Histochemistry and Cell Biology (2025) 163:29
https://doi.org/10.1007/s00418-024-02351-4

ORIGINAL PAPER

Distinct TYRO3 and PROS1 expression levels contribute to preeclampsia pathogenesis

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Accepted: 14 December 2024

Abstract

Preeclampsia (PE) is a severe placental complication occurring after the 20th week of pregnancy. PE is associated with inflammation and an increased immune reaction against the fetus. TYRO3 and PROS1 suppress inflammation by clearing apoptotic cells. Disruptions in TYRO3/PROS1 signaling may increase the risk of PE. This study investigated the role of TYRO3/PROS1 signaling in the development of PE using healthy placentae (HP) and preeclamptic placentae (PP) of six pregnant women each. Tissue morphology using hematoxylin and eosin (H&E), *TYRO3*, *MERTK*, *PROS1*, and *GAS6* mRNA levels using qPCR and localization and expression levels of TYRO3 and PROS1 using immunohistochemical staining (IHC) were evaluated. The study results show that the levels of TYRO3, MERTK, PROS1 and GAS6 mRNA, as well as TYRO3 protein, increased in PE. TYRO3 expression was observed in extravillous trophoblast (EVTs) and syncytiotrophoblast cells (SCTs). PROS1 was observed in HP fetal vessels through IHC while absent in PP. The reduced presence of PROS1 in the cytotrophoblast layer in PE may indicate a compromised blood-placental barrier. The absence of PROS1 in fetal vessels may suggest potential complement activation and thrombosis. TYRO3, MERTK, PROS1 and GAS6 may help balance impaired inflammation, apoptosis, thrombosis, complement activation and the blood-placental barrier in PE.

Keywords Preeclampsia · Placenta · TYRO3 · MERTK · PROS1 · GAS6

Abbreviations

PE	Preeclampsia
TAM	TYRO3, AXL and MERTK
RTK	Receptor tyrosine kinase
H&E	Hematoxylin and eosin
PROS1	Protein S
GAS6	Growth arrest specific 6
CS	Cesarean section
µm	Micrometer
EVTs	Extravillous trophoblast cells
CTBs	Cytotrophoblast cells
SCTs	Syncytiotrophoblast cells
BBB	Blood-brain barrier
NK	Natural killer
HLA	Human leukocyte antigen
C4BP	C4b-binding protein
SEM	Standard error of the mean

Introduction

The placenta is a vital organ during pregnancy, facilitating the exchange of gases, nutrients and fetal waste products between the mother and fetus. Additionally, it produces essential hormones such as hCG, progesterone and estrogen, which are crucial for sustaining the pregnancy and supporting fetal growth (Evain-Brion and Malassine 2003, Dong and Theunissen 2022). The placentation starts with the embryonic implantation into the decidualizing maternal endometrium. The outer cell mass of the embryo consists of trophoblastic stem cells, which rapidly proliferate and

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differentiate to form villous cytotrophoblasts (CTBs) or extravillous cytotrophoblasts (EVTs). Then, CTBs differentiate into multinucleated syncytiotrophoblasts (SCTs) through the fusion of multiple CTBs. SCTs form an epithelial monolayer that lines the surface of the villi, acting as a physical barrier between maternal blood and fetal tissue, and are responsible for substance exchange and hormone production. At the same time, interstitial and endovascular EVTs are differentiated from EVTs and invade the maternal decidua and spiral arteries, respectively, until they reach one-third of the myometrium. Endovascular EVTs participate in the arterial remodeling process by replacing the spiral artery endothelium (Ozmen et al. 2022).

Shallow EVT invasion, which leads to inadequate arterial remodeling, is widely recognized as one of the main mechanisms triggering the later development of preeclampsia (PE). As one of the main causes of maternal and fetal mortality and morbidity, PE is a placental complication characterized by maternal hypertension and proteinuria, generally occurring after the 20th week of pregnancy (Al-Jameil et al. 2014, Evain-Brion and Malassine 2003, Pei et al. 2022, Dong and Theunissen 2022). Inadequate arterial remodeling results in limited dilation of maternal spiral arteries, thereby reducing blood flow and causing placental ischemia. This abnormal placenta development can release molecules including soluble FLT1, soluble endoglin and/or interleukin 1 and 6, etc., into the maternal bloodstream, causing endothelial dysfunction in the maternal vasculature (Ozmen et al. 2023, Patel et al. 2024, Torres-Torres et al. 2024). This dysfunctional vascular reactivity increases the tendency to blood clot formation as well as to inflammation and oxidative stress, which further worsen endothelial dysfunction and cause systemic damage at both decidual and chorionic sites (Al-Jameil et al. 2014, Mao et al. 2024, Schofield et al. 2024).

TYRO3 belongs to the TAM (TYRO3, AXL and MERTK) family of receptor tyrosine kinases (RTKs). It plays a significant role in various biological functions, including regulating the immune system, inflammation, epithelial-mesenchymal transition, blood coagulation, tissue repair, cell growth and cell survival. The binding of TYRO3 by its ligands, protein S (PROS1) or growth-arrest specific 6 (GAS6), triggers autophosphorylation of the receptor and activates downstream signaling pathways. This leads to the modulation of cellular responses, including the clearance of apoptotic cells, inhibition of inflammation and promotion of cell survival and growth (Al Kafri and Hafizi 2019, Suleiman et al. 2013, Chen et al. 2020, Zagórska et al. 2014, Lemke and Rothlin 2008). PROS1 is also an anticoagulant crucial for preventing blood clotting by inhibiting Factor Xa, Va and IXa and serving as a cofactor for other anticoagulants, activated protein C and tissue factor pathway inhibitor (Pilli et al. 2016). TYRO3 also promotes tumor growth, metastasis and cancer cell survival. Overactivation of TYRO3 has been observed in certain types of cancer. However, blocking the nuclear translocation of TYRO3 or inhibiting its nuclear function has been shown to effectively prevent colorectal cancer metastasis. Moreover, insufficient or dysfunctional expression of TYRO3 may contribute to the development of autoimmune diseases (Hsu et al. 2023, Blades et al. 2018, Chen et al. 2020).

