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Title: Altered Expression of ADAMTSs and HAPLNs in Preeclamptic Placenta

Running Head: ADAMTSs and HAPLNs in Preeclampsia

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ABSTRACT

Objective: Preeclampsia (PE) is a pregnancy-specific complication defined by the new onset of hypertension and proteinuria during the second trimester of pregnancy. The pathogenesis of PE remains poorly understood. Revealing the key factors involved in placental dysfunction is critical for the understanding the pathogenesis of PE. The aim of this study was to determine the expression levels of ADAMTSs and their molecular partners, TIMP-3 and HAPLNs in the placental tissues of women with PE.

Materials and Methods: Experimental research was conducted on control and preeclamptic placentas. A total of 10 control and 10 preeclamptic placentas were included in the present study. The expression levels of ADAMTSs, HAPLNs, and TIMP-3 were analyzed in two groups by Western blot.

Results: The expression levels of ADAMTS-4, -8, -10, -12, -13, -14, -16, and -19 were considerably lower, whereas the expression levels of HAPLN-1, -2, and -4; ADAMTS-18; and TIMP-3 were significantly higher in preeclamptic placentas than in controls.

Conclusion: Altered expression levels of ADAMTSs and their molecular partners, TIMP-3 and HAPLNs, may contribute to the pathogenesis of PE.

Keywords: ADAMTSs, TIMP-3, HAPLNs, preeclampsia, placenta

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INTRODUCTION

Preeclampsia (PE) is one of the major causes of perinatal and maternal morbidity and mortality affecting 5%–10% of pregnant women worldwide. PE is a pregnancy-specific complex condition defined by the new onset of hypertension (blood pressure >140/90 mmHg) accompanied by a significant proteinuria (>0.3 g/24 h) emerging after 20 weeks of gestation (1). Severe progression of the disease has been associated with maternal renal damage, liver dysfunction, and eventually seizures and death (2). Although the pathophysiology of PE is unclear, abnormal placentation appears to be a key factor accounting for the development of PE.

Implantation and placentation are crucial processes in the development and maintenance of a successful pregnancy (3). During normal pregnancy, the trophoblast cells that are highly invasive shift to the myometrium and decidua, thereby attacking the muscularis tunica media together with the endothelium of spiral arteries. Trophoblast invasion contributes to the loss of smooth muscle tissues from the uterine spiral arteries' distal part. The uterine artery's terminal branches become vessels that withstand low resistance and increased capacity, thereby facilitating the blood flow required for proper placental development (4, 5). This invasive process is disrupted in PE. In PE, several factors hinder the invasion of trophoblasts into the uterine wall leading to inadequate remodeling of the spiral arteries and consequently poor placental perfusion (6, 7).

Activation of distinct extracellular matrix (ECM) components and/or proteolytic degradation as well as the controlled variation in the cell–ECM and cell–cell interactions are important factors involved in inadequate remodeling of the spiral arteries and abnormal invasion of the trophoblast in PE (8, 9). The definitive molecular mechanisms regulating trophoblast migration/invasiveness during gestation and their relationship with feto-placental development remain largely unknown; however, a number of cytokines, proteinases as well as growth factors appear to be involved in the invasive behavior of trophoblast (3, 10, 11).

A disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) is a family of ECM proteinases comprising 19 secreted proteolytic enzymes that are structurally and functionally related to matrix metalloproteinases (MMPs) (12). Enzymatic activities of these proteases are inhibited by tissue inhibitor of metalloproteinase-3 (TIMP-3) (12). ADAMTSs play a critical role in the remodeling of ECM as well as many other physiological processes, including embryonic development, cell migration, and angiogenesis (13, 14). ADAMTS family members have been implicated in the pathologies of many diseases, including cancer, inflammatory conditions, especially arthritic diseases, and atherosclerosis (14). A limited number of studies have shown the expression pattern of some ADAMTS subtypes in human placenta and their possible implication in gestational trophoblastic diseases (15, 16); however, a more comprehensive study is required to show the expression pattern of all ADAMTS family members in preeclamptic placentas.

