Summary

The presence of unexplained amenorrhea for more than six months with elevated gonadotropin (FSH>40IU/l) levels is defined as premature ovarian failure (POF). The complete absence of menses is defined as primary amenorrhea (PA). A total of 196 patients with ovarian failure were recruited at the Infertility Institute and Research Centre (IIRC), Hyderabad and the Institute of Reproductive Medicine (IRM), Kolkata. The recruited patients include POF cases (n=133), PA cases (n=63). Normal healthy females with regular menstrual history, normal FSH levels and successful pregnancies were recruited as controls (n=200). Recruitment of the controls was entirely population-based to support the study. Genomic DNA was isolated from all the patients and controls for subsequent genetic analysis. Present study is an endeavor to establish germline status of key regulators involved in inhibin mediated FSH down-regulation. Inhibins downregulate the FSH levels whereas activins upregulate the FSH production by gonadotrophs. Inhibins complexed with its receptor betaglycan compete activins for the availability of their type II receptor. This inhibin mediated mechanism lessens the downstream signaling and FSH synthesis by gonadotrophs. ‘Activin receptor type I’ is the last receptor which gets activated by binding activin/activin receptor type II complex and sends intracellular Smad signaling. Any loss of function mutation in inhibin (INHA) gene or its receptor betaglycan (TGFBR3) gene would lead to FSH elevation and subsequent depletion of ovarian follicles. Similar results are expected due to the presence of
gain of function mutations in the activin type I receptor (ACVR1B) gene. The production of inhibins is positively mediated by two oocyte derived growth factors, GDF9 and BMP15. These growth factors are also imperative for the growth and differentiation of ovarian follicles. The loss of function mutation in these genes would cause defects in ovarian follicle development and FSH upregulation.

Inhibins are dimer of inhibin-α chain dimerized with either inhibin-βA or inhibin-βB chain. The alpha chain contains binding domain for the betaglycan receptor located on gonadotrophs. The complete coding region of inhibin alpha gene (INHA) and mature peptide regions of inhibin-βA gene (INHBA), inhibin-βB gene (INHBB) were amplified and sequenced for all the patients and controls. The mutational screening of the INHA gene revealed a total of nine variants including five novel variants. These variants are c.1-124A>G, c.1-16C>T, c.275G>A (p.Ser92Asn, novel), c.327C>T (novel), c.487G>A (p.Val163Met, novel), c.525C>G (p.His175Gln, novel), c.531C>T, c.545C>A (p.Ala182Asp, novel) and c.769G>A (p.Ala257Thr). The variant c.769G>A was significantly associated with ovarian failure with its presence in 10.5% cases of POF (fourteen out of 133, Fisher's exact test P value = 0.000017), 9.5% cases of PA (six out of 63, Fisher’s exact test P value = 0.0013) with its presence in one control out of 200 controls. All the mutant individuals were heterozygous except one homozygous patient. The presence of c.769G>A variant was further confirmed by RFLP using BbvI restriction enzyme. All the patients with this variation were below the age of 25 years at the time of clinical diagnosis. The remaining four
missense variants were located in pro-region. Out of these four, three missense variants c.275G>A (p.Ser92Asn), c.525C>G (p.His175Gln), c.545C>A (p.Ala182Asp) were present in three discrete patients whereas one missense variant c.487G>A (p.Val163Met) was present in a control. The \textit{INHBA} and \textit{INHBB} genes did not reveal any association except the following rare variants. The \textit{INHBA} gene revealed a missense variant c.896A>C (p.Gln299Pro) in an individual with PA. The \textit{INHBB} gene revealed a silent variant c.942C>T in an individual with POF.

The complete coding region of the \textit{GDF9} gene was sequenced for all the patients and controls. The sequencing analysis revealed eight variants out of which five are novel. Five novel variants were c.1-8C>T (promoter region), c.199A>C (p.Lys67Glu), c.205C>T (silent), c.646G>A (p.Val216Mat), c.1353C>T (silent) and three documented variants were: c.398-39C>G (intronic), c.447C>T (silent), c.546G>A (silent). Both the missense variants were less frequent but associated with ovarian failure with their complete absence in controls. The c.199A>C variant was present in four out of 133 POF cases. The c.646G>A variant was present in two out of 133 POF cases. These missense variants were located in pro-region of GDF9 protein and therefore may affect the processing of pro-region cleavage. Haplotype analysis was performed for two frequent polymorphisms c.398-39C>G, c.447C>T. The occurrence of CT haplotype is significantly higher in patients (p value = 0.03) whereas CC haplotype is representative of control group (p value = 0.004).

