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Soluble CD146 is increased in preeclampsia and interacts with galectin-1 to regulate trophoblast migration through VEGFR2 receptor

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Objective: To explore the regulatory role of soluble CD146 (sCD146) and its interaction with galectin-1 (Gal1) in placenta-mediated complications of pregnancy.

Design: Prospective pilot and experimental studies.

Setting: University-affiliated hospital and academic research laboratory.

Patient(s): One hundred fifteen women divided into three groups: 30 healthy, nonpregnant women, 50 women with normal pregnancies, and 35 with placenta-mediated pregnancy complications.

Intervention(s): Wound-healing experiments were conducted to study trophoblast migration.

Main Outcome Measure(s): Quantification of sCD146 and Gal1 by enzyme-linked immunosorbent assay. Analysis of trophoblast migration by wound closure.

Result(s): Concomitant detection of sCD146 and Gal1 showed lower sCD146 and higher Gal1 concentrations in women with normal pregnancies compared with nonpregnant women. In addition, follow-up of these women revealed a decrease in sCD146 associated with an increase in Gal1 throughout pregnancy. In contrast, in women with preeclampsia, we found significantly higher sCD146 concentrations compared with women with normal pregnancies and no modification of Gal1. We emphasize the opposing effects of

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S.M.B. and W.T. should be considered similar in author order.

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sCD146 and Gal, since, unlike Gal1, sCD146 inhibits trophoblast migration. Moreover, the migratory effect of Gal1 was abrogated with the use of an anti-CD146 blocking antibody or the use of small interfering RNA to silence VEGFR2 expression. This suggests that trophoblast migration is mediated through the interaction of Gal1 with CD146, further activating the VEGFR2 signaling pathway. Significantly, sCD146 blocked the migratory effects of Gal1 on trophoblasts and inhibited its secretion, suggesting that sCD146 acts as a ligand trap.

Conclusion(s): Soluble CD146 could be proposed as a biomarker in preeclampsia and a potential therapeutic target.

Clinical Trial Registration Number: NCT 01736826. (Fertil Steril Sci® 2022;3:84–94. ©2021 by American Society for Reproductive Medicine.)

Key Words: CD146/sCD146, galectin-1, preeclampsia, trophoblast migration

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Preeclampsia, a placenta-mediated pregnancy complication affecting 4% to 5% of pregnancies worldwide (1), is associated with high maternal and fetal morbidity and mortality (2). Preeclampsia is defined as the presence of new-onset hypertension and proteinuria or other end-organ damage occurring after 20 weeks of gestation (3, 4). A major cause of preeclampsia is believed to be insufficient trophoblast migration, resulting in poor spiral arterial remodeling and, consequently, inadequate placental perfusion (5). During normal placentation, placental extravillous trophoblasts differentiate from an epithelial to an endothelial phenotype, also called “pseudo-vasculogenesis,” and invade the spiral arteries to accommodate the blood flow required for the developing fetus (6). In preeclampsia, this process, which is tightly regulated by a complex network of soluble factors and signaling pathways, is incomplete (5).

The Fetal Medicine Foundation algorithm is used during the first trimester of pregnancy to calculate the risk of preeclampsia, using the placental growth factor (PlGF) level, among other parameters (7). The ratio between soluble receptor for vascular endothelial growth factor (sFlt1), an antiangiogenic factor, and PlGF also has a negative predictive value for the short-term prediction of preeclampsia (8). sFlt1 and PlGF are mostly derived from the maternal vascular system rather than the placenta (9).

Among the newly described placenta-regulating factors, we propose soluble CD146 (sCD146) as a molecule that delays trophoblast migration and, consequently, placental vascular development. However, its involvement in preeclampsia remains unknown.

CD146, an adhesion molecule of the immunoglobulin superfamily, is expressed in several cell types, including endothelial cells, TH17 lymphocytes, and extravillous trophoblast cells (10, 11). CD146 and sCD146, as generated by membrane proteolysis through metalloproteases (12), are mainly involved in regulating inflammation and angiogenesis. It has recently been reported that CD146 potentiates the angiogenic function of VEGFR2 (13), which is expressed in the placenta at term (14). During pregnancy, CD146 is expressed by extravillous trophoblasts and also by the endometrium, the cumulus oocyte complex, and the preimplantation embryo (11, 15). In preeclampsia, reduced or absent expression of CD146 has been reported at the extravillous trophoblast membrane, in association with a decrease in its invasive capacity (16). The role of sCD146

in extravillous trophoblast migration and placental development has been proposed based on the following data (17): in vitro, sCD146 inhibits extravillous trophoblast migration and proliferation and pseudocapillary formation; in ex vivo placental explants, sCD146 decreases the invasive potential of extravillous trophoblasts; in a pregnant rat model, repeated injections of sCD146 decreased the pregnancy rate and the number of embryos per litter; and placental histologic analysis has shown that these effects are accompanied by a decrease in the migration of glycogen cells, which are equivalent to extravillous trophoblasts in women. Moreover, interest in sCD146 as a biomarker has been shown in various diseases associated with vascular defects or recurrent miscarriages. A decrease in the level of serum sCD146 during normal pregnancy has been reported, depending on gestational age (17). In addition, sCD146 concentrations have been described as elevated in 100 women who had at least one unexplained fetal loss compared with 100 women who had at least one viable child (18).

Recently, we showed that sCD146/CD146 was detected at very early stages of human embryonic development (day 2) and that embryos secrete sCD146 into the medium in which they are cultured, suggesting that sCD146 could be involved in embryo implantation (15). Furthermore, CD146 is a highly glycosylated protein, suggesting that this protein could interact with sugar-binding proteins, such as lectins; a previous study by our team showed that CD146 specifically binds to galectin-1 (Gal1) (19).

Gal1 is one of the CD146 ligands described on endothelial cells (18). Gal1, a member of the carbohydrate-binding protein family, is involved in the protein interactions that modulate cell growth, differentiation, and apoptosis (20). Similar to CD146, Gal1 is expressed in extravillous trophoblast cells (21) and in the placenta at term (22). It is also detected at very early stages of human embryonic development (23), and embryos secrete Gal1 into the medium in which they are cultured (24). In mice, blocking Gal1-mediated angiogenesis results in a spontaneous preeclampsia-like syndrome, mainly by dysregulating placentation (25). In addition, the role of Gal1 in trophoblast invasion has been proposed (21, 26), but without demonstrating its regulatory function on extravillous trophoblast cell migration.

