Regulating role of fetal thyroid hormones on placental mitochondrial DNA methylation: epidemiological evidence from the ENVIRONAGE birth cohort study

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Abstract

Background: Fetal development largely depends on thyroid hormone availability and proper placental function with an important role played by placental mitochondria. The biological mechanisms by which thyroid hormones exert their effects on mitochondrial function are not well understood. We investigated the role of fetal thyroid hormones on placental mitochondrial DNA (mtDNA) content and mtDNA methylation. We collected placental tissue and cord blood from 305 mother–child pairs that were enrolled between February 2010 and June 2014 in the ENVIRONAGE (ENVIronmental influence ON early AGEing) birth cohort (province of Limburg, Belgium). Placental mtDNA content was determined by qPCR and placental mtDNA methylation by bisulfite-pyrosequencing in two regions, i.e., the D-loop control region and 12S ribosomal RNA (MT-RNR1). The levels of free thyroid hormones (FT³, FT⁴) and thyroid-stimulating hormone (TSH) were measured in cord blood.

Results: Cord blood FT³ and FT⁴ were inversely associated with placental mtDNA methylation at the MT-RNR1 (p ≤ 0.01) and D-loop (p ≤ 0.05) regions, whereas a positive association was observed for both hormones with placental mtDNA content (p ≤ 0.04). Assuming causality, we estimated that MT-RNR1 and D-loop methylation mediated, respectively, 77% [indirect effect +14.61% (95% CI 2.64 to 27.98%, p = 0.01)] and 47% [indirect effect +8.60% (95% CI 1.23 to 16.50%, p = 0.02) of the positive association between FT³ and placental mtDNA content. Mediation models with FT⁴ gave similar results but the estimated effect proportions were smaller compared with those of FT³ (54% and 24%, respectively).

Conclusions: We showed that epigenetic modification at specific loci of the mitochondrial genome could intervene with the thyroid-dependent regulation of mitochondrial DNA copy numbers.

Keywords: DNA methylation, Epigenetics, Mitochondria, Mitochondrial DNA content, Placenta, Fetal thyroid hormones

Background

Thyroid hormones are known to have profound effects on mitochondrial energetics and biogenesis [1, 2]. Under the control of thyroid-stimulating hormone (TSH), the thyroid gland produces thyroxine (T₄), the major form of thyroid hormone, and triiodothyronine (T₃), the active form. On account of its biological activity, unbound or free T₃ (FT₃) regulates mitochondrial biogenesis most likely in two different cell compartments: (i) binding of FT₃ to thyroid receptors (TRs) that consecutively bind to response elements in the nucleus, activating the expression of mitochondria-related genes such as peroxisome proliferator-activated receptor γ-coactivator 1α (PPARGC1A) or (ii) direct binding to specific TRs in the mitochondrial matrix stimulating transcription of the mitochondrial genome [3].
The placenta is a relevant tissue to investigate the interplay between thyroid hormones and mitochondria. This metabolically active organ contains a high number of mitochondria and regulates nutrient, oxygen, and hormonal transfer, allowing for fetal growth. It is also well established that thyroid hormones are critical for placental [4] and fetal development [5]. FT₃ stimulates the production of factors that control trophoblast growth and development [6]. The placenta expresses two types of iodothyronine deiodinases (D2 and D3) that are capable of metabolizing FT₄ to FT₃ [7], and thus, plays an important role in thyroid hormone homeostasis.