The mutational analysis of coding region of the \textit{BMP15} gene revealed a total of 18 variants out of which 16 were novel. Novel variants (n=16)
include one intronic insertion variant c.328+44_328+45insG, one 3' flanking duplication variant c.*40dupG, one silent variant c.381A>G and missense variants (n=13) namely c.181C>T, c.182G>A, c.226C>T, c.227G>A, c.308A>G, c.538G>A, [c.538G>T(+)c.539C>T], c.588T>A, c.617G>A, c.631C>T, c.661T>C, c.727A>G and c.788_789insTCT (Figure 11). 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. None of these variants were observed in the controls. Out of these 11, one missense variant c.631C>T was homozygous, while the remaining 10 were heterozygous. These 11 variants were present in different patients discretely. The remaining two missense variants, c.308A>G and c.788_789insTCT were present both in the cases and the controls. Haplotype analysis was performed for three frequent variants viz. c.9C>G, c.308A>G and c.852C>T. Haplotype G-G-C found to be significantly associated with ovarian failure (P value=0.0075). The functional study of first human missense variant in the BMP15 gene which was also located in pro-region illustrated the complete functional loss of mutant BMP15 protein using GC proliferation assays in vitro. Results also corroborated abnormal processing of the mutant protein, which antagonizes the activity of wild type protein in a dominant negative fashion (Di Pasquale et al. 2004). This missense mutation results in the secretion of unprocessed monomers and abnormal dimeric products, which probably antagonize the normal protein functions via receptor interference.
The mutational analysis of coding region of TGFB3 gene revealed a total of 46 sequence variants including 22 novel variants. The novel variants include six exonic variants and sixteen intronic variants. Two novel coding variants were missense, c.550A>G (p.Iso184Val) in a control and c.2323C>T (p.Pro775Ser) in a POF case. Based on the Fisher’s exact test, allelic frequencies for three variants were significantly diverse in cases of ovarian failure than in controls as given below {i} c.382-81C>T (p value for POF=0.0136, p value for PA=0.0606), {ii} c.382-77T>C (p value for POF=0.0136, p value for PA=0.0606) {iii} c.1200G>A (p value for POF=0.0669, p value for PA=0.0875). The genotypic distribution for these three variants was also significantly varied (95% confidence level) in patients as compared with controls. Notably the C allele of c.382-81C>T variant was always linked with the C allele of c.382-77T>C and vise versa. 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). The two haplotypes CCAGT and TTAGT were significantly higher in the controls than in patients (p value=0.046, 0.001 respectively). Other less frequent haplotypes like CCGAC was only present in the patients (p value=0.0096), TTAAT was only present in the PA cases (p value=0.0003) and TTAAC was only present in the controls (p value=0.019).
The sequence analysis of coding region of the \textit{ACVR1B} gene revealed a total of ten 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. The variants $\text{c.389T>C (p.Iso130Thr), c.392T>G (p.Iso131Ser), c.505T>G (p.Cys169Gly), c.581G>A (p.Gly194Glu)}$ were associated with ovarian failure cases while $\text{c.356C>T (p.Pro119Leu)}$ variant was present in a control. The $\text{c.505T>G and c.356C>T}$ variants were present in the regions of unknown functions. The $\text{c.389T>C, c.392T>G and c.581G>A}$ variants were significantly associated with ovarian failure with their complete absence in controls. Both the $\text{c.389T>C and c.392T>G}$ variants were completely linked in all the patients. The $\text{c.389T>C and c.392T>G}$ variants were observed in $7/133$ cases of POF (Fisher's p value = 0.0015) and $4/63$ cases of PA (Fisher's p value = 0.003) whereas $\text{c.581G>A}$ variant was present in $4/133$ cases of POF (Fisher's p value = 0.025). The $\text{c.389T>C and c.392T>G}$ variants are probably of gain of function nature based on previous literature.

Overall the studied candidate genes which are involved in the genetic network of inhibin mediated FSH down-regulation are important for the understanding of the POF etiology. The genes which are laterally linked with the presently studied candidate genes and support the hypothesis of \textit{"The defects which impair inhibin mediated FSH down-regulation are major cause of POF etiology"} should be studied. Our selected candidate genes were studied for the first time among Indian women with ovarian failure for their germline status and association.