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Galectin-3, a potential predictor and contributor of placenta accreta spectrum pathogenesis by inducing local vascular cell adhesion molecule-1 expression: A longitudinal study

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Keywords

- Galectin-3 (Gal-3)
- Placenta accreta spectrum, VCAM-1
- Macrophage
- Trophoblastic invasion.

Abstract

Background: Galectin-3 (Gal-3) is a unique glycoprotein expressed in different tissues with several biological functions. Vascular cell adhesion molecule-1 (VCAM-1) is a cell adhesion molecule, expressed from vascular endothelium, and is implicated in the process of tissue angiogenesis. Both Gal-3 and VCAM-1 are expressed normally in placental tissue to achieve successful trophoblastic invasion. Placenta accreta spectrum (PAS) is a serious pregnancy-related complication, with a progressively rising incidence worldwide. Massive trophoblastic invasion is a major contributor to its pathogenesis, and its diagnosis prenatally depends solely on Doppler ultrasonography. **Aim:** This study aimed to investigate the possible predictive value of serum Gal-3 in the occurrence of PAS and to highlight the potential involvement of Gal-3, macrophage recruitment, and VCAM-1 in its pathogenesis. **Methods:** This longitudinal study included 62 pregnant women; divided into group N, comprising31 pregnant women with normal placenta, and group P, comprising 31 pregnant women with PAS. **Results:** placental Gal-3 increased from 2.597 ± 0.061 to 4.392 ± 0.181 ng/mg protein, placental VCAM-1 increased from 2.901 ± 0.096 to 41.911 ± 1.885 ng/mg protein, and placental macrophage count increased from 2.323 ± 0.106 to 11.174 ± 0.643 /10 HPF. Serum Gal-3 had a significant predictive value for PA with a cutoff value ≥ 8.012 ng/ml. **Conclusion:** The overexpression of placental Gal-3 and VCAM-1 can be regarded as important key players in the pathogenesis of PAS. Remarkably, the detection of high serum levels of Gal-3 as early as the second trimester can predict the occurrence of PAS.

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1. Introduction

Galectin-3 (Gal-3) is the only chimera-type galectin expressed in several cells, including vascular endothelium, macrophages, endometrium, and trophoblastic cells [1]. It is involved in different biological activities, including cell growth, apoptosis, adhesion, and angiogenesis, and through these rampant effects, it contributes to the pathogenesis of inflammation, thromboembolic conditions, and cancers [2].

Vascular cell adhesion molecule-1 (VCAM-1) is a cell adhesion molecule predominantly expressed in endothelial cells, tissue macrophages, and placental trophoblastic cells [3]. It is implicated in cell migration and adhesion; thus, its role is obvious in the pathogenesis of inflammation and tumor metastasis [4]. Regarding pregnancy, both Gal-3 and VCAM-1 participate in placental formation by inducing trophoblastic invasion and angiogenesis [5].

Placenta accreta spectrum (PAS) is an abnormal adherence of the placenta to the myometrium [6]. The global incidence of PAS ranged from 1:533 to 1:731 deliveries in 2002 and increased to 1 in 272 deliveries in 2016 [7]. A recent Egyptian single-center study estimated a relatively high incidence of PAS (i.e., 9:1000 deliveries) [8]. Approximately more than half of the maternities with a history of elective cesarean section develop PAS[9]. Decidual maldevelopment and excessive trophoblastic invasion are considered the main pathogenic mechanisms of PAS[10]. One of the proven contributors is increased placental macrophage recruitment, which is considered the main source of local VCAM-1, which subsequently promotes excessive trophoblastic invasion of PAS[11]. The prenatal diagnosis of PAS depends largely on ultrasonography with Doppler examination, which is considered the gold standard tool for diagnosing PAS in the third trimester [12]. However, to the best of our knowledge, there is no identifiable biochemical marker that can precisely predict the occurrence of PAS.

This study was designed to highlight the potential predictive value of Gal-3 as an early biochemical marker for the occurrence of PAS and investigate the possible involvement of Gal-3 in the pathogenesis of PAS by affecting the local placental expression of VCAM-1.

Material and Methods:

- **Ethics:**

Before the recruitment, ethical approval was obtained from the Ethics Committee of the Faculty of Medicine, Aswan University (approval NO: saw/477/9/20), and informed consent was obtained from all eligible participants after explaining the nature of the study.