All these data led us to hypothesize that the regulatory role of sCD146 in trophoblast migration involved its interaction with Gal1 and that it may be defective in preeclampsia.

Therefore, sCD146 may be of great interest in preeclampsia. With this in mind, we performed a pilot study to quantify sCD146 and Gal1 in women with placenta-mediated pregnancy complications (preeclampsia and/or intrauterine growth retardation [IUGR]) in comparison with women with normal pregnancies. In parallel, we analyzed the combined action of sCD146/CD146 and Gal1 in regulating trophoblast migration and investigated the underlying mechanisms of action.

MATERIALS AND METHODS

Patients

Serum from healthy, nonpregnant women and pregnant women were obtained from a prospective cohort study performed in the Department of Gynecology and the Hematology Outpatients Department of Nîmes University Hospital (France) from June 2015 to December 2018 ("Grosspath" study; clinicaltrials.gov identifier: NCT 01736826). The estimation of sample size was based on the primary objective, which was to compare plasma nucleosomes and cell-free DNA levels between women with normal pregnancies and women with a placenta-mediated pregnancy complication (27). Recruitment of participants has been previously described (27). Briefly, we recruited 30 healthy, nonpregnant volunteers (group HV), 50 women with a normal pregnancy (group NP), and 35 pregnant women with a placenta-mediated pregnancy complication (preeclampsia and/or IUGR) (group PMPC).

The 30 healthy, nonpregnant women were recruited after a general appeal to all hospital staff; 15 of them were also tested every 4 weeks for 2 months (group HVx). The ages of the controls were matched with the ages of the pregnant women. The exclusion criteria were pregnancy and postpartum period (up to 3 months after childbirth) and any history of thrombotic events, chronic disease, chronic or recent infection, or cancer.

The 50 pregnant women who finally experienced a normal pregnancy were recruited during their initial consultation in the Department of Gynecology during the 12th week of gestation (WG). Among them, 17 were randomly sampled for testing every 4 weeks for 6 months from the 3rd month of pregnancy to delivery (group NPx). The 33 remaining women (group NP minus patients directed to group NPx) gave a single blood sample at the time of delivery. The exclusion criteria were any previous placenta-mediated complication in pregnancy and any history of thrombotic events, chronic disease, chronic or recent infection, or cancer. Any NP or NPx subjects in whom a pregnancy complication subsequently developed during follow-up were then transferred to the abnormal pregnancy group and analyzed in that group.

A total of 35 pregnant women in whom a placenta-mediated pregnancy complication such as early-onset (<34 WG) or late-onset (≥34 WG) preeclampsia and/or early-onset IUGR (<3rd percentile) had developed were included at the onset of the complication. Among these 35 patients, one woman came from group NPx after the development of preeclampsia during the 7th month of pregnancy. Blood samples were obtained in order to compare biologic markers

between groups NP and PMPC just before delivery to avoid biases caused by differences in labor and delivery between the 2 groups. Preeclampsia and IUGR were diagnosed according to international criteria (3, 4, 28). For preeclampsia, if 24-hour urine collection was not available, the protein:creatinine ratio was measured from a random urine sample (29).

The study was approved by the institutional review board and the ethics committee of Nîmes University Hospital and by the local Comité de Protection des Personnes Soumises à la Recherche Biomédicale (CPP Sud Méditerranée III). This clinical investigation was performed in accordance with the Declaration of Helsinki of 1975 as revised in 1996. All participants gave informed consent to participate (clinicaltrials.gov identifier: NCT 01736826).

All pregnancies were singletons. We collected the patients' clinical data: age, smoking habits, blood pressure, and information about current and previous pregnancies and delivery and perinatal outcomes.

Preparation of Participants' Samples

Nonanticoagulated blood samples (serum separator gel tube) were collected by a clean venipuncture procedure, avoiding the use of a tourniquet. A centrifugation procedure was performed with 2500 g of blood for 15 minutes at 15°C within 1 hour after sampling. The samples were frozen at −80°C until quantification by enzyme-linked immunosorbent assay (ELISA).

Measurement of sCD146 and Gal1

Soluble CD146 and Gal1 were assayed in duplicate by commercially available ELISA kits (CY-QUANT ELISA sCD146, Biocytex, Marseille, France, and R&D systems, Minneapolis, MN, respectively) according to the manufacturer's recommendations and were quantified in nanograms per milliliter. All serum samples obtained from a given patient (all visits from inclusion to delivery) were defrosted and assayed at the same time using the same standard curve.

Cell Culture

HTR8/SVneo (extravillous trophoblasts) were cultured in RPMI 1640 (Gibco In Vitrogen, Cergy-Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (Biotech GmbH, Berlin, Germany) under standard conditions (37°C, 5% CO₂, humidified atmosphere).

Immunocytochemical Staining

We performed immunocytochemical staining of CD146 in HTR8/SVneo. The cells were fixed with formaldehyde for 30 minutes at room temperature, rinsed in phosphate-buffered saline-bovine serum albumin (PBS-BSA) 0.5% (3 washes), and permeabilized with Triton 0.5X. After 3 rinses in PBS-BSA 0.5%, the cells were incubated for 60 minutes at 37°C with the anti-CD146 monoclonal antibody (S-Endo 1, 1 mg/L; Biocytex) at 1:200 dilution or an irrelevant antibody anti-IgG (mouse IgG1, 0.1 mg/L; Biocytex) at 1:20 dilution. After 3 rinses in PBS-BSA 0.5%, the cells were incubated

for 60 minutes at 4°C with a secondary anti-mouse antibody associated with a fluorescent probe, Alexa Fluor 488 (1:500; Life Technologies, Villebon sur Yvette, France), then rinsed 3 times in PBS-BSA 0.5%. Nuclear DNA monoclonal antibody was labeled with 4,6-diamino-2-phenylindole (Fluoroprep; Biomérieux, Craponne, France). The cells were placed on glass slides with cover slips in an aqueous mounting medium with 4,6-diamino-2-phenylindole to preserve their fluorescence. The cells were then examined by fluorescence microscopy using an LSM 510 Zeiss confocal microscope.

