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Effect of sex hormones on n-3 polyunsaturated fatty acid biosynthesis in HepG2 cells and in human primary hepatocytes

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Abstract

Female humans and rodents have been shown to have higher 22:6n-3 status than males. Sex hormones are involved, but it is unclear whether higher 22:6n-3 status and biosynthesis in females is mediated by the action of estrogen or progesterone. We investigated the specificity of the effects of physiological concentrations of sex hormones on the mRNA expression of genes involved in polyunsaturated fatty acid (PUFA) biosynthesis and on the conversion of [d5]-18:3n-3 to longer chain fatty acids in HepG2 cells. Progesterone, but not 17 α -ethynylestradiol (EE₂) or testosterone, increased the mRNA expression of FADS2, FADS1, ELOV1 5 and ELOV1 2. Progesterone induced similar changes in primary human hepatocytes, although the increase in expression was only significant for FADS2. This was accompanied by reduced methylation of specific CpG loci in the FADS2 promoter located within a putative cAMP response element binding protein binding domain in HepG2 cells. Progesterone, but not EE₂ or testosterone, increased conversion of [d5]-18:3n-3 to 20:5n-3, 22:5n-3 and 22:6n-3. Together these findings show that progesterone up-regulates n-3 PUFA biosynthesis by increasing the mRNA expression of genes involved in this pathway, possibly via changes in the epigenetic regulation of FADS2, in human liver cells *in vitro*.

Keywords

Omega-3 polyunsaturated fatty acids; eicosapentaenoic acid; docosahexaenoic acid; estrogen; progesterone; testosterone; FADS; elongase

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6. Author contributions

GCB and KAL designed the study. CMS, SPH, R C-H, PL and JTB carried out the experiments and analysed the data. GCB wrote the first draft of the manuscript with subsequent input from all authors. The authors declare no conflict of interest.

1. Introduction

The relative proportions of polyunsaturated fatty acids (PUFA) are an important influence on cell function as they influence the biophysical properties of the phospholipid bilayer and provide substrates for phospholipase-mediated signalling pathways. 20:5n-3 and 22:6n-3 can be obtained preformed from dietary sources such as oily fish. However, most mammalian species have some capacity for the synthesis of these fatty acids from the n-3 essential fatty acid 18:3n-3 which is present in specific plant oils. Such molecular transformation involves desaturation at the 6 position by 6 desaturase to form 18:4n-3, elongation of the carbon chain by elongase-2 activity to form 20:4n-3, and desaturation at the 5 position by 5 desaturase to form 20:5n-3(1). Conversion of 20:5n-3 to 22:6n-3 involves further elongation by elongase -2 or -5 activity to form 22:5n-3 and elongation by elongase-2 activity to form 24:5n-3. 24:6n-3 is then formed by desaturation at the 6 position by 6 desaturase. 24:6n-6 is then translocated to peroxisomes where one cycle of fatty acid β -oxidation converts 24:6n-3 to 22:6n-3 which is then returned to the endoplasmic reticulum(1).

Sex appears to be an important influence on 22:6n-3 status and capacity for synthesis of 20:5n-3 and 22:6n-3 from 18:3n-3 in humans. A meta-analysis of 51 studies of the PUFA content of plasma, erythrocyte or adipose tissue in 8,541 subjects has shown the proportion of 22:6n-3 to be 37% lower in men than women (2). In addition, a meta-analysis of 2,907 subjects also showed the proportion of 22:6n-3 to be 47% lower in men than in women (3). Such sex differences in 22:6n-3 status appear to be independent of dietary background (1, 4). Female rodents have also been shown to have higher plasma and liver 22:6n-3 status than males (5–8). Studies in which dietary 18:3n-3 intake was increased in men have shown a dose-related increase in 20:5n-3 and 22:5n-3, but not in 22:6n-3. This implies constraint in the reactions that follow 22:5n-3 synthesis (5). The effect of 18:3n-3 supplementation on 20:5n-3 and 22:6n-3 status in women is not known. Studies that have used 18:3n-3 labelled with stable isotopes to measure its conversion to longer chain metabolites have confirmed the findings of the observational and dietary intervention studies which showed that women were shown to convert more 18:3n-3 to 22:6n-3 than men(9–11). In rats, the mRNA expression of Fads2, which encodes 6 desaturase, and Fads1, which encodes 5 desaturase, have been shown to be higher in the liver of females than males (6, 12) which demonstrates that at least part of the effect of sex hormones on n-3 PUFA metabolism is mediated through differences in mRNA expression. Together the findings of these studies indicate that sex hormones play a central role in the regulation of PUFA synthesis and status.