Although some studies are questioning the presence of DNA methylation in the mitochondrial genome [8, 9], in the last few years, mtDNA methylation has emerged as a next-generation biomarker that might be a diagnostic tool for several diseases such as cardiovascular disease [10]. Here, we examined two specific mtDNA loci, i.e., the displacement loop (D-loop) and MT-RNR1. DNA methylation levels in the D-loop control region are suggested to play an important role in modulating either the replication or transcription of mtDNA since nearly the entire mitochondrial genome transcribes from this region [11]. Our other studied locus MT-RNR1 encodes for the 12S rRNA protein which is critical for normal function and integrity of the mitochondrial ribosome [12]. In this mother–newborn study, we aimed to elucidate possible mechanisms involved in the regulation of mitochondrial biogenesis by investigating the association between fetal FT₃, FT₄, and TSH with mtDNA content and mtDNA methylation in placental tissue. We hypothesized that methylation at specific loci of the mitochondrial genome could interfere with thyroid hormone-dependent regulation of mitochondrial biogenesis.

### Results

**Mother–newborn characteristics and demographics**
The study included 305 mother–child pairs (mean maternal age, 29.1 yr; range, 18–42 years). Demographic and prenatal lifestyle factors are reported in Table 1. Briefly, mean (10th–90th percentile) pre-pregnancy body mass index (BMI) of the participating mothers was 24.1 (19.7–29.7) kg/m². Fifty-two mothers (17.1%) reported to have smoked during pregnancy and smoked on average 7.8 cigarettes per day. Most women (64.9%, n = 198) never smoked cigarettes. The majority (>50%) of the mothers were highly educated. Nearly half of the newborn population were boys (n = 151; 49.5%). The overall mean gestational age was 39.2 weeks (10th–90th percentile 18–41) and included a vast majority of primiparous (51.8%, n = 158) or secundiparous (36.7%, n = 112) newborns.

| Characteristics | Mean (10th–90th percentile) or frequency (%) |
|-----------------|--------------------------------------------|
| **Mother**      |                                            |
| Age, years      | 29.1 (23–36)                               |
| Pre-pregnancy BMI, kg/m² | 24.1 (19.7–29.7)                        |
| Net weight gain, kg | 14.7 (7.5–22.0)                          |
| Maternal education<sup>a</sup> |                   |
| Low             | 37 (12.1%)                                 |
| Middle          | 107 (35.1%)                                |
| High            | 161 (52.8%)                                |
| Self-reported smoking habit |                 |
| Never smoker    | 198 (64.9%)                                |
| Cessation before pregnancy | 55 (18.0%)                        |
| Smoker during pregnancy | 52 (17.1%)                             |
| Parity          |                                           |
| 1               | 158 (51.8%)                                |
| 2               | 112 (36.7%)                                |
| ≥3              | 35 (11.5%)                                 |
| **Newborn**     |                                            |
| Sex             |                                           |
| Male            | 151 (49.5%)                                |
| Ethnicity<sup>b</sup> |                                     |
| European-Caucasian | 269 (88.2%)                         |
| Gestational age, weeks | 39.2 (38–41)                           |
| Season at delivery |                                         |
| Winter (Dec–Mar) | 80 (26.2%)                                 |
| Spring (Mar–Jun) | 89 (29.2%)                                 |
| Summer (Jun–Sep)| 60 (19.7%)                                 |
| Autumn (Sep–Dec)| 76 (24.9%)                                 |
| Apgar score after 5 min |                                    |
| 7 or 8          | 20 (6.6%)                                  |
| 9               | 90 (29.5%)                                 |
| 10              | 195 (63.9%)                                |
| Cord plasma insulin, pmol/L<sup>c</sup> | 46.4 (15.3–85.4)          |
| Birth weight, g  | 3421 (2880–3985)                           |

<sup>a</sup>Mother’s education: low (no high school diploma), middle (high school diploma), high (college or university diploma)
<sup>b</sup>Based on the native country of the newborn’s grandparents. European-Caucasian when two or more grandparents were European, or non-European when at least three grandparents were of non-European origin
<sup>c</sup>Geometric mean

### Levels of fetal thyroid hormones, placental mtDNA methylation, and mtDNA content

The geometric means (10th–90th percentile) of thyroid hormone levels in cord blood were 2.63 (2.15–3.22) pmol/L for FT₃, 15.66 (13.26–18.53) pmol/L for FT₄, and 11.65 (5.16–20.59) mU/L for TSH (Table 2). A positive correlation was observed between the FT₃ and FT₄
values ($r = 0.25; p < 0.0001$) and between the FT$_3$ and TSH values ($r = 0.19; p = 0.0008$) (Fig. 1).

