ANNUAL REPORT
2009-2010

INSTITUTE OF PATHOLOGY
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CONTENTS

EXECUTIVE SUMMARY

Institute of Pathology (IOP) being one of the premier institutes of Indian Council of Medical Research is committed to carryout cutting edge research on both communicable and non-communicable diseases with an attempt to translate the knowledge generated from laboratory to medical practice. The thrust areas of research include leishmaniasis, genital chlamydial infection, cancers, environmental toxicology and bioengineering of skin for clinical applications. Scientists at the institute have made significant efforts and pioneer contributions in understanding disease processes, development of preventive markers, new diagnostic tools and establishment of novel therapeutic strategies during the year under report.

In Tumor Biology Division, scientists are working on understanding molecular pathology, identification of diagnostic, predictive and prognostic biomarkers and drug targets for ***breast, urogenital, hematological and brain cancers.*** Investigation on the **gene-environmental link** responsible for very high incidence of several malignancies, especially those associated with tobacco and pesticide (oral, esophageal, gastric, lung and breast cancers) in north-eastern states in India, is also a major area for research. The field of cancer epidemiology has now emerged as one of the core disciplines.

Analysis of gene expression and hypermethylation profiles associated with early onset breast cancer showed 300 differentially expressed genes in tumor tissue compared with the adjacent normal tissue. Human whole genome methylation analysis showed total 5192 differentially CpG sites where 2395 were hyper-methylated sites. Two breast cancer cell lines have been established from primary breast tumours from young Indian women which may serve as a good *in vitro* model for understanding early breast tumorigenesis and mechanisms underlying breast tumorigenesis in Indian population. The study on the role of type 1 growth factor receptor genes- EGFR, c-erbB-2, c-erbB-3 along with MDR1 and AR genes in predicting response to neo-adjuvant chemotherapy in locally advanced breast cancer cases showed AR gene to have independent prognostic value. Study investigating the role of androgen signaling (AR, PSA) and metabolizing (CYP19) genes, p53 codon 72 polymorphisms and mutations/expression of mismatch repair genes MLH1 and MSH2 in genetic susceptibility and progression of prostate carcinoma showed significant association of short AR CAG repeats (<24) (OR 1.75(1.00-3.09),

0.05) and p53 codon 72 Pro/Pro genotype with carcinoma prostate risk. A C/T transition at -93 position of the core promoter region of MLH1 gene was also identified and CC genotype was found as a genetic predisposing factor for prostate cancer development.

Multicentric studies on association of tobacco with high incidence of oral, esophageal, gastric and lung cancers in northeast region did not show significant association of GSTM1 and GSTT1 null genotypes with risk of these cancers while they appeared to work as a protective factor for lung cancer. Genome-wide analysis of chromosomal changes using single nucleotide polymorphism array in ESCC showed gains at 3q and 5p while regions on 3p, 8p, 13q and 18q were the most common sites for loss. The amplified regions include gene pathways involved in cellular migration and proliferation, tumor cell metastasis and invasion, anchorage independent growth, inhibition of apoptosis and MAPK pathway. Deleted regions included genes involved in suppression of tumor including those involved in Wnt signaling and focal/ cell adhesion pathway.

The study on identification of genes associated with the presence of fusion gene (BCR-ABL) in chronic myeloid leukemia using oligonucleotide microarray showed upregulation of MAPKKK cascade, G protein coupled receptor internalization, interleukin-1 beta secretion, regulation of T-cell differentiation, signal transduction by p53 class mediator resulting in induction of apoptosis, regulation of GTPase activity, cell proliferation and of I-kappaB kinase/NF-kappaB cascade. Downregulated genes included regulation of caspase activity, oxygen transport, B-cell apoptosis, sodium ion transport and regulation of progression through cell cycle. In study on NF-kB pathway in Acute Leukemias (AL), non-responder patients of AML showed lower p53 and higher IKK-alpha expression suggesting that loss of p53 function leads to transcriptional induction of IKK-α which results in NF-kB mediated gene expression. This appears to be an important mechanism for activation of NF- kB in AL.

A high-throughput Tissue Microarray (TMA) chip containing 300 brain tumors from archival paraffin blocks at IOP according to subtypes and histological grades based on WHO classification has been constructed using manual tissue arrayer with core diameter of 1.0 mm and used to study the protein expression of the differentially expressed genes identified by cDNA microarray at National Cancer Institute using immunohistochemistry TMA-IHC for analysis of potential diagnostic and prognostic biomarkers.

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen worldwide. In women, infection with *C. trachomatis* causes pelvic inflammatory disease (PID), infertility and ectopic pregnancy, thus having a devastating impact on the reproductive health of

women. Further it has been reported that infection with *C. trachomatis* increases chances of co-infection with HIV and HPV-induced cervical neoplasia.

Study on role of chlamydial HSP60 and cHSP10 in pathogenesis of genital tract infection in women showed significant modulation of genes involved in apoptosis in primary cervical epithelial cells. Significant upregulation of interleukin (IL)-1β-convertase, caspase-3, -8 and -9 genes was found by cDNA microarray upon stimulation with cHSP60 and cHSP10 suggesting thereby their role in apoptosis.The studies conducted to understand the mechanism of DC antigen presentation and the role of sex hormones in modulating the immune responses during chlamydial infection showed that estradiol treatment significantly reduces the release of proinflammatory cytokines IL-12 and TNF-α at the highest concentration, however, a non-significant decrease in levels of IL-6 was observed while IL-10 was found significantly increased. The expression of a number of Toll-like receptor signalling genes such as IRAK4, MyD88 and nF-κB were upregulated following chlamydial infection. Estradiol pretreatment significantly reduced expression of TLR4 as compared to untreated cells.

Evaluation of antimicrobial activity of novel polyherbal formulation BASANT against *C. trachomatis* showed its potential clinical utility for the prevention of *C. trachomatis* infection. In the study to determine the drug sensitivity profile of *C.trachomatis* isolates from patients with treatment failure and recurrent infection, decreased antibiotic susceptibility was observed in isolates obtained from recurrently infected patients towards the current first line drugs (azithromycin and doxycycline) and it was seen that its complete eradication could not occur even at higher concentration (8μg/ml). However, the number and size of inclusion bodies were found to be decreased with increasing concentration of antibiotics which reflects the presence of heterotypic resistance. In presence of doxycycline, a significant decrease in the levels of IL-1β and IL-6 was observed in *Chlamydia* positive fertile women whereas no significant decrease in the levels of cytokines was observed in *Chlamydia* positive infertile women. In recurrent infection, a significant decrease in the IL-1β, IL-6, IL-8, IL-10 and TNF-α was observed in both *Chlamydia* positive fertile and infertile women in presence of azithromycin in *C. trachomatis* stimulated cells while in presence of doxycycline, no significant decrease in the levels of cytokines was observed in both the groups.

Studies on role of Chlamydia pneumoniae in coronary artery disease showed high prevalence of *C. pneumoniae* (29.6%) in CAD patients in India with higher seropositivity for *C. pneumoniae*-specific IgA than the *C. pneumoniae* IgG. Study on cHSP60 mediated signaling cascade in CAD patients showed significant ($p<0.001$) upregulation of VCAM1, NF-kB1, IL-8,

ICAM1, FASLG, EGR1, BAX, and MMP7 genes and significant ($p<0.001$) downregulation of c-JUN, IkkB, BIRC1genes in cHSP60 positive CAD patients compared to cHSP60 negative CAD patients.

In studies on *leishmanial* infection, studies on identification of a novel ubiquitin-like system in the protozoan parasite *Leishmania donovani* have established the presence of an Ufm1-Uba5-Ufc1 ubiquination like pathway in trypanosomatids. The cloning and characterization of *Ld*Uba5 (Ufm1 activating enzyme 5) showed homology with human Uba. $Cys^{217.}$ Further, a *Leishmania* ubiquitin-fold modifier 1 (*Ld*Ufm1) was identified with an exposed C-terminal glycine which is essential for subsequent activation by its cognate E1 protein (*Ld*Uba5). Functional role of *Ld*Uba5 activity in *Leishmania* growth was evident since overexpression of mutants of *Ld*Uba5 (Cys^{217} to Ser/Ala) resulted in reduction of the parasite growth, indicating the importance of *Ld*Uba5 activity in *Leishmania* growth The unique feature of *Leishmania* Ufm1 pathway was its localization in the mitochondria as human Ufm1 pathway is found in cytoplasm. As *Ld*Uba5 is functionally important for the growth of *Leishmania*, it is possible this pathway may be involved in both protein turnover and /or transcriptional regulation like ubiquitin and other Ubls.

To understand the mechanism of natural antimony resistance using field isolates from kala-azar, expression of nine genes in natural SAG resistant and sensitive field isolates of *Leishmania donovani* showed expression of PSA- 2, HSP83 and histones H1, H2A and H4 low in all sensitive isolates while MAPK1 high in sensitive isolates. Of these, PSA-2 and H2A genes showed a strong correlation of expression with drug susceptibility. Further, these genes showed increased expression at protein level in all resistant isolates. The data indicates that antimony resistance in field isolates is a multifactorial phenomenon. The high correlation of PSA-2 and H2A gene expression with antimony susceptibility makes them attractive candidates as biomarkers for distinguishing the resistant and sensitive parasites in the endemic area.

The baseline data on natural susceptibility of Indian *L. donovani* isolates towards new upcoming antileishmanial drugs, Paromomycin (PMM) and Sitamaquine (SIT) which are currently under Phase IV/III trials respectively was also established. The data highlights the potential of PMM as an alternative anti-leishmanial drug with similar sensitivities in parasites from zones of low resistance (LR) and high resistance (HR) to antimony and showing no cross resistance towards SAG and miltefosine resistant *L.donovani* parasites. The percentage parasite killing correlated significantly with the nitric oxide release ($r=0.76$, $p<0.05$) for PMM and SIT ($r=0.48$, $p<0.05$) treated macrophages. For SIT, upon addition of NO inhibitor, there was a

decrease in NO production with decrease in the parasite killing suggesting that cytotoxic activity of SIT against *L.donovani* amastigotes may involve NO pathway. In case of PMM, there was very little effect on the parasite killing in the presence of NO inhibitor suggesting that activity of PMM against *L.donovani* may not be solely dependent on nitric oxide.

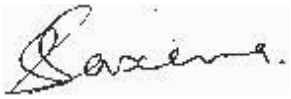
Study on association between parasite burden and localized immune response in patients of Cutaneous Leishmaniasis (CL) caused by *Leishmania tropica* showed significantly up-regulated IL-4 in early lesions ($p<0.02$) which was also found correlated with higher parasite burden and may be involved in pathogenesis of CL by inhibiting protective immune response.

Studies on attenuation conditions for 3T3 fibroblasts for use as feeder cells led to establishment of a foolproof irreversible growth arrest of 3T3 cells with concomitant optimal stimulation of keratinocyte proliferation depending on numerical dosing of mitomycin-C for which a patent has been filed through IPR Division of ICMR. Studies on utility of Mebiol gel as a supportive matrix towards developing a feeder free culture system for human keratinocytes showed that human epidermal keratinocytes seeded as a concentrated pellet in between two discs of gels showed marked migrations of certain specific cell types towards the periphery of the discs with different time lapse. These cells are isolated and are now being tested for their specific markers.

Studies on health hazards of phthalate vis-à-vis idiopathic male infertility showed that with the increasing potential of occupational phthalate exposure, there was a significant corresponding decline in the testosterone levels. The testosterone levels also showed corresponding decline with sperm count and motility and ultrastructral changes in sperm morphology.The results on hormonal, morphological and functional studies are being correlated with the levels of phthalates and their metabolites estimated by GC-MS. Assessment of pesticide exposure in tea garden workers of north-eastern state of India (HEBM) has revealed the presence of malathion, endosulfan, fenverate, dimethoate, primiphos methyl, tebuconazole, delta BHC, lindane, ethion, heptachlor, aterazine, chlorpyriphos, quinalphos, flufenacet, DDT, deltametharin, cypermetharin, glyphosate, paraquat, naphthalene, pyrene, dicofol, phosalone and monochrotophos in the placental tissue extract and blood extract analysed so far in one or more samples.

Institute is proud of academic achievements of its scientists. **Dr. Sunita Saxena**, Director, IOP was awarded the **Novartis Oration Award** for research in the field of cancer by ICMR for the year 2006 and **Dr. Aruna Singh**, Scientist 'F' was awarded **Lala Ram Chand Kandhari Award** of ICMR for research in the field of *Chlamydia* for the year 2007 on 18th Sept.

2009. Dr. S. Raju Bagadi has been awarded ACSDI fellowship of AACR. The continuous efforts of scientists by way of national and international publications and research grants add new dimensions to the academic activities. The institute continues to contribute significantly to human resource development by conducting various academic activities, *viz.*: DNB, Ph.D, summer fellowships and WHO training programs. I take this opportunity to convey my thanks to Dr. V. M. Katotch , Director General, ICMR a nd Secretary, Department of Health Research for his support for infrastructure development and encouragement for scientific and academic programs. I acknowledge my sincere thanks to my scientific, technical and administrative staff for their contribution towards enhancement of infrastructure and scientific activities.



(Dr. Sunita Saxena)

Director

RESEARCH ACTIVITIES

Tumor Biology

TUMOR BIOLOGY

I. BREAST CANCER

1. STUDY ON GENE EXPRESSION AND HYPER-METHYLATION PROFILES IN EARLY- ONSET BREAST CANCER

Scientific staff	:	**Dr. Sunita Saxena, Dr. S. A. Raju Bagadi, Dr. Sujala Kapur, Ms. Shreshtha Malvia**
In collaboration with	:	**Dr. A. Bhatnagar, Consultant, Dr. Chintamani, Dr. R. S. Mohil, Department of Surgery, Safdarjung Hospital, New Delhi**
Technical Staff	:	**Mrs. Valsamma Matthew**
Duration	:	**2007-2011**

Aims, Objectives & Background:

Breast cancer is a heterogeneous disease encompassing a wide variety of pathological entities and a range of clinical behavior. These are underpinned at the molecular level by a complex array of genetic and epigenetic alterations that impinge on cellular processes. Breast cancer diagnosed at young age is well recognized as clinically different as breast cancers diagnosed at older age. Differences in clinical behavior and molecular profile of early onset breast cancer suggest the need for understanding the risk factors and molecular mechanisms involved in development of breast cancer in young women. Hence, molecular signatures of early-onset breast cancer are required for better understanding of pathogenesis which will result in better disease management. Study of gene expression profile and methylation status unique to early-onset breast cancer may lead to identification of risk factors and various cellular pathways involved in pathogenesis. This would help in identification of biological prognostic/predictive markers and targets for therapeutic intervention.

