Chapter 7

To evaluate the prevalence and association of PDE8B polymorphisms with Subclinical hypothyroidism and female infertility

And to study the possible genotype-phenotype correlation with the cause of SCH and infertility in Gujarat infertile female population

7.1 Introduction

The sharing of 99.5% genomic sequence level identity of humans implies that the phenotypic diversity arises on account of the remaining 0.5% difference as well as epigenetic modifications. The sequence difference is due to variable number tandem repeats, insertion or deletion polymorphisms, and single nucleotide polymorphisms (SNPs). Variation at single nucleotide adenine (A), thymine (T), cytosine (C), or guanine (G) in a DNA sequence in the genome or other shared sequence differs between members of a species or paired chromosomes in an individual is termed as SNP (Mccarroll *et al.*, 2006; Orr *et al.*, 2008). Due to their most frequent occurrence, easy analysis and less expensive genotyping as well as the possibility to carry out association studies, SNPs are considered as the most useful biomarkers for the diagnosis or prognosis of disease (Srinivasan*et al.*, 2016). Thyroid diseases in women with reproductive age are very common due to the complex interplay of various hormones (Silva *et al.*, 2018). Abnormal thyroid functions of hyper or hypothyroidisms are symptomatic and they may have an adverse effect on the reproductive health contributing to infertility (Weiss *et al.*, 2014; Saran *et al.*, 2016). However, subclinical hypothyroidism (SCH) is silent and hence it is often undiagnosed. SCH is a common thyroid disorder often found to coexist with various other morbidities. It is an asymptomatic condition where the patient has a normal serum free T4 (fT4/thyroxin) levels, but high thyroid stimulating hormone/thyrotropin (TSH) levels. TSH is considered as a sensitive indicator of the thyroid status and SCH. Normal TSH levels in serum are finely regulated in humans. Nevertheless, serum thyroid parameters show substantial inter- individual variability (Practice Committee of the American Society for Reproductive Medicine, 2015), in which genetic variations are proved as the major factors in several populations. A number of genes have recently been identified that are associated with altered thyroid function (Arnaud-Lopez*et al.*, 2008; Panicker*et al.*, 2008, Peeters*et al.*, 2003).It has been shown that altered TSH levels are related to genetic factors in up to 65% of the cases (Bernadette *et al.*, 2013; Panikar *et al.*, 2011; Malinowski *et al.*, 2014).

Cohort studies reported phosphodiesterase 8B gene (*PDE8B*) as a genetic modulator of TSH levels. *PDE8B* gene encodes a cyclic adenosine monophosphate (cAMP) specific phosphodiesterase (PDE) enzyme (Medici*et al.*, 2015). Figure 7.1 depicts the intracellular signaling pathways following activation cyclases. The cAMP and cGMP synthesized act as second messengers in the cellular responses. *Phosphodiesterases (PDE)* in turn inactivate

cAMP and cGMP. Eleven different PDE families including several isoforms and splice variants are known. Some PDE families are specific for cAMP (PDE4, PDE7 and PDE8), others are specific for cGMP (PDE5, PDE5 and PDE9) and several (PDE1, PDE2, PDE3, PDE10 and PDE11) show dual specificity (Bender *et al.*, 2006).

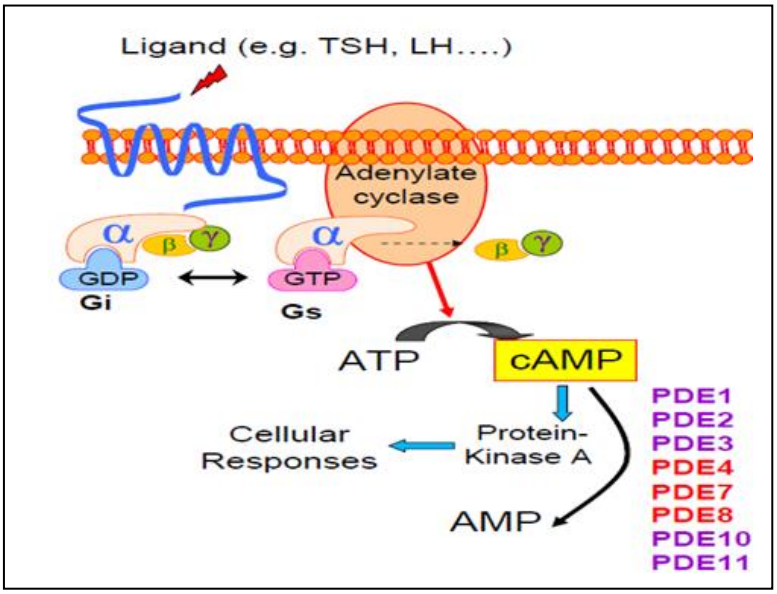

Figure 7.1The intracellular Signaling pathways following activation of cyclases: The cAMP and cGMP synthesized act as second messengers in the cellular responses. Phosphodiesterases (PDE) in turn inactivate cAMP and cGMP.