There have been relatively few studies of the effects of sex hormones on 22:6n-3 status or synthesis. Women who use an oral contraceptive pill have higher 22:6n-3 status (13) and greater conversion of 18:3n-3 to 22:6n-3 than those who do not. Male transsexuals undergoing hormone therapy treated with synthetic estrogens combined showed increased 22:6n-3 levels in blood compared to untreated men, while those treated with cyproterone acetate, and anti-androgen and weak progesterone mimetic, showed no difference to untreated men (13). Treatment of postmenopausal women with conjugated estrogens and medroxyprogesterone acetate also increased 20:4n-6 and 22:6n-3 levels in blood compared to untreated individuals (14). Furthermore, female transsexuals who were treated with

testosterone showed a reduction in 22:6n-3 status (13). Such findings point to a specific positive effect of females sex hormones estrogens on 22:6n-3 synthesis. However, it is not clear which hormone is involved. One recent study in rats has shown that the increase in 22:6n-3 status associated with pregnancy was associated positively with progesterone concentration (6). Such effects were associated with increased mRNA expression of Fads2. These findings imply that the regulation of PUFA synthesis by sex hormones involves effects on the transcription of genes that encode key genes in this pathway.

In order to investigate the specificity of the effects of hormones on the regulation of n-3 PUFA synthesis, we treated the human hepatocarcinoma cell line HepG2 with a physiologically relevant estrogen (17 α -ethynylestradiol (EE₂) which has similar potency to naturally occurring 17 β -estradiol (15)), progesterone and testosterone. Because sex hormones have been shown previously to alter Fads2 mRNA expression in rat liver, we measured the effect of treatment with these hormones on the mRNA expression of FADS and ELOVL genes. We then determined whether any changes in mRNA levels were associated with altered synthesis of 20:5n-3 and 22:6n-3. In addition, we investigated whether any effects of sex hormones on genes involved in n-3 PUFA metabolism in HepG2 cells were also induced in primary human hepatocytes.

2. Materials and methods

2.1 Materials

HepG2 cells and primary hepatocytes were obtained from ECACC and Invitrogen. [17,17,18,18,18-d₅]-18:3n-3 (98%) was purchased from Cambridge Isotope Laboratories. Primers for real time PCR were from Biomers (FADS2) and Qiagen (FADS1, ELOVL2, ELOVL5 and cyclophilin). Pyrosequencing and PCR and sequencing primers were synthesised by Biomers. All other reagents were obtained from Sigma and PAA, with noted exceptions.

2.2 Cell culture

Cells were maintained at 37°C under 5% CO₂. HepG2 cells were grown in DMEM-high glucose (Sigma) supplemented with 2 mM L-glutamine, penicillin/streptomycin (100 U/ml and 100 ug/ml, respectively) and 10% (v/v) fetal bovine serum. HepG2 cells were transferred to 90 mm culture dishes (Greiner) and left to adhere overnight before treatment with hormones. Primary hepatocytes were grown as instructed by the manufacturers (Invitrogen). All media was warmed to 37°C and primary hepatocytes were thawed in CHRM medium at 37°C and then added to collagen coated (5 ug/cm²) 6 well plates (Greiner) in 2 ml of plating medium (Williams E medium containing: 5% Fetal Bovine Serum, 1 μ M DMSO and 18 ml of thawing/plating cocktail) per well. The plates were incubated for 4–6 hours after which they were agitated to loosen debris and the media was then removed and replaced with incubation medium (Williams E medium containing: 0.1 μ M of DMSO and 20 ml of cell maintenance cocktail). Primary hepatocytes could not be sub-cultured so were incubated overnight and then used directly in experiments.

HepG2 cells were incubated for 72 hours with EE₂ (0.07, 0.7 or 7 nmoles/l), progesterone (10, 25 or 50 nmoles/l) or testosterone (10, 25 or 50 nmoles/l) or no hormone supplement

(untreated). Primary human hepatocytes were incubated for 72 hours with the highest concentration of EE₂ or progesterone, 7 nM and 50 nM respectively or remained untreated. The media and hormone supplements were replenished daily. These concentrations of hormone were used as they represent physiological relevant concentrations observed in plasma.

