TR
INONU UNIVERSITY
INSTITUTE OF SCIENCES

INVESTIGATION THE ROLES OF SIRTUIN 1, 2, AND 7 IN PLACENTA ACCRETA PATHOGENESIS

MASTER THESIS

Sevim GÜRBÜZ

Department of Molecular Biology and Genetics

Thesis Advisor: Asst. Prof. Dr. Irmak İÇEN TAŞKIN

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ACKNOWLEDGEMENTS

I would like to thank;

My advisor Asst. Prof. Dr. Irmak İÇEN TAŞKIN, for her moral support from the beginning to the end of this work process, and for giving me the opportunity to improve myself with her valuable suggestions,

Assoc. Prof. Mehmet Sait İÇEN, for the supply of tissues and serum samples,

Prof. Dr. Engin DEVECİ for his contribution immunohistochemical staining of placental tissues,

My dear mother and family, who supported me on this path I have embarked on,

Scientific and Technological Research Council of Turkey (TUBITAK), and Inonu University Scientific Research Projects Unitfor their contribution and support with 220S416 and TYL-2021-2348 project numbers respectively in the execution of my work.

WORD OF HONOR

This thesis titled "Investigation the Roles of Sirtuin 1, 2, and 7 in Placenta Accreta Pathogenesis", which I submitted as a Master's Thesis, is my own work. I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work. I confirm this with my honor.

Sevim GÜRBÜZ

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ABBREVIATIONS

CTB : Cytotrophoblast
D : Decidua
EMT : Epithelial Mesenchymal Transition
EVT : Extravillus Trophoblast
HDAC : Histone Deacetylase
M : Myometrium
MMP : Matrix Metalloproteinase
PAS : Placenta Accreta Spectrum
PC : Placenta Accreta/creta
PI : Placenta Increta
PP : Placenta Previa
PPT : Placenta Previa Totalis
S : Serosa
SIRT1 : Sirtuin 1
SIRT2 : Sirtuin 2
SIRT7 : Sirtuin 7
TGF-β : Transforming Growth Factor-β
ZEB2 : Zinc Finger E-Box 2

ABSTRACT

Master Thesis

INVESTIGATION THE ROLES OF SIRTUIN 1, 2, AND 7 IN PLACENTA ACCRETA PATHOGENESIS

Sevim GÜRBÜZ

Inonu University
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45+XI

2022

Thesis Advisor: Asst. Prof. Dr. Irmak İÇEN TAŞKIN

The Placenta Accreta Spectrum (PAS) is defined as the adhesion of the placenta to the uterine wall to varying degrees. The normal placenta does not pass beyond one-third of the myometrium, but the invasive placenta acts in a similar way to a malignant tumor, multiplying its local structures and invading the myometrium. Uterine scar, history of cesarean section, and presence of placenta previa are considered the clinical risk factors, but studies on the molecular mechanisms responsible in PAS are very limited. It is known that SIRT1, 2, and 7 affect epithelial plasticity by reprogramming EMT, leading to invasion and metastasis. Although EMT's markers level are high in PAS, there are no studies on the role of SIRT1, 2, and 7 in the pathogenesis of the disease. For this purpose, 30 PAS, 30 PPT and 30 controls were included in our study. The expression levels of SIRT1, 2 and 7 in the placentas of these groups were determined by Western blot method. Serum levels of SIRT1, 2, and 7 in maternal and fetal cord blood were determined by the ELISA. The localization of SIRT1, 2, and 7 in the placental tissues of the patient and control groups were determined by immunohistochemical staining. In our studies, we found that SIRT1 and SIRT7 were significantly lower in PAS compared to the control in placentas, while SIRT1, 2, and 7 levels in serum samples were not significantly different when compared between the 3 groups.

In conclusion, our results suggest that SIRTs may play an important role in the pathogenesis of the PAS in the aspect of contribution to invasiveness features.

Keywords: Placenta Accreata Spectrum, Epithelial Mesenchymal Transition, Sirtuin 1, Sirtuin 2, Sirtuin 7, Placenta Previa Totalis, Trophoblast Invasion.

ÖZET

Yüksek Lisans Tezi

SİRTUİN 1, 2 VE 7'NİN PLASENTA AKRETA PATOGENEZİNDEKİ ROLLERİNİN ARAŞTIRILMASI

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2022

Tez Danışmanı: Dr. Öğr. Üyesi Irmak İÇEN TAŞKIN

Plasenta Akreata Spektrumu (PAS) plasentanın uterus duvarına değişen derecelerde yapışması olarak tanımlanmaktadır. Normal plasenta miyometriyumun üçte birlik bölümünden ilerisine geçmez, ancak invaziv plasenta, malign bir tümöre benzer şekilde davranarak lokal yapılarını çoğaltıp miyometriyumu istila eder. Uterus skarı, sezeryan geçmişi ve plasenta previa varlığı klinik risk faktörleri arasında sayılsa da PAS'ta artmış trofoblast invasyonundan sorumlu moleküler mekanizmalar ile ilgili çalışmalar oldukça sınırlıdır. SIRT1, 2 ve 7'nin EMT'yi yeniden programlayarak epitel plastisitesini etkilediği, invazyona ve metastaza yol açtığı bilinmektedir. PAS'ta EMT belirteçleri yüksek olmasına rağmen, EMT'de etkinliği kanıtlanmış SIRT1, 2 ve 7'nin hastalığın patogenezindeki rolü ile ilgili herhangi bir çalışma mevcut değildir. Bu amaç doğrultusunda çalışmamıza 30 PAS ,30 PPT ve 30 kontrol dahil edilmiştir. Bu grupların plasentalarında SIRT1, 2 ve 7'nin ekpresyon seviyesi Western blot yöntemi ile belirlenmiştir. Maternal ve fetal kord kanına ait serumlarda SIRT1, 2 ve 7 seviyeleri ELISA yöntemi kullanılarak belirlenmiştir. Hasta ve kontrol gruplarının plasental dokularında SIRT1, 2 ve 7'nin lokalizasyonları immünohistokimyasal boyama ile belirlenmiştir. Çalışmamızda plasenta örneklerinde PAS'da SIRT1 ve SIRT7 seviyesinin kontrole göre anlamlı derecede düşük olduğu saptanırken, serum örneklerinde ise her 3 grup arasında da SIRT1, 2 ve 7 seviyelerinin istatistiksel olarak anlamlı bir fark göstermediği saptanmıştır.

Sonuç olarak elde ettiğimiz bulgular invaziv özelliklerine katkısı açısından Sirtuinlerin PAS patogenezinde önemli bir rol oynayabilecegini düşündürmektedir.

Anahtar kelimeler: Plasenta Akreta Spektrumu, Epitel Mezenkimal Geçiş, Sirtuin 1, Sirtuin 2, Sirtuin 7, Plasenta Previa Totalis, Trofoblast İnvazyonu.

1. INTRODUCTION

1.1 Placenta Accreta Spectrum

The term “placenta accreta” refers to the excessive trophoblast invasion of part or all of the placenta into the uterine wall's myometrium [1]. Irving and Herting first described it in 1937 as the abnormal adhesion, in whole or in part, of the afterbirth to the underlying uterine wall [2]. According to the decision taken by the International Federation of Obstetrics and Gynecology (FIGO) in 2018; The general name of the adhesion of the placenta to the uterus and the organs close to the uterus at different degrees is called the Placenta Accreta Spectrum (PAS) [3]. While a healthy placenta continues to develop and grow normally, it does not attach to the myometrium. However, abnormal adhesion to the myometrium is observed in placentas with PAS. Due to unhealthy adhesion, injuries (such as bladder and ureter) occur as the adherent placenta separates from the myometrium and other tissues during delivery. This situation leads to lose of blood [4]. Blood loss appears to be around of 3000-5000 ml on average. Moreover, while up to 90% of blood transfusions are required, approximately 40% of patients require more than 10 units of blood [5]. Although PAS can develop in the first trimester on rare instances, it is more commonly detected in the second and third trimesters [6]**.**

1.2 Incidence of PAS

PAS, and particularly its invasive forms, has a serious effect on maternal health all over the world. According to studies, the incidence of PAS is on the rise [7]. In the 1980s, it was just observed in 1 out of every 2510 pregnancies (0.03 %) [8]. From 1982 to 2002, it was 1 in every 533 pregnancies (0.18 %) [9]. According to recent research, it is 1 in 272 pregnancies in 2016 (0.36 %), (**Figure 1**) [6]. The stated maternal mortality rate is 7 % due to PAS today [10].

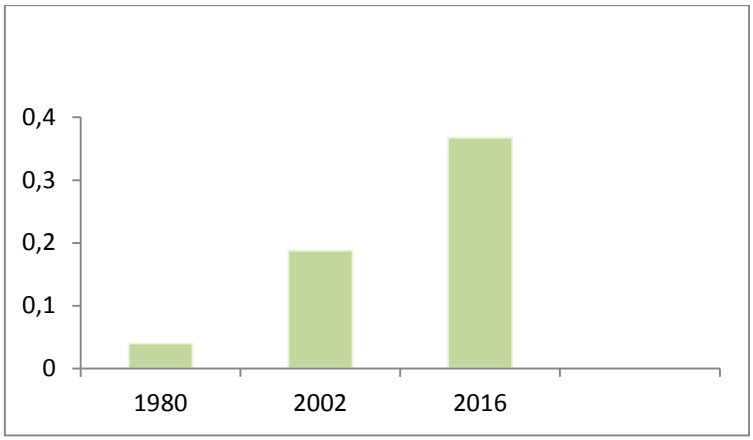

Figure 1: The percentage of incidence of PAS between 1980-2016 [6].

1.3 Classification

The PAS is histopathologically categorized into three types based on the degree of attachment to the myometrium [11]. In placenta accreta, chorionic villi (part of the placenta), attaches directly to the myometrium. In increta, chorionic villi attacks to myometrium and in the percreta it invades the adjacent organs by passing through the myometrium (**Figure 2**). The incidence rates of these three categories are as follows: Placenta accreta accounts for 79%, increta for 14%, and percreta for 7% of the 138 samples [9]. Increta and percreta are less common than accreta in PAS cases [12].

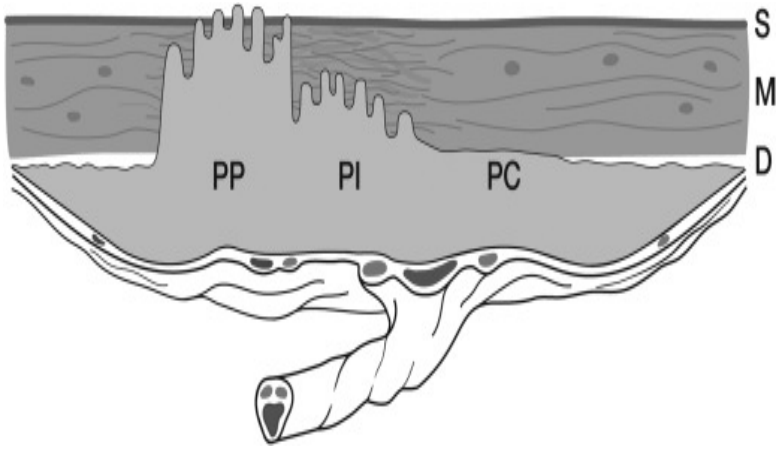

Figure 2: Types of PAS. **M**: myometrium, **D**: decidua, and **S**: serosa. **PC**: Placenta creta=accreta, **PI**: Plasenta Increta and **PP**: PIasenta Percreta. Abnormal placenta adherence and assault to the uterine wall [13].

1.4 Risk Factors of PAS

There are many risk factors available for the PAS. These include presence of placenta previa, previous uterine surgery, maternal age, placental position, previous uterine abortion, Asherman syndrome, uterine anomalies, and hypertensive pregnancy disorders. These main risk factors are explained below [14].

Presence of placenta previa: Placenta previa is defined as the cover of the cervix completely or partially by the placenta (**Figure 3**). It has been reported that the incidence of placenta previa in patients with placenta acreta is 11.10% [15].

Previous uterine surgery: The risk of developing PAS during pregnancy mostly increases if patient has had previous cesarean section and other uterine damage [16]. Furthermore, the probability of placenta previa and PAS development increases as the number of cesarean sections rises [17]. In addition, availability of the both previa and previous cesarean section increase the risk of the PAS. In placenta previa diagnosed women, the risk of PAS during the first cesarean section was 3% and, the risk of PAS during the second cesarean section increased by 11%, 40%, 61% and, 67% during the third, fourth and the fifth cesarean delivery respectively [18].

