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Preserved efficiency of Sickle Cell Disease placentas despite altered morphology and function.

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Abstract

Introduction: Pregnant women with sickle cell disease (SCD) are at high risk for sickle cell-related complications, obstetrical complications, and perinatal morbidity. Chronic inflammation and the proangiogenic environment associated with SCD have been associated with endothelial damage.

It is unknown whether SCD complications could be associated with placental dysfunction or abnormal placental morphology. Moreover, circulating angiogenic factors in pregnant women with SCD are unexplored.

Methods: Clinical records, placental and blood samples were collected at term delivery for 21 pregnant patients with SCD and 19 HbAA pregnant controls with adapted to gestational age birth weight newborns. Histological and stereological analyses and scanning electron microscopy (SEM) of the placenta, and PlGF and sFlt1 measurements in blood were performed.

Results: In the SCD group, the parenchyma-forming villi of placentas were thinner than in controls, and increased fibrinoid necrosis and an overabundance of syncytial knots were seen. SEM revealed elongated intermediate villous endings with a reduction in the number of terminal villi compared to controls, indicating a significant branching defect in SCD placentas. Finally, SCD patients had an imbalance in the angiogenic ratio of sFlt1/PlGF ($p=0.008$) with a drop of PlGF concentrations.

Discussion: We evidence for the first time both abnormal placenta morphology and altered sFlt1/PlGF ratio in SCD patients, uncorrelated with maintained placental efficiency and fetal growth.

Keywords: fetal growth, pregnancy outcome, sFlt1, PlGF, placenta, sickle cell-related complications

INTRODUCTION

Sickle cell disease (SCD) is the most common monogenic disorder worldwide and is associated with significant morbidity and mortality, especially during pregnancy[1–5]. SCD includes both sickle cell HbSS disease (homozygous genotype) and various compound heterozygous genotypes (HbSC, sickle cell β -thalassemia disease).

The complications related to SCD include acute pain crisis (57%), anaemia requiring blood transfusion (26%), infection, admission to a critical care unit, and death[1–4,6–10].

Pregnancy in SCD is supposed to be at very high risk, because it exacerbates the pre-existing pathophysiological state [11] and the clinical manifestations of the disease, associated with an overall increase of oxygen requirement by the fetoplacental unit.

Several authors have reported obstetrical and neonatal risks, including stillbirth (RR 3.94), small-for-gestational-age infants (RR 3.72), preeclampsia (RR 2.43) and preterm delivery (RR 2.21)[1–5]. Fortunately, most newborns have adapted to gestational age (AGA) birth weights despite the increased risk for having a smaller baby[12].

The placenta is a temporary organ vital for fetal development, and its integrity is critical for fetal-maternal exchange. Abnormal placenta, particularly with low villosities branching, has been associated with pathological pregnancy outcomes[13]. Numerous findings support the hypothesis that complicated pregnancies, notably small-for-gestational-age and pre-eclampsia, are associated with angiogenic factors ratio impairment [14–16]. Indeed, the balance of angiogenic factors, particularly placental growth factor (PlGF) and soluble Fms-like tyrosine kinase 1 (sFlt1), affects normal and pathological development of the placenta[17,18]. It is known that SCD non pregnant patients present increased levels of circulating PlGF and sFlt1[19–21] correlating with clinical state. Blood concentrations of these factors secreted by placenta during SCD pregnancies are presently unknown.

To date, few studies have described the histological features of SCD placentas[22–26], and none have focused on the structure of the villous tree in correlation with the outcome of these pregnancies. We undertook this study in SCD obstetrically uncomplicated pregnancies with AGA newborns. Hence, for the first time, we studied and gathered data concerning SCD placental morphology and compared the status of angiogenic factors between control and SCD patients at term. We hypothesize that SCD pregnant women environment will impact placental development.

MATERIALS AND METHODS

Study design

The study obtained approval from our local ethics committee (CPP 2015-Mai-13909). All patients gave their informed written consent to participate in the study.

Patients who gave birth between January 2016 and August 2017 in obstetric units of Ile de France hospitals (Bicêtre, Antoine Bécère, Tenon, St Denis, Créteil) were included in a prospective study. We included 21 pregnant patients with SCD and 19 HbAA as healthy pregnant controls. Inclusion criteria were delivery of an adapted for gestational age (AGA, birthweight between 10th and 90th percentile) newborn after 36 weeks of gestation (WG) without obstetrical complications during pregnancy. Exclusion criteria were pre-existing maternal pathology (hypertension, diabetes, chronic illness) and proven obstetrical complications (pre-eclampsia, growth restriction).

Data collected included age, smoking habits, parity, body mass index (BMI) at the first trimester and blood pressure at the third trimester. We recorded the medical background and SCD-related complications (frequency of blood transfusion, vaso-occlusive crisis (VOC) requiring hospitalization or acute chest syndrome (ACS)). Laboratory data including capillary haemoglobin electrophoresis results, haemoglobin concentrations at the first and third

trimesters, haemoglobin S and reticulocyte count were collected. The mode of delivery and neonatal parameters (term, birth weight, sex) were recorded. Both placenta and serum samples were obtained for all SCD patients but two, and compared to 10 placentas and 19 blood samples for controls.