PDE8B affects cAMP levels in the thyroid gland resulting in changes in the levels of thyroid hormones, which in turn affects the release of TSH from the pituitary gland. *PDE8B* is mainly expressed in thyroid and brain (Vezzosi*et al.*, 2011; Lakics *et al.*, 2010). *PDE8B* influence serum TSH levels through its effect on TSH dependent thyroid hormone synthesis and secretion (Mariotti *et al.*, 2010). Several single nucleotide polymorphisms (SNPs) for *PDE8B* havebeen demonstrated to be associated with increased levels of serum TSH. More than 360,000 SNPs were tested for their associations with serum TSH levels with an additive model. The obtained results revealed three SNPs (i.e. rs4704397, rs6885099 and rs2046045) with genome-wide significance (P<10-10). These three SNPs were reported to be in strong linkage disequilibrium. Of the three SNPs, rs4704397 showed strongest association and it could explain 2.3% of the variations in TSH levels (Arnaud-Lopez *et al.*, 2008). *PDE8B* mutation leading to increased intracellular cAMP has been described in pituitary adenomas (Persani *et al.*, 2001), and in Cushing's syndrome with bilateral hyperplasia (Hovarth *et al.*, 2008). Increased *PDE8B* activity was reported in autonomous hyper functioning thyroid adenomas and interpreted as a compensatory mechanism to the constitutively activated cAMP pathway typical of these tumors (Persani *et al.*, 2000). *PDE8B* up regulation has also

been reported in Alzheimer's brain (Pérez-Torres *et al*., 2003). *PDE8B* rs4704397polymorphism apart from regulating thyroid homeostasis, has been also found to be associated with myocardial infarction and body height (Jorde *et al.*, 2014), obesity in children (Grandone *et al.*, 2012) and to insulin secretion (Dov *et al.*, 2008). Another *PDE8B* polymorphism, rs6885099 has also been shown to increase TSH levels, but to a lesser extent, in different populations (Arnaud-Lopez *et al.*, 2008). The relevance of human reproduction to PDE has been well-documented (Hayashi *et al.*, 2002; Soderling *et al.*, 1998; Gamanuma *et al.*, 2003, Horvath *et al.*, 2008). *PDE8B* rs4704397 polymorphism has been reported for subclinical hypothyroidism in pregnancy (Shields*et al.*, 2009; Yang*et al.*, 2015) and for recurrent miscarriage (Granfors *et al.*, 2012). But there is no report on the role of *PDE8B* polymorphisms for subclinical hypothyroidism in female infertility. We therefore, aimed to find out the prevalence rate of *PDE8B* polymorphism and explore association of *PDE8B* rs4704397 and rs6885099 polymorphisms with subclinical hypothyroidism and infertility and to see if we can correlate the *PDE8B* polymorphism with being the cause of SCH and infertility in primarily infertile females of Gujarat.

7.2 Material and Methods

7.2.1 Ethical consideration

It was ensured that the study design complies with the ethical standards of the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/BC/PR/1) set up as per guidelines of the Indian Council of Medical Research (ICMR) and with the 1964 Helsinki declaration.

7.2.2 Study Population

The present retrospective study is a matched, case-control study. The study population consists of parous control and IF-SCH female subjects with primary infertility as case subjects, (as was screened and reported in the first objective of the present study. Detail of the study population is as mentioned in chapter 2(2.2.2).

7.2.3 Blood collection and sample preparation

A volume of 1 ml blood samples aliquot was taken from the total 5 ml of the blood sample taken during the objective-1 study parameters from 110 control and 270 IF-SCH (Infertile

females with subclinical hypothyroidism) subjects. These aliquots of blood samples were stored for genotyping.

7.2.4 Genotyping *PDE8B* rs4704397 and rs6885099 polymorphisms

DNA was extracted from peripheral blood mononuclearcells (PBMCs) using 'IAamp DNA Blood Kit (QIAGENInc., USA) as per manufacturer's instructions. *PDE8B* rs4704397 (A/G) genotyping was done by polymerasechain reaction-restriction fragment length polymorphism (PCR-RFLP) while *PDE8B* rs6885099 (G/A) genotyping was done by amplification refractory mutation system (ARMS)-PCR. Sequence for SNPs rs4704397and rs6885099 in PDE8B gene are as follows:

