Synopsis

The presence of unexplained amenorrhea for more than six months with elevated gonadotropin (FSH >40IU/l) levels is defined as premature ovarian failure (POF). Several factors were studied to explain the etiology of this disorder including genetic, immunological, nutritional and environmental factors. Various chromosomal and DNA based association studies as well as mice models of ovarian failure strengthened the polygenic nature of the etiology of the ovarian failure. Previous studies showed chromosomal abnormalities involving X chromosome and this led to the hypothesis of the presence of critical region in Xq arm which was believed to be responsible for maintenance of female fertility. Later, studies reported the chromosomal aberrations out of critical regions and included autosomes as well. Hence all these studies could not establish any specific region at chromosomal level which brings out the importance of studies essentially to be carried out at DNA level. The candidate genes of hypothalamus-pituitary-ovarian axis, FSHB (Follicle Stimulating Hormone Beta gene), FSHR (Follicle Stimulating Hormone Receptor), LHB (Luteinizing Hormone Beta gene), were largely studied by many researchers for their association with ovarian failure. This axis was primarily involved in ovarian regulation by pituitary and hypothalamus. The FSHR gene was reported to have a strong association with disorder in Finnish population but the mutations in this gene were uncommon in other world populations. So far, no study showed mutations in FSHB and LHB genes to have any association with ovarian failure. The genetic screening of the FMR1, FMR2 and DIA genes was reported by
several researchers due to their presence in the critical region of X chromosome and expression in gonads. The *Dia* knockout mice also exhibited similar phenotype as of ovarian failure in human. No association was established for the *FMR2* and *DIA* genes with ovarian failure. The premutation status (50-200 repeats) of CGG repeats in the promoter region of the *FMR1* gene was found to have association with the disorder in various populations. The mechanism of association of premutation status of CGG repeat is unknown.

The elevation of FSH levels is the most important clinical outcome of POF which is considered as first line of diagnosis for ovarian failure. The inhibins and associated gene network are responsible for the regulation of FSH secretion from gonadotrophs in the anterior pituitary. The inhibins downregulate production and secretion of FSH hormone by receptor mediated mechanism on gonadotrophs. This negative regulation is opposed by activins which in turn are responsible for FSH upregulation. Both activins and inhibins are produced by granulosa cells, which surround the oocyte. The presence of elevated serum FSH levels and low serum inhibin levels among majority of POF patients provides strong evidence of the involvement of inhibins in the patho-etiolog of POF (Petraglia *et al.* 1998, Shahara *et al.* 1998). There are three inhibin genes i.e. inhibin alpha (*INHA*), inhibin beta A (*INHBA*), inhibin beta B (*INHBB*). The dimerization of alpha subunit with either of the beta subunits results in the inhibins. The dimerization of beta A and beta B subunits results in the activins. The first study was reported for the association of inhibin alpha (*INHA*) gene mutation c.769G>A with POF in New Zealand population (Shelling *et al.* 2000). The INHA gene product
(inhibin) is involved in negative regulation of FSH production by gonadotrophs. The presence of any loss of function gene mutation in this gene would impair the regulatory role of inhibins and result in the upregulation of FSH levels. This study was the first study involving inhibins and we further extended the genetic screening of inhibin genes and associated receptors/factors among Indian cases of ovarian failure. The study of the INHA gene in Indian population revealed the similar results as reported by Shelling et al. (2000). We carried our mutational analysis of complete coding region of the INHA gene and mature peptide region of the INHBA and INHBB genes for all patients and controls (Chapter 5). The mutational analysis of the INHA gene revealed five missense variants, two promoter polymorphisms, two silent variants and one 3’ UTR variant. The most important missense variant c.769G>A (p.Ala257Thr) was significantly associated with ovarian failure with its presence in ~10% of cases (p value = 0.000017 for POF cases, p value = 0.0013 for PA cases). This variant was located in mature peptide region and may exert its effect by impairing the inhibin binding to its receptor. All patients with this variation were below the age of 25 yrs at the time of clinical diagnosis. These data strongly associate the presence of c.769G>A variant with an earlier age of menopause. We have experimented a confirmatory RFLP testing for the detection of this variant using INHα1F and INHα1R primers and BbvI restriction enzyme. This RFLP testing is a user-friendly and quick method for the detection of c.769G>A variant in clinical laboratories. Three other missense mutations in the INHA gene were present in three discrete patients. These three variants were located in the pro-region and may affect the secretion and stability of
inhibin dimers. The mutational analysis of the *INHBB* gene revealed one silent variant. The mutational analysis of *INHBA* gene revealed a c.896A>C (p.Gln299Pro) missense variant present in one PA patient.

