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## Impaired trophoblast invasion and increased numbers of immune cells at day 18 of pregnancy in the mesometrial triangle of type 1 diabetic rats

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## ABSTRACT

**Introduction:** Type 1 diabetes (T1D) is associated with adverse pregnancy outcome, usually attributed to hyperglycemia. Recently, we showed that pregnancy outcome in normoglycemic T1D rats was characterized by decreased fetal and placental weight, suggesting impaired placental development. In the present study, we tested the hypothesis that trophoblast invasion and spiral artery (SA) remodeling is impaired in T1D rats and that this is associated with aberrant local presence of NK cells and macrophages in the mesometrial triangle (MT).

**Methods:** Placentae with MT from pregnant biobreeding diabetes-prone (BBDP; T1D model) rats, control biobreeding diabetes-resistant (BBDR) and Wistar-rats were dissected at day 18 of gestation and stained for trophoblast invasion, SA remodeling, uNK cells and macrophages.

**Results:** Interstitial trophoblast invasion and SA remodeling was impaired in BBDP-rats vs. control rats, coinciding with increased presence of NK cells and an increased iNOS<sup>+</sup>/CD206<sup>+</sup> ratio of macrophages.

**Discussion:** Decreased fetal and placental weight in BBDP-rats was associated with diminished interstitial trophoblast invasion and less optimal SA remodeling, increased numbers of NK cells and increased iNOS<sup>+</sup>/CD206<sup>+</sup> macrophage ratio in the MT of BBDP-rats.

**Conclusions:** The impaired trophoblast invasion and SA remodeling may be due to an aberrant local immune-response and may result in damage to the fetal placenta and insufficient supply of nutrients towards the fetus with eventually decreased fetal weight as a consequence.

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### 1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease and associated with an increased incidence of pregnancy complications, like pre-eclampsia, preterm birth, macrosomia and perinatal death [1,2]. The mechanisms behind the increased incidence of these complications is not fully understood, but hyperglycemic events play a role [3,4]. Recent work from our lab showed that also in an animal-model for T1D, biobreeding diabetes-prone (BBDP) rats, more complications occurred during pregnancy, even during normoglycemia [5]. This suggests that other mechanisms may be involved.

We observed decreased litter size, increased fetal resorptions and reduced fetal and placental weight in BBDP-rats compared to healthy control rats [5]. The reduced fetal and placental weight may suggest that the development of the placenta is impaired in pregnant rats with T1D.

Similar to humans, rats have a haemochorial placenta, with deep trophoblast invasion into the uterine wall [6]. Invasion of trophoblast cells follow an endovascular and interstitial route and both ways may be involved in the extensive vascular remodeling of spiral arteries (SA) in the mesometrial triangle (MT). However, it is thought that the endovascular cells play a more prominent role [7,8]. The SA remodeling starts with invasion of endovascular trophoblasts into the vascular wall, leading to breakdown of vascular endothelium and smooth muscle cells (VSMCs), followed by the development of a fibrinoid layer and re-endothelialization [7,8]. Besides fetal trophoblast cells, uterine Natural Killer (uNK) cells and macrophages are involved in the SA remodeling [6,9]. uNK-cells

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start to 'cuff' the SA around day 11 of gestation and are involved in initiating trophoblast invasion and SA remodeling [10,11]. The exact role of macrophages in SA remodeling is unclear, but it is thought that they play a role in creating a tolerant immune environment [12]. As SA remodeling is important for placental blood flow and function and, therefore, for fetal growth and development, we hypothesized that the SA remodeling is impaired in rats with T1D as compared to healthy rats, and associated with differences in immune cell populations in the MT.

To test our hypothesis, we used the biobreeding diabetes-prone (BBDP) rat. Pregnant congenic non-diabetic biobreeding diabetes-resistant (BBDR) and Wistar-rats (as the parent strain of BBDP- and BBDR-rats) served as controls. To test our hypothesis, we sacrificed rats at day 18 of pregnancy, which is the day of maximal trophoblast invasion [7,8]. If there is an effect of T1D on trophoblast invasion and SA remodeling, we believe that this would be most obvious at this day. Trophoblast invasion, SA remodeling and the presence of uNK-cells and macrophages in the MT were quantified using immunohistochemistry and 2D techniques for analysis.

## 2. Methods

### 2.1. Experimental design

For the present experiments, placentae were used from the pregnant rats used in our previous paper in which we studied the peripheral immune-response during pregnancy [5]. We included pregnant rats, which were sacrificed at day 18 (BBDP:  $n = 6$ ; W:  $n = 5$ ; BBDR:  $n = 6$ ). BBDP-rats were kept normoglycemic by using insulin implants. At sacrifice, placentae and fetuses were dissected and weighed. Of each rat, three placentae with MT were fixed and stained for trophoblast invasion, SA remodeling and presence of uNK-cells and macrophages. From each mother (as far as possible), we fixed 3 individual placentas, 2 in the buffered zinc solution and 1 in PFA. From each placenta we cut many consecutive sections and selected all sections in which a maternal channel was observed. From the selected sections of buffered zinc solutions fixed placentas, we stained 3 sets of consecutive sections for cyto-keratin, alpha smooth muscle actin, ANK-61 and iNOS. From the selected sections from the PFA fixed placentas, we stained 3 sets of consecutive sections for CD68 and CD206. All sections were scanned and viewed using Aperio Scanscope. Similar staining patterns were observed in the section sets. Therefore we randomly selected 1 set, which was analyzed using Aperio as described in detail in the [Supplementary File](#).

