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Smoking and sVEGFR-1: Circulating maternal concentrations and placental expression

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ABSTRACT

Smoking reduces the expression of VEGFR-1 in non-pregnant women. In pregnant women it reduces the risk of pre-eclampsia, which in turn is associated with increased placental expression of VEGFR-1 and increased maternal circulating soluble VEGFR-1 (sVEGFR-1). We therefore hypothesized that smoking might affect VEGFR-1 expression in pregnant women. In maternal plasma sVEGFR-1 concentrations during the third trimester in both smoking (median 1088, range 834–1362 ng/L, $n = 20$) and non-smoking (728, 719–1336 ng/L, $n = 19$) women were higher than during the second trimester (smokers 374, 291–683 ng/L, $n = 6$, $p > 0.05$; non-smokers 375, 290–667 ng/L, $n = 22$, $p < 0.001$). No difference was observed between smokers and non-smokers. Secretion of sVEGFR-1 into the culture medium, as well as the pattern and intensity of immunostaining in first trimester placenta were similar in tissue from smoking ($n = 22$) and non-smoking ($n = 20$) women. Thus, contrary to our hypothesis, smoking does not affect circulating maternal sVEGFR-1 concentrations or placental secretion of sVEGFR-1 or expression of VEGFR-1 *in vitro*.

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1. Introduction

Smoking is a world wide problem also among pregnant women. In many developed countries 15–30% of pregnant women smoke (Hammoud et al., 2005). Smoking increases the risk of pregnancy complications, such as spontaneous abortion, foetal growth restriction, preterm premature rupture of membranes, placenta previa, placental abruption, and preterm delivery (Castles et al., 1999; Higgins, 2002; Hammoud et al., 2005). Women who actively smoke also have an increased risk of failing to conceive (Hull et al., 2000). Paradoxically, the risk of pre-eclampsia, a major cause of maternal and foetal morbidity and mortality, is markedly reduced among smokers (Conde-Agudelo et al., 1999; Hammoud et al., 2005). Common risk factors of pre-eclampsia are primiparity, low or high maternal age, diabetes mellitus and essential hypertension. Endothelial dysfunction is thought to be a pathophysiological feature (Roberts, 1998; VanWijk et al., 2000). However, the exact cause and molecular mechanism behind the disease remain a mystery.

The well-known angiogenic vascular endothelial growth factor (VEGF) and its receptors; VEGF receptor-1 (VEGFR-1) and VEGFR-2 are essential for maintenance of pregnancy, as mice deficient in their genes die *in utero* due to maldevelopment of the vasculature (Fong et al., 1995; Shalaby et al., 1995; Ferrara et al., 1996). VEGF promotes neovascularization, reduces blood pressure, and is critical for the formation and maintenance of the glomerular filtration barrier (Neufeld et al., 1999; Ferrara and Gerber, 2001; Eremina et al., 2003).

The placenta secretes VEGF and VEGFR-1 both into the foetal and maternal circulations, but mainly to the maternal side (Brownbill et al., 2007). The soluble form of VEGFR-1 (sVEGFR-1) binds VEGF and is a potent negative regulator of VEGF activity *in vivo* (Kendall and Thomas, 1993). sVEGFR-1 is known to leak from early trophoblasts into maternal circulation as early as 30 days after conception (Molskness et al., 2004). In women with clinically manifest pre-eclampsia increased concentrations of sVEGFR-1 have been detected in the placenta, amniotic fluid, and maternal serum (Tsatsaris et al., 2003; Vuorela et al., 2000a; Koga et al., 2003). Increased concentrations in maternal serum are observed already at 16–20 weeks of pregnancy, about 13 weeks before the onset of disease (Wathen et al., 2006).

Cigarette smoking has been shown to decrease circulating sVEGFR-1 concentrations in non-pregnant women and in men (Belgore et al., 2000; Schmidt-Lucke et al., 2005). *In vitro*, cigarette

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Table 1
Clinical characteristics of women from whom blood samples were collected. Data are given as mean ± S.D. or number.