The ECM structural substrates, such as versican, aggrecan, and collagen are degraded by the ADAMTS family members (14). On the other hand, hyaluronan, an ECM's basic component, is aided by versican in stabilizing the matrix (17). Circulating hyaluronan concentration has been shown to be elevated in women suffering from PE (18). Hyaluronan and proteoglycan link proteins (HAPLN) are glycoproteins located in the ECM of various tissues, such as brain, cartilage, as well as heart (19). HAPLN genes are

responsible for the generation and stabilization of the hyaluronan and proteoglycan aggregates degraded by proteases, including ADAMTS subtypes (20). Therefore, comprehending the ECM elements and factors concerned with the remodeling of ECM in PE is crucial for revealing the new therapeutic targets and treatment methods. Based on this objective, we assessed the expression patterns of ADAMTSs and their potential molecular partners, TIMP-3 and HAPLN gene family members, in both control and preeclamptic placentas.

MATERIALS and METHODS

Study subjects

This study was approved by the local ethical committee. Informed consent was obtained from all participants included in the study. Overall, 10 preeclamptic placentas from women diagnosed with preeclampsia and 10 control placentas from healthy pregnant women were included in the present study. PE pregnant women were selected based on an elevated systolic and diastolic blood pressure (>140/90 mm Hg) that emerged after 20 weeks of gestation, accompanied by proteinuria (300 mg/24 h) that was detected after urine analysis. Preeclamptic pregnant women with infection, chronic hypertension, or any other chronic diseases were excluded from the study. Patients with intrauterine growth restriction were also excluded from the study. To match the gestational ages of PE placentas and control placentas, asymptomatic patients who had spontaneous preterm delivery induced by uterine distension have been included as control. Control women had no chronic and gestational hypertension, proteinuria, infection, and any other chronic diseases during pregnancy. Table 1 shows the demographic and clinical features of control women and women with preeclampsia.

Placental tissue collection

Samples of placental tissue (1 cm × 1 cm × 1 cm) obtained from healthy pregnant women and women with PE immediately after cesarean deliveries were cut out from the maternal side around the umbilical cord in a sterile condition and immediately flash frozen using liquid nitrogen. The flash-frozen placental tissues were stored at −86 °C until Western blot analysis.

Antibodies for Western blot

Antibodies against TIMP-3, HAPLN subtypes (-1, -2, and -4), and ADAMTS proteases (-4, -8, -10, -12, -13, -14, -16, -18, and -19) were purchased from Santa Cruz Biotechnology. Anti-β-actin, HRP-conjugated goat anti-mouse, and HRP-conjugated goat anti-rabbit antibodies were obtained from Abcam.

Western blot analysis

The snap frozen placenta was grinded to a fine powder in a chilled mortar in the presence of liquid nitrogen. Immediately after grinding, the placenta powder was lysed on ice in RIPA buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific). Total cellular protein concentration was determined using a BCA protein assay kit according to the manufacturer's instructions (Pierce, Thermo scientific). Total cellular proteins (20 µg) were separated using 10% SDS-PAGE gel, and the separated proteins were transferred onto polyvinyl difluoride (PVDF) membrane (Bio-Rad). Nonspecific binding was blocked by incubation of the membrane in PBS with 5% nonfat dried milk and 0.1% Tween-20 for 1 h at room temperature. The membranes were probed with primary antibodies for 2 h at room temperature. β-actin was used as loading control. Appropriate HRP-conjugated secondary antibodies were used to visualize the specific bands. The protein bands were

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visualized using ECL (Bio-Rad) according to the manufacturer's instruction. The images were taken using ChemiDoc™ MP (Bio-Rad). Densitometry analyses were performed using Image Lab 5.1 (Bio-Rad).

Statistical analysis

The density of each band on the Western blots was measured using Image Lab 5.1 software (Bio-Rad). Quantitative data obtained from Western blots were subjected to statistical analysis. Significance of the differences between control and PE was calculated by Student's *t*-test using the Sigmaplot 12 software package (Systat Software Inc, California, USA). A p-value of <0.05 was considered as statistically significant.