Wound-Healing Assay

HTR8/SVneo cells (5×10^4) were seeded in a 96-well plate in complete growth medium. After 24 hours, the cells were starved overnight in serum-free medium. Adherent cells were then pulsed with Gal1 (1 ng/mL and 1 μ g/mL), galectin-2 (Gal2) (1 ng/mL), recombinant human sCD146 (100 ng/mL), or a combination of Gal1 and recombinant human sCD146 (1 ng/mL and 100 ng/mL, respectively) in serum-free medium (RPMI 1640) for 6 hours. Gal2 was used as a control because of its structural similarity to Gal1 and because it had been previously shown that it did not interact with CD146 on endothelial cells (14). Two doses of Gal1 (1 ng/mL and 1 μ g/mL) were tested according to previous data obtained with Gal1 in trophoblast invasion (16, 18). Concerning the concentration of sCD146 (100 ng/mL), we previously showed that CD146 inhibited trophoblast migration at this concentration (12). The cells were scratched with a sterile tip to create an artificial wound and allowed to heal for the next 6 hours. Photographs were taken at 0 and 6 hours with an Olympus inverted microscope. The wound size was measured on photographs from 3 separate experiments using ImageJ analysis software (<http://rsb.info.nih.gov/ij>). Cell wound repair was calculated using the ratio $(t_0 - t_6 \text{ h})/t_0$, in which t_0 represents the wound area measured at the time of wounding and t_6 represents the wound area measured after 6 hours of incubation. The results were expressed as a percentage of the area of the control condition (RPMI 1640), considered as 100%. In some experiments, a blocking antibody (S-Endo 1, 5 μ g/mL, IgG1; Biocytex) or an irrelevant IgG (5 μ g/mL; Biocytex) was added to determine whether the effect of Gal1 depended on the CD146 membrane.

Coimmunoprecipitation Assays

HTR8/SVneo cells were solubilized in lysis buffer as previously described (14). Briefly, the cells were washed in phosphate-buffered saline, submitted to trypsin action, and extracted with 500 μ L of ice-cold radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris[hydroxymethyl]aminomethane [Tris] HCl, pH 7.4, 2.4 mM ethylenediaminetetraacetate, 1% ethoxylated octylphenol (NP40, Thermo Scientific, Waltham, MA) P-40, 0.5 mM phenylmethylsulfonyl fluoride) for 30 minutes at 4°C. The cell lysates were incubated and immunoprecipitated with anti-CD146 (S-Endo 1, IgG1, 5 μ g; Biocytex) or irrelevant IgG1 (5 μ g; Biocytex) followed by incubation with protein G sepharose beads (Invitrogen/Life Technologies, Villebon sur Yvette,

France). The immune complexes underwent Western blot analyses with 4%–12% NuPage sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen/Life Technologies) and were transferred onto nitrocellulose membrane (Invitrogen/Life Technologies) with antibodies to human Gal1 (R&D, Abington, United Kingdom) or human CD146 (S-Endo 1, IgG1; Biocytex) and detected by horseradish peroxidase-coupled goat anti-mouse or rabbit anti-goat, respectively (Thermo Scientific, Waltham, MA).

RNA-Mediated Silencing of VEGFR2 in HTR8/SVneo

HTR8/SVneo cells (8×10^3) were seeded in a 96-well plate in complete growth medium. After 12 hours, transfection of siRNA directed against VEGFR2 (20 μ M; In Vitrogen/Life Technologies) or control small interfering RNA (siRNA) (10 μ M; Santa Cruz Biotechnology, Dallas, TX) was performed by magnetofection (Silence Mag™; OzBiosciences, Marseille, France) according to the manufacturer's instructions. After 72 hours, adherent cells were pulsed with Gal1 (1 ng/mL) in serum-free medium (RPMI 1640) for 6 hours to perform wound-healing assays, as previously described. We verified the efficacy of siRNA by Western blot, as previously described. After blocking with 4% BSA in Tris buffered saline-Tween 20 (TBST), the membranes were incubated with primary antibodies (anti-VEGFR2, 1:500, or anti-actin, 1:3000; Cell Signaling, Saint-Quentin-en-Yvelines, France), diluted in TBST, overnight at 4°C with constant shaking. Horseradish peroxidase-coupled goat anti-rabbit was obtained from Thermo Scientific and diluted at 1:5000. Membranes were scanned and analyzed by G:Box-Chemi-XT4 (Syngene, Cambridge, United Kingdom).

Enzyme-Linked Immunosorbent Assays in Supernatants

HTR8/SVneo cells (5×10^5) were seeded in six-well plates in complete growth medium. After 24 hours, the cells were starved overnight in serum-free medium. Adherent cells were then pulsed with Gal1 (1 ng/mL) or recombinant human sCD146 (100 ng/mL) in serum-free medium (RPMI 1640) for 6 hours. The supernatants were collected.