Myeloid-epithelial-reproductive tyrosine kinase (MERTK) is another TAM RTK member essential for regulating immune responses and clearing apoptotic cells. Its expression in macrophages supports anti-inflammatory responses. The ligand PROS1 binds to phosphatidylserine on apoptotic cells and to MERTK, facilitating their clearance and promoting the secretion of anti-inflammatory cytokines, which helps prevent chronic inflammation and maintain immune tolerance (Wu et al. 2021, Rovati et al. 2021, Lemke 2013, Zagórska et al. 2014).

Development of maternal immune tolerance, which protects the fetus from the maternal immune cell attacks, sufficient vascular supply to the developing fetus, removal of apoptotic cells and suppression of severe inflammation are crucial for a healthy pregnancy and reducing the risk of complications. If there are problems with this signaling, there may be an increased risk of developing a condition called PE, a dangerous pregnancy complication (Schofield et al. 2024, Brown et al. 2018, Kanellopoulos-Langevin et al. 2003, Straszewski-Chavez et al. 2005). In this context, we hypothesize that TYRO3 and/or PROS1 is differentially expressed in placentas from PE-complicated pregnancies. Thus, we investigated expression levels of TYRO3 and PROS1 in different trophoblastic cell types of placental specimens obtained from gestational age-matched PE-complicated versus normal pregnancies.

Materials and methods

Ethical statement: patient and sample collection

This study was approved by the Clinical Research Ethics Committee of the Akdeniz University Faculty of Medicine on 24 June 2020 under decision number KAEK-434. After written informed consent was obtained, this study included women ($n = 6$) at ≥ 32 weeks of pregnancy who exhibited symptoms of hypertension (systolic blood pressure > 140 mmHg and diastolic blood pressure > 90 mmHg), proteinuria (> 0.3 g/day) and edema from the Department of Gynecology and Obstetrics, Akdeniz University Faculty of Medicine. Pregnant women ($n = 6$) without hypertension, proteinuria and edema were included as a control group. All placenta specimens were obtained at term from preeclamptic pregnancies (PP) and healthy pregnancies (HP). Patients with chronic hypertension, diabetes, kidney disease, polycystic ovary syndrome, fetal anomalies, multiple pregnancies, reproductive tract infection, chromosomal anomaly, endocrine system disease, reproductive system structural abnormality and/or immune system disease were excluded from this study. Only late-onset PE cases and mothers delivering by cesarean section (CS) were included in experiments to minimize sample processing bias. There were no significant differences between the two groups regarding age, gestational age, parity or other baseline data. Collected placental samples were promptly transported to the laboratory on ice and washed with a physiological saline solution containing heparin. Placental samples, measuring 1 cm^3, were taken from the central and peripheral regions of the placental disc using a scalpel, with infarcted areas excluded. Homogeneous villous tissue pieces were sliced longitudinally from the decidual to the chorionic side. Tissue samples fixed in 4% paraformaldehyde solution were processed for paraffin embedding to use hematoxylin and eosin (H&E) or immunohistochemistry staining. Some placental pieces were placed in Trizol reagent (Life Technologies, Darmstadt, Germany) and stored at −80 °C to isolate mRNA for subsequent quantitative polymerase chain reaction (qPCR) analysis.

H&E staining

Tissue sections were cut to 5 µm thickness and incubated overnight at 60 °C. The following day, the slides were passed through xylene (1 and 2), a series of alcohol concentrations (100%, 90%, 80% and 70%) and distilled water, with each step lasting 10 min. The slides were then immersed in hematoxylin (AMRESCO-0701, AMRESCO Inc., Solon, OH, USA) for 1 min, excess dye was removed with water, and the slides were subsequently dipped in eosin (AMRESCO-0109, AMRESCO Inc.). After excess dye was removed with water, the slides were passed through the alcohol series and placed in xylene, with each step lasting 10 min. They were mounted with Entellan® (107,961, Merck, Darmstadt, Germany). Slides were then examined and photomicrographed using ZEN 2.5 lite software (blue edition-version 2.5.75.0) and a Zeiss Primo Star light microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The preferred objective lens magnification for the photographs was 40×/0.65 (Zeiss, Plan-ACHROMAT). The images were acquired at 90% intensity with 9.5-ms exposure.

RNA isolation and quantitative real-time PCR (qPCR)

After removing the tissues from the nitrogen tank, they were placed on a cold glass surface at + 4 °C under sterile conditions and quickly dissected using a scalpel. RNA was isolated as described previously (Kirimlioglu et al. 2024). Subsequently, the cDNA was obtained by reverse transcription of equal amounts of RNA for each specimen using RETROScript kit according to the manufacturer's instructions (AM-1720, Ambion-Invitrogen, USA). The concentrations of mRNA and cDNA were determined by measuring absorbance at 260 and 280 nm using the EPOCH NanoDrop system. The mRNA levels of *TYRO3*, *MERTK*, *PROS1*, *GAS6* and *GAPDH (*as control) genes in the experimental groups were measured by qPCR. The following cycles were applied to amplify the genes using a qPCR instrument (Rotorgene-Qiagen): initial denaturation for 5 min at 95 °C and then 30 cycles of 30 s at 92 °C, 20 s at 65 °C and 1 min at 72 °C. The experiment involved using gene-specific primers listed in Table 1 and SYBR Green PCR Master Mix from Applied Biosystems (170-8880, Bio-Rad, Hercules, CA, USA). The quantitative expression levels of *TYRO3*, *MERTK*, *PROS1*, *GAS6* and *GAPDH* genes were determined using the threshold cycle method. Delta Ct values were calculated by subtracting the Ct value of *GAPDH* from the Ct value of the tested genes in the samples. Using the $2^{-\Delta\Delta CT}$ method, transcription levels for all genes were calculated, and the results are presented as fold changes.