	Second trimester		Third trimester	
	Non-smokers $n = 19$	Smokers $n = 20$	Non-smokers $n = 22$	Smokers $n = 6$
Maternal age (year)	30 ± 4	26 ± 6	32 ± 4	27 ± 7
Gestational age at sample collection (week)	19 ± 0.7	19 ± 0.6	38 ± 3	35 ± 5
Nulliparous (n)	8	11	7	2
Amount of cigarettes smoked per day (n)		10 ± 4		11 ± 6
Gestational age at delivery (week)	40 ± 1	41 ± 2	40 ± 1	41 ± 1
Birth weight (g)	3448 ± 415	3577 ± 512	3768 ± 513	3445 ± 329
Placental weight (g)	598 ± 109	689 ± 96	651 ± 150	631 ± 165
Umbilical artery pH	7.26 ± 0.1	7.29 ± 0.3	7.28 ± 0.1	7.29 ± 0.1

smoke exposure, as well as nicotine and its major metabolite, cotinine, up-regulate cellular VEGF and VEGFR-1 gene expression (Wright et al., 2002; Conklin et al., 2002). VEGF immunostaining is increased in pulmonary arteries and bronchial mucosa of smokers (Santos et al., 2003; Calabrese et al., 2006) and VEGF and VEGFR-2 gene expression is increased *in vivo* in the rat trachea (Wright et al., 2002).

As smoking reduces the incidence of pre-eclampsia and affects the expression of VEGFR-1 and, as VEGFR-1 expression increases in pre-eclampsia, we hypothesized that smoking might affect the expression of VEGFR-1 in pregnant women. We therefore studied whether smoking affects circulating sVEGFR-1 concentrations in pregnant women, its expression in the placenta and secretion by placental tissue in culture.

2. Materials and methods

The study was approved by the Ethical Committee of Helsinki University Central Hospital. All women volunteered for the study and gave written informed consent. All were healthy, all had singleton pregnancies, and attended at the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital. From all women the amount of smoked cigarettes was asked at enrolment.

2.1. Plasma samples

Plasma samples were collected from the antecubital vein from a total of 67 women: 39 women ($n = 19$ non-smokers, $n = 20$ smokers) during the second trimester and 28 women ($n = 22$ non-smokers, $n = 6$ smokers) during the third trimester. Gestational age was determined according to ultrasound performed for routine screening of nuchal translucency at gestational weeks 11–13. Women who gave second trimester samples were recruited at the routine visit for ultrasound screening of foetal structures at gestational weeks 19–20. Women who gave third trimester samples were recruited at their antenatal visit offered for counselling of women with fear of vaginal delivery. The enrolment period was 6 months. Clinical data are given in Table 1.

2.2. Placental samples

Placental samples were collected by curettage from 42 healthy, normotensive women ($n = 20$ non-smokers, $n = 22$ smokers) during legal termination of pregnancy performed for psychosocial reasons during the first trimester. Gestational age was determined by transvaginal ultrasound, by which a normal intrauterine pregnancy was also verified. In some cases the amount of tissue collected allowed only either culturing ($n = 11$ non-smokers, $n = 19$ smokers) or analysis by immunohistochemistry ($n = 16$ non-smokers, $n = 21$ smokers). The numbers of successful samples as well as clinical data are given in Table 2.

Table 2
Clinical data on women from whom placental samples were collected. Data are given as mean ± S.D. or number.

	Smokers $n = 22$	Non-smokers $n = 20$
Age (years)	29 ± 8	28 ± 6
Gestational age at sample collection (week)	9 ± 1	9 ± 1
Amount of cigarettes smoked per day (n)	12 ± 5	
Successful samples obtained (n)		
Immunohistochemistry	21	16
Culture	19	11

Following curettage, samples were immediately immersed in sterile PBS (PBS; 0.14 mol/l NaCl, 2.7 mol/l KCl, 0.01 mol/l Na_2HPO_4, 1.76 mol/l KH_2PO_4, pH 7.4) for a maximum of 2 h until either cultured or dehydrated for paraffinization. All samples were coded and processed blinded to clinical data.

2.3. Analysis of sVEGFR-1

Both plasma and cell culture medium samples were stored at −80 °C until analysis of VEGFR-1 concentrations by a commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA). Intra- and inter-assay coefficients of variation were <5.0% and <8.2%, respectively. The detection limit of the assay was 5 ng/L. To reduce the impact of inter-assay variation samples from both smoking and non-smoking women were analyzed in the same batch. Samples of cell culture medium samples were diluted 1:10 to fall within the measurable range of the assay.

2.4. Tissue culture

Samples were washed twice in PBS and once in culture medium (GIBCO™ Amniomax™ C100 Supplement liquid). Small pieces of villi (2–5 mm^3) were prepared by cutting and dissected villous tissues were placed into 24-well culturing plates in a volume of 0.5 ml (in triplicate wells). Tissue samples were cultured at 37 °C, at 5% CO_2 in a humidified incubator for 24 h. After culturing, media were collected for ELISA analysis and the wet weight of the tissue samples recorded.

