lack of polymorphism in the PLA2G4D gene.

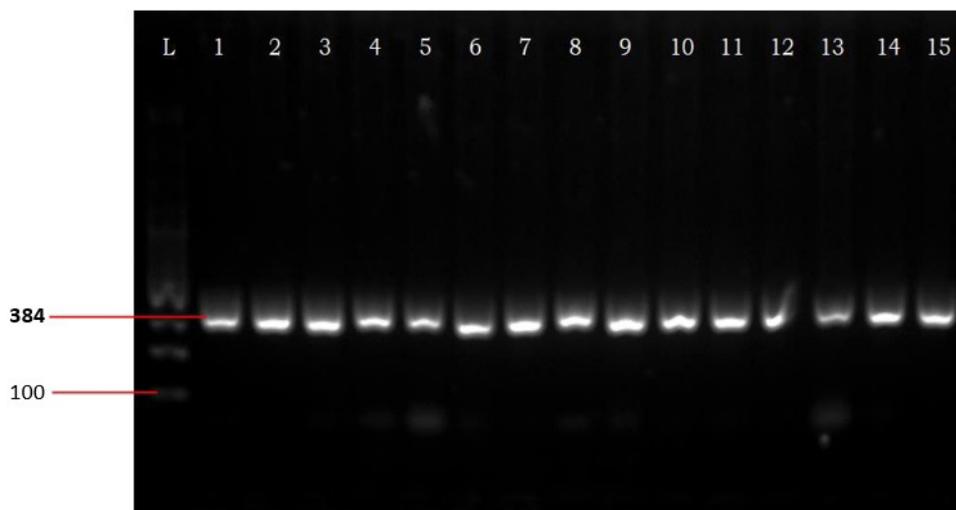


Figure 8 Electrophoretic separation of PCR samples of 174bp predicted length, amplified with the use of primers allowing for detection of SNP in PLA2G4D gene, on 1,5% agarose gel. Control group. The picture was taken using the G:Box transilluminator. L – sample mass template, 1-5 – numbers of PCR samples from the control group

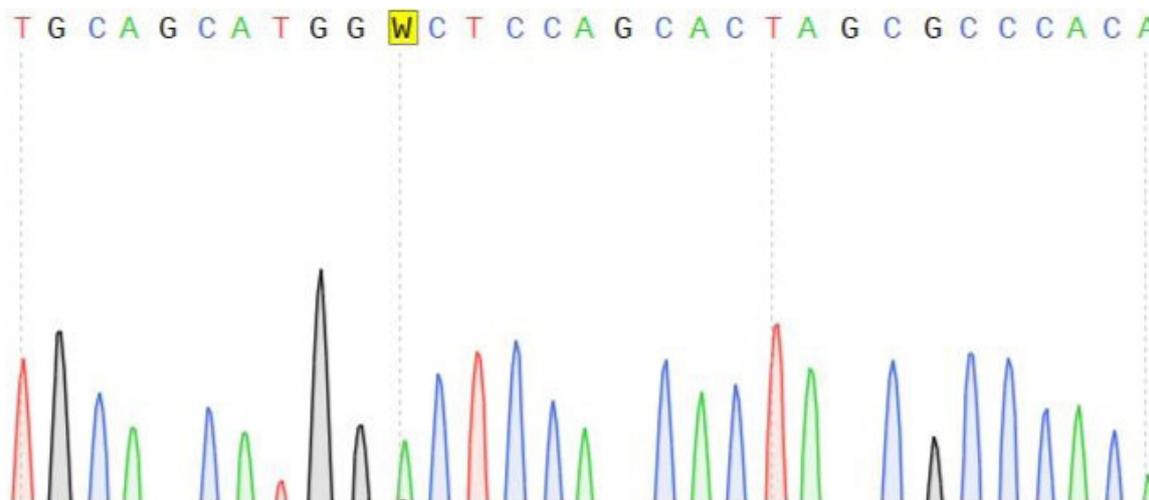


Figure 9 Sample PLA2G4C sequencing results in patient from the study group. SNP was marked with the letter K. [A/C]