7.2.4.1 SNP rs4704397 in PDE8B gene

CTCCAGCTTGAATTCCAGCTCTGCCCTGTACAAACTTTGTGACTTTGGAG
AAGCTGCATGATTTCTCCTTGCACGGTAGGGATAATGCATCTCTAAAAGG
ATTGGTACAACCTAGAGGTACAGTCCCTGTAAACCCGCTAGGTTCTCAAG
TACGTGTGTGTGTATTTTCTGCCTTATACTAAGGGCGCTACTCTAGGTTTG
GAGTGATAAAAAAAATGAATATGATGGCCGGGCATGGTGGCTCATGCCT
GTAAACCCAGCACTTTGGGAGGCTGAGGCGGGTGGATCACGAGGTCAGG
AGTTCGAGACTATCCTGACCAACATGGTGAAACCCCATCTCTCCTAAAAA
TACAAAAATTAGCTGGGCGTGGTGGCTTGCACCTGTAATCCCAGTTACTC
AGGAGGCTGAGACAGGAGGATTACTTGAATCTGGGAGGCAGCCTGGGTG
ACAGAGTGGAGACTCCATCTCAAAAAACAGAATATGACATAGTTCCACG
TTTC**[A/G]**GGAGCTTCCATTGTTTGTGCCCTATATTGTCGTCTTCTGTGGG
AAGGCAATGTGTTGACTGAGTGCCGGCTGGCTGGCAGGAGTTACGGATT
GGGAATTGGGTTCTTCCAGGTGAGACACTCCTACTGGGAGAGCATGAAG
AACTGTGAGCCTGAGGGGTAAGTCAGCTGAAGACTGTAAGGAAAAGCCA
AGGAGCAGACAACTGTACGGCCACAGGCAGGGGCCAGAGGCAGAATTA
AGGTCAGGCTTTCTAAGCCTGGAGCTCAAAGCTGCCAGACATAAATAGG
ATCCAAGAGAACTGATTAAGAAGGACTCTAAAGTCTGGTGGAGTTGATG
GCTGAGAATACTCTTGGGTGTTATGGTTCAGAGACTGCCAGATAAGGCTT
GCTTTGGGTTGAAGCTCTAAAGCACATTACCCAATAGAAATAAAATGTG
ACCCAGATGTAACCTGCACATGTAATTGTAAAATTTTTTATGAATCACAT
TTTTAAAAGTCAAA

GTTCCAGAATCTTGACCGCACCTCCTAATGACGATGTGGTACACGTACC
CAGCATTTTGCATGATTTCCTAGTATTGACCTTCTGTAGGGAATGTCAC
CTTTGTTGGTAAAATGGCCCCCAGAACCTAGACCTGGAAGGACTGGGC
AGCTCTTGACTCCATCTATGGCATTTTATCTTCTGCTTTGATCTCCCTAC
TCACCCTCACCCAACTCCACATGGGCTTCAGGGTGAGAGAAGGGGCCC
TCCTCCCCTCGTTAATATCCTAGCCAACTCATACACATGAGGTTGTATT
TAGTTCCCAGAGCAGAGGTGAGTCAAGACTCGCATGTTCAAGAGGACT
TTACCGA**[G/A]**CCTCAGAGATGACAGAATAGAACAGGCCGAGGGAGCA
CTCTGAAACTAGACCCTCTGGTTTGCCCTTGGGTGCTGCAGTACTTGTC
TAGTCTACCAGAGACTCCAGTCTGGCCTTCCCTACATAGTACCTTTCCA
TAGAAGTTTGACCTTTATCCTCTCTCCTCCAGATTCTTCCCCAAGATTCC
TAGACCCCTGACGTCCAGACCAGGTGATTCTAAATCCCTCCCAGGGTA
AATGTTATCTTTGGAAGTAAGTGATTGA

Amplification was performed using MastercyclerGradient PCR (Eppendorf, Germany) according to the following protocol: initial denaturation at 94°Cfor 10 minutes, followed by 30 cycles of denaturation at94°Cfor 45 seconds, annealing at 60°C for 45 seconds and72°C for 1 minute. The amplified products were analyzed by electrophoresis in a 2.0% agarose gel stained with ethidium bromide. The respective primers and restriction enzyme (RE) used for genotyping are shown in Table 7.1. 15 µl of the amplified products was digested for 16 hoursat 37°C, using 1 U restriction enzyme. For PCR-RFLP based genotyping, the digested products (300 bp and 219bp) with 100 bp DNA ladder (Bioron, Germany) were loaded in 3.5% agarose gels stained with ethidium bromideand visualized under UV transilluminator. Furthermore, genotyping of PDE8B rs6885099 G/A was done by Amplification refractory mutation system (ARMS-PCR) in 60 IF-SCH females and 76 control females. Human growth hormone (HGH) was used, as a reaction control in the ARMS-PCR (Jadeja *et al*., 2017). Amplification was performed using Mastercycler Gradient PCR according to the following protocol: initial denaturation at 94°C for 10 minutes, followed by 35 cycles of 94°C for 30 seconds, primer dependent annealing for 30 seconds and 60°C for 1 minute. The amplified products were analyzed by electrophoresis in a 3.5% agarose gel stained with ethidium bromide using 100 bp DNA ladder.