### 2.2. Animals

Approval of the institutional Animal Care Ethics Committee, University of Groningen was obtained; all animals received care in compliance with Dutch Law on Experimental Animal Care. Rats (age 3–6 months and weighing 200–250 gr) were kept in temperature- and light-controlled environment (lights on from 6 AM to 6 PM). BBDP-rats were used as model for T1D, for control, we used two groups of female rats. The first controls are congenic BBDR-rats, which do not develop diabetes spontaneously. The others are Wistar-rats which are the parent strain of BBDP- and BBDR-rats. This additional Wistar-control was deemed necessary as BBDR-rats are sensitive to diabetes induction due to viral infection and to depletion of regulatory T cells or Toll-like receptor ligation [13,14]. BBDP- and BBDR-rats were bred and nurtured at the Central Animal Facility of the UMCG [13]. For all rats (in BBDP-rats after establishment of diabetes), vaginal smears were taken daily to assess ovarian cyclicity. Pregnancy was achieved by housing female rats on the night of pro-estrus with a fertile male. The next day, when spermatozoa were detected in the smear, was designated as day 0 of pregnancy. Animal handling was described more extensively in the [Supplementary Files](#).

Rats were sacrificed at day 18 of pregnancy. At the day of sacrifice, rats were anesthetized with isoflurane/O<sub>2</sub>, after which the abdomen was opened. Immediately after opening the abdomen, zinc-buffer solution was sprinkled over both uterine horns. The number of living fetuses and resorptions were counted and the uterus, including placentae and fetuses, was dissected. All fetuses were weighed and checked for major abnormalities. Of each rat, all placentae were dissected and weighed, without the MT, except for three placentae. These three placentae, including MT, were dissected as described previously [15] and immediately fixed in a buffered Zinc solution (2 placentas) [16] or 4% paraformaldehyde (1 placenta) for 24 h after which they were transferred to 70% alcohol at room temperature for at least 24 h. Thereafter, the placentae were dehydrated and embedded in paraffin according to standard procedures.

### 2.3. Immunohistochemical staining

Zinc or PFA sections of 4  $\mu\text{m}$  were cut (Reichert–Jung autocut 2055 microtome) parallel to the mesometrial–fetal axis. Sections containing a maternal channel were

stained with Periodic-Acid-Schiff's reagent (PAS) [17]), and stained for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; as marker for VSMC), cyto-keratin (MN-116), Natural Killer cells (ANK-61), CD68 (monocytes/macrophages), iNOS (identifying type 1 macrophages) and CD206 (identifying type 2 macrophages). For all staining procedures, we used slides omitting the first antibody as our negative control. They were consistently negative. Details of the staining can be found in the [Supplementary File](#). After staining, all slides were digitized by Aperio ScanScope (Aperio, Vista, CA, USA) and shade-corrected according to standard methods. 2D Analyses, as described in the [Supplemental Files](#), were made using Aperio's ImageScope Viewer (version 11.1.2.760, Aperio Technologies, Vista, CA, USA). To analyze depth of invasion of endovascular trophoblast cells, spiral artery remodeling and the presence of uNK cells in the MT, we divided the MT into three concentric zones (see [Supplementary Fig. 1A](#)).

### 2.4. Statistical analysis

Continuous parameters were expressed as median ( $Q_1$ – $Q_3$ ). Mann–Whitney  $U$ -test was used to compare differences in continuous parameters, while Chi-square test was used for analyzing potential differences in categorical variables. To evaluate correlations between trophoblast invasion and placental and fetal weight, Spearman's correlation coefficients were calculated. For all analyses, a  $p$ -value  $< 0.05$  was considered as significant. Analyses were performed using SPSS version 20.0 (SPSS, Inc., Chicago, IL).