Immunohistochemistry Staining

Placental sections of 5 µm thickness were preprocessed for immunohistochemistry analysis as we described previously (Katirci et al. 2024). Briefly, the sections were dried overnight at 60 °C in an oven, then deparaffinized in xylene and rehydrated via descending alcohol concentrations. The antigenic retrieval was performed by steam-boiling of the slides in preheated Tris-EDTA buffer at pH:9 for 20 min. The tissue was treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. To prevent non-specific antibody binding, the sections were incubated with 5% Normal Goat Serum (Vector S-1000, Vector Labs, Newark, CA, USA). The sections were then incubated overnight at 4 °C with rabbit polyclonal anti-TYRO3 (1/400, ab109231, Abcam, Cambridge, UK) or anti-PROS1 (1/400, ABIN603703, Antibodies Online, Aachen, Germany). For negative control, normal rabbit IgG (Vector I-1000, Vector Labs) was

Table 1 Primers used in qPCR

TYRO3 forward primer	5′-CCTGGCCGACAACCTGTAT-3′
TYRO3 reverse primer	5′-TCCATTCGCAGACAAGTAAAGC-3′
MERTK forward primer	5′-CCCCCTCCGTGCTAACTGTT-3′
MERTK reverse primer	5′-TGTGGGCCTCACAACTGAAG-3′
GAS6 forward primer	5′-CCTTCCATGAGAAGGACCTCGT-3′
GAS6 reverse primer	5′- GAAGCACTGCATCCTCGTGTTC-3′
*PROS*1 forward primer	5′-GGCTCCTACTATCCTGGTTCTG-3′
PROS1 reverse primer	5′-CAAGGCAAGCATAACACC AGTGC-3′
GAPDH forward primer	5′ -GTCTTCACCACCATGGAGAA- 3′
GAPDH reverse primer	5 -ATCCACAGTCTTCTGGGTGG-3

used at the same concentration of primary antibodies. After washing with PBS, the sections were incubated with Rabbit IgG specific SignalStain® Boost IHC Detection Reagent (8114-HRP, Cell Signaling, Danvers, MA, USA) for 30 min at room temperature. The staining was developed using the DAB Substrate Kit (ab64238, Abcam), and the tissues were subsequently counterstained with Mayer's hematoxylin (Vector H-3404, Vector Labs). Finally, the slides were covered with a coverslip using Entellan®, and photomicrographs were captured with the ZEN 2.5 lite software on a Zeiss Primo Star light microscope (Zeiss). The preferred objective lens magnification for images obtained at 90% density and 9.5-ms exposure was 40×/0.65 (Zeiss). Immunohistochemical evaluations included Image-J software (Kirimlioglu et al. 2024) for total immunoreactivity levels of proteins and H-SCORE analysis for distinct trophoblast types as described (Katirci et al. 2024).

Statistical analysis

Numerical data values obtained from Image J, H-SCORE or qPCR analyses were transferred to Prism (GraphPad, version 10; Boston, MA, USA) for statistical analysis. The Kolmogorov-Smirnov test was used to determine whether the data from the two groups were normally distributed. Differences in means between groups were analyzed using the independent two-sample *t*-test for normally distributed data, while the Mann-Whitney *U* test was used for non-normally distributed data. Data with $P < 0.05$ were considered statistically significant. Results are presented as mean ± standard error of the mean (SEM).

Results

Histopathological evaluation

Morphological evaluation of placental chorionic villi stained with H&E revealed that in placentas from PPs compared to HPs, SCTs exhibited an increased number of nuclei, predominantly arranged as syncytial node nuclear accumulation foci. Furthermore, PPs showed fewer villous vessels with irregular dilation and constriction in different parts of the villi. An increase in fibrotic deposits and a decrease in fetal stromal cells were also observed in the connective tissue area of the PPs' villi compared to those from HPs (Fig. 1).

Transcriptional comparison of TAM receptor kinases and their ligands in placentas from PPs versus HPs

Analysis of mRNA isolated from placental specimens by qPCR revealed significantly higher transcriptional levels of *TYRO3* ($P < 0.05$), *MERTK* ($P < 0.05$) and their ligands *PROS1* ($P < 0.05$) and *GAS6* ($P < 0.05$) expression in the PP group compared to the HP group (Fig. 2). To determine whether these transcriptional changes correspond to protein levels, we conducted immunohistochemical analyses using paraffin sections of placental tissues.

Immunohistochemical comparison of TYRO3 receptor kinase and its ligand PROS1 in placentas from PPs versus HPs

Immunohistochemical analysis of placental sections revealed varying immunoreactivity levels for TYRO3 and PROS1 in different cell types, including EVTs, SCTs, villous vessels, and stromal cells. Initially, we assessed the overall expression levels of TYRO3 and PROS1 in placental sections using Image-J software. Total TYRO3 levels were significantly elevated in placental specimens from PPs compared to HPs (Fig. 3), while PROS1 levels showed no significant difference between the two groups (Fig. 4).

Our further cell-specific evaluations in immunostained placental sections revealed stronger TYRO3 immunoreactivity and lower PROS1 immunoreactivity in EVTs at the decidual-chorionic interface in the PP group compared to the HP group (Figs. 3, 4 and Supplementary Fig. 1). H-SCORE analysis confirmed a significant increase in TYRO3 expression ($P < 0.0001$) and a decrease in PROS1 expression ($P < 0.01$) in EVTs from PPs compared to HPs. TYRO3 expression in EVTs predominantly showed a membranous pattern in the PP group, whereas it was cytoplasmic in the HP group (Fig. 3). Conversely, PROS1 expression in EVTs was cytoplasmic and membranous in the HP group, but predominantly cytoplasmic in the PP group (Fig. 4).

In the SCT layer of placenta villi from PPs versus HPs, we observed weaker TYRO3 and stronger PROS1 immunoreactivity (Figs. 3 and 4). H-SCORE analyses confirmed significantly decreased TYRO3 immunoreactivity ($P < 0.0001$) and increased PROS1 immunoreactivity ($P < 0.0001$) in the SCT layers of placental villi from PPs compared to HPs. In both HP and PP groups, SCTs displayed cytoplasmic TYRO3 and PROS1 expressions (Figs. 3 and 4). Endothelial and stromal cells in villi showed similar weak levels of TYRO3 immunoreactivity in both groups (Fig. 3). However, fetal vessels in villi from HPs exhibited strong PROS1 immunoreactivity, which was notably absent in fetal vessels from PPs (Fig. 4).