Table 7.1 Primers and Restriction Enzymes (REs) used for genotyping of *PDE8B* rs4704397 and rs6885099 polymorphisms

Gene	SNP	Primers	RE	Products	Cut products
PDE8B	rs4704397	FP: GGCGCTACTCTAGGTTTGGA RP: GTCTGCTCCTTGGCTTTTCC	519bp	*Bsl1*	300bp 200bp
PDE8B	rs6885099	FP1:GTTCAAGAGGACTTTACCGAG FP2:GTTCAAGAGGACTTTACCGAA RP:ACCTGGTCTGGACGTCAG	231bp	-	
HGH	-	FP: TCAGTTCCTCCTTACTCATGG RP: CACCTGTAACTGGCTGTTTG	428bp	-	

SNP, Single Nucleotide Polymorphism; RE, Restriction Enzyme; bp, base pair

7.2.5. Sampling method

The sampling method for selecting the participants was purposive (also called convenience method) sampling method as this provides the best information by the members of the selected community. Sample size for the present study was calculated using G-Power software with Alpha 0.05 and effect size of 0.9.

7.3 Statistical analysis

Hardy-Weinberg equilibrium (HWE) test was evaluated for the polymorphisms using chi-square test equating the observed and expected genotype frequencies. The genotype and allele risk associations were calculated by chisquaretest using Prism 5 software (GraphPad SoftwareInc, USA; 2007). For genetic analysis, Bonferroni's correctionwas applied and statistical significance was consideredat P-value less than 0.025. The linkage disequilibrium (LD) and haplotype analysis were carried out using http://analysis.bio-x.cn/myAnalysis.php (Barrett *et al.*, 2004). Levels of TSH and thyroid hormones were analyzed by non-parametric unpaired t-test and one-way ANOVA using Prism 5 software (GraphPad Software Inc.; 2007).

7.3.1 In-silico analysis

Web-based in-silico prediction tool HaploReg v4.1(https://www.pubs.broadinstitute.org/mammals/haploreg/haploreg.php) was employed to predict the effect of noncodingrs4704397 polymorphism. Tissue specific effectof rs4704397 was assessed by an eQTL database-GTeXportal (https://www.gtexportal.org).

7.4 Results

7.4.1*PDE8B* rs4704397 SNP in infertile females with subclinical hypothyroidism

Genotyping *PDE8B* rs4704397 polymorphism was carried out in 60 IF-SCH females and 76 healthy fertile females, Fig.7.2A). Other variables such as age (p=0.419), BMI (p=0.309), smokers (0%) and Hemoglobin, Hb (p=0.117) levels were not significantly different between the subjects of each genotypes, Table 7.2. Clinical parameters of Thyroid function Test other than TSH were also compared for all the three genotypes, which were not significantly different between the subjects of each genotype, fT_3 (p= 0.353), fT_4 (p=0.403), anti TPO-Abs prevalence rate was not statistically significant. Reproductive hormonal profile of the IF-SCH females carrying three different genotypes did not report any significance with PRL (p=0.302), LH (p=0.753) and FSH (p=0.951) as listed in the Table 7.2. Oxidative stress biomarkers studied were MDA (p=0.184), CAT (p=0.501) and SOD (p=0.773) showed no significant difference between the three genotypes. Lipid profile alteration between the three genotypes studied were also not statistically significantly different with TG (P=0.904), TC (p=0.152), LDL (p=0.255) and HDL (p=0.632), Table 7.2. Unequal sample numbers for the three genotypes in the present study might be the reason for the statistically not significant results obtained for all the demographic and clinical parameters.

Table 7.2 Demographic and Clinical characteristics of the studied population according to the *PDE8B* rs4704397 genotypes

Demographic/Clinical characteristics	**AA**	**AG**	**GG**	***p*-value**
Age, years	29.39 ± 0.73	30.43 ± 0.45	30.20 ± 0.74	0.419 (ns)
BMI, Kg/m²	23.92 ± 0.30	23.41 ± 0.29	23.18 ± 0.42	0.309 (ns)
Hb g/dl	11.47 ± 0.30	12.09 ± 0.16	11.53 ± 0.30	0.117 (ns)
Smokers (%)	0	0	0	-
fT_3 (pg/dl)	3.02 ± 0.07	3.083± 0.1	2.868 ± 0.11	0.353 (ns)
fT4 (ng/dl)	1.16 ± 0.04	1.09 ± 0.05	1.05 ± 0.07	0.403 (ns)
Anti-TPO Abs prevalence	34%	39%	27%	ns
PRL (ng/ml)	25.91 ± 3.01	24.11 ± 4.40	13.55 ± 3.34	0.302 (ns)
LH (mIU/ml)	7.67 ± 0.94	6.44 ± 0.60	7.78 ± 4.76	0.753 (ns)
FSH (mIU/ml)	9.59 ± 1.09	9.87 ± 1.26	8.61 ± 3.03	0.921 (ns)