To achieve the aim, gene expression and promoter methylation profiles were analyzed using micro-array chips designed separately for these specific applications.

Work done during the year:

RNA from 18 breast cancer specimens was used for gene expression profiling. In brief, RNA has been labeled and hybridized on to Illumina Sentrix Human Whole Genome (WG-6) expression bead chip. The data has been average normalized, and gene expression changes with respect to normal RNA were calculated and gene list was generated on the Bead Array studio. About 300 differentially expressed genes were obtained either up-regulated or down-regulated.

Human whole genome methylation analysis was performed on Illumina Human methylation27 chip with total of 24 samples including adjacent normal tissues as controls. In brief, DNA was isolated using 10-20mg of tissue using tissue DNA isolation kit, following the manufactures instructions. Quality of the DNA was checked on 0.8% agarose gel and quantity was checked on Picogreen. 1 µg of genomic DNA is treated with bisulfite conversion kit. After bisulfite conversion there was a whole genome amplification followed by enzyme fragmentation, hybridization staining and scanning. The level of methylation is determined at each locus by the intensity of the two possible fluorescent signals, from the C (methylated) and T (un-methylated) alleles. Differential methylation analysis performed between early and late cases, showed total 5192 differentially methylated CpG sites of which 2395 were hyper-methylated. (Fig. 1).

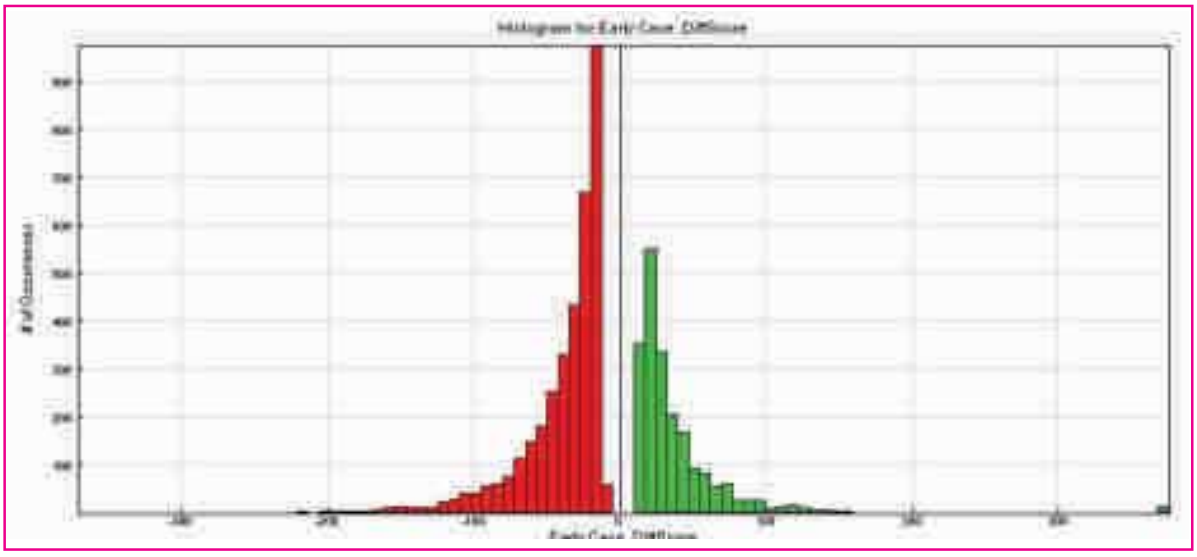


Fig. 1: Histogram showing frequencies of diff exp genes at different diff scores (early-onset cases versus late-onset cases).

Pathway analysis on preliminary data has shown many deregulated genes involved in various pathways (Fig. 2).

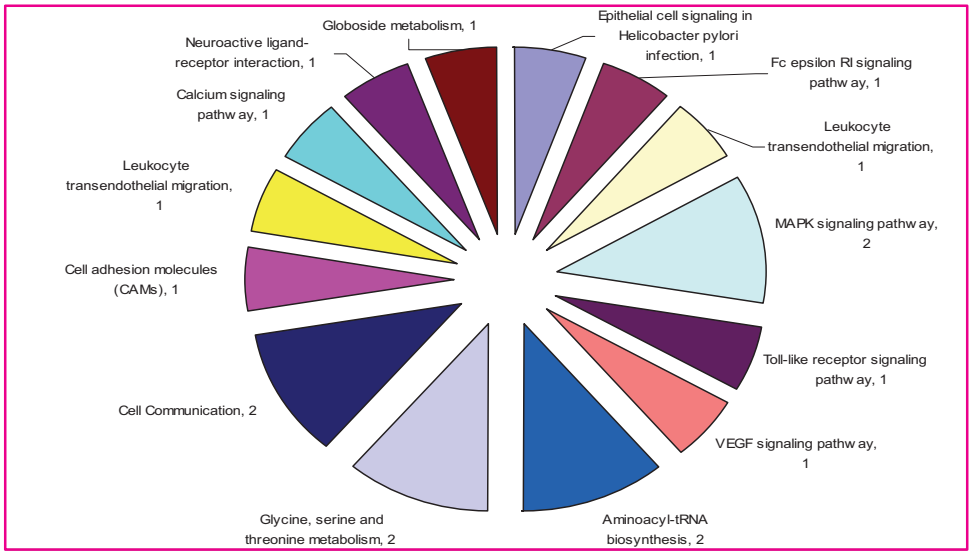


Fig. 2: Various pathways with deregulated gene expression

Further, validation for up-regulated and down-regulated genes will be done by real time quantification and tissue micro-array (TMA).

2. ESTABLISHMENT AND CHARACTERIZATION OF BREAST CANCER CELL LINES FROM PRIMARY BREAST CANCERS

Scientific staff	**:**	**Dr. Sunita Saxena, Dr. S. A. Raju Bagadi, Dr. Sujala Kapur, Ms. Shanti latha P.**
In collaboration with	**:**	**Dr. A Bhatnagar, Consultant, Dr. Chintamani, Dr. R. S. Mohil Department of Surgery, Safdarjung Hospital, New Delhi**
Technical Staff	**:**	**Mr. Jagdish Pant**
Duration	**:**	**2007-2011**

Aims, Objectives & Background:

Although the incidence of breast cancer in India is low as compared to the western countries, the incidence of breast cancer in younger women remains more or less same suggesting a high proportion of early onset of breast cancer in Indian women. The molecular mechanism associated with early breast cancer is not well understood. Therefore, there is a need to establish cell lines from Indian population, which may be useful for understanding molecular mechanisms involved in breast tumourigenesis in Indian population. Cell lines provide an unlimited, self-replicating source of cells that can be widely distributed to facilitate comparative studies. The majority of breast carcinoma cell lines have been established from tumour metastases, in particular malignant pleural effusions, while relatively few have been established from primary tumours. Thus, the present study is aimed to develop breast cancer cell line from primary breast tumours that may serve as a good *in vitro* model for conducting studies to investigate breast tumourigenesis in Indian population. The objectives of the project include: a. Establishment of cell line(s) from human primary breast tumours, b. Characterisation of the established breast cancer cell lines.

Work done during the year:

During the period under report, 50 breast tumours were collected and used for initiation of primary cultures. Primary cultures were established by enzymatic disaggregation (using trypsin and collagenase). All the primary cultures established were subjected to purification by differential trypsinsation and or Magnetic Activated Cell Sorting (MACS) using cytokeratin or EpCAM antibodies (Fig. 1). Two breast cancer cell lines, PCB20 (72 passages) and PCB36 (54 passages) have been established from patients with age of 38 and 39 years respectively using triple negative primary tumours (Fig. 2 & 3). They are characterized for epithelial markers, anchorage independent growth, ultrastructural analysis and cytogenetic studies (Fig. 4 & 5). Karyotyping analysis of PCB20 has shown that the cell line is near tetraploid with a modal number of 58 to 62 chromosomes. PCB36 cell line was found to be nearly tetraploid to pentaploid complement and the chromosomal numbers ranged from 107 to 110. Most of the chromosomes of both the cell lines exhibited several translocations and several marker

chromosomes. The doubling time of PCB20 cell line is 30 hrs; whereas for PCB36 cell line it is 27 hrs.

Fig. 1: Establishment and Purification of PCB36 Cell Line

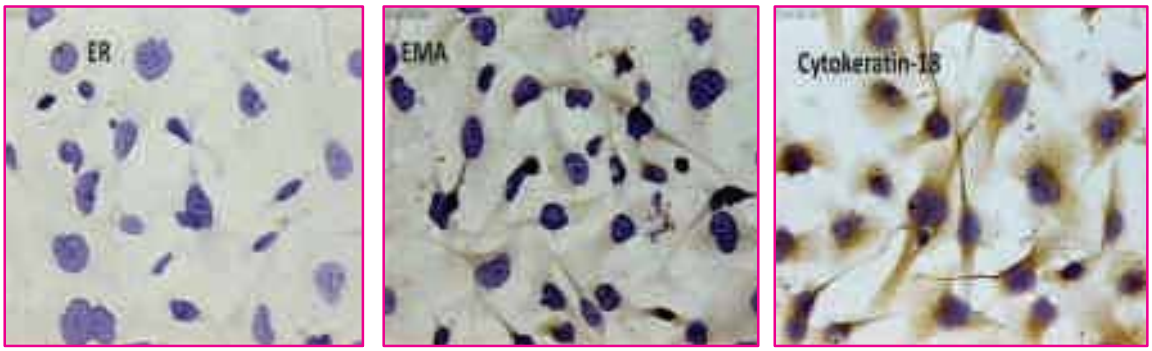


Fig. 2: Expression of Biological Markers in PCB36 Cell Line

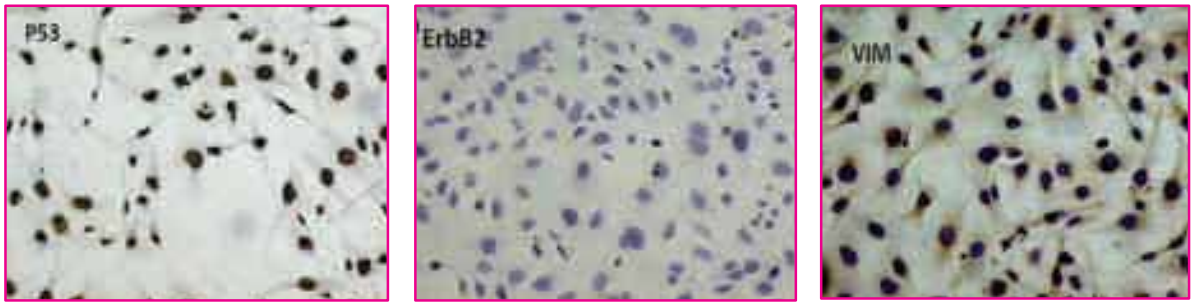


Fig. 3: Representative Karyogram of PCB20

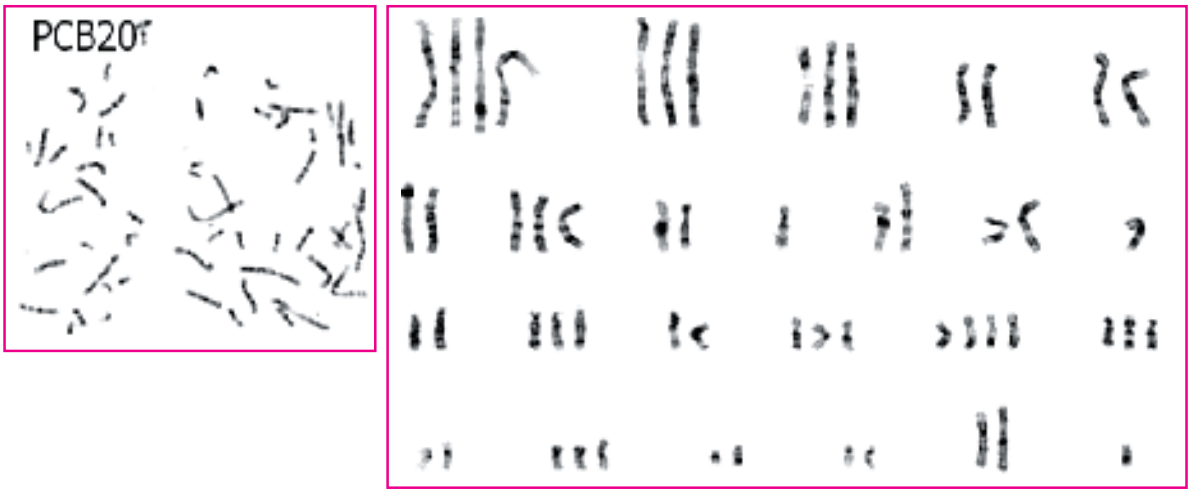


Fig. 4: Representative Karyogram of PCB20 cell line

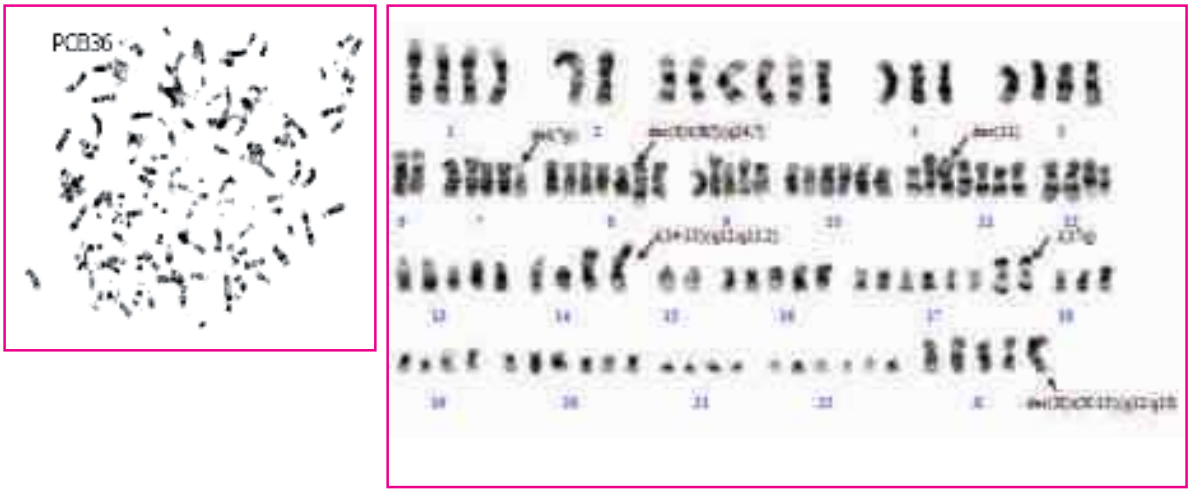


Fig. 5: Representative Karyogram of PCB36 cell line

3. TYPE 1 GROWTH FACTOR RECEPTOR FAMILY : EXPRESSION AND CORRELATION WITH RESPONSE TO NEO-ADJUVANT CHEMOTHERAPY IN LOCALLY ADVANCED BREAST CANCER

Scientific staff	**:**	**Dr. Sunita Saxena, Dr. L.C. Singh, Dr. Sujala Kapur, Dr. Anurupa Chakraborty**
In collaboration with	**:**	**Dr. Dinesh Bhatnagar, Dr. Chintamani, Dr. Nidhi Sugandhi, S J Hospital, New Delhi**
Duration	**:**	**2006-2010**

Aims, Objectives & Background:

Locally Aadvanced Breast Cancer (LABC) accounts for a sizeable number (30-60%) of breast cancer cases and is a common clinical scenario in developing countries. The standard care for LABC is Neoadjuvant Chemotherapy (NACT) followed by surgery in the form of modified radical mastectomy and subsequent three more cycles of CAF (cyclophosphamide, adrimycin, 5-fluorouracil adjuvant chemotherapy. Based on therapy response the patients are grouped into responders and non-responders.