## 3. Results

### 3.1. Adverse pregnancy outcome in type 1 BBDP-rats

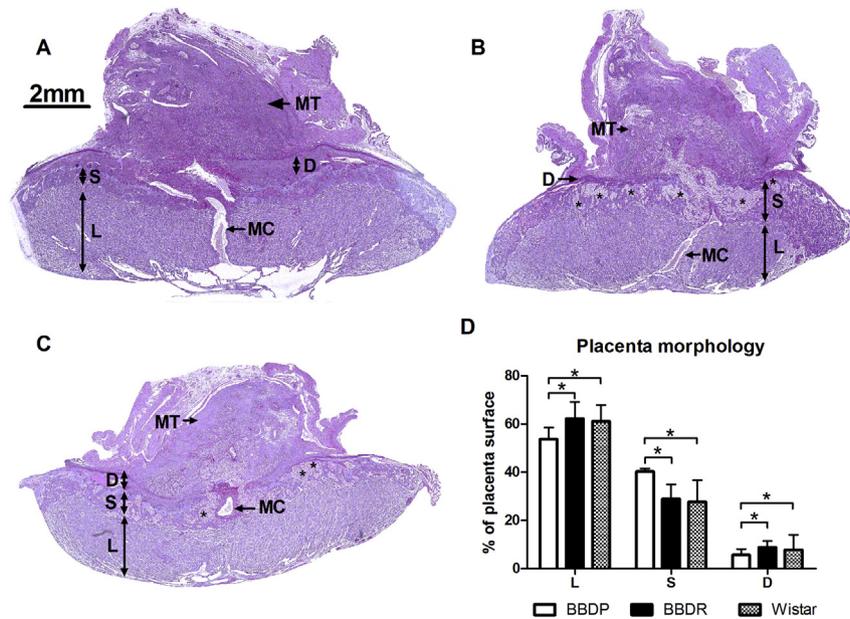
We observed decreased litter size, increased fetal resorptions, and reduced fetal and placental weight in BBDP-rats compared to healthy controls ([Supplementary Table 2](#)). This dataset has been previously published under a Creative Commons Attribution [5].

### 3.2. Placental morphology and size

[Fig. 1](#) shows placentae from a Wistar- (A), a BBDP- (B) and a BBDR-rat (C) at day 18 of pregnancy. The largest part of the placenta in all rats at this day is the labyrinth; the fetal part of the placenta in which exchange of nutrients and gasses takes place. The percentage of surface area of the labyrinth was significantly smaller in BBDP-rats as compared to Wistar- and BBDR-rats ( $p < 0.05$ ). In Wistar-rats, the spongiotrophoblast mainly consists of giant trophoblast cells and a few glycogen islands, while, in BBDP-rats, this layer contains many large glycogen islands, filled with trophoblast cells. Although, in BBDR-rats, such islets are present, they are smaller in size and number vs. BBDP-rats. The percentage surface area of the spongiotrophoblast layer is significantly larger in BBDP-rats as compared to the control groups ( $p < 0.01$ ). In BBDP-rats, the decidua is significantly smaller than in the control groups ( $p < 0.05$ ). Although the ratio placenta/MT surface area was similar between BBDP- and Wistar-rats (2.50 (2.12–2.61) vs. 2.40 (2.15–3.00);  $p = 0.792$ ), this ratio was decreased in BBDP-rats as compared to BBDR-rats (2.50 (2.12–2.61) vs. 2.96 (2.70–3.67);  $p < 0.01$ ).

### 3.3. Impaired trophoblast invasion in T1D rats

[Fig. 2B–C](#) shows representative overviews of placentae from a Wistar- and BBDP-rat stained for the presence of trophoblasts. Trophoblasts invaded the MT by an endovascular and interstitial route. Interstitial trophoblasts migrated through the interstitium of the MT and were mainly located around SA. It can be seen that interstitial trophoblast invasion in the Wistar-rat almost reached the mesometrium and thus covered almost the entire MT. In BBDP-rats, however, interstitial trophoblasts invaded the MT to a lesser extent. This is reflected in the quantitative analysis of trophoblast invasion showing that the trophoblast surface area was decreased in BBDP- vs. Wistar-rats, which was due to both a decreased depth and width of invasion ([Fig. 2D](#)). In all rat strains, the trophoblast



**Fig. 1.** Photomicrographs of representative placentae of the different rat strains following a PAS staining according to standard methods. A: a placenta of a day 18 pregnant Wistar-rat. B: a placenta of a day 18 pregnant BBDP-rat. C: a placenta of a day 18 pregnant BBDR-rat. D: surface area of the labyrinth, spongiotrophoblast and decidua as percentage of the total surface of the placenta measured by 2D techniques. \*Significant difference, Mann–Whitney *U*-test,  $p < 0.05$ . Abbreviations in photomicrographs: L = labyrinth, S = spongiotrophoblast, D = decidua, MC = maternal channel and MT = mesometrial triangle. Asterisks: large vacuole like structures in the spongiotrophoblast in the placenta of the BBDP- and BBDR-rats.

surface area correlated with fetal weight ( $R = 0.691$ ,  $p = 0.002$ ) and placental weight ( $R = 0.571$ ,  $p = 0.041$ ). We also looked at endovascular trophoblast invasion. In all control rats (Wistar-rats), trophoblast invasion (both interstitial and endovascular) progressed into zone 3, the MT was either fully invaded by interstitial and endovascular trophoblast or a very small part of the MT was not invaded. Interestingly, in the rats in which the MT was not fully invaded, endovascular trophoblast invasion was not observed beyond interstitial trophoblast invasion, i.e. we observed no endovascular trophoblast in the outer layer of the mesometrial triangle in which there was no trophoblast invasion. This was different in BBDP rats. Although interstitial trophoblast invasion was less deep and wide, endovascular trophoblast invasion extended beyond interstitial trophoblast invasion and progressed into the third zone even into arteries at the outer part of the MT. Also in BBDR-rats there was endovascular trophoblast invasion beyond interstitial trophoblast invasion, which also resulted in endovascular trophoblast invasion into the third zone. Therefore, we found no differences in migration of endovascular trophoblast cells between the three strains (results not shown).