MDA(nmoleformed/mgHb)	192 ± 9.06	218.6 ± 9.20	218.4 ± 27.54	0.184 (ns)
CAT(Unit/mgHb)	205 ± 11.49	224.2 ± 12.69	189.9 ± 34.45	0.501 (ns)
SOD(Unit/mg Hb)	6.51 ± 0.36	6.40 ±0.54	5.83 ± 0.71	0.773 (ns)
TG (mg/dl)	118.8 ± 8.05	119.7 ± 9.84	110.3 ± 12.06	0.904(ns)
TC (mg/dl)	147 ± 4.6	157.4 ± 9.40	172.5 ± 14.13	0.152 (ns)
LDL (mg/dl)	110.2 ± 7.9	89.88 ± 6.14	100.3 ± 13.17	0.255 (ns)
HDL (mg/dl)	44.18 ± 7.8	40.53 ± 4.2	47.34 ± 4.18	0.632 (ns)

Data represents Mean ± SEM values

The observed genotype frequencies of *PDE8B* rs4704397 SNP in IF-SCH females were slightly deviated from HWE (p=0.049; Table 7.3), whereas the control population was under HWE (p=0.062; Table 7.3). Ancestral allele 'A' and genotype 'AA' were considered as the reference allele and genotype respectively. The frequency of AG and GG genotypes were significantly lower in IF-SCH females, compared to controls (p=<0.0001 and p=0.006 respectively; Table 7.3). The frequency of 'G' allele was also significantly lower in IFSCH females, compared to the control females (23% vs. 47%, P<0.0001, OR=0.34). Hence, "G" allele was identified to have a protective effect and ' A' allele was identified as the risk allele for SCH and infertility in females.

Table 7.3 Distribution of genotype and allele frequencies for *PDE8B* rs4704397 A/G polymorphism

Genotype or allele	**IF-SCH females (Freq. %)**	**Control females (Freq. %)**	***p*-value**	**Odds Ratio**	**95% CI**	***p*-value HWE**
Genotype	n=60	n=76				
AA	38 (63%)	17(22%)	R	1	0.07 - 0.35	0.062 (C)
AG	16 (27%)	46(61%)	<0.0001[a]	0.16	0.07 - 0.63	
GG	06 (10%)	13(17%)	0.006 [a]	0.21		
Allele						0.049 (P)
A	92 (77%)	80(53%)	R	1	-	
G	28 (23%)	72(47%)	<0.0001[b]	0.31	0.19-0.57	

n; number of IF-SCH females/Control females, R; reference groupCI; Confidence Interval, P;IF-SCH females,C; Control females,[a]IF-SCH female vs. control females (genotype) using chi-squared test with 2 × 2 contingency table,[b]IF-SCH females vs. control females (allele) using chi-squared test with 2 × 2 contingency table.

7.4.2*PDE8B* rs6885099 SNP in infertile females with subclinical hypothyroidism

Genotyping of *PDE8B* rs6885099 polymorphism was carried out in 60 IF-SCH and 76 control females, figure7.2B. The observed genotype frequencies of *PDE8B* rs6885099 polymorphism among the Control and IF-SCH females were in accordance with HWE (p=0.248 and p=0.134 respectively; Table 7.4). Distribution of genotype as well as allele frequencies revealed no significant difference among the IF-SCH and control females, Table 7.4.

Table 7.4 Distribution of genotypes and alleles for *PDE8B* rs6885099 G/A polymorphism

Genotype or allele	IF-SCH females (Freq. %)	Control females (Freq. %)	*p*-value	Odds Ratio	95% CI	*p*-value HWE
Genotype	n=60	n=76				
GG	17 (28%)	32 (42%)	R	1	0.82 – 3.65	0.248 (C)
GA	35 (58%)	38 (50%)	0.1914[a]	1.73	0.74 – 8.42	
AA	08 (13%)	06 (8%)	0.2145 [a]	2.51		
Allele						0.134 (P)
G	51 (42%)	50 (33%)	0.1292[b]	1.51	-	
A	69 (58%)	102 (67%)	R	1	0.92-2.47	

n; number of IF-SCH females/Control females, R; reference groupCI; Confidence Interval, P;IF-SCH females,C; Control females,[a]IF-SCH female vs. control females (genotype) using chi-squared test with 2 × 2 contingency table,[b]IF-SCH females vs. control females (allele) using chi-squared test with 2 × 2 contingency table.