2.3 Measurement of mRNA expression

Total RNA was isolated from cells using Tri-reagent (Sigma). Complementary DNA was prepared and amplified using real-time RT PCR, which was performed using SYBR Green Jumpstart Ready Mix (Sigma) in a final volume of 25 µl. Samples were analysed in duplicate and expression was normalized to the housekeeping gene cyclophilin. Cycle parameters were 95°C for 2 minutes then 40 cycles of 95°C for 30 seconds, 55°C (cyclophilin, FADS1, ELOVL2 and ELOVL5) or 52°C (FADS2) for 1 minute and 72°C for 1 minute. Expression was determined by the standard curve method.

2.4 Measurement of DNA methylation

HepG2 cells were incubated for 72 hours with 50 nmoles/l progesterone or left untreated. Cells were removed from plates in HBSS and cell pellets were then frozen for subsequent DNA extraction. Bisulfite conversion was carried out using the EZ DNA methylation kit (ZymoResearch). The pyrosequencing reaction was carried out using primers listed in Table 1. Modified DNA was amplified using KAPA2G Robust Hot Start Taq DNA polymerase (Labtech). PCR products were immobilised on streptavidin–sepharose beads (GE Healthcare UK Ltd.), washed, denatured and released into annealing buffer containing the sequencing primers. Pyrosequencing was carried out using the SQA kit on a PSQ 96MA machine (Biotage, Uppsala, Sweden), and the percentage methylation was calculated using the Pyro Q CpG software (Biotage).

2.5 Measurement of PUFA synthesis

HepG2 cells were incubated for 48 hours with 10 µmoles/l [d₅]-18:3n-3 and EE₂ (7 nmoles/l), progesterone (50 nmoles/l) and testosterone (50 nmoles/l) or left untreated. Cells were scraped from plates 0.9% (w/v) NaCl (0.8ml). Total lipids were extracted with chloroform and methanol (16) and converted to fatty acid methyl esters (FAMES) by incubation with methanol containing 2% (v/v) sulphuric acid (17). The concentration of individual n-3 PUFA was determined by gas chromatography (18). Incorporation of deuterium label into n-3 PUFA was determined by gas chromatography-mass spectrometry (19). Briefly, ions corresponding to the d₀ and d₅ isotopologues of the four major n-3 fatty acids (18:3, 20:5, 22:5, and 22:6) were integrated. Ratios of the d₅ to d₀ were calculated and used as measures of net conversion over the 48 h incubation period. Total cell protein was extracted from a 10 µl aliquot of cells in 0.9% (w/v) NaCl by Pierce® BCA Protein Assay kit (Thermo Scientific).

2.6 Statistical analysis

Statistical analysis was carried out using SPSS (Version 21.0, IBM, Armonk, NY: IBM Corp.). Data from dose-response experiments was analysed by 1-way ANOVA with

Dunnett's post hoc test to compare the effect of each concentration of hormone to control. Experiments involving single concentrations of hormones were analysed by Student's unpaired t test. Associations between data sets were tested using linear regression analysis. Statistical significance was assumed at probabilities less than 0.05. The sample size of ten replicates was sufficient to detect a 10% difference in the primary outcome measures, mRNA expression and synthesis of deuterated fatty acids, with a statistical power of 85% and probability of $P < 0.05$.

3. Results

Treatment of HepG2 cells with progesterone for 48 hours induced a linear ($r = 0.67$, $P < 0.0001$) dose-related increase in FADS2 mRNA expression which reached statistical significance compared to control at 25 nM (2.1-fold) and 50 nM (Figure 1). However, maximum mRNA levels of FADS1, ELOVL 5 and ELOVL 2 were induced at the lowest concentration of progesterone tested (10 nmole/l); 2.1-fold, 2.5-fold and 3.9-fold, respectively (Figure 1). There was no significant effect of treatment with EE₂ or testosterone on the mRNA expression on any of the genes measured in HepG2 cells (Figure 1).

In order to investigate whether the effect of sex hormones of FADS and ELOVL gene expression in HepG2 cells could be induced in primary human hepatocytes, these cells were treated with the highest concentration of progesterone or EE₂ to which HepG2 cells were exposed. Because of the slow replication rate of primary hepatocytes, sufficient cells could not be generated to test the effect of testosterone. Treatment of primary human hepatocytes with progesterone induced a significant increase in FADS2 mRNA expression (2.4-fold) and non-significant ($P < 0.1$) trends towards higher mRNA expression of FADS1, ELOVL5 and ELOVL 2 (Figure 2). There was no significant effect of treatment with EE₂ on the expression of any of the genes measured in primary hepatocytes (Figure 2).