Discussion

TYRO3 and MERTK modulate critical biological processes like immunomodulation, cell proliferation, differentiation and survival. PROS1 and GAS6 proteins act as ligands within the TAM RTK family, playing essential

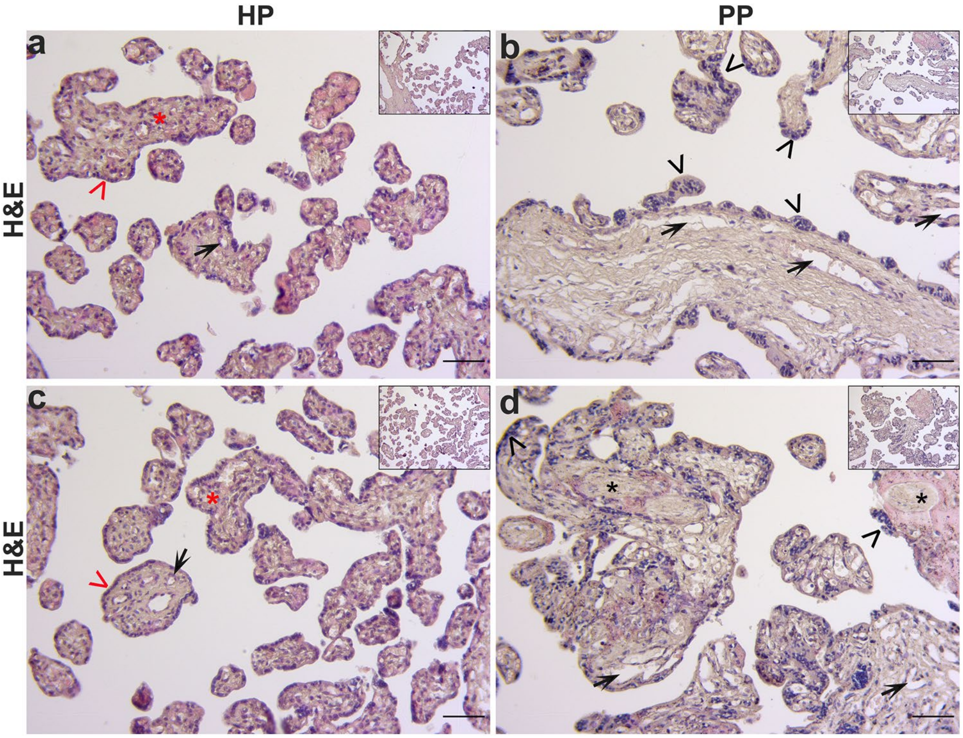

Fig. 1 Enhanced syncytial node nuclear accumulation foci and fibrosis in placentas from preeclampsia-complicated pregnancies vs. healthy pregnancies. **a** and **c** H&E-stained images of healthy placentae (HP); **b** and **d** images of preeclamptic placentae (PP). The density of fetal stromal cells (red star) is higher in the HP vs. PP. Placental villi from HP show fetal vascularization regularly distributed with no fibrin foci, whereas irregular distribution of fetal vessels (black arrow) and fibrin accumulations (black star) can be observed in the areas within the chorionic villi in placentas from the PP group. In HP, nuclei of syncytiotrophoblast cells (SCTs) are arranged regularly around the villi (red arrowhead), whereas the nuclei of SCTs increase in number and form syncytial node nuclear accumulation foci (black arrowhead) in the PP. Images were obtained at 20 × magnification; the thumbnails in the upper right corner were taken at 10×. Scale bars = 100 μm

roles in activating and regulating these receptors' functions. Despite their shared membership, these receptors exhibit distinct functions. The binding of GAS6 and PROS1 to TAM receptors depends on factors such as ligand concentration, biological context and the presence of phospholipids (Prieto and Lai 2024, Lemke and Rothlin 2008, Zagórska et al. 2014, Suleiman et al. 2013). TYRO3 regulates the functions of antigen-presenting cells (APCs), particularly in the innate immune system, as well as inflammatory responses, immune tolerance, cell survival and proliferation (Lu et al. 2021, Jiang et al. 2021). PROS1-TYRO3 binding enhances cell proliferation, differentiation and anti-inflammatory responses (Al Kafri and Hafizi 2019). Our results may suggest elevated mRNA and protein levels of TYRO3 and increased mRNA but decreased protein levels of PROS1 expression in placenta specimens from the PP group compared to the HP group. To our knowledge, this study represents the first report demonstrating the placental expression levels of TYRO3 and PROS1 in PE.

Given the functions attributed to TYRO3 mentioned above, its presence in the healthy placentas likely contributes to maintaining maternal immunotolerance and supports the survival and proliferation of trophoblast cells, which is crucial for normal placental development. It may also play a role in suppressing inflammatory responses to safeguard placental tissue from damage. Our findings might point towards increased expression of TYRO3 in the placenta of the PP group, suggesting an adaptive response to counteract the excessive inflammation associated with PE. This response may protect decidual-chorionic tissues by supporting survival and potentially enhancing the proliferation of trophoblast cells, which are susceptible to apoptosis.

We also found significantly increased mRNA levels of placental *GAS6* expression in addition to *PROS1* mRNA levels in PP *vs.* HP. Inflammation suppression may involve decreased PROS1 levels in EVTs in the maternal tissue. Additionally, our results may indicate that the placental GAS6 mRNA level is expressed much higher than PROS1. This indicates that GAS6, rather than the decreased PROS1, plays a role in suppressing PE-associated excessive inflammation and helps maintain the inflammatory balance. These

