

## 2 INFERTILITY AND REPRODUCTIVE DISORDERS

The Institute's infertility experts, both basic and clinical, have been engaged in carrying out extensive research to identify genetic causes of male and female infertility and to develop tools based on state-of-the-art molecular techniques for diagnosis and treatment of reproductive disorders and infertility. These tools can provide valuable adjuncts to clinicians for evidence based treatment, for screening inherited disorders and prevent transmission through assisted reproductive techniques.

### 2.1 Molecular Studies Related to Hyperandrogenemia of Polycystic Ovary Syndrome

Principal Investigator: **Anurupa Maitra**  
Project Associates: Heena Shirwalkar, C Sarkar, C Saravanan  
and A Golani  
Duration: 1995-2007

Polycystic ovary syndrome is the most commonly encountered condition of infertility in females with heterogeneous phenotypes. An important hallmark feature of the syndrome is hyperandrogenicity, pathophysiology of which is still not understood well. Studies have therefore been undertaken with rat models to elucidate the mechanisms at molecular level underlying abnormal steroidogenesis and ovarian development seen in the syndrome. Studies undertaken with E2V injected rat model was described last year (Annual Report 2005-06, p 43). Results of the study indicated that exposure to exogenous estrogen in adulthood can have deleterious effects on the ovarian physiology and endocrinology which may ultimately lead to cystogenesis, loss of follicle pool and early senescence. The study was continued during this year to assess the gonadotropin and steroid profile in these rats (Figs. 24 and 25). The hormones were analysed at one week, two weeks, three weeks and four weeks following subcutaneous administration of 2 mg of estradiol valerate (E2V). Suppression in serum LH levels was seen in a time dependant manner. The levels were marginally reduced at two-week post treatment with significant decline at third and fourth week as compared to controls (Fig. 24 A). In contrast, serum FSH levels were significantly decreased at first two weeks as compared to controls, following which a recovering trend was seen (Fig. 24 B). Thus a time dependent suppressive effect of E2V appeared to be mainly on FSH rather than LH at initial time points and on LH rather than FSH at later time points. This was reflected by

relative increase in LH:FSH ratio at first and second week post E2V treatment, followed by a significant decrease at third and fourth week (Fig. 24 C).

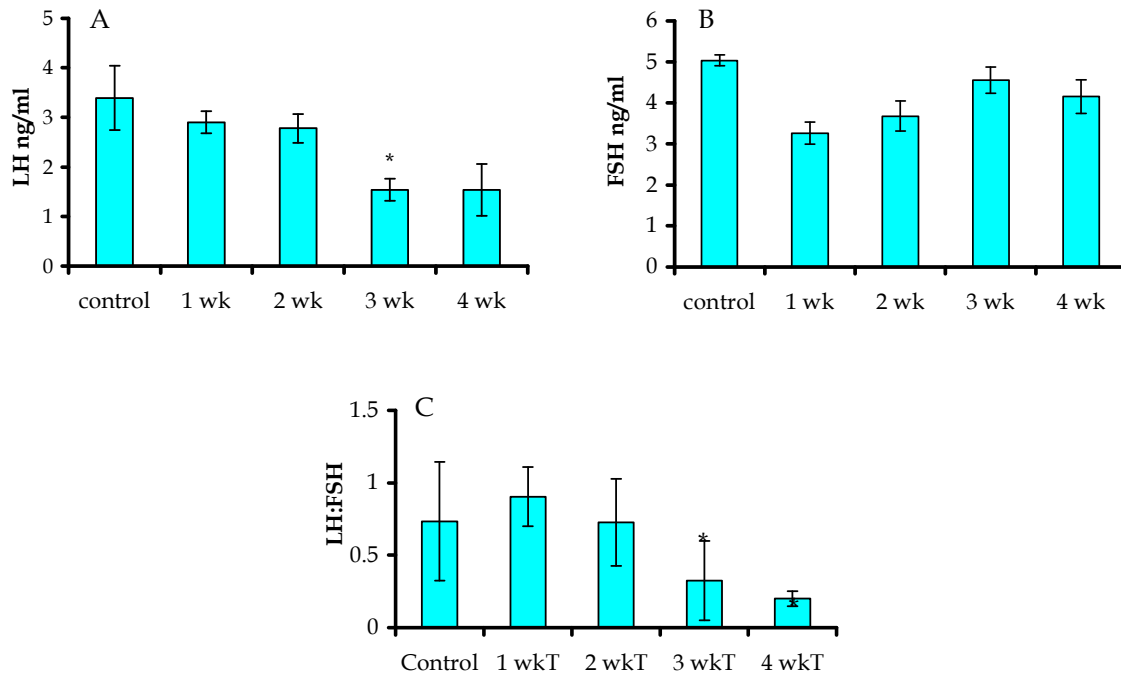


Fig.24: Changes in serum levels of LH (1A), FSH (1B) and LH: FSH ratios (1C) in adult female rats exposed to a single subcutaneous injection of E<sub>2</sub>V. Data represent mean ± S.E. (n=10 animals/group).

\* p < 0.05 is considered significant compared to control.

With regard to steroid hormones, serum progesterone levels post treatment were increased at first two weeks compared to controls, which was significant at first week ( $P \leq 0.05$ ) (Fig. 25A). However at the end of third week, the circulating levels reduced in the treated animals reaching significantly low levels at four weeks. Serum Androstenedione levels were significantly elevated over controls from first week through third week, which later dropped to 50 percent of the control levels at four-week post exposure (Fig.25B). The circulating levels of Estradiol also showed a biphasic pattern as that of Progesterone and Androstenedione. The levels were significantly elevated over controls from first week through third week and later dropped significantly at fourth week post exposure (Fig. 25C). Overall results with the E2V injected rat model show deleterious effects on the ovarian physiology and endocrinology, which may ultimately lead to cystogenesis, loss of follicle pool and early senescence.

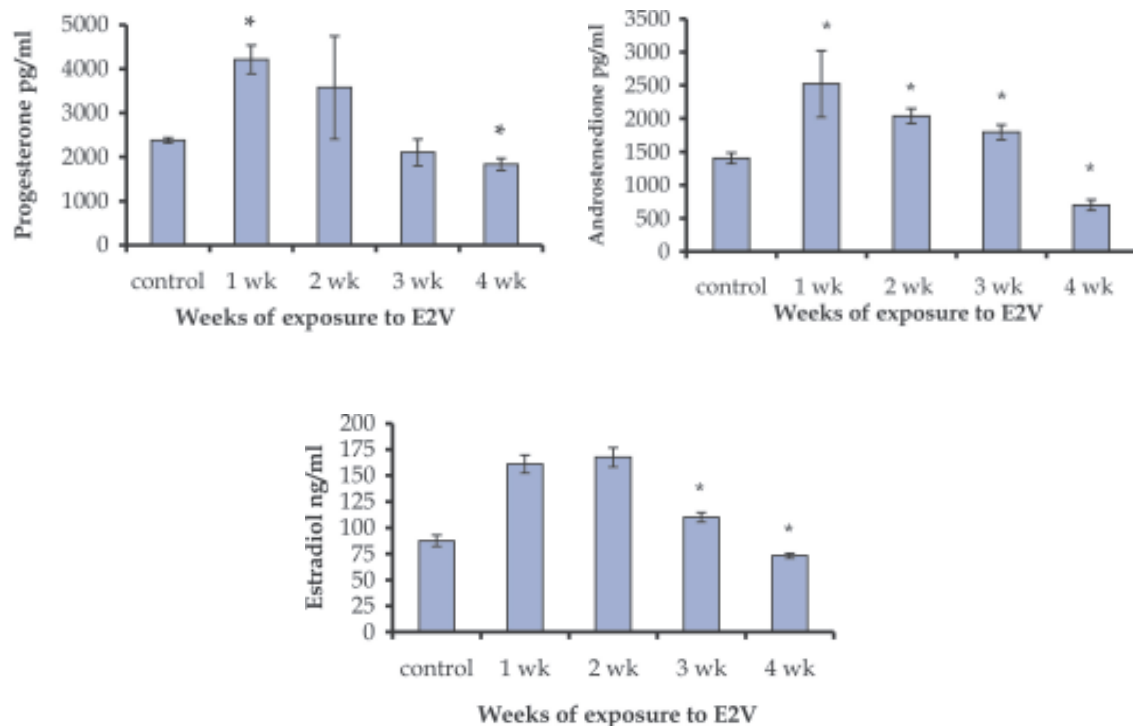


Fig. 25: Changes in the serum levels of Progesterone (2A), Androstenedione (2B) and Estradiol (2C) in adult female rats exposed to a single subcutaneous injection of E<sub>2</sub>V. Data represent mean ± S. E. (n= 10 animals/group) \*, #p < 0.05 was considered significantly different from control.