- **Study design:**

From October 1, 2020, to October 1, 2021, a double-center longitudinal study registered at **ClinicalTrials.gov (NCT04573452**) was conducted by recruiting 62 pregnant women with normal placenta and PAS from Assiut Woman's Health Hospital and Aswan University Hospital, Egypt. The sample size was assigned according to the study's primary outcome with an estimated power of 80%, using G*Power, version 3.1.9.7.

Eligible participants were enrolled according to the following inclusion criteria: gestational age between 24 and 36 weeks, age between 20 and 40 years, singleton pregnancy, and high probability of PAS according to two-

dimensional (2D) gray-scale imaging and color Doppler flow mapping (Table **1)**. Meanwhile, women known to have metabolic syndrome; those with hypertension; those with bleeding disorders; those with cardiac, renal, endocrinal, and autoimmune diseases, and those on anticoagulant therapy were excluded from the study. Then, according to the results of 2D gray-scale imaging, color Doppler flow mapping, and definite intraoperative diagnosis, the study participants were divided into two groups (n = 31): ***Group N***, comprising pregnant women with normal placenta, and ***Group P***, comprising pregnant women with PAS.

- **Technical information:**

1. Blood sampling and analysis:

Venous blood samples were collected from all participants of both groups at two gestational phases: the second trimester (24–28 weeks; N_{T2} and P_{T2}) and the third trimester (32–36 weeks**;** N_{T3} and P_{T3}). Then, the samples were centrifuged, and the clear non-hemolyzed supernatant sera were analyzed for Gal-3 levels using Gal-3 enzyme-linked immunosorbent assay (ELISA) kit purchased from Shanghai Korain Biotech Co., Ltd (**catalog no**: E1951Hu) and for VCAM-1 levels using the corresponding ELISA kit purchased from Shanghai Korain Biotech Co., Ltd (**catalog no**: E0203Hu), according to the manufacturer's protocol.

2. Tissue sampling and analysis:

At the time of delivery, placental tissue samples were collected in the form of two rectangular sections measuring 1.5–3.5 cm in size from the decidual surface of the placenta of each participant.

Half of the collected sections were fixed in 10% formalin for 24 h; then, they were processed for both histopathological examination using hematoxylin and eosin (H&E) staining [13] and evaluation of macrophage count by immunohistochemical analysis**,** using a CONFIRM™ anti-CD68 (KP-1) primary monoclonal antibody (VENTANA catalog no: 790-2931(Y10630)) and automated Ventana auto Stainer (SN 8320039 - Ventana Medical System, Inc, Tucson, Arizona - USA). Using a research microscope (Olympus BX 43), The expression of macrophages (CD68 positive cells) in the stroma of fetal and maternal parts of the placenta was first scanned at low power (10x) to determine 3 areas having the largest number of CD68 expression. Then, CD68-positive cells were counted in ten consecutive high-power fields (HPFs) by (40x) magnification. For each slide, the mean number of macrophages was calculated in these 10 chosen HPFs and expressed as macrophages count per 10 HPF (macrophage count / 10 HPF). Images were obtained by the use of a digital camera (ToupCamTM - XCAM Full HD Camera - XCAM Family 1080P HDMI) [14].

The other half of the placental sections were suspended in cold 1× phosphate buffer saline with a pH of 7.4 and homogenized using a General Laboratory Homogenizer (GLH 650) roto-stator homogenizer; then, the homogenate was centrifuged, and the supernatant was analyzed for Gal-3 and VCAM-1 levels using the corresponding ELISA kits, according to the manufacturer's protocol. Protein levels in the tissue supernatant samples were analyzed using the protein determination kit purchased from ChemaDiagnostica (catalog no: TP0100CH).

Then, the tissue supernatant results of both Gal-3 and VCAM-1 were recalculated and expressed in nanogram (ng) per milligram (mg) protein of the tissue homogenate supernatant in each sample.

- **Statistics:**

All statistical analyses were performed using Statistical Package for the Social Sciences, version 20 (IBM Corp., Armonk, NY, USA). The results were expressed as means ± standard errors of the mean. Based on the results of the normality test, analysis was performed between the groups using the Kruskal–Wallis H test, followed by the Mann–Whitney U-test. The relationship between the study parameters was analyzed using linear regression analysis. The possible predictive value of Gal-3 was assessed using the receiver operating characteristic (ROC) analysis. *P-values* ≤ *0.01* were used to denote statistical significance [15].