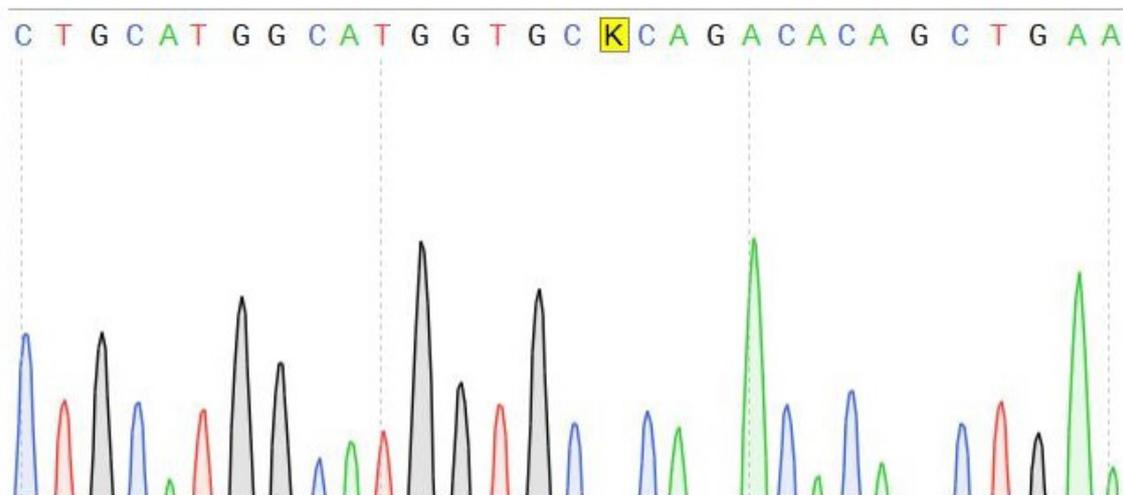


Figure 10 Sample PLA2G4D sequencing results in patient from the study group. SNP was marked with the letter W. [A/C]

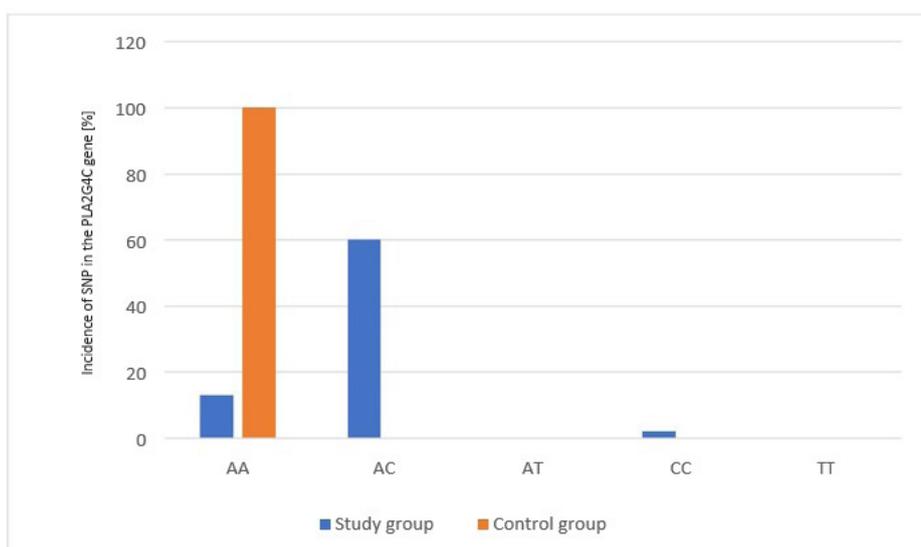


Figure 11 Comparison of rate of appearance of SNP in PLA2G4C gene in study and control groups

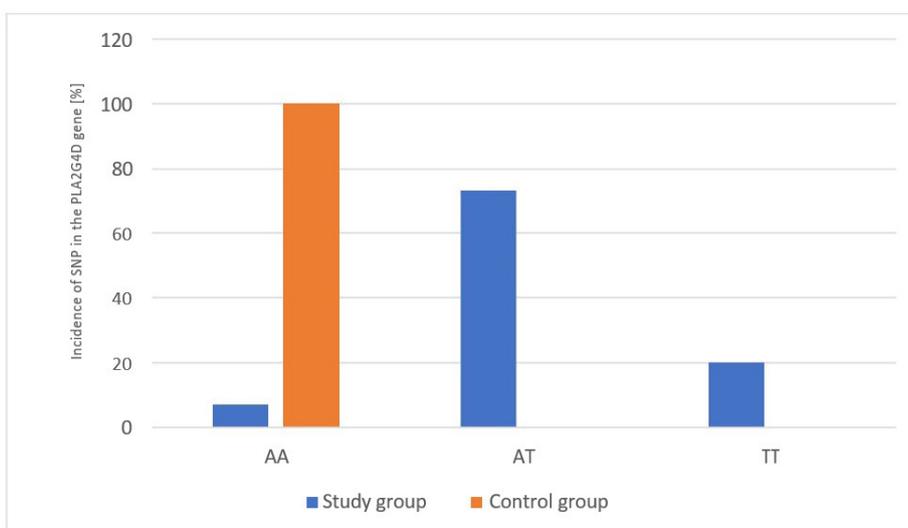


Figure 12 Comparison of rate of appearance of SNP in PLA2G4D gene in study and control groups

Table 5 Distribution of rate of the appearance of SNP [A/T] in the PLA2G4D gene. Study and control groups

	N	Variants [A/C/T]									
		AA		AC		AT		CC		TT	
		N	%	N	%	N	%	N	%	N	%
Study group	15	2	~13	9	60	0	0	4	~2	0	0
Control group	5	5	100	0	0	0	0	0	0	0	0

Table 4 Distribution of rate of the appearance of SNP [A/C/T] in the PLA2G4C gene. Study and control groups

	N	Variants [A/T]					
		AA		AT		TT	
		N	%	N	%	N	%
Study group	15	1	~7	11	~73	3	20
Control group	5	5	100	0	0	0	0

Discussion

As is known, a number of factors can influence premature birth, starting with general socio-economic and ending with specific genetic factors.