## 2.2 Studies on Genetic Aspects of Polycystic Ovary Syndrome (Partly funded by Indian Council of Medical Research under Genomics and Molecular Medicine)

Principal Investigator: **Anurupa Maitra**

Project Associates: Madhavi Pusalkar, Pervin K. Meherji, Jyotsna S. Gokral, C. Saravanan and S.D. Rai

Collaborators: Duru Shah, Programme Director  
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Duration: 2002-2007

Polycystic ovary syndrome, the most common cause of infertility affects 6-7 percent of women of reproductive age. Marked by an ovulatory cycles and hormonal disturbances, etiology of the syndrome still remains enigmatic. Its signs and symptoms are expressed as bewildering combinations in women from all ethnic groups and racial backgrounds. Yet its phenotype is readily transmitted between generations, strongly suggesting a genetic basis. Studies have therefore been undertaken to elucidate this genetic basis using pathway specific candidate genes. In the present study emphasis is on candidate genes associated with two important features of the syndrome viz. hyperandrogenicity and obesity (Annual Report, 2005-06, p 62-64). Specific objectives of the study are to i) screen a defined group of women with PCOS for variations in genes in the pathways of hyperandrogenicity and obesity and ii) determine their association with the phenotype.

Diagnosis of PCOS was carried out as per the Rotterdam Consensus (2005) and candidate genes selected were CYP11A1 and CYP17 in hyperandrogenicity pathway and Leptin in the obesity pathway. Screening has been completed in 95 PCOS cases and 55 controls for the following loci i) CYP11A1 - Coding exons and pentanucleotide repeats in promoter region ii) CYP17 - T>C polymorphism in promoter region iii) Leptin gene - Coding exons and non coding exon1.

Assessment of androgen profile showed a trend similar to that described last year (Annual Report 2005-06, p 48). A significant increase in levels of Testosterone, Androstenedione as well as Leptin was seen in the PCOS group as compared to controls. Screening of the genetic loci showed a higher prevalence of T>C polymorphism in CYP17 in PCOS cases compared to controls. With regard to CYP11A1, a novel polymorphism (G>A) was identified in exon 5 (GeneBank accession no. DQ358147; Fig. 26). Screening of promoter region of the gene showed alleles with varying number of pentanucleotide repeats (4, 6, 8, 9, 10, 12). Frequency of higher repeat alleles ( $\geq 6$ ) was found to be greater in PCOS group compared to controls. Fig 27. shows an electropherogram of the pentanucleotide repeat region with heterozygous 6/9 polymorphism. Leptin gene analysis did not reveal any variations in the coding sequences. Non coding exon1 showed an A>G variation frequency of which was higher in our population compared to that reported in West. The study is being continued to confirm the genotype phenotype association.

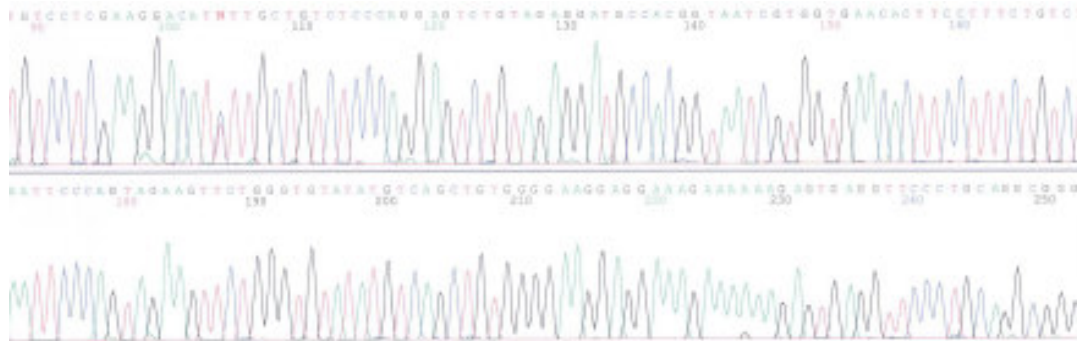


Fig. 26: A novel heterozygous GA variation observed in exon 5 of CYP11A1 (GeneBank accession no. DQ358147) A - Sense strand ; B - Antisense strand.

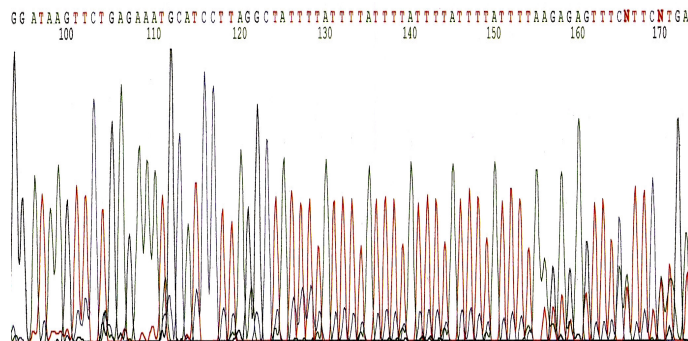


Fig. 27: A heterozygous 6/9 (ttta)<sub>n</sub> repeat polymorphism in CYP11A1 promoter

### 2.3 A Genetic Analysis of Polycystic Ovary Syndrome with Special Emphasis on Genes involved in Insulin Resistance (Partly funded by Department of Science and Technology)

Principal Investigator: **Srabani Mukherjee**

Project Associates: Anurupa Maitra, Pervin K Meherji,  
Gayatri Shinde, Sushma Khavale,  
C. Saravanan, P.P. More.

Collaborator: Nalini Shah, Padma Menon,  
Department of Endocrinology,  
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Mumbai.

Duration: 2006-2009

Polycystic ovary syndrome (PCOS) is a common heterogeneous disorder characterized by hyperandrogenism and chronic anovulation. The syndrome is frequently associated with an increased risk of insulin resistance and type 2 diabetes mellitus (T2DM); obesity exacerbates insulin resistance and favors the progression from impaired glucose tolerance to diabetes in these patients. The consequences of polycystic ovary syndrome extend beyond reproductive axis: women with the disorder are at substantial risk of developing metabolic and cardiovascular abnormalities. The familial clustering of women with PCOS suggests that heredity is implicated in the origin of the syndrome. PCOS is a polygenic disorder. Studies indicate that PCOS and T2DM could share genetic susceptibility factors. The present study has been undertaken to determine the role of variants in the genes associated with insulin resistance viz. insulin receptor, insulin receptor substrates, calpain 10, calpain 5, peroxisome proliferator-activated receptor gamma (PPR $\gamma$ ) and delta (PPRD) and paraoxonase in the pathogenesis of PCOS.

The recruitment of study subjects is ongoing. During the reporting year forty seven PCOS women and thirty control women have been recruited. A type 2 diabetic group has also been recruited as diabetic control. Glucose, insulin and other hormonal estimations were carried out in these recruited subjects (Fig. 28). Conventionally fasting glucose to insulin ratio < 4.5 is a marker of insulin resistance which has been detected in 55 percent of PCOS subjects.

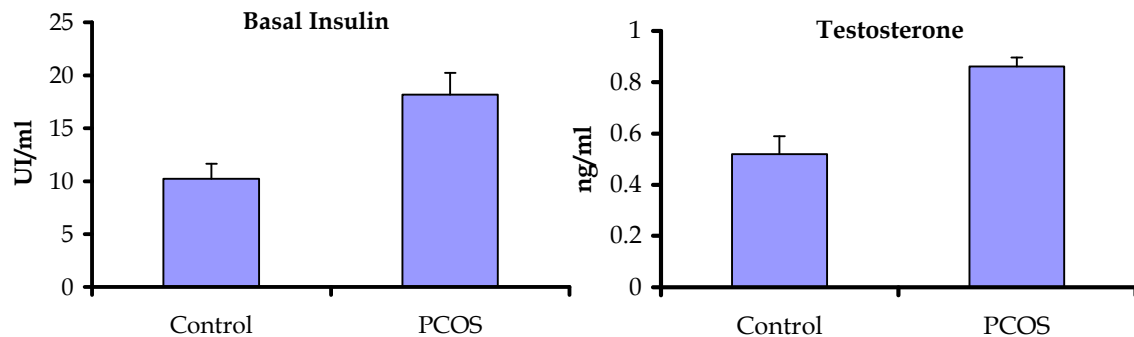


Fig. 28: Comparison of insulin and testosterone levels between control and PCOS groups

Sequencing of insulin receptor and PPR $\gamma$  gene has been initiated (Annual Report 2005-2006, p 64-66). A polymorphism C/T at His 1058 of Exon 17 of INSR gene was detected. Frequency of the polymorphic T allele was found to be higher in PCOS women (49 percent) compared to controls (25 percent). Screening also revealed the absence of the T/C polymorphism at Cys 1008 site reported in Chinese population.