2.5. Immunohistochemistry

Serial 5 μm sections of formalin-fixed, paraffin-embedded placental tissue were dewaxed, rehydrated, placed in 10 mM citrate buffer (pH 6) and treated in a microwave oven at 550 W for 5 min four times to retrieve antigens. After cooling at room temperature for 20 min, the sections were washed in phosphate buffered saline (PBS; pH 7.4) twice. To remove endogenous peroxidase activity, sections were kept in hydrogen peroxide (Perhydrol, Merck, Darmstadt, Germany) in methanol (3.2 ml H_2O_2 and 200 ml MeOH) for 30 min at room temperature and were washed in PBS twice. The sections were blocked with Cashblock (Zymed Laboratories Inc., San Francisco, CA, USA; 100 μl per section) for 20 min at room temperature and reacted with polyclonal rabbit anti-VEGFR-1 (0.5 μg/ml; R&D Systems, Inc., Minneapolis, MN, USA) or with goat IgG (negative controls; 0.5 μg/ml, DAKO A/S, Glostrup, Denmark) in Chem Mate (Glostrup, Denmark) 100 μl per section). Primary antibodies were incubated at +4 °C overnight, followed by 30 min incubation with biotinylated anti-goat secondary antibody (Vector Laboratories, Burlingame, CA, USA; 25 μl antibody/5 ml PBS + 75 μl normal rabbit serum; 100 μl/section). Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories) according to manufacturer's instructions. The sections were counterstained with Mayer's hemaluminum solution (Merck, Darmstadt, Germany) and mounted with Aquamount Improved mounting medium (BDH laboratory supplies, Poole, England). Between each step, excluding those between Cashblock and primary antibody the sections were washed twice for 5 min each in PBS, and after immunohistochemistry and Mayer's hematoxylin solution the sections were washed with sterile water.

The intensity of immunoreactivity was semi-quantitatively evaluated as follows. Positively stained cells were classified into the following categories: – (no staining), 1+ (weak but detectable), 2+ (moderate or distinct) and 3+ (intense). For each category, over 25% of stained cells should stain with the same intensity. The sections were analyzed by two investigators (T.S. and R.B.) blinded to clinical data.

2.6. Statistical analysis

Normally distributed variables were compared between groups using Student's *t*-test, while non-normally distributed variables were analyzed by the non-parametric Mann–Whitney *U*-test. Values are expressed as mean ± standard deviation (S.D.) for patient data and median and inter-quartile range (IQR) for experimental results. *P*-values less than 0.05 were considered statistically significant. Statistical calculations were performed using the Statistical Package for Social Sciences (SPSS 13.0 for Windows, Chicago, IL, USA).

Table 3
Concentrations of sVEGFR-1 (ng/L) in maternal plasma. Data are given as median (IQR).

	Non-smokers	Smokers
Second trimester	375 (290–667)	374 (291–683)
Third trimester	1088 (834–1362)[a]	728 (719–1336)[b]

[a] $P < 0.001$ compared to second trimester.
[b] $P < 0.05$ compared to second trimester.

3. Results

Smoking and non-smoking women from whom plasma samples were collected were comparable with respect to parity, gestational age at sample collection and delivery, infant birth weight and umbilical arterial pH. Among women who gave second trimester plasma samples those who smoked, were slightly younger and had higher placental weights than those who did not smoke (Table 1). Smoking and non-smoking women from whom placental samples were collected were comparable with respect to age, duration of pregnancy at curettage and sample collection (Table 2).

Soluble VEGFR-1 concentrations were measurable in all maternal plasma samples and they were higher in the third than in the second trimester in both smoking and non-smoking women. Median concentrations did not differ between smoking and non-smoking women either during the second or third trimesters (Table 3).

Soluble VEGFR-1 was measurable in all culture media samples. The amount of sVEGFR-1 secreted was calculated from the measured concentration and the volume of medium. The ratio of total sVEGFR-1 to the weight of the cultured tissue was calculated. This ratio, total sVEGFR-1/tissue weight, was similar in smoking and non-smoking women (Fig. 1).

Immunohistochemical staining located sVEGFR-1 expression mainly into the villous cytotrophoblasts. The staining of cytotrophoblasts was negative in only one case in each group. In the other cases the staining intensity ranged from 1+ (weak but detectable) to 3+ (intense). The variation in staining intensities of cytotrophoblasts was similar in the two groups (Fig. 2A–D).