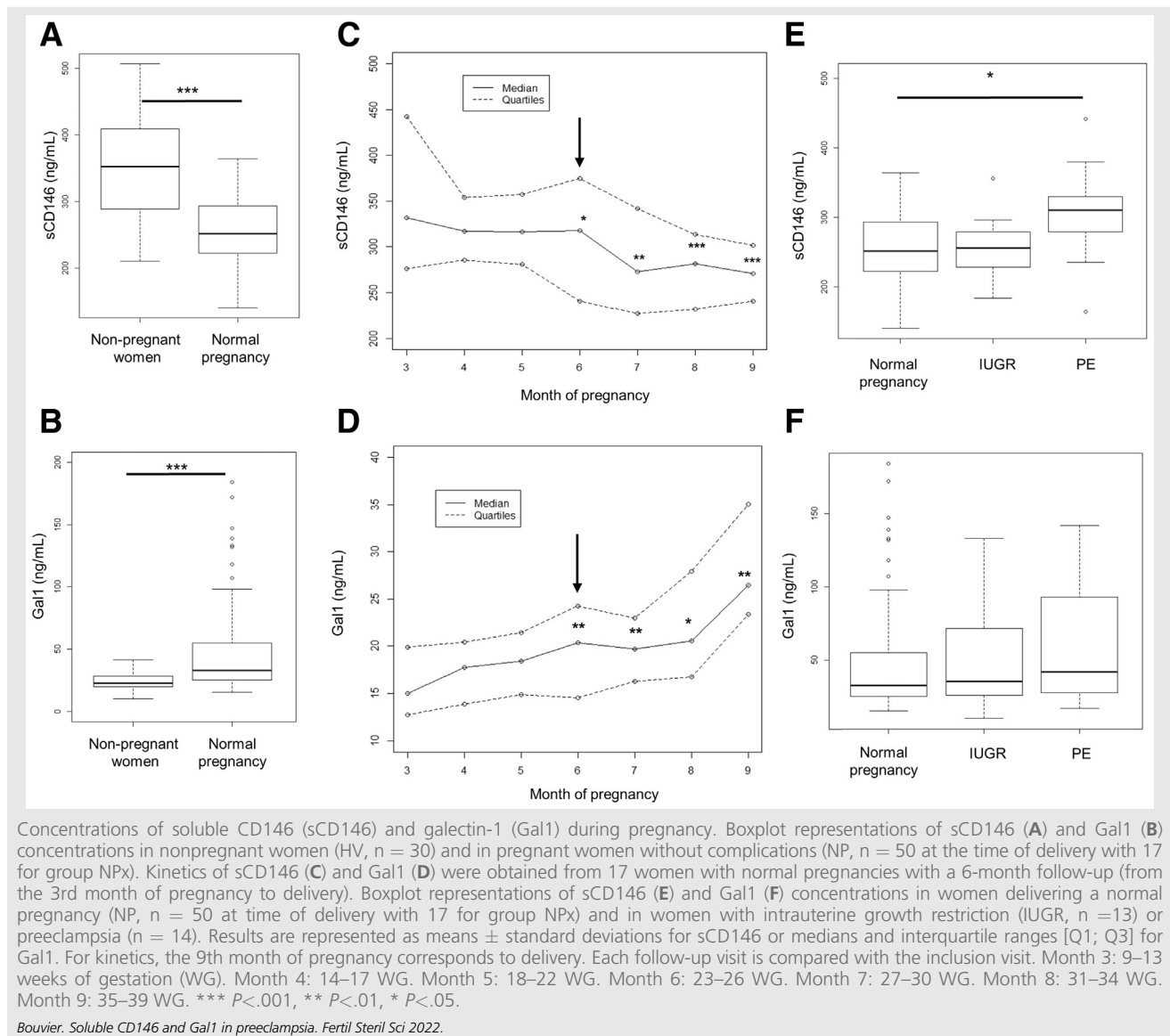
The levels of sCD146 in culture supernatants were examined by CY-QUANT ELISA provided by Biocytex. The sCD146 levels were also quantified after pretreatment with a metalloproteinase inhibitor, GM6001 (25 μ M; Sigma-Aldrich, Saint-Quentin Fallavier, France) for 30 minutes at 37°C to determine whether the effect of Gal1 on sCD146 secretion was metalloproteinase dependent.

Human Gal1 levels were measured with a specific sandwich ELISA protocol, as previously described (18). The results were expressed as percentages of the sCD146 and Gal1 concentrations in the control condition (RPMI 1640), considered as 100%.

Statistical Analysis

For patient data, statistical analysis was performed with R 3.5.1 software (R Development Core Team [2018]; R

FIGURE 1



Foundation for Statistical Computing, Vienna, Austria). Quantitative data were expressed as mean and standard deviation or median and interquartile range, according to their distribution. Qualitative data were expressed as absolute numbers and frequencies (%). To compare groups, we used ANOVA or the Kruskal-Wallis test for three-group comparisons and Student's *t* test, Wilcoxon's test, the chi-squared test, or Fisher's exact test for two-group comparisons, as appropriate. Comparisons between more than 2 visits used mixed models, and comparisons between 2 visits used the paired Student's *t* test or Wilcoxon's signed rank test, as appropriate. A *P*-value $< .05$ was considered to indicate statistical significance.

Data on cell culture were analyzed with GraphPad (San Diego, CA) statistical software (version 5.01), using a nonparametric Wilcoxon test for paired data and the

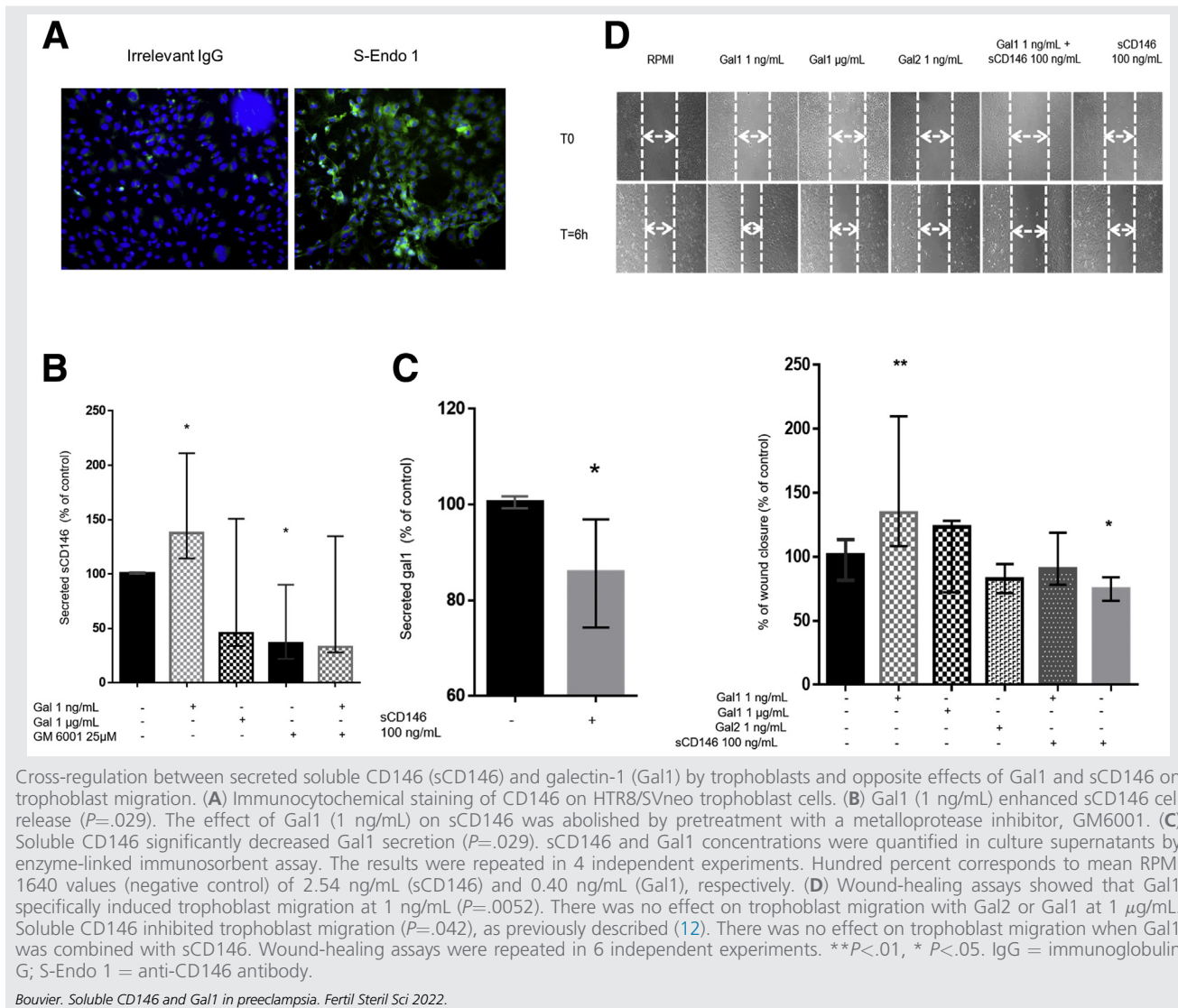
nonparametric Mann-Whitney test for unpaired data. Data were expressed as median \pm interquartile range. A *P*-value $< .05$ was considered to indicate statistical significance. All experiments were conducted in triplicate, and normalization was performed among all values of the controls.