RESULTS

In this study, we investigated the expression patterns of ADAMTSs and HAPLNs gene family in preeclamptic and control placentas. Patients were approximately matched for age, gestational age, and body mass index with control pregnant women (Table 1). The expression levels of ADAMTS-4, ADAMTS-8, ADAMTS-10, ADAMTS-13, and ADAMTS-14 were found to be significantly lower in preeclamptic placentas than in control placentas (Figure 1). Moreover, the expression level of ADAMTS-16 was found to be higher in control placentas than in PE placentas (Figure 2A, 2B). There was also a statistically significant decrease in the expression levels of ADAMTS-12 and ADAMTS-19 in preeclamptic placentas compared with those in control women placentas (Figure 2C, 2D, 2E, 2F). Although most of the ADAMTS subtypes exhibited a similar pattern with decreased expression levels in preeclamptic placentas, the expression level of ADAMTS-18 appeared to be significantly higher in preeclamptic placentas than in controls (Figure 3A, B). We also examined the expression levels of

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ADAMTS-1, ADAMTS-2, ADAMTS-7, and ADAMTS-15, but they were not expressed at detectable levels in both control and preeclamptic placentas (data not shown).

ADAMTSs show restricted susceptibility to inhibition by the four tissue inhibitors of metalloproteinases (TIMPs). TIMP-3 is the only member of the TIMP family that specifically inhibits the enzymatic activities of ADAMTS proteases. We examined the expression level of TIMP-3 to understand the reason behind reduced expression of the ADAMTS proteases. The result showed that while there was no detectable expression of TIMP-3 in control placentas, the expression level of TIMP-3 dramatically increased in preeclamptic placentas (Figure 3C).

Then, we investigated whether the expression of HAPLNs (HAPLN-1, -2, and -4), which are the molecular partners of ADAMTSs involved in the remodeling of ECM, was altered in preeclamptic placentas. The result revealed that preeclamptic placentas exhibited high levels of HAPLN-1 and HAPLN-2; however, a very low basal expression of HAPLN-1 and HAPLN-2 was detected in control placentas (Figure 4). In addition, while high expression of HAPLN-4 was found in preeclamptic placenta, there was no observable expression of HAPLN-4 in control placenta (Figure 4).

DISCUSSION

In the present study, we reported here for the first time the expression patterns of all ADAMTS subtypes and their molecular partners, TIMP-3 and HAPLNs, in preeclamptic placentas. We showed that the expression levels of HAPLNs (-1, -2, and -4), ADAMTS-18, and TIMP-3 were significantly higher, whereas the expression levels of ADAMTS-4, -8, -10, -12, -13, -14, -16, and -19 were significantly lower in preeclamptic placentas than in controls.

Placentation requires release of special MMPs, trophoblast invasion, and spiral arteries remodeling, as well as the eventual embodiment of ECM structure (3). Two interrelated but discrete factors, poor

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trophoblast invasion and inadequate spiral artery remodeling, are typical characteristics of PE (21, 22). ADAMTSs are involved in the degradation and reassembly of the ECM process (14), which is required for trophoblast invasion and spiral arteries remodeling in normal placental development. The expression of ADAMTS-1, -2, -4, -5, -6, -7, -9, -10, and -12 subtypes has been observed in the human placenta (15, 16, 23–26). Owing to their expression in the placenta and their functional significance during ECM remodeling, ADAMTSs and their molecular partners are likely to be implicated in the pathogenesis of PE.

A study reported that ADAMTS-12 is abundantly expressed in invasive human trophoblastic cells, and the decreased or abolished expression of ADAMTS-12 leads to a decrease in the invasive behavior of the trophoblastic cells, indicating that ADAMTS-12 is critical in the regulation of trophoblast invasion (10). They also showed that ADAMTS-12 promotes trophoblast invasion independently from its enzymatic activity as the catalytically dead ADAMTS-12 expression is shown to elevate the invasive capacity of the trophoblast cells (10). A further study reported that the levels of ADAMTS-12 in the serum of patients with PE are considerably reduced (27). Consistent with earlier results about ADAMTS-12, our results revealed that not only ADAMTS-12 but also ADAMTS-4, -8, -10, -13, -14, -16, and -19 protein levels were reduced in preeclamptic placentas. To the best of our knowledge, this is the first comprehensive study that evaluated ADAMTS subtype protein levels in preeclamptic placental tissues.