In recent years, the family of epidermal growth factor receptors including EGFR, ERBB2, ERBB3 has attracted great attention in the literature. Although expression of these receptor genes has been studied in several types of human tumours, only EGFR and ERBB2 have been proven to play major roles in different types of breast cancer. EGFR and ERBB2 co-expression in breast cancer was recently associated with reduced overall survival and disease free survival. Over-expression of ERBB3 is also frequently reported in ERBB2 altered breast cancers. Human breast cancer cell lines commonly co-over-express both ERBB2 and ERBB3, further supporting their role in breast carcinogenesis.

The Androgen Receptor (AR) is detectable in the majority of tumor specimens from patients undergoing mastectomy for breast cancer. AR expression in breast cancer tissue samples has been associated with an improved response to hormone therapy and longer survival in patients with breast cancer. Multidrug Resistance (MDR1) is a significant challenge in the

treatment of breast cancer. MDR1 expression is also associated with the expression of the ERBB2 oncogene. Hence, this study aimed to define mRNA expression level of growth factor receptor genes (EGFR, ERBB2, ERBB3), hormone receptor gene AR and multidrug resistant gene -MDR1 gene and their association with response to NACT in locally advanced breast cancer to identify possible candidate gene(s) which may predict response to treatment and help in assessing the successful drug based therapy.

Work done during the year:

Fifty matched samples of pre-and post-NACT tumor tissues have been collected during the year under report. Total RNA (TRIzol method) was isolated and RNA quality was assessed further on Agilent 2100 Bioanalyzer (*Agilent Technologies, Waldbronn, Germany*). Samples having RNA integrity Number (RIN) 6 and above were selected for real time experiment. Then, cDNA was generated using High Capacity cDNA Archive kit (*Applied Biosystems*) according to the manufacturer's protocols.

The relative quantitation of expression levels of EGFR, ERBB2, ERBB3, AR and MDR1 genes in pre and post NACT breast tissue with human normal breast total RNA(*Ambion*) as a control sample was carried out by real time RT-PCR (ABI 7000 SDS, *Applied Biosystems*) with cDNA as template using TaqMan probe Assay.

Clinically, 37 out of 50 patients were responders and 13 were non-responders. 19 out of 50 were pre-menopausal and 31 were post-menopausal.

Result

In view of pre-therapeutic expression, the AR mRNA expression level was found comparatively higher in responder group than non-responders and the outcome was found statistically significant (p=0.049). However, no significant difference in expression levels of EGFR, ERBB2, ERBB3 and MDR1 genes was observed in responders in comparison to non-responders.

On the contrary, the expression levels of target genes in post-therapeutic samples did not show significant differences among responders.

In responders (37/50), down-regulation of AR expression (*27 cases [72.9%])* (Fig. 1) and up-regulation of EGFR (*23 cases [62.2%*], MDR1 (31 cases [83.7%]) expression were significant (*p=0.020*, *p=0.045 and p= 0.001, t-test*). The down regulation of ERBB2 expression (26 cases [70.2%]) was remarkable but not significant (p=0.335).

In non-responders (13/50), up-regulation of EGFR (*10 cases [76.9%*]) and MDR1 (*13 cases [100%*]) was statistically significant (*p=0.039, p=0.004, t-test*). For AR, the upregulation was notable i.e. *84.6% (11/13),* but not statistically significant *(p=0.290).*

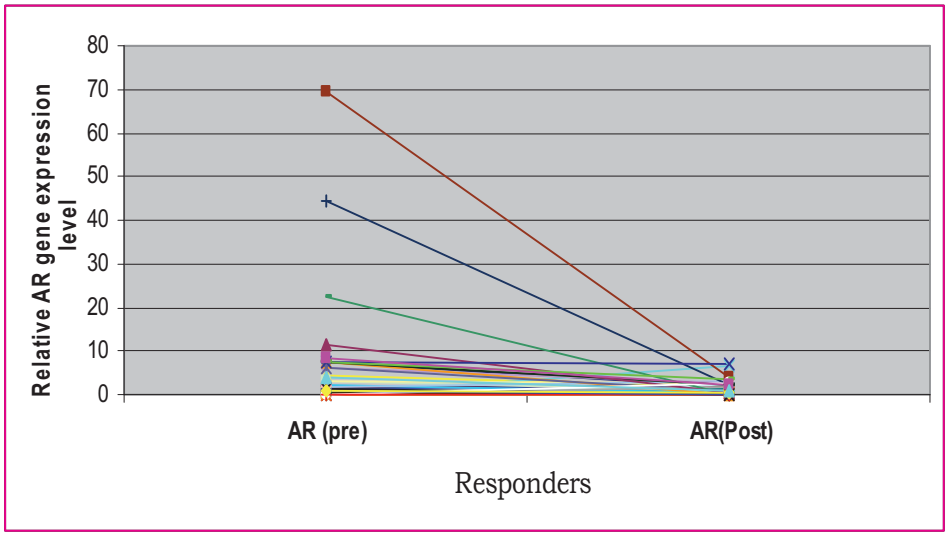


Fig. 1: Expression levels of AR measured in pre-therapeutic tumor biopsy specimens and post-therapeutic tumor resection samples in responders

Conclusion

Responders showed a significantly high level of pre-NACT AR gene expression (p=0.049) which reduced following NACT (p=0.020). This indicates an independent predictive role of AR with response to NACT and helps in the prediction of the success of neo-adjuvant chemotherapy in individual cancer patients with locally advanced breast carcinoma.

A significant post-therapeutic increase in the expression levels of EGFR and MDR1 genes in responders ($p=0.045$ and $p=0.001$) as well as in non-responders ($p=0.039$, $p=0.004$) suggests that expression of these genes changes during therapy but they don't have any role in drug response.

Future Work

To study the role of AR gene with other AR associated genes (TGF β, PSA, ARA70 and SMAD3) & its correlation with EGFR, ERBB2 and ERBB3.

II UROGENITAL MALIGNANCIES

1. STUDY ON MICROSATELLITE INSTABILITY IN ANDROGEN RECEPTOR GENE, P53 GENE POLYMORPHISMS AND EXPRESSION PROFILE OF MISMATCH REPAIR GENES IN PROSTATE CARCINOMA

Scientific staff	**:**	**Dr. Sunita Saxena, Dr. Anju Bansal, Ms Abha Soni**
In collaboration with	**:**	**Dr. N.K. Mohanty, Safdarjung Hospital, New Delhi**
Duration	**:**	**2005-2010**

Aims, Objectives & Background:

Prostate Cancer (CaP) is the sixth most common cancer in the world and the third leading cause of cancer in men. In India, it is the fifth most common cancer among men. Knowing the widespread prevalence of CaP in the world and palliative nature of treatment after the disease spreads, the search for reliable molecular biomarkers is as important as ever. (Molecular markers that can accurately predict the presence, metastasis and serve as prognosticators that are desired to foretell the course of CaP.)

The present study was to indentify microsatellite in stability in androgen receptor (*AR*) and *CYP19* genes, polymorphisms in *PSA* gene, mutations/polymorphisms in and two major MMR genes hMLH1 and hMSH2 genes and gene expression profile of Mismatch Repair Genes *hMLH1, hMSH2, PMS1, PMS2, hMSH3* and *hMSH6,* in prostate cancers that would help in understanding molecular mechanisms underlying occurrence and progression of prostate cancer along with their propensity for development in certain individuals.

Work done during the year:

For polymorphism studies:

Genomic DNA was obtained from 105 prostate cancer patients and 120 BPH cases along with 106 normal healthy individuals as controls.

For expression study:

Tissue samples from 32 cases of CaP and 30 cases of BPH were collected.

For mutational analysis of p53 gene:

DNA extraction was done from formalin fixed paraffin embedded (FFPE) tissue blocks of biopsy samples from 48 CaP, 24 PIN and 24 BPH cases. PCR-based GeneScan analysis was performed for *AR* CAG and *CYP19* TTTA analysis. For mutational analysis in hot spot exons 5, 6, 7 and 8 of p53 gene, PCR amplification followed by direct sequencing was done on DNA extracted from biopsy specimens obtained as FFPE tissue sections. Using PCR-RFLP assay p53 codon 72 polymorphism was determined. By Quantitative RT-PCR the expression of *AR* and *PSA* was studied.

Results

***AR* CAG repeat polymorphism:**

A highly statistically significant association of short AR CAG repeats (⩽ 24) has been found with CaP risk OR, 2.98 (95% CI 1.69-5.24, p <0.001) showing nearly three-fold risk among men with short (⩽ 24) *AR* CAG repeats compared to men with long *AR* CAG repeats (>24). Similar high frequency of short AR CAG repeats (⩽ 24) was found in BPH cases when compared with controls OR, 1.96 (95% CI 1.15-3.32, p= 0.01), however risk conferred was found to be lesser than that for CaP.

***PSA* gene polymorphism:**

About two to three-fold increased risk of BPH and CaP was found with the GA and AA genotype respectively. For GA genotype ORs were 2.07 (95% CI, 1.13-3.77, p=0.018) and 2.47 (95% CI, 1.07-5.68, p=0.03) for CaP and BPH respectively. The risk associated with AA genotype was 2.68 (95% CI 1.20-5.98, p=0.016) and 3.46 95% CI (1.92-6.25, p<0.001) for CaP and BPH respectively. AA genotype was found to confer greater risk than GA genotype.

***CYP19* TTTA repeat polymorphism:**

Homozygosity for the A2 (8 repeats) allele was more frequent in CaP (23.8%) compared to BPH (14.2%) and controls (12.5%). Heterozygosity for A1 and A2 alleles (A1A2 genotype) was less common in CaP (16.2%) compared to BPH (22.6%) and controls (20.0%). None of the *CYP19* genotypes conferred significant risk for CaP or BPH.

AR and *PSA* gene expression

The mRNA expression of both *AR* and *PSA* was found to be lower than constitutively expressed genes. However, the gene expression of both *AR* and *PSA* was significantly higher in CaP as compared to BPH cases. The mean (mean ± SD) expression of *AR* was -0.756 ± 4.591 in CaP while in BPH cases was -4.042± 3.569 and the differences in the expression found to be statistically significant (p=0.004). Similarly, the mean *PSA* mRNA expression was observed to be -6.653 ± 4.990 and -9.402 ± 5.390 in CaP and BPH respectively and the difference between the two was significant (p=0.0491). Both *AR* and *PSA* gene expression showed nearly 3 fold increased expression in CaP as compared to BPH (Fig. 1).

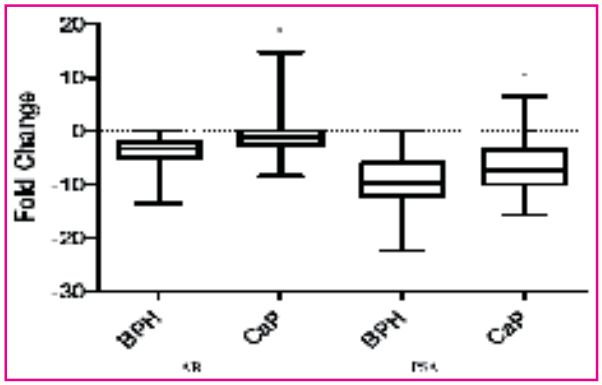


Fig. 1: Gene expression of AR and PSA in CaP and BPH cases

Correlation between *AR, PSA* gene expression and clinicopathological parameters

The mRNA expression was correlated with clinicopathological parameters (age, serum PSA, Gleason score and tumor volume), however, neither of these markers (*AR* and *PSA*) was significantly correlated with any of clinicopathological parameters like age, serum PSA and tumour volume in both CaP and BPH cases. However, the expression of *PSA* was found to be positively correlated with the *AR* expression (p <0.001).

Functional consequences of risk genotypes

To substantiate the possible functional consequences of the polymorphisms in *AR* and *PSA* gene expression, we examined the association between risk genotypes and gene expression differences in CaP and BPH cases. The median mRNA expression of *AR* and *PSA* with different genotypes was assessed, with *AR* gene expression and dichotomous *AR* (CAG) repeats a statistically significant difference was observed in CaP cases (p=0.002) (Figure. 2 & 3) while with *PSA* gene expression and *PSA* genotypes (GG, GA or AA) no significant difference was observed in CaP. No significant difference in the expression of either *AR* or *PSA* was found among different genotypes in BPH cases.