Study subjects were also screened for a major variant Pro12Ala SNP in PPR $\gamma$  gene. Twenty control and twenty-four PCOS samples were sequenced. The preliminary data showed absence of Ala variant, which is known to protect insulin sensitivity. All samples were found to be of wild type. Additional analysis of other SNPs in PPR $\gamma$  gene and PPRD gene has been initiated.

The variations in the gene encoding a cysteine protease CAPN10 are also associated with insulin resistance and influence predisposition to T2DM. To investigate role of CAPN10 in PCOS, two SNP sites i.e. SNP 19 and SNP 63 have been amplified. SNP 19 contains an insertion deletion polymorphism; allele1 of 155bp has two repeats of 32 bp sequence and allele 2 of 187 bp with three repeats (Fig. 29). Our preliminary data shows the frequency of allele 1 to be 49 percent and 38 percent and allele 2 to be 51 percent and 62 percent in control and PCOS subjects. The SNP 63 was PCR amplified and subjected to restriction fragment length polymorphism with HhaI enzyme (Fig.30). Out of twenty controls and eighteen PCOS subjects analyzed for SNP63, no variants have been detected so far. Allele specific primers have been designed for other two SNPs in CAPN10 gene and analysis is in progress.

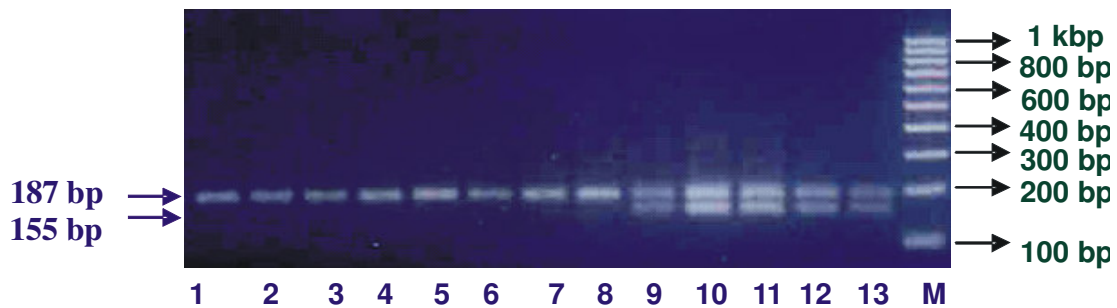


Fig. 29: Amplification of SNP-19 of CAPN10 gene. Lane 1 – 6: controls, Lane 7- 13 PCOS and M: 100 bp Ladder. Lane 1-8 samples are homozygous for allele 1 (187 bp) and lane 9-12 samples are heterozygous (187 bp and 155bp).

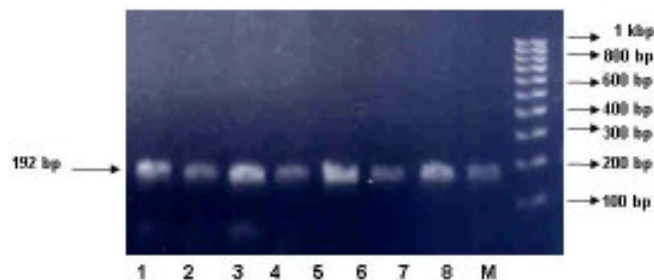


Fig. 30: Amplification of SNP-63 of CAPN10 gene. Lane 1 – 3: controls, Lane 4 - 7 PCOS and M: 100 bp Ladder

Findings so far reveal a higher frequency of T polymorphism in insulin receptor gene in PCOS compared to control women. The Pro12Ala polymorphism in exon 2 of PPRγ gene and SNP63 in CAPN10 gene is absent in the study population analyzed so far.

## **2.4 Genetic Studies in Women with Premature Ovarian Failure**

Principal Investigator: **Pervin K. Meherji**

Project Associates: Anurupa Maitra, Zareen Patel, Jyotsna Gokral, Seema Kadam, Suparna Chatterjee

Duration: 2002-2007

The study aims to determine the association of mutations/premutations in the FMR1, inhibin and FOXL2 genes in women with idiopathic premature ovarian failure (POF) and their families and in women with poor ovarian reserve and also to identify the best candidate gene for determining predisposition to this condition. In the reporting year eleven additional cases of POF (women under the age of forty years with secondary amenorrhoea, serum FSH levels >40 mIU/ml) eight more family members and thirteen cases of POR were enrolled and studied along with additional ten regularly cycling fertile women.

Mutations in the FOXL2 and Inhibin genes screened by RFLP and SSCP analyses revealed a silent substitution of nucleotide and a co-existing C>G mutation in the coding region of the FOXL2 gene. No mutations were detected in the Inhibin alpha and beta A genes.

Sizing of CGG repeats in the FMR1 gene by PCR and polyacrylamide gel electrophoresis was carried out. Till date all patients and controls have been found to have CGG repeats in the normal range of 5-50.

Exons 2,3,4,5 and 6 of the gene were amplified using three sets of primers. Exon 1 of the gene but yet not been identified. Screening of the NOBOX gene was continued Primer set 1 amplifies the predicted exon 2 comprising of 556 bp (Fig 31A), primer set 2 amplifies the predicted exons 3 and 4, comprising of 562 bp (Fig 31B), and primer set 3 amplifies the predicted exon 5 and 6, comprising of 544 bp (Fig. 31C). These PCR products were subjected to heteroduplex analysis to screen for mutations. No mutations could be identified. Thus, direct DNA sequencing of this gene has now been undertaken and is under progress (Fig. 31D, E, and F).

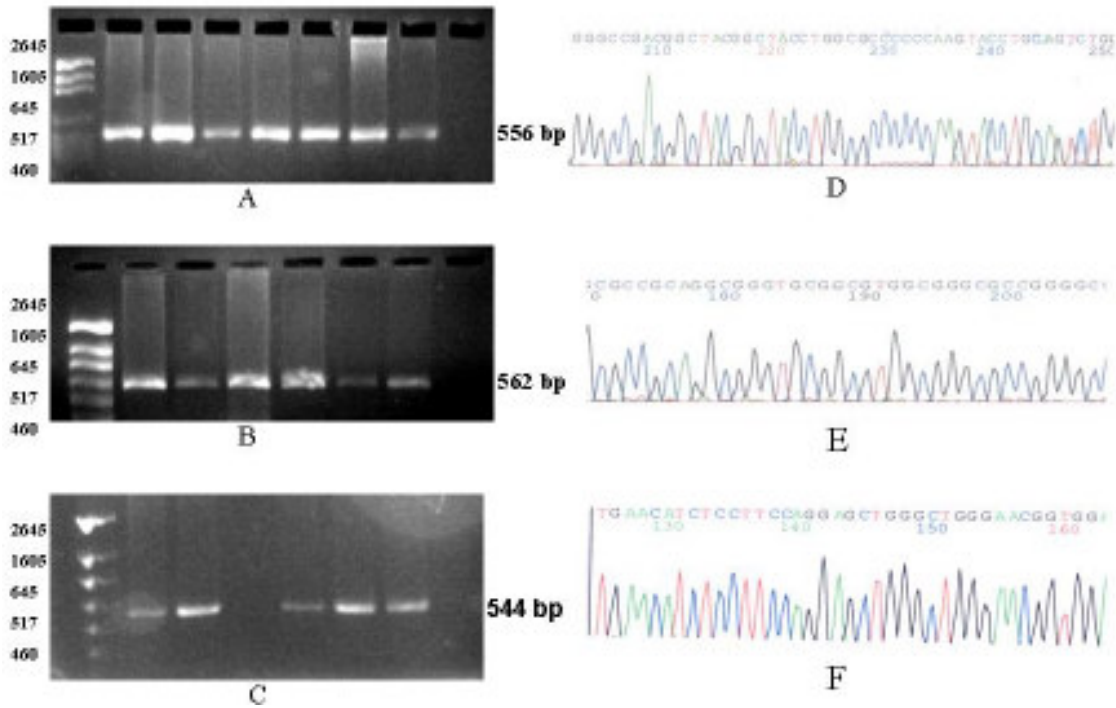


Fig. 31: (A) PCR amplification of exon 2 Lane 1: Molecular weight marker, Lanes 2-5:POF case, Lanes 6-8 : controls Lane 9 : Negative control (B) PCR amplification of exons 3,4 Lane 1:Molecular weight marker, Lane 2-5:POF cases Lanes 6-8 :controls, Lane 8 : Negative control. (C) PCR amplification of exons 5,6 Lane 1: Molecular weight marker, Lane 2,3 : controls, Lane 5,6,7: POF cases, Lane 4 : Negative control. (D)Sequencing of exon 2 of NOBOX gene using primer set 1. (E). Sequencing of exons 3,4 of NOBOX gene using primer set 2.(F) Sequencing of exons 5,6 of NOBOX gene using primer set 3.