Table 2 also shows mtDNA methylation and mtDNA content values measured in placental tissue. Placental mtDNA methylation averaged (10th–90th percentile) 9.51% (4.46–14.67) for MT-RNR1 and 3.61% (1.94–5.24) for D-loop. The methylation levels of MT-RNR1 and D-loop were strongly correlated ($r = 0.85, p < 0.0001$) (Fig. 1 and Additional file 1: Figure S1). The geometric mean of placental mtDNA content was 1.11 (0.49–2.49) (unitless).

In this subset, and as previously published [13], we report a strong inverse correlation between placental mtDNA methylation and mtDNA content ($r = -0.63, p < 0.0001$ for MT-RNR1 and $r = -0.40, p < 0.0001$ for D-loop) (Fig. 1).

Association of fetal thyroid hormones with placental mtDNA methylation and mtDNA content

Both fetal FT$_3$ and FT$_4$ were inversely correlated with mtDNA methylation at the MT-RNR1 and D-loop region, whereas a positive correlation was observed for both hormones with mtDNA content in placental tissue (see Fig. 1 for correlations). Fetal TSH levels did not correlate with either placental mtDNA methylation or mtDNA content.

The associations remained significant after adjustment for maternal age, pre-pregnancy BMI, gestational age, newborn’s sex, smoking status, parity, maternal education, ethnicity, and cord plasma insulin level. A 10th–90th percentile increment (53%) in cord blood FT$_3$ (log$_{10}$ values) was associated with a lowering in absolute methylation of $-1.50\%$ (95% CI $-2.70$ to $-0.30\%$, $p = 0.01$) for MT-RNR1 and $-0.46\%$ (95% CI $-0.83$ to $-0.09\%$, $p = 0.02$) for D-loop. A similar association, but with smaller estimates, was observed for FT$_4$ (Table 3). On the other hand, a 10th–90th percentile increment in cord blood FT$_3$ (53%) or FT$_4$ (13%) was associated with a higher placental mtDNA content (relative change) of $+20.35\%$ (95% CI 0.47 to 44.17%, $p = 0.04$) for FT$_3$ and $+11.04\%$ (95% CI 4.00 to 18.54%, $p = 0.002$) for FT$_4$.

Mediation analysis

We performed mediation analysis to estimate the proportion of the associations between cord blood thyroid hormones and placental mtDNA content that might be mediated by changes in mtDNA methylation if the underlying causal assumptions of the mediation analysis are valid. We selected both MT-RNR1 and D-loop as potential mediators because methylation levels at both regions were significantly associated with FT$_3$ and FT$_4$ as well as with placental mtDNA content (Fig. 1). While adjusting for the aforementioned variables, we estimated that MT-RNR1 methylation mediated 77% [indirect effect $+14.61\%$ (95% CI 2.64 to 27.98%, $p = 0.01$)] and D-loop methylation mediated 47% [indirect effect $+8.60\%$] of the association between FT$_3$ and placental mtDNA content.
(95% CI 1.23 to 16.50%, \( p = 0.02 \)) of the positive association between FT3 and placental mtDNA content (Fig. 2). Mediation models with FT4 gave similar results but the estimated effect proportions were smaller compared with those of FT3 (54 and 24%, respectively) (Additional file 1: Figure S2).

Sensitivity analysis

Certain CpGs were more associated with fetal thyroid hormones compared to others, underscoring the importance of methylation levels at specific CpG (Additional file 2: Table S1). For example, FT3 was associated with methylation levels at all three CpG sites of D-loop (the transcription start site of the mitochondrial genome).