We measured the methylation status of a region between -1661 and -18 bp upstream of the FADS2 transcription start site (Figure 3). The region was characterised by highly methylated CpG loci between -1661 and -1156 bp, loci with intermediate methylation at loci -718 and -669 and an essentially unmethylated region (average methylation $< 5\%$) proximal to the transcription start site (Figure 4). Treatment of HepG2 cells with progesterone (50 nmoles/l) induced reduction in the methylation status of specific CpG dinucleotides located at -1661 (3%, $P = 0.02$) and -1665 (14%, $P = 0.007$) bp relative to the transcription start site (Figure 4).

In order to determine whether the changes in gene expression were associated with altered activity of the PUFA synthesis pathway, HepG2 cells were incubated with [d₅]-18:3n-3 in the presence or absence of sex hormones. Progesterone induced a significant decrease (38%) in the amount of labeled 18:3n-3 in cell total lipids, and significant increases in the amounts of labeled 20:5n-3 (5-fold), 22:5n-3 (7-fold) and 22:6n-3 (36-fold) (Figure 5). There was no significant effect of treatment with EE₂ or testosterone on the incorporation of stable isotope label into any of the n-3 PUFA that were measured (Figure 5).

4. Discussion

Although there is compelling evidence from studies of humans and of rodents that males and females differ in n-3 PUFA status, in particular 22:6n-3, and in capacity for conversion of 18:3n-3 to 22:6n-3, there remains uncertainty in nature of the endocrine regulation of the PUFA biosynthesis. While some studies have implicated estrogen as an agonist (9, 13), there is also evidence for positive regulation by progesterone (6). In contrast, testosterone appears to antagonise conversion of 18:3n-3 to its longer chain metabolites (13). The findings of this study provide evidence that progesterone is an endocrine agonist for n-3 PUFA biosynthesis and that such effects are mediated at the level of transcription.

Treatment of HepG2 cells with physiological concentrations of progesterone induced an increase in the mRNA expression of FADS2 and 1, and ELOVL 5 and 2, while there was no significant effect of treatment with EE₂ and testosterone on the level of these transcripts. Treatment of primary hepatocytes with progesterone induced a significant increase in FADS2 mRNA expression, while there were only non-significant trends towards higher expression of FADS1 and ELOVL 2 and 5. Together these findings suggest that while progesterone acts as an agonist for PUFA biosynthesis in both the cancer cell line and in primary hepatocytes, FADS1 and ELOVL 2 and 5 appear to be less responsive to progesterone in primary hepatocytes than in HepG2 hepatocarcinoma cells. This may reflect adaptation of HepG2 cells to *in vitro* culture or the transformed nature of the cells. One implication is that the findings of studies in which HepG2 cells are used as a model of PUFA biosynthesis should be treated with caution. However, as in HepG2 cells, in primary hepatocytes estrogen treatment did not alter the mRNA expression of FADS 2 and 1 or ELOVL 2 and 5..

It has been suggested that higher 22:6n-3 status and greater capacity for 22:6n-3 biosynthesis in females than in males reflects a metabolic adaptation that facilitates supply of 22:6n-3 from the mother to the developing offspring. The concentration of 22:6n-3 in plasma phospholipids increases during pregnancy in women and in liver and plasma of pregnant rats. This increase in 22:6n-3 status has been associated with greater FADS 2 and 1 mRNA expression in the liver of pregnant rats. Furthermore, both estrogen and progesterone have been shown to be positively associated with hepatic FADS2 mRNA expression in pregnant rats, but negatively associated with the concentrations of other metabolites of 18:3n-3 (6). The findings of the present study support a potential role for progesterone in the pregnancy-associated increase in FADS2 mRNA expression and in 22:6n-3 concentration. Whether or not estrogen also contributes to this change 22:6n-3 in pregnancy cannot be deduced from these *in vitro* findings.