## 2.5 Assessment of Gene Mutations Associated with Congenital Adrenal Hyperplasia *(Partly funded by Indian Council of Medical Research under Genomics and Molecular Medicine)*

Principal Investigator: **Anurupa Maitra**

Project Associates: Heena Shirwalkar, Zareen M. Patel,  
C. Saravanan, Sudhisha Dubey and S.D. Rai

Collaborator: Sudha C Rao  
Bai Jerbai Wadia Hospital for Children,  
Mumbai

Duration: 2000-2008

Congenital Adrenal Hyperplasia (CAH) is a group of disorders caused by inborn errors of steroid metabolism and is the most common cause of ambiguous genitalia. It is a treatable disorder with an autosomal recessive trait and affects adrenal cortex and its development, leading to defective synthesis of cortisol. The abnormally low rate of cortisol synthesis causes secondary elevation of ACTH and subsequent hyperplasia. Five different forms of CAH have been described depending on position of enzyme block in the pathway of cortisol synthesis, *viz.* 21-hydroxylase, 3 $\beta$ -hydroxysteroid dehydrogenase, 11 $\beta$ -hydroxylase, 17 $\alpha$  hydroxylase and Steroidogenic acute regulatory (StAR) protein. The most common form among these is the deficiency of enzyme 21-hydroxylase. Mutations in the gene encoding the 21-Hydroxylase enzyme (CYP21) are now known to be responsible in about 95 percent of cases of CAH. This syndrome displays a wide spectrum of phenotypes ranging between the severely affected form called "salt wasting" (SW) with a concurrent defect in aldosterone biosynthesis, a less severe form with apparently normal aldosterone biosynthesis i.e. simple virilizing (SV) and a mild non-classical form that may be asymptomatic or with symptoms of androgen excess developing during childhood/puberty. A large number of mutations in CYP21 gene have been reported by various workers in these cases which have been found to be population specific. Information on Indian population is however not available. Knowledge of these mutations has important implications for early treatment and counseling for the disease. The present project has been undertaken with the aim to determine i) mutations in the CYP21 gene in clinically confirmed Indian cases of CAH and ii) association of these mutations with the phenotypes.

A PCR-sequencing based method developed in the laboratory to screen the entire CYP21 gene has been described in last year's report (Annual Report 2005-06, p 53). The method is based on direct analysis of DNA extracted from peripheral blood and involves following key steps : i) Amplification of the active CYP21 gene into 2 fragments of sizes 1.1 and 2.2kb using specific primers and ii) nucleotide sequencing of both the fragments in multiple steps to accurately identify the mutations. A total of 32 index cases with classical CAH have been screened so far. The mutations observed were as follows : i) Active gene deletion ii) Nonsense mutation Q318X iii) Missense mutations - R356W, I172N, Exon6 Cluster mutation and Frame shift mutation iv) Intron2Splice mutation A,C >G. A novel missense mutation Phe>Val was also observed in one case which was submitted to Gene Bank (Accession no. EF563986). Fig. 32 shows electropherogram of this novel variation observed. Fig. 33 shows the frequencies of various mutations observed. All but two of these mutations *viz.* I172N and Intron2Splice lead to complete depletion of the 21-hydroxylase enzyme activity

and were found to be associated with the severe salt wasting form of the disease. I172N and Intron2 Splice mutations that result in 1 percent and 5 percent residual activity of the enzyme respectively in homozygous form, were found to be associated with simple virilizing form of the disease. Thus, a good genotype phenotype correlation has been shown by the molecular analysis carried out so far in the limited number of cases.

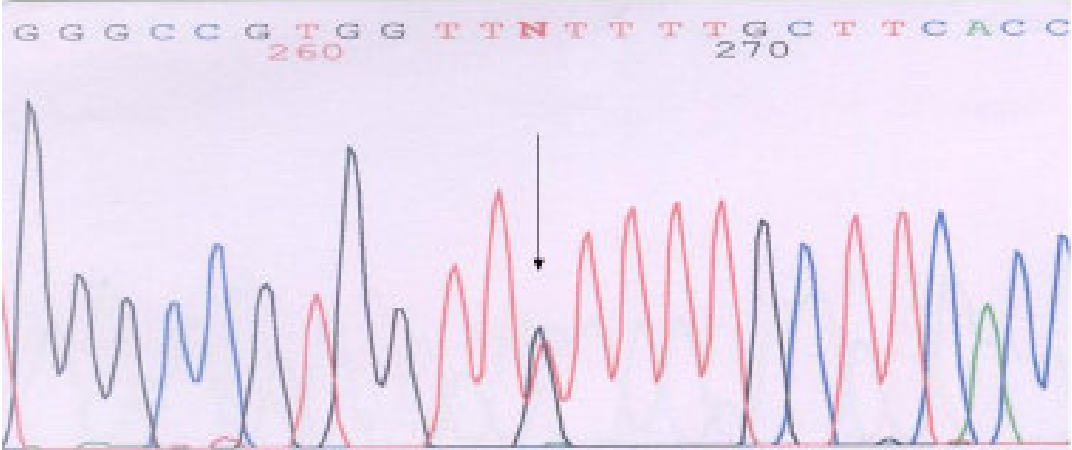


Fig. 32: A novel missense mutation Phe>Val observed in a case of CAH (Gene Bank Accession no. EF563986).

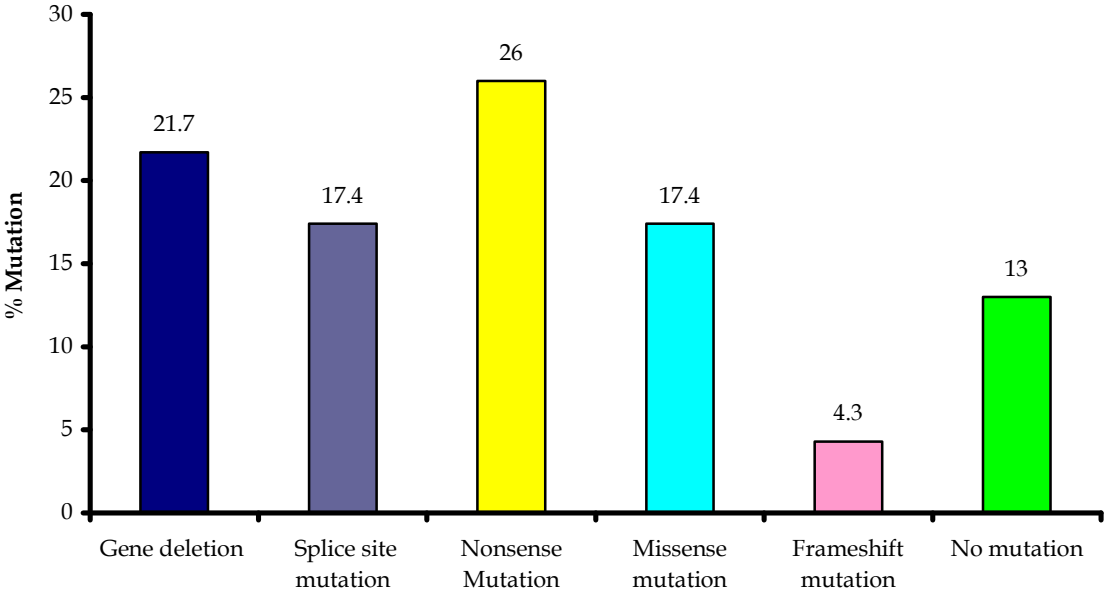


Fig. 33: Frequencies of CYP21 mutations observed in CAH cases.