Results:

- **Analysis of Gal-3 (serum and placental Gal-3)**

Collectively, group P (groups P_{T2} and P_{T3}) showed significantly higher serum levels of Gal-3 than group N (groups N_{T2} and N_{T3}). Notably, the serum levels of Gal-3 in the third trimester of both groups exhibited higher values than those in the second trimester (Table **2)**. Furthermore, the placental tissue expression of Gal-3 in group P was higher than that in group N (Figure **1A)**.

Interestingly, in the second trimester, the serum levels of Gal-3 in both groups were a significant predictor of the occurrence of PAS (*P <0.001*), at a cutoff value ≥8.012 ng/mL, with a sensitivity of 93.5%, a specificity of 96.8%, a negative predictive value of 93.7%, and a positive predictive value of 96.7% (Table **3)**.

The serum and placental levels of Gal-3 of all study groups showed a statistically significant positive correlation (r = 0.395; *P =0.002*).

- **Analysis of VCAM-1 (serum and placental VCAM-1)**

Similarly, in both the second and third trimesters, the serum levels of VCAM-1 were significantly higher in group P than in group N. Unlike serum Gal-3, the serum levels of VCAM-1 in the third trimester were lower than those in the second trimester (Table **2)**. Additionally, the placental levels of VCAM-1 were significantly higher in group P than in group N (Figure **1B)**. Moreover, the placental levels of Gal-3 had a statistically significant positive correlation with the placental levels of VCAM-1 (r = 0.810, *P < 0.001*).

Table (1): Criteria for diagnosis of PAS according to two-dimensional (2D) gray-scale imaging and color Doppler flow mapping [13]:

Two-dimensional (2D) gray-scale imaging	Color Doppler flow mapping
1. Loss of the retro-placental clear zone.	1. Sub placental hypervascularity.
2. Thinning of the myometrium.	2. Uterovesical hypervascularity/bridging vessels.
3. Abnormal placental lacunae.	3. Vascular lacunae with turbulent flow/feeder vessels.
4. Bladder wall interruption.	
5. Placental bulge.	
6. Exophytic placental mass.	
7. Loss of the retro-placental clear zone.	
8. Thinning of the myometrium.	
9. Abnormal placental lacunae.	

Table (2): Mean serum levels of Gal-3 and VCAM-1 in all groups under study (n = 31).

Outcomes	Group N			Group P			Inter-groups comparison *P-Value*	
	2nd trimester N_{T2}	3rd trimester N_{T3}	*P-value*	2nd trimester P_{T2}	3rd trimester P_{T3}	*P-value*	N_{T2} & P_{T2}	N_{T3} & P_{T3}
Gal-3	6.306 ± 0.159 ng/mL	6.969 ± 0.056 ng/mL	*0.008**	8.935 ± 0.127 ng/mL	10.546 ± 0.320 ng/mL	*0.000***	*0.000***	*0.000***
VCAM-1	25.903 ± 0.372 ng/mL	10.983 ± 0.147 ng/mL	*0.000***	110.924 ± 3.929 ng/mL	68.159 ± 3.068 ng/mL	*0.000***	*0.000***	*0.000***

Data were expressed as means ± standard errors of the mean.Gal-3, galectin-3; VCAM-1, vascular cell adhesion molecule1; group N, pregnant women with normal placenta; group P, pregnant women with placenta accreta spectrum;N_{T2}, pregnant women with normal placenta in the 2nd trimester; N_{T3}, pregnant women with normal placenta in the 3rd trimester; P_{T2}, pregnant women with placenta accreta spectrum in the 2nd trimester; P_{T3}, pregnant women with placenta accreta spectrum in the 3rd trimester.

*, statistically significant difference ($P \leq 0.01$).

**, statistically significant difference ($P \leq 0.001$).

Table (3): Receiver operating characteristic (ROC) curve showed the area under the ROC curve (AUC) and the predictive value of serum Gal-3 levels in the 2nd trimester.

Variable	Sensitivity	Specificity	AUC, 95%CI	*P-value*	+PV	-PV	Cutoff point
Normal placenta vs placenta accreta spectrum	93.5%	96.8%	0.974	*< 0.001***	96.7	93.7	8.012 ng/mL

ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval, +PV: positive predictive value, -PV: negative predictive value.