#### 3.4. Diminished spiral artery remodeling in T1D rats

Fig. 3 shows representative examples of SA. In all animals, spiral arteries were identified by the presence of endovascular trophoblast, presence of smooth muscle actin (which may be intact or remodeled), a cuff of NK cells around the arteries or a combination of these characteristics. Fig. 3 shows representative examples of SA which were A) completely remodeled, almost lacking VSMC, showing endovascular trophoblast and dilated and the vascular wall was (partly) re-endothelialized; B) unremodeled, as can be observed from the presence of VSMC (red staining) throughout the complete vascular wall and the narrow lumen of the artery; C) partly remodeled, in which VSMC has almost disappeared, endovascular trophoblasts were present, and parts of vascular wall were re-endothelialized. Quantitative analysis showed that in the total

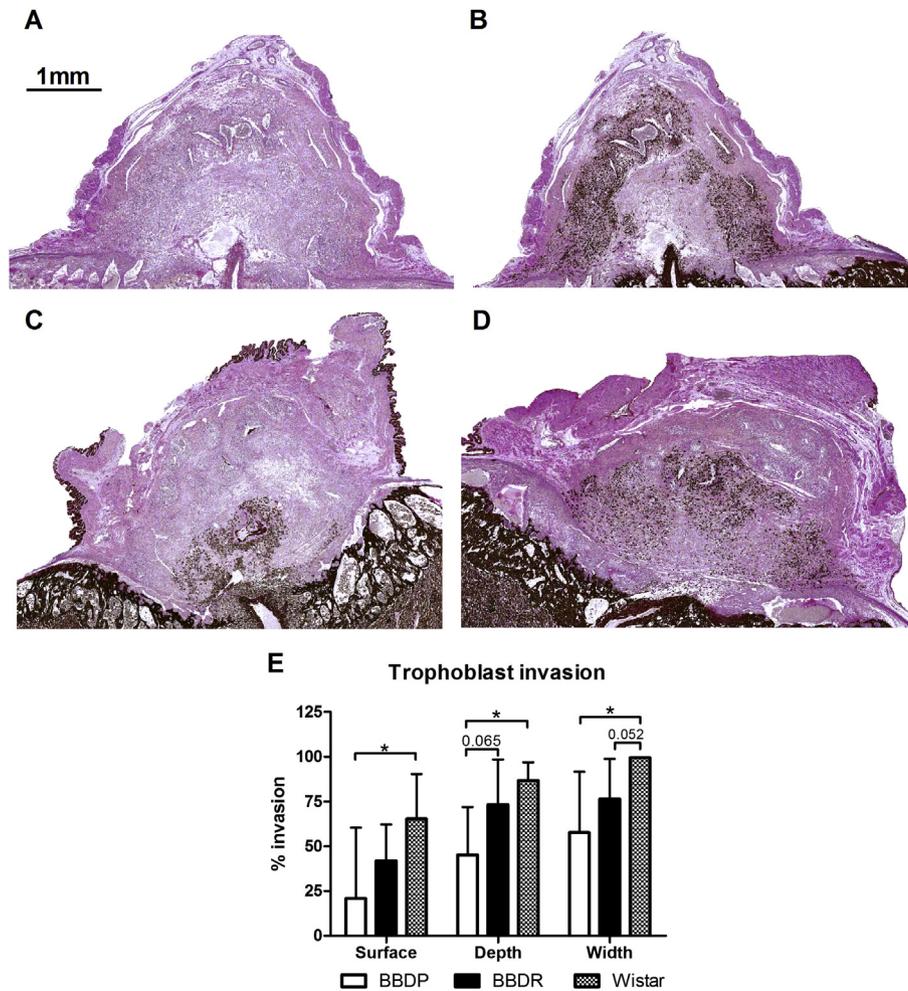
MT, BBDP-rats had less arteries of the fourth category (i.e. highly remodeled with 67–100% disappearance of VSMC; Fig. 3E) as compared to Wistar-rats. Further analysis of the differences in SA remodeling per zone revealed that most differences were observed in zone 3: in this zone, BBDP-rats showed a trend towards an increased number of SA of the first category (i.e. no remodeling, 0–3% VSMC disappearance) and a significantly decreased number of SA of the fourth category (highly remodeled) as compared with Wistar-rats. In zone 2, a decreased number of SA of the second category (i.e. 3–33% VSMC disappearance) was observed in BBDP- vs. Wistar-rats. In zone 1, no differences in SA remodeling were found between the three rat strains (not shown). SA remodeling of BBDR-rats was very similar to SA remodeling in Wistar-rats in all three different zones.

#### 3.5. Increased accumulation of uNK-cells in T1D rats

Fig. 4B–C shows representative overviews of placentae of a Wistar- and BBDP-rat, stained for the presence of uNK-cells. For both rat strains, uNK-cells are present in the MT and are mainly located around the SA, which have not been (completely) remodeled. It can be seen that, in Wistar-rats, uNK-cells are mainly present in zone 3 of the MT, while uNK-cells can be found throughout the whole MT of BBDP-rats. Quantitative analysis showed that this pattern of invasion was significantly different between Wistar- and BBDP-rats (Fig. 4D). We also observed increased numbers (i.e. surface area) of uNK-cells in the MT of BBDP-rats as compared to Wistar-rats (Fig. 4E).