Maternal age: While the rate of placenta accreta is 3.2% in women under the age of 25, this rate is 6.2% in women between the ages of 25-29. The incidence of PAS in women aged 30-34 is 10.2%, and the incidence of PAS in women aged 34 and over is 14.6%. According to these data, the rate of PAS formation increases as maternal age increases (>35)[19].

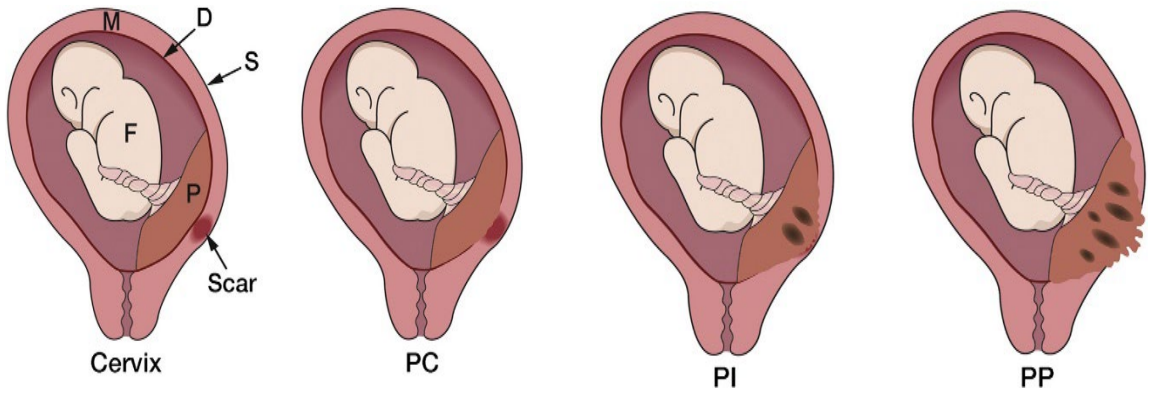

Figure 3: Types of previa accreta. Anterior of placenta previa (**P**) on cesarean scar and different types of previa accreta=creta (**PC**) villi adhere to myometrium (**M**). Placenta increta (**PI**) where villi invade myometrium. Placenta percreta (**PP**) the villi spread across the myometrium and cross the uterine serosa (**S**) [13].

1.5 Molecular Biology of the PAS

The most accepted hypothesis about the molecular mechanism of trophoblast invasion affecting PAS is that a defective in the endometrial-myometrial interface causes the disruption of the normal decidualization structure in the uterine scar region [20]. This abnormal structuring allows for deep placental anchoring villi and excessive trophoblastic invasion [13]. Studies in this area show that disruptions in the uterine cavity damage the endometrial-myometrial interface and affect the development of scar tissue [21].

Trophoblast cells are the first cell type to differentiate from the zygote formed in the early stages of pregnancy and make up most of the placenta. As the placenta continues to develop, the presence of an extremely invasive trophoblast causes placental aberrations (PAS). Insufficient invasion of the trophoblast may cause preeclampsia [22]. When invasive placenta samples were examined, remodeling trophoblasts deep in the myometrial artery and abnormal arterial enlargement in the same region were observed. According to extravillus trophoblast (EVT) histopathology examinations in hysterectomy samples taken from a total of 49 patients, 38 of whom were diagnosed with placenta accreta and 11 of whom were not diagnosed with accreta; Accreta specimens, decreased rate of remodeling vasculature and physiological changes were observed in most vessels. In addition, vascular

remodeling of the myometrium and interstitial trophoblast invading the uterine wall more deeply were observed [21]. Furthermore, placental villi advancement to the myometrium is linked to aberrant trophoblast cell development. Upregulation of a variety of angiogenic growth factor such as VEGF (vascular endothelial growth factor) and Angiopoietin-2 (Ang-2) was detected in PAS samples compared to normal placenta, according to Tseng et all., studies [23]. Epidermal growth factor receptor (EGFR) has been shown to be up-regulated in syncytiotrophoblasts from PAS patients [24].

Epithelial mesenchymal transition (EMT) is a developmental program that causes quiescent epithelial cells to transform into migratory mesenchymal cells [25]. It is very critical for the healthy and correct adherence of the placenta to the myometrium during the first trimester. In the second and third trimesters, normal EMT binding should not be continued [26]. It has been shown that if EMT is present in the second and third trimesters, it contributes to the formation and development of PAS [27]. DaSilva-Arnold et all., showed that the difference in cytotrophoblast CTB-EVT during the first trimester causes the formation of the EMT [28].

Matrix metalloproteinase (MMP) is a family of enzymes that breakdown the extracellular matrix and have a role in trophoblast cell penetration [29]. MMP-2 and MMP-9 levels in PAS samples have been demonstrated to rise with time when compared to control samples [30]. A higher expression pattern of the MMP-2 in PAS samples was detected than in normal placental samples in a research conducted by Kocarslan et all., [31]. The loss of E-cadherin is the well defined event during EMT. It was observed that E-cadherin's expression in the chorionic villi of the invasive part was lower in placentas with placenta accreta than in the non-invasive parts, and the expression of Snail and TGF-β increased in the decidual cells of the invasive part [32].

1.6 Sirtuins

The silent information regulatory (SIR) family of proteins and their mammalian homologues, sirtuins, catalyze the covalent exchange of histones by deacetylation, which results in chromatin remodelling [33]. Classified as histone deacetylases (HDACs), sirtuins are proteins that repress gene transcription by removing acetyl moieties from the acetamido group on lysine residues within histones [34]. This gene was originally identified in *Saccharomyces cerevisiae* by Klar et all., [35]. Sirtuins catalyze the deacylation of proteins at acyl-lysine residues in the presence of NAD^+. From bacteria to eukaryotes, these proteins may be found in a wide range of species [36]. Sirtuin genes have been found in bacteria, worms, flies, plants, and mammals [37]. There are seven protein members in the mammalian (SIRT1-7). They can be found in a variety of locations within the cell. Furthermore, the activities and roles of these proteins differ from one another [38]. SIRT1, SIRT6, and SIRT7 are generally located in the nucleus, SIRT2 is mostly found in the cytoplasm, while SIRT3, SIRT4, and SIRT5 are mostly found in the mitochondria (**Figure 4**) [39]. They are classified as cofactors based on the NAD requirements [40]. The control of gene expression is the most critical function of the SIRT protein family [41]. SIRT1 is primarily limited to the nucleus because it controls gene expression by deacetylating lysine amino acid residues, lowering the effectiveness of p53 transcription and inhibiting apoptosis in response to oxidative stress and/or DNA damage [42].

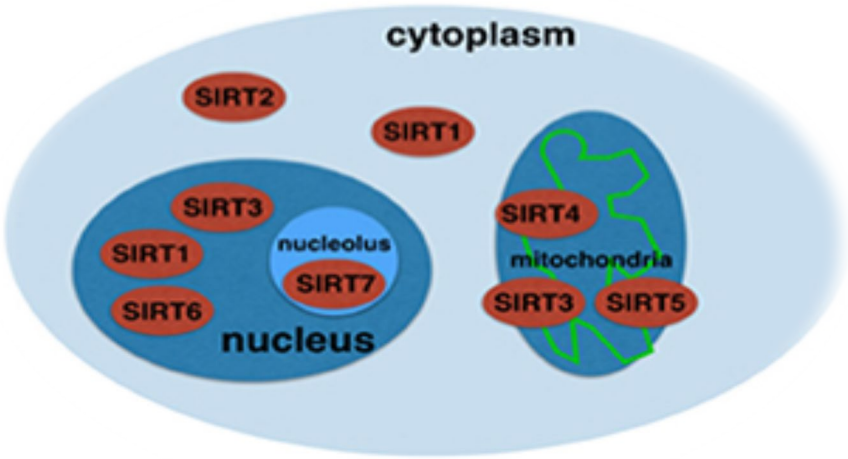

Figure 4: Intracellular location of Sirtuins [43].

Sirtuin 1 (SIRT1) is one of the protein from sirtuin family [37]. It's gene is found on chromosome 10q21.3, and its genomic sequence spans 33,660 base pairs with a single genome location. SIRT1, which is encoded by 9 exons and containing 747 amino acids [44]. It is a NAD-dependent deacetylase that removes acetyl groups from a variety of histone and nonhistone proteins [45]. SIRT1 is involved in the formation of heterochromatin by deacetylating histone H1, K9 in histone H3 and K16 in histone H4. It is also govern in nonhistone protein deacetylation. These include transcription factors, DNA repair proteins, and signaling proteins. Because of its capacity to deacetylate a number of substrates, SIRT1 is engaged in a variety of physiological processes, including the regulation of gene expression, metabolism, cancer, and senescence [38]. Studies in mice have also shown that SIRT1 is involved in development and is also important for embryogenesis and gametogenesis [46]. They modulate epithelial plasticity via induction of the transcriptional reprogramming that lead to EMT, invasion and metastases [47]. The most fundamental event that occurs in EMT is the disappearance of the E-cadherin. Its loss is associated with lower survival, worse prognosis and higher metastasis rate [48]. It has been shown that SIRT1 is functioning in EMT activation through deactivating E-cadherin expression and acting a crucial role in the regulation of EMT in pancreatic cancer [49]. SIRTs used as tumor suppressors are known as negative regulators of EMT. Similarly, it has been shown that overexpression of SIRT1 in breast cancer cells decreases EMT in mice, whereas suppression of SIRT1 increase EMT [50].

Sirtuin 2 (SIRT2) is a histone deacetylase that depends on nicotinamide adenine dinucleotide (NAD^+) [51]. The SIRT2 gene has 18 exons and is found on human chromosome 19 [52]. It is mostly found in the cytoplasm, although it can also be detected in the nucleus [42]. SIRT2 can switch between nucleus and cytoplasm [53]. SIRT2 interacts with the HOXA10 in both the cytoplasm and the nucleus, and so plays a role in mammalian development [54]. Human SIRT2 expression is predominant in brain, heart, testis and skeletal muscle tissues [55]. The mammalian central nervous system (CNS), including the cortex, striatum, hippocampus, and spinal cord, has been demonstrated to be strongly expressed SIRT2 [56]. Also SIRT2 plays a critical role in the regulation of many vital activities, including apoptosis, aging, gene transcription, inflammation and metabolism [57]. In addition, SIRT2 is known to have a role in EMT. An increase in

MMP-9 was observed in SIRT2-null cells, while a decrease in E-cadherin, which promotes cellular migration and invasion [58].

Sirtuin 7 (SIRT7) is found mostly in the nucleolus and it is the last of the mammalian Sirtuins in *Homo sapiens*. SIRT7 localizes to chromosome 17q25.3 and encodes a 400 amino acid protein that acts as a class III histone deacetylase [51]. SIRT7 has been demonstrated to collaborate with upstream binding factor (UBF) and RNA polymerase I (RNA Pol I). It is expressed higher in metabolically active testis, spleen and liver tissues and less in nonproliferating tissues such as heart, brain, and skeletal muscle [59]. SIRT7 is required for the transcription of rDNA [60]. Furthermore, SIRT7 reduces DNA damage while assisting cellular survival in genomic stres situations [61]. SIRT7 is known to modulate EMT by transforming growth factor-β (TGF-β) signaling. SIRT7 deficiency activates TGF-β signaling and increases EMT. It has also been demonstrated that resveratrol promotes SIRT7 deacetylase activity, inhibits breast cancer lung metastases and improves survival, as well as antagonizes TGF-signaling [62].

2. MATERIALS AND METHODS

2.1 Chemical Materials

Western Bright ECL (Advansta), TGX FastCast Acrylamide Kit 10% (BIO-RAD), Sodium chloride (Sigma), Disodium hydrogen phosphate (Sigma), Sodium dodecyl sulfate (Sigma), Potassium chloride (Sigma), Glycine (Sigma), Trizma base (Sigma), Potassium dihyrogen phosphate (Sigma), Bromophenol blue (Fisher bioreagents), Tween-20 (Fisher bioreagents), Rıpa buffer (Thermo), Protease inhibitör (Thermo), EDTA (Thermo), Nuclease (Thermo), Benzonase nuclease (Sigma), Human SIRT1 ELISA kit (Elabscience), Human SIRT2 ELISA kit (Sunred), Human SIRT7 ELISA kit (Sunred), Ultravision-TP-125-HL (Thermo) and Ultravision-TA-125-HD (Thermo).

2.2 Tools and Equipments

Millipore brand pure water unit, electrophoresis power supply (Bio-rad), pH meter (Mettler Toledo), Blotting cassette (Thermo), PVDF Transfer membrane 0.45/30 cmx3M (GVS), Western blotting filter paper 7cm-8.4 cm (Thermo), Syngene G:Box device, Precision balance (Ohaus) and positively charged slide (OrLab).

