Genetic and microscopic assessment of the human chemotherapy-exposed placenta reveals possible pathways contributive to fetal growth restriction.

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1 **Introduction:**

2 Fetal growth restriction (FGR) can be caused by maternal, placental and fetal factors, or by an imbalance
3 in the complex interaction between these three compartments [1]. There is evidence to suggest that
4 impaired vasculogenesis is the most striking pathology identified in obstetrical complications and more
5 specifically in FGR [2]. In up to 90% of all growth-restricted infants the underlying cause is placental
6 disturbed angiogenesis and villous formation [3]. Disorders in the very precise elaboration of the
7 uteroplacental compartment may result in an impaired trophoblast invasion and inadequate perfusion,
8 which are known as underlying mechanisms leading to preeclampsia and FGR [4]. Correct diagnosis of
9 FGR is important since FGR carries an increased risk of perinatal morbidity and mortality. Preterm
10 birth, neonatal hypothermia, hypoglycemia, morbidities and even perinatal mortality can occur in the
11 acute setting, while more cardiovascular and metabolic diseases are seen in the long-term follow-up of
12 these children [5-7].

13 Studies on the outcome of children after prenatal exposure to chemotherapy show an increased risk of
14 FGR [8-12], up to 21%. The high incidence of FGR in pregnant cancer patients may have multiple
15 causes: diminished caloric intake, anemia, increased incidence of thrombosis, toxic (treatment) exposure
16 and negative impact on the uteroplacental blood flow, relative older maternal age, high maternal stress
17 levels, and/or chronic disease/inflammatory response. Animal experiments have shown that tumor
18 growth in pregnant rats has deleterious effects on placenta and fetus [13-14]. Studies reported impaired
19 fetal growth, changes in placental weight and protein content as well as increased hemorrhage and edema
20 with high fetal resorption [13]. It is hypothesized that through competition for nutrients the rapid tumor
21 growth damages the placental development and fetal growth [15]. Substances synthesized by the tumor
22 may cause oxidative stress reactions resulting in an increased ratio of apoptotic trophoblast [14]. In 2009
23 Abellar R.G. et al described the pathologic findings in 13 placentas exposed to chemotherapy and
24 observed histologic findings suggestive of placental underdevelopment when chemotherapy was
25 administered during 2nd and 3rd trimesters of pregnancy [16]. However, they also indicated that other
26 detrimental factors (malnutrition, stress, immune suppression) might have had an additional impact.

27 In this study, we investigated the placental physiology and pathology in a small prospective exploratory
28 cohort study of patients diagnosed and treated for cancer during pregnancy to identify possible
29 mechanisms of chemotherapy-associated FGR. With the use of whole transcriptome shotgun sequencing
30 (WTSS) we explored the presence of important activated or depressed pathways in the placental tissue
31 after chemotherapy-exposure. In addition immunohistochemical analyses were performed to explore
32 some factors related to the differential expressed pathways (oxidative DNA damage, apoptosis,
33 proliferation).

34

35 **Methods:**

36 *Patients and data collection*

Cancer patients and controls were prospectively recruited during pregnancy. Between January 2014 and September 2016, cancer patients, all treated with chemotherapy during pregnancy, were recruited from Belgium (n=19), the Netherlands (n=3), the Czech Republic (n=2), and Luxembourg (n=1). Controls (n=66) were recruited from the University Hospital Leuven and the University Hospital of Louvain-la-Neuve. All newly registered cancer patients were entered in the study after obtaining a written informed consent; allocation to the FGR or no FGR group took place after delivery. Birth weight percentiles were calculated considering the gestational age at birth, birth weight, sex, ethnicity, parity, and maternal length and weight (www.gestation.net, v6.7.5.7(NL),2014). A percentile <10 was considered as FGR. FGR controls were recruited based on sonographic Estimated Fetal Weight (EFW) below the 10th percentile, measured after 30 weeks of pregnancy. If after delivery the birth weight percentile turned out to be above 10, the patient was excluded from the FGR control group. Normal weight (NW) controls were recruited at admission to the delivery room. Exclusion criteria for all controls were: maternal medical disorders (Crohn's Disease, colitis, congenital heart disorders, auto-immune disease) and presence of Doppler abnormalities mostly due to early (<30 weeks GA) and severe preeclampsia. The cancer (1) and control (2) patients were subdivided in FGR (A) and NW (B). The study was approved by the Ethical Committee of University Hospital Leuven (Belgian number B322201421061/ S56168). Detailed general, obstetric and oncological information was available from the online registry database (www.incipregistration.be) and from patient files. Recorded patient characteristics included maternal age, ethnicity (Caucasian, North-African, African, Asian or Latin-American), maternal body mass index (BMI), cigarette smoking during pregnancy (yes/no), obstetrical complications (including hypertensive disorders, diabetes, preterm labor, maternal infection, or cholestasis (yes/no)), parity (nulli- or multiparous), gender of the neonate, gestational age (GA) at birth (days), birth weight and percentile.
The placentas were weighted immediately after delivery. Placental samples from each patient were taken from 4 different cotyledons (4 quadrants) at the maternal side of the placenta. Each sample was divided in 2 parts and rinsed in phosphate buffered saline (PBS) before storage in RNAse buffer (RNA stabilization reagent, Qiagen, Hilden, Germany) and fixation in 4% buffered formaldehyde respectively. Samples in RNAse buffer were stored for maximally 4 weeks at 4°C until analysis., while fixed samples were processed for microscopic examination. All laboratory analyses were performed after validation of the methods and, where possible, samples were tested simultaneously to minimize inter-essay variability.