RESULTS

Participants

One hundred fifteen participants were included in the study: 15 healthy, nonpregnant women in group HV; 15 healthy, nonpregnant women in group HVx; 50 pregnant women without complications in group NP, including 17 in group NPx; and 35 pregnant women with complications in group PMPC (Supplemental Table 1, available online). One patient originally included in group NPx was transferred to group

FIGURE 2



PMPC after preeclampsia developed during the 7th month of pregnancy. For this patient, only the blood sample taken just before delivery was analyzed.

In group NPx ($n = 17$), 8 women missed one follow-up visit, including one whose 8-month visit corresponded to the delivery date due to premature delivery. This woman delivered at 34 + 2 weeks of gestation because of preterm premature membrane rupture. Her pregnancy follow-up had been normal, with no signs of preeclampsia or IUGR, so she was analyzed in group NPx. The flowchart is shown in Supplemental Figure 1 (available online).

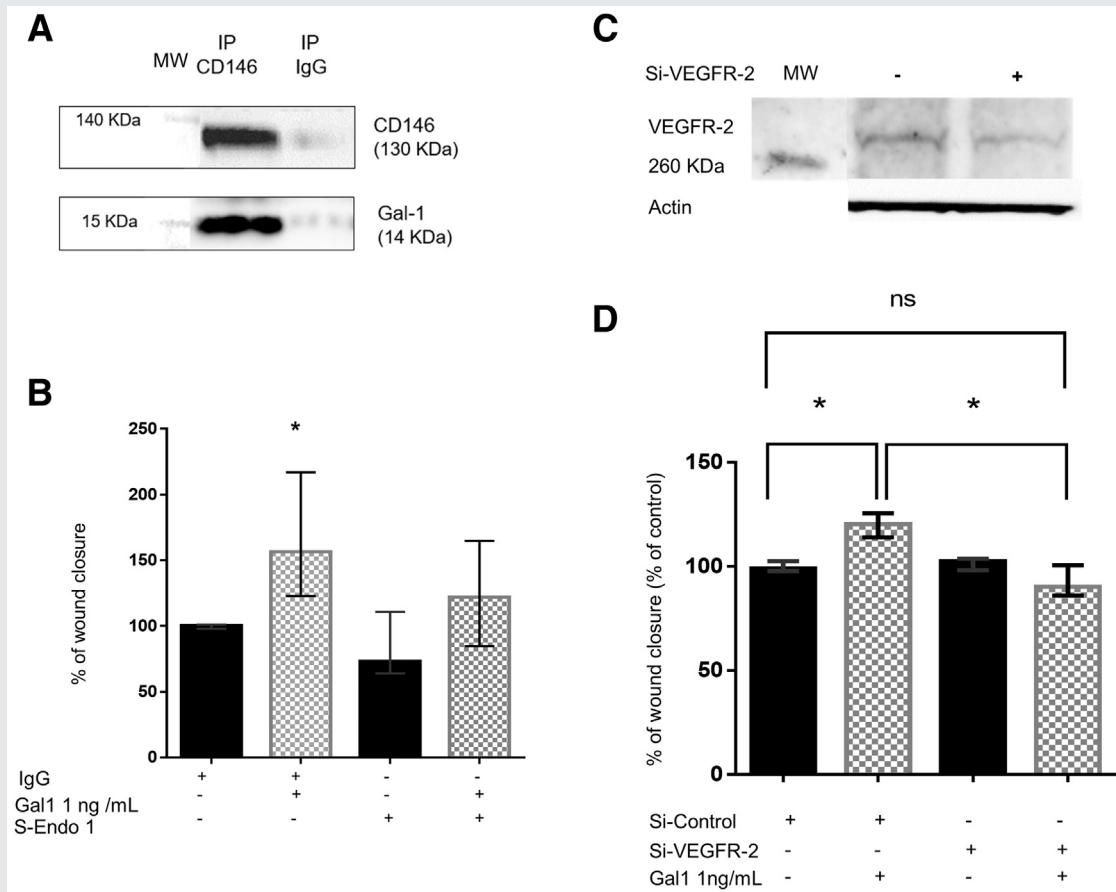
Among placenta-mediated pregnancy complications, preeclampsia alone developed in 14 women (38%), early-onset IUGR (<3rd percentile) in 13 women (35%), and both complications (preeclampsia and IUGR) in 8 women (22%).