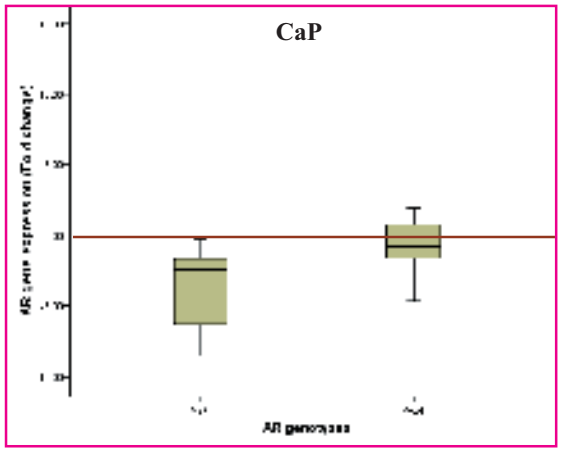


Fig. 2: Impact of *AR CAG* genotypes on gene expression in CaP

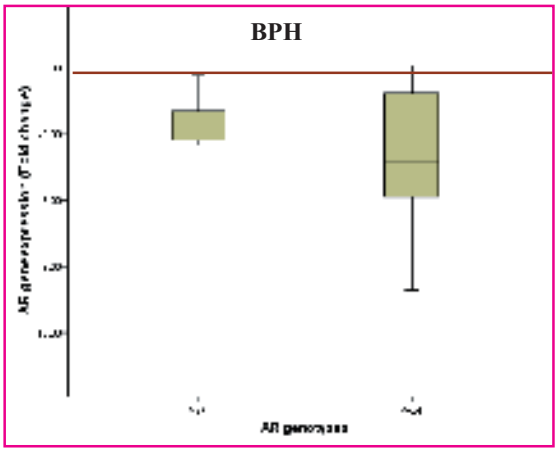


Fig. 3: Impact of *AR CAG* genotypes on gene expression in BPH

2. CHARACTERIZATION OF HOST IMMUNE PROFILE ASSOCIATED WITH PROGRESSION OF SUPERFICIAL TCC OF BLADDER BY MICROARRAY ANALYSIS

Scientific staff	**:**	**Dr. Sunita Saxena, Dr. Sujala Kapur, Dr. Usha Agrawal, Dr. Saurabh Verma, Ms. Nitu Kumari**
Name of Collaborator	**:**	**Dr. N K Mohanty, S J Hospital, New Delhi**
Duration of the project	**:**	**2009-2012**

Aims, Objectives & Background:

Bladder cancer is the sixth most common cancer, surpassed in frequency only by cancers of the lung, larynx, tongue prostate and esophagus according to the Delhi Cancer Registry, 2003. The majority of the patients present with superficial disease and are treated by transurethral resection of the bladder tumor. More than half of these patients experience recurrence, with about 20% progressing to muscle invasive disease. The natural history of bladder cancer is largely unpredictable owing to tumor heterogeneity.

Earlier reports have shown that patients having bladder malignancies were immunodeficient manifesting as impaired cell-mediated and humoral immunity as well as by impaired non-specific host defence mechanisms. Identification of defects in host antitumour immunity responsible for recurrences can help in predicting tumor behavior. It would also help in understanding the biologic profile responsible both for recurrence and resistance to treatment.

Cytokines are a unique family of growth factors secreted primarily from leukocytes. The characteristic cytokine products of Th1 and Th2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. This cross-regulation may partly explain the strong bias towards either Th1 or Th2 responses associated with many pathological conditions. The evaluation of cytokine profile at the local and systemic level will further help in planning therapeutic strategies which at present include only local (intravesical) immunotherapy. The project was undertaken with the aim to identify host immune determinants for recurrence and progression of Superficial Transitional Cell Cancer (TCC) of bladder.

Work done during the year:

Urine and blood samples were collected a day prior to surgery and tissue samples including tumour tissue and adjacent normal appearing mucosa of bladder in each case for paired control were collected in RNA Later (*Ambion*) for RNA and DNA extraction and stored at 70°C following histopathological confirmation of malignancy.

In the year under report, blood and urine samples from 38 cases of bladder cancer have been collected and tumour tissue collected from 35 cases which include: 7 invasive, 28 non-muscle invasive tumours. RNA has been isolated from all the samples and quantitation was carried out using Nanodrop (*Thermo Scientific*) and RNA quality checked on agarose gel electrophoresis. RNA samples were pooled according to the following sub-groups: 4 samples from patients with High Grade Non-Muscle invasive urothelial cancer (HGNI), 4 samples from patients with Low Grade Non-muscle Invasive urothelial cancer (LGNI), 4 samples from patients with High Grade Muscle Invasive urothelial cancer (HGI). RNA was converted to cDNA by reverse transcription. For each group, pooled RNA from adjacent normal appearing tissue samples was used as control.

Pathway-focused expression profiling was performed by Real-Time RT-PCR on tumor and normal cDNA for human Th1-Th2-Th3 cytoki ne/chemokine genes (*SABiosciences*). For first strand cDNA synthesis, SYBR green qPCR master mix was used by using 96-well PCR array plate (*SABiosciences*) and the appropriate program as per manufacturers instructions was run followed by Absolute Quantitation. The results of the fold-changes derived from the C_t values are as depicted below (Fig. 1).

Preliminary analysis of the data show that several chemokines (CCL11, CCR4, CCR5) and cytokines (IL23A, IL10, IL12B, IL4R, IL5, IL13RA1 and IL1R2) are up-regulated more than 4-fold in tumour tissue compared to normal adjacent mucosa and were markedly downregulated in the invasive tumours. Genes of the JAK-STAT pathway and MAP kinases are also upregulated in tumour tissue when compared to adjacent normal appearing mucosa.

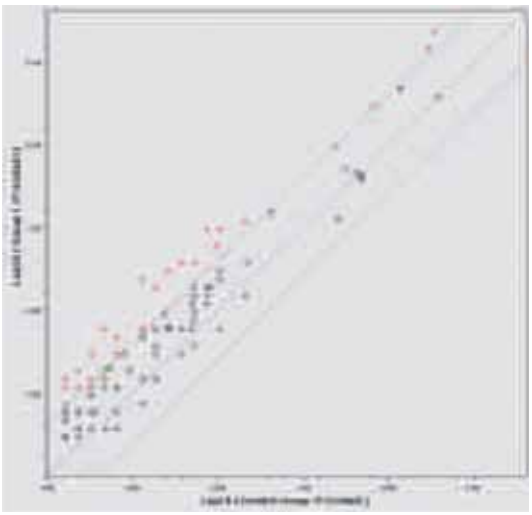


Fig. 1: Scatter plot showing the cytokines which are upregulated (>4-fold) in tumour compared to normal adjacent mucosa.

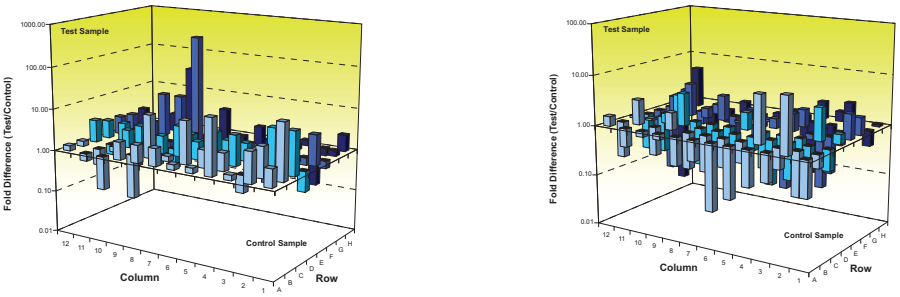


Fig. 2: 3-D profile of the up-regulated and down-regulated genes in the non-muscle invasive and invasive bladder tumour

Future plan of action

Differential upregulated or downregulated genes between invasive and non-invasive cancers will be identified and validated by Taqman assay on larger number of samples. Serum and urine cytokines will be assayed on multiplex bead array to understand the local and systemic immune responses in bladder cancer.

3. TO STUDY THE ROLE OF CYCLOOXYGENASES IN CYTOKINES DYSFUNCTION OF INVASIVE AND NON-INVASIVE TCC OF HUMAN BLADDER.

Scientific staff	:	**Dr. S. Varma, Dr . Usha Burra, Dr. S. Saxena**
Collaborators	:	**Dr. N K Mohanty, Safdarjung Hospital**
Technical Staff	:	**Mr. P.D. Sharma**
Duration	:	**2007-2010**

Aims, Objectives & Background:

Cyclooxygenase (COX) is an enzyme that is responsible for formation of important biological mediators called prostanoids including prostaglandins, prostacyclin and thromboxane which is responsible for inflammation, angiogenesis and tumour progression. Cyclooxygenases exist in two isoforms, *viz.*: Cox-1 and Cox-2 are rate limiting enzymes in the formation of prostaglandins from arachidonic acid and are up-regulated in multiple types of solid tumors including urinary bladder in humans. Prostagland in has been a major cox product involved in tumor development and progression. Cyclooxygenase derived prostaglandins contribute to tumor cell resistance to apoptosis, new blood vessel formation and tumor cell proliferation. In this study, we evaluated the role of cyclooxygenases and associated cytokines in PBMCs and its gene expression in invasive and non-invasive TCC of human bladder.

Work done during the year:

Heparinized blood samples from normal healthy individuals and TCC patients from Urology Department, Safdarjung hospital were collected for the study of Cox-1 and Cox-2 gene expression using Real Time PCR and their expression in correlation with cytokines studied by Flow Cytometer and CLSM. Out of total 33 cases, 3 cases were invasive while 30 were non-invasive. Among 3 invasive cases, 1 (33.3%) and 2 (66.6%) were from grade II and grade III respectively while among 30 non-invasive cases, 4 (13.3%), 19 (63.3%) and 7 (23.3%) were from grade I, II and III respectively.

Total RNA isolation was done from biopsy ti ssues of TCC of human bladder by Qiagen RNA minikit in accordance with manufacturer's instructions and followed by generation of cDNA using high capacity cDNA archI've kit according to manufacturer's controls. The relative quantitation of expression levels of Cox1 and Cox 2 genes was carried out by real time RT-PCR using Taqman probe assay. Expression levels were normalized to individual GAPDH (internal control gene). Primers and probe for the target genes and internal control gene were:

Cox-2 forward primer 5'-GCTCAAACATGATGTTTGCATTC-3', reverse primer 5'-GCTGGCCTCGCTTATGA-3' with Cox- 2 probe 5'-TGCCCAGCACTTCACGCATCAGTT-3'. Cox-1 forward primer was 5'-GTTCCGAGCCCAGTTCCAA-3', Cox-1 reverse primer 5'-CATCTCCTTCTCTCCTGTG-3' and probe 5'-CGTGCAGCAGCTGAGTGGCTATTT-3' GAPDH forward and reverse primers were 5-'CCCATGTTCGTCATGGGTGT-3', 5'-TGGTCATGAGTCCTTCCACGATA-3' respectively with probe 5'-CTGCACCACCAACTGCTTAGCACCC-3' were used in experiments.

A singleplex reaction mix was prepared according to the manufacturer's protocol of assay on demand gene expression products and included 10µl of taqman universal PCR master mix, 1µl of 20x assay on demand gene expression assay mix and 5µl of cDNA (50ng) diluted in RNAse free water, in total 20µl volume.

The cut-off range for Cox-1 and Cox-2 expression in TCC was taken arbitrarily and the normal value falls in range of 0.75 to 1.25. Out of 33 samples, 6 non- invasive cases did not show consistency with internal control gene GAPDH, hence these samples were excluded. Cox-2 gene expression was found upregulated in all the 3 (100%) invasive cases and in 20 (83.3%) cases out of 24 non-invasive cases and 4 cases showed normal expression (Fig. 1).

The flow cytometric studies on separate d PBMCs from TCC patients showed the increased expression of IL-1β and IL-6 in comparison with normal healthy individuals. A significant increase in double positive cells of IL-1β and IL-6 along with Cox-2 was found in cancer patients (18.3 ± 8.7 to 41.7 ± 11.9, $p>0.003$ and 23.08± 7.32 to 37.87± 6.14, $p>0.01$) respectively in comparison to normal healthy groups (Fig.3). Increased expression of Cox-2 in tumor tissue was found in comparison to normal tissue by immunofluoresecence (Fig 4). Cox 2

expression was seen in all the 3 (100%) invasive and in 18 of 30 ((60%) non invasive TCC samples.

No significant difference in expression of Cox 1 was found in malignant and normal bladder epithelium both by real time PCR (Fig. 2) and immunofluoresecence.