FADS2 transcription is regulated epigenetically by DNA methylation of its promoter (18, 20–22). We show for the first time the pattern of DNA methylation in a region of the human FADS2 gene that is located between the transcription start site and -1661 bp upstream (Figure 3). This region was characterised by a relatively highly methylated domain located distal to the transcription start site, a region of transition in the level of methylation between -718 to -669 bp and a region proximal to the transcription start site in which methylation was below the detection limit for analysis by pyrosequencing of 5% (23) which we assumed

to be essentially unmethylated (Figure 4). A number of putative transcription factor binding sites were identified throughout the region that was analysed (Figure 3). However, no sequence was identified that corresponded to the progesterone receptor response element, although a putative estrogen receptor response element was identified between 1325 and 1345 bp relative to the transcription start site (Figure 3). Treatment of HepG2 cells with progesterone induced reduction in methylation of CpG loci at -1661 and -1665 bp relative to the FADS2 transcription start site. Reduction in the methylation of individual CpG loci has been shown to increase FADS2 transcription in rat liver and aortae (18, 21). However, it is unclear from the present findings whether changes in DNA methylation of 3% or 14% are sufficient to account for the magnitude of the increase in FADS2 mRNA expression. The CpG dinucleotides located at -1661 and -1655 bp lie within a putative cAMP response element binding protein binding site (CREB) (Figure 3). Progesterone has been shown to increase transcription by up-regulation of the cAMP signaling pathway (24, 25) and CREB-1 has been shown to increase the level of transcription of steroyl-CoA desaturase-1 (26). Therefore it is possible that the action of progesterone on FADS2 transcription may be mediated via CREB activity. If so, then occupancy of the CREB response element may facilitate demethylation of the CpG dinucleotides at -1661 and -1655 bp by blocking the action of DNA methyltransferases (27). However, the elucidation of the mechanism by which progesterone treatment induced altered methylation of the FADS2 promoter was beyond the scope of this study.

Progesterone treatment induced a significant increase in the synthesis of 20:5n-3, 22:5n-3 and 22:6n-3 from [d₅]-18:3n-3. These findings confirm that induced changes in the mRNA expression of genes that encode the four major enzymes involved in conversion of 18:3n-3 to longer chain metabolites affect the capacity of cells for n-3 PUFA biosynthesis. The decrease in the [d₅]-18:3n-3 concentration in HepG2 cells treated with progesterone probably reflected the increase in conversion to longer chain n-3 PUFA. There was no effect of treatment with EE₂ or testosterone on the synthesis of 20:5n-3, 22:5n-3 and 22:6n-3 which is consistent with the absence of an effect of these hormones on gene transcription.

The findings of this study show a specific positive effect of progesterone on the mRNA expression of genes involved in PUFA biosynthesis and on conversion of 18:3n-3 to longer chain n-3 PUFA in human hepatocarcinoma cells or primary hepatocytes at physiological concentration in vitro. Previous studies have shown that estrogen, but not progesterone or testosterone, can increase plasma n-3 PUFA status (13, 14), in particular DHA, in women who took a combined estrogen and progesterone contraceptive pill had greater capacity for conversion of 18:3n-3 to 22:6n-3 than those who did not (9). Similarly, combined treatment of post-menopausal women with estrogen and progesterone increased their blood 20:4n-6 and 22:6n-3 status (14). Furthermore, blood progesterone concentration has been associated positively with hepatic Fads2 mRNA expression in rats (6) and estrogen treatment of ovariectomised rats increased brain 22:6n-3 concentration (28). While the current findings show a specific effect of progesterone on n-3 PUFA metabolism and the expression of associated genes, they do not resolve the debate about the relative roles of estrogen and progesterone in regulating PUFA metabolism. The main difference in the design of the current study from those reported previously is that the effects of sex hormones on PUFA

metabolism were tested *in vitro*. While the use of a liver cancer cell line may be a limitation, we also tested the effects of hormones in primary human hepatocytes and found similar changes in gene expression. One advantage of this approach is that the hormone exposure was controlled which cannot be achieved *in vivo*. Furthermore, the design of the present study allowed n-3 PUFA metabolism to be measured directly, rather than using proxy measures such as plasma n-3 PUFA status. This may be important as estrogens can modify phospholipid metabolism (29, 30) in a manner that may mask any direct effects on PUFA biosynthesis. Furthermore, cross-talk by estrogen and progesterone signaling pathways (31, 32) may also produce ambiguous findings with respect to the specificity of the effect of sex hormones on n-3 PUFA metabolism.

Together the findings of this study show that progesterone up-regulates n-3 PUFA biosynthesis and the mRNA expression of genes involved in this pathway, possible via changes in the epigenetic regulation of FADS2, in human liver cells *in vitro*. Further studies, including the use of hormone receptor knockout models, are needed to clarify the relative role of progesterone and estrogen in regulating PUFA biosynthesis *in vivo*.

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Fig. 1.