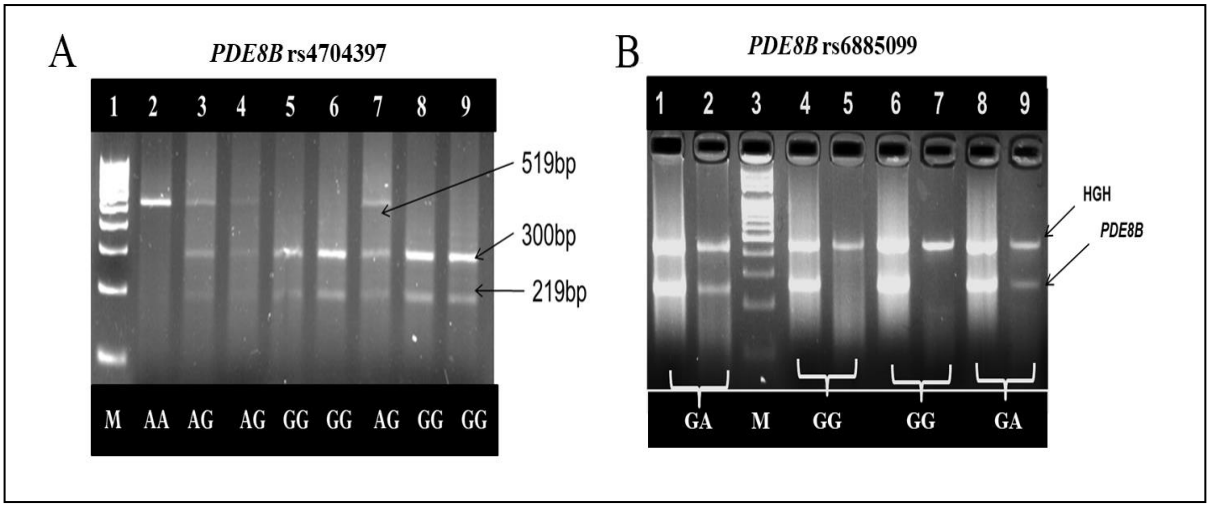

Figure 7.2 Representative gel images for *PDE8B* rs4704397 and rs6885099 genotyping: A. PCR-RFLP analysis of *PDE8B* rs4704397 SNP on 3.5% agarose gel. Lane 1 shows 100 bp ladder, lane 2 shows homozygous (AA) genotype, lanes 3, 4 and 7 show heterozygous (AG) genotypes, lanes 5, 6, 8 and 9 show homozygous (GG) genotypes. **B.** ARMS-PCR analysis of *PDE8B* rs6885099 SNP on 3.5% agarose gel. Lanes 1 and 2 show homozygous (GA); lane 4, 5, 6 and 7 show homozygous (GG) genotypes and lane 3 shows 100 bp ladder, lanes 8 and 9 show heterozygous (GA) genotypes. PCR-RFLP;Polymerase chain reaction-restriction fragment length polymorphism.

7.4.3 Linkage disequilibrium and haplotype analysis

Linkage disequilibrium (LD) analysis revealed thattwo investigated *PDE8B* polymorphisms (i.e. rs4704397and rs6885099) were in low LD association (D'=0.060,r2=0.003), figure 7.3. Haplotype analysis revealed that the frequencyof 'AA' haplotype was significantly higher in the patientsand risk of IF-SCH females was increased by 3.84fold (P=0.0001, OR=3.84; CI=1.86-8.01; Table 7.5). Thefrequency of 'GG' haplotype was significantly lower inIF-SCH females, compared to the controls suggestingits protective effect (P=0.0023, OR=0.33; CI=0.16-0.69; Table 7.5).

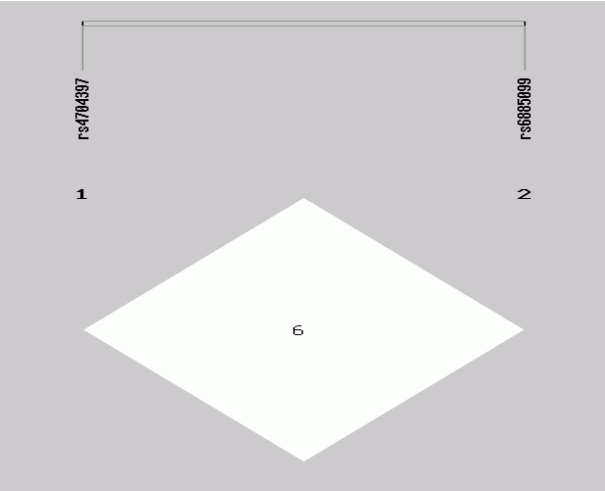

Figure 7.3Linkage Disequilibrium Analysis:
The linkage disequilibrium (LD) coefficient D'= D/Dmax for the pair of the most common alleles at each site were estimated using the SHEsis Online software.
LD analysis revealed that both SNPs studied are in lowLD association.