**, statistically significant difference ($P \leq 0.001$).

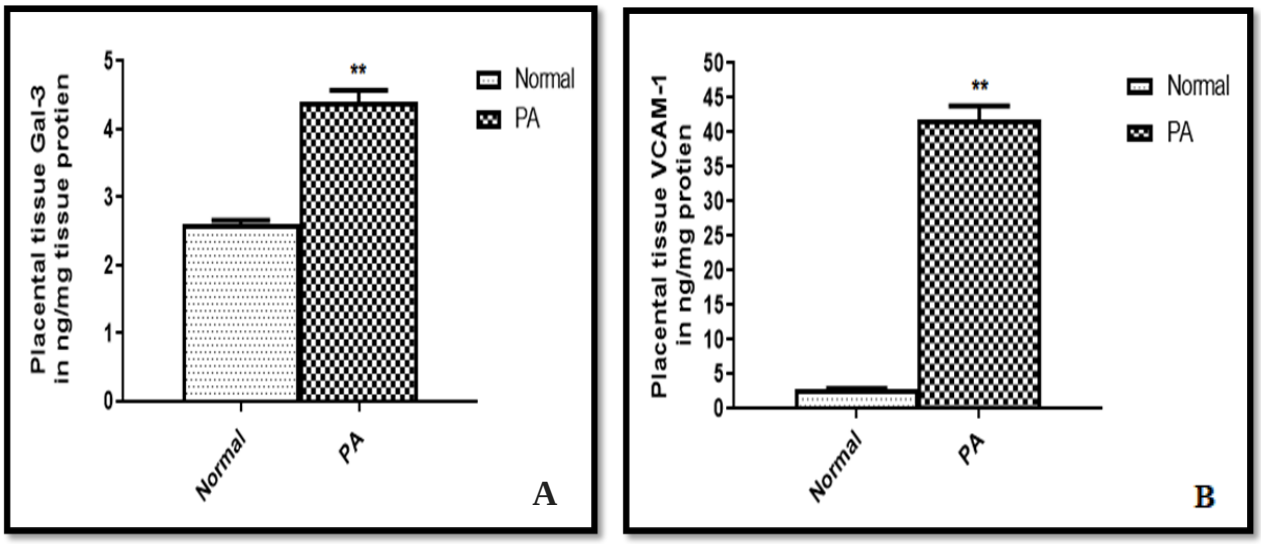

Figure (1) A: Placental tissue galectin-3 (Gal-3) levels of both the normal pregnancy and placenta accreta spectrum (PAS) groups.

B: Placental tissue vascular cell adhesion molecule-1 (VCAM-1) levels of both the normal pregnancy and placenta accreta spectrum (PAS) groups.

**, statistically significant difference compared with the normal group ($P \leq 0.001$).

- **Histopathological examination:**

Microscopic examination of H&E-stained placental sections obtained from group N showed normally structured decidua, separating the basal plate of chorionic villi from the uterine muscle (Figure **3 A&B**). In contrast, the placental sections obtained from group P (Figure **4 A&B**) showed chorionic villi lying on the fibrin layer separating it from the uterine muscle without intervening decidua.

- **Immunohistochemical analysis:**

The placental sections obtained from group N showed only a few fetal and decidual stromal CD68-positive macrophages with round to oval nuclei (Figure **3 C&D**). In contrast, the placental sections obtained from group P revealed much more stromal giant CD68-positive macrophages (having more than one nucleus) than those obtained from group N in both the villi and fibrin layers (Figure **4 C&D&E**). Macrophage density was significantly higher in group P (11.174 ± 0.643) than in group N (2.323 ± 0.106) (*P <0.001*) (Figure **2)**.

Concerning the relationship between placental Gal-3 and macrophage recruitment, a statistically significant positive correlation (r = 0.754; *P < 0.001*) was observed between placental Gal-3 and macrophage count in all study groups. Simple linear regression analysis to predict the placental expression of VCAM-1 based on placental macrophage recruitment showed a significant regression equation (F = (1,60) = 90.852; *P < 0.001*) with an R^2 of 0.602, and the placental expression of VCAM-1 increased by 3.172 ng/mg for each 0.333 increase in macrophage count.