Placenta collection

We collected 21 placentas from the 21 SCD patients with AGA neonates (11 HbSS and 10 HbSC) and ten placentas from the 19 HbAA patients with AGA neonates called control placentas. After delivery, membranes and cord were removed from the placenta, which was weighed. Placental efficiency was determined by calculating the ratio between the birth weight of the neonate and that of the placenta[27]. Five samples per placenta (1cm² blocks from the chorionic to the basal plate) for histological and stereological analyses and chorionic villi for microscopy were collected within 30 minutes after delivery via systematic uniform random sampling[28]. All the conditions cannot be made on all the placentas because the time and the localisation of delivery was not compatible with the complexity and timing of some of the experiments in the laboratory (supplementary figure).

Histological and stereological analyses

The blocks of placenta were fixed in 4% formaldehyde and then processed for inclusion in Paraplast (Sigma-Aldrich, Saint-Quentin Fallavier, France). For each placenta, 5 random fragments were collected taking into account the entire thickness of the placenta. Each fragment was processed for inclusions, sections of 5 µm thickness and stained by haematoxylin and eosin (Mayer Merck, Germany; Sigma-Aldrich, France) for general visualization.

Histology

Twenty-one placentas of SCD patients (11 HbSS - 10 HbSC) with AGA birth weight were compared with ten AGA control placentas. Five slides were analyzed blindly on a photonic microscope (NIKON Eclipse E800M) by two experienced medical pathologists in the same centre (AB and JM).

Stereology

Seven placentas (4HbSS - 3 HbSC) from the 21 SCD patients with AGA birth weight infants and seven control from the 19 placentas at term were analyzed.

Five slides per placenta (one per random sample) were scanned with a whole-slide Hamamatsu NanoZoomer Digital Pathology® scanner (Hamamatsu Photonics, France).

Sections (vertical uniform random) were analysed at a $\times 200$ magnification. As shown in figure 2, for each fragment, around 30 counting frame were defined ($600 \times 600 \mu\text{m}$ every $900 \mu\text{m}$) and 30 linear dipole probes were randomly distributed over the section [29]. Components crossed by the linear dipole were computed as trophoblast, intervillous space, mesenchyme, fetal vessel, fibrin and knots [30]. All data presented are expressed as relative values as the absolute volume of the placenta has not been determined before sample collections. Thus, their relative surface ($S_v, \mu\text{m}^{-1}$) and relative volume ($V_v, \%$) were calculated using One Stop Stereology software (MercatorPro®, ExploraNova (Reed)).

Scanning electron microscopy (SEM)

We analyzed eleven placentas from the 21 SCD patients (6 HbSS-5 HbSC) with AGA newborns and compared them with three of the 10 controls placentas. Three random samples of chorionic villi per placenta were fixed in a solution of 2% paraformaldehyde (EMS, France) and 3% glutaraldehyde (EMS, France) in 0.1 M phosphate buffer (Sorensen, France), pH 7.4, for 2 hours. They were then washed in 0.1 M cacodylate buffer (EMS, France), CaCl_2 (Prolabo, France), pH 7.4, and post-fixed in 1% OsO_4 (EMS, France) for one hour at 4°C .

After washing and dehydration in graded ethanol, villi were dried in HMDS (Sigma Aldrich, USA) for 5 minutes, mounted, and metalized using palladium in a Jeol JFC-1300 Autofine Coater. Villi were then photographed in a Jeol 100S SEM microscope (Jeol, France) at different magnifications.

Biochemical analysis

Blood samples from 19 of the 21 SCD patients (2 were not available) and the 19 controls were collected before delivery. Whole blood was allowed to clot for 45 minutes at room temperature and then centrifuged at 3,000 rpm for 15 minutes at 4°C. Samples were stored at -20°C until assayed. Free PIGF and sFlt1 were measured with quantitative sandwich ELISA (R&D Systems, Minneapolis) according to the manufacturer's instructions. All samples were tested in duplicate. Variability was controlled with an independent standard curve and internal quality controls on each plate.

Statistical analysis

For the description of the population, results are presented as median [min, max]. Comparison between SCD patients and controls was performed using Mann-Whitney tests. The results were considered significant at $p < 0.05$.

For stereological analysis, data are expressed as median and presented as boxplots (median and interquartile range (IQR) [Q1; Q3]) and analyzed using a linear model, with random effect of mother adjusted for group (control or SCD) and number of sample collected per placenta. Linear Mixed Effects Model (nlme) was used from R package (Jose Pinheiro, Douglas Bates, Saikat DebRoy, Deepayan Sarkar and the R Development Core Team 2013. nlme. R package version 3.1-111) in R statistical software ([29]).