#### 3.6. Increased iNOS/CD206+ macrophage ratio in the MT of T1D rats

CD68+ cells (i.e. total macrophage population) were located throughout the interstitium and around SA. iNOS positive macrophages were mainly located around SA, which are remodeled or being remodeled, while CD206+ macrophages were mainly



**Fig. 2.** Trophoblast invasion parameters in the three rat strains. A: control staining for trophoblast omitting the first antibody (MMF116). B, C and D: Photomicrographs of a representative mesometrial triangle after staining for trophoblast cells of a Wistar rat (B) and BBDP-rat (C) and BBDR-rat (D). E: The parameters of trophoblast invasion into the MT of the 3 rat strains, i.e. median surface, depth and width of trophoblast invasion measured by 2D techniques. \*Significant difference, Mann–Whitney *U*-test,  $p < 0.05$ .

observed around arteries being remodeled or around unremodeled SA in the outer regions of the MT close to the longitudinal myometrial muscle layer (see [Supplemental Fig. 2](#)). Quantitative analysis of the positive area of the reaction product after staining for CD68, CD206 and iNOS showed no differences in the percentage of CD68+ area between the three rat strains ([Fig. 5A](#)). However, BBDP-rats showed a trend towards an increased percentage of iNOS+ macrophages as compared to Wistar-rats, while a trend towards a decreased percentage of CD206+ macrophages was found in BBDP-rats as compared to Wistar-rats ([Fig. 5B–C](#)). This led to a trend towards an increased iNOS/CD206 ratio (percentage area of positive reaction product after iNOS staining/percentage area of positive reaction product after CD206 staining) in BBDP-rats as compared to Wistar-rats (3.82‰ (2.83–39.65) vs. 0.18‰ (0.12–3.38);  $p = 0.082$ ). BBDR-rats were mainly similar to Wistar-rats.

#### 4. Discussion

Using immunohistochemistry and 2D analysis techniques, the present data suggest that decreased fetal and placental weight in T1D pregnant rats was associated with a relatively small fetal part of the placenta, decreased interstitial trophoblast invasion and impaired SA remodeling in the MT. The decreased trophoblast invasion and SA remodeling was accompanied by increased presence of uNK-cells and an increased iNOS+/CD206+ macrophage ratio in

the MT of BBDP-rats. Our data led to the following hypothetical model in BBDP rats: A relatively small fetal part of the placenta may result in insufficient nutrient supply towards the fetus. Impaired SA remodeling may result in decreased dilatation of the SA, which in turn is associated with increased velocity of the maternal blood towards the placenta [18]. This may lead to damage and functional inhibition of the placenta. Together, this may explain the observed decreased fetal and placental weight. Of note, we used 2D techniques to analyze our data. Therefore, future studies should include stereological analysis to confirm the present results. The advantage of stereology above 2D techniques is that it offers the opportunity to measure total volume, surface and length of placental structures or numbers of cells. It thus helps in finding differences in the 3D structure between healthy and diseased placentas.