2.3 Solutions and Buffers

10X TBS (Tris-Buffered Saline) with Tween-20 (TBS-T) 1L

- ◊ 30 gr Tris Base
- ◊ 2 gr KCI
- ◊ 80 gr NaCl
- ◊ 10 ml Tween-20

10X Running Buffer (SDS-PAGE) 1L

- ◊ 10 gr of SDS
- ◊ 30 gr Tris Base
- ◊ 144 gr Glycine

10X Transfer Buffer Working Solution 1L

- ◊ 144 gr Glycine
- ◊ 30 gr Tris Base

10X Phosphate Buffered Saline (PBS) 1L

- ◊ 80 gr NaCI
- ◊ 2 gr KCI
- ◊ 14.4 gr Na_2HPO_4
- ◊ 2.4 gr KH_2PO_4

2X SDS Loading Dye Buffer

- ◊ 125 mM Tris-HCI, pH=6.8
- ◊ 20 % Glycerol
- ◊ 4.0 % SDS
- ◊ 0.02 % Bromophenol Blue

Blocking and primary/secondary antibody dilution buffer

- ◊ 5 % non fat dry milk powder or 5% BSA
- ◊ 1X TBS-T (0.1% Tween-20)

2.4 Antibodies

Table 1: Primary Antibodies.

Antibody	Species	Dilution	Application	Vendor
SIRT-1	Rat	1:1000	WB	BioLegend
SIRT-2	Rabbit	1:1000	WB	St John's Lab.
SIRT-7	Rabbit	1:1000	WB	St John's Lab.

Table 2: Secondary Antibodies.

Antibody	Species	Dilution	Application	Vendor
HRP anti mouse	Goat	1:5000	WB	Advansta
HRP anti rabbit	Goat	1:5000	WB	Advansta

2.5 Selection of the People Included in the Study

Patients who applied to Dicle University Faculty of Medicine, Department of Obstetrics and Gynecology were included in our study. Three groups of patients were formed. The first group consists of patients with no previous history of cesarean section, uterine intervention or uterine surgery but diagnosed with placenta previa without invasion were included, and this group was named the placenta previa totalis (PPT) group. In the second group, patients who had at least one previous cesarean section and had placenta previa and invasion were included, and this group was called the placenta accreta spectrum (PAS) group. The third group was the control group, and those with similar demographic features and with no known disease were included. The patients in the control group (as the recommended delivery method of PAS and PPT were cesarean section) were formed from patients who delivered by cesarean section. Patients with placenta previa marginalis or inferior placenta, those who underwent surgery before the 24th week of pregnancy or patients who gave birth under 500 grams, individuals under the age of 18, multiple pregnancy, patients with pregnancy complications in the past, thyroid dysfunction, hypertension, epilepsy, those with gestational diabetes mellitus, those with type 1 and type 2 diabetes mellitus, any known systemic disorder, patients using any medication that may affect the cardiovascular system, pregnant women with kidney disease were not included in the study. For preoperative diagnosis, abdominal, transvaginal and Doppler ultrasonography were used. PAS diagnosis (as stated in the literature); a) With the pathology result in patients who underwent hysterectomy b) It was placed with the failure of spontaneous separation of the placenta or a part of it from the uterine wall during the operation and active bleeding of the placental bed due to this. Age of patients, gravidity, parity, pregnancy week, baby's gender, baby's weight and patient information was recorded. Care was taken to ensure that the age, number of pregnancies and gestational weeks of the patients were compatible with the control group. The patients who took part in the research and the individuals in the control group were informed. Written consent of the volunteers was obtained.

The research was done out in the Inonu University's Molecular Biology and Genetics department's laboratory. This study was conducted with the decision of Inonu University Clinical Research Ethics Committee dated **2020/51-13.05.2020.** The research and study process was carried out according to the protocol of the clinical research ethics committee.

2.6 Collecting Placental Samples

Placental tissues were obtained from the parts that faced the maternal surface in all groups. They were collected immediately after cesarean section and stored at -80 degrees until Western blot analysis and also stored in 10% formaldehyde solution for immunohistochemical staining.

2.7 Determination of Total Protein Levels of SIRT1, 2, and 7 in Placenta Samples

2.7.1 Preparation of samples

In placental samples obtained from 20 PPT, 30 PAS, and 30 control individuals, SIRT1, 2, and 7 protein levels were determined using western blot method. For western analysis, placenta samples were removed from -80 degrees and crushed in liquid nitrogen. After that, cold RIPA buffer containing protease-phosphatase inhibitor cocktail and nuclease was added to milled sample. It was vortexed on ice at 15 minutes intervals and incubated for one hour.

2.7.2 Measuring protein concentration

The quantity of total protein was determined using the BCA protein assay kit. Total protein concentration was determined in µg/ml by measuring at 562 nm using BSA (Bovine Serum Albumine) standards and following the kit's instructions for use.

2.7.3 SDS-PAGE

APS was prepared 10% fresh. The separation gel is prepared and poured into the tank. Immediately afterwards, the stacking gel was prepared and poured over the separating gel and the comb was placed. The loading buffer was thoroughly mixed with β-mercoptoethanol. The protein samples and the loading buffer were thoroughly mixed. After denaturation, it was loaded into the wells then 1X working buffer was added to the tank (**Figure 5**). Lastly, the proteins were operated at 120V for 120 minutes.

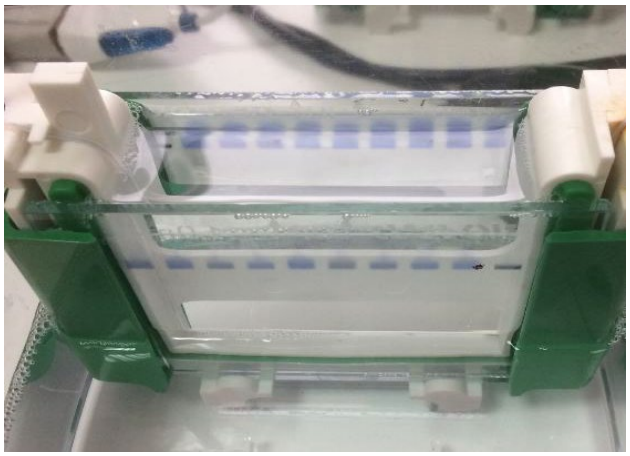

Figure 5: Loading of protein samples into gel wells.

2.7.4 Transfer of the gel to the mebrane

The membrane was first soaked in methanol for 2 minutes. 4 filter papers and the membrane that moistened with methanol were kept in 1X transfer buffer for 5 minutes. For transfer of gel to the membrane; First, 2 filter papers were placed and air bubbles were removed. The membrane was placed on filter papers and the gel removed from the tank and placed on the membrane. Two more filter papers were placed on top of the gel. The proteins in the gel were transferred to the membrane.

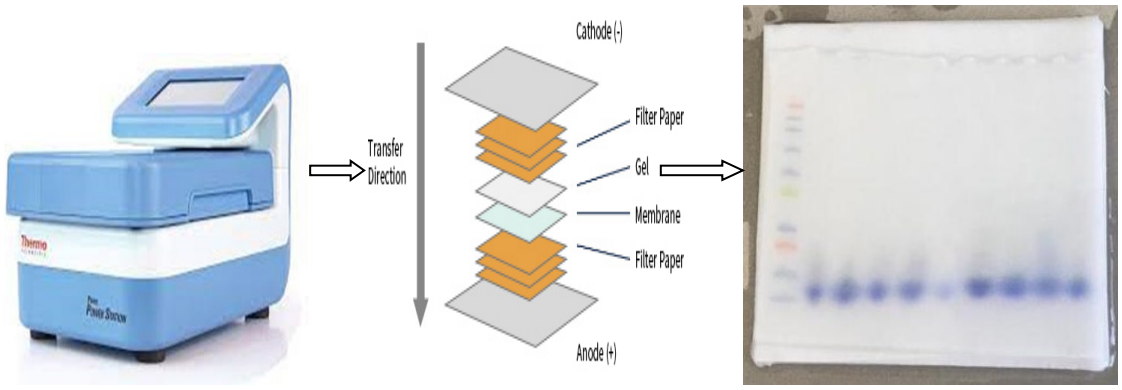

Figure 6: Western blot transfer system.

After transfer, the membrane was treated with the primary antibody for 60 minutes at 20 to 25°C. Then, membrane was shaken overnight at +4 °C. It was also shaken additionally for 120 minutes at 20 to 25°C. The membrane was take out from the primary antibodies and washed. The membrane was treated with the secondary antibody for 90

minutes at 20 to 25°C. After antibody treatment, the membrane was washed with TBST. ECL (Enhanced Chemiluminescent) and WB (Western Bright) were thoroughly mixed with peroxide. The entire surface of the membrane was treated with ECL and kept in a lightless environment and then, imaging was performed.

2.8 Measuring Maternal and Fetal Cord Blood Serum Level

Protein levels in maternal and fetal cord serums were determined by ELISA method. The serums of maternal venous blood and fetal cord artery blood kept at -80°C and were removed and thawed on ice at the day of analysis.

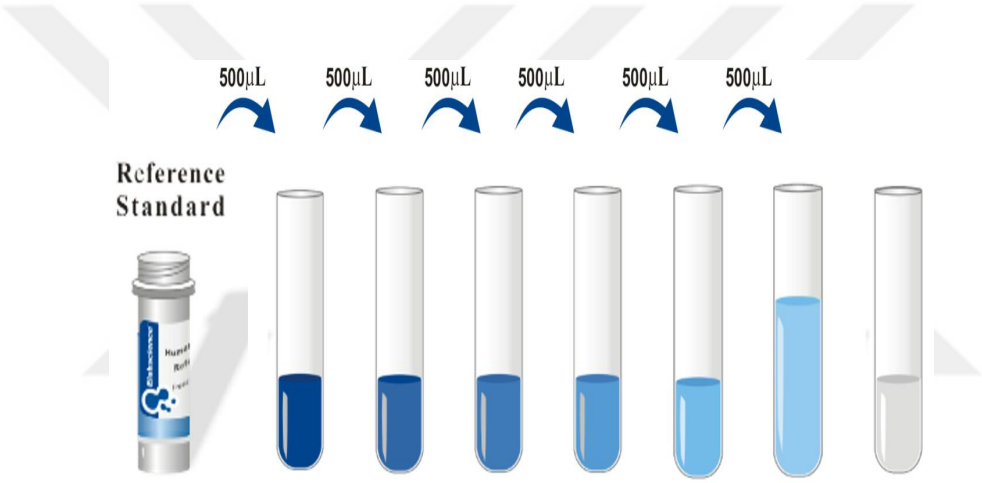

Figure 7: Preparation of the main standards by dilution.

1. Standards of SIRT1, 2, and 7 were prepared by diluting the main standards as indicated in the contents of each kit.
2. Relevant solutions were prepared.
3. 50 µl of standards and 40 µl of serum samples were added to the wells.
4. 10 µl of SIRT1, 2, and 7 antibodies were added to wells other than standard and blank.
5. All wells received 50 µl of Streptavidin-HRP. Excluding the blank.
6. The plate was enclosed with foil, mixed slowly, and it was incubated at 37°C for 60 minutes.
7. Then, the plate was washed 5 times with 1X washing solution.

8. 50 µl of chromogen solution A and chromogen solution B were added to the all wells. It was mixed and covered with foil and kept at 37°C for 10 minutes.
9. Then, 50 µl of stop solution was added to all wells.
10. The optical density (OD value) of each well was identify with a microplate reader at 450 nm.

2.9 Determination of SIRT1, 2, and 7 Localisation in Placental Tissues

SIRT1, 2, and 7 expression localizations in placental tissues were determined by immunohistochemical staining. Tissue parts of the maternal face from the placentas of the individuals included in the PPT, PAS and control groups were taken into 10% neutral buffered formalin solution for routine tissue follow-up. It was kept in the fixation solution for 48 hours. After fixation, tissue pieces were removed from formalin solution and washed with water. Then the tissue samples were passed through increasing alcohol series (50%, 70%, 80%, 90%, 96% and absolute ethyl alcohol series) and were in filtrated in paraffin at 58 °C after clearing (3x30 minutes in xylene). After the infiltration stage, the tissues were embedded in paraffin blocks and the sections with 4-6 µm thick were taken from the blocks with the help of a microtome and prepared for Hematoxylin-eosin and immunohistochemical staining.