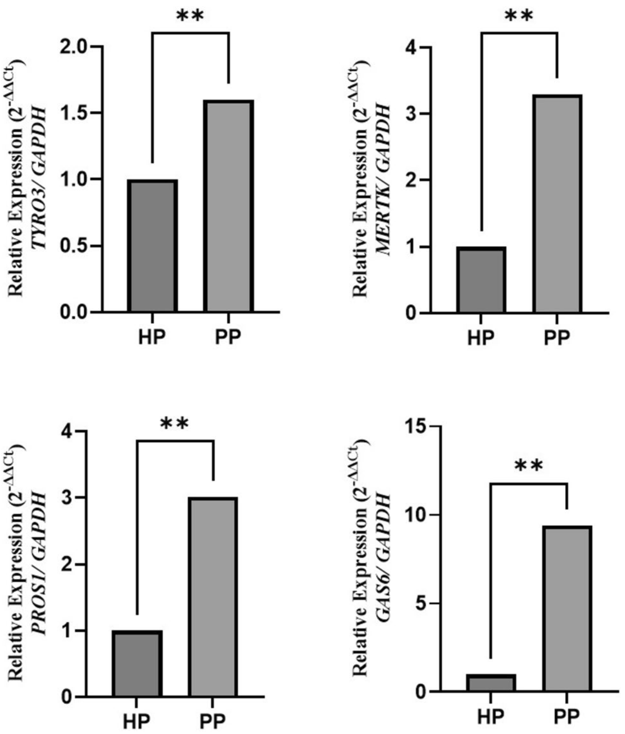

Fig. 2 Elevated levels of *TYRO3*, *MERTK* and their ligand *PROS1*, *GAS6* mRNA expressions in placentas from preeclampsia-complicated pregnancies. Transcriptional analysis of *TYRO3*, *MERTK*, *PROS1*, *GAS6* mRNA levels normalized to *GAPDH* in placental specimens from preeclampsia-complicated pregnancies and healthy pregnancies. Healthy placentae (HP), preeclamptic placentae (PP). Bars represent mean ± SEM, $n = 6$/each, $^{**}P < 0.01$

findings may suggest that GAS6 may take over the role of the ligand of TYRO3.

GAS6 is a vitamin K-dependent protein, sharing structural and functional similarities with PROS1, but does not have anticoagulant activity. A previous study reported that GAS6 levels increase significantly following vitamin K1 prophylaxis in preterm newborns, although this increase is not seen in term infants (Eksi Alp et al. 2018). PROS1 is a glycoprotein synthesized by the liver and found in the blood either free or bound to C4b-binding protein (C4BP). C4BP regulates the complement system by blocking excessive complement activation. PROS1 plays a role in preventing excessive blood clotting, thereby reducing the risk of thrombosis. PE is associated with increased complement activation, which contributes to enhanced inflammatory responses in women with PE. Additionally, PE is linked to hypercoagulability, resulting in an increased tendency to clot. C4BP binds to PROS1, reducing free PROS1 levels, which may impact on the effectiveness of anticoagulant mechanisms (Regal et al. 2017,Suleiman et al. 2013). One study concluded that relatively high levels of free PROS1, averaging 40% in the blood of fetuses between 15 and 35 weeks, may help prevent blood clotting and compensate for low levels of protein C (Melissari et al. 1988). In another study, PROS1 increased vascular permeability by activating p38 MAPK and Rho/ROCK pathways through MERTK and TYRO3 receptors (Joussaume et al. 2024). Our results, which show intense PROS1 expression in areas within the fetal vessels in HPs but not in PPs, align with Mellissari et al.'s observations and support a regulatory mechanism to reduce the possibility of clotting during pregnancy (Fig. 5a and b).

Anticoagulant factor Protein S deficiency in mice causes fatal coagulopathy, ischemic/thrombotic injuries, vascular dysgenesis and disruption of the blood-brain barrier (BBB) with intracerebral hemorrhages. PROS1 can also maintain BBB integrity through TYRO3 and sphingosine-1 phosphate receptor (Zhu et al. 2010). In our results, the disappearance of the PROS1 protein level in areas within the fetal vessels and the decrease in the TYRO3 protein level in the STC layer in PE can be considered a sign of PE-related disruption of the placental blood barrier, in accordance with PROS1 observations in the BBB (Fig. 5a and b).

MERTK has a critical role in the clearance of dead cells and cellular debris as well as in cell survival, proliferation and differentiation (Post et al. 2021, Nguyen et al. 2013). These properties suggest that MERTK may also play a role in maintaining cellular homeostasis in the placenta. Our qPCR results showed a significant increase in MERTK mRNA expression in PE. This increase may contribute to immune tolerance and support the healthy functioning of the placenta. MERTK may be involved in maintenance of balance between rapid cell renewal and apoptosis in the placenta. Elevated MERTK levels in PE may participate in regulating the immune response by suppressing excessive inflammation and supporting placental immune tolerance, thus preventing the maternal immune system from harming the fetus. Since differentiation and renewal processes occur constantly in the placenta (Asanoma et al. 2012), this process also enhances apoptosis in PE (Horvat Mercnik et al. 2024). Thus, the increase in MERTK expression in placentas complicated with PE is a compensatory mechanism to preserve the functionality and integrity of the placenta to maintain the pregnancy.

Human leukocyte antigen (HLA) molecules have a role in helping the immune system recognize and respond to non-self-cells (Medhasi and Chantratita 2022). Non-classical HLA molecules in the placenta, especially HLA-G, are important sensors of maternal immunotolerance. HLA-G protects fetal cells by inhibiting the activity of natural killer (NK) cells (Vincze et al. 2024). TYRO3 enhances NK cell cytotoxicity (Lu et al. 2021), and MERTK regulates immunomodulatory function (Huelse et al. 2020). NK cells may enhance their cytotoxicity by acquiring TYRO3 from EVTs expressing high TYRO3 in placental tissue via trogocytosis, a fast, cell-cell contact-dependent uptake of membrane