Excluding women who had undergone a cesarean section (\( n = 13 \)) slightly increased our effect estimates for FT3 (Additional file 2: Table S2), whereas excluding small for gestational age infants (\( n = 22 \)) slightly decreased our effect estimates for FT3 (Additional file 2: Table S3). However, we verified whether the association between fetal thyroid hormones and placental mtDNA content was still present in the initial group of 547 mother–child pairs (flowchart Additional file 1: Figure S3). Adjusting for the aforementioned variables, the association between cord blood FT3 and placental mtDNA content was stronger compared with the reported association in the main analysis, i.e., relative change of +29.22% (95% CI 16.22 to 43.67%, \( p < 0.0001 \)) for a 10th–90th percentile increment in cord blood FT3, whereas the association for FT4 was less (+6.65%, 95% CI 2.23 to 11.26%, \( p = 0.003 \)).

Furthermore, additional adjustments for cord blood plasma estradiol (\( n = 304 \)), passive indoor tobacco smoke exposure (\( n = 295 \)), alcohol consumption (\( n = 295 \)), or pH of the arterial cord blood (indicator of hypoxemia) (\( n = 264 \)), did not alter our associations between fetal thyroid hormones and mtDNA content or mtDNA methylation (data not shown).

Mitochondrial biogenesis relies on a tightly coordinated process between the nuclear and mitochondrial genome. For example, the nuclear gene PPARGC1A is a central regulator of mitochondrial gene expression and biogenesis that is also controlled by FT3 [14]. We observed an inverse association between fetal FT3 levels and placental PPARGC1A promoter methylation, i.e., −2.31% (95% CI −4.04 to −0.59, \( p = 0.009 \)) for a 10th–90th percentile increment in FT3 (Additional file 2: Table S1). No association was observed between placental PPARGC1A promoter

### Table 3: Associations of placental mtDNA methylation and content with cord blood thyroid hormones

| Variable          | FT3, pmol/L | FT4, pmol/L | TSH, mU/L |
|-------------------|-------------|-------------|-----------|
|                   | \( \beta \) (95% CI) | \( \beta \) (95% CI) | \( \beta \) (95% CI) |
| mtDNA methylation |             |             |           |
| MT-RNR1           | -1.50 (-2.70 to -0.30) | -0.60 (-1.04 to -0.16) | 0.38 (-0.21 to 0.97) |
| D-loop            | -0.46 (-0.83 to -0.09) | -0.14 (-0.28 to -0.002) | 0.11 (-0.07 to 0.29) |
| mtDNA content     | 20.35 (0.47 to 44.17) | 11.04 (4.00 to 18.54) | -5.34 (-13.35 to 3.40) |

\( \beta \) represents an absolute change in placental mtDNA methylation percentage (%) for a 10th-90th percentile increment of cord blood thyroid hormone.

\( \beta \) represents a relative change (%) in placental mtDNA content for a 10th-90th percentile increment of cord blood thyroid hormone.

All models are adjusted for maternal age, pre-pregnancy BMI, gestational age, newborn’s sex, smoking status, parity, maternal education, ethnicity, and cord plasma insulin level.

\(* p\)-value < 0.05, \(** p\)-value < 0.005.
methyltransferases making the mtDNA less accessible to structural changes making the mtDNA less accessible to DNA methylation and mtDNA content \( (p = 0.23) \) or mtDNA methylation \( (D\text{-}loop, p = 0.54; \text{MT-RNR1}, p = 0.74) \).

**Discussion**

In this study, we report for the first time associations between fetal thyroid hormones and epigenetic modification at specific loci in the mitochondrial genome that could, at least in part, mediate the fetal thyroid-dependent regulation of mitochondrial biogenesis in placental tissue.