## 2.6 Antigens Involved in Ovarian Autoimmunity

Principal Investigator:	<b>Vrinda Khole</b>
Project Associates:	E.S. Pires, Purvi Mande, Manish Ghosalkar Pervin K. Meherji
Collaborators:	Rama Vaidya, Dean, Bhavans SPARC, Mumbai Padma Menon, Emeritus, Dept of Endocrinology, Seth G S Medical College and KEM Hospital, Mumbai Firuza Parikh, Director, Jaslok Hospital
Duration:	2002-2010

Premature Ovarian Failure (POF) is described as a condition in which onset of amenorrhoea occurs in women under the age of 40 and is associated with hypergonadotropinism and hypoestrogenism. In a significant number of such idiopathic cases, POF is known to be associated with autoimmunity. Detection of specific autoantibodies therefore remains the most practical clinical and research marker of autoimmune disease. Approximately 10-15 percent of couples who desire children suffer from infertility. Even after a thorough evaluation, the cause of the inability to conceive remains unknown in at least 10 percent of these cases. Despite treatment, including *in-vitro* fertilization and embryo transfer (IVF-ET), many of these couples remain childless. Recently, the search for answers to unexplained infertility and failed IVF has concentrated on the possible role of immunity in reproductive failure. Autoimmunity is an immune reaction to self. In simple terms, it is considered a failure or loss of tolerance to self tissue. Autoimmune abnormalities have been investigated for possible associations with reproductive failure and have led many clinicians to recommend immunologic testing, specifically autoantibodies to screen women with infertility. Certain autoimmune abnormalities have been associated with recurrent pregnancy loss, including antiphospholipid antibodies and lupus anticoagulant, antithyroid antibodies, antisperm antibodies, antizonal antibodies, antinuclear antibodies and antiovarian antibodies (AOA) are under investigation as possible adverse factors involved in implantation and reproductive failure, and thus failed IVF. Also repeated IVF attempts have been speculated to induce AOA, both by repeated hormonal stimulation and by repeated microtrauma during oocyte retrieval that can cause the release of altered immunogenic proteins from internal ovarian layers. Most couples seeking children attempt to do so by visiting IVF clinics. The presence of AOA could impair the development of the egg and embryo, downstream to oocyte collection, and thus after its removal

from the ovary. Identifying women with AOA before recruiting them in the IVF-ET program would not only benefit the treating clinician in disease management but also aid the registered couple investing a large amount of money for the procedure.

Earlier we have published a non-invasive, simple, specific and sensitive novel test to identify women with AOA. Using this test (Western blotting and Immunohistochemistry) we have been able to identify a large number of these women with AOA. Of the 581 serum samples screened, 121 were AOA positive (22 percent) as seen in Fig. 35. Majority of these antibodies reacted to the oocyte although some of them did show reactivity to various other somatic cells such as adrenal or thyroid (Fig. 36). There is involvement of not just a single antigen but multiple antigenic targets are involved (Fig. 34). Of these 121 AOA positive patients 60 had antibodies to a 90 kDa immunodominant protein. This protein shows predominant ooplasm of the oocyte localization.

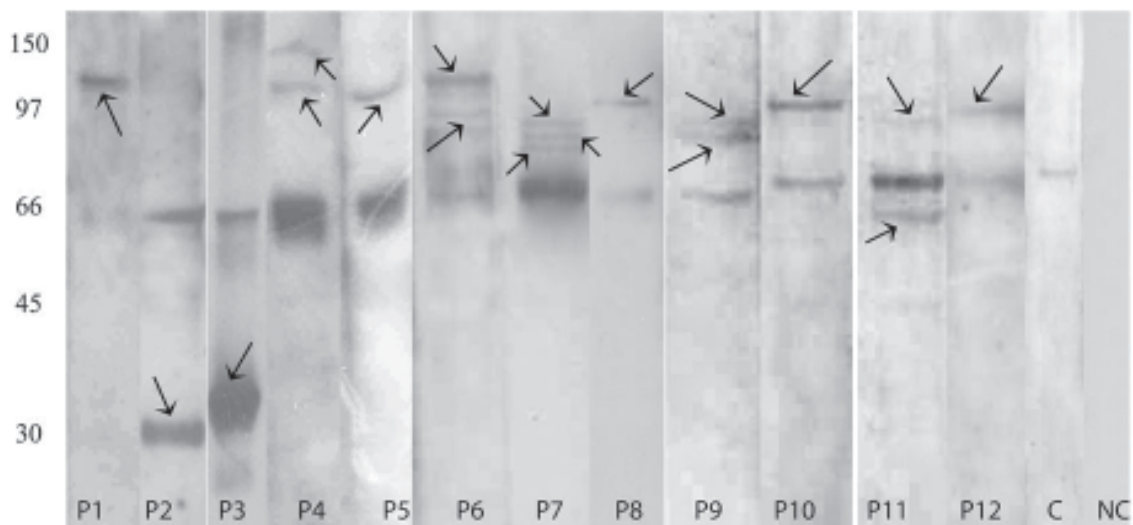


Fig. 34: This figure shows multiple antigenic targets using sera from POF as well as IVF-ET patients. As seen various proteins between molecular weights 30 kDa to 150 kDa have been targeted. None of the controls show immunoreactivity to these molecular targets except the 66 kDa which is seen in controls as well in patients. Negative controls do not react to any targets.

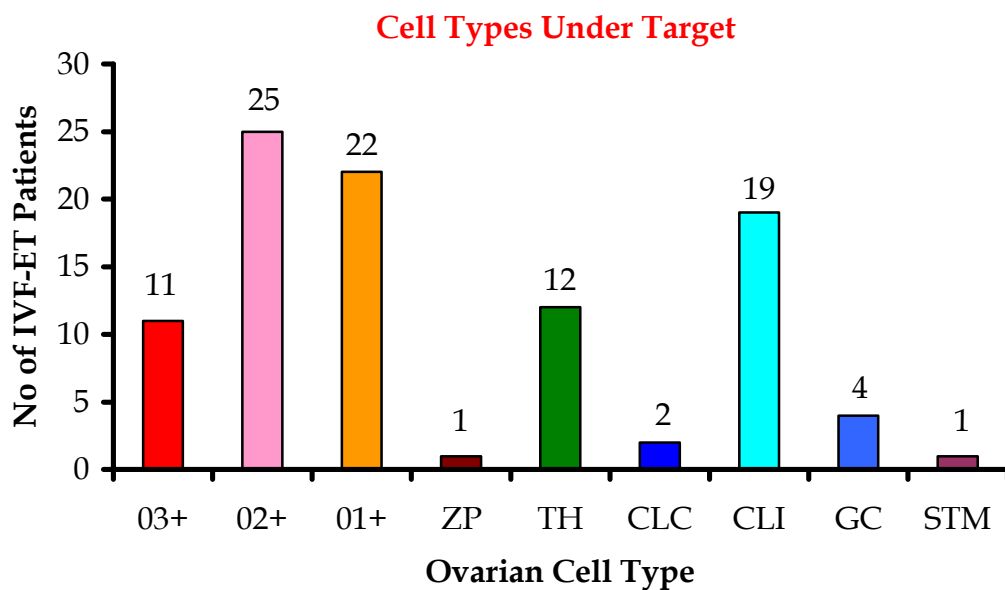
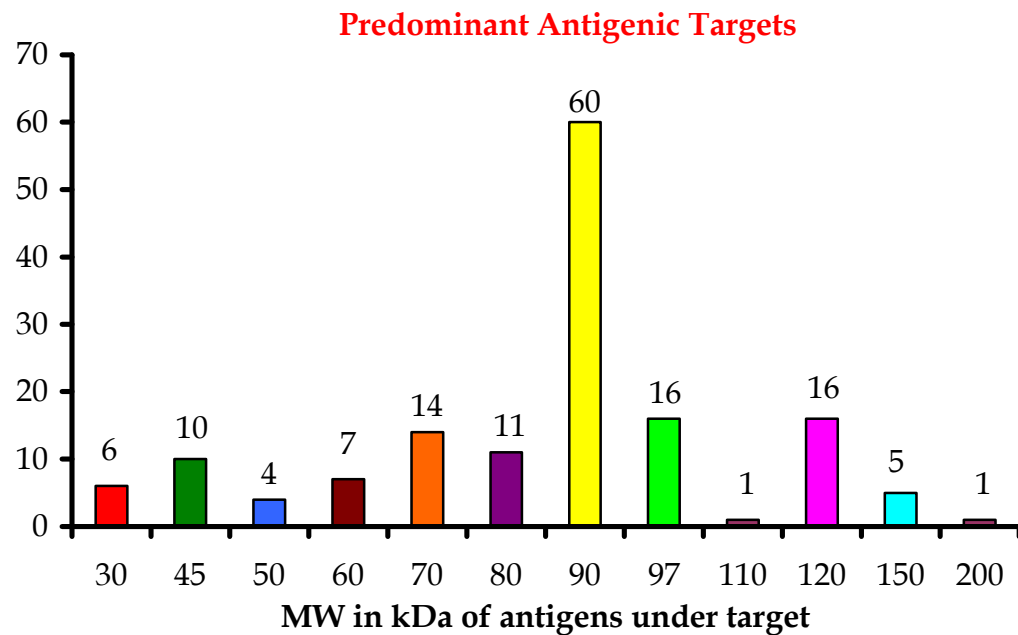


Fig.35: This figure shows the prevalence of anti-ovarian antibodies to various multiple targets as seen using the sera of patients in the study group. Of all these targets a 90 kDa protein is the most immunodominant antigen targeted by the humoral arm of the immune system. In terms of cellular targets, one can clearly see that the oocyte is the immunodominant cellular target however; other somatic components of the ovary are also under target.