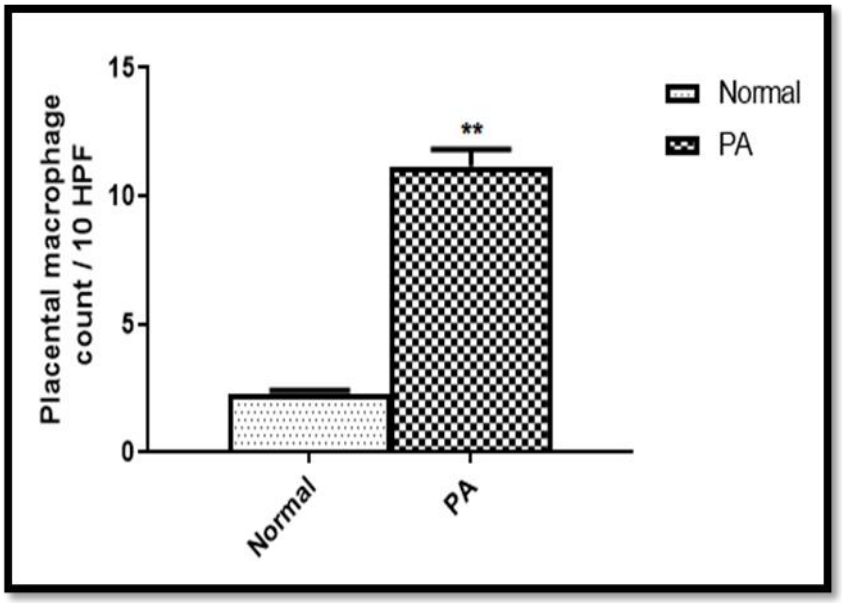

Figure (2): Placental macrophage recruitment in both the normal pregnancy and placenta accreta spectrum (PAS) groups. **, statistically significant difference compared with the normal group (*P ≤ 0.001*).

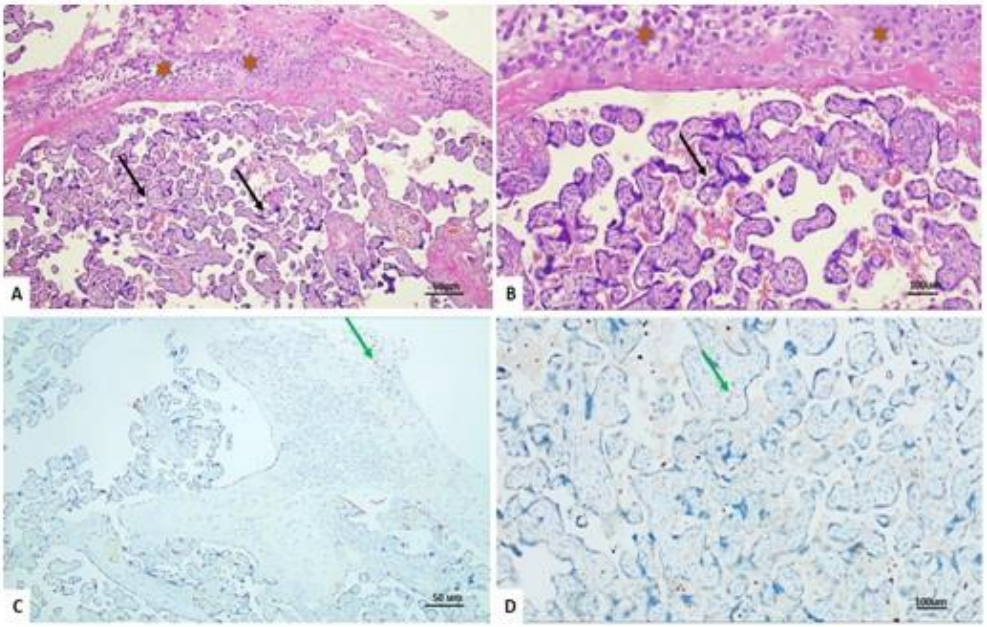

Figure (3): Normal placenta: **(A&B)**: Hematoxylin and eosin-stained sections showing that the decidua (brown asterisk) is attached to the basal plate of chorionic villi (black arrows) (40× and 100×, respectively). **(C&D)**: Immunohistochemical-stained sections for CD68-positive cells showing only a few positive cells in the decidua and core of the chorionic villi (green arrows) (40× and 100×, respectively).