A study reported that ADAMTS-13 has the capacity to facilitate angiogenesis when it is in its full-length form and promotes tube formation, proliferation, and migration of human umbilical vein endothelial cells (28). In the present study, we have identified that ADAMTS-13 was highly expressed in control placenta, whereas preeclamptic placentas showed a drastically reduced expression level of ADAMTS-13. Failure of placental angiogenesis and vasculogenesis leads to abnormal placental development

associated with the pathogenesis of PE. This result suggests that reduced ADAMTS-13 expression level plays a role in impaired angiogenesis in preeclamptic placentas.

In reference to genome-wide association studies, ADAMTS-16 has been perceived as a candidate locus linked with hypertension (29). Targeted disruption of ADAMTS-16 gene in a rat model shows a significant function of ADAMTS-16 in the regulation of blood pressure (30). In our study, we found that the expression of ADAMTS-16 was downregulated in preeclamptic placenta. Further investigation is required to clarify the link between ADAMTS-16 and hypertension in patients with preeclampsia.

Both malignant tumors and trophoblast implantation employ identical biochemical mediators in overseeing invasion (11). Dysregulation of the finely controlled process of trophoblast invasion can lead to a wide spectrum of pregnancy abnormalities including PE (11). Mutations in ADAMTS-18 gene, and methylation of promoter region of ADAMTS-18 gene are highly linked to several tumors, indicating that ADAMTS-18 acts as a tumor suppressor gene (31–33). In our study, ADAMTS-18 was the only ADAMTS subtype with significantly elevated expression in preeclamptic placentas. Our result suggested that ADAMTS-18 have an active role in the prevention of trophoblast invasion in a way similar to the mechanism of tumor suppression. However, a study reported that the maternal serum concentrations of ADAMTS-18 do not differ between women with preeclampsia and controls (27). It is possible that the variation in ADAMTS-18 expression level resulted from different tissue samples, such as maternal blood and placenta. Thus, further investigation may be helpful to clarify the exact role of ADAMTS-18 in preeclampsia.

ADAMTSs show restricted susceptibility to inhibition by four TIMPs. TIMP-3 is the only family member that most efficiently inhibits the enzymatic activities of ADAMTS proteases (12). There are a number of placenta-related diseases associated with the overexpression of TIMP-3, and abnormal TIMP-3 methylation in preeclampsia is capable of revealing the engagement of TIMP-3 in trophoblast invasion

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(34–36). As the failure of trophoblast invasion has been associated with PE, it is likely that TIMP-3 is involved in the pathophysiology of PE, which is supported by our study which shows overexpression of TIMP-3 in preeclamptic placentas. However, in another study, the expression of TIMP-3 is shown to be lower in preeclamptic placentas than in normal placentas (37). It is possible that the variation in TIMP-3 expression could result from different gestational ages of the placentas derived from patients with preeclampsia. In addition, these different results might be attributed to the effect of patient properties and different laboratory conditions. Further investigations are needed to clarify the exact role of TIMP-3 in the pathophysiology of PE.

The expression of ADAMTS subtypes and their relation with the expression levels of HAPLNs in preeclamptic placentas were examined for the first time in the present study. Our study revealed that HAPLN-1, HAPLN-2, as well as HAPLN-4 were predominantly expressed in preeclamptic placentas in comparison with control placentas. Current evidence indicates that HAPLNs may be the key components in the hyaluronic acid (HA)-based matrix scaffold organization (38). HAPLN-1 is highly renowned in stabilizing HA–proteoglycan interactions (39), for instance, versican and aggrecan degraded by ADAMTSs (20). Nonetheless, elevated expression of HAPLNs and lecticans within the adult central nervous system spatially and temporally relates with variations in ECM solubility as well as with entrance of ECM aggregates within neuron subsets, identified as “perineuronal nets.” Such deviations have been linked with limited cellular motility and minimized synaptic plasticity (40). Remarkably, both HAPLN-2 and HAPLN-4 link proteins have only been identified in neural tissue (19). Furthermore, attenuated expression of HAPLNs in malignant gliomas (38) results in similar characteristics with trophoblastic invasion, suggesting that HAPLNs are an important factor for normal placentation, which failures in PE.