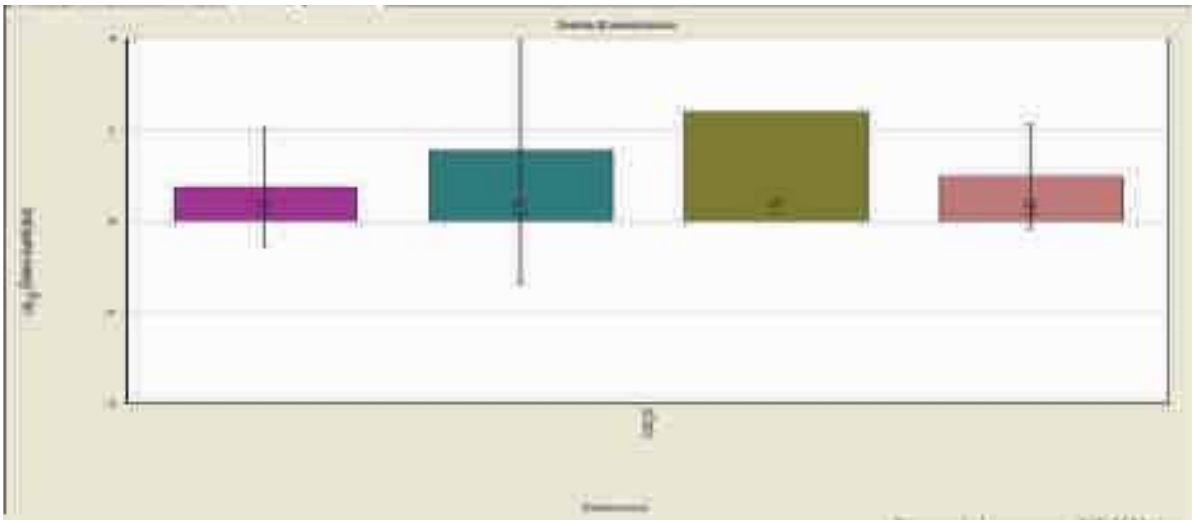


Fig. 1: Showing Cox-2 gene expression

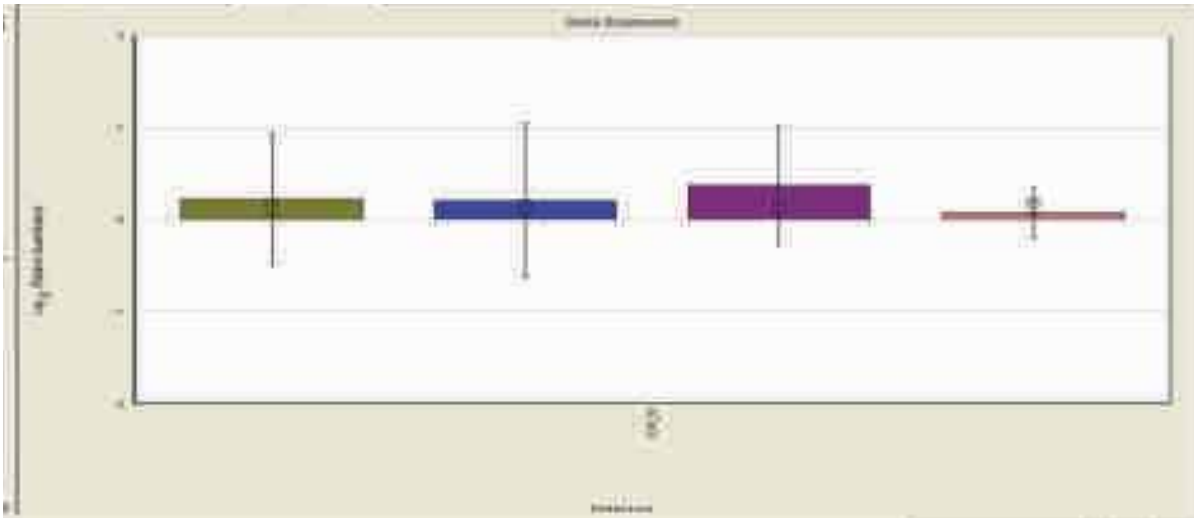
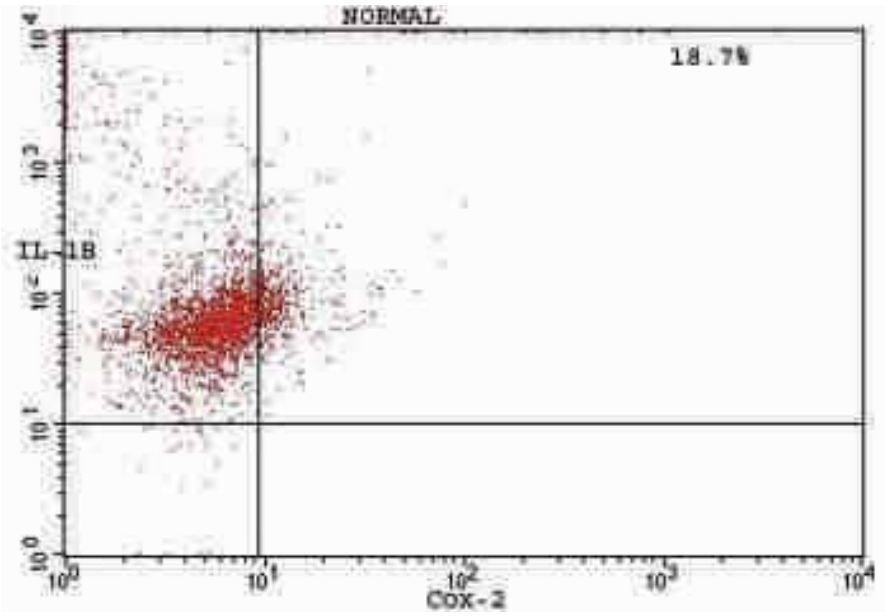


Fig. 2: Showing Cox-1 gene expression.

A.



B.

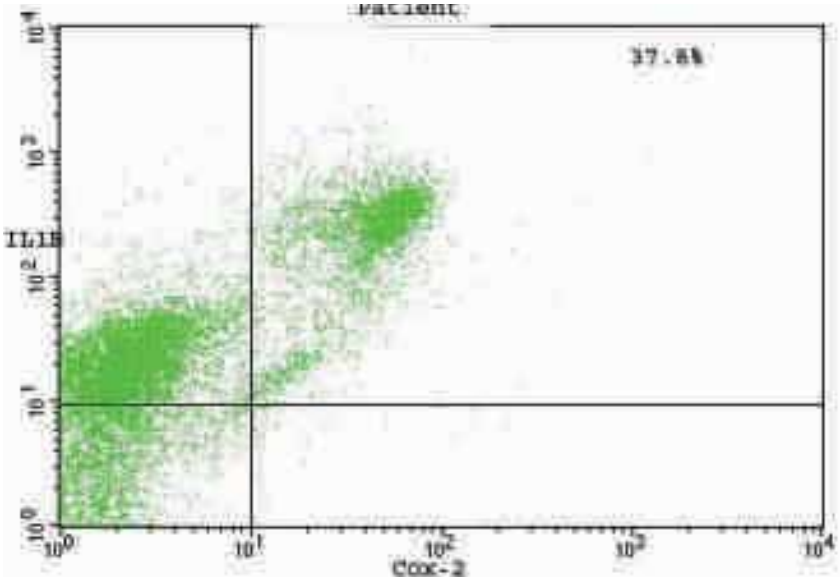
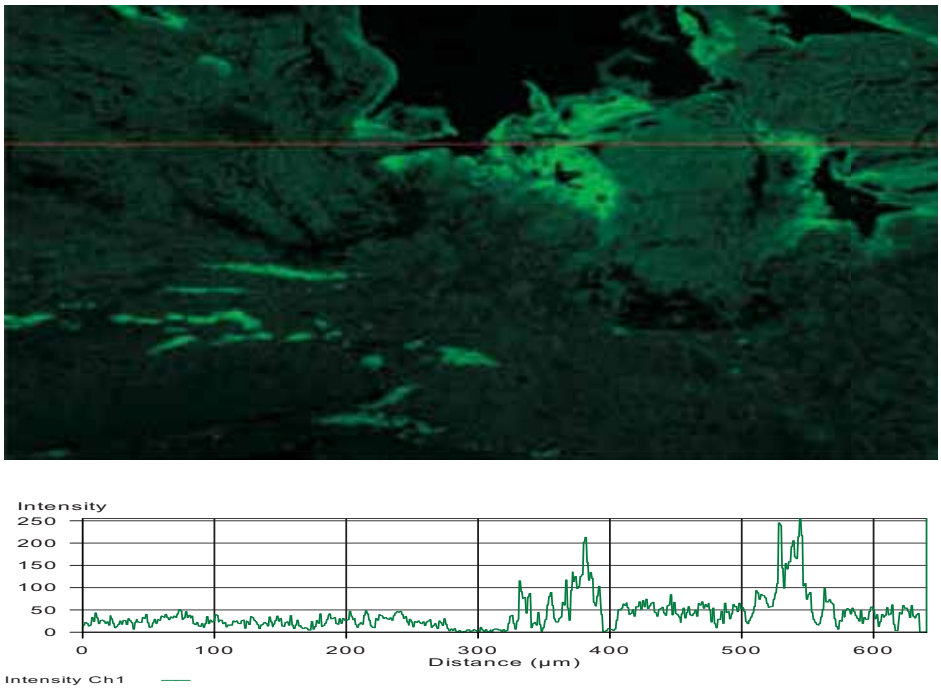


Fig. 3: Dot plot showing the Cox-2 cells expressing IL-1Beta in Normal (A) and TCC patient (B)

A.



B.

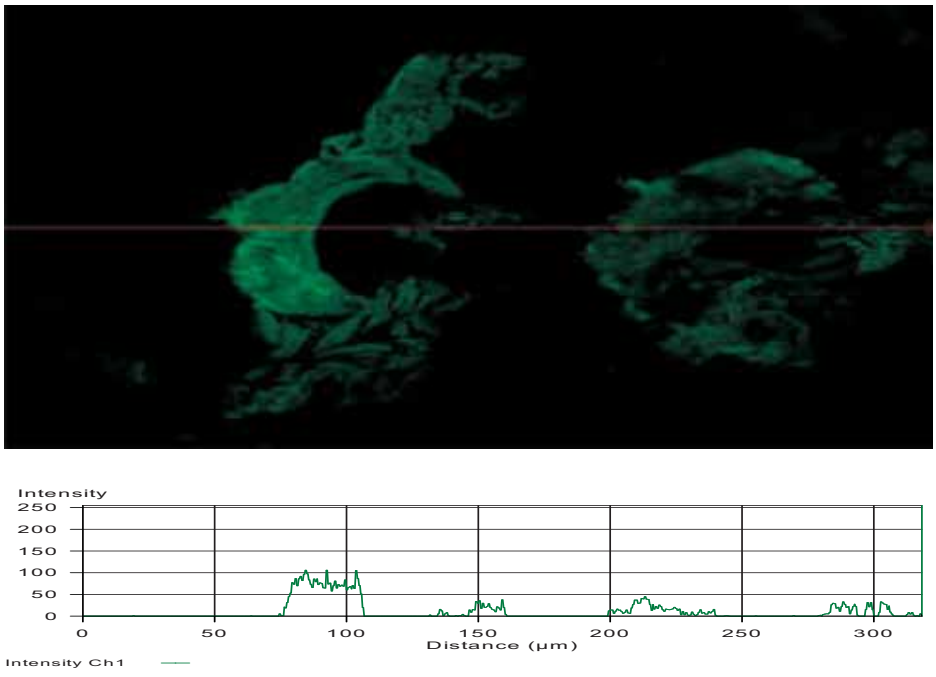


Fig. 4: Image showing expression of Cox-2 in invasive (A) and non invasive (B) TCC

III. HEMATOPOIETIC- LYMPHOID MALIGNANCIES

1. EXPRESSION OF FUSION ONCOPROTEIN AND GENE EXPRESSION PROFILING IN ACUTE AND CHRONIC LEUKEMIA

Scientific staff	:	**Dr Sujala Kapur, Mr. Pradeep Singh Chauhan**
Collaborators	:	**Dr Sumita Saluja, Safdarjung Hospital**
Duration	:	**2007 – 2010**

Aims, Objectives & Background:

BCR-ABL is a classic cytogenetic abnormality and a fusion oncoprotein in chronic myeloid leukemia. It is a signal transduction molecule involved in cell cycle pathways. Its role in ALL and AML is, however, uncertain. The present study had been undertaken to evaluate the presence of fusion oncoproteins BCR-ABL in the patients of acute leukemia.

Work done during the year:

In this study, 120 cases of acute leukemia were screened by the multiplex RT-PCR for the BCR/ABL translocation for the presence of fusion oncoproteins BCR-ABL in the leukemic cells of patients with acute leukemia including 53 cases of Acute Lymphoblastic Leukemia (ALL) and 67 cases of Acute Myloid Leukemia (AML) .

Among 53 ALL patients, there were 20 adults and 33 children. Fifteen patients (28.3%) were found to be positive for the translocation BCR/ABL fusion gene: Eight (15%) were found to be positive for p210 (b3a2/b2a2) and seven (13.2%) were positive for p190 (e1a2). Among sixty seven AML patients screened for the presence of BCR/ABL fusion gene at presentation, there were 51 adults and 16 children. 8(12%) cases were found to be positive for the translocation BCR/ABL fusion gene: seven were positive for p210 (b3a2/b2a2) and one was positive for p190 (e1a2). Response rates were lower in BCR/ABL positive cases compared to negative cases (66% vs 88%) in ALL, while there was no significant difference in the response rate to chemotherapy in patients with or without BCR/ABL translocation in AML. This suggests that BCR-ABL may be a prognostic indicator in ALL but not in AML.

2. PREVALENCE AND PROGNOSTIC VALUE OF FLT3 AND OTHER GENETIC ALTERATIONS IN ACUTE MYELOID LEUKEMIA

Scientific Staff	:	**Dr. Sujala Kapur, Mr. Pradeep Singh Chauhan**
Collaborators	:	**Dr. Sumita Saluja, Safdarjung Hospital**
Duration	:	**2008-2011**

Aims, Objectives & Background:

Activating mutations on FLT3 receptor are one of the most common genetic alterations reported. However, the prevalence and prognostic significance of FLT3 genetic alterations in AML patients with cytogenetically normal karyotype is still controversial. In our earlier study, we analyzed FLT3/ITD and FLT3/D835 mutations in patients of *de novo* AML with normal cytogenetics by genomic PCR assay. FLT3 internal tandem duplication (ITD) and FLT3/D835 mutations were detected in 20% and 3% samples respectively. Although statistically insignificant, the frequency of FLT3/ITD was higher in >15 year age group as compared to <15 year group (23% vs.13%, p=0.2). The white blood count (WBC) was found to be significantly higher in patients with FLT3/ITD mutation as compared to those without the mutation (40 x 10^9/L vs. 20x10^9/L, p=<0.002) or those with FLT3/D835 mutations (30 x 10^9/l). Aberrant expression of CD7 was observed more frequently in patients with FLT3/ITD mutation (p<0.002). There was no significant difference in the response rate to chemotherapy in patients with or without FLT3/ITD mutation (67% and 64% respectively). FLT3/ITD mutation was found to be associated with the age, leukocytosis, and aberrant expression of CD7 although no influence of FLT3/ITD mutation was seen on the clinical outcome of AML patients with normal cytogenetics.