D'	rs6885099
rs4704397	0.060

Table 7.5: Distribution of haplotype frequencies for *PDE8B* rs4704397 and rs6885099 polymorphisms

Haplotype [rs4704397(A/G): rs6885099 (G/A)]	IF-SCH Female Freq. (%)	Control females Freq. (%)	*p* value for association	*p* value (Global)	Odds Ratio [95% CI]
AG	48 (46%)	49 (21%)	0.4434	7.5×10^{-5}	1.230 [0.72-2.09]
AA	31 (30%)	12 (10%)	0.0001		3.84 [1.86-8.01]
GG	12 (12%)	34 (28%)	0.0023		0.33 [0.16-0.69]
GA	13 (12%)	25 (21%)	0.0876		0.53 [0.25-1.10]

Freq.; Frequency, CI; Confidence interval (Frequency <0.03 in both control and case has been dropped and it was ignored in the analysis), and IF-SCH; Infertile females with subclinical hypothyroidism

7.4.4 Genotype-phenotype correlation analysis

TSH levels in IF-SCH females were analyzed with respectto the genotypes of *PDE8B* rs4704397 A/G andrs6885099 G/A. No significant difference in TSH levelswas observed with respect to genotypes of the both SNPs, figure 7.4.

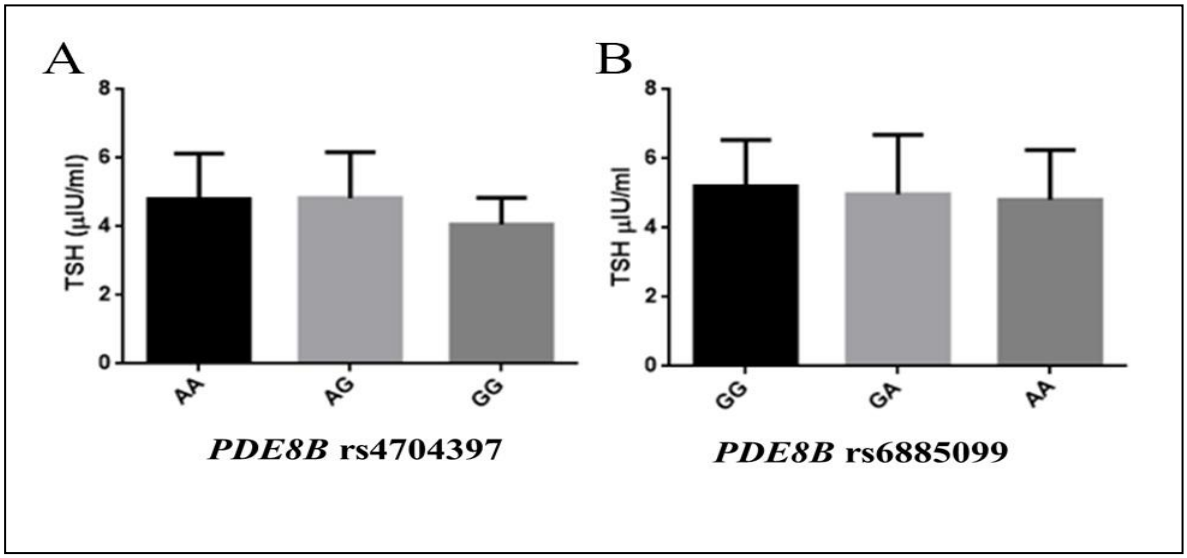

Figure 7.4 Correlation of *PDE8B* rs4704397 and rs6885099 with TSH levels in IF-SCH females: No significant difference of TSH levels was observed withrespect to *PDE8B* polymorphisms **A.** rs4704397 and **B.** rs6885099.TSH;Thyroid stimulating hormone, IF-SCH; Infertile females with subclinicalhypothyroidism

7.4.5 In-silico analysis

Analysis of functional consequences of *PDE8B*rs4704397 by HaploReg v4.1 predicted that *PDE8B*rs4704397 could alter heat shock factor-type (HSF)motif and enhancer state by H3K27 acetylation(H3K27ac) in inferior temporal lobe of brain (https://www.pubs.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs4704397).eQTL database GTExportal showed significantly elevated PDE8B transcriptsin thyroid tissue of individuals carrying 'A' allele, comparedto 'G' allele (https://www.gtexportal.org/home/snp/rs4704397).

7.5 Discussion

The present case-control study report that (i) Phosphodiesterase 8B (*PDE8B)* polymorphism rs4704397 is associated with infertility in Subclinical Hypothyroid females of Gujarat region. The study found 'A' allele (rs4704397 polymorphism) as the risk allele for SCH and infertility in females with 63% prevalence rate of rs4704397 polymorphism ('AA' genotype)

in the selected population while, (ii) No association was observed between *PDE8B*rs6885099 polymorphism and infertility in subclinical hypothyroid females(Mansuri *et al.*, 2020).