2.9.1 For hematoxylin-eosin staining;

1. Placenta tissue sections with 4-6 µm thickness taken from paraffin blocks were transferred a bain-marie set at 37 °C.
2. Sections were held in the oven for 6 hours at 58-62 °C to dissolve any remaining paraffin on the slide.
3. It was deparaffinized in xylene for 3x15 minutes.
4. For 10 minutes, sections were run through areduce series of alcohol (100%, 96%, 90%, 70%, 50% ethyl alcohol) followed by distilled water for 5 minutes.
5. Tissue sections were soaked in Harris Hematoxylin stain for 8 minutes and then washed under running water for 5 minutes.
6. Tissue sections were rinsed and soaked in alcoholic eosin stain for 6 minutes.
7. Tissue slices were swiftly dipped in increasing alcohol series (80%, 90%, 96%) and held in alcohol for 2 minutes.
8. Lastly, the sections were kept in xylene for 3x15 minutes and covered with a coverslip by dripping Entellan onto the tissue.

2.9.2 For immunohistochemical staining;

1. Placental tissue sections of 4-6 μm thickness taken from paraffin blocks were placed in a bain-marie set at 37 °C. It was then transferred to polylysine slides.
2. Sections were held in the oven for 6 hours at 58-62 °C to dissolve any remaining paraffin on the slide surface.
3. Tissue sections that were deparaffinized for 3x15 minutes in xylene were then passed through a series of decreasing alcohol (100%, 96%, 90%, 70%, 50% ethyl alcohol) for 10 minutes and kept in distilled water for 5 minutes.
4. Tissue sections were washed in phosphate buffer solution (PBS) for 3x5 minutes.
5. Then, sections were taken into Ethylenediamine tetraacetic acid (EDTA) solution (pH: 8.0) for antigen retrievial treatment and heated in a microwave oven for 3x6 minutes.
6. After warming, the tissue sections were kept at 20 to 25°C for 20 minutes and the sections were transferred to PBS.
7. Immunohistochemistry bar, where the sections will be arranged, was prepared and the humidity and temperature of the bar were controlled. Tissue sections were lined up on the prepared immunohistochemistry bar and tissue borders were drawn with a hydrophobic pen.
8. Hydrogen peroxide solution was dripped onto the tissue sections and left for 20 minutes.
9. Then the tissue sections were washed with PBS for 3x5 minutes and the sections were left for 8 minutes by dripping Ultra V Block solution.
10. The V Block solution was removed from the sections. SIRT1, 2, and 7 primary antibodies were prepared at a 1:100 dilution and dropped onto the sections, and the sections were stood overnight at +4°C.
11. The next day, the sections were kept at room temperature for 30-60 minutes and washed with PBS.
12. Then, secondary antibody with biotin was dripped onto the tissue sections lined up on the bar and incubated for 14 minutes. After streptavidin-peroxidase was dripped onto the sections and waited for 15 minutes, the sections were washed with PBS again.
13. 3,3'diaminobenzidine (DAB) was dripped onto the washed sections and left for 5-10 minutes. Tissue sections were washed with PBS for 3x5 minutes and counter-stained with Harris hematoxylin.

14. Afterwards, the sections were closed with entellan and evaluated under Zeiss Imager A2 photomicroscope and imaged.

2.10 Statistical Analysis

Band intensities obtained as a result of Western blot analysis evaluated by the Image J. OD values obtained as a result of ELISA analyzes were analyzed via Myassays program. Statistical analyis was performed by SPSS® 11.5 (SPSS Inc; Chicago, IL, USA) program. As a result of the analyses, mean±standard deviations of the numerical data were given. Normally distributed data in the comparisons between multiple groups were analyzed with a one-way ANOVA test. Mann Whitney-U was used to examine SIRT1, 2, and 7 levels with newborns gender. Pearson's correlation coefficient analysis was applied to explore the correlation. (p<0.05) value was considered statistically significant.

3. RESULTS

3.1 Demographic and Clinical Characteristics of Individuals Included in the Study

Demographic and clinical properties of control, PPT and PAS groups are given in (**Table 3**). All group shows similar features in terms of age, birth weight, birth week, hemoglobin, hematocrit and thrombocyte.

Table 3: Demographic and clinical characteristics of patient groups.

Demographic and clinical characteristics	**Control**		**PPT**		**PAS**		***p* value**
	mean,	± std	mean,	± std	mean,	± std	
Age	33.3	± 4.8	33.4	± 6.0	33.9	± 4.9	0.883
Gravidity	4.7	±1.9	3.2	± 2.3	5	± 1.8	*0.009
Parity	3.2	±1.4	1.45	± 1.5	3.3	± 1.7	**0.0002
Previous cesarean section	2.7	±1.1	0		2.3	± 1.0	0
Birth weight (g)	3076	±420	2954	±574	2788	± 430	0.062
Birth week	37.2	± 1.2	36.5	± 2.4	36.3	± 1.2	0.106
Hemoglobin	11.7	± 1.4	11.3	± 1.6	11.5	± 1.2	0.689
Hematocrit	36.1	± 3.6	36.5	± 4.7	34.9	± 3.5	0.505
Thrombocyte	224	± 58	197	± 40	227	± 51	0.103

One-Way ANNOVA test was used. (*p<0.05, **p<0.01).

3.2 Determination of the Total Protein Levels of SIRT1, 2, and 7 in Placenta Samples

The presence of SIRT1, 2, and 7 were examined by Western blot analysis in the placental tissues viausing anti-SIRT1, anti-SIRT2 and anti-SIRT7 primary antibodies. Our result showed that SIRT1 expression levels were significantly decreased in PAS patients compared to the control and PPT groups (*p<0.037), (**Figure 8, Figure 9**). In addition, SIRT7 expression levels were lower in PAS patients compared to the control and PPT groups (*p<0.024) (**Figure 8, Figure 11**). However, even though SIRT2 expression levels were found to be decreased in PAS patients compared to control and PPT groups this decreation was not statistically significant (p>0.05). (**Figure 8**, **Figure 10**).

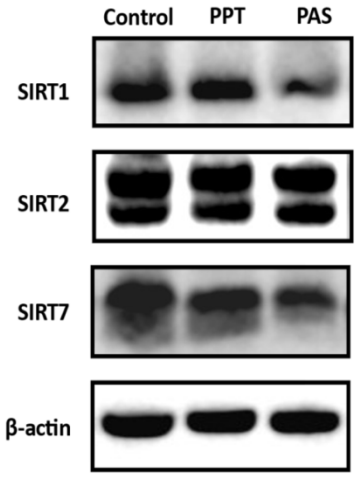

Figure 8: Protein levels of SIRT1, 2 and 7 were determined by Western blotting. β-actin was used as a loading control.

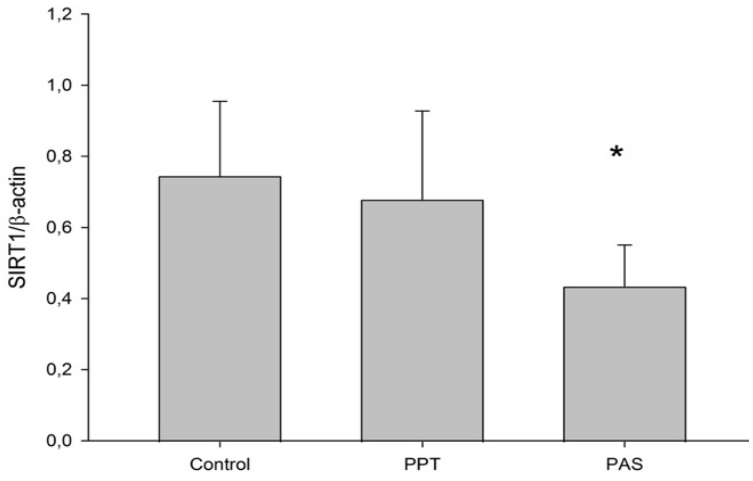

Figure 9: Densitometry analyses of the intensity of the bands of SIRT1 were presented as a ratio to the total level of β-actin. Quantitative data indicate the mean ± standard deviation (error bars), (* $p<0.05$).

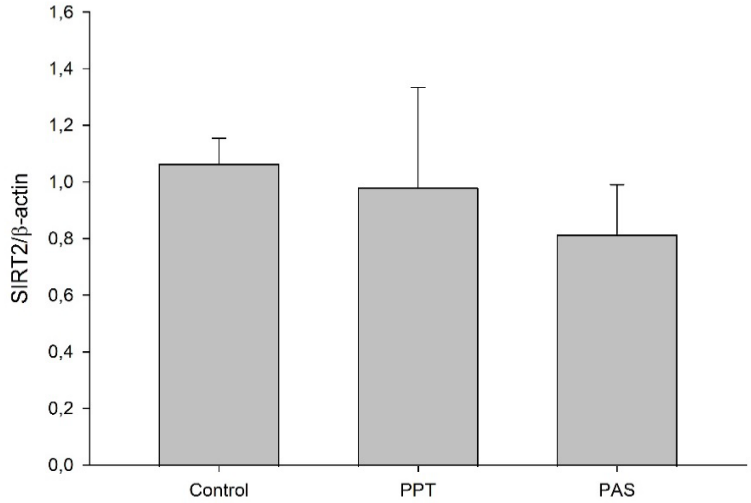

Figure 10: Densitometry analyses of the intensity of the bands of SIRT2 were presented as a ratio to the total level of β-actin. Quantitative data indicate the mean ± standard deviation (error bars).

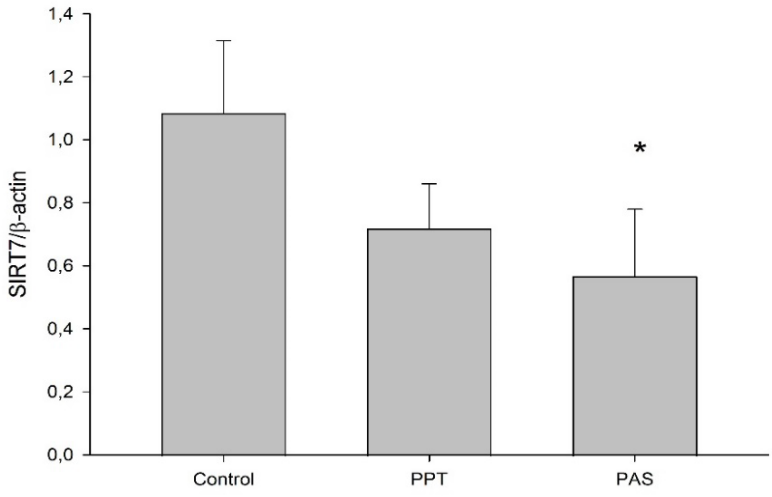

Figure 11: Densitometry analyses of the intensity of the bands of SIRT7 were presented as a ratio to the total level of β-actin. Quantitative data indicate the mean ± standard deviation (error bars), (* $p<0.05$).

3.3 Determination of the Protein Levels of SIRT1, SIRT2, and SIRT7 in Maternal and Fetal Cord Serums

This study was consisted of 30 individuals in control (30 maternal and 30 fetal cord serums), 20 patients in PPT group (20 maternal and 20 fetal cord serums), and 30 patients in PAS group (30 maternal and 30 fetal cord serums). SIRT1, SIRT2 and SIRT7 protein level were identified by ELISA in all serums.

30 maternal and 30 fetal cord serum were included in the study for SIRT1 analysis. However, the majority of the control and patient groups could not be identified because they were out of the kit's range. Our result showed that there was not any statistically difference between SIRT1 level in both maternal serum samples of control (n=30, mean: 8.6ng/ml, ±std: 5.4), PPT (n=20, mean: 11.3ng/ml, ±std: 3.0) and, PAS (n=30, mean: 5.7ng/ml, ±std: 5.2), (p=0.1145) (**Figure 12.A**) and fetal cord serums of control (n=30, mean: 7.0ng/ml, ±std: 5.2), PPT (n=20, mean: 7.1ng/ml, ±std: 5.4) and PAS (n=30, mean: 3.5ng/ml, ±std: 2.6), (p=0.1838), (**Figure 12.B**).