Work done during the year:

Gene expression profiling in our earlier study showed deregulation of pathways associated with cell proliferation, inflammatory response and immune mechanism. In the current year, gene expression of multidrug resistance genes (MDR, MRP, LRP and BCRP) were analyzed in blood samples of patients with acute leukemias. Drug resistance is a major obstacle

in the successful treatment and an important cause of death in acute leukemia. Such resistance may be present before beginning treatment or may develop during chemotherapy. Drug resistance that extends to structurally and functionally unrelated drugs is termed multidrug resistance (MDR). Drug resistance in leukemias frequently involves overexpression of ABC transport proteins P-glycoprotein (Pgp), or the Multidrug Resistant Related Protein (MRP), which provides tumor cells with the capacity of resisting lethal doses by actively pumping drugs out from the inner cell environment. P-glycoprotein (PGP), also named P-170, is a product of the multidrug resistance1 gene (MDR1) and is an ATP-dependent pump capable of expelling drugs out of cancer cells. PGP is a transmembrane glycoprotein conferring cross-resistance to a variety of mechanistically and structurally unrelated cytotoxic drugs, such as anthracyclines, taxanes, vinca alkaloids and epipodophyllotoxins.Another protein, the Multidrug Resistant Related Protein (MRP), is structurally similar to PGP and belongs to the same transmembrane transporter super family. In addition to these two proteins, a 110-kDa protein has been identified in a PGP-negative MDR lung cancer cell line. This protein was termed the Lung Resistance Protein (LRP) and acts as a major vault protein in humans. Recently, a new ABC protein ABCG2 [Breast Cancer Resistance Protein (BCRP)] was described in MDR1-negative, ABCC1 (MRP1)-negative cell lines resistant to anthracycline and mitoxantrone. Information regarding drug resistance mechanisms is likely to increase the chances of cure either through development of new drugs or by means of strategies that may modulate or reverse resistance. Drug resistance gene expression has been studied in acute leukemia and reports that determination of MDR gene expression at diagnosis provides useful prognostic information for acute leukemia patients.

Expression of multidrug resistance genes (MDR, MRP, LRP and BCRP) genes in samples of patients with AL was done by Real Time RT-PCR (*TaqMan probe assay*) The study has so far been conducted on 20 samples of FAB diagnosed acute leukemia patients (Table1).

Table I: Immunophenotypic Distribution of Acute Leukemia Samples

Type of Acute Leukemia	No. of Samples (N=20)
B-ALL	10
T-ALL	3
AML	7

Preliminary results shows that:

- Expression level of MRP gene was found to be downregulated in AML (0.5 ± 0.3) compared to normal bone marrow.
- The expression level of MDR was higher in non-responder group of AML compared to responder group (p=0.06).
- No significant difference was seen in the expression level of LRP in non-responder and responder group of ALL and AML (p=0.7 and p=0.5).
- Expression of BCRP was found to be lower in non-responders compared to responders in ALL patients (p=0.7).

Future Plan

This study is continuing. Approx. 50 more samples (25 AML and 25 ALL) will be collected and data will be analyzed to reach a conclusion.

3. EXPRESSION OF ACTIVATOR AND TARGET GENES OF NUCLEAR FACTOR-KAPPA B (NF-KB) TRAN SCRIPTION FACTOR IN ACUTE LEUKEMIA

Scientific Staff	:	**Dr. Sujala Kapur, Dr. Bharat Bhushan**
Collaborators	:	**Dr. Sumita Sa luja, Safdarjung Hospital, New Delhi**
Duration	:	**2006-2009**

Aims, Objectives & Background:

NF-kB signalling is known to be aberrantly activated in acute leukemia (AL) making it a suitable target pathway for therapeutics. Several inhibitors of the NF-kB pathway, including some common synthetic (e.g., aspirin), and traditional (e.g., green tea, curcumin) remedies, have been identified and some inhibitors are entering clinical trials. However, molecular pathways involved in NF-kB dysregulation remain controversial. Knowledge of the signaling pathways in NF-kB-activated samples with AL may enable the development of more specific and potent inhibitors that not only enhance the efficacy of anticancer treatments but may also be helpful in designing individualized therapy. Abnormalities in th e regulation of the nuclear factor-kappa B (NF-kB) pathway are frequently seen in a variety of human malignancies including leukemias,

lymphomas, and solid tumors. This study has been under taken to identify such molecules that can specifically block the activation of NF-kB pathway and NF-kB-regulated gene expression.

Work done during the year:

The relative quantitation of expression levels of selected genes involved in upstream (AKT, IKK-α, IKK-β, IKK-γ) and downstream (BCL-2, cyclin D1, IkB-α, cIAP-2, Survivin and TRAF-2) NF-kB were analyzed by Real time RT-PCR.

Samples of AML were significantly more likely to have activated NF-kB pathway compared to ALL (81.2% vs. 50% of cases respectively). In AML, aberrant activation of NF-kB was common in children and adults, however, in ALL, activation of NF-kB pathway was found to be higher in children (66.6%) compared to adults (35.7%). Expression level of IkB-α (reporter of NF-kB pathway activation) was found to be upregulated in both AML and ALL samples compared to normal bone marrow sample (calibrator). Expression level of P53 was found to be significantly lower in nonresponders of AML compared to responders (0.7 ± 0.3 and 1.1 ± 0.3 respectively, $p=0.04$). Expression level of IkB-α was found to be upregulated in responders of AML compared to nonresponder of AML, however, such difference was not seen in ALL samples. These findings suggest that overexpression of IkB-α may be a good prognostic marker in AML. Expression level of cIAP-2 was found to be higher in AML samples showing NF-kB activation compared to samples in which NF-kB pathway was not activated (4.1 ± 3.0 versus 1.4 ± 0.8 fold, $p=<0.01$), however, similar results were not observed in ALL samples suggesting that cIAP-2 is also an important marker in AML samples showing NF-kB activation. No significant difference was seen in the expression level of IKK-A, IKK-B and IKK-G between responder and nonresponder of AML patients.Overall, in ALL samples, IKK-A, IKK-B genes were found to be higher in samples showing NF-kB activation comparison to samples in which pathway was not activated while in samples of AML, cIAP-2 was found to be significantly upregulated in samples showing NF-kB activation compared to samples in which the pathway was not activated.

IV. CANCER IN NORTH-EAST REGION OF INDIA

A high incidence of several cancers such as those of esophagus, oral, lung, stomach, breast and non-Hodgkin lymphoma (NHL) have been reported from NE India. These cancers are often associated with exposure to tobacco and pesticides. NE Indians have diverse ethnic groups, customs, food habits and life style. In addition, familial aggregation of esophageal cancer has been reported in Assam. The form in which tobacco is consumed is different in NE region. Moreover, there is extensive use of pesticides in tea gardens in these regions. Two projects, in collaboration with Dr. Bhubaneshwar Borooah Cancer Institute, Guwahati, Assam, Regional Medical Research Centre, ICMR, Dibrugarh, Assam, Institute of Cytology and Preventive Oncology, ICMR, Noida, U.P., National Institute of Occupational Health, Ahmedabad, National Cancer Registry Programme, Bangalore, and the Population Based Cancer Registers (PBCRs) of NE India, were completed in the year under report.

1. ROLE OF TOBACCO USE IN CAUSATION OF CANCER IN NORTH-EAST INDIA

Scientific Staff	**:**	**Dr. Sunita Saxena, Dr. Sujala Kapur, Dr. Usha Agrawal, Ms. Th. Regina Devi, Mr. Dhirendra Singh Yadav, Ms. Rakshan Ihsan**
Collaborators	**:**	**Dr. J Mahanta, RMRC, Dibrugarh, Dr. H.N. Sayed, Dr. P. Nag, NIOH, Ahemedabad Dr. B.C. Das, Dr Alok Bharti, ICPO, Noida Dr. A. Nand Kumar, NCRP, Bangalore Dr. Eric Zomwia, Aizawl, Dr. Yogesh Varma, Sikkim**
Duration	**:**	**2005-2010**

Aims, Objectives & Background:

The carcinogenic potential of tobacco depends on its carcinogenic contents and also on its pattern of use. The form in which tobacco is consumed in NE region is different from that of the rest of the country. A Multicentric study was designed with NCRP, Bangalore (to design and

prepare questionnaire to relate epidemiological data), RMRC Dibrugarh (for statistical analysis of data and its interaction with other factors to evaluate the risk of cancer) and with NIOH Ahmedabad (for estimation of metabolites of nicotine and pesticides in serum and urine samples). The role of IOP, New Delhi and ICPO, Noida was to identify risk of developing cancers with relation to allelic alteration in microsatellite markers, mutation/polymorphisms and gene expression pattern. Blood and tissue samples were sent to IOP from three PBCR Centers at Dr. B. Boroowah Cancer Hospital, Guwahati, Civil Hospital, Aizawl and Sir TNM Hospital, Gangtok for oral, esophageal, gastric and lung cancers.

Work done during the year:

Oral Cancer

Ninety-two percent of Oral Squamous Cell Carcinoma (OSCC) in men and 61% of OSCC in women are attributable to tobacco usage. The quantitative absorption, distribution, metabolism, and excretion of carcinogenic tobacco constituents depends on the activity and efficiency of metabolic and enzymatic detoxification pathways. The enzymatic detoxification process is mainly divided into three phases. Phase I enzymes such as cytochrome p450 A1 are involved in activation of toxic compounds predominantly by oxidation into more reactive intermediates that are neutralized and conjugated by Phase II family of enzymes such as glutathione-S-transferase (GST) and N-acetyltransferase (NAT). The resultant water-soluble and less-toxic conjugated product can easily be eliminated from the cell by phase III transport mechanisms for the elimination of conjugates.

GST polymorphism

The detoxification efficiency of GST enzymes is determined by the presence, amount, and nature of the isoenzymes coded by GSTT1, GSTM1 and GSTP1 genes. The allelic polymorphism of GSTT1 and GSTM1 are characterized by the deletion of a part of the gene. GSTP1 polymorphism is a single base pair substitution where adenine is replaced by guanine, resulting in an amino acid change in which isoleucine (I105) is replaced by valine (V105). Electrophilic compounds are reported to be detoxified less efficiently in individuals with null

genotypes of GSTT1 and GSTM1 or variant genotypes of GSTP1 (Ile/Val and Val/Val) when compared with those with wild-type genotype. The presence of GSTT1 and GSTM1 null genotypes have been reported to be associated with increased risk for several cancers including skin, lung, bladder, prostate, colorectal, and oral cancers. However, several other reports have failed to confirm this association. The prevalence of polymorphism in GST genes in tobacco-associated cancer patients from NE India is not well known. The individual difference in susceptibility to chemically induced carcinomas may possibly be attributed to the genetic differences in the activation or detoxification of carcinogens due to polymorphic variants of GST genes.

Of 136 patients with oral cancer (78 from Assam, 33 from Sikkim and 25 from Mizoram) and 270 healthy controls (108 from Assam, 73 from Sikkim, and 89 from Mizoram), 103 (76%) cases with cancer and 152 (56%) controls were tobacco chewers, 81 (60%) cases and 133 (49%) controls were tobacco smokers and 106 (78%) cases and 169 (63%) controls were betel quid chewers. The conditional logistic regression analysis further revealed that the risk of developing oral cancer significantly increased in tobacco chewers (OR =2.44, 95% CI: 1.47–4.05, p=0.001), tobacco smokers (OR=1.72, 95% CI: 1.08–2.73, p=0.02), and betel quid chewers (OR=2.20, 95% CI: 1.29–3.76, p=0.004). The frequency of GSTT1 and GSTM1 null genotype was 31% and 49% in samples obtained from patients with oral cancer and 31% and 44% in controls, respectively. No significant association was found for GSTM1 and GSTT1 null genotype independently or in combination with oral cancer risk. The frequency of the variant genotypes of GSTP1 (heterozygous Ile/Val and homozygous Val/Val) was higher in samples of patients with oral cancer (43%) when compared with controls (36%); however, this difference was not statistically significant (OR=1.35, 95% CI: 0.86–2.13, p=0.19). Although no significant independent association of oral cancer with null genotypes of GSTT1, GSTM1, and the variant alleles of GSTP1 was found, individuals with both GSTM1 null genotype and variant alleles of GSTP1 were found to have marginal increased risk for developing oral cancer (OR=1.84, 95% CI: 0.91–3.72, p=0.08).

When data were analyzed for each geographical region, GSTT1 null genotype was found to be significantly higher (OR=2.58, 95% CI: 1.01–6.61, p=0.05) in oral cancer cases (26%) when compared with controls (12%) for the Assam region (Table 1).

Risk Factors	Oral cancer Cases N=136 n (%)	Controls N=270 n (%)	Adjusted* OR (95% C.I.)	P
Tobacco Chewing*	103 (76)	152 (56)	2.44(1.47-4.05)	0.001
Tobacco Smoking*	81 (60)	133 (49)	1.72(1.08-2.73)	0.02
Betel quid Chewing*	106 (78)	169 (63)	2.20(1.29-3.76)	0.004
GSTT1				
Present	94 (69)	185 (69)	1.00	
Null*	42 (31)	85 (31)	1.02(0.61-1.71)	0.93
GSTM1				
Present	70 (51)	150(56)	1.00	
Null*	66 (49)	120(44)	1.18(0.74-1.88)	0.48
GSTP1				
Ile/Ile	78 (57)	173 (64)	1.00	
Ile/Val or Val/Val*	58 (43)	97 (36)	1.35(0.86-2.13)	0.19

**** Adjusted with all other risk factors.***

Table 1: Distribution and association of tobacco and betel quid consumption habits and GST genotypes

CYP1A1 (Msp1 and Noc1) Polymorphisms

The CYP1A1 gene produces a Phase I enzyme, aromatic hydrocarbon hydroxylase, which is responsible for the first oxidative step in the metabolism of many substrates including polycyclic aromatic hydrocarbons like benzo(a)pyrene from tobacco smoke, steroids with a phenol ring, combustion products from meats and fats and caffeine. Combined with Phase II enzymes, steroids and xenobiotics are “detoxified” for excretion; however, some of the initial resulting metabolites are highly reactive oxygen species that can damage DNA and potentially promote cancer development. Thus, there has been considerable interest in the relationship between CYP1A1 and cancer, including whether polymorphic variants of the gene may influence cancer risk.