RESULTS

Characteristics of the population and outcome

Characteristics of SCD patients (11 HbSS and 10 HbSC) and controls (HbAA) are summarized in Table 1. The SCD and control groups were similar in the mean age and parity of mothers. BMI at the first trimester was slightly lower in SCD patients than in controls ($p=0.03$). In HbSS patients, 45.5% had already developed ACS and 45.5% retinopathy and/or bone involvement, compared with 10 and 30%, respectively, in HbSC patients ($p=0.07$ and $p=0.46$).

Mean arterial blood pressure at the third trimester was within the normal range and similar in the two groups. Haemoglobin concentrations at the first and third trimesters were significantly lower in HbSS patients than in HbSC patients and between SCD and controls ($p=0.01$ and <0.005 , respectively). Eight out of 21 SCD patients (38.1%) needed a transfusion during pregnancy (7 S/S and 1 S/C), and 15 out of 21 suffered from a vaso-occlusive crisis with hospitalization (71.4%; 10 of the 11 S/S and 5 of 10 S/C). These complications were significantly more frequent in HbSS patients than HbSC patients ($p=0.01$ and $p=0.03$, respectively).

A significant difference was observed between SCD and control newborns in the mean length of gestation ($p=0.0002$). Furthermore, HbSS patients gave birth earlier than HbSC patients ($p=0.03$). SCD newborn birth weight did not differ between HbSS and HbSC patients, but was significantly lower in SCD than in control newborns ($p=0.008$). However, none of the birth weights was under the 10th percentile, in accordance with the inclusion criteria.

No significant difference in fetal-placental weight ratio was observed between-groups (HbSS vs HbSC 6.72 vs 6.65; SCD vs controls 6.69 vs 7.21, $p>0.05$).

Histological and stereological analysis

Histological analysis (Figure 1) of 21 3rd trimester SCD placentas showed generally smaller villi diameter (<40-50 μm in 84% of villi), abundant syncytial knots and increased intervillous fibrin. Villous agglutination and focal infarctions resulted in reduced volume of intervillous space. In addition, thickened decidual vessels, and excessive fibrin in the basal plate were observed in 16 out of 21 placentas (76.2%). Those alterations were present irrespectively in both HbSS and HbSC placentas.

Quantification of these structures was made by stereology (Figure 2). SCD placentas showed only a significant higher volume fraction of knots (0.01 [0.005-0.02] vs 0.027 [0.017-0.043], $p=0.013$) compared to the control (Figure 2B). Analysis of the surface density of the structure of the placenta indicated a significantly increase of fibrin (0.002 [0.001-0.003] vs 0.0043[0.003-0.006] μm^{-1} , $p= 1.519^{e-05}$) and a tendency of decrease of the surface density of intervillous space (0.026 [0.024-0.03] vs 0.024[0.023-0.028] μm^{-1} , $p=0.075$) in SCD placentas compared to control. Moreover, we observed a significantly drop of trophoblast (0.045[0.04-0.052] vs 0.039[0.035-0.043] μm^{-1} , $p=0.034$) and mesenchyme tended to decrease (0.025[0.021-0.028] vs 0.022[0.019-0.025] μm^{-1} , $p= 0.075$) in SCD placentas.

Scanning electron microscopy

In term controls placentas, terminal villous structures presented a dense population of multiple small, budlike, villi-containing projections, similar in size and shape (one representative control in Figure 3A&D). In term SCD placentas of normal birth weight newborns (representative HbSS (Figure 3B&E) and HbSC (Figure 3C&F)), we observed elongated intermediate villous endings with reduced branching with less terminal villi. Images of representative villosities of both groups are shown with no evident differences to differentiate HbSS and HbSC placentas.

PlGF and sFlt1 analyses

PlGF concentrations were significantly decreased in SCD patients (both HbSS and HbSC) compared with controls (mean 170.8 and 140.5 vs. 254.6 pg/mL, $p=0.05$ and $p=0.009$ respectively; Figure 4), whereas no significant difference was observed for sFlt1. The sFlt1/PlGF ratio was significantly increased in SCD patients (103.7 and 147.8 vs. 29.1, $p=0.02$ and 0.04 , respectively, and $p=0.008$ when HbSS and HbSC were grouped compared with controls).

DISCUSSION

Here we report clinical data, placental structure and the angiogenic environment of SCD pregnant women. We focused on AGA birth weight newborns to specify placental features in SCD pregnancies without obstetrical complications. SCD patients did not differ from controls concerning the fetal-placental weight ratio, an indicator of the efficiency of the placental unit[27,28,31]. However, in our cohort without obstetrical complications, placental histology and morphology in SCD patients were consistent with abnormal placental development. We found that placentas of SCD patients had thickened decidual vessels, numerous infarctions in the parenchyma and subchorionic hematoma and in villi that were small for the term. A moderate-to-severe overabundance of syncytial knots (aged syncytial nuclei that accumulate locally and later extrude into the maternal circulation) and excessive fibrin deposits were also observed as previously shown[22–25]. The stereological results confirmed quantitatively these statements.