From mid-gestation onwards, the fetus starts secreting small amounts of thyroid hormones in addition to the transplacental supply of maternal thyroid hormones [15]. The optimal concentration of fetal thyroid hormones at each stage of development is maintained by the placenta, in conjunction with the fetal thyroid gland, liver, and kidneys [4]. The placenta expresses two types of iodothyronine deiodinases (D2 and D3) that are capable of metabolizing FT3 and FT4 [7], and thus, plays an important role in thyroid hormone homeostasis. Besides regulating fetal development [5], thyroid hormones have profound effects on mitochondrial energetics and biogenesis [1, 2].

Despite the spectacular progress in the knowledge of the T4-T3 nuclear pathway, a clear answer to how it regulates mitochondrial biogenesis is lacking. While it is known that an upregulation of nuclear-encoded respiratory genes including PPARGC1A occurs within hours after injection of T3 in hypothyroid rats, other non-genomic direct effects are detectable within minutes suggesting another mode of action [16, 17]. T3 binds directly to specific receptors inside the mitochondria. A 43 kDa c-Erb A alpha1 protein (p43), located exclusively in the mitochondrial matrix, acts as a T3-dependent transcription factor and specifically binds to four mitochondrial DNA sequences with a high similarity to nuclear T3 response elements [18, 19]. Overexpression of p43 increases mitochondrial genome transcription and protein synthesis, stimulating mitochondrial biogenesis in a T3-dependent manner [19]. Interestingly, of the four mitochondrial DNA sequences, two response elements are located in the D-loop region and one in the MT-RNR1 gene. We hypothesized that DNA methylation at these mitochondrial hotspots could interfere with thyroid hormone-dependent regulation of mitochondrial biogenesis. First, we have shown that fetal thyroid hormones, especially FT3, are positively associated with placental mtDNA content and inversely associated with placental methylation levels at the D-loop and MT-RNR1 region. Next, we underscored a substantial mediating role of placental mtDNA methylation between the association of fetal thyroid hormones and placental mtDNA content. Hence, we postulate that high DNA methylation levels in the mitochondrial genome are related to conformational or structural changes making the mtDNA less accessible to proteins and transcription factors such as the T3-dependent transcription factor p43. Previously, we have shown that exposure to particulate matter (PM2.5) during pregnancy is associated not only with changes in fetal thyroid hormones [20] but also with higher levels of methylation of the D-loop and the MT-RNR1 sequence, affecting mtDNA content in placental tissue [13]. An increase in mtDNA methylation was observed in blood leukocytes of steel workers exposed to air pollution [21] and in an experimental study of human cultured cells treated with ethidium bromide [22]. The ethidium-bromide-exposed cells recovered from mtDNA depletion and showed increased overall methylation levels indicating that mtDNA was less packed with proteins during active mtDNA replication [22].

Our mediation analysis revealed that the effect of FT3 on mtDNA content is mediated for 77% by MT-RNR1 and for 54% by D-loop methylation levels, meaning that there are alternative routes, most likely nuclear pathways, by which thyroid hormones exert their action on mitochondrial biogenesis. T3 controls the expression of PPARGC1A [16], a transcriptional co-activator of several nuclear-encoded transcription factors including mitochondrial transcription factor A (TFAM) that regulates mitochondrial biogenesis [23] (Fig. 3). In addition to specific mitochondrial actions of FT3, we also observed an inverse association between placental PPARGC1A promoter methylation and fetal FT3 levels. Most likely this is indicative of upregulated PPARGC1A mRNA levels in the presence of high FT3 concentrations since promoter hypomethylation is usually associated with increased gene expression due to unwound DNA that is available for transcription factors. Interestingly, our interrogated region is a putative binding site for CREB transcription factors. The transcriptional activity of CREB is critical for the establishment and maintenance of energy homeostasis in mice neonates [24] and appears to be involved in the regulation of PPARGC1A. It is noteworthy to mention that the promoter region of PPARGC1A was largely unmethylated in cord blood but not in placental tissue [25], suggesting differential regulation of PPARGC1A in cord blood and placental tissue. Even though nuclear-encoded genes that are involved in mitochondrial function are regulated by epigenetic mechanisms [26], it would be interesting to determine the direct functional significance at mRNA expression level in response to FT3 levels. Unfortunately, we did not measure mRNA expression due to lack of suitable RNA samples and consider this a limitation of the study. We also have no clear information on “threshold” changes in mtDNA methylation that lead to functional effects of mitochondrial biogenesis. The mitochondrial genome, as far as we observed, shows a far less DNA methylation level (range 0 to 40%) compared with nuclear DNA (range 0 to 100%). Hence, small changes in mtDNA methylation might still
have functional effects. It has been shown that small changes in single CpG sites and regional methylation changes interfere with gene/protein expression [27] by altering the affinity of transcription factors to their binding sites or chromosome looping events [28]. Even when small changes in methylation would not impact functional effects, mtDNA methylation can provide invaluable help in developing new and useful markers of exposure and disease [29]. However, our findings should first be confirmed in an experimental study and we need to investigate the health consequences in later life before we can translate it into a clinical meaning.