It is important to note that the placental tissue was consistently obtained from the same site of the placenta in both patient and control groups. The limitation of the present study is that the tissue samples were collected from only one site of the placenta which may not be a good cross-sectional representation of the whole placenta.

CONCLUSION

Our study suggests that ADAMTSs and their molecular partners, TIMP-3 and HAPLNs, might be related to the placental dysfunction in the context of PE.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Dicle University.

Informed Consent: Written informed consent was obtained from patients and control groups who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Conceived and designed the experiments: SI. Performed the experiments: İİT, GP, MAT. Analyzed the data: SI, İİT, KD. Wrote the paper: SI, İİT. All authors have read and approved the final manuscript.

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Table 1. Demographic and clinical characteristics of the study groups

Characteristics	Preeclampsia (n = 10)	Control (n = 10)	*p
Maternal age (years)	28.4 ± 5.7	29.4 ± 3.8	0.518
Gestational age (week)	32.6 ± 3.35	33.7 ± 4.2	0.197
Body mass index (kg/m^2)	23.85 ± 3.63	22.7 ± 2.62	0.521
Systolic blood pressure (mm Hg)	161.3 ± 18	119.6 ± 6.4	**<0.001**
Diastolic blood pressure (mm Hg)	105.5 ± 15	73.6 ± 5.9	**<0.001**
Proteinuria (g/24 h)	0.42 ± 0.13	–	–
Mode of delivery	Cesarean section	Cesarean section	

Data are shown as mean ± s.d.

*p-Values <0.05 are represented in boldface.

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Figure 1: The expression levels of ADAMTS-14, ADAMTS-10, ADAMTS-8, ADAMTS-13, and ADAMTS-4 were significantly lower in preeclamptic placentas than in healthy women placentas. The cell lysates were examined for the expression of ADAMTS-14, ADAMTS-10, ADAMTS-8, ADAMTS-13, and ADAMTS-4 using antibodies against the indicated proteins by Western blot. The lowest panel represents loading control (β-actin). The image shown represents a single representative example of 10 separate experiments.

Figure 2: ADAMTS-16, ADAMTS-12, and ADAMTS-19 expression levels were significantly reduced in preeclamptic placentas. A, C, E) The cell lysates were examined for the expression of ADAMTS-16, ADAMTS-12, and ADAMTS-19 using antibodies against the indicated proteins by Western blot. The lowest panels represent loading control (β-actin). The images shown represent a single representative example of 10 separate experiments. B, D, F) Densitometry analyses of the intensity of the bands of ADAMTS-16, ADAMTS-12, and ADAMTS-19 were presented as a ratio to the total level of β-actin. The mean ± s.d. (n=10) is shown. *$p < 0.05$.

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Figure 3: Both ADAMTS-18 and TIMP-3 expression levels were increased in preeclamptic placentas. A, C) The cell lysates were examined for the expression of ADAMTS-18 and TIMP-3 using antibodies against the indicated proteins by Western blot. The lowest panels represent loading control (β-actin). The images shown represent a single representative example of 10 separate experiments. B) Densitometry analysis of the intensity of ADAMTS-18 was presented as a ratio to the total level of β-actin. The mean ± s.d. (n=10) is shown. *$p < 0.05$.

Figure 4: The expression levels of HAPLN-1, HAPLN-4, and HAPLN-2 were significantly higher in preeclamptic placentas than in control placentas. The cell lysates were examined for the expression of HAPLN-1, HAPLN-4, and HAPLN-2 using antibodies against the indicated proteins by Western blot. The lowest panel represents loading control (β-actin). The image shown represents a single representative example of 10 separate experiments.