The major and unexpected finding of this study was that SEM analysis of term SCD placentas with AGA foetuses showing a decrease in villous branching, with a reduction in the density of tertiary villi. Again, the stereology confirm the tendency of a drop of trophoblast and mesenchym surface density. These results were observed despite an uncomplicated outcome and an adapted for gestational age newborn birth weight.

The birth of a healthy infant at term is dependent upon normal placental development and function, notably the branching angiogenesis of immature intermediate villous vessels[32]. Restricted branching patterns are usually associated with placental vascular impairment, mimicking pre-eclampsia [13,33] and associated with early IUGR[34–38]. Macara et al [39] described a decrease in the volume of the terminal villi in placentas of newborn with early IUGR.

In IUGR and PE, etiology is hypothesized to be disturbed remodelling of spiral arteries. It is possible to interpret morphology and angiogenic factors in IUGR and PE as being an adaptation to the context with compensatory efforts (though unsuccessful) of the placenta for earlier damage. The poorly developed placenta might be responsible for impaired exchanges associated with this disorder, as intermediate and terminal villi represent the majority of the placental volume in the third trimester and are the primary sites for gas and nutrient exchanges.

In our cohort, there was an apparent discordance in between the favorable outcomes of SCD included pregnancies with adapted for gestational age newborn birthweights and the pathologic histology and morphology of their placentas. Effectively, results highlight a paradox between branching defects in the SCD placentas and fetal growth. We evoke a totally unknown (but successful) mechanism that compensate SCD-stress and IUGR/PE-morphology in parallel. We agree that this view would be unusual and not fitting with the views on the role of placentopathy and factor levels already described in IUGR/PE. However, the neonatal outcome are different. Our study is not showing any direct evidence what this mechanism might be, but we can make some hypothesis.

The first hypothesis concerns the implication of angiogenic factors.

Proper coordination between trophoblasts and villous fetal endothelial cell development is essential in the early stages for a healthy pregnancy. These mechanisms are mediated by growth factors such as PlGF, which is in turn regulated by cytokines, oxygen pressure, and mechanical stimuli[37]. Previous studies have reported an association between the balance in the angiogenic ratio of maternal PlGF to sFlt1 and villous branching[40,41]. Indeed, PlGF stimulates angiogenesis directly via intracellular mechanisms and by increasing the bioavailability of VEGF-A[42]. Free PlGF concentrations throughout pregnancy are

decreased in patients with pre-eclampsia by binding to excess circulating sFlt1[14,15]. Besides, decreased PlGF is associated with histological lesions in placenta from small for gestational age fetuses[16,43].

We found a significant decrease in PlGF with a concomitant significant increase in the sFlt1/PlGF ratio. This result is in agreement with the branching defects in the SCD placentas. PlGF gene expression is inhibited under hypoxic conditions[44,45] so, in SCD pregnancies, low PlGF could result from the overall systemic hypoxic conditions.

A likely hypothesis of our paradoxical observation is the existence of specific mechanisms in SCD to ensure placental efficiency and fetal growth.

By this way, an alteration of the ST barrier, together with the development of a fibrin layer, could lead to facilitated transport through the placenta that could modify nutrient transport as described by Nelson et al [46].

Another mechanism could involve oxygen transport. SCD fetuses can be less hypoxic than those with IUGR because of HbS's lower affinity for oxygen. Indeed, SCD hemoglobin (HbS) has a particular oxygen dissociation curve, adapted to the chronic hypoxia that release oxygen easier than HbA. The diffusion of oxygen to the fetus should be facilitated in SCD placentas and the consequences of chronic anemia partly compensated in contrary to iron anemia. Furthermore, chronic maternal anaemia, which we observed in our patients, may induce an adaptative response in fetal-placental vascularization leading to an increase in the blood vessel surface area of exchange[47].

Finally, one can hypothesize an adaptation of transporters expression and activity in the trophoblast layer, promoting nutrient transfer to the fetus and regulating fetal growth in SCD. An increased activity of glucose transporters and placental systems A and L has been described in pregnant women with diabetes and fetal overgrowth [60-62]. In contrast, an

altered functional activity of transporters (SLC7A7, SLC38A5, SLC19A1, SLC19A2) has been seen in IUGR foetus placentas[48–50][63-68]. Such differences in the expression and activity of transporters may account for the differences in fetal growth observed between pregnant patients with SCD and those with IUGR, despite their similarities in villous branching structure.

Another hypothesis posits changes in syncytiotrophoblast metabolism especially in the mitochondrial energy pathway[51].

The strengths of our study of SCD pregnancies is the compilation of clinical data, placental histology and morphology specifically by SEM, and laboratory parameters, including both SS and SC patients and controls. On the other hand, the sample size is small and this is a pilot study that will need to be confirmed in a larger cohort.