Physiologic Variations of sCD146 and Gal1 Concentrations During Normal Pregnancy

We first verified the reproducibility of sCD146 and Gal1 in group HVx by showing that there were no significant differences among the 3 blood samples in detection of sCD146 and Gal1 ($P = .294$ and $P = .298$, respectively), suggesting that sCD146 and Gal1 levels were relatively stable and reproducible in the absence of any clinical event (Supplemental Table 2, available online).

We then compared the sCD146 and Gal1 concentrations between the HV and NP groups. The mean sCD146 concentration was significantly lower in the NP group than in the HV group (252.81 ± 55.4 vs. 350.93 ± 77.34 ng/mL; $P<.001$) (Fig. 1A). On the contrary, the median Gal1 concentration

FIGURE 3



Involvement of membrane CD146 and VEGFR2 receptor in galectin-1 (Gal1)-induced trophoblast migration. (A) Precipitation with anti-CD146 (S-Endo 1, 5 μ g/mL) proved the interaction between Gal1 and CD146 in HTR8 cells. Irrelevant mouse IgG1 was used as a negative control. (B) The effect of Gal1 on trophoblast migration was dependent on membrane CD146; the use of a blocking anti-CD146 antibody (S-Endo 1) abolished the effects of Gal1 at 1 ng/mL on trophoblast migration. An IgG isotypic control was used at the same concentration for control. (C) Western blot analysis of the efficacy of VEGFR2 small interfering RNA (siRNA). (D) The effect of Gal1 on trophoblast migration was completely abrogated by VEGFR2 siRNA on HTR8/SVneo trophoblast cells. Wound-healing assays were repeated in 5 independent experiments. * $P < .05$. IP = immunoprecipitation; MW = molecular weight; ns = not significant.

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was significantly higher in the NP group than in the HV group (32.90 [25.43; 54.93] vs. 22.85 (19.80; 28.45) ng/mL; $P < .001$) (Fig. 1B).

We further analyzed the kinetics of sCD146 and Gal1 concentrations from the 3rd month of pregnancy until delivery during normal pregnancy (Fig. 1C and D; Supplemental Table 3, available online). For sCD146, a significant decrease was observed throughout normal pregnancy ($P < .001$). The comparison of each follow-up visit with the first inclusion visit showed that concentrations of sCD146 were significantly lower from the 6th month of pregnancy (Fig. 1C). A significant increase in Gal1 was observed throughout normal pregnancy ($P < .001$). The concentrations of Gal1 were significantly higher, once again, from the 6th month of pregnancy until delivery (Fig. 1D).

Soluble CD146 Concentration Is Significantly Altered in Preeclampsia

We then compared serum concentrations of sCD146 and Gal1 between the NP and the PMPC groups according to complication subtypes (preeclampsia or IUGR). In the PMPC group, we found higher concentrations of sCD146 in women with preeclampsia than in women with a normal pregnancy (mean value for NP group, 252.81 \pm 55.40 ng/mL; mean value for PMPC group with preeclampsia, 306.90 \pm 66.80 ng/mL; $P = .012$) (Fig. 1E). In contrast, we found no significant difference in sCD146 levels between PMPC women with an IUGR and women with a normal pregnancy (mean value for NP group, 252.81 \pm 55.40 ng/mL; mean value for PMPC group with IUGR, 252.00 \pm 46.20 ng/mL; $P = .96$) (Fig. 1E). We found no significant difference between the groups in Gal1 concentration (Fig. 1F).

The difference in sCD146 concentrations was even more significant in women with late-onset preeclampsia than in those with early-onset preeclampsia compared with women with a normal pregnancy (mean value for NP group, 252.81 ± 55.40 ng/mL; mean value for late-onset preeclampsia, 296.80 ± 56.60 ng/mL; mean value for early-onset preeclampsia, 320.50 ± 79.00 ng/mL; $P = .021$ and $P = .051$, respectively) (Supplemental Fig. 2A, available online). There was a tendency for higher concentrations of Gal1 in women with late-onset preeclampsia compared with women with normal pregnancies (median value for NP group, 32.90 ng/mL [25.43; 54.93]; median value for women with late-onset preeclampsia, 74.95 ng/mL [40.20; 112.50]; $P = .28$), but this difference remained nonsignificant (Supplemental Fig. 2B).

Soluble CD146 and Galectin 1 Shedding Is Inversely Regulated

The concomitant up-regulation and down-regulation of sCD146 and Gal1 observed in normal pregnancies led us to search for a potential inverse regulation of their shedding in trophoblasts. To this end, extravillous trophoblast cell lines were first treated with Gal1, and the sCD146 level was determined by ELISA.

Because it was known that Gal1 is expressed by HTR8/SVneo (21), we first verified that CD146 is also expressed by this cell line (Fig. 2A). After preliminary kinetics experiments (data not shown), we performed quantification 6 hours after stimulation. Gal1 stimulation induced a significant increase in sCD146 secretion after 6 hours of treatment ($P = .029$) compared with the control condition. Interestingly, pretreatment for 30 minutes at 37°C with GM6001, a broad-spectrum metalloproteinase inhibitor, reduced sCD146 release upon Gal1 stimulation (Fig. 2B). This effect was observed at a concentration of 1 ng/mL but not at a concentration of 1 μ g/mL. These findings suggest that Gal1 at low doses is involved in regulating CD146 shedding by trophoblast cells. We next analyzed the effect of sCD146 (100 ng/mL) on Gal1 secretion by HTR8/SVneo cells after 6 hours of treatment. Our results show that sCD146 negatively regulates Gal1 secretion ($P = .029$) (Fig. 2C).

Soluble CD146 and Gal1 Exert Opposite Effects on Trophoblast Cell Migration

Because insufficient trophoblast migration is one of the major causes of preeclampsia, we studied the individual and combined effects of sCD146 and Gal1 on HTR8/SVneo trophoblast cell migration. In agreement with previous findings (17), we showed that sCD146 at 100 ng/mL significantly decreased trophoblast cell migration (Fig. 2D). In contrast, we observed an increase in trophoblast cell migration after stimulation with 1 ng/mL of Gal1 ($P = .005$) but not with 1 μ g/mL of Gal1. Gal2, used as a control for Gal1 specificity effect, had no effect on HTR8/SVneo trophoblast cell migration. We also found that Gal1-mediated cell migration is specifically influenced by sCD146. Indeed, when HTR8/SVneo trophoblast cells were treated with both Gal1 and sCD146, the cells

showed a loss of migration ability compared with Gal1-treated trophoblast cells (Fig. 2D).