In the villous syncytiotrophoblasts weak VEGFR-1 immunostaining was seen in two samples of non-smoking women and in three samples of smoking women. The syncytium was negative in all other samples. Villous vascular endothelium as well as villous stroma was negative for VEGFR-1 (Fig. 2).

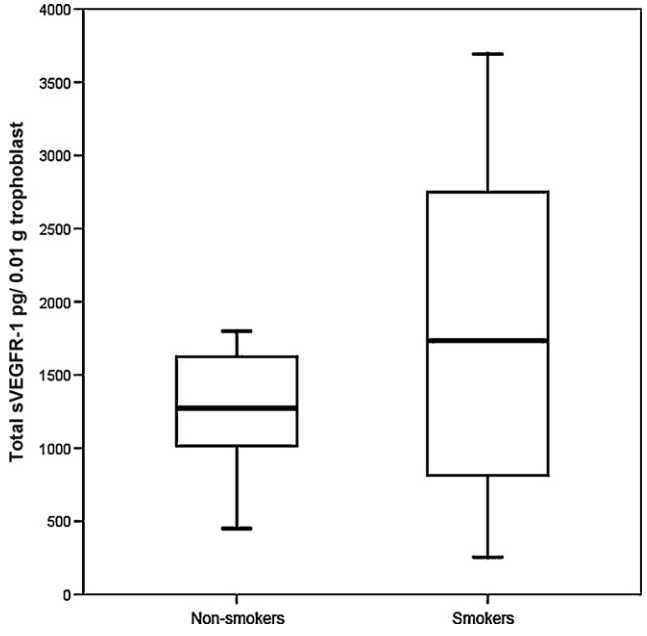


Fig. 1. Soluble VEGFR-1 (sVEGFR-1) in culture media of first trimester placental villi from non-smoking and smoking women. The ratio of total sVEGFR-1 in culture media to the wet weight of cultured tissue is shown. There was no statistically significant difference between the two groups. Box plot demonstrates 10th, 25th, 50th, 75th, and 90th percentiles.

4. Discussion

The present study shows that circulating maternal VEGFR-1 concentrations are not affected by maternal smoking and thus the protective effect of smoking on pre-eclampsia does not seem to be mediated by VEGFR-1. These results differ from those of Powers et al., who demonstrated that smokers have lower serum sVEGFR-1 concentrations during the second and third trimesters than non-smokers (Powers et al., 2005). The reason for different results might be due to different smoking habits of the study populations. Another possibility is the small number of smokers in the present study, as smoking during pregnancy has become rare obviously due to the intensive anti-smoking information given in Finnish maternal care, which almost all women attend regularly.

We also studied the effect of VEGFR-1 expression at tissue level as smoking, and particularly nicotine and its stable metabolite cotinine, have direct toxic effects on the placenta, which they readily cross (Pastrakuljic et al., 1998). Maternal smoking affects placental development also by decreasing cytotrophoblast proliferation and differentiation (Genbacev et al., 1995). Furthermore, nicotine and hypoxia *in vitro* and maternal smoking *in vivo* impair cytotrophoblast transit through the cell cycle (Genbacev et al., 2000). As shown before, placental tissue secretes sVEGFR-1 (Vuorela-Vepsäläinen et al., 1999; Gu et al., 2007; Mehendale et al., 2007). Secretion of sVEGFR-1 by first trimester placental tissue collected from smoking women tended to be higher than that from non-smoking women, but the difference was not statistically significant. Recently, Mehendale et al. showed that under hypoxic conditions cigarette smoke extract reduces the secretion of sVEGFR-1 from term placental villous explants collected from healthy non-smoking women (Mehendale et al., 2007). Taken together, based on these results we cannot exclude an effect of smoking on placental sVEGFR-1.

By immunohistochemistry we observed VEGFR-1 protein expression in first trimester placenta mainly in the cytotrophoblasts and in some samples also in the syncytiotrophoblasts and decidua. However, the immunostaining pattern or staining intensity did not appear to be affected by smoking. The staining pattern is in line with earlier work by us and others showing first trimester expression of placental VEGFR-1 protein in villous cyto- and syncytiotrophoblasts and Hofbauer cells, as well as in decidual stromal, extravillous trophoblast, macrophage and glandular epithelial cells (Clark et al., 1996; Cooper et al., 1996; Shiraishi et al., 1996; Vuorela et al., 2000b). We could not detect immunostaining in villous stromal cells. Stromal cell VEGFR-1 immunopositivity has earlier been demonstrated by some (Clark et al., 1996; Cooper et al., 1996), but not by other groups (Jackson et al., 1994; Ahmed et al., 1995; Shiraishi et al., 1996). Our own previous work, in which different antibodies were used than in the present one, showed either faint or no immunostaining in villous stromal cells (Vuorela et al., 2000b). The vascular endothelium was negative for VEGFR-1 in the present and other studies (Jackson et al., 1994; Ahmed et al., 1995; Clark et al., 1996; Cooper et al., 1996; Shiraishi et al., 1996; Vuorela et al., 2000a).