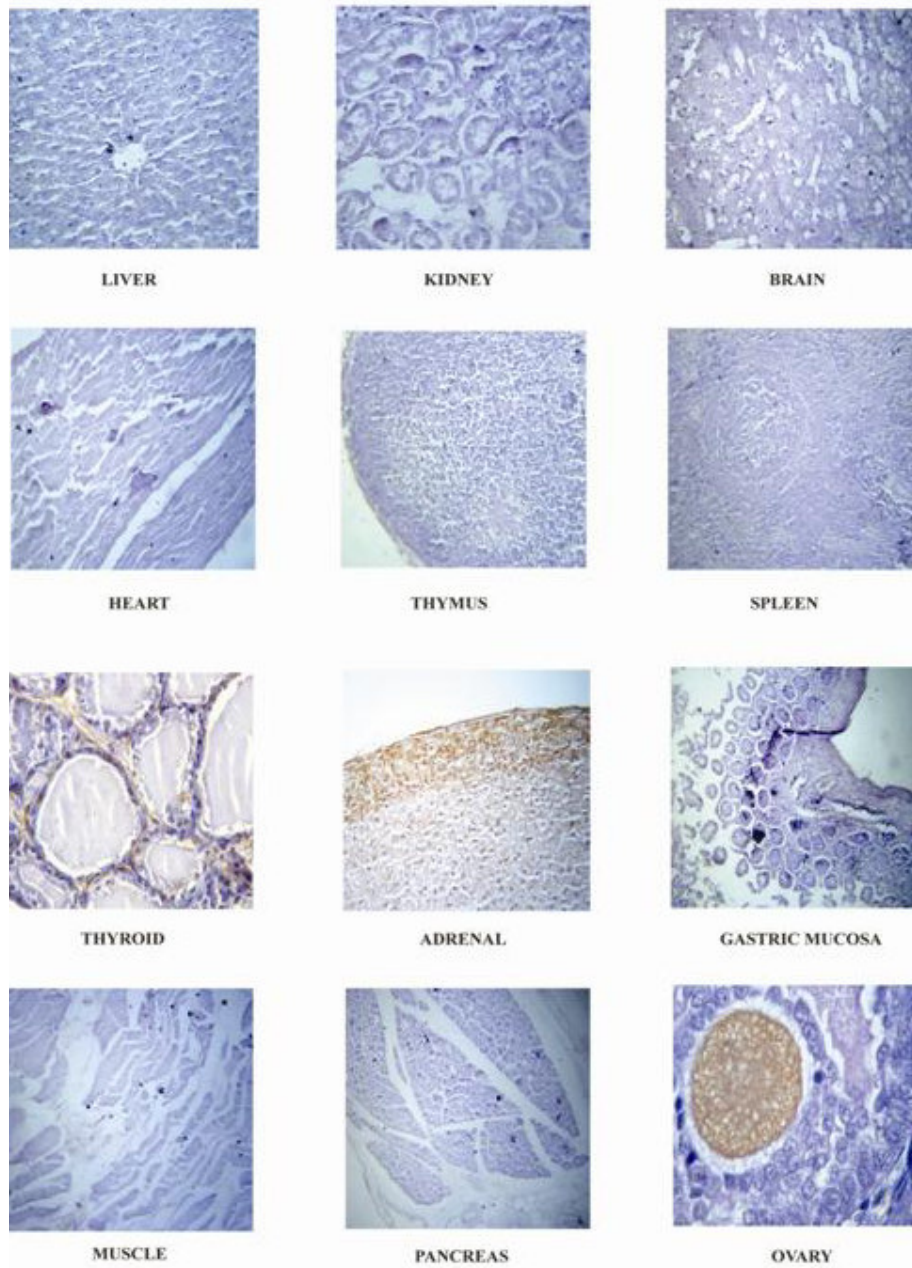


Fig. 36: This figure shows the immunoreactivity of all AOA positive sera with other somatic tissues. It can be clearly seen that all AOA positive sera show reactivity to the ovary. However, as ovarian autoimmunity now being classified as a part of the Autoimmune Polyglandular Syndrome, some of the positive sera show reactivity to either adrenal and / or thyroid antigens. Controls and negative control show no immunoreactivity to any cell types.

## 2.7 Expression of Integrins on Human Endometrium: A Possible Marker for Uterine Receptivity and Implantation

Principal Investigator: K.V.R. Reddy

Project Associate: S. Mangale

Duration: 1998-2006

A fully functional decidua is an absolute prerequisite for successful implantation and a full term delivery. Abnormalities in decidual function have been associated with various pregnancy related complications like mid-term abortion, pre-eclampsia, intra-uterine growth retardation. Integrin cell adhesion molecules and their extracellular matrix (ECM) ligands assume prime importance in this context as decidualization involves extensive cell proliferation, differentiation and apoptosis. Integrin  $\alpha v\beta 3$  and its ligands vitronectin and osteopontin are upregulated in mouse decidual tissue (Annual Report 2005-06, p 75). The role of  $\alpha v\beta 3$  integrin in decidual function was further investigated using an *in-vitro* model by blocking  $\alpha v\beta 3$  integrin. cDNA array analysis revealed downregulation of cell cycle genes and upregulation of apoptosis related genes along with protooncogenes.

During this year, work was initiated to validate the results of cDNA array analysis by Real-time PCR. RNA was isolated from the decidual cells where  $\alpha v\beta 3$  integrin was blocked. Primers used were designed against genes which were found to be differentially expressed by cDNA array analysis. Genes such as cyclin D<sub>3</sub>, cyclin G, cyclin F, Retinoblastoma, E2F, p53 were selected. These results were matched with cDNA array data reported in the previous year. In both the experiments, cell cycle genes were downregulated whereas protooncogenes and apoptotic genes were upregulated after blockade of  $\alpha v\beta 3$  integrin *in vitro* (Fig. 37).

Further analysis of the expression of these genes *in vivo* in a mouse model was carried out (Fig. 38). Decidua from day 6.5, 8.5 and 13.5 of pregnancy was collected; RNA was extracted and analyzed for the expression of above mentioned genes by Real time PCR. A progressive increase was observed in the expression of cyclins on 8.5 dpc of pregnancy, with a decrease in the expression of protooncogenes and apoptotic genes. This coincides with the high proliferative activity in the decidua during this period. Hence, it is evident that expression and interaction of  $\alpha v\beta 3$  integrin with its ligands may be responsible for modulating the expression of genes crucial for cell proliferation and death occurring in the decidua during pregnancy. These findings may provide us important insight in the treatment of various pregnancy related disorders.

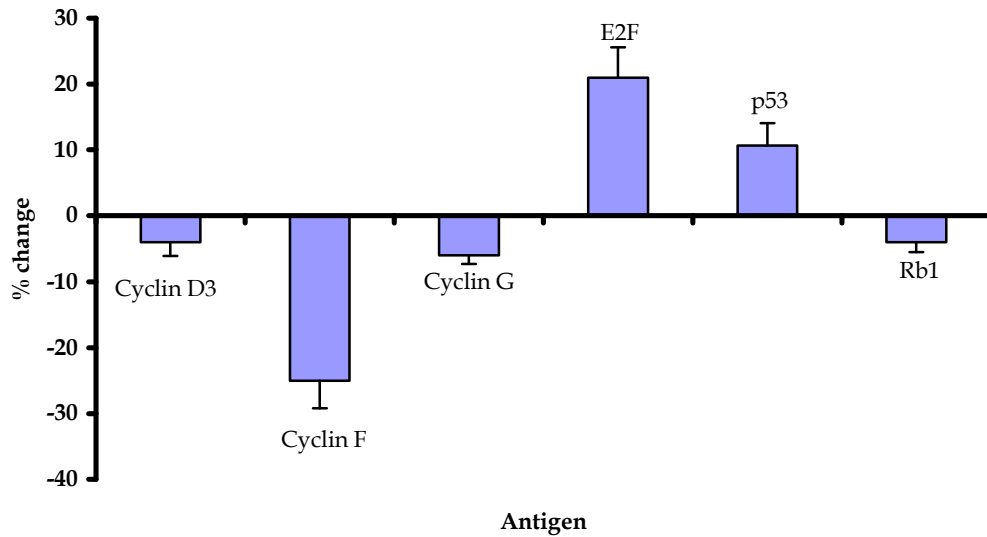


Fig. 37: *In vitro* validation of cDNA micro array results by Real time PCR in mice on day 8.5 pregnancy. The values represent the fold difference in the expression of cyclin D3, cyclin F, Cyclin G, E2F, p53 and Rb1 in treated cells ( $\alpha\nu\beta 3$  blocked by antibody) vs control ( $\alpha\nu\beta 3$  allowed to interact with vitronectin). Values are  $\pm$  SD of six observations. (Statistically significant at  $p < 0.05$ ).