In summary, we observed in SCD patients an abnormal placental structure and altered PIGF and sFlt1/PIGF ratio that did not correlate with fetal growth. To identify therapeutic options in pathologies due to abnormal placentation, it is crucial to unravel the molecular mechanisms underlying the dissociation between placenta structure and efficiency for fetal growth. Targeted studies on possible mechanisms may enable future treatment strategies for pregnancies lacking these unknown pathways.

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STATEMENT OF AUTHOR CONTRIBUTIONS:

AGC, SG, and AB conceived the study; SG, TF and KP supervised the work; JM performed the histological analysis. AGC, FV, and RLK performed SEM experiments and analyzed data; AGC and ACT performed stereological experiments, and AGC, ASB, ACT and EC analyzed data; AGC, SG, and KP wrote, reviewed and edited the manuscript. All authors edited and approved the final draft of the manuscript.

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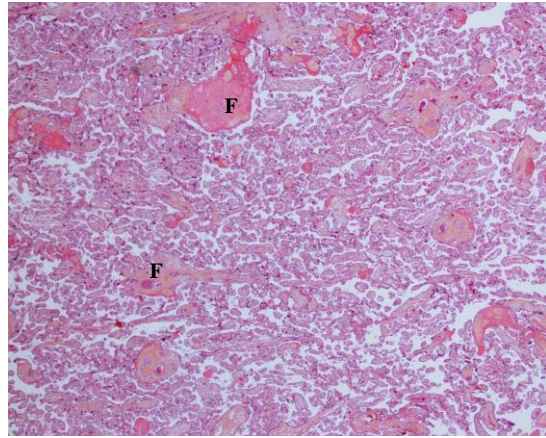
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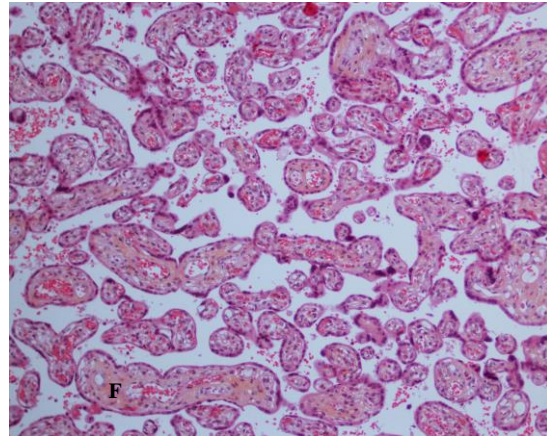
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Figure 1:

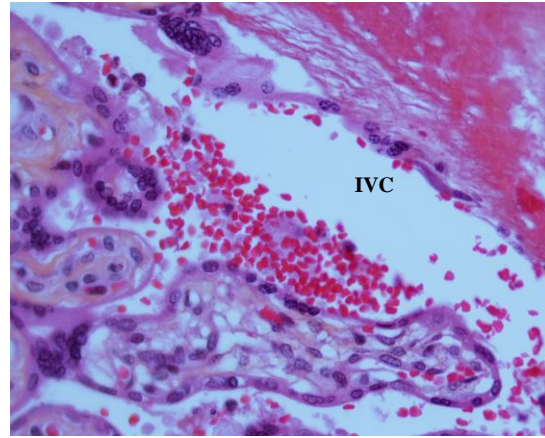
Control



A

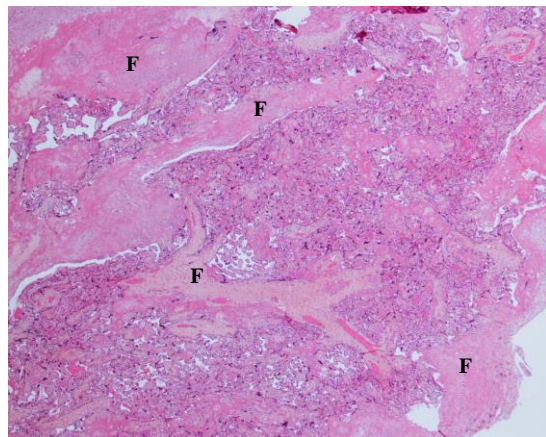


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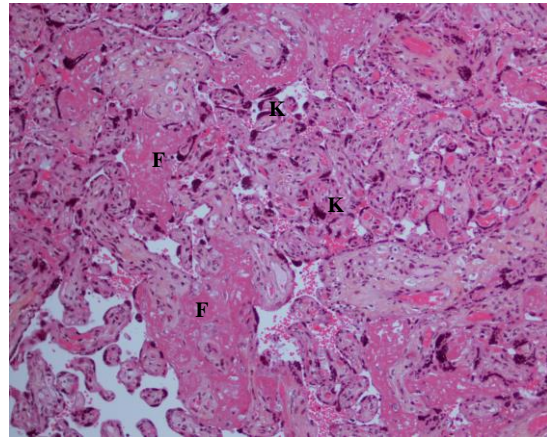