Interaction Between Gal1 and CD146 Induces Trophoblast Migration Through the VEGFR2 Receptor Signaling Pathway

In a previous study, we showed that Gal1 is a ligand of CD146 on endothelial cells (19). To confirm that Gal1 may act as a ligand to CD146 on trophoblast cells, we performed coimmunoprecipitation experiments to detect the interaction of CD146 and Gal1 in HTR8 cells. As shown in Figure 3A, Gal1 was immunoprecipitated by anti-CD146 antibody in HTR8 cells, confirming an interaction between these molecules.

To investigate the function of membrane CD146 in Gal1-promoted trophoblast migration, we performed experiments in the presence of anti-CD146 blocking antibody (S-Endo 1 clone, 5 μ g/mL; Biocytex). The results showed that the effect of Gal1 on trophoblast migration was abrogated by CD146-blocking antibody (Fig. 3B), suggesting that CD146 is involved in trophoblast cell migration induced by Gal1.

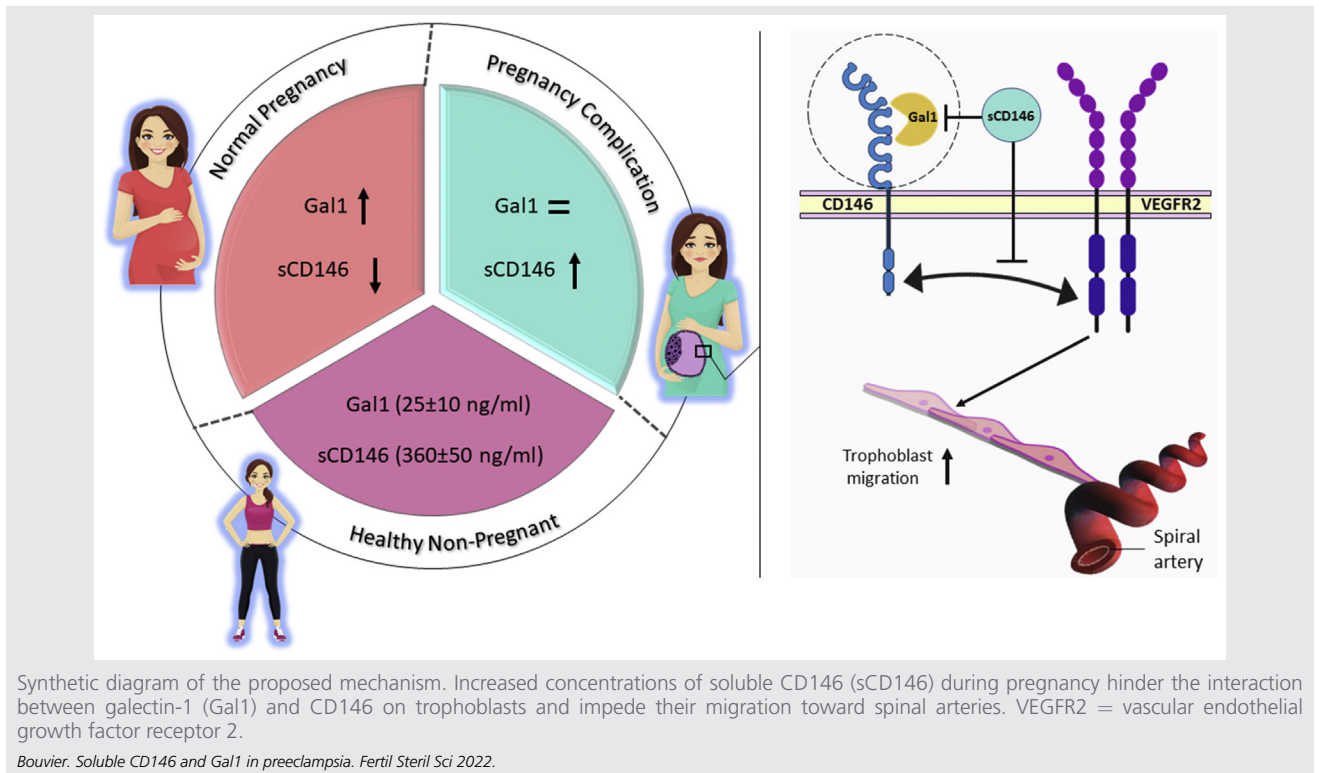
Because CD146 has been described as associated with VEGFR2 (13), we next investigated whether the effect of Gal1 on trophoblast migration could involve this receptor. For this purpose, we used Gal1 and VEGFR2 siRNA to evaluate the effect of Gal1 on the trophoblast migration signaling pathway. The results showed that the effect of Gal1 on trophoblast migration was abolished when VEGFR2 was down-regulated (Fig. 3C and D).

DISCUSSION

We previously identified sCD146 as an inhibitor of trophoblast migration, but the mechanism involved and the pathologic impact in placental complications has never been extensively studied (17). In this study, we showed that sCD146 is up-regulated in women with preeclampsia and that it inhibits trophoblast migration by inactivating the effect of the CD146 ligand, Gal1, which promotes cell migration through VEGFR2 signaling. Our data led us to suppose that sCD146 plays an essential role in trophoblast cell migration and that the dysregulation of sCD146 may be associated with preeclampsia.