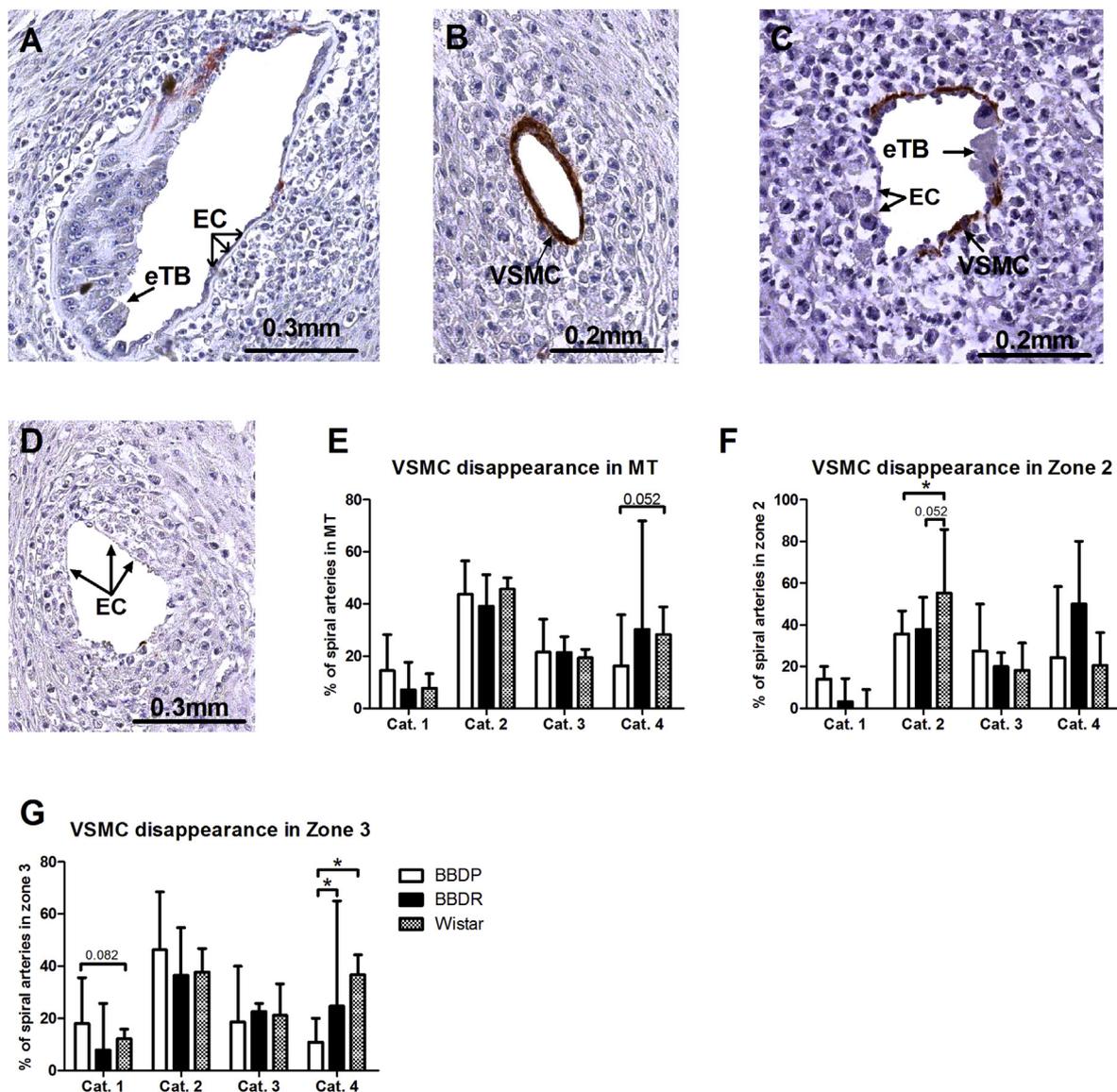
The combination of a decreased surface area of the labyrinth and an increased surface area of the spongiotrophoblast and decidua in BBDP- vs. healthy rats may suggest that the diabetic placenta could be relatively insufficient in facilitating supply of nutrients and gasses to the fetus. The placenta also seems to be relatively immature in BBDP-rats, since in healthy rats the labyrinth size increases during the course of pregnancy, relative to the size of decidua and spongiotrophoblast. The labyrinth in the rat is the equivalent of the villous structure in humans [19]. Villous immaturity was also observed in women with T1D, as characterized by decreased formation of terminal villi and increased presence of

immature and mature intermediate villi and could be equivalent to findings in our study [20,21].

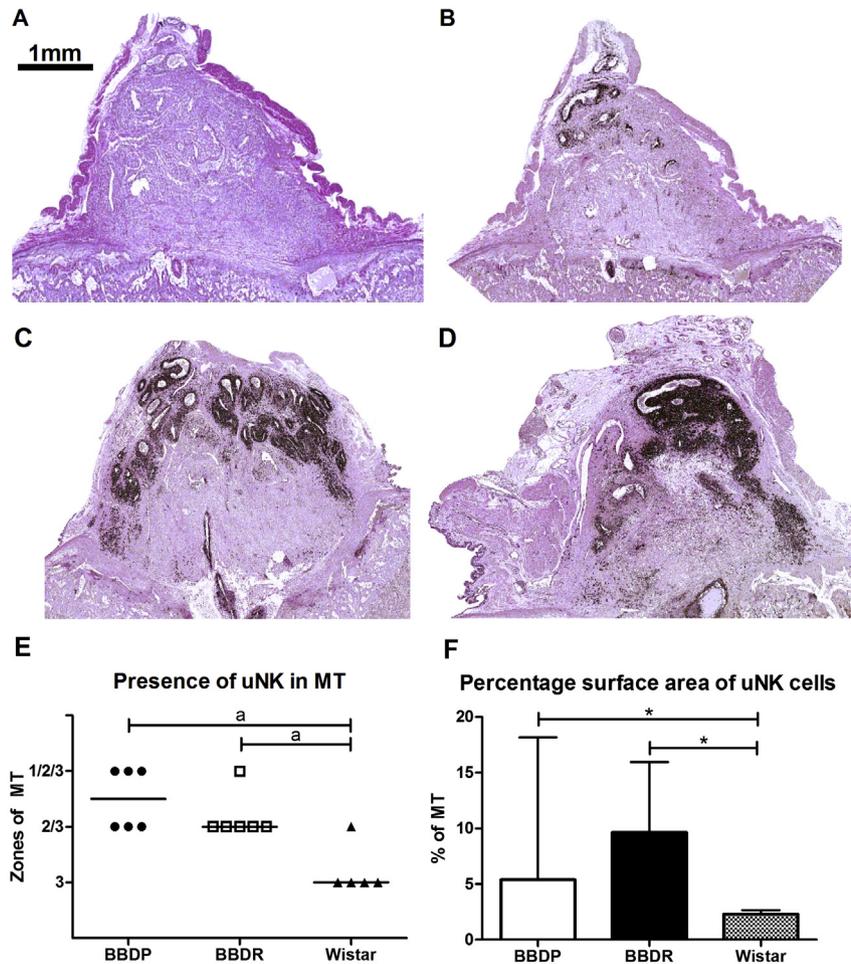
We observed increased numbers and size of glycogen islands in the spongiotrophoblast layers in placentae from BBDR-rats. These glycogen islands contain a large pool of trophoblast cells, which are able to migrate into the MT as interstitial trophoblasts around day 16 of gestation [8]. In healthy pregnant rats, interstitial trophoblast invasion appears to be maximal around day 18–19 of pregnancy and the spongiotrophoblast and glycogen islands start to degenerate [8]. The increased presence of glycogen islands and trophoblast cells in the spongiotrophoblast layer in BBDR-rats are thus in line with decreased interstitial trophoblast invasion into the MT in

BBDR-rats and supports our suggestion of immaturity of placentation in BBDR-rats.