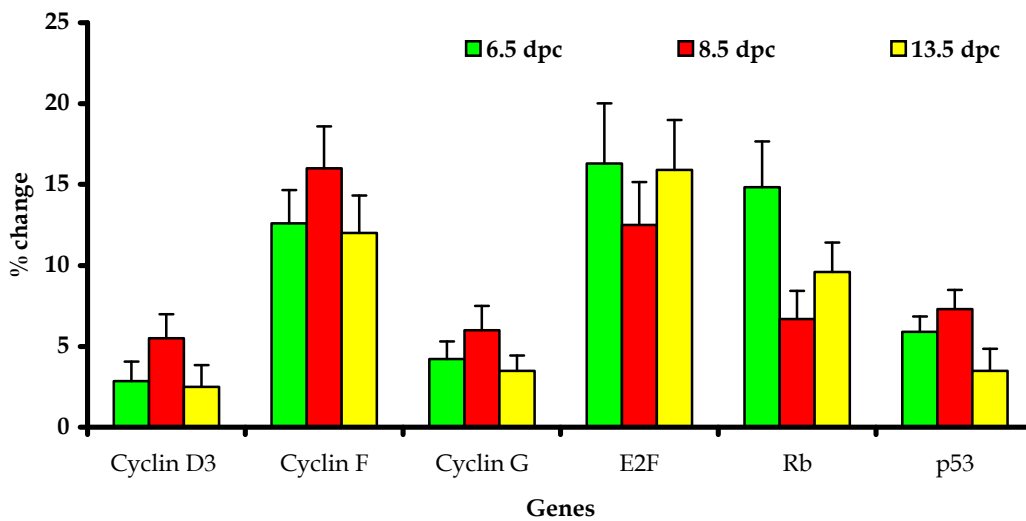


Fig. 38: Expression of differentially expressed genes in mouse decidua on different days of pregnancy. The values represent fold difference in the expression in 8.5 dpc and 13.5 dpc in comparison with 6.5 dpc. Each value is the mean  $\pm$  S.D. of six observations. Values are statistically significant at  $p < 0.05$ .

## **2.8 Studies to Elucidate the Mechanisms of Endometrial Receptivity and Implantation in Primates**

Principal Investigators: **Geetanjali Sachdeva and C.P. Puri**

Project Associates: Tanu Bajaj, Shruti Nimbkar, R.R. Katkam, Sushma Gadkar, D.D. Manjramkar, S. Metkari, Serena D'Souza, Pervin K. Meherji and Lalita Savardekar

Collaborator: Vinita Salvi, Seth G S Medical College and KEM Hospital, Parel, Mumbai

Duration: 2005-2010

Endometrial receptivity, implantation and maintenance of pregnancy is the result of several factors, dysfunction of which could contribute to significant percentage of infertility and recurrent pregnancy losses in women. These factors can be of endometrial or embryonic origin and need to be identified on a global scale. Our focus has been to identify the factor, which are derived from the endometrium and are of potential functional relevance. Toward this, studies are being pursued to identify factors which are differentially expressed in endometrial tissues/secretions during the receptive or midsecretory phase in nonconceptional and conceptional cycle in primates. It is anticipated that the data derived from differential expression profiling of endometrial tissues/secretions across the menstrual cycle will yield information on identification of those hormonally regulated factors, which are of significance in the endometrial preparation for receptivity. Studies aim to scan the endometrial profile during a conceptional cycle which will advance our understanding of the factors that dictate the success of implantation.

### **Identification of the major components of human uterine fluid using 2D proteomics approach**

Last year studies were initiated to map some of the major proteins present in human uterine fluid (Annual Report 2005-06, p 78-79) by 2D proteomics approach. Transferrin precursor, Alpha 1 Antitrypsin precursor, ApoA1 protein fragment and haptoglobin were found to be present in human uterine fluid. These studies were continued. Some additional spots were cored from 2D gels and subjected to MALDI-TOF-TOF analysis. Studies revealed the presence of Heat shock protein 27 (hsp 27), fragment of beta actin, Apolipoprotein A4 in human uterine fluid. hsp 27 was not detected in the 2D map of human serum (Fig. 39).

Heat shock protein-27 (hsp27) is a member of the small Hsp (sHsp) family of proteins. In addition to its role as a chaperone, it has also been reported to have effects on the apoptotic pathway, cell movement and embryogenesis. Preliminary analysis revealed its equivalent representation in the 2D maps of human samples collected during proliferative and secretory phases, whereas apolipoprotein A1 fragment and Apolipoprotein A4 appeared to be more abundant in the secretory phase as compared to that in proliferative phase (Fig 40).

### Investigation of the structural and molecular profile of the endometrium during embryo implantation

Embryo implantation involves a coordinated interaction between the blastocyst and a receptive endometrium. The endometrium undergoes dramatic alterations in its structural and molecular profile, in preparation for blastocyst implantation. However, not much is known about the precise sequence of events occurring at the embryo-maternal interface during the early stages of implantation in primates.

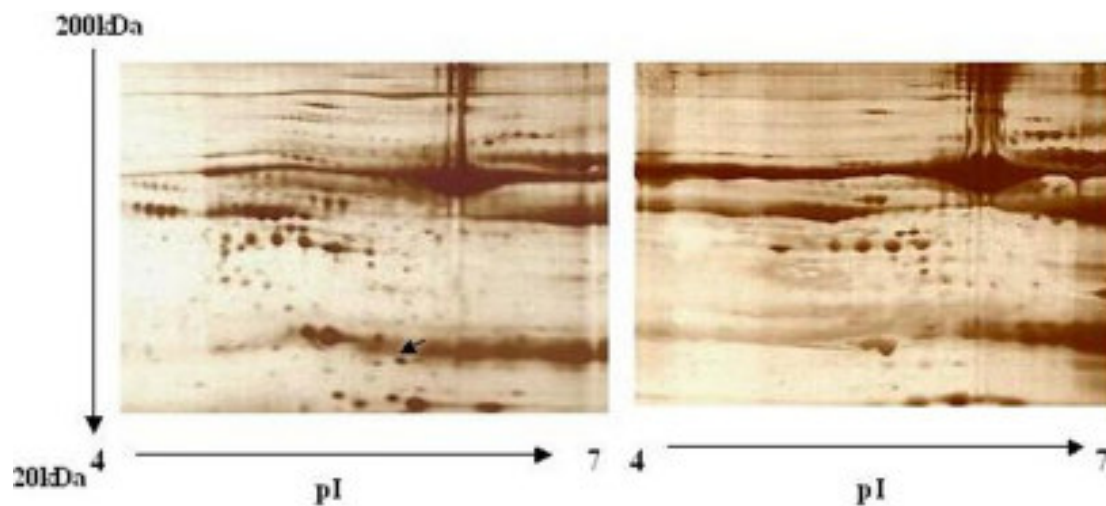


Fig. 39: Protein profiling of human uterine fluid (A) and serum (B) collected during receptive phase. Total proteins from both samples were focused in the range of pH 4-7 and then resolved on 10% SDS-PAGE. Arrow represents the location of hsp27 protein.

Delineation of these endometrial modifications during early pregnancy in a primate species may help decipher the causes of pregnancy losses and other implantation related disorders. Studies have been previously conducted by us to scan the structural and molecular profile of the pre-implantation phase endometrium during day 6 of pregnancy in bonnet monkeys (*Macaca radiata*).

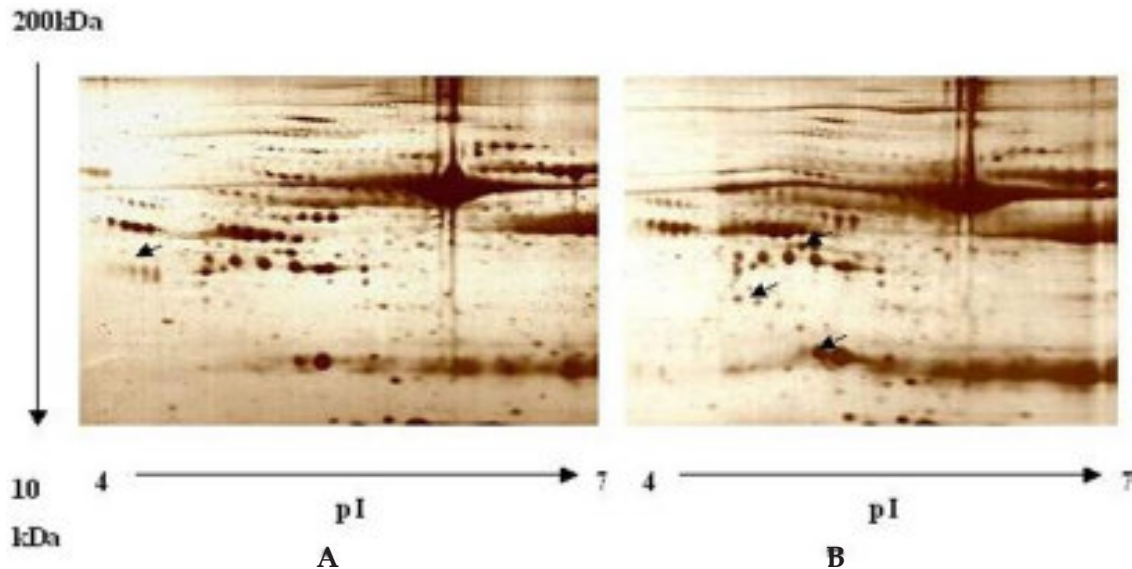


Fig. 40: 2 D protein spots detected in human uterine fluid during the proliferative phase (A) and during the receptive phase (B). Total proteins (200ug) were resolved in linear range of 4-7 in the first dimension and 10 percent polyacrylamide gel in the second dimension. Arrows represent proteins which were found upregulated either in proliferative phase or in the receptive phase