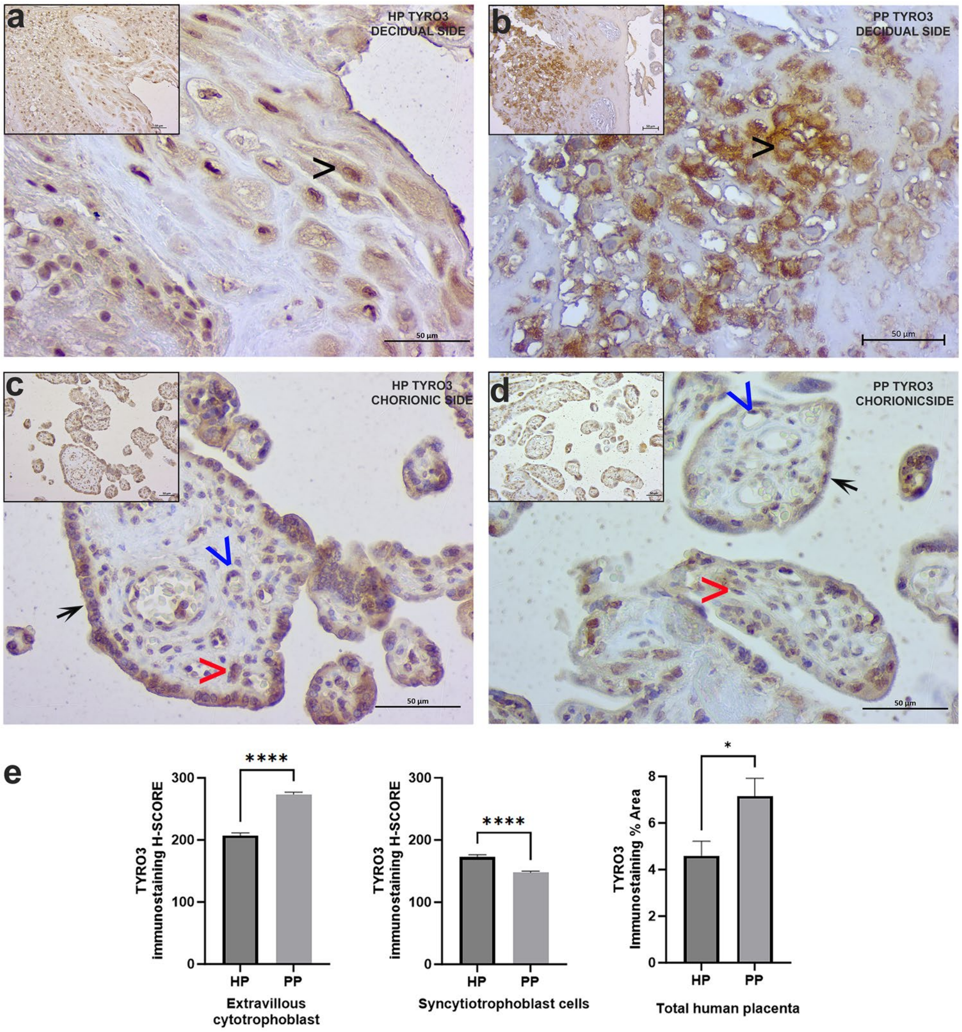

Fig. 3 Immunohistochemical evaluation of placental expressions and localizations of TYRO3 in preeclampsia-complicated vs. healthy pregnancies. At the decidual-chorionic interface, higher TYRO3 immunoreactivity in EVT (black arrowhead) in the preeclampsia-complicated vs. the healthy pregnancies. TYRO3 showed nuclear and cytoplasmic expression in the EVT in the HP and predominantly membranous and cytoplasmic expression in the PP group. In the placental villi, expression of TYRO3 in the syncytiotrophoblast layer (black arrow) was significantly lower in the PP group compared to the HP group. Additionally, TYRO3 expression in fetal endothelial cells (blue arrowhead) is strong in the HP group but absent in the PP group. TYRO3 expression in fetal stromal cells (red arrowhead) in the PP and HP groups. TYRO3 expression in placental tissue: **a** HP decidual side; **b** PP decidual side; **c** HP chorionic side; and **d** PP chorionic side. Graphs **e** show extravillous trophoblast cells (EVT)—or syncytiotrophoblast cells (SCTs)—or total expression of TYRO3 in the PP and HP groups. Healthy placentae (HP), preeclamptic placentae (PP). Bars represent mean ± SEM, $n = 6$/each $^{*}P < 0.05$; $^{****}P < 0.0001$. Scale bars = 50 µm

patches and associated molecules by one cell from another. On the other hand, they may also regulate the reduction of inflammation by promoting apoptotic cell phagocytosis through high placental MERTK expression. The increases observed in the levels of TYRO3 and MERTK in the PP group, as well as the increase in TYRO3, observed in the protein level in EVTs, may be a mechanism that will balance NK cytotoxicity and prevent fetal rejection in maternal tissue. The possible role of TYRO3 and MERTK in this process may help strengthen immunotolerance. Further studies using co-culture systems are required to test this hypothesis.

PROS1 promotes phagocytosis and immune tolerance, mostly by interacting with MERTK (Giroud et al. 2020, Cabezón et al. 2015), while GAS6 increases cell proliferation and survival by activating TYRO3 (Apostolo et al. 2024). The relationship between TYRO3 and PROS1 is particularly important for cell survival and enhancing anti-inflammatory responses (Vago et al. 2021, Al Kafri and Hafizi 2019). While MERTK and PROS1, which increase with PE, may support the increased phagocytosis due to PE, they may also support immune tolerance to protect the fetus. TYRO3 and PROS1 mRNA increase in the total