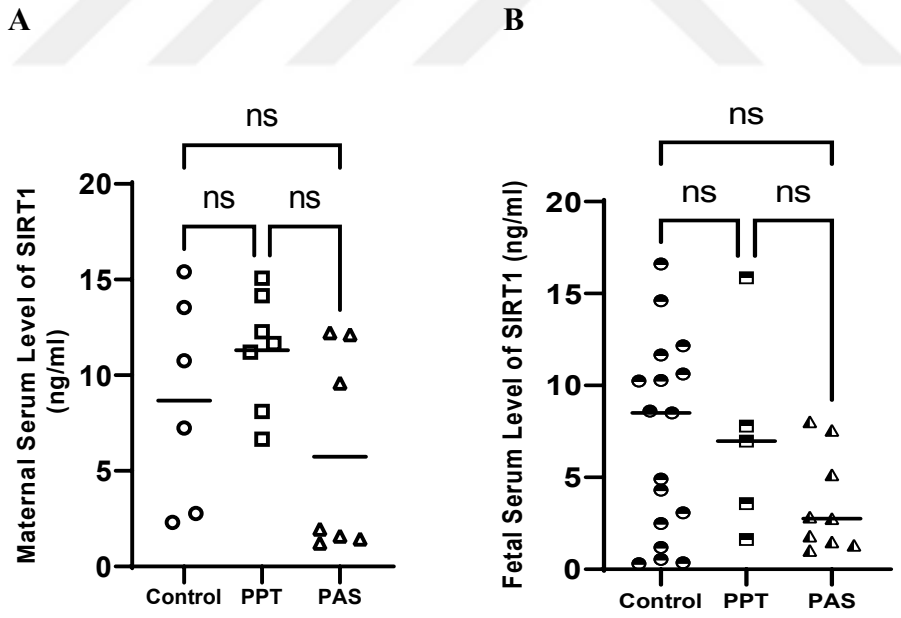

Figure 12: Maternal and fetal serum levels of SIRT1 in control, PPT and PAS. (**A**) Comparison of SIRT1 levels in control (n=30), PPT (n=20) and PAS (n=30) groups in maternal serums. (**B**) Comparison of SIRT1 levels in control (n=30), PPT (n=20) and PAS (n=30) groups in fetal serums. One way ANOVA was used for comparisons among the three groups.

Our result showed that there was not any statistically difference between SIRT2 level in both maternal serum samples of control (n=30, mean: 1.7ng/ml, ±std: 1.0), PPT (n=20, mean: 1.5ng/ml, ±std: 0.8) and PAS (n=30, mean: 2.3ng/ml, ±std: 1.7), (p=0.0549) (**Figure 13.A**) and fetal cord serums of control (n=30, mean: 4.5ng/ml ,±std: 3.13), PPT (n=20, mean: 3.85ng/ml, ±std: 2.91) and PAS (n=30, mean: 4.14ng/ml, ±std: 3,09) (p=0.6932), (**Figure 13.B**).

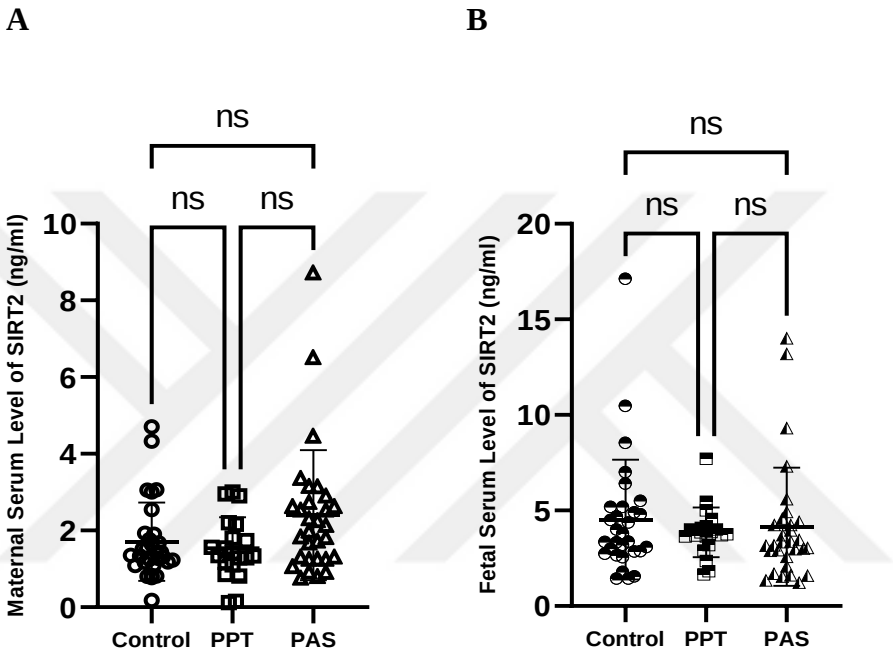

Figure 13: Maternal and fetal serum levels of SIRT2 in control, PPT and PAS. (**A**) Comparison of SIRT2 levels in control (n=30), PPT(n=20) and PAS (n=30) groups in maternal serums. (**B**) Comparison of SIRT2 levels in control (n=30), PPT (n=20) and PAS(n=30) groups in fetal serums. One way ANOVA was used for comparisons among the three groups.

When we compare SIRT7 level in maternal serums, there was not any differences between control (n=30, mean: 2.2ng/ml, ±std: 1.2), PPT (n=20, mean: 2.3ng/ml, ± std: 1.2) and PAS group (n=30, mean: 2.5ng/ml, ±std: 1.6) (p=0.6574), (**Figure 14.A**). We also evaluated SIRT7 level in fetal cord serum but there is a similar trend in 3 groups as in maternal serums of control (n=30, mean: 2.2ng/ml, ±std: 1.0), PPT (n=20, mean: 2.0ng/ml, ±std: 0.7) and PAS (n=30, mean: 2.4ng/ml, ±std: 1.2), (p=0.5880), (**Figure14.B**). As a result, no significant difference was found between the serum levels of the 3 groups.

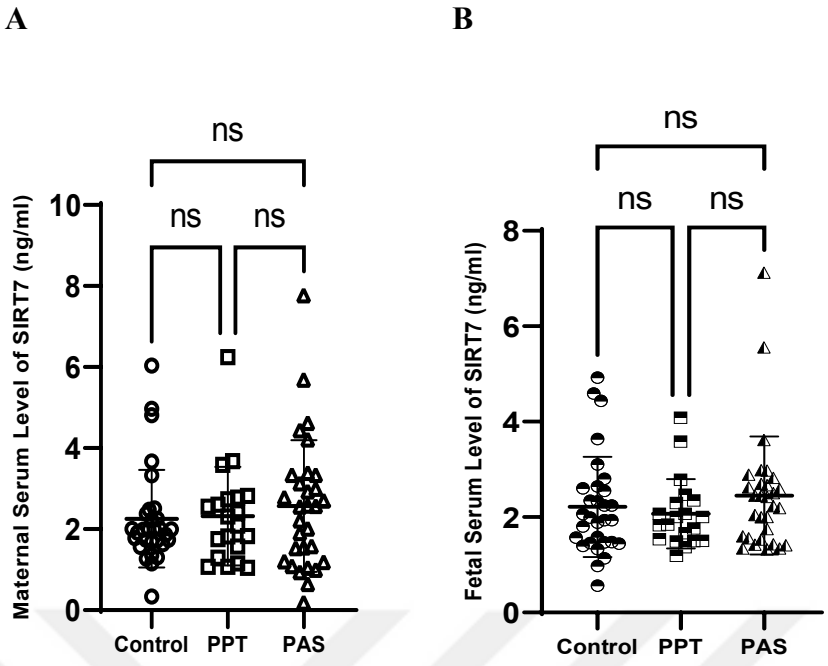

Figure 14: Maternal and fetal serum levels of the SIRT7 in control, PPT and PAS. (**A**) Comparison of SIRT7 levels in control (n=30), PPT (n=20) and PAS (n=30) groups in maternal serums. (**B**) Comparison of SIRT7 levels in control (n=30), PPT (n=20) and PAS (n=30) groups in fetal serums. One way ANOVA was used for comparisons among the three groups.

3.4 Correlation of Demographic and Clinical Parameters of Patients with SIRT1, SIRT2, and SIRT7 Levels

The correlation between maternal SIRT1, SIRT2, and SIRT7 levels and age, gravity, parity, cesarean section number, birth week, birth weight, hemoglobin, hematocrit and thrombocyte levels of the patients in the PAS (n=30) group was tested and the relevant values were respectively are given in (**Table 4**). As a result of the analysis parity, hematocrit and thrombocyte levels were negative correlation with SIRT1 levels while a positive correlation was found between age, gravitiy, cesarean section number, birth week, birth weight and hemoglobin levels but no statistically significant difference was found ($p>0.05$) (**Table 4**). As a result of the analysis, a negative correlation was found between SIRT2 levels and age, gravity, parity, birth week, birth weight, hemoglobin, hematocrit and thrombocyte levels, but no statistically significant difference was found ($p>0.05$) (**Table 4**). Likewise, age, gravity, parity, birth week, birth weight and thrombocyte levels were negatively correlated with SIRT7 levels, while a positive correlation was found between

cesarean section number, hemoglobin and hematocrit levels. However, a statistically significant negative correlation was found only in terms of week of birth and birth weight (birth week (*p=0.002) and birth weight (*p=0.040)), **(Table 4**).

Table 4: The relationship between demographic and clinical characteristics of the PAS group and serum levels of SIRT1, SIRT2 and SIRT7.

PAS (n=30)		**SIRT1**	**SIRT2**	**SIRT7**
Age	p=	0,468	0.097	0.227
	r=	0.331	-0.309	-0.227
Gravidity	p=	0,381	0.859	0.514
	r=	0.394	-0.34	-0.124
Parity	p=	0.672	0.967	0.661
	r=	-0.197	-0.08	-0.083
Previos cecarean section	p=	0.177	0.403	0.698
	r=	0.575	0.158	0.074
Birth week	p=	0.612	0.231	*0.002
	r=	0.235	-0.225	**-0.537
Birth weigth	p=	0.198	0.274	*0.040
	r=	0.553	-0.206	*-0.378
Hemoglobin	p=	0.857	0.886	0.667
	r=	0.084	-0.027	0.082
Hematocrit	p=	0.725	0.738	0.731
	r=	-0.164	-0.064	0.066
Thrombocyte	p=	0.284	0.867	0.194
	r=	-0.472	-0.032	-0.244
****Correlation is significant at the 0.01 level (2 tailed). *Correlation is significant at the 0.05 level (2tailed).**				

Pearson correlation test was applied.

In addition, the correlation between maternal SIRT1, SIRT2, and SIRT7 levels and age, gravity, parity, cesarean section number, birth week, birth weight, hemoglobin, hematocrit and thrombocyte levels of the patients in the PPT (n=20) group was tested, and the relevant values were respectively are given in (**Table 5**). As a result of the analysis, a negative correlation was found between SIRT1 levels and birth week, birth weight, hematocrit and thrombocyte levels. However, no statistically significant difference was

found between these features (p>0.05), (**Table 5**). As a result of the analysis, a negative correlation was found between SIRT2 levels and gravity, parity, birth week, birth weight and hematocrit levels, but no statistically significant difference was found (p>0.05) **(Table 5)**. Similarly, while a negative correlation was found between age, gravity, parity and hemoglobin levels and SIRT7 levels in the PPT group, a positive correlation was found between birth week, birth weight, hematocrit and thrombocyte levels. However, no statistically significant difference was found between these features (p>0.05), (**Table 5**).

Table 5: The relationship between demographic and clinical characteristics of the PPT group and serum levels of SIRT1, SIRT2, and SIRT7.

PPT (n=20)		**SIRT1**	**SIRT2**	**SIRT7**
Age	p=	0.624	0.598	0.858
	r=	0.206	0.126	-0.043
Gravidity	p=	0.157	0.952	0.439
	r=	0.551	-0.015	-0.183
Parity	p=	0.503	0.717	0.161
	r=	0.206	-0.087	-0.326
Previos cecarean section	p=	0	0	0
	r=	0	0	0
Birth week	p=	0.362	0.605	0.276
	r=	-0.374	-0.123	0.256
Birth weigth	p=	0.238	0.647	0.616
	r=	-0.471	-0.109	0.12
Hemoglobin	p=	0.981	0.909	0.974
	r=	0.01	0.027	-0.008
Hematocrit	p=	0.802	0.753	0.618
	r=	-0.106	-0.075	0.119
Thrombocyte	p=	0.937	0.446	0.404
	r=	-0.034	0.181	0.198
****Correlation is significant at the 0.01 level (2 tailed)** ***Correlation is significant at the 0.05 level (2 tailed)**				

Pearson correlation test was applied.

In the PAS group (n=30), there was no statistically significant difference in SIRT1, SIRT2 and SIRT7 level and infant gender (p=0.724, p>0.05, p=0.918, p>0.05 and p=0.275, p>0.05 respectively), (**Table 6**).

Table 6: The relationship between maternal SIRT 1, 2, and 7 levels and infant gender in the PAS group.

PAS	gender	n	mean	std	*p* value
SIRT1	Boy	3	5.226	±5.981	0.724
	Girl	4	6.122	±5.629	
SIRT2	Boy	16	2.695	± 2.186	0.918
	Girl	14	2.058	± 0.806	
SIRT7	Boy	16	2.333	± 1.168	0.275
	Girl	14	2.842	± 1.575	
The Mann-Whitney U Test was applied.					