The effect of treatment with sex hormones on the mRNA expression of genes involved in n-3 PUFA biosynthesis in HepG2 cells. Values are mean $\pm$ SD (n = 10 replicates per treatment). Statistical analysis was by 1-way ANOVA with Dunnett's post hoc test. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$ compared to untreated cells. ANOVA probability for the effect of progesterone treatment was for FADS1 and 2 $P < 0.0001$, ELOVL 5 $P = 0.008$ and ELOVL 2 $P = 0.09$). There were no statistically significant effects of treatment with EE_2 or testosterone on the mRNA expression of any of the genes measured.

Fig. 2.

The effect of treatment with sex hormones on the mRNA expression of genes involved in n-3 PUFA biosynthesis in primary human hepatocytes. Values are mean $\pm$ SD (n = 10 replicates per treatment). Statistical analysis was by Student's unpaired t-test. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$ compared to untreated cells. There was a statistically significant effect of progesterone treatment on FADS2 mRNA expression ($P = 0.01$) and a non-significant trend towards an effect of progesterone on FADS1 expression ($P = 0.08$). There were no statistically significant effects of treatment with progesterone on ELOVL 5 or 2 expression or of EE_2 or testosterone on the expression of any of the genes measured.

Fig 3.
 Nucleotide sequence of the 5' region analysed by pyrosequencing. CpG dinucleotides as indicated by distance (bp) from the transcription start site. Putative transcription factor response elements are indicated by bars beneath the DNA sequence.

Fig. 4.

The effect of treatment of HepG2 cells with progesterone on the methylation of specific CpG loci in the FADS2 promoter. Values are mean $\pm$ SD (n = 6 replicates per treatment). Statistical analysis was by Students unpaired t test. * P<0.05; **P<0.01 compared to untreated cells.

Fig. 5.

The effect of treatment with sex hormones on n-3 PUFA biosynthesis in HepG2 cells. Values are mean $\pm$ SD (n = 5 replicates per treatment). Statistical analysis was by 1-way ANOVA with Dunnett's post hoc test. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$ compared to untreated cells. There was a statistically significant effect of progesterone treatment on FADS2 mRNA expression ($P = 0.01$) and a non-significant trend towards an effect of progesterone on FADS1 expression ($P = 0.08$). There were no statistically significant effects of treatment with progesterone on ELOVL 5 or 2 expression or of EE₂ or testosterone on the expression of any of the genes measured.

Table 1

Oligonucleotide primers used in real time RTPCR assays			
Gene	Forward primer	Reverse primer	
FADS1	CCAGACTCCACTTCTCAA	GCCAGTTCAACCAATCAGC	
FADS2	Quantitect Primer Assay HS_FADS1_1_SG		
ELOVL2	Quantitect Primer Assay HS_ELOVL2_1_SG		
ELOVL5	Quantitect Primer Assay HS_ELOVL5_1_SG		
Oligonucleotide primers used in DNA methylation assays			
PCR primers			
Start CpG	End CpG	Length of amplicon (bp)	
-1661	-1665	168	GTATGGTGGTTTTGAGGATGTGT
-1337		290	TTGTTGTGAAATTTAGATTGGGTAGG
-1278		290	TTGTTGTGAAATTTAGATTGGGTAGG
-1156		90	TTGTTGTGAAATTTAGATTGGGTAGG
-119	-1101	319	ATTTGAGGGTTTATATTTTCTTTAGTGAT
-1071	-1056	218	ATTTGAGGGTTTATATTTTCTTTAGTGAT
-1013	-975	323	ATTTGAGGGTTTATATTTTCTTTAGTGAT
-914	-855	199	GGTAGTTTATTTGTTGGAGTTTGTAT
-817	-775	167	ATTGAGTTTATTTGAGATTAGGGTAAGG
-718	-667	116	ATTGTTTGATTAGAGGGTTTAAAGTT
Sequencing primers			
			TGGTTTTGAGGATTGTTAA
			AGATTGGGTAGGGTT
			GGTTTTTTATTTTAAAGTGAGATG
			GGGTTTTATATAATTTTTTTTAGTGAT
			AAAATAAACCTAACTACATCC
			CCTCAAAACCCCAACT
			ATTATACAAACTCCAAACAAAT
			ATTATGTTTAAATGATGTTTG
			CCTCTAATCAAAACAATCTTAAAA

Oligonucleotide primers used in DNA methylation assays				
PCR primers				
Start CpG	End CpG	Length of amplicon (bp)	Forward primer	Reverse primer
			AGAGGGTTTAAAGTTTTTAAT	