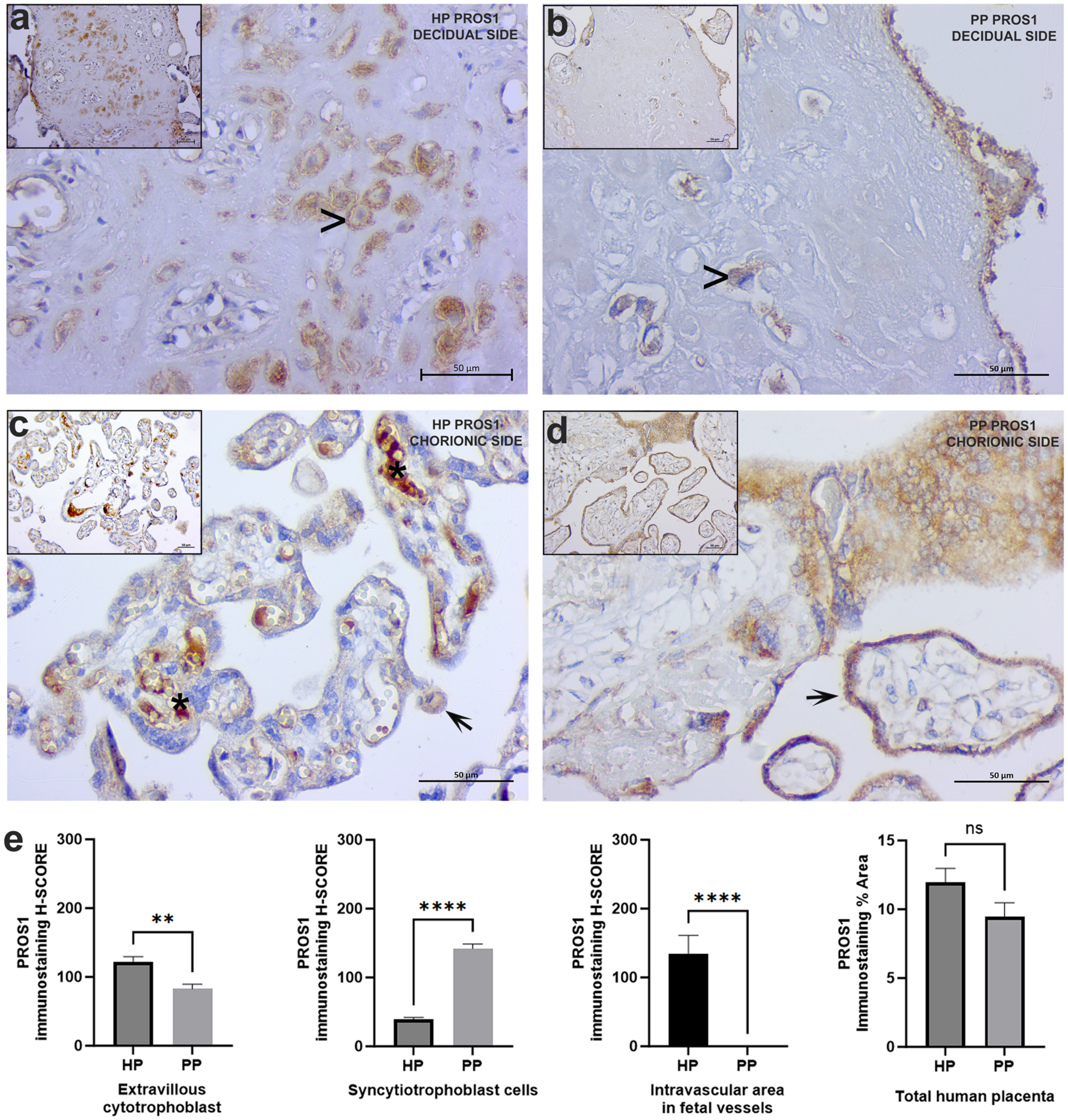

Fig. 4 Immunohistochemical evaluation of placental expressions and localizations of PROS1 in preeclampsia-complicated vs. healthy pregnancies. PROS1 immunoreactivity was weakly expressed in both preeclampsia-complicated vs. healthy pregnancies. At the decidual-chorionic interface, EVTs (black arrowhead) show predominantly membranous and cytoplasmic PROS1 expression in the HP group and weak perinuclear PROS1 expression in the PP group with a significant decrease ($P < 0.01$) for EVTs in the PP group compared to the HP group. In the villi, the syncytiotrophoblast layer (black arrow) showed PROS1 immunoreactivity with significantly higher levels ($P < 0.0001$) in the PP group compared to the HP group. Notably, strong PROS1 immunoreactivity (asterisk) was detected in fetal vascular lumens of placental villi from the HP group but not in the PP group. PROS1 expression in placental tissue: **a** HP decidual side; **b** PP decidual side; **c** HP chorionic side; **d** PP chorionic side. Graphs **e** show extravillous trophoblast cells (EVT)—or syncytiotrophoblast cells (SCTs)—or intravascular or total expression of PROS1 in the PP and HP groups. Healthy placentae (HP), preeclamptic placentae (PP). Bars represent mean ± SEM, $n = 6$/each. **$P < 0.01$; ****$P < 0.0001$. Scale bars = 50 µm

tissue and may support the survival of PE-dependent cells while supporting the balancing of inflammation and supporting the fetus in the maternal tissue. Increased TYRO3 and decreased PROS1 in SCTs following PE can be considered as an indicator of disruption of the placenta-blood barrier (Fig. 5b).

A previous study (Al Kafri et al. 2022) has shown that the laminin G-like (LG1) domain of PROS1 is critical in activating intracellular TYRO3 signaling, which triggers Erk kinase activity and activates pathways that promote cell survival, migration and proliferation. In our study, TYRO3 expression in EVT cells exhibited