We are aware of the issue of placental tissue heterogeneity but it is difficult to assess the impact of this variation on our methylation results. To our knowledge, there is only one study that explored genome-wide DNA methylation patterns in the two main cell types of the placenta (cytotrophoblasts and fibroblasts) [30]. These cell types did not demonstrate genome-wide differentiation in DNA methylation but some specific genes showed differential promoter methylation. Although these authors demonstrated that the methylation profile of the placenta is mainly driven by cytotrophoblasts, there is a possibility that our epigenetic data is confounded by variation in cell type distributions. To overcome this obstacle, one should correct for differences in tissue composition using algorithms that estimate cell type proportions based on DNA methylation signatures derived from Illumina microarrays [31, 32]. However, statistical adjustments of DNA methylation data for cell distributions require the availability of reference epigenomes for the component cell types created by cell sorting [33]. Currently, there is no algorithm available to estimate the cell type proportions in placental tissue. Another limitation of our study is that we were not able to measure thyroid hormones in placental tissue.
but we used cord blood levels as a proxy since it circulates through the fetal side of the placenta.

Conclusions
Given our epidemiological findings and other experimental research data, it seems that there exist coordinated events between mtDNA methylation, mtDNA content, and thyroid hormones. We summarized our findings in a simplified scheme (Fig. 3) indicating that epigenetic modification at specific loci of the mitochondrial genome could intervene with thyroid-dependent regulation of mitochondrial biogenesis. Our findings could contribute to further epidemiological understanding of mitochondrial disorders. Whether alterations in mitochondrial function or newborn’s thyroid hormone levels have health consequences later in life should be elucidated.