RNA extraction from placental tissue, Whole Transcriptome Shotgun Sequencing (WTSS) and Pathway analysis

Total RNA was isolated using Tripure Isolation Reagent (Sigma-Alldrich, Bornem, Belgium). The quantity of the extracted RNA was photometrically tested (NanoDrop - Isogen Life Science, Temse, Belgium). The quality of the extracted RNA was evaluated by an RNA integrity assay system (Experion RNA StdSens Analysing kit - Bio-Rad, Nazareth Eke, Belgium: good quality RNA samples included an

RNA integrity number > 7 for all samples). One μg of total RNA was used as input material for sequencing library preparation which was performed with the Illumina TruSeq Stranded mRNA Sample Preparation Kit according to the manufacturers protocol. RNA was denaturated at 65°C in a thermocycler and cooled down to 4°C. Samples were indexed to allow for multiplexing. Sequencing libraries were quantified using the Qubit fluorometer (Thermo Fisher Scientific, Massachusetts, USA). Library quality and size range was assessed using the Bioanalyser with the DNA 1000 kit (Agilent Technologies, California, USA) according to the manufacturer's recommendations. Each library was diluted to a final concentration of 2nM and sequenced on Illumina HiSeq2500 according to the manufacturer's recommendations generating 50 bp single-end reads. Adapters from raw reads were filtered with software ea-utils v1.2.2 [17]. Raw reads were aligned to the reference human genome hg19 with Tophat v2.0.13 [18]. Quantification of reads per gene and downstream inference analyses with DeSeq2 [19] were performed using the software Array Studio V10.0 (Qiagen, Hilden, Germany). Fold change of gene expression among different groups, and the corresponding corrected p-value or False discovery rates, were used to select differentially expressed genes. Cut offs for FDR were set at 0.1. The resulting list of genes were uploaded into Qiagen's Ingenuity Pathway Analysis (IPA) software (Qiagen, Hilden, Germany) to predict possible dysregulated biological pathways.

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

cDNA was synthesized (1 μg) using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts, US). The reactions were incubated at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min and then instantly cooled on ice to 4°C. 25ng of input cDNA was used for RT-qPCR analysis. Using TaqMan probes for IGFBP6, eNOS, and PCNA (all from Thermo Fisher Scientific, Waltham, Massachusetts, US), the relative abundance of each target transcript was normalized to the expression level of YWHAZ, UBC, CYC1, and GADPH as endogenous controls and assessed with the Applied Biosystems StepOne Software v2.1. Cycle conditions were: Holding stage of 20 sec at 95°C followed by 40 cycling stages of 1 sec at 95°C and 20 sec at 60°C, for 40 min in total. Critical threshold values of the target genes were normalized to the geometric mean of the 4 endogenous controls and the mean normalized expression of the target genes was calculated using Q-Gene software.

Immunohistochemistry

Placental tissue samples were fixed for 24h in 4% buffered formaldehyde, thoroughly rinsed in phosphate-buffered-saline (PBS), brought to ethanol 70% and further processed to paraffin blocks according to standard procedures. Four-μm sections were cut for hematoxylin and eosin (HE) staining and other immunohistochemical studies (8-hydroxy-2'-deoxyguanosine (8-OHdG; marker for oxidative