However, in PPT (n=20) group, even though there was no difference between maternal SIRT1 and SIRT2 levels and infant genders (p=0.739, p>0.05 and p=0.197, p>0.05 respectively), (**Table 7**), a significant difference was detected between maternal SIRT7 level and infant gender (*p=0.042) (**Table 7**).

Table 7: The relationship between maternal SIRT1, 2 and 7 levels and infant gender in the PPT group.

PPT	gender	n	mean	std	*p* value
SIRT1	Boy	6	10.43	±4.635	0.739
	Girl	2	9.475	±3.966	
SIRT2	Boy	15	1.376	± 0.522	0.197
	Girl	5	1.024	± 0.543	
SIRT7	Boy	15	2.082	± 1.435	*0.042
	Girl	5	2.83	± 0.513	
The Mann-Whitney U Test was applied. ***p<0.05**					

3.5 Determination of the SIRT1, SIRT2 and SIRT7 Localizations in Placenta

The expression localizations of SIRT1, 2 and 7 in placental tissues were evaluated by immunohistochemical staining method.

3.5.1.Hematoxylin-eosin staining of the control tissues

When the control group's Hematoxylin-eosin stained placenta sections were analyzed, the villus structures were found to be normal in general. However, free erythrocyte cells were detected in the villous structures and intervillous areas. It was concluded that syncytiotrophoblast and cytotrophoblast cells were in normal structure and there was no obvious pathological condition (**Figure 15**).

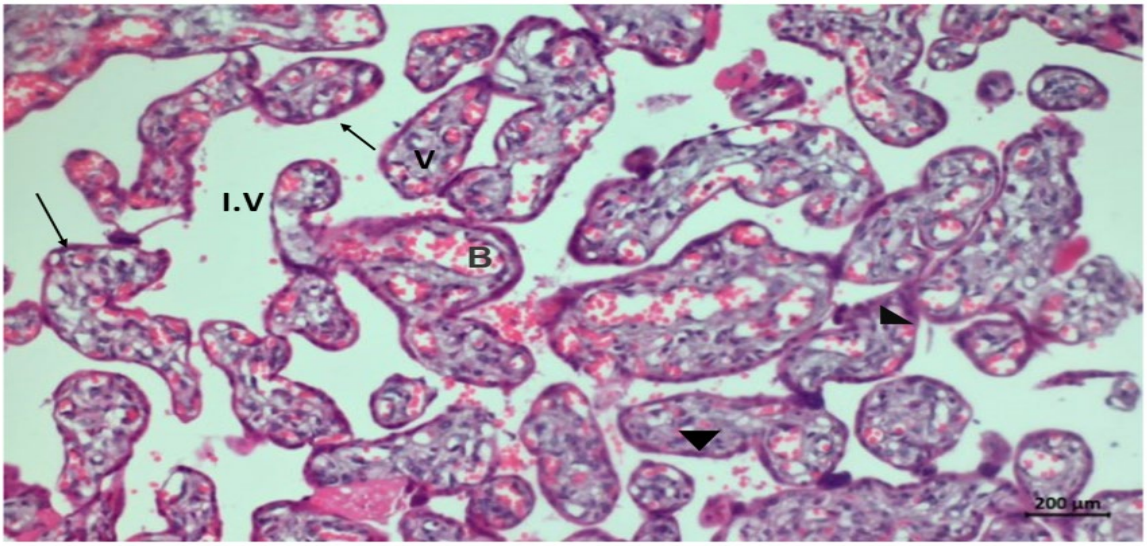

Figure 15: Hematoxylin-eosin staining of placenta sections of the control group. Normal structured villi structures (**V**), intervillous area (**I.V**), blood vessel (**B**), syncytiotrophoblast cells (**black arrow**) located in the outermost part of the villi structures of the control group placental section and cytotrophoblasts located under these cells (**arrowhead**). (Bar:200 µm).

3.5.2 Hematoxylin-eosinstaining of the PPT tissues

Hematoxylin and eosin staining in the placenta totalis group reveal that there were distinct degenerations in the villus structures. In the sections belonging to this group, tissue thickening was observed in the syncytial nodes along with an increase in fibrinoid tissue (**Figure 16**).

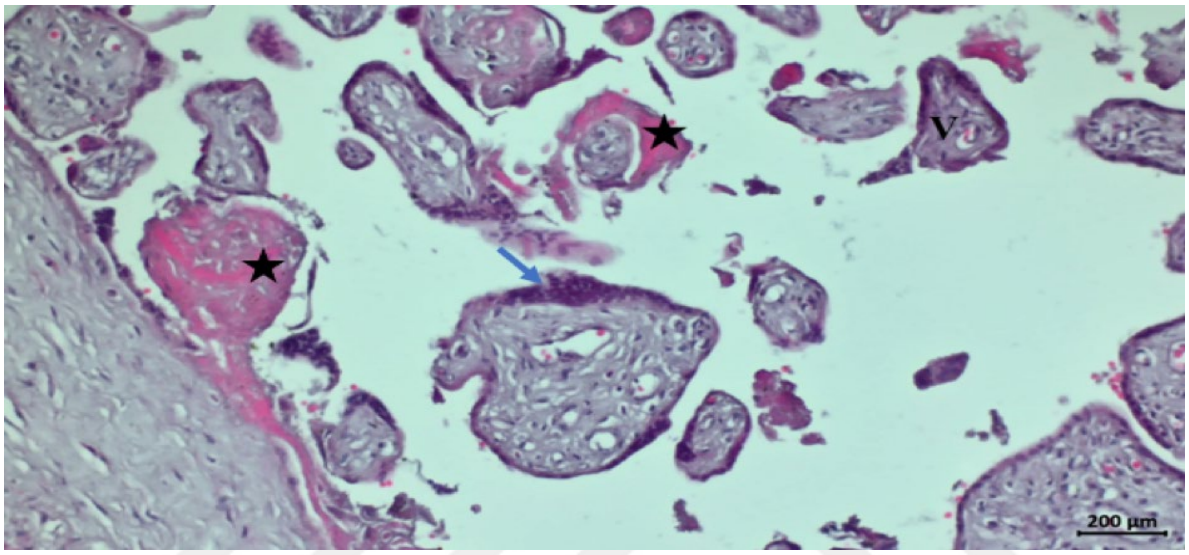

Figure 16: Hematoxylin-eosin staining of placenta sections of the PPT group. Degenerated villus structures (**V**), ın the sections of the more enlarged chorionic villi, a large increase of fibrinoid tissue (**star**) and fibrinoid structures were also observed in the intervillous area and an increase in the number of syncytial nodes was observed due to the fusion of the nucleus membranes in the syncytial zone (**blue arrow**), (Bar:200 µm).

3.5.3 Hematoxylin-eosin staining of the PAS tissues

It was detected that there were significant degenerations in the villus structures in the sections stained with Hematoxylin eosin in the group with placenta accreta invasion. In general, it was observed that there were many free red blood cells in the tissue structure and blood vessels. In addition, intense fibronid increases and the presence of hyalinized tissues were detected (**Figure 17**).

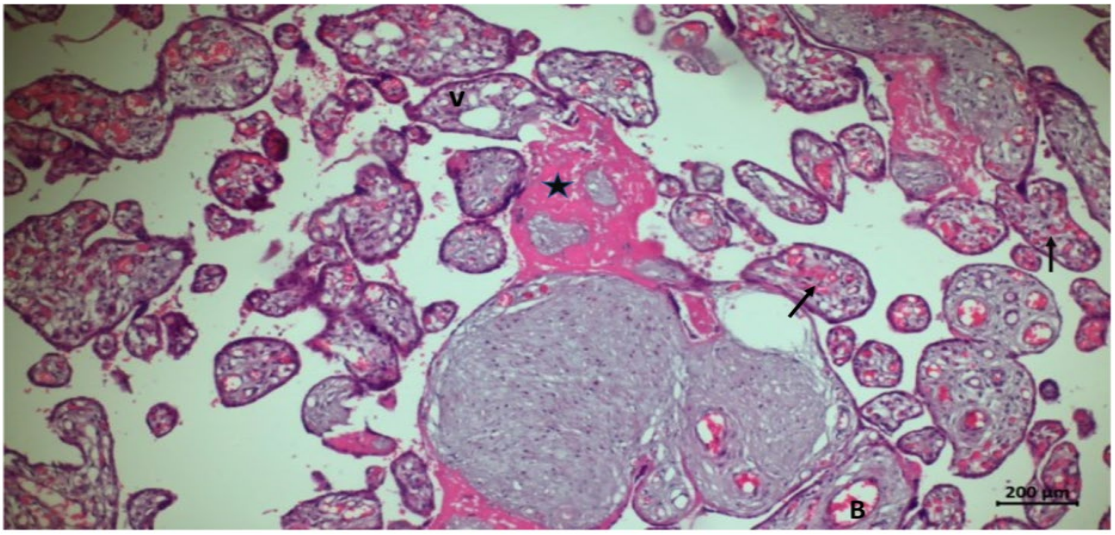

Figure 17: Hematoxylin-eosin staining of placenta sections of the PAS group. Degenerations in the villus structures (**V**), congestion and enlargement in blood vessels (**B**), many free red blood cells (**black arrow**) in the villus structures, and hyalinized tissue structures (**star**) with an increase in fibronoid structures in the tissue section belonging to the group with placenta accreta invasion (Bar:200 µm).

Control placenta and placenta totalis SIRT1 immunohistochemically stained sections showed that SIRT1 expression was not intense (**Figure 18**, **Figure 19**).

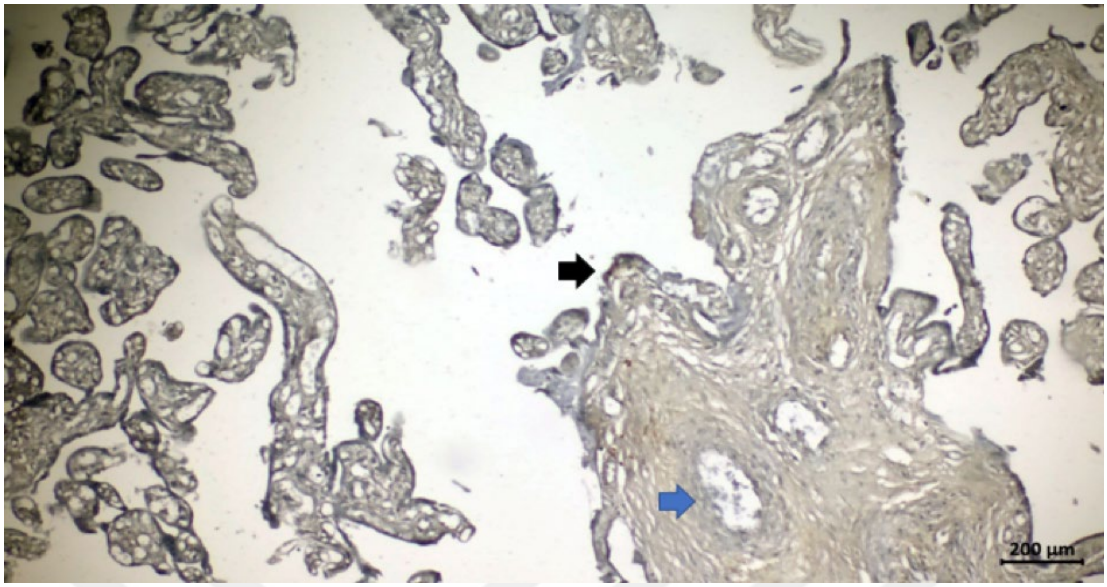

Figure 18: Immunohistochemical staining of SIRT1 in the placenta of the control group. Positive SIRT1 expression (**black arrow**) in cytotrophoblast cells and negative SIRT1 expression (**blue arrow**) in the blood vessel in the villous structure (Staining: SIRT1 immunohistochemistry, Bar: 200 µm).