Inhibins as well as activins after secretion from granulosa cells, through the blood stream, reach to the anterior pituitary where gonadotrophs are located. Both the growth factors have specific receptors located on gonadotrophs through which they process downstream signaling. Activins bind to their type II receptors (Activin type II receptor A and B) and activin/type II receptor complex further recruit type I receptor (*ACVR1B*) to stimulate FSH production by gonadotrophs. The downstream signaling is mediated by Smad 2/4 which after dimerization reaches nucleus and elevate FSH gene expression. The inhibins bind to their type III receptor betaglycan (or *TGFBR3*) and inhibin/TGFBR3 complex further recruits activin type II receptors. This complex of inhibin/betaglycan/activin type II receptor does not bind to type I receptor but limits the availability of activin receptors for activins. This phenomenon results in the down-regulation of the FSH production by not allowing activins to process their receptor mediated signaling. Inhibin/TGFBR3 complex has the equal binding affinity for activin type II receptor as of activins. Our study objective included the genetic screening of the *TGFBR3* gene for the presence of any possible mutation which may have reduced binding affinity with inhibins and may cause FSH upregulation. The study was also designed for the genetic analysis of the *ACVR1B* gene due to its central role in transmitting the intracellular signals for FSH hormone production.
The involvement of betaglycan gene (TGFBR3) in ovarian physiology was further strengthened by sharp fluctuations in the expression of betaglycan mRNA and receptor in gonadotrophs with menstruation cycle stages, inhibin, FSH and estradiol levels (Chapman et al. 2003). In situ hybridization analyses of rat tissues confirmed localized expression of the betaglycan mRNA and protein in anterior and intermediate lobes of pituitary, oocytes, granulosa cells and theca cells. We carried out mutational analysis of complete coding regions of the TGFBR3 gene in all the cases and controls (Chapter 8). The study revealed a total of 46 sequence variants including 22 novel variants. The novel variants include six exonic variants and sixteen intronic variants. Two novel variants were missense, c.550A>G (p.Iso184Val) in a control and c.2323C>T (p.Pro775Ser) in a POF case. Based on the high frequency of minor allele (>5%) and the significant differences in genotype distribution at 80% confidence level (p value <0.2), five variants viz. c.382-81C>T, c.382-77T>C, c.566-216G>A, c.1200G>A, c.2022T>C were chosen for haplotyping. The CCAAT haplotype was significantly higher in the patient population as compared with the controls (p value = 0.00007). Overall, the TGFBR3 gene was found to be of highly polymorphic nature and the strong association of CCAAT haplotype might partially contribute in the etiology of ovarian failure in milieu of other genetic and environmental factors.

The mutational analysis of coding region of the ACVR1B gene was performed in all patients and controls (Chapter 9). The study revealed a total of 10 variants including eight novel variants. The novel variants included five missense variants, three intronic variants and one 3' UTR variant. Four
missense variants were exclusively present in patients while one was present in a control. Two missense variants c.389T>C (p.Iso130Thr), c.392T>G (p.Iso131Ser) were completely linked with each other and significantly associated with ovarian failure with their complete absence in controls. All the patient associated variants are of probable gain of function nature hence may contribute to the enhanced activity of the ACVR1B receptor.

The hypothesis and work objectives were extended for two oocyte derived growth factor genes, GDF9 (Growth Differentiation Factor 9) and BMP15 (Bone Morphogenetic Protein 15), which positively regulate the inhibin production by granulosa cells. These both growth hormones are also involved in follicle development and differentiation. Like other TGFβ family members, the GDF9 and BMP15 can form homo and hetero-dimers with each other. Mature peptide dimers bind to specific receptors located on granulosa cells of follicles and activate the Smad signaling. The above described important roles of both the genes urged the screening of these genes among patients with ovarian failure. We carried out mutational analysis of the coding regions of both the genes in all patients and controls (Chapter 6 and 7). The germline screening of the GDF9 gene revealed eight variants out of which five are novel. Two missense variants c.199A>C (p.Lys67Glu) and c.646G>A (p.Val216Mat) were present exclusively in patients. The haplotype analysis of two frequent variants c.398-39C>G and c.447C>T showed significant association of CT haplotype with ovarian failure. The mutational analysis of the BMP15 gene revealed a total of 18 variants including 16 novel variants and two earlier reported variants. The
novel variants include one intronic insertion variant, one 3' flanking duplication variant, one silent variant and 13 missense variants. Out of these 13 missense variants, 11 variants viz. c.181C>T, c.182G>A, c.226C>T, c.227G>A, [c.538G>T(+)c.539C>T], c.538G>A, c.588T>A, c.617G>A, c.631C>T, c.661T>C, c.727A>G were present only in ovarian failure cases. The associated missense variants of the GDF9 and BMP15 gene are located in pro-region. These associated mutations may cause impaired post-translational activity and defective secretion of the dimers. Another possibility is that the missense variants can lead to misfolding of the protein during protein synthesis itself. Overall the present study is a collective study of genetic screening of inhibin genes (INHA, INHBA, INHBB), GDF9, BMP15, TGFBR3 and ACVR1B genes among Indian women with ovarian failure.