DNA damage), endothelial nitric oxide synthase (eNOS; marker for oxidative stress), proliferating cell nuclear antigen (PCNA; marker for proliferation) and Cleaved Caspase 3 (marker for apoptosis)). For PCNA and eNOS, antigen retrieval was performed by immersion in citrate buffer (pH6) at 90-95°C for 30 and 60 minutes respectively. Antigen retrieval for 8-OHdG and Cleaved Caspase 3 was performed in Tris-EDTA buffer (pH9) at 95°C for 60 and 120 minutes respectively. Sections were incubated for 2h (PCNA) or overnight at 4°C (8-OHdG, eNOS, Cleaved Caspase 3) with the following primary antibodies: 8-OHdG (clone 15A3, 10µg/ml, Abcam, Cambridge, UK), eNOS (polyclonal ab5589, 10µg/ml, Abcam, Cambridge, UK), PCNA (clone PC10, 1.635µg/ml, Agilent Technologies, California, USA) and Cleaved Caspase 3 (polyclonal Asp175, 0.315µg/ml, Cell Signaling, Massachusetts, US). After incubation with secondary antibodies, binding was visualized with Dako DAB+ Chromogen (K3467, Agilent Technologies, California, USA). All sections were counterstained with Harris' haematoxylin. Negative controls were performed by omitting the primary antibody in the first incubation step. To minimize inter-assay variability tissue micro array (TMA) was used. The nuclear staining of 8-OHdG and PCNA was quantified as follows: from each patient, 5 good quality cores were evaluated by counting all positive and negative nuclei in syncytio- and cytotrophoblast respectively and for each core the percentage of positive nuclei was calculated with the following equation [20]:

$$\text{percentage of nuclei}^{+} = \frac{\text{No. of nuclei}^{+}}{\text{No. of nuclei}^{+} + \text{No. of nuclei}^{-}} \times 100$$

then the mean total percentage was calculated per patient. Similarly the cytoplasmic eNOS and 8-OHdG staining in both trophoblast types were evaluated. Since in the syncytiotrophoblast there are no separate cells, we counted the nuclei which were surrounded by positive staining and calculated their percentage versus the total number of syncytial nuclei for each evaluated field. The endothelial cell layers were scored for circumferential completeness in 5 percentage categories: 0 (absent), 25 (1–25%), 50 (26–50%), 75 (51–75%) and 100 (76–100%). For this, again 5 cores were scored and the total mean percentage was calculated. Throughout the evaluation we used the Zen image analysis system linked to an Axioskop 50 microscope fitted with an Axiocam MRc5 camera (all from Carl Zeiss, Zaventem, Belgium). Photographs were taken at a 25x magnification at the highest resolution possible (2584 × 1936 Pixels).

Statistical analysis

Median values and range of distribution of continuous variables of the different groups were compared using the Kruskal-Wallis test followed by Dunn's post-test for multiple comparisons and Mann-Whitney U test for single comparisons. Non-parametric Mann-Whitney U test was used to compare the median of the percentage of positive cells calculated per group between the chemo-exposed placentas and the non-exposed controls. $P<0.05$ was considered statistically significant.

Results

A total of 25 cancer patients (10 FGR and 15 NW) and 66 controls (24 FGR and 42 NW) were included. Table 1 shows the recorded patient characteristics of the included patients and controls. There was a statistically significant difference between the distributions of GA at delivery, birth weight, birth percentile, and placental weight between cancer and control groups (Kruskall-Wallis: $P<0.01$). Placental weights were clearly lower in the FGR than in the NW groups, with a median weight of 340g vs 414g in cancer patients (Mann-Whitney U: $P=0.015$), and 380g vs 595g in controls (Mann-Whitney U: $P<0.01$).

Whole Transcriptome Shotgun Sequencing (WTSS)

Different comparisons between the groups were made to explore the differentially expressed genes and their possible relation to FGR after chemotherapy-exposure vs. FGR not related to chemotherapy (Supplementary appendix, tables 1a – 1c, figure 1a). Comparing the chemotherapy-exposed groups (1A-B) with the control groups (2A-B), 69 out of 20314 genes were significantly up- or downregulated (FC ≥1.5 or ≤-1.5; FDR ≤ 0.1). Out of these 69 genes, only one was downregulated, C-type lectin domain family 4, member M (CLEC4M). In table 2 we show the 10 most dysregulated genes. Most genes were associated to the following 5 network functions: cellular development, cellular growth and proliferation, organ development, cell-to-cell signaling and interaction, and inflammatory response. Comparing the FGR groups and NW groups exposed or not exposed to chemotherapy, 161 genes were significantly up- or downregulated between 1A and 2A but only 7 genes between 1B and 2B. Associated network functions were: cellular movement, tissue development, cardiovascular system development and function, free radical scavenging, and small molecule biochemistry. We compared the highest dysregulated genes (FC ≥1.5 or ≤-1.5) from the comparisons of chemotherapy-exposed patients vs the NW controls (1A vs 2B and 1B vs 2B) and observed that only 2 genes were concordant: poly(A) binding protein, cytoplasmic 3 (PABPC3) and delta-aminolevulinate synthase 2 (ALAS2) (Supplementary appendix, figure 1c). Two genes involved in (mRNA) metabolism. Exploring more specifically the differences between FGR related to chemotherapy exposure and FGR without exposure, we compared group 1A and 1B, and 2A and 2B, but no significantly up- or downregulated genes (FC ≥1.5 or ≤-1.5; FDR ≤ 0.1) were observed. To assess if other factors besides chemotherapy could be related to the increased incidence of FGR after chemotherapy-exposure, we explored the differences in cancer types (breast cancer vs hematological malignancies), trimester at diagnosis, maternal age and delivery mode (vaginal birth vs cesarean section). The start of chemotherapy had an additional impact on the gene expression, with 86 genes significantly differential expressed between early start of exposure (< 196 days GA) and late start of exposure (> 196 days GA). (Supplementary appendix, Table 1d) One of the network functions in which these genes were involved is the superoxide radicals degeneration network. For all other comparisons no significantly differential expressed genes were observed.