Among the diverse factors contributing to the development of preeclampsia, aberrant trophoblast migration is considered the underlying major cause of its pathogenesis. Trophoblast migration is a complex, tightly controlled physiologic process at the fetomaternal interface, involving various molecules with balancing regulatory effects. For example, the soluble form of vascular endothelial growth factor receptor 1 (sVEGFR1, also called sFlt1) suppresses VEGF-mediated and PlGF-mediated signaling, and the soluble form of endoglin (sEng, also called sCD105) disturbs transforming growth factor β (TGF- β)-mediated signaling (30, 31). In our study, we demonstrated an inverse regulation of sCD146 and Gal1 levels in pregnancy. Concomitant detection of sCD146 and Gal1 serum levels showed lower sCD146 concentrations associated with higher Gal1 concentrations in women with normal pregnancies compared with

FIGURE 4



nonpregnant women. In addition, the follow-up of women with normal pregnancies revealed that, unlike Gal1 concentrations, which increased, sCD146 concentrations decreased throughout pregnancy until delivery, with a significant effect on both molecules from the 6th month of pregnancy. The opposing kinetics between sCD146 and the ligand of its membrane form, Gal1, led us to propose a balanced regulation between them. Corroborating our hypothesis, we showed that sCD146 reduced the secretion of Gal1 by trophoblast cells, whereas Gal1 induced sCD146 release, probably by stimulating its cleavage by metalloproteinases, as evidenced by the abolition of the Gal1 effect by pretreatment with a metalloproteinase inhibitor, GM6001. Recently Gal1 has been shown to up-regulate metalloproteinase expression, which could lead to sCD146 release (32). All these findings led us to propose a negative feedback loop in regulation of the shedding of Gal1 and CD146.

In addition to a balanced reverse regulation of sCD146 and Gal1, our study emphasizes the opposite effects of sCD146 and Gal1 on trophoblast migration. sCD146 inhibited whereas Gal1 enhanced trophoblast migration. No effect of Gal1 at 1 $\mu\text{g}/\text{mL}$ was observed, in agreement with a biphasic effect of Gal1 described before (33), suggesting a fine regulation of the partners (sCD146 and Gal1) that is concentration dependent. Importantly, sCD146 blocked the migratory effects of Gal1 by blocking its secretion, suggesting that sCD146 acts as a ligand trap and antagonizes the effects and signaling mediated by the membrane molecule.

To investigate the mechanism further, we first confirmed the interaction between Gal1 and membrane CD146 on the trophoblasts. We then showed that the enhanced migration of trophoblasts, as induced by Gal1, was abrogated upon the use of a blocking anti-CD146 antibody and upon silencing VEGFR2 expression by siRNA, suggesting that the Gal1 migratory effect is associated with the binding to CD146 that subsequently activates the VEGFR2 signaling pathway. The obtained effects could be related to the fact that CD146 and Gal1 are proangiogenic factors that play a crucial role in pregnancy development, embryo implantation, and the occurrence of placental vascular diseases (16, 25, 34). The use of an anti-CD146 blocking antibody was shown to prevent implantation of the embryo in vitro and in vivo in mice (34). In preeclampsia, the expression of CD146 is greatly reduced or absent, together with a decline in the invasive capacity of trophoblast cells (16). Moreover, VEGFR2 is a functional receptor for the growth factor VEGF, and in many patients with preeclampsia, the VEGF-VEGFR2 interaction is disturbed by excess amounts of soluble VEGFR1 (also named sFlt1), a natural PlGF/VEGF antagonist (35).

One major finding of our study is that, for the first time to our knowledge, we showed a significantly higher concentration of sCD146 in women with preeclampsia than in women with normal pregnancies. This up-regulation could be due to the absence of the physiologic decrease in sCD146 observed during normal pregnancies. Because no parallel change in Gal1 concentration was observed, we

can speculate that sCD146 regulation is unbalanced in women with preeclampsia. No differences were found in sCD146 concentrations in women who only developed IUGR. This discrepancy between the 2 placental pathologies agrees with results showing that placental lesions are different between preeclampsia and IUGR; preeclampsia is mainly associated with maternal lesions, whereas IUGR is associated with fetal findings (36). The difference in sCD146 concentrations was more significant in women with late-onset preeclampsia than early-onset preeclampsia compared with women with normal pregnancies, probably due to the small number of women with early-onset preeclampsia. Data from the literature on Gal1 are controversial, and different tests have been used to quantify Gal1. In 2013, Freitag and colleagues reported that levels of Gal1 in late-onset preeclampsia were higher than in normal pregnant controls and that the levels of Gal1 in early-onset preeclampsia were not different from those in normal pregnancies (25). Recently, Hirashima and colleagues reported that the concentrations of Gal1 in women in whom late-onset and early-onset preeclampsia developed were higher than those in women with normal pregnancies (37). In our study, we did not find any significant difference between women with normal pregnancies and women with preeclampsia (either early or late-onset preeclampsia). However, the data seem to tend toward an increase in cases of preeclampsia and particularly in cases of late-onset preeclampsia. We did not find any difference in Gal1 concentrations in women with IUGR, as described by Hutter and colleagues (38). On the contrary, Jin and collaborators recently found a low expression of Gal1 in the serum and placenta of pregnant women with IUGR (39), highlighting the heterogeneity of data concerning Gal1 in placenta-mediated pregnancy complications. Furthermore, it has been reported that the expression of some galectin molecules could be gender specific, particularly galectin-2 and galectin-13, which are expressed in normal and preeclamptic placentas. However, Gal1 expression does not seem to be gender specific (38).

Our study has certain limitations. It was a pilot monocentric study with a small sample size, mainly of women with preeclampsia, based on our recruitment capacities in the main “Grosspath” study (4). Placenta-mediated complications often present as a clinical emergency with women under stress, making inclusion of these patients complicated. In any case, our sample size was large enough to show significant differences among the 3 groups.

Our study also has several strengths. As shown for other pairs of angiogenic factors (35), our findings demonstrate a balancing regulatory effect between sCD146 and Gal1 in trophoblast migration via VEGFR2 receptor engagement (Fig. 4). A defect in this regulation, as observed in preeclampsia, leads to a change in sCD146 kinetics during pregnancy. Therefore, throughout pregnancy, a constant concentration or even an increased concentration of sCD146 might signal a trophoblastic migration defect and represent a predictive marker of placenta-mediated pregnancy complication. Although prospective studies are now necessary to confirm these data, these results led us to propose the follow-up of

sCD146 concentrations during human pregnancies as an innovative tool in pregnancy management.

CONCLUSION

Soluble CD146 may be an attractive biomarker of placental angiogenesis and also a therapeutic target in placenta-mediated pregnancy complications. These preliminary results now require confirmation in a multicentric prospective study including women at the beginning of pregnancy to evaluate sCD146 as a prognostic biomarker of preeclampsia.

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