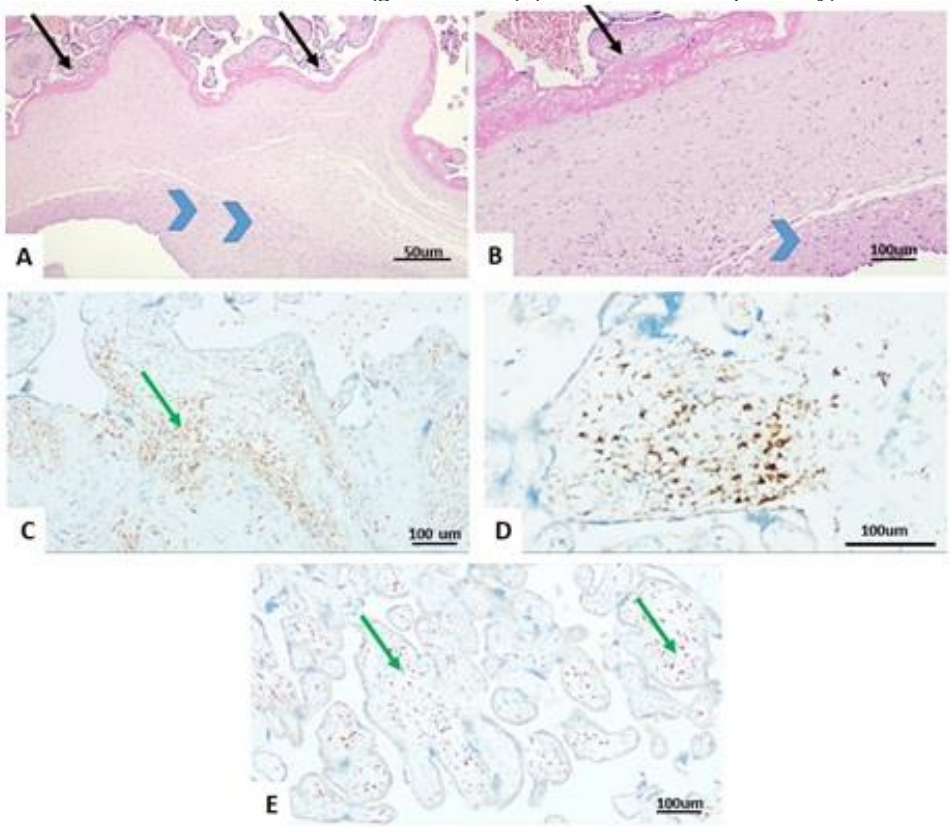

Figure (4): Placenta accreta spectrum: **(A&B)**: Hematoxylin and eosin-stained sections showing that the chorionic villi (black arrow) lie on the fibrin layer, which separates them from the muscle layer (blue arrowhead) without intervening decidua (40× and 100×, respectively). **(C)**: Immunohistochemical-stained sections for CD68-positive cells showing many cells in the fibrin layer (green arrow) (100×). **(D)**: Higher power of the previous section showing positive cells for CD68 (200×). **(E)**: Immunohistochemical-stained sections for CD68-positive cells showing that many cells in the chorionic villi core are positive for CD68 (green arrows) (100×).

Discussion:

This study was designed to investigate the role of Gal-3 as a predictor and contributor in the pathogenesis of PAS by inducing macrophage recruitment and VCAM-1 production. To achieve this aim, Gal-3 and VCAM-1 levels were estimated using the ELISA technique in pregnant women with PAS and those with normal placenta. Meanwhile, placental macrophages were quantified by immunohistochemical analysis.

Our results showed that in the serum, both Gal-3 and VCAM-1 were significantly higher in group P than in group N. In detail, the third-trimester samples of all groups exhibited higher Gal-3 levels than the second-trimester samples. In contrast, the second-trimester samples exhibited higher VCAM-1 levels than the third-trimester samples in all study groups. In the placenta, group P showed higher placental levels of both Gal-3 andVCAM-1 than group N. Likewise, the macrophage count was markedly higher in group P than in group N.

Studies on the changes in both serum and placental expressions of Gal-3 in PAS are still lacking. **However, Gao and Fang (2014)** and **Than et al. (2015)** showed a linear increase in both serum and placental Gal-3 levels in group N along with the progression of pregnancy [2,16]. The increase in estrogen and progesterone levels along with the progression of pregnancy explains this incremental rise in Gal-3, by stimulating trophoblastic production of both placental and serum Gal-3 [2]. Gal-3 contributes to placentation by promoting trophoblastic proliferation and inhibiting its apoptosis [17] and enhancing the adhesion of trophoblastic cells to maternal decidua [18]. In PAS, decidual defects create a hypoxic environment that encourages further trophoblastic production of Gal-3 [19], which subsequently enhances further trophoblastic invasion [18].Additionally, the associated exaggerated inflammatory state, particularly the overproduction of interleukin (IL)-6 and IL-33, activates circulating macrophages, which in turn secrete Gal-3 [20,21].