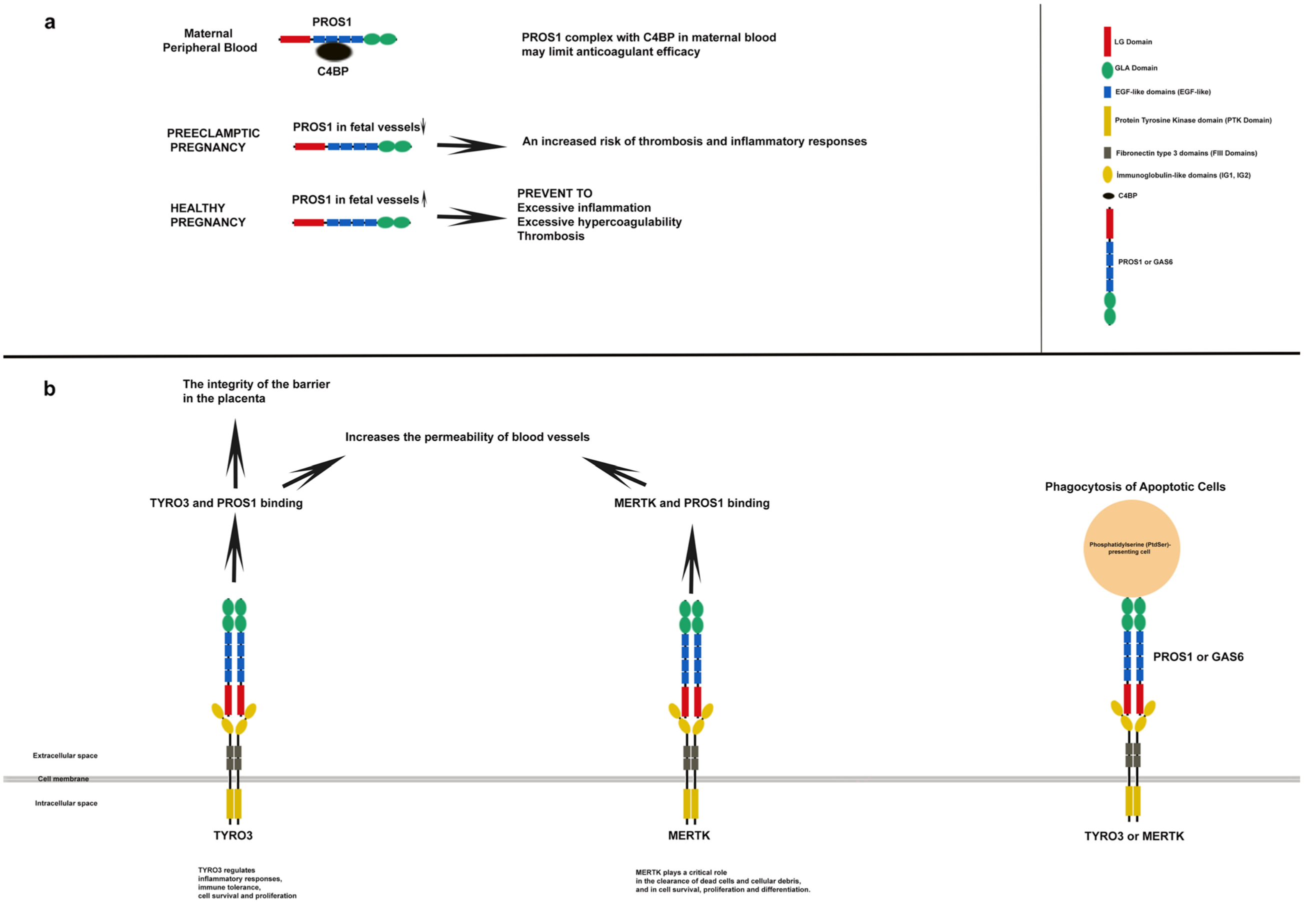

Fig. 5 Schematic representation of current study findings in the context of previous literature. **a** In maternal blood, PROS1 may form a complex with C4BP, reducing free PROS1 and limiting its anticoagulant effect. In healthy pregnancies, PROS1 may be expressed intensively in fetal vessels. It may support anticoagulant mechanisms, whereas, in preeclamptic pregnancies, decreased PROS1 levels may be associated with disruption of placental blood barrier integrity and increased thrombosis risk. **b** MERTK plays a critical role in the clearance of dead cells and cellular debris, cell survival, proliferation and differentiation, while TYRO3 regulates inflammatory responses, immune tolerance, cell survival and proliferation. TYRO3 and MERTK contribute to the clearance of apoptosis of cells presenting phosphatidylserine (PtdSer) via PROS1 or GAS6. TYRO3-PROS1 binding may support placental barrier integrity during pregnancy. In contrast, TYRO3-PROS1 and MERTK-PROS1 binding may play a role in maintaining placental functions by increasing the permeability of blood vessels and regulating blood flow to the placenta

a predominantly membranous pattern in the PP group, whereas PROS1 expression was cytoplasmic. This suggests that EVT cells may respond to preeclampsia-associated endothelial injury by differentiating and migrating toward blood vessels to control and repair the injury.

Based on our results, TYRO3 and MERTK receptors may contribute to immune tolerance in the placenta, and PROS1 and GAS6 ligands may play critical roles in the healthy development of placental tissue and maintenance of immune homeostasis by supporting the functions of MERTK and TYRO3 receptors. Further functional studies are needed to support all these data.

Conclusion

Based on our results, PE cases may display a reduced protein level of PROS1 in the cytotrophoblast layer, likely indicating a disrupted blood-placental barrier. Moreover, the absence of PROS1 in fetal vessels in PE strongly suggests enhanced risk for complement activation and thrombosis. Further functional studies are needed to investigate the crucial role of TYRO3, MERTK, PROS1 and GAS6 interactions in impaired inflammation, immune response, cellular survival and apoptosis associated with preeclamptic placentas (Fig. 5a and b).

Limitations

This study has several limitations that should be considered. First, the small sample size may limit the generalizability of our findings and reduce the statistical power. Larger studies are needed to confirm these results in other populations. Second, as an observational study, causal relationships cannot be established. While certain biological changes are correlated, a direct cause-effect link is unclear. Further experimental studies are necessary to clarify this. Lastly, only late-onset preeclampsia cases were examined, which may restrict the findings to this subgroup. Early-onset cases could yield different results. Given these limitations, our findings should be interpreted cautiously and validated with further research.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00418-024-02351-4.

Author contributions Esma Kirimlioglu (Esma K.) established and supported the project's hypothesis with Akdeniz University TSA-2021-5488 funding. She optimized the immunohistochemistry techniques and qPCR protocol. She did İmmunohistochemistry, QPCR and H-SCORE and Image J analysis. She performed data interpretation and evaluation. She statistically analyzed all experiments and created all figures and tables. She drafted the manuscript. Ertan Katirci (E.K.) collected the placentas, washed them with physiological saline, turned them into blocks and took sections from paraffin blocks and stained them with H&E. He worked on the immunohistochemical protocol together with Esma K. and performed the H-SCORE analysis. Mehmet Simsek (M.S.) collected healthy and preeclamptic placenta samples and obtained written consent from the volunteers.

Funding Akdeniz University BAP supported this study with TSA-2021-5488 Project number.

Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflicts of interest The authors declare no competing interests.

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