176 **Immunohistochemistry**

177 We were specifically interested in exploring the effect of chemotherapy on the oxidative DNA damage,
178 proliferation and apoptosis. By immunohistochemical analyses we explored the expression of 8-OHdG,
179 eNOS, PCNA and Cleaved Caspase 3. The quantitative data are shown in table 3.

180 There was significantly more nuclear expression of 8-OHdG in the trophoblast of the placentas in groups
181 1A and B vs groups 2A and B (*P=0.003*) (Figure 1). In the FGR control placentas (group 2A) the nuclear
182 expression of 8-OHdG was clearly lower, but there was marked cytoplasmic staining which was
183 probably due to the presence of mitochondrial DNA damage [21]. Most of the normal weight controls
184 (group 2B) had very limited expression of 8-OHdG.

185 While the expression of eNOS in the endothelial cells of the groups 1A and B was high and comparable
186 to the expression in groups 2A and B (*P=0.702*) (Figure 2), the expression of eNOS in the
187 syncytiotrophoblast of groups 1A and B was significantly lower when compared to groups 2A and B
188 (*P=0.015*).

189 PCNA immunoreactivity was present in villous cytotrophoblast of all groups (Figure 3). We observed a
190 decreased percentage of positive cells in groups 1A and B vs groups 2A and B (*P=0.135*). In our
191 observation FGR placentas showed lower expression compared to the controls without FGR, but this
192 was not statistically significant (data not shown).

193 The rate of apoptosis as detected by the expression of Cleaved Caspase 3 showed a very limited
194 expression in all groups.

195 **Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

196 We verified the immunohistochemical expression of eNOS and PCNA by RT-qPCR. The expression of
197 eNOS by RT-qPCR showed comparable results between both groups (*P=0.804*) (Figure 4). The
198 decreased percentage of proliferation (PCNA) was confirmed by RT-qPCR (*P=0.175*) (Figure 4).

199 To verify the expression levels observed by WTSS, we verified by RT-qPCR the significant upregulation
200 of IGFBP6, being an important factor in growth processes. A comparable significant upregulation
201 (*P=0.001*) was observed (Figure 4).

202 **Discussion**

203 Whole transcriptome sequencing and immunohistochemical analysis revealed an increase in oxidative
204 DNA damage, which might impact the growth and developmental processes in chemotherapy-exposed
205 placentas compared to placentas of healthy pregnancies. Genes connected to the oxidative damage
206 pathway were more expressed in chemotherapy-exposed as well as in the control FGR placentas with

207 an increased expression of the oxidative DNA damage marker 8-OHdG in the trophoblast. No increased
208 rate of apoptosis could be detected.

209 The etiology underlying FGR is divided in three groups with maternal, fetal or placental factors
210 involved. Up to 90% of events is caused by placental dysfunction, which may result from abnormal
211 placental bed development with poor spiral artery remodeling, physical separation at the maternal
212 interface, or from dysregulated metabolic adaptations [22-24]. The placental weight was shown to be
213 lower in growth restricted fetuses [25] and also in our study lower placental weights were recorded in
214 the FGR groups. This lower placental weight in the cancer patients might indicate a potential negative
215 impact caused by the tumor growth, chemotherapeutic agents or other detrimental factors (malnutrition,
216 stress, immune suppression) on the placental development.