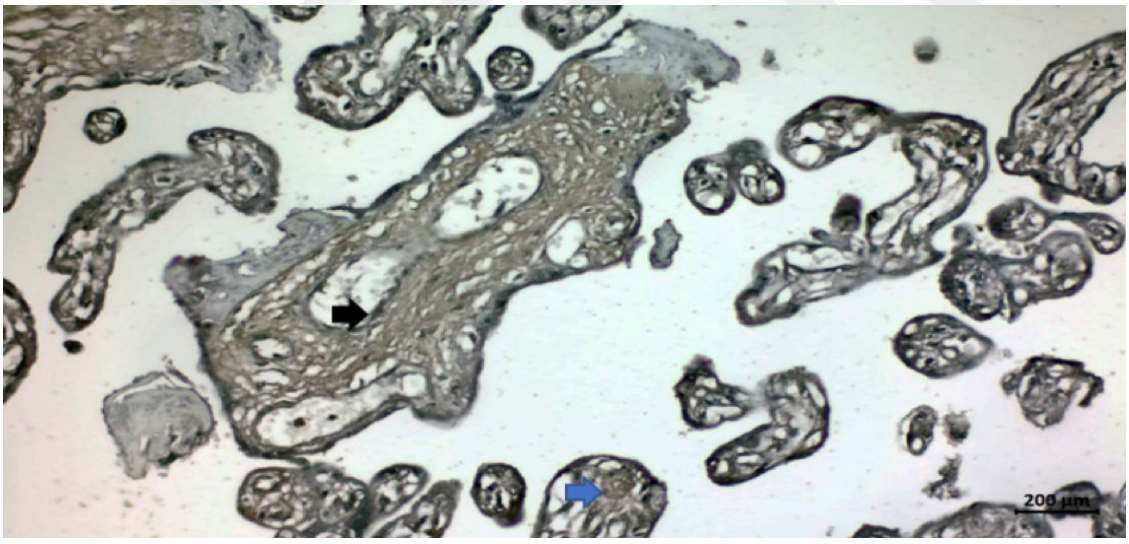

Figure 19: Immunohistochemical staining of SIRT1 in the placenta of the PPT group. Positive SIRT1 expression (**black arrow**) around the blood vessel and negative SIRT1 expression (**blue arrow**) in the villus structure (Staining: SIRT1 immunohistochemistry, Bar:200 µm).

It was observed that SIRT1 expression was increased especially in blood vessels and syncytiotrophoblast cells in the placenta accreta invasion (**Figure 20**).

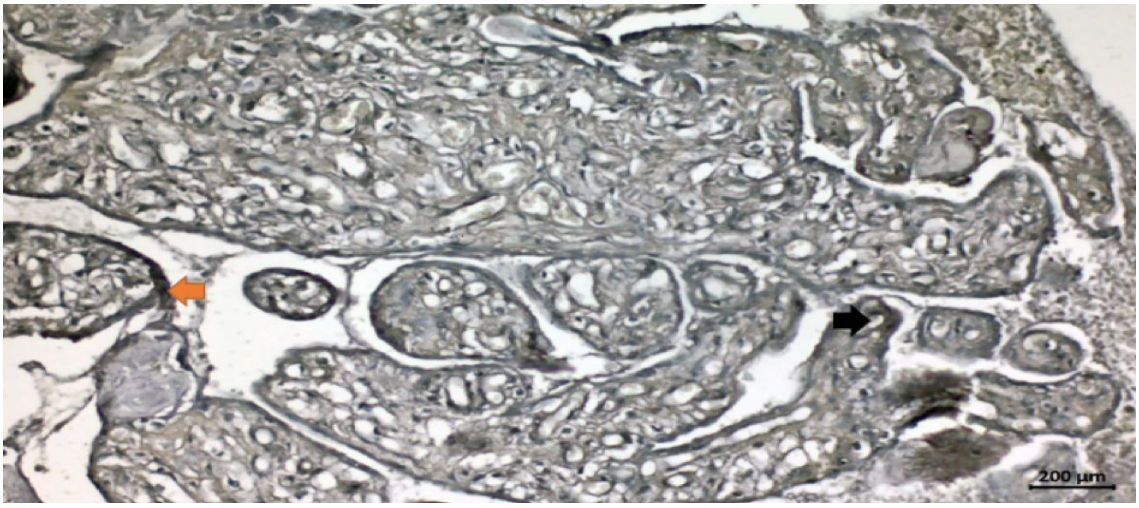

Figure 20: Immunohistochemical staining of SIRT1 in the placenta of the PAS group. Positive SIRT1 expression (**black arrow**) around the blood vessel and positive SIRT1 expression (**orange arrow**) in syncytiotrophoblast cells (Staining: SIRT1 immunohistochemistry, Bar:200 µm).

When we analysed SIRT2 expression in 3 groups, the control placenta was partially positive in the sections stained with SIRT2 immunohistochemistry (**Figure 21**).

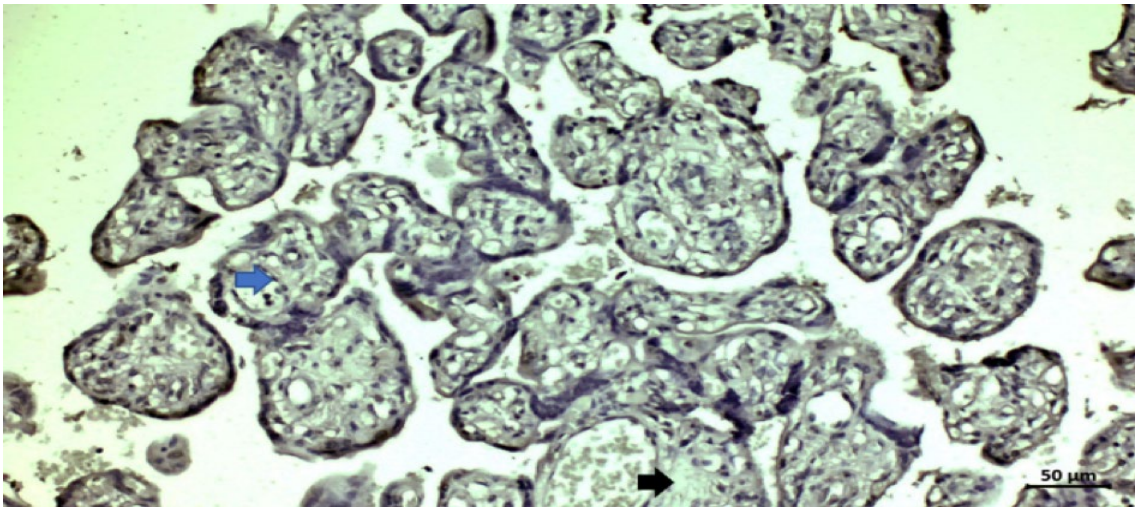

Figure 21: Immunohistochemical staining of SIRT2 in the placenta of the control group. Weak SIRT2 expression in the villi areas (**black arrow**) and negative SIRT2 expression in the endothelial cells of blood vessels were detected (**blue arrow**) (Staining: SIRT2 immunohistochemistry, Bar: 50 µm).

It was observed that SIRT2 expression was at similar intensities in the placenta totalis and placenta accreta invasion groups (**Figure 22, Figure 23**). In contrast to the control group, SIRT2 expression was increased especially in the placenta accreta invasion group.

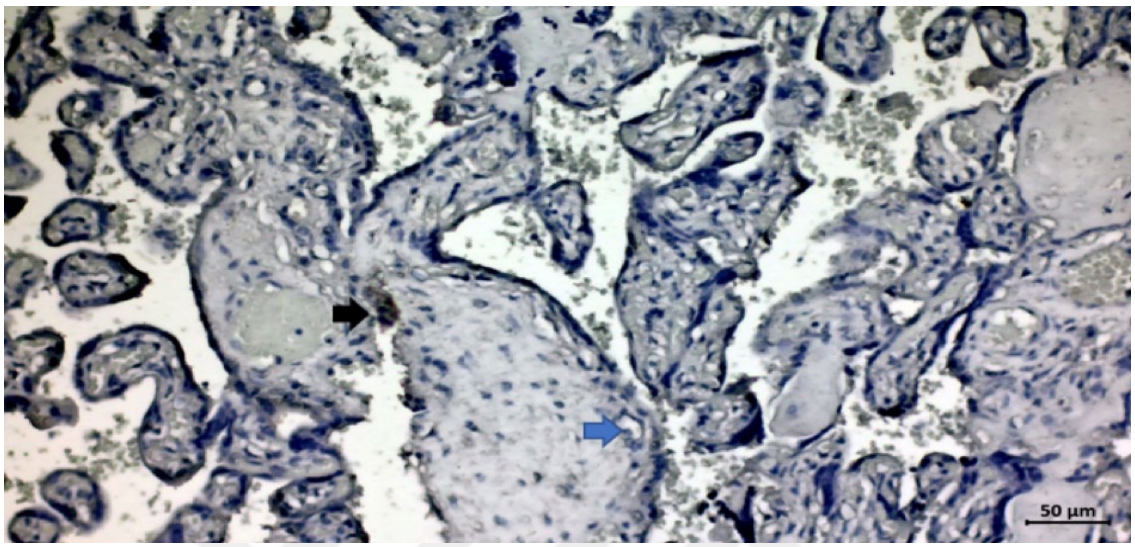

Figure 22: Immunohistochemical staining of SIRT2 in the placenta of the PPT group. SIRT2 expression was intense in the syncytial bridge and cytotrophoblasts (**black arrow**) and negative SIRT2 expression around the blood vessel (**blue arrow**) (Staining: SIRT2 immunohistochemistry, Bar: 50µm).

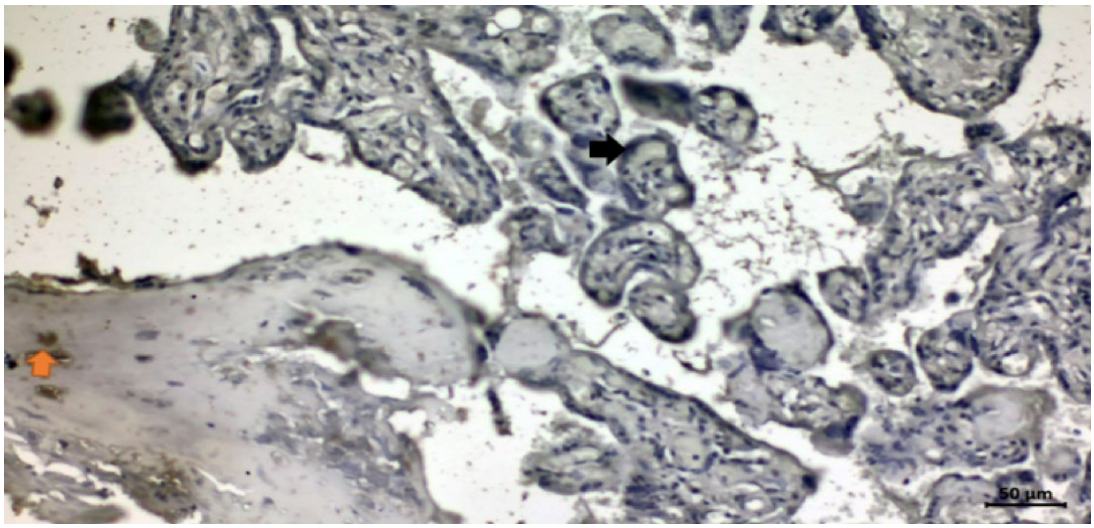

Figure 23: Immunohistochemical staining of SIRT2 in the placenta of the PAS group. Positive SIRT2 expression (**black arrow)** in cytotrophobalast cells and positive SIRT2 expression (**orange arrow)** in villi (Staining: SIRT2 immunohistochemistry, Bar:50 µm).

When the SIRT7 immunohistochemically stained sections were examined, it was observed that SIRT7 expression was weak in the sections of the control and placenta totalis groups (**Figure 24, Figure 25**) but SIRT7 expression was intense especially in the syncytiotrophoblast cells in the group with placenta acreata (**Figure 26**).

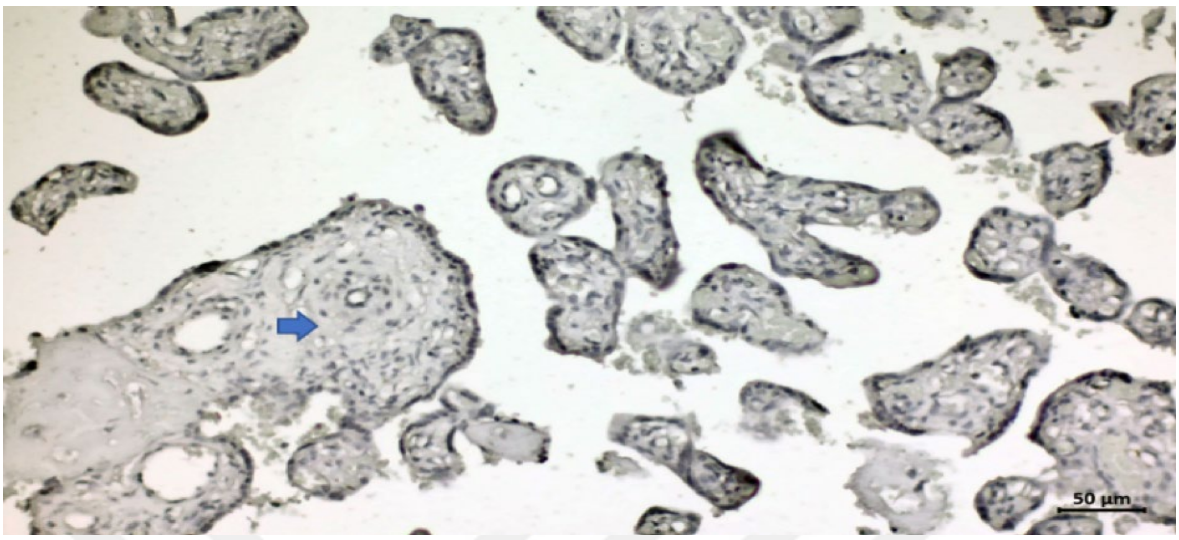

Figure 24: Immunohistochemical staining of SIRT7 in the placenta of the control group. Negative SIRT7 expression (**blue arrow**) in villi areas (Staining: SIRT7 immunohistochemistry, Bar: 50 µm).