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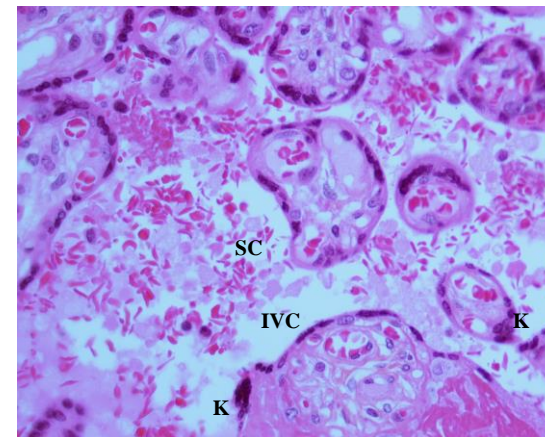
SCD



D



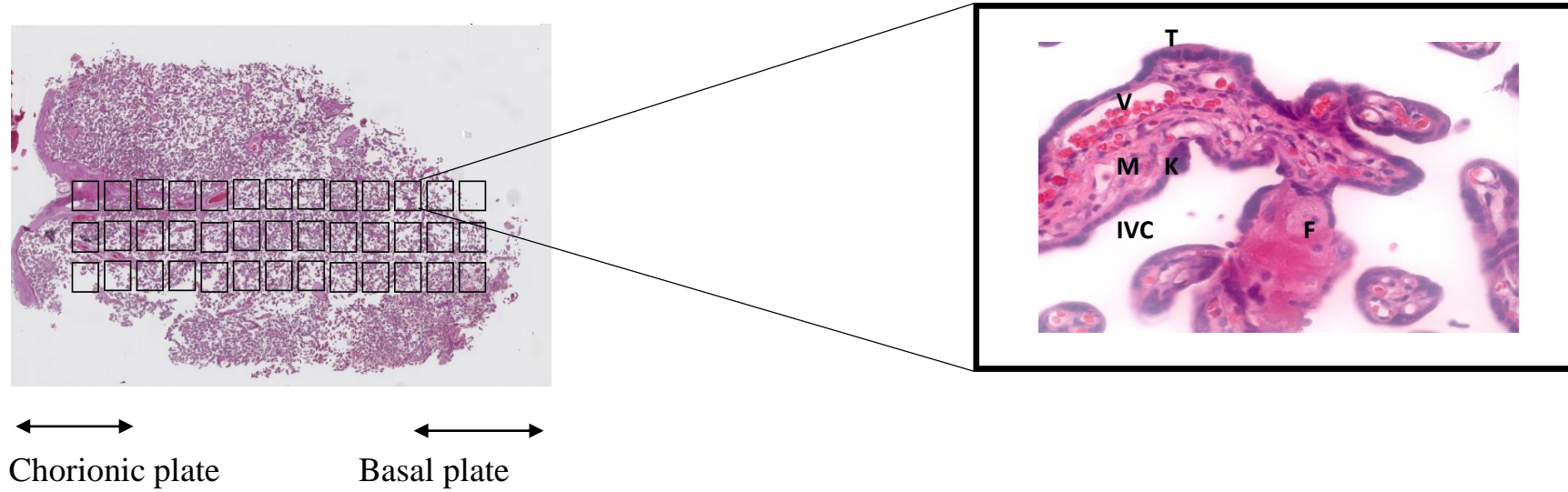
E



F

Figure 2:

A:



B:

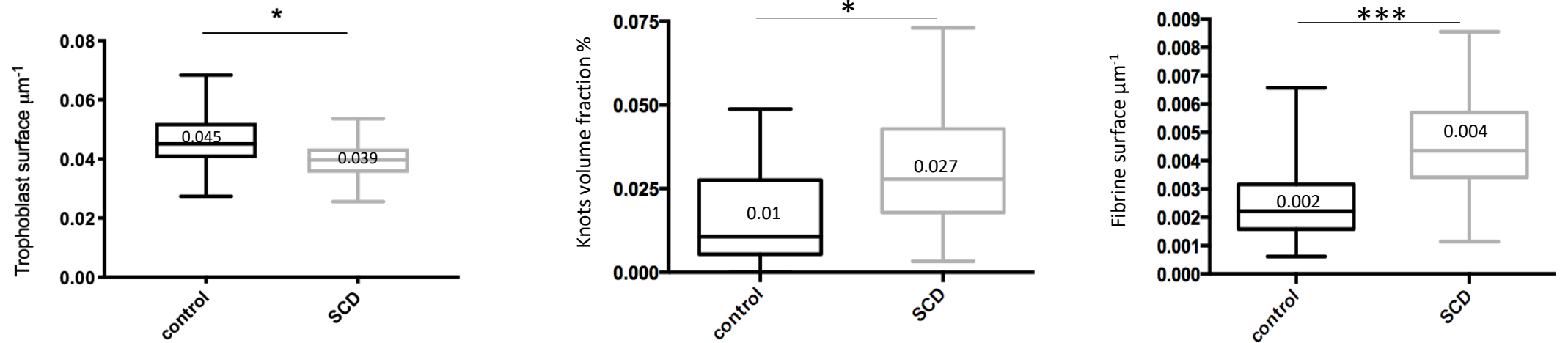


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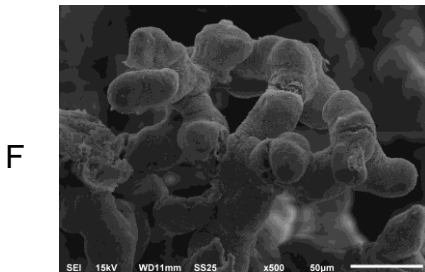
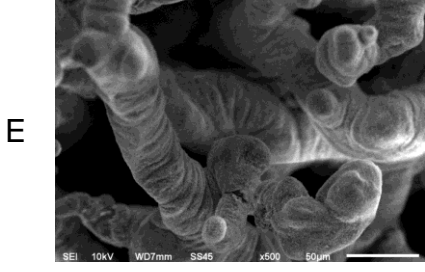
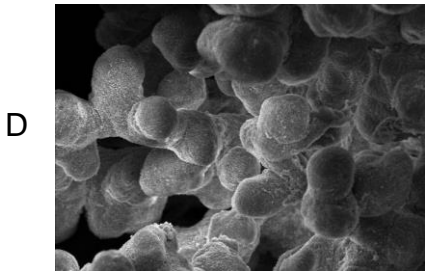
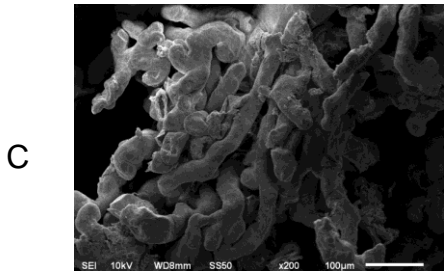
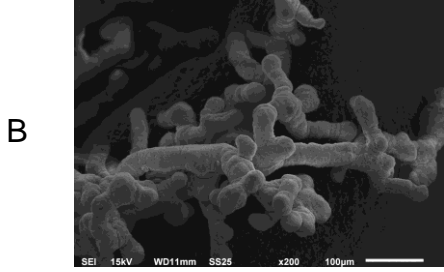
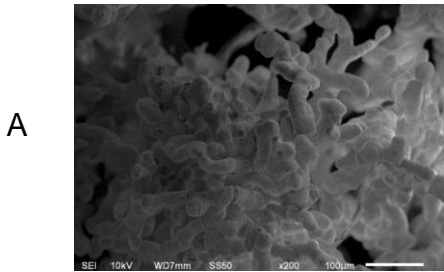


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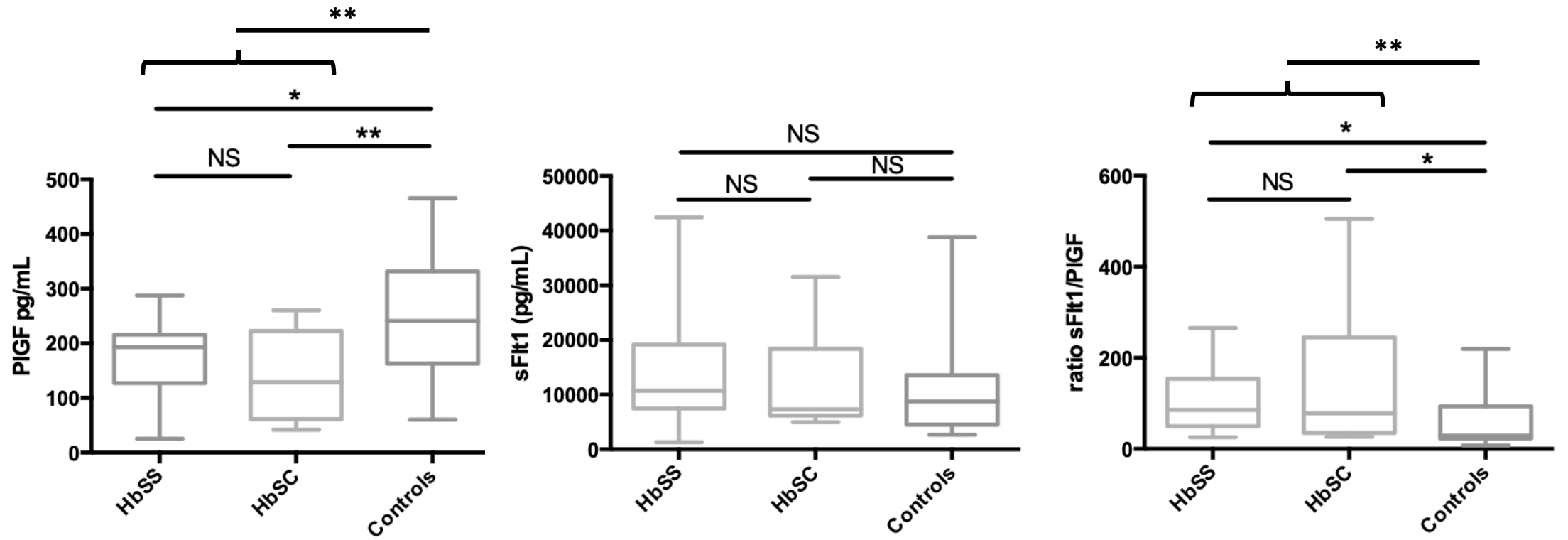


Table 1: Maternal characteristics in SCD and Control groups

*: mean [min-max]; BMI: Body Mass Index; WG: Week of gestation, T1: trimester 1; T3: trimester 3. NA: not applicable. NS: not significant.