Remarkably, this is the first study to investigate the predictive value of serum Gal-3, and the results showed that high serum Gal-3 levels (≥8.012) in the second trimester can predict the occurrence of PAS with a sensitivity of 93.5% and a specificity of 96.8%. This result is expected considering the role of Gal-3 in the pathogenesis of PAS as Gal-3 plays an important role in angiogenesis and trophoblastic invasion [18].

Regarding serum VCAM-1, the results of this study coincide with those of **Raynor and Parthasarathy (1997)** and **Daniel et al.(2000),** who showed an inverse relationship between serum VCAM-1 level and gestational age [22,23]. In pregnancy, serum VCAM-1 is produced from the vascular endothelium and trophoblastic cells [22,24]. At term, the decrement in trophoblastic production of VCAM-1 helps trophoblastic separation to achieve delivery [25]. In **2018,Korkmazer et al.** showed that the placental expression of VCAM-1 was significantly higher in PAS than in normal pregnancy, which agrees with our results [11]. To the best of our knowledge, until now, no studies have investigated the serum levels of VCAM-1 in pregnant women with PAS. The amplified inflammatory condition associated with PAS largely explains the increase in both the placental and serum levels of VCAM-1. It increases macrophage recruitment, which then induces the

production of both serum and placental levels of VCAM-1 [11,26]. VCAM-1 and Gal-3 play a crucial role in placentation by promoting trophoblastic invasion through their proangiogenic and cellular adhesion effects [27].

Regarding placental macrophage recruitment, in line with our results, **Hecht et al.(2020)** found that placental macrophage recruitment was markedly higher in PAS than in normal placenta [26]. Pregnancy-associated inflammation is considered the prime stimulus for macrophage entrapment in both the fetal and maternal parts of the placenta [28]. This helps with successful trophoblastic invasion and spiral artery remodeling [28,29].

The relationship between Gal-3, VCAM-1, and placental macrophage count was analyzed by correlation analysis. A positive correlation was found between the serum and placental levels of Gal-3 in all study groups. This result agrees with those of **Pankiewicz et al. (2020),** who detected the same relationship between the serum and placental expressions of Gal-3 [30]. This can be because both serum and placental Gal-3 have many placental sources, such as trophoblastic cells, endometrial cells, and uterine smooth muscle [1]. Placental Gal-3 exhibited a significant linear relationship with placental VCAM-1 expression. It is agreed that Gal-3 is tightly bound to VCAM-1 indirectly, by sharing the same ligand binding—α4β1 integrin—particularly in inflammation [31,32]**,** where its expression in eosinophils facilitates its rolling and adhesion to VCAM-1 [31], and through the presence of the N-glycosylation site of VCAM-1 that binds to Gal-3,andGal-3 induces the endothelial expression of VCAM-1 [33].

Outstandingly, placental Gal-3 levels showed a direct relationship with the macrophage count in the placenta. This relationship could be clarified by the mutual relationship observed between Gal-3 and macrophages during inflammation [34]. Gal-3 enhances macrophage recruitment through its proinflammatory chemoattractant effect [35,36], and the recruited macrophages in turn produce Gal-3 [34].Comparably, placental VCAM-1 level showed a similar direct relationship with macrophage recruitment in the placenta in all groups under study. This result coincides with those of **Korkmazer et al.(2018),** who showed that placental macrophages and trophoblasts are the main sources of placental VCAM-1 [11]. The relationship between macrophages and VCAM-1 is because placental macrophages are one of the sources of local VCAM-1, along with the vascular endothelium [3].

Conclusively, we propose that Gal-3 is an important player in PAS as high serum levels of Gal-3 in the second trimester can predict the occurrence of PAS in healthy women. Furthermore, the increased trophoblastic production of Gal-3 in PAS cases is largely responsible for massive trophoblastic invasion both directly and indirectly through increased macrophage recruitment. These macrophages produce VCAM-1, which in turn enhances trophoblastic invasion through the promotion of angiogenesis. Simultaneously, the recruited macrophages viciously increase the trophoblastic secretion of Gal-3.

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