SCH occurs due to multiple factors. Some of them include congenital agenesis, defect in synthesis due to iodine deficiency or anti-thyroid drugs, autoimmune diseases, post-surgery, hypopituitarism, TSH deficiency, environmental pollutants, mutations and SNPs (Biondi et al., 2008). Of these factors, the present study focuses on the SNPs (genetic factor) as the etiological factor for SCH and subsequently for the infertility in the primarily infertile female population. To evaluate possible correlation between the polymorphisms associated with increased TSH levels and infertility, two SNPs (rs4704397 and rs6885099) of the *PDE8B* were studied in healthy controls and IF-SCH females. Higher frequency of the "A" allele for *PDE8B* rs4704397 polymorphism in SCH related infertile patients which revealed "A" as a risk allele for infertility in IF-SCH females. A high prevalence rate (63%) of rs4704397 polymorphism was observed in primarily infertile females with subclinical hypothyroidism in the selected population suggesting a genetic factor contributing to SCH and consequently to infertility. However, *PDE8B*rs6885099 polymorphism was not associated with infertility. Different cohort studies reported *PDE8B* as a genetic modulator of TSH levels. The human PDE8B gene is located at human chromosome 5q14.1 in intron 1 and encodes a high affinity cyclic adenosine monophosphate (cAMP) specific nucleotide phosphodiesterase (Gamanuma *et al.*, 2003; Hayashi *et al.*, 2002). It is reported that*PDE8B* is found in the thyroid but not pituitary. In addition, given the importance of cAMP activity in TSH signaling, it is suggested that the *PDE8B* rs4704397 polymorphism could reduce cAMP levels in the thyroid resulting in adecreased response of thyroid gland to TSH stimulation, which leads to an increase of TSH set point for the same free T3 and T4 levels (Grandone et al., 2012). Polymorphism in*PDE8B*, rs4704397 results in an increase in *PDE8B*enzyme expression. We propose that this could result in a faster degradation of cAMP, which decreases the synthesis and release of T3 and T4. In such a scenario, the negative inhibition of Thyrotropin-releasing hormone (TRH) will not take place and this will result in increased levels of TRH and hence TSH. As a consequence, T_3 and T_4 levels become normal. The increased level of TSH results in development of SCH.*PDE8B* rs4704397 polymorphism might induce phosphodiesterase activity in *PDE8B*, thereby reducing the ability of thyroid gland to generate free T4 when stimulated by TSH. This results in SCH, which can be the cause of infertility in IF-SCH patients. Arnaud *et al.* (2008) in a GWAS study reported that *PDE8B* rs4704397 could affect

plasma TSH levels. Each copy of the minor allele "A" may lead to a mean increase of 0.13 mIU/ml TSH levels; an effect size equating to around a 0.42 SD difference between the AA and GG genotypes. This genetic variation in TSH concentrations, within the normal population, is likely to result in altered "normalranges" for the three different genotypes (Arnold-Lopez *et al.*, 2008). However, we did not observe significant correlation of the *PDE8B* rs4704397 SNP with circulating TSH levels. This might be due to the limited sample size in the present study. *PDE8B* rs4704397 SNP was also found to be associated with various conditions like cardiovascular and body height, obesity in children and insulin secretion (Jorde *et al.*, 2014; Grandone *et al.*, 2012; Dov *et al*., 2008). Reproductive disruptions like SCH inpregnancy (Shields*et al.*, 2009; Yang*et al.*, 2015) and recurrent miscarriage (Granfors *et al.*, 2012) have been linked to rs4704397 polymorphism, but no reports are found in the literature study for the association of this SNP with female infertility at both national and international levels. Though the exact underlying mechanism of *PDE8B* rs4704397 SNP affecting TSH levels is not clear, in-silico tools predicted that this variation might lead to enhancement of*PDE8B* expression by influencing epigenetic level. The role of *PDE8B* in human placenta and ovaries is still to be understood, while human reproduction relevance to *PDE* has been proposed (Hayashi *et al.*, 2002; Soderling *et al.*, 1998; Gamanuma *et al.*, 2003, Horvath *et al.*, 2008).Because *PDE8B* is undetectable in the pituitary (Persani *et al.*, 2001), Meriotti *et al.* (2010) proposed that it could act primarily in the thyroidby inactivating cAMP produced after TSH stimulation. Indeed, of the 5 major isoforms of *PDE8B*, themajor isoform *PDE8B1* and minor isoforms *PDE8B2* and *PDE8B3* are abundantly expressed in the thyroid. *PDE8B* could therefore influence serum TSH levels through its effect on TSH dependent thyroid hormone synthesis and secretion. The underlying mechanism of regulating oocyte maturation is not clearly documented yet, but the second messenger cAMP role in oocyte maturation is well known (Shu *et al.*, 2008). Thus, investigating the role of rs4704397 in the oocyte maturation could be an interesting area of research as far as female infertility is concerned. The high prevalence and association of *PDE8B* rs4704397 polymorphism with subclinical hypothyroidism and infertility reported in the present study emphasis on elucidation of the cAMP role in understanding the mechanism of oocyte maturation as well as its mechanism, and henceforth research investigating the role of rs4704397 in the mechanism of oocyte maturation might give an insight to primary infertility caused by subclinical hypothyroidism.

7.6 Conclusion

The present study establishes an association of *PDE8Brs4704397* with infertility in subclinical hypothyroid primarily infertile females and reiterates the importance of screening Subclinical Hypothyroidism, as a diagnostic tool in infertility management.

7.7 References

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