These studies demonstrated that different endometrial compartments of pregnant animals exhibit distinctive features, such as markedly decondensed and irregular shaped cells of luminal epithelium, enlarged multi-nucleated cells of glandular epithelium showing loss of secretory activity and presence of diffused gap junctions (Annual Report 2005-06, p 79-81). These studies have thus shown that the embryonic stimuli trigger pronounced structural and molecular alterations in the endometrium as early as in pre-implantation phase.

This year studies were pursued to investigate the endometrial profile during successive events of embryo attachment and invasion in bonnet monkeys. To study the events occurring at the embryo-attachment stage, the endometrial samples were collected on day 7 post-ovulation from pregnant and non-pregnant animals (n=3 each). Pregnancy in mated animals was confirmed using the Pre-implantation Factor (PIF) bioassay (Annual Report 2002-03, p 67). In pregnant animals, a very pronounced pre-epithelial plaque (PEP) reaction was observed only in those luminal epithelial cells, which were just beneath the attachment site (Fig. 41B). The luminal epithelial cells in non gestational endometrium were single layered (Fig. 41A). In pregnant animals, the endometrial stroma was highly

compact (Fig. 41D, except in the area just beneath the attachment site. The stroma beneath the attachment site appeared edematous as observed in the stroma of non-pregnant animals (Fig. 41C). In pregnant animals, secretory activity in most of the glands was absent and the glandular epithelial cells exhibited a PEP reaction similar to that seen in the luminal epithelium (Fig 41D). In contrast, in non-pregnant animals, the glandular epithelial cells were single layered with basifixed nuclei and with secretions being present in the glandular lumen (Fig. 41E). Thus, the endometria of pregnant animals exhibited characteristic structural alterations quite distinct from those seen in the endometria of non-pregnant animals.

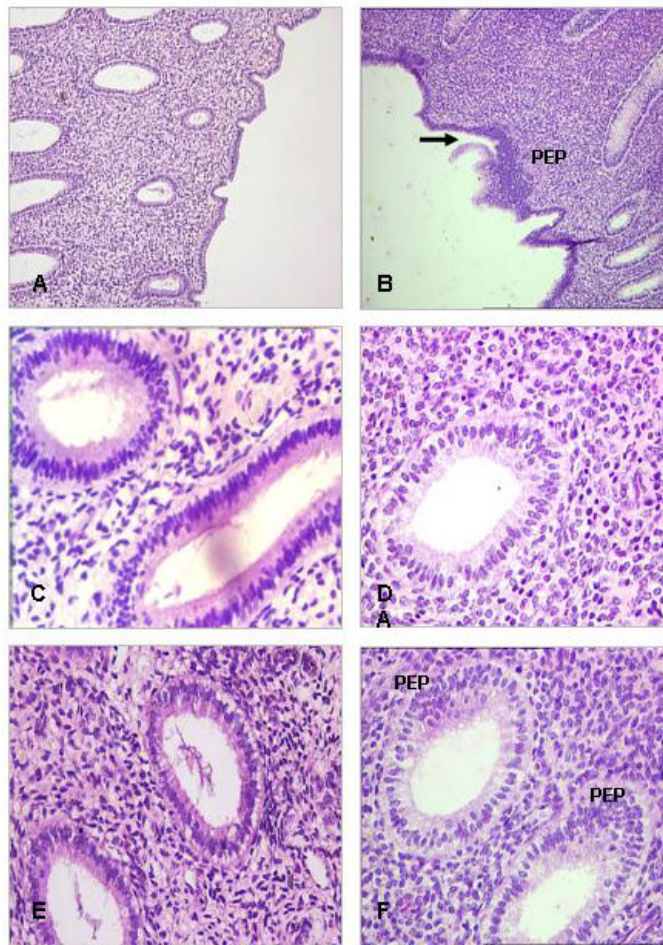


Fig. 41: Histomorphological analysis of endometria from non-pregnant (A, C and E) and pregnant (B, D, F) bonnet monkeys. PEP indicates pre-epithelial plaque reaction. The embryo attachment site observed in the endometrial section is indicated by arrow in B.

Immunohistochemical studies were also performed in order to investigate the localization pattern of OCT-4 and Insulin like Growth Factor Binding Protein-1 (IGFBP-1) in the endometrial sections of pregnant and non-pregnant bonnet monkeys on day 7 post-ovulation. OCT-4 was localized only in the embryonic cells present at the attachment site (Fig. 42A, C). IGFBP-1 expression pattern was studied in order to investigate whether decidualization had occurred in endometria of pregnant and non-pregnant animals on day 7 post-ovulation. IGFBP-1 localization was found to be zone-specific in pregnant animals. IGFBP-1 expression was particularly higher in the stromal cells in the functionalis region just beneath the attachment site, whereas in the basalis region only glandular epithelial expression was observed (Fig. 43A, C and D). In non-pregnant animals, IGFBP-1 expression was restricted to the glandular epithelial cells (Fig. 43B, D). These studies show that during embryo attachment, remarkable modulations occur at the structural and molecular level at the attachment site. Further attempts will be made to investigate the global gene expression profile of the endometrium on day 7 of pregnancy.

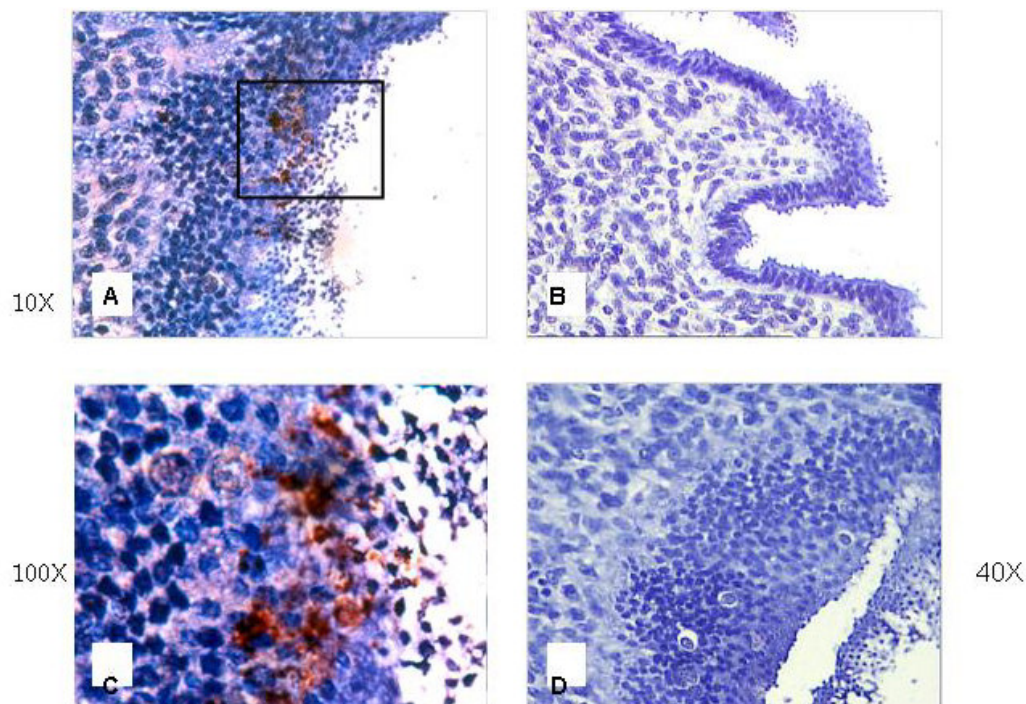


Fig. 42: Immunolocalization of OCT-4 in the embryonic cells at the attachment site. The expression was seen only in the embryonic cells present at the attachment site as shown in A and C. OCT 4 localization was not observed in non-pregnant animals as shown in B. Negative control is shown in D.