Among four CYP1A1 variants described, only two have been extensively studied in relation to cancer risk. The Msp1 restriction site polymorphism is found at the 3’end of the

noncoding region of the gene, and Ile/Val is a point mutation at position 4889 in exon 7 resulting in a change from an Ile to Val residue, with Ile as the more common form. In our study, we sought to examine the relationship between oral cancer and the Msp1 and Ile/Val (Nco1) polymorphisms of CYP1A1 and the interaction between these polymorphisms and various exposures, which might either be related to oral cancer risk or be affected by the CYP1A1 metabolic pathway.

The CYP1A1 Msp1 polymorphism was detected by PCR-RFLP using Msp1 enzyme which yielded 340 bp PCR product. The T (wildtype) allele is not cut by the enzyme, whereas the C (mutant) allele yields 200 and 140 bp products. Wildtype (TT or WW) produced a 340 bp band; homozygote mutation (CC or VV) produced two bands of 200 and 140 bp, while heterozygote (TC or WV) samples exhibited a digestion pattern of all three bands when digested with Msp1.

CYP1A1 C2455 A>G gene polymorphism was determined using Nco1 enzyme which yielded 370 bp product. The wild-type allele (Ile) was distinguished by the presence of a 232-bp fragment, whereas a 263-bp fragment identified the variant allele (Val). Heterozygous samples (Ile/Val) showed two bands at 263 bp and 232 bp (Fig. 1).

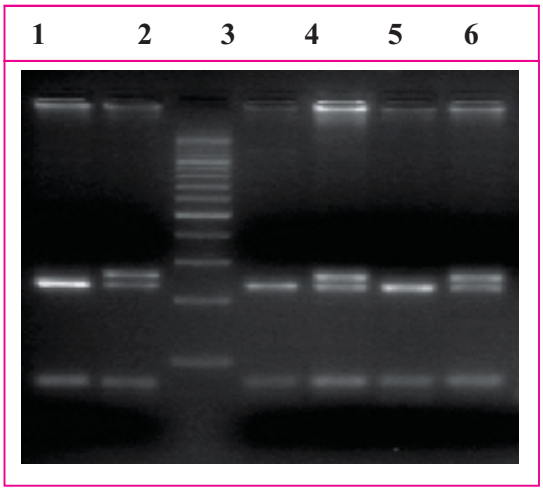


Fig. 1: RFLP image of CYP1A1 (Nco1 polymorphism) in 3.5% agarose gel. Lane 3: 100bp DNA ladder, Lane1, 4 & 6: Ile/Ile (Wild type), Lane 2, 5 & 7: Ile/Val (Heterozygous) of image 2

Frequencies for CYP1A1 (Msp1) polymorphism was analyzed in 160 oral cancer cases and 169 age and sex matched controls from the same ethnic group. Genotypes TT (Wildtype), TC (Heterozygote) and CC (Homozygote mutant) in cases and controls were 65 (41%), 79 (49%), 16 (10%) and 67 (40%), 78 (46%), 24 (14%) respectively. When analyzed by using med cal software, no significant association was observed in the variant genotypes of either CYP1A1 (Msp1) or CYP1A1 (Nco1). Multiple gene interaction analysis is required to understand the role of different genes involved in the complex detoxification pathways

NAT2 polymorphisms

Arylamine N-acetyltransferase 2 (NAT2) exhibits single nucleotide polymorphisms (SNPs) in human populations that modify drug and carcinogen metabolism. Two arylamine N-acetyltransferase isozymes, NAT1 and NAT2, have been identified in humans. The NAT1 and NAT2 are 290 amino acid products of intron less 870 base pair open reading frames located within 170 kb on chromosome 8p22. A pseudogene (NATP) resides between the NAT1 and NAT2 coding exons. NAT1 and NAT2 open reading frames are 87% identical and the NAT1 and NAT2 proteins differ only in 55 amino acids. NAT2 phenotype is assigned based on co-dominant expression of rapid and slow acetylator NAT2 alleles or haplotypes. Individuals homozygous for rapid NAT2 acetylator alleles are deduced as rapid acetylators, individuals homozygous for slow acetylator NAT2 alleles are deduced as slow acetylators, and individuals possessing one rapid and one slow NAT2 allele are deduced as intermediate acetylators.

NAT2 was amplified using primers: 5- CCT GGA CCA AAT CAG GAG AG -3 and 5-ACA CAA GGG TTT ATT TTG TT C C-3, the NAT2 genotypes GG (wildtype) produced three bands, a 170-bp, 142-bp and 109-bp band, NAT2 AA (homozygote mutation) produced two bands of 279-bp and 142-bp, while NAT2 G590A GA (heterozygote) samples exhibited a digestion pattern of all four bands (279, 170, 142 and 109 bp) when digested with Taq I (Fig. 2).

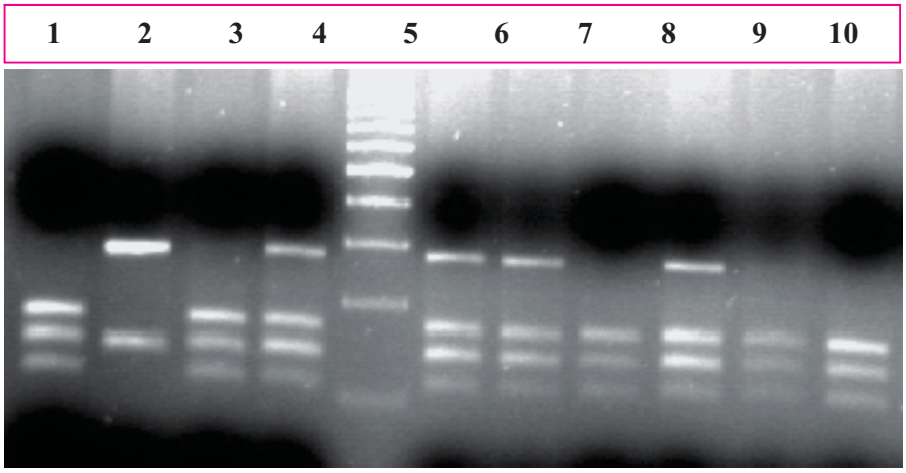


Fig 2: RFLP digested PCR product in 3.5% agarose gel electrophoresis
Lanes 1,3, 8, 10 11: GG (Wild type)
Lanes 4, 6, 7, 9: GA (Heterozygous)
Lanes 2: AA (Homozygous mutant)

In our results for NAT2 polymorphic in oral cancer, we have not found any significant association of NAT2 polymorphic variants with oral cancer. These results are similar to few other studies which have shown no association of oral cancer with NAT2 variants. To analysis it for multiple gene interactions, experiments are undergoing.

Esophageal Cancer

Analysis of EPHX1 Polymorphisms:

Microsomal epoxide hydrolase (mEH) is an enzyme that hydrolyzes epoxides yielding corresponding trans-dihydrodiols. Usually, this hydrolysis acts as a detoxifying step, although in some instances, transdihydrodiols generated from PAHs are highly toxic and mutagenic. Therefore, mEH plays a dual role in the detoxification and activation of procarcinogens, and its role in carcinogenesis may depend on exposures to different environmental substrates. There are two polymorphic sites that affect the enzyme activity in human mEH gene. One variant is characterized by substitution of histidine for tyrosine at the amino acid position 113, the other is characterized by substitution of arginine for histidine at the position 139. The proteins encoded by polymorphic alleles demonstrated different enzyme activities *in vitro*.

PCR reaction for both exon 3 and 4 of EPHX gene was carried out in a volume of 20µl. Restriction digestion for exon 3 and exon 4 was carried out by enzymes EcoRV and RsaI respectively. The digested products were loaded on 2.5% agarose gel and subjected to gel electrophoresis and visualized under UV (Fig. 3 ,4)

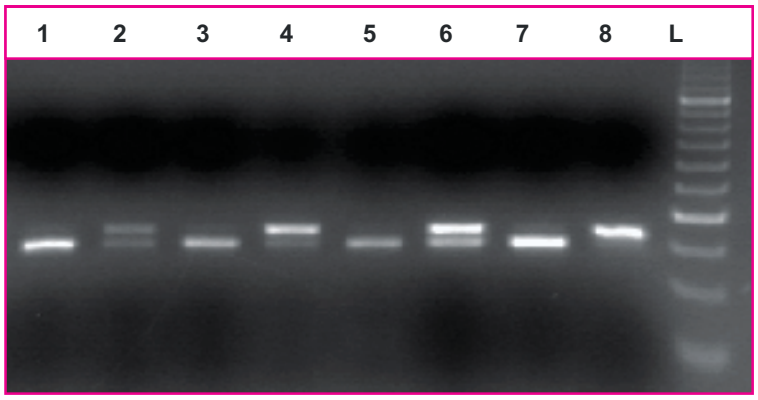


Fig 3: EtBr stained agarose gel electrophoresis for RFLP products of exon 3 of EPHX1 gene. Lanes 8 shows the undigested product (162bp) representing His/His allele. Lanes 1, 3, 5 and 7 show the digested products (140bp +22bp, not visible) representing the homozygous Tyr/Tyr allele. Lanes 2, 4 and 6 show the presence of two bands representing the heterozygous condition of His/Tyr allele. Lane L shows 50bp ladder.

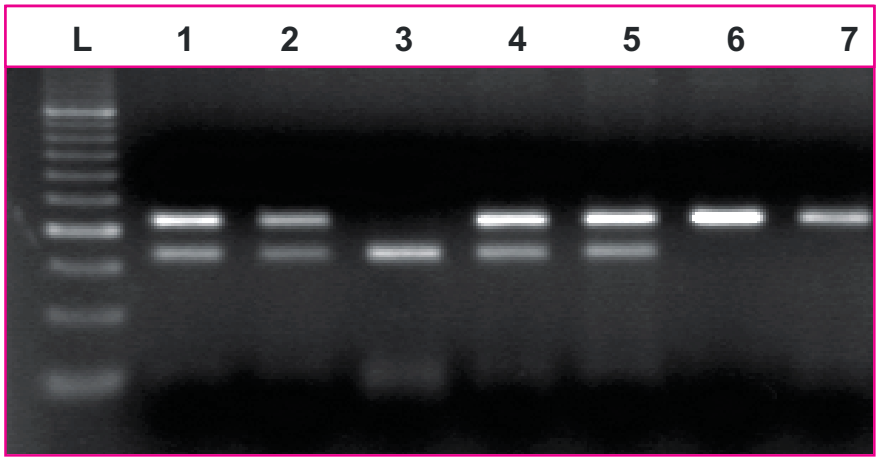


Fig. 4: EtBr stained agarose gel electrophoresis for RFLP products of exon 4 of EPHX1 gene. Lanes 6 and 7 show the undigested product (210bp) representing His/His allele. Lane 4 show the digested products (164bp + 46bp, not visible) representing the homozygous Arg/Arg allele. Lanes 1, 2, 4 and 5 show the presence of two bands representing the heterozygous condition of His/Arg allele. Lane L shows 50bp ladder.

A total of 142 cases and 185 controls were screened for EPHX1 polymorphisms. In the control group, the frequency of exon 3 heterozygous genotype 113Tyr/His was higher (51.89%) compared to other homozygous 113Tyr/Tyr (29.73%) and 113His/His (18.38%) genotypes. In the exon 4 polymorphism, 74.59% controls had the 139His/His genotype, 24.32% had the 139His/Arg genotype and the 1.08 % had 139Arg/Arg genotype (Table 2). The variant of the

mEH Arg139Arg genotype was rare in the controls (n = 2). In esophageal cancer cases, the frequency of exon 3 heterozygous 113Tyr/His genotypes was lower in the cancer group compared to the control group (34.50% vs 51.89%; OR 0.462, 95% CI = 0.267–0.800, P = 0.006); the difference was statistically significant. The frequency of exon 3 homozygous 113His/His genotypes was slightly higher than in the controls (26.76% vs 18.38%; OR = 1.254, 95% CI = 0.664–2.369, P = 0.486). Patients with exon 3 heterozygous 113Tyr/His genotypes were at a lower risk of ESCC. In contrast, the frequencies of exon 4 heterozygous 139His/Arg and homozygous 139Arg/Arg genotypes were higher in the cases than in the controls (33.80% vs 24.32% and 4.93% vs 1.08%.

Genotype	Total (327) (C %)	ESCC (142) (C%, R %)	Controls (185) (C%, R %)	OR* (95%CI), P-value
Exon3 (Tyr113His)				
Tyr113Tyr	110 (33.6)	55 (38.73, 50)	55 (29.73, 50)	1.0
Tyr113His	145 (44.3)	49(34.50, 33.8)	96 (51.89, 66.2)	0.462 (0.26-0.80), 0.006
His113His	72(22.0)	38(26.76, 52.8)	34 (18.38, 47.2)	1.254 (0.66-2.36), 0.486
Exon4 (His139Arg)				
His139His	225 (68.80)	87(61.27, 38.7)	138(74.59, 61.3)	1.0
His139Arg	93 (28.84)	48 (33.80,51.6)	45(24.32,48.24)	1.887 (1.11-3.20), 0.019
Arg139Arg	9 (2.8)	7 (4.93, 77.8)	2 (1.08,22.2)	7.140(1.27-393.95), 0.025

*Age-ender and Exon4 adjusted odds ratio.
**Age-gender and Exon3 adjusted odds ratio.
(Column %, Row %)

Table 2: Association of EPHX1 exon3 (Tyr113His) and exon4 (His139Arg) polymorphisms and alleles with risk of esophageal cancer

Gastric Cancer

In Mizoram, NE India, according to NCRP report, a very high prevalence of Gastric Cancer (GC) (AAR of 47.7/105 in males and AAR of 25.7/105 in females) has been observed. However, knowledge about the molecular mechanisms underlying tumor development and

progression is limited and molecular features of GC are not commonly used for diagnosis and treatment.

Cytochrome p450 (CYP) and glutathione S-transferase (GST) enzymes are involved in activation and detoxification of many potential carcinogens. Genetic polymorphisms in these enzymes have been found to influence interindividual and interethnic susceptibility to cancer. Although CYP and GST enzymes are involved in the activation and detoxification of N-nitrosamines and related compound, studies on the relationship between genetic polymorphisms of CYPIA1, GSTT1, and GSTM1 and the risk of gastric carcinoma (GC) have not been carried out in this high risk region of India.