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Figure 1)

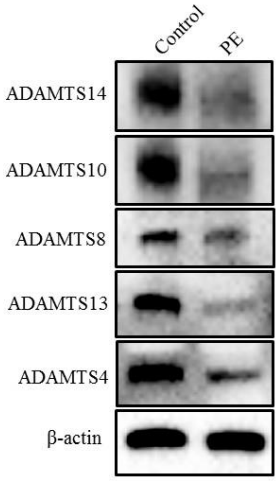

Figure 2)

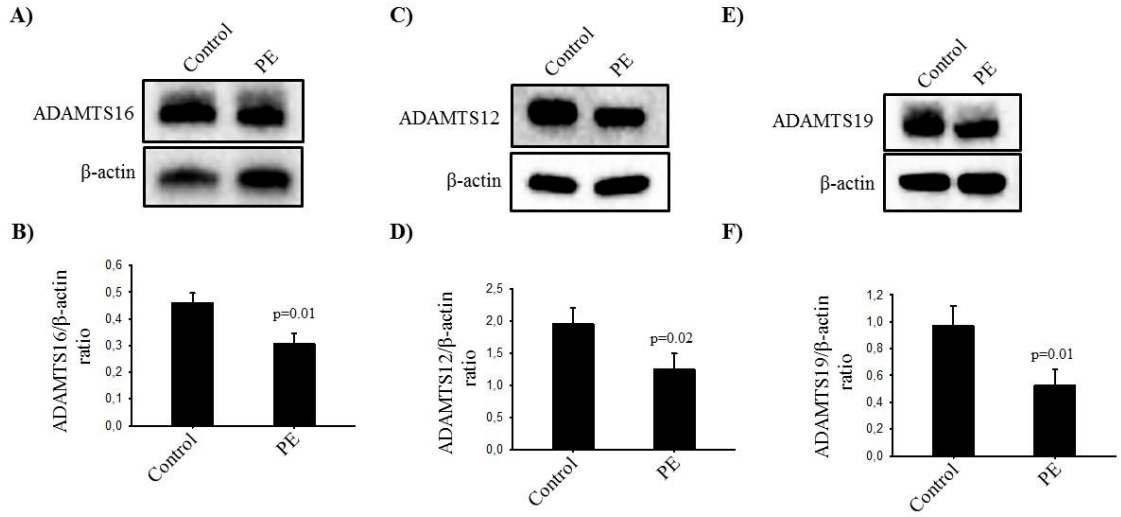

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Figure 4)

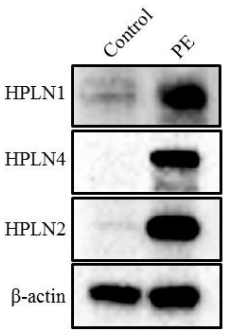
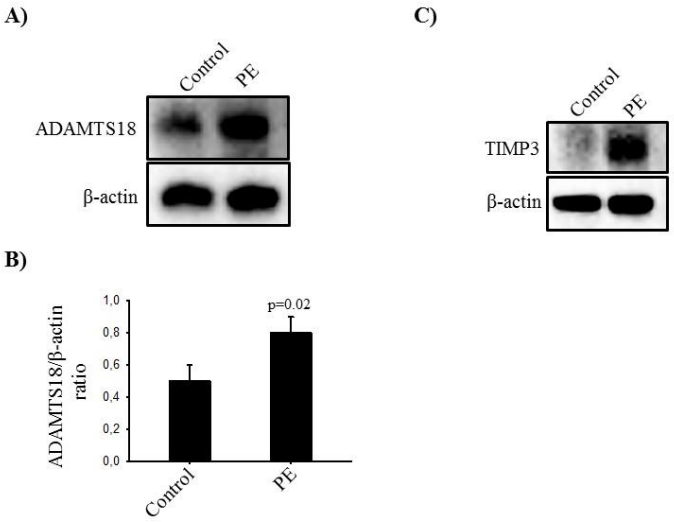


Figure 3)



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