When studying the influence of inflammation on premature pregnancy, the topic of prostaglandins and their direct link with inflammation cannot be overlooked. In 1987, the effect of prostaglandins on an earlier time of delivery was examined. Roberto Romero conducted an experiment to investigate the effect of prostaglandin concentration in amniotic fluid, intrauterine infection, and premature delivery on increase of the risk of premature rupture of membranes. Prostaglandins E2 and F2α, in samples taken from female term and premature mothers, were measured by radioimmunoassays. Premature delivery in the absence of intrauterine infection was not associated with a significant increase in the concentration of prostaglandins in the amniotic fluid. However, women with pre-term labor and intra-amniotic infections had a higher concentration of prostaglandins in the fluid than women with preterm delivery and an absence of infection, or a those with an intra-amniotic infection in the absence of preterm delivery. This demonstrates the direct effect of prostaglandins on preterm delivery [11].

Currently, the relationship between single nucleotide polymorphisms and premature birth is also increasingly sought. An example of such research is the work of B. J. Wang et al. Wang and his group

examined 12 different polymorphisms of a single nucleotide in 11 genes involved in the folic acid metabolism pathway. The research shows that genotypes of complex mutations may increase the risk of preterm delivery. The study lists: MTHFD-G1958A, MTR-A2756G, RFC1-G80A, MTHFD-G1958A, MTR-A2756G and CBS-C699T [12]

However, the presence of SNP in candidate genes does not always have a negative effect on the length of pregnancy. In studies conducted in 2013 by Dr. Wei Wu, the reverse conclusions were drawn. Due to the role of inflammation in premature delivery (PTB), polymorphisms in the interleukin 6 gene and nearby (IL6) were the targets of these associative studies. It was found that IL6 SNP rs1800795 CC genotype protects against PTB in women of European descent and is irrelevant in fetal genotype analysis [13].

In this study, SNPs in PLA2G4C and PLA2G4D genes, that encode phospholipase A2, were examined. Studies have shown the lack of SNP in both genes in each woman in the control group. However, the study group showed a different case, where in the majority of premature mothers the results of sequencing tests showed the presence of SNP in the anticipated places. Nevertheless, this is not the first work on these specific genes and SNPs in their area.

In 2017, similar studies were conducted among the Chinese population. The results showed that SNP (rs4924618), found in the maternal PLA2G4D gene, was associated with a reduced risk of SPTB.

In turn, for the SNP in the PLA2G4C gene analyzed in the study, a zero association with premature delivery was observed [14]. Interestingly, a similar result was obtained by a US research group in 2010. A case-control study involving different clinical populations showed that SNP (rs1366442) in PLA2G4C was detrimental to PTB in the Hispanic population in the USA [15]

In studies dedicated to this work, SNPs in the PLA2G4C and PLA2G4D genes seem to have an association with preterm delivery. However, the results may be falsified by the small number of the test and control samples. An additional negative effect may be the disproportion between the test and the control sample. However, it should be remembered that although the same genes and sites where SNPs were presented for consideration, the research was conducted on different populations: Chinese, Latin American and Polish.

This fact calls for consideration of heterogeneity and characteristics specific to each population and ethnic group. It is possible that this is only additional evidence for the fact that the differences in the genetics of different populations and ethnic groups are the cause of different statistics on preterm delivery.

It is swayed that the research contained in this paper has the potential to contribute to a deeper understanding of the etiology of preterm delivery. However, it should be repeated in a larger test and control group. The reliable collection of data on women taking part in the research will also be indispensable. Deficiencies in questionnaires filled out by patients can often be an obstacle in analyzing statistical data.

Premature systolic uterine activity is still a topic worth constant exploration. By discovering new aspects of this issue, we will be able to predict some of the complications associated with earlier births, provide a basis for improving care for premature newborns, and shed new light on the mechanisms of the perfect machine that is the human body.

Abbreviations

AB1: washing buffer; CBS: β -cystathionine synthase; COX: cyclo-oxygenase; DNA: deoxyribonucleic acid; EDTA: EthyleneDiamine-Tetraacetic acid; MTHFD: C-1-tetrahydrofolate synthase; MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase; PCR: polymerase chain reaction; PGG: prostaglandin G; PLA2G4C: phospholipase A2 group IVC; PLA2G4D: phospholipase A2 group IVD; PTB: preterm birth; RFC1: reduces folate carrier; SNP: single nucleotide polymorphism; TAE: Tris Acetate Electrophoretic buffer.

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Conflict of interest

The authors declare that they have no conflict of interest.

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