Polymorphisms of GSTM1, GSTT1, GSTP1

A hospital-based case-control study was conducted to investigate whether variations in these genes affect the risk of developing GC. Subjects included 133 GC patients and 270 unaffected controls. Of these, 86 (65%) cases with gastric cancer and 152 (56%) controls were tobacco chewers, 53 (40%) cases and 133 (49%) controls were tobacco smokers, 95(71%) cases and 169 (63%) controls were betel quid chewers.

Peripheral white blood cell DNA was obtained from all subjects. Genotyping of GSTP1 was performed using a PCR-based restriction fragment length polymorphism assay. Deletion of GSTT1 and GSTM1 genes was assessed by multiplex PCR.

Risk Facors	Cases N=133n (%)	Controls N=270n (%)	OR (95% C.I.)	P
GSTT1				
Present	83 (62)	185 (69)	1.00	
Null*	50 (38)	85 (31)	1.24(0.76-2.03)	0.39
GSTM1				
Present	84 (63)	150(56)	1.00	
Null*	49 (37)	120(44)	0.74(0.46-1.17)	0.19
GSTP1				
Ile/Ile	75 (56)	173 (64)	1.00	
Ile/Val or Val/Val*	58 (44)	97 (36)	1.25(0.77-2.03)	0.37

Table 3: GST polymorphism in gastric cancer

The frequency of GSTT1 and GSTM1 null genotype was 38% and 37% in samples obtained from patients with GC and 31% and 44% in controls respectively. Variant genotypes (Ile/Val and Val/Val) of GSTP1 were found more frequently in samples from patients with gastric cancer (44%) as compared to controls (36%), but the difference was not statistically significant (OR=1.25, 95% CI 0.77-2.03, P=0.37). GST genes polymorphism was not found to be associated with risk of gastric cancers.

CYP1A1 gene polymorphisms

One hundred and fifty six cases of gastric cancer and 169 contrds were screened for the presence of CYP1A12A and CYP1A12C polymorphisms. For the analysis of CYP1A1 gene polymorphisms, DNA samples were extracted from blood cells and then subjected to restriction fragment length polymorphism and polymerase chain reaction. Gastric cancer risk was estimated as odds ratios (OR) and 95% confidence interval.

The percentage frequencies of CYP1A12A genotypes WW, WV and VV were found to be 37, 47, 16 and 40, 46, 14 in cases and in controls respectively. None of the genotypes showed significant difference in the distribution between the gastric cancer patient group and normal healthy controls.

The percentage frequencies of CYP1A12C genotypes II, IV and VV were 71, 20, 14 and 70, 29, 1 respectively. There was a significant difference between the case and control groups in the VV genotype (p=0.005) but not in II (p=0.891) and IV (p=0.078). The odds ratio of the CYP1A12C V/V polymorphism was 8.232 (95% CI=1.839-36.836) in cases compared with the control groups indicating that it might act as a risk factor for the development of gastric cancer in this high prevalence region of India .

Lung Cancer

Lung cancer accounts for around 9.42% of total cancer cases among males and 2.55% cases among females. Cigarette smoking is responsible for 90% of all lung cancers, the leading cause of cancer deaths in the United States and the world. In India too, a history of active tobacco smoking is present in 87% of males and 85% of females, showing smoking as the prime cause of the disease. High incidence of lung cancer has been reported in NE states in India especially in Mizoram where smoking is quite common in females also.Tobacco smoke contains potentially carcinogenic chemicals such as benzo[a]pyrene and polycyclic aromatic hydrocarbons (PAH). *In vitro* studies indicate various mechanisms for tobacco smoke components to induce lung carcinogenesis. Components of cigarette smoke induce cell death in normal bronchial airway by mitochondrial damage and later also induce necrosis and increase proliferation of tumor cells. The role of Phase I and Phase II detoxifying enzymes in individual susceptibility to develop lung cancer is, however, controversial.

GST Polymorphisms:

A multiplex PCR method was used to detect the presence or absence of the GSTM1 and GSTT1 genes in the genomic DNA samples. This method had both GST primers sets in the same PCR reaction and included a third primer set for β-globin as internal control to ensure proper functioning of PCR. The PCR products were electrophoresed in 2.5% agarose gels containing ethidium bromide, prepared and run in 0.5X TBE buffer. The absence of 480 bp band indicates GSTT1 null and the absence of 215 bp indicates GSTM1 null genotypes (Fig. 5).

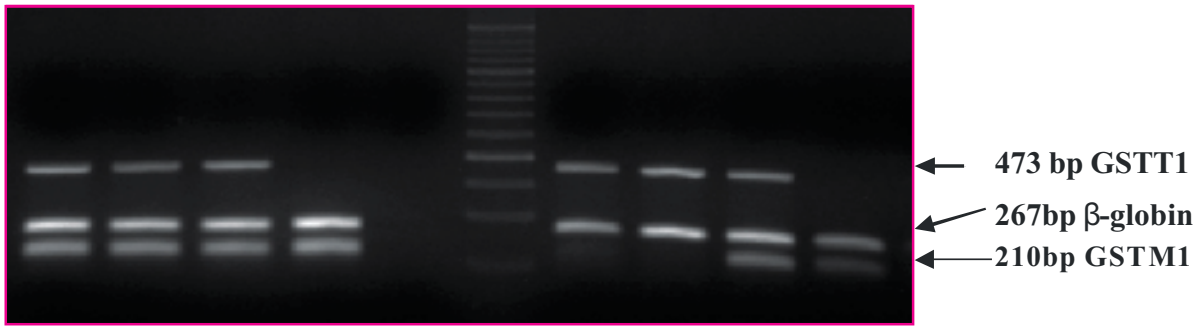


Fig. 5: EtBr stained agarose gel picture showing PCR products of GST genes. The presence of wild type GSTT1 gene, 210 bp PCR product shows presence of wild type GSTM1 gene. β-globin gene was used as internal control detected by the presence of 267 bp PCR product.

A total of 100 lung cancer cases and 248 controls were included in the study. The cases included 73% of tobacco users. Null genotype of GSTT1 was present in 19% of the cases and 32% of the controls. The odds ratio for the GSTT1-null genotype was 0.42 (95% C.I. 0.21-0.84; p value 0.02), which was statistically significant. Likewise, null genotype of GSTM1 was higher in cases (45%) as compared to controls (33%). The odds ratio for the GSTM1-null genotype was also less than 1 and came out to be marginally significant, O.R. = 0.53 (95% C.I. 0.28-0.99; p value 0.04) (Table 4).

Null genotypes of both GSTM1 and GSTT1 were associated with lung cancer. Odds ratio for null genotype of both the genes showed that the absence of the genes was a significant protective factor towards the disease. In other words, absence of the GSTT1 and GSTM1 enzymes could render a person 58% and 47% less chance of developing lung cancer. The results imply that certain procarcinogens or other chemicals can be activated by GSTs and the metabolites are carcinogenous. It was reported that GSTT1 could activate dicholoromethane into mutagen, inducing lung and liver cancer in mice.

Genotype	Cases (100) N (%)	Controls (248) N (%)	OR (95% C.I.)	P-value
GSTT1				
Present	82 (81)	168 (68)	1.00	
Null	19 (19)	80 (32)	0.42 (0.21-0.84)	0.02
GSTM1				
Present	68 (67)	136 (55)	1.00	
Null	33 (33)	112 (45)	0.53 (0.28-0.99)	0.04

Table 4: Frequency of GSTM1 and GSTT1 genotypes in lung cancer cases and controls

Genotypes of GSTP1 were detected using PCR-RFLP method. PCR reactions for GSTP1 gene were carried out in a volume of 20µl and 5µl of PCR product was digested with BsmA1 restriction enzyme at standardized conditions. The products were then separated by electrophoresis in 2.5% agarose gel. The genotypes were classified based on the band pattern obtained after enzyme digestion. The undigested 189bp product represented the Ile/Ile (A/A)

allele, the completely digested product yielding two bands of 148 and 41 bps represented the val/val (G/G). The heterozygous genotype Ile/Val (A/G) had all the 3 bands (Fig. 6).

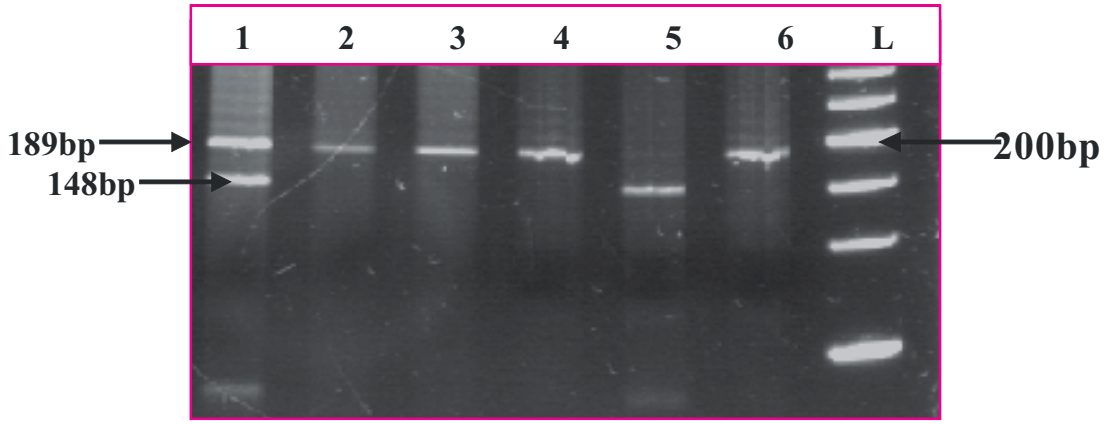


Fig. 6: EtBr stained PAGE of RFLP products of GSTP1 gene: Lanes 2,3,4,6 show the undigested product representing the A/A genotype. Lane 5 shows the homozygous mutant genotype G/G (41bp not visible). Lane 1 shows th heterozygous genotype A/G. Lane L shows 50bp ladder.

The wild type genotype of GSTP1 (Ile/Ile) was found to be more prevalent in controls (64%) as compared to the cases (54%). Variant genotypes of GSTP1 (Ile/Val and Val/Val) were found more frequently in lung cancer cases (47%) as compared to controls (36%) but the difference was insignificant. There was no significant association of variant alleles of GSTP1 with lung cancer (Table 5). The role of GSTP1 polymorphisms in lung cancer is reported to be conflicting, showing it as both, a risk factor and having no association with the disease.

GSTP1	Cases (100) N (%)	Controls (248) N (%)	OR (95% C.I.)	P-value
Ile/Ile	54 (54)	158 (64)	1.00	
Ile/Val + Val/Val	46 (46)	90 (36)	1.51 (0.83-2.77)	0.18

Table 5: Frequency of GSTP1 genotypes in lung cancer cases and control

p53 Gene Codon 72 Polymorphism

Genotypes of p53 codon 72 gene were detected using PCR-RFLP method. Restriction digestion of the amplified fragments was carried out by enzyme Bsh12361. The restriction digestion was carried out on 10µl PCR product in 15µl final reaction in a water bath at 37ºC, overnight. RFLP products were loaded on 2.5% agarose gel and subjected to gel electrophoresis and stained with ethidium bromide and visualized under UV (Fig. 7).

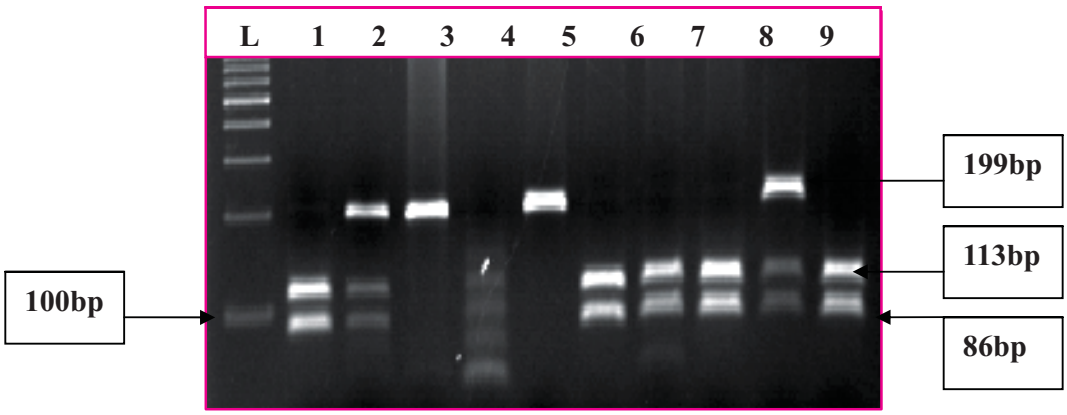


Fig.7: EtBr stained agarose gel electrophoresis for RFLP products of p53 gene. Lane L shows 100bp ladder. Lanes 3 and 5 show the undigested bands (199bp) representing Pro/Pro allele. Lanes 1,6,7,8 and 10 show the digested products (113bp +86bp) representing the homozygous Arg/Arg allele. Lanes 2 and 9 show the presence of all the three bands representing the heterozygous condition of Arg/Pro allele.

A total of 134 lung cancer cases and 185 controls were included in the study. The frequency of the three genotypes of the p53 gene in the patients with lung cancer and controls was determined (Table 6).

p53 genotypes	Cases (134) N (freq)	Controls (180) N (freq)	OR (95% C.I.)	p-value
Pro/Pro	30 (0.22)	44 (0.24)	0.892 (0.527-1.510)	0.689
Arg/Pro	71 (0.52)	93 (0.51)	1.054 (0.674-1.648)	0.820
Arg/Arg	33 (0.24)	43 (0.23)	1.041 (0.620-1.749)	0.895

Table 6: Frequency of Pro/Arg polymorphism at p53gene loci in lung cancer cases and controls

The frequencies of genotypes Pro/Pro, Arg/Pro and Arg/Arg were found to be 0.22, 0.52, 0.24 and 0.24, 0.51, 0.23 in cases and in controls respectively. Arg/Pro and Arg/Arg genotypes showed marginal risk towards lung cancer (OR=1.054 and OR= 1.041). However, these results were not statistically significant. None of the genotypes showed significant difference in the distribution between the lung cancer patient group and normal healthy controls.

EPHX1 Polymorphisms:

PCR reaction for both exon 3 and 4 of