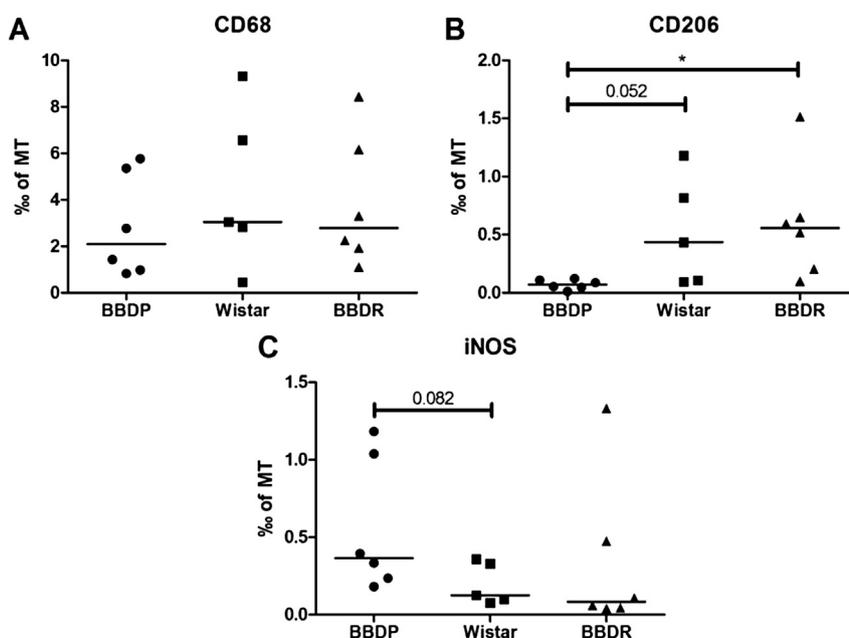
Although endovascular trophoblast invasion in the rat, which precedes interstitial trophoblast invasion, has been associated with SA remodeling [7], the role for interstitial trophoblast invasion in rat placental development is less clear [8]. As our data show that decreased interstitial trophoblast invasion was correlated with lower fetal and placental weight, this route of trophoblast invasion may be important for fetal and placental development in our model. Moreover, our data also suggest that decreased SA remodeling in BBDR-rats is associated with decreased interstitial trophoblast invasion and not with decreased endovascular



**Fig. 3.** Spiral artery remodeling in the three different rat strains. A–C: photomicrographs of examples of degrees of spiral artery remodeling in the third zone of the MT in the different rat strains. The degrees of spiral artery remodeling occur in each rat strain, however, the proportions differ. A: an almost completely remodeled spiral artery from a day 18 placenta of a Wistar rat, which is dilated and lacks vascular smooth muscle cells (VSMC), the vascular wall is covered with endothelial cells (EC's) (category 4). B: an unremodeled spiral artery from a day 18 placenta of a BBDR-rat, VSMC (red staining) is present throughout the vascular wall and the lumen of the artery is narrow (category 1). C: a partly remodeled spiral artery from a day 18 placenta of a BBDR-rat, in which VSMC has almost disappeared, endovascular trophoblasts (eTB) are present, and part of vascular wall is re-endothelialized (category 3). D: an example of a non-invaded SA, which was not invaded by endovascular trophoblast, but almost completely lost its vascular smooth muscle layer. E–G: quantification of the degree of VSMC disappearance in the spiral arteries in the complete mesometrial triangle [E], in zone 2 [F] and in zone 3 [G]. Degree of VSMC disappearance was classified into 4 groups; category (cat.) 1 indicated 0.0–3.0% disappearance, cat. 2 3.1–33.3% disappearance, cat. 3 33.4–66.7% disappearance and cat 4 66.8–100% disappearance. Since the major part of the spiral arteries were located in zone 2 and 3, the results of zone 1 were not shown. 2D techniques were used. \*Significant difference, Mann–Whitney *U*-test,  $p < 0.05$ .



**Fig. 4.** uNK cells in the mesometrial triangle of the three different rat strains. A: control staining for trophoblast omitting the first antibody (ANK-61). B, C and D: representative photomicrographs showing the presence of uNK cells throughout the mesometrial triangle from a pregnant day 18 Wistar-rat [B], a pregnant day 18 BBDR-rat [C], and a pregnant day 19 BBDR-rat [D]. E: quantitative presence of uNK cells in the three different zones of the mesometrial triangle for the three rat strains measured using 2D techniques. F: percentage surface area of uNK cells in the mesometrial triangle of the three rat strains measured using 2D techniques. <sup>a</sup>Significant difference, Chi-square test,  $p < 0.05$ . <sup>\*</sup>Significant difference, Mann–Whitney  $U$ -test,  $p < 0.05$ .