217 WTSS and IPA analysis revealed different processes that might be involved to the increased incidence
218 of FGR in chemotherapy-exposed pregnancies. Most differentially expressed genes between
219 chemotherapy-exposed and controls were related to growth and developmental processes, and radical
220 scavenging networks. Comparative analyses between the FGR and no FGR cases in both the
221 chemotherapy-exposed and control groups, did not reveal statistically significant differentially
222 expressed genes. Therefore we cannot describe a causal link between the differential gene expression
223 and FGR. We compared some other variables to investigate if these factors might be contributive to the
224 increased incidence of FGR. We observed that the duration of chemotherapy exposure had an additional
225 impact on the expression of genes related to superoxide radicals degeneration. Because in the FGR cases
226 the median start of treatment was earlier than in the NW cases (131 days vs 163 days) and also the
227 duration of chemotherapy was longer (104 days vs 76 days). This adds to our hypothesis that
228 chemotherapy exposure might be the dominant factor towards the increased incidence of FGR in
229 pregnant cancer patients. Large-scale longitudinal comparative analyses on the expression levels of
230 genes would however be more informative. No significantly dysregulated genes were found when we
231 compared different cancer types (breast cancer vs hematological malignancies), maternal age, maternal
232 BMI, and delivery mode (vaginal birth vs cesarean section).
233

234 Normal physiological conditions in aerobic organisms provide a balance between endogenous oxidants,
235 which lead to tissue damage, and several enzymatic and non-enzymatic mechanisms of tissue defense.
236 Imbalances between these processes will lead to extensive oxidative damage. DNA damage leads to the
237 formation of several DNA products. Among them is a hydroxyl product of deoxyguanosine generated
238 in vivo by oxidative damage, which can be measured quantitatively as 8-OHdG lesions, both nuclear
239 and cytoplasmic (mitochondrial DNA damage) [21,26]. Previous studies already mentioned the presence
240 of this marker in pregnancies complicated by preeclampsia and FGR [27,28]. In the present study an
241 increase of 8-OHdG expression in the cyto- and syncytiotrophoblast was present in the chemotherapy-

exposed groups (*P=0.003*). This might be indicative for direct toxicity of the chemotherapeutic agents to the trophoblast layers.

WTSS revealed an increased association to the radical scavenging function in FGR after chemotherapy-exposure. The nitric oxide radical is generated from the metabolism of L-arginine by the enzyme nitric oxide synthase (NOS) in the endothelium, which diffuses into the underlying vascular smooth muscle and by its activation of guanylate cyclase, leads to vascular relaxation [29]. There are three isoforms of NOS: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), of which the latter two are expressed in the placenta, mainly in syncytiotrophoblast and endothelial cells. iNOS peaks at mid gestation whereas eNOS expression increases towards the end of the third trimester [30,31]. The main stimulus for NO release in placental vessels and for angiogenesis via eNOS activation is shear stress (frictional force between blood flow and endothelium) [30]. Although not statistically significant, we observed an increased expression of eNOS in the endothelial cells between chemotherapy-exposed placentas and controls, but significantly lower expression of eNOS in the syncytiotrophoblast (*P=0.015*) and comparable levels assessed by RT-qPCR (*NS*). Oxidative stress has been related to a decreased activity and expression of eNOS in the fetal endothelial cells, impairing the NOS-dependent relaxation [32], compared to an increased expression at the trophoblast activating the pathologic angiogenesis [3]. Our results indicate that no increased oxidative stress (as evaluated by eNOS at the DNA and protein level) is present in the fetal endothelial cells and syncytiotrophoblast after chemotherapy exposure. However, earlier Toledo et al. described the increase of the malondialdehyde content as an oxidative stress biomarker in the placentas of tumor-bearing rats [14].

IGFBP1 have been described as negatively correlated with birth and placental weight [33]. By WTSS and verified by RT-qPCR, we revealed a significant upregulation of IGFBP6 in the chemotherapy-exposed placentas (P=0.001). Up till now, no data are published on the expression levels of IGFBP6 in the placenta and its possible correlation with birth weight. Investigating the expression of IGF's and IGFBP's in our specific patient population would therefore be very interesting.

We used PCNA as a commonly used proliferation marker to examine the effect of chemotherapy on the cellular proliferation processes. In the human placenta, the most intense expression of PCNA has been identified in villous and invasive cytotrophoblasts [34] with lower expression seen in FGR placentas [35]. We noticed a decreased expression of PCNA (IHC and RT-qPCR) in the chemotherapy-exposed placentas vs the controls *(NS*). Apoptosis was not increased in the chemotherapy-exposed placentas as observed by the limited expression of Cleaved Caspase 3. Elmore described the complexity of apoptosis and its different interacting pathways [36]. The intrinsic, extrinsic and perforin/granzyme pathways all contribute to DNA degradation and cleavage, but not all of them activate caspases. Therefore we speculate that in the placental tissues studied, the caspase-pathway may not have been involved in apoptosis.