Methods
Study population
Within the on-going ENVIRONAGE birth cohort (ENVIRonmental influence ON early AGEing) [34], we conducted our investigation in a group of 589 singleton pregnancies for which the mothers agreed withdrawal of cord blood, the collection of the placenta after delivery, and the use of information from their medical files. The placenta could not be collected for ten newborns, four placentas had insufficient DNA yield, 12 placentas had missing measurements of mtDNA content, and 16 mothers with thyroid gland complications were excluded. Because mtDNA methylation was measured only in a subset of this large study sample (n = 547), we ended with a final sample size of 305 newborns for the main analysis (see flowchart; Additional file 2: Table S4). PCR amplification of regions of interest prior to pyrosequencing was performed in a total reaction volume of 30 µl, using 15 µl GoTaq Hot Start Start Green Master Mix (Promega, Madison, WI, USA), 10 pmol forward primer, 10 pmol reverse primer, 1 µl bisulfite-treated genomic DNA, and water. PCR products were purified and sequenced by pyrosequencing using the PyroMark Q96 MD Pyrosequencing System (Qiagen, Inc., Germantown, MD, USA). The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Samples were run in duplicate on two different plates from which the average methylation levels were used. The coefficient of
variation was 3.7% for MT-RNR1, 5.8% for D-loop, and 8.1% for PPARGC1A. The efficiency of the bisulfite-conversion process was assessed using non-CpG cytosine residues within the sequence. We used 0% (PSQ-T oligo: 5′-TTGCAGATACGCGGAAACACGGTTGAATTC-3′) and 100% (PSQ-C oligo: 5′-TTGCGATACGCGGAAACACGGTTGAATTC-3′) DNA methylation control oligos. The sequencing primer for the control oligo was 5′-AAACGTTGAATTC-3′. We mixed the PSQ-C oligo (or PSQ-T oligo) with the sequencing entry in PyroMark Annealing Buffer (Qiagen, Inc., Valencia, CA, USA) and performed pyrosequencing with the sequencing oligo in PyroMark D-loop (AACGTTTGTTCCCGT-3′). We determined the ratio of two mitochondrial gene copy numbers (MTT3212/R3319 and MT-ND1) to two single-copy nuclear control genes (RPLP0 and ACTB) using a quantitative real-time polymerase chain reaction (qPCR) assay as previously described [35] but with small modifications. Briefly, 2.5 μl diluted genomic DNA (5 ng/μl) was added to 7.5 μl mastermix consisting of Fast SYBR Green I dye 2× (5 μl/reaction), forward and reverse primer (each 0.3 μl/reaction), and RNase free water (1.9 μl/reaction). Primer sequences (Additional file 2: Table S5) were diluted to a final concentration of 300 nM in the master mix. Samples were run in triplicate in 384-well format. Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling profile: 20 s at 95 °C (activation), followed by 40 cycles of 1 s at 95 °C (denaturation) and 20 s at 60 °C (annealing/extension), ending with melting curve analysis (15 s at 95 °C, 15 s at 60 °C, 15 s at 95 °C). qBase software (Biogazelle, Zwijnaarde, BE) was used to normalize data and correct for run-to-run differences [36].

mtDNA content analysis

mtDNA content was measured in placental tissue by determining the ratio of two mitochondrial genome copy numbers (MTT3212/R3319 and MT-ND1) to two single-copy nuclear control genes (RPLP0 and ACTB) using a quantitative real-time polymerase chain reaction (qPCR) assay as previously described [35] but with small modification. Briefly, 2.5 μl diluted genomic DNA (5 ng/μl) was added to 7.5 μl mastermix consisting of Fast SYBR Green I dye 2× (5 μl/reaction), forward and reverse primer (each 0.3 μl/reaction), and RNase free water (1.9 μl/reaction). Primer sequences (Additional file 2: Table S5) were diluted to a final concentration of 300 nM in the master mix. Samples were run in triplicate in 384-well format. Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling profile: 20 s at 95 °C (activation), followed by 40 cycles of 1 s at 95 °C (denaturation) and 20 s at 60 °C (annealing/extension), ending with melting curve analysis (15 s at 95 °C, 15 s at 60 °C, 15 s at 95 °C). qBase software (Biogazelle, Zwijnaarde, BE) was used to normalize data and correct for run-to-run differences [36].