**Fig. 5.** Percentage surface area of positive staining of the mesometrial triangle after staining for CD68 [A], CD206 [B] and iNOS [C] positive macrophages in the mesometrial triangle of the three different rat strains measured using 2D techniques. <sup>\*</sup>Significant difference, Mann–Whitney  $U$ -test,  $p < 0.05$ .

trophoblast invasion. Therefore, our findings indicate a role for interstitial trophoblast invasion in spiral artery remodeling in the present model. Interestingly, in contrast to findings of Caluwaerts et al. [7], in our study, in both control and BBBDP rats, we observed non-invaded arteries, which are outside the area of interstitial trophoblast invasion, and thus can be found in zone 3, and which were not invaded by endovascular trophoblast. However, they almost completely lost their vascular smooth muscle layer (see Fig. 3D). Such arteries were always surrounded by a cuff of uterine NK cells, suggesting that these cells play a role in the vascular remodeling.

The increased presence of uNK-cells in the MT of BBBDP-rats may play a role in the decreased trophoblast invasion and/or SA remodeling. The following mechanism may be proposed for healthy pregnant rats: uNK-cells increase in number within the MT [10]. From about day 13 of pregnancy, they surround unremodeled SA, forming so-called 'cuffs' [7]. By producing proangiogenic factors (like VEGFs) and cytokines, they induce trophoblast invasion and initiate SA remodeling [10]. As trophoblast invasion and SA remodeling progresses from the decidual to the myometrial site of the MT, uNK-cells disappear from the decidual site of the MT towards the myometrial site of the MT during the course of pregnancy [22,23]. This is followed by the invasion of trophoblast cells into the MT [7,8,10,11]. In BBBDP-rats we observed not only increased numbers of uNK-cells in the MT, but also a decreased disappearance of uNK-cells from the MT. It may be speculated that the increased presence of uNK-cells in the MT may inhibit interstitial trophoblast invasion. Moreover, as we found an increased number of NK cells in the MT of BBBDP rats, and at the same time decreased spiral artery remodeling, the question arises whether NK cells in BBBDP-rats are less able to affect spiral arteries, resulting in less spiral artery remodeling. Such a suggestion may be in line with the differences in circulating NK cells, we found in these rats [5].

Macrophages also play a role in trophoblast invasion [24]. Although the exact role of macrophages in the MT of rats is unknown, in humans, macrophages in the placental bed are mainly of the type 2 phenotype and create a tolerant immune environment, facilitating trophoblast invasion and spiral artery remodeling [12]. We evaluated the presence of type 1 (M1) and type 2 (M2) macrophages in the MT by counting iNOS+ and CD206+ cells, which represent M1 and M2 macrophages respectively [25]. Unfortunately, we have been limited to the use of iNOS and CD206 as markers for M1 and M2 macrophages, due to the lack of antibodies for other markers for M1 and M2 macrophages subsets in the rat. It may be speculated that the increased presence of iNOS+ and the increased iNOS+/CD206+ ratio in the MT of BBBDP-rats is involved in the decreased interstitial trophoblast invasion and SA remodeling in BBBDP-rats. This suggestion is in line with the finding that M1 macrophages are overrepresented in the placental bed of complicated pregnancies associated with decreased SA remodeling, such as preeclampsia [26].

The question remains as to what comes first: defective trophoblast invasion or increased numbers of immune cells? Although hyperglycemia may affect trophoblast invasiveness [27], in our study, BBBDP-rats were kept normoglycemic before and during pregnancy. Therefore, an effect of hyperglycemia on trophoblast invasion is unlikely. Data on trophoblast invasion in human T1D pregnancy are scarce, but it has been shown that SA remodeling may be decreased in 25% of pregnant women with T1D, independent of glycemic control [28]. Earlier, we have shown that major differences exist in the peripheral immune-response between pregnant BBBDP- and control rats [5]. This was characterized by a more proinflammatory phenotype and increased activation of NK-cells, which was already observed at day 10 of pregnancy [5]. It may, therefore, be speculated that in our BBBDP model also in the

uterine wall differences in immune cells may be present before the start of trophoblast invasion, resulting in impaired trophoblast invasion and SA remodeling. However, increased presence of immune cells in the MT of BBBDP rats, may also be related to general alterations in placental growth and structure in the rats.

In conclusion, we showed that decreased fetal and placental weight in BBBDP-rats may be associated with relative placental immaturity and diminished interstitial trophoblast invasion and less optimal spiral artery remodeling using 2D techniques. We hypothesize that this could be induced by increased numbers of uNK-cells and an increased presence of M1 macrophages in the MT of BBBDP-rats. Although placental immaturity and decreased SA remodeling have been shown in human T1D placentae, the role of the immune-response in this defective placentation in human T1D placentae requires further investigation.

### Conflict of interest

We wish to confirm that there are no known conflicts of interest.

### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.placenta.2014.12.004>.

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