278 There are some limitations in this study. Most importantly, the groups are small with only 10 cases of
279 FGR in the patients exposed to chemotherapy. Additional comparative analyses between potential
280 confounding variables (cancer types, start of treatment, maternal age and BMI, delivery mode) showed
281 that a longer treatment exposure has an additional impact on the differential expression of 86 genes of
282 which a number was related to the network of superoxide radicals degeneration. Despite these extended
283 comparative analyses, there is still a large heterogeneity in the chemotherapeutic agents, and number of
284 cycles administered within the study groups, Although we could not detect significant differentially
285 expressed genes between the breast cancer patients compared to the hematologic malignancies, 4
286 patients of the FGR group had a hematologic malignancy whereas only 1 patient in the NW group. This
287 might still suggest that the kind of malignancy (local vs systemic) influences fetal growth. Additionally,
288 we cannot exclude the effect of maternal habits during the course of pregnancy (smoking [37], alcohol
289 intake, stress [38] or other toxic exposures) affecting the placenta and its expression of different
290 markers/genes.
291
292 **Conclusion**
293
294 This is the first case cohort study exploring the effects of cancer and chemotherapy on the placental
295 tissue, investigating placental changes and underlying mechanisms for FGR. Here we observed that the
296 placental tissue of cancer patients treated with chemotherapy shows an increase in oxidative DNA
297 damage. This might have an impact on the placental cellular growth and development. Early start of
298 chemotherapy treatment and a longer exposure resulted in increased incidence of FGR. To examine
299 whether this increased oxidative DNA damage explains the increased incidence of FGR, larger
300 prospective cohort studies are required.
301
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396

Tables and Figures:

Table 1

	Chemotherapy; FGR (n=10) (Group 1A)	Chemotherapy; no FGR (n=15) (Group 1B)	No cancer; FGR (n=24) (Group 2A)	No cancer; no FGR (n=42) (Group 2B)	P Value (between all groups)
Age (Yr): median [range]	33 (25-39)	31 (22-36)	30 (22-42)	31 (18-41)	0.465
BMI: median [range]	25.6 (18.7-29.4)	24.1 (16.4-35.9)	22.0 (18.6-28.7)	21.6 (16.9-38.3)	0.101
Parity: nulli-multi	3-7	8-7	13-11	17-25	
Ethnicity					
- Caucasian	9	14	20	34	
- North-African	1	1	2	2	
- African			1	2	
- Latin-American				1	
- Asian			1	3	
Smoking: y-n	1-9	1-14	5-19	3-39	
GA (days) at delivery: median [range]	261 (240;270)	261 (215;272)	262 (217;277)	274 (243;288)	<0,01
Type of delivery: vaginal-cesarean section	5-5	10-5	12-12	27-15	
Gender child: male-female	7-3	8-7	8-16	20-22	
Birth Weight (g): median [range]	2395 (1880;2820)	2735 (1600;3360)	2275 (1370;2750)	3285 (2380;4110)	<0,01
Birth Percentile: median [range]	2.9 (0.1;8.6)	29.2 (11.8;74.6)	2.6 (0.01;9.3)	42.9 (10.1;95.8)	<0,01
Placental Weight (g) (median; range)[a]	340 (211-440)	414 (252-640)	380 (161-580)	595 (390-800)	<0,01
Obstetrical complications					
- Hypertensive disorders[b]: y-n	1-9		4-20	3-39[c]	
- Gestational diabetes[b]: y-n	2-8	1-14[c]		3-39[c]	
- Cholestasis: y-n		1-14[c]			
GA (days) at diagnosis: median [range]	126 (20;185)	140 (19;217)			
Tumor type					
- Breast	6	10			
- Lymphoma: HL-NHL	4: 0-4	1: 1-0			
- Leukemia		1			
- Brain		1			
- Gastro-intestinal		2			
Cancer Treatment					
- Surgery + CT	4	5			
- CT	2	8			
- CT + RT	0	1			
- CT + targeted therapy (Rituximab)	4	1			

GA (days) at start chemotherapy: median [range]	131 (91;198)	163 (92;222)
GA (days) at end chemotherapy: median [range]	235 (220;260)	242 (190;259)
Type of chemotherapy: n; median number of cycles [range]		
- Alkylating agents	10; 4 (1;6)	12; 3 (2;7)
- Anthracyclines	10; 4 (1;6)	12; 3 (1;7)
- Taxanes	4; 3 (3;12)	5; 8 (3;11)
- Platinum-based	/	2 (3;5)
- Vinca Alkaloids	4; 4 (4;6)	1 (7)
- Antimetabolites	4; 3 (3;4)	6; 3 (1;6)
- Targeted Therapy	4; 5 (4;6)	/

Recorded patient characteristics.

Abbreviations: FGR= Fetal Growth Restriction; GA= Gestational Age; BMI= Body Mass Index; HL= Hodgkin lymphoma; NHL= non-Hodgkin Lymphoma; CT= Chemotherapy; RT= Radiotherapy; y= yes; n= no; NS = not significant.

[a]Data available of respectively 9, 9, 20 and 34 placentas of the different groups. [b]In any of the included patients, medication was required. No patient was diagnosed with preeclampsia. [c]In one patient 2 complications were registered.

Table 2

IPA name	Full name	Main function	1A-B vs 2A-B		1A vs 2A	
			FC	FDR	FC	FDR
CLEC4M	C-type lectin domain family 4, member M	transmembrane receptor, involved in the immune system, probable pathogen-recognition receptor	-1.53	<0.01		
HBB	hemoglobin, beta	the hemoglobin chains, each with its own heme moiety, cooperate in binding and release of oxygen	1.81	<0.01		
HBQ1	hemoglobin, theta 1	found in human foetal erythroid tissue, research supports a transcriptionally active role possibly in early erythroid tissue	1.73	<0.01		
IGFBP6	insulin-like growth factor binding protein 6	modulates IGF-mediated growth and developmental rates	1.69	<0.01		
ALAS2	aminolevulinate, delta-, synthase 2	erythroid-specific mitochondrially located enzyme, catalyzes the first step in the heme biosynthetic pathway	1.67	<0.01		
HBG2	hemoglobin, gamma G	gamma globin genes are normally expressed in the foetal liver, spleen and bone marrow, together with two alpha chains constitute foetal hemoglobin (HbF)	1.66	<0.01		
S100A4	S100 calcium binding protein A4	localized in the cytoplasm and/or nucleus of a wide range of cells, involved in the regulation of cell cycle progression and differentiation	1.66	<0.01		
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	localized to the nucleus of endothelial cells, induced by IL-1 and TNF-α stimulation, functions as a transcription factor in endothelial cell activation	1.66	<0.01		
HBA1	hemoglobin, alpha 1	the hemoglobin chains, each with its own heme moiety, cooperate in binding and release of oxygen	1.65	<0.01		
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	proteins in this family are involved in the breakdown of extracellular matrix in normal physiological processes (embryonic development, reproduction, tissue remodeling), as well as in disease processes (arthritis, metastasis)	1.65	<0.01		

ADORA3	adenosine A3 receptor	belongs to the family of G-protein-coupled receptors involved in a variety of intracellular signaling pathways and physiological functions, mediates both cell proliferation and cell death	1.64	<0.01		
KLHL14	kelch-like family member 14	member of the Kelch-like gene family, cell cycle regulators, play a role in mitosis			-1.69	0.08
LRP2	low density lipoprotein receptor-related protein 2	critical for the reuptake of lipoproteins, sterols, vitamin-binding proteins, and hormones, but also has a role in cell-signaling processes			-1.68	0.03
SLC4A4	solute carrier family 4 (sodium bicarbonate cotransporter), member 4	encodes a sodium bicarbonat cotransporter, also related to other transport pathways (glucose, bile salts and organic acids, metal ions and amine compounds)			-1.63	0.10
NRXN3	neurexin 3	neuronal cell surface protein that may be involved in cell recognition and cell adhesion, may mediate intracellular signaling and may play a role in angiogenesis			-1.62	0.09
F5	coagulation factor V (proaccelerin, labile factor)	central regulator of hemostasis, critical cofactor for activated factor Xa resulting in the activation of prothrombin to thrombin			-1.61	0.06
FBXO27	F-box protein 27	member of the F-box protein family, interact with ubiquitination targets through other protein interaction domains, related to the immune system pathway			-1.58	0.09
GLUD2	glutamate dehydrogenase 2	acts as a homohexamer to recycle glutamate during neurotransmission			-1.57	0.10
KDR (VEGFR2)	kinase insert domain receptor (vascular endothelial growth factor receptor 2)	promotes proliferation, survival, migration and differentiation of endothelial cells, required for VEGFA-mediated induction of NOS2 and NOS3, leading to the production of the signaling molecule nitric oxide (NO) by endothelial cells			-1.55	0.10
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	promotes activation of caspases and apoptosis, contributes to p53/TP53-dependent apoptosis after radiation exposure			-1.52	0.10
LAMA2	laminin, alpha 2	thought to mediate the attachment, migration, and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components			-1.51	0.09

CRIP1	cysteine-rich protein 1 (intestinal)	role in zinc absorption, may function as an intracellular zinc transport protein			1.94	0.03
C19orf33	chromosome 19 open reading frame 33	found primarily in the nucleus, may play a role in placental development and diseases such as pre-eclampsia			1.93	0.03
ATOH8	atonal homolog 8 (Drosophila)	regulates endothelial cell proliferation, migration and tube-like structures formation, modulates endothelial cell differentiation through NOS3 (eNOS)			1.93	0.03
ST6GAL2	ST6 beta-galactosamide alpha-2,6-sialyltranferase 2	involved in the generation of the cell-surface carbohydrate determinants			1.90	0.04
HES4	hes family bHLH transcription factor 4	transcriptional repressor, negative regulator of myogenesis, may play a role in response pathways to DNA cross-link damage			1.87	0.05
SPON2	spondin 2, extracellular matrix protein	cell adhesion protein, promotes adhesion and outgrowth of hippocampal embryonic neurons			1.86	0.05
B3GNT8	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	plays a role in the elongation of specific branch structures of multiantennary N-glycans			1.82	0.03
FXYD1	FXYD domain containing ion transport regulator 1	regulated ion channel activity, may have a functional role in muscle contraction			1.82	0.06
IFITM10	interferon induced transmembrane protein 10	IFN-induced antiviral protein which disrupts intracellular cholesterol homeostasis, inhibits the entry of viruses to the host cell cytoplasm by preventing viral fusion with cholesterol depleted endosomes			1.82	0.06
ADRA2C	adrenoceptor alpha 2C	member of the G protein-coupled receptor superfamily, have a critical role in regulating neurotransmitter release			1.81	0.06

Highest statistically significant down- and upregulated genes between patients (1A-B) and controls (2A-B). The 5 most network functions associated to the dysregulated genes between all chemotherapy exposed and control cases were: cellular development, cellular growth and proliferation, organ development, cell-to-cell signaling and interaction, and inflammatory response. The networks for the subanalysis of FGR cases were: cellular movement, tissue development, cardiovascular system development and function, free radical scavenging, and small molecule biochemistry. A positive respectively negative FC means up- and downregulated in the chemotherapy-exposed placentas. IPA: Ingenuity Pathway Analysis, FC: fold change, FDR: false discovery rate, FGR: fetal growth restriction.

Table 3

Staining	Parameters	Group 1A (n=10)	Group 1B (n=15)	Group 2A (n=24)	Group 2B (n=42)	P-value (group 1A vs 2A)	P-value (group 1B vs 2B)	P-value (group 1A-B vs 2A-B)
8-OHdG (nuclear/cytoplasmic, trophoblast)	Min	48.0	24.0	5.2	10.0	0.061	0.044*	0.003*
	Median	86.8	90.6	62.5	58.0			
	Max	100.0	100.0	100.0	100.0			
	IQR	40.0	61.0	63.5	33.6			
eNOS (cytoplasmic, endothelial cells)	Min	0.0	15.0	0.0	0.0	0.940	0.358	0.702
	Median	72.5	30.0	57.5	45.0			
	Max	95.0	100.0	100.0	100.0			
	IQR	56.0	35.0	50.0	40.0			
eNOS (cytoplasmic, syncytiotrophoblast)	Min	0.0	0.0	4.0	0.0	0.066	0.102	0.015*
	Median	45.0	34.0	64.5	64.0			
	Max	96.0	86.0	100.0	99.0			
	IQR	66.0	78.0	42.0	54.0			
PCNA (nuclear, cytotrophoblast)	Min	0.2	0.4	2.2	0.6	0.076	0.710	0.135
	Median	11.0	12.6	15.7	20.1			
	Max	21.2	72.0	58.0	81.0			
	IQR	18.4	38.4	19.2	37.0			

Quantitative evaluation of 8-OHdG, eNOS and PCNA: minimum (min), maximum (max), median and interquartile range (IQR) of the percentages of positive trophoblasts or endothelial cells were shown (Mann-Whitney U test). For Cleaved Caspase 3 the total number of positive cytotrophoblast was low in all groups. Of all evaluated cores per placenta we calculated in total respectively 1 to 6 positive cytotrophoblasts (group 1A); 0 to 14 (group 1B); 0 to 8 (group 2A); and 0 to 9 (group 2B). 1A = chemotherapy-exposed with FGR; 1B = chemotherapy-exposed with NW; 2A = FGR control; 2B = NW control. *$P<0.05$ is considered significant. Abbreviations: FGR= Fetal Growth Restriction, NW= Normal Weight.