Maternal characteristics		SCD HbSS (N=11)	SCD HbSC (N=10)	P between HbSS and HbSC	Whole SCD (N=21)	Control HbAA (N=19)	P between SCD and Control
General characteristics	Maternal age (years) *	35.2 [27-42]	30.1 [22-40]	NS	32.7 [22-42]	31 [24-40]	NS
	Parity *	2.3 [1-5]	2.2 [1-4]	NS	2.3 [1-5]	2.3 [1-5]	NS
	Tobacco use	0	0	NS	0	0	NS
	BMI (g/m ²) *	22.6 [19.8-29.7]	24.5 [17.3-29.7]	NS	23.5 [17.3-29.7]	26.8[17.8-38.8]	0.03
	Diastolic blood pressure T3 (mmHg) * Systolic blood pressure T3 (mmHg)*	76 [61-90] 118 [111-124]	72 [60-79] 118 [104-138]	NS NS	73.9 [60-90] 117.6 [104-138]	71 [51-81] 117 [104-128]	NS NS
Laboratory data	Haemoglobin S level T1 (%) *	79.1 [51-93]	45.8 [42-47]	0.002	62.4 [42-93]	NA	NA
	Haemoglobin F T1 (%) *	5.4 [1-11]	2.7 [1-6.6]	NS	4.3 [1-11]	NA	NA
	Haemoglobin T1 (g/dL) *	8.6 [7.5-9.6]	10.1 [7.2-11.8]	0.01	9.3 [7.2-11.8]	11.3 [8.7-13.5]	<0.0001
	Haemoglobin T3 (g/dL) *	7.9 [6.8-8.8]	9.7 [7.9-10.8]	0.0004	8.8 [6.8-10.8]	11.6 [9.5-13.1]	<0.0001
	Reticulocytes T1 (G/dL) *	243 [144-424]	136 [97-157]	0.02	193 [97-424]	NA	NA
Background	Obstetrical complications N (%)	3 (27.3)	1 (10)	0.07	4/21 (19)	0/19	NA
	Acute chest syndrome N (%)	5 (45.5)	1 (10)	0.46	6/21 (28.6)	NA	NA
	Retinopathy or bone involvement N (%)	5 (45.5)	3 (30)	0.46	8/21 (38.1)	NA	NA

Table 2: Maternal complications and pregnancy outcomes in SCD and Control groups

*: mean [min-max]; BMI: Body Mass Index; WG: Weeks of gestation; NA: not applicable; NS: not significant.

Characteristics		SCD HbSS (N=11)	SCD HbSC (N=10)	P between HbSS and HbSC	Whole SCD (N=21)	Control HbAA (N=19)	P between SCD and Control
Complications during pregnancy	Blood transfusion total N (%)	7 (63.6)	1 (10)	0.01	8 (38.1)	0	NA
	Targeted transfusion N (%)	3 (27.3)	0		3 (14.3)		
	Prophylactic transfusion N (%)	4 (36.4)	1 (10)	0.03	5 (23.8)		
	Hospitalization for VOC N (%) ACS N (%)	10 (90.9) 4 (36.4)	5 (50) 0	0.04 0.03	15 (71.4) 4 (19)	0	NA
Delivery data	Mode of delivery Vaginal N (%) Caesarean section N (%)	3 (27.3) 8 (72.7)	6 (60) 4 (40)	0.13	9 (42.8) 12 (57.1)	9 (47.3) 10 (52.6)	0.77
	Term (WG) *	37.4 [36.1- 39.1]	38.8 [37.4- 41.2]	0.03	38.1 [36.1-41.2]	39.6 [38-41.6]	0.0002
	Birthweight (g) *	2929.3 [2500- 3470]	3104 [2640- 3620]	NS	3012.5 [2500- 3620]	3363.1 [2780- 4050]	0.008
	Placenta weight (g)	447.4[360- 603]	483.9[335- 597]	NS	462.8 [335-603]	474.7 [380-612]	NS
	Placental/newborn weight ratio	6.72 [5.28- 7.61]	6.65 [5.49- 8.28]	NS	6.69[5.28-8.28]	7.21[5.64-8.89]	NS
	Sex (Female/Male)	5/6	4/6	NS	9/12	11/8	0.34