In conclusion, we did not find support to our hypothesis that smoking would affect maternal circulating sVEGFR-1 or placental

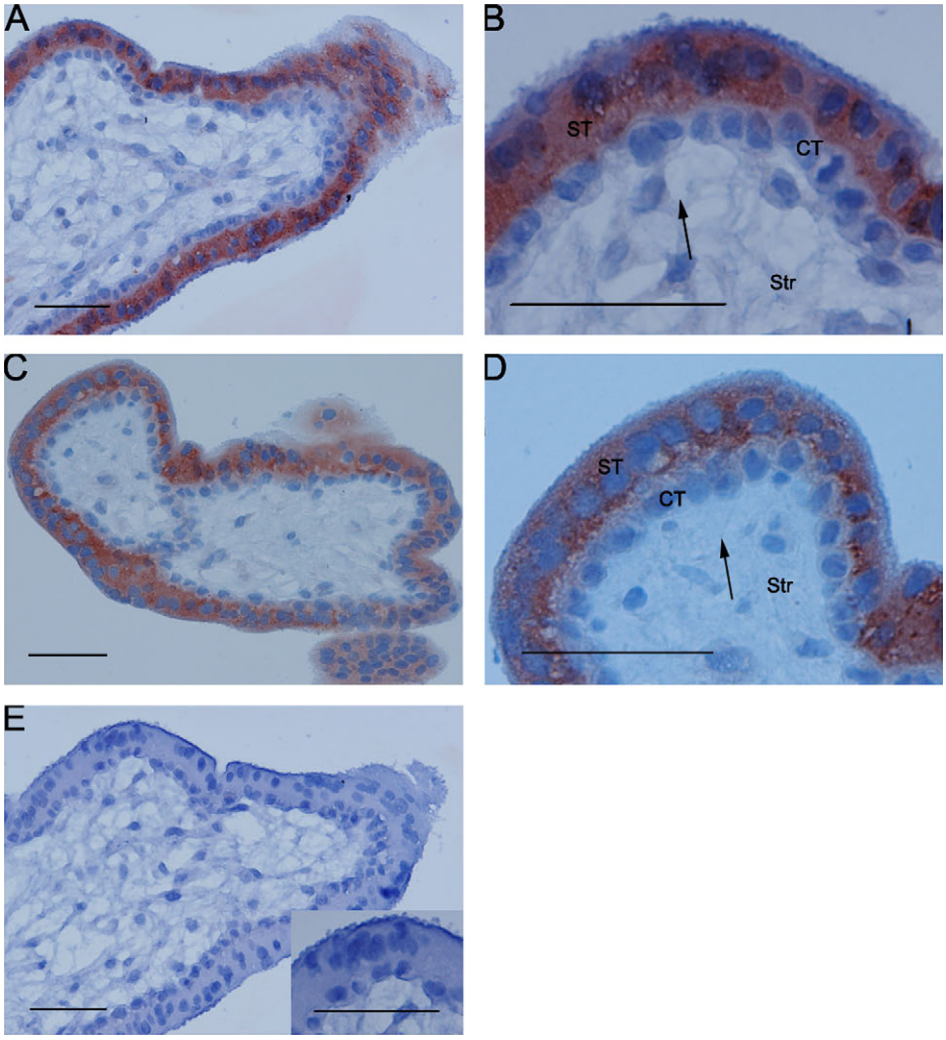


Fig. 2. Immunolocalization of VEGFR-1 in first trimester placental villi of non-smoking (A and B) and smoking (C and D) women. Positive staining was observed in the syncytiotrophoblast (ST) layer with similar intensity in both non-smoking and smoking women. The cytotrophoblasts (CT), as well as villous stroma (Str) and vascular endothelium (arrow) remained negative. The negative control is shown (E). Scale bar 50 μm.

VEGFR-1 expression. It is possible that the peculiar association between smoking and the reduced risk of pre-eclampsia is explained by some other mediators.

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