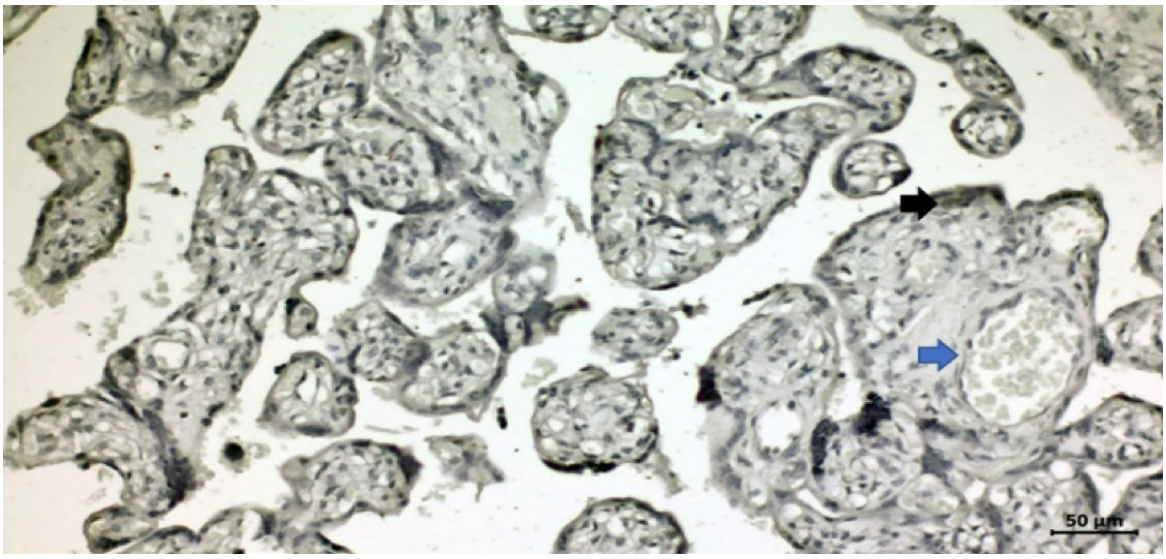

Figure 25: Immunohistochemical staining of SIRT7 in the placenta of the PPT group. Positive SIRT7 expression **(black arrow)** is seen around the syncytiotrophoblast cells and negative SIRT7 expression **(blue arrow)** in the blood vessel. (Staining: SIRT7 immunohistochemistry, Bar: 50µm).

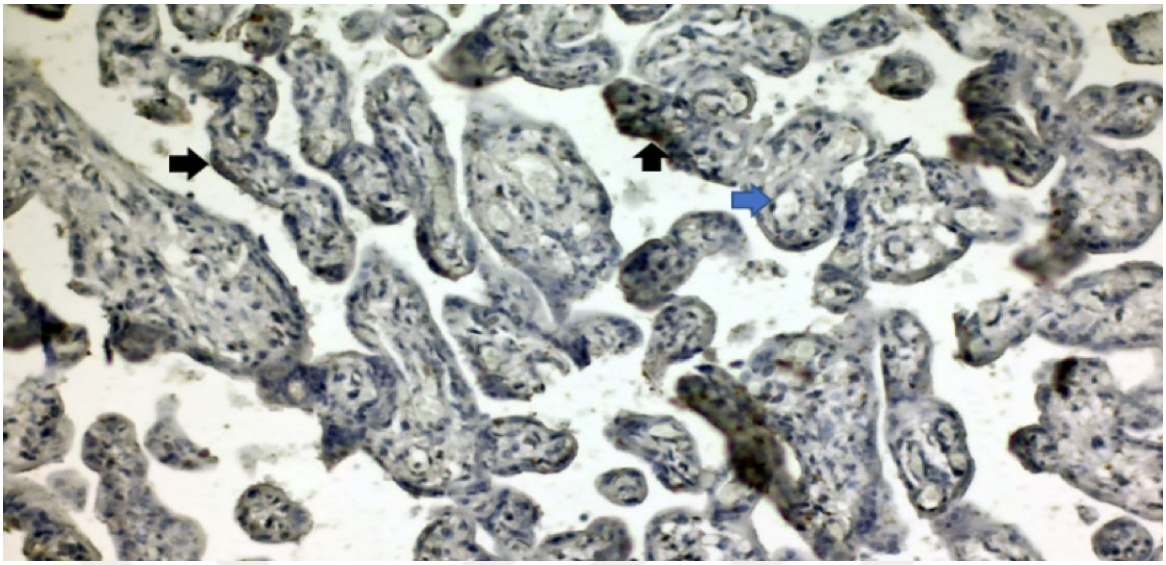

Figure 26: Immunohistochemical staining of SIRT7 in the placenta of the PAS group. Positive SIRT7 expression (**black arrows**) in syncytiotrophoblast cells in the villous structure and negative SIRT7 expression (**blue arrow**) in endothelial cells of blood vessels (Staining: SIRT7 immunohistochemistry, Bar: 50 μm).

4. DISCUSSION

Placental adhesion anomaly is a condition in which the placenta adheres to the uterine wall to various degrees. The development of PAS is a complex and multi-factor process. A healthy placenta does not go beyond one third of the myometrium with tight spatial and temporal regulation, but an invasive placenta acts like a malignant tumor, replicating its local structures and invading myometrium [26]. The molecular mechanism behind placental invasion is unknown. The lack of the decidua or basal layer, improper maternal revascular patterning, and excessive EVT invasion are among postulated theories [21]. Because they have an invasive feature, a comparison can be made between PAS and tumor behavior. In both cases, cells escape immune system elements, invasion is activated and angiogenesis is triggered [26]. EMT is a cellular event that helps the EVT phenotype transform into an invasive one [63]. Although EMT is necessary for proper placental invasion and attachment to the myometrium in the first trimester, it should not continue throughout pregnancy [26]. It's been proven that excessively vigorous EMT that persists during pregnancy contributes to the development of PAS [27]. N-cadherin, ZEB1 and Snail are markers of EMT [63]. The loss of the crucial E-cadherin is the most visible symptom of EMT. The expression of E-cadherin was decreased in the chorionic villi of the invasive part of the placenta seeded by the placenta, whereas the expression of Snail and TGF-β increased in the decidual cells of the invasive region [32]. These data imply that the cause of PAS may be due to epithelial-mesenchymal transition. SIRTs are govern a variety of cellular processes [37]. Sirtuins may play acritical role in tumorigenesis regulation. In particular, sirtuins have been shown to affect epithelial plasticity by reprogramming transcription at the epithelial-mesenchymal transition, leading to invasion and metastasis. SIRT1 controls trophoblast cell invasion. By knockdown of SIRT1, invasive EVTs express FasL to avoid maternal immune attacks, and FasL binds to Fas, a cell surface death receptor, to induce apoptosis. FasL promotes apoptosis of Fas-bearing maternally activated lymphocytes by conferring an immune privileged phenotype to EVTs, thereby promoting EVT invasion and spiral artery remodeling. Then, invading EVTs can effectively evade maternal immune attacks by increasing FasL expression, and increasing migration into ascending spiral arteries. Moreover, loss of SIRT1 in EVTs promoted invasion by upregulation of EMT, MMP-9, MMP-2 and Akt/p38MAPK pathways. Increased invasion and induction of EMT's markers such as N-cadherin, Snail and ZEB1 were shown to result from activation of Akt and p38MAPK signaling pathways [64]. SIRT2 has been shown to

favorably regulate metastatic potential via activating the Akt/GSK/-catenin signaling pathway in EMT [65]. According to the results obtained, the high EMT's markers seen in PAS suggested that the SIRT family with proven effectiveness in EMT may have an active role in the pathology of PAS.

Based on these findings, we determined the total expression level of the SIRT1, 2, and 7 in 3 groups; control, previa totalis and placenta accreta. In addition, we examined the localization levels of these in control, PPT and PAS by immunohistochemical staining, and finally in serum samples from the maternal and fetal cord blood. We found that 3 members of the sirtuin family had less expression in PAS than the control and previa totalis groups. However even though SIRT1 and SIRT7 were significantly lower in placenta accreta than in the control, SIRT2 was not statistically significant compared to the control.

PAS molecular biology has been found to share numerous properties with cancer biology [26]. SIRT1 protein levels are lower in hepatocellular carcinoma than in healthy liver specimen, and similarly SIRT1 levels have also been shown to be lower in breast cancer specimen than in healthy tissue [66]. Its levels also lower in BRCA1 mutant breast cancers, and overexpression of SIRT1 in BRCA1 mutant breast cancer cell lines reduces xenograft tumor growth [67]. Likewise, another study showed that SIRT1 expression was lower in high-grade colon cancer specimen and knockdown of SIRT1 in colon carcinoma cell lines accelerated xenograft tumor growth in mice [68]. Furthermore, prostate carcinoma samples revealed reduced expression of SIRT1 compared with normal tissue, and this expression was even lower in metastatic lesions. An analysis of glioblastoma samples revealed reduced expression of SIRT1 in tumors compared to normal tissue [66]. In addition, according to a study in mice, SIRT2 deficiency in the liver was reported to increase the occurrence of hepatocellular carcinoma (HCC). The same study found that human HCC samples showed low levels of SIRT2 expression compared to non-tumorogenic tissue. Subsequent histological studies demonstrated downregulation of SIRT2 in human breast tumors compared to healthy tissue [69]. SIRT2 expression was decreased in the colon in colorectal carcinoma specimen, and this decrease was even greater in metastatic tumors. The anti-tumor effects of shikonin have been shown to be mediated by SIRT2 overexpression [70]. In addition, histological studies in the prostate showed that SIRT2 is downregulated in human tumors and lower SIRT2 is associated with higher grade tumors and worse prognosis [71]. Likewise, it has been shown that another member of sirtuin family, SIRT7 modulates EMT by transforming growth factor-β (TGF-β) signaling. SIRT7 is significantly downregulated in breast cancer lung metastases in

humans and mice, it deacetylates, β-TrCP1-mediated SMAD4 degradation and SIRT7 deficiency activate TGF-β signaling and increases EMT. They also showed that resveratrol antagonizes TGF-β signaling by activating SIRT7 deacetylase activity, inhibiting breast cancer lung metastases and increasing survival. They then also showed that SIRT1 can regulate SMAD4 with SIRT7 in breast cancer metastasis [72]. Interactions between the TGF-β protein family have been shown to contribute greatly to the regulation of EVT invasion [62]. SIRT7 expression was found to be low in the placenta of the PAS group in our study, and the serum level of TGF-β expression was found to be significantly higher in placenta accreta group compared to the healthy female group by another research [73] those findings suggest that SIRT7 may play an effective role in PAS reveals.

As a result of our study, it was determined that total SIRT1 and SIRT7 levels were lower in groups with PAS compare to the other groups, and localizations of the SIRT1, 2 and 7 which were determined by immunohistochemical staining were observed especially in the syncytiotrophoblasts of individuals with PAS compared to control and PPT. However, no difference was detected in the maternal and fetal cord serums. In summary, based on many studies; It has been shown that decreasing SIRT1, 2 and 7 expression levels in tumors and metastatic lesions contribute to tumor formation and progression. Similarly, our findings suggest that SIRT1 and SIRT7 may contribute to the invasive features of PAS.

Resveratrol, the most potent of the sirtuin activating compounds (STACs), has been identified in SIRT1 [74]. Many reports have demonstrated that treatment of mouse cancer models with STACs can decrease tumor growth. Activation of sirtuin prior to tumor appearance has been consistently shown to prevent or delay the onset of cancer [75]. Our findings reveal that the Sirtuin family is effective in PAS. Our data may contribute to the understanding of the pathogenesis of PAS and may lead to the prevention of maternal death and dysfunctions caused by PAS. With this aspect, it is predicted that it can contribute to clinicians in guiding the treatment (for example, recommending the use of SIRT1 activators such as resveratrol). However, additional studies are needed to prove the efficacy of SIRT1, 2, and 7 in PAS.

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