Statistical analysis

For database management and statistical analysis, we used the SAS software program (version 9.4; SAS Institute Inc., Cary, NC, USA). mtDNA content and thyroid hormone levels were log10-transformed to improve normality. For each subject, we measured in placental tissue methylation levels of CpG sites at two regions of the mitochondrial genome and at the promoter region of PPARGC1A. The pyrosequencing-based DNA methylation analysis produced a methylation value (%) for each CpG of MT-RNR1 (two CpGs), the D-loop region (three CpGs), and PPARGC1A (three CpGs). In the main analysis, we used the average methylation levels of the different CpGs. Pearson correlation coefficients were calculated between the different thyroid hormone levels in cord blood (FT3, FT4, and TSH), mtDNA methylation (MT-RNR1 and D-loop), and mtDNA content in placental tissue using R software packages. We performed multiple linear regression to determine the association between thyroid hormones and placental mtDNA methylation (both MT-RNR1 and D-loop) and between thyroid hormones and placental mtDNA content. Thyroid hormones were fitted as linear variables in the models, and effect estimates on mtDNA methylation and mtDNA content were calculated for a 10th–90th percentile increment in thyroid hormones, which corresponds to a 52% change in FT3, a 12% change in FT4, and a 84% change in TSH. All models were adjusted for maternal age, pre-pregnancy BMI, gestational age, newborn's sex, smoking status, parity, maternal education, ethnicity, and cord plasma insulin level. The Shapiro-Wilk statistic and Q-Q plots of the residuals were used to test the assumptions of all linear models.

We used mediation analysis to investigate potential associations that may underlie the relation between the exposure variable (FT3 or FT4) and the continuous outcome variable (mtDNA content) by examining how they relate to a third variable, the mediator (mtDNA methylation) [37]. We accomplished this by decomposing the total effect into a direct effect (DE; exposure effect on outcome at a fixed level of the mediator) and an indirect effect (IE; exposure effect on outcome that operates through the mediator).

Sensitivity analysis

To underscore the importance of single CpG sites, we performed separate linear regression analyses between levels of FT3 or FT4 and the methylation levels at each CpG site of MT-RNR1 and D-loop. Furthermore, it is known that cord blood thyroid hormone levels are influenced not only by different external factors such as fetal stress exemplified by cesarean section [38] but also by other factors such as cord plasma estradiol [39], passive smoking [40], alcohol consumption [41], and pH of arterial cord blood [42]. Besides adjusting the main model with the aforementioned variables, we performed additional analyses in which we excluded women who had undergone a cesarean section or excluding infants born with a birth weight less than the 10th percentile. Lastly, we used linear regression analyses to investigate the association between fetal thyroid hormones and placental promoter methylation of the nuclear gene PPARGC1A.
Additional file 1: Additional tables. Table S1. Associations of cord blood thyroid hormones FT3 and FT4 with CpG-specific mtDNA methylation and PPARGC1A promoter methylation. Table S2. Associations of fetal thyroid hormones with placental mtDNA methylation and mtDNA content while excluding women who had undergone a cesarean section (n = 13). Table S3. Associations of fetal thyroid hormones with placental mtDNA methylation and mtDNA content while excluding small for gestational age infants (n = 22). Table S4. Bisulfit- pyrosequencing primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser. Table S5. Mitochondrial and nuclear primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser. (DOCX 54 kb)

Availability of data and materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Funding

The ENVIRANGE birth cohort is supported by the European Research Council (ERC-2012-StG-306908), by the Flemish Scientific Fund (FWO, G.0.733.15.N), and the Special Research Fund (BOF) of Hasselt University. This work was also supported by funding from the National Institute of Environmental Health Sciences (R21ES022694 and R01ES021733).

Acknowledgements

The authors thank the participating mothers and neonates, as well as the staff of the maternity ward, midwives, and the staff of the clinical laboratory of East-Limburg Hospital in Genk.

Authors’ contributions

TSN coordinates the ENVIRANGE birth cohort and designed the current study together with BGJ and AAB. WG and BGJ gave guidance to the midwives and did the quality control of the database. BGJ performed the experiments with the help of HMB and BGJ carried out the statistical analysis. JP performed the thyroid hormone analysis. BGJ, HMB, AAB, and TSN did the interpretation of the data. BGJ wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All procedures were according to the principles outlined in the Helsinki Declaration for investigation of human subjects and approved by the Ethical Committee of Hasselt University and the East-Limburg Hospital. All participants provided written informed consent.

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Received: 21 September 2016 Accepted: 12 June 2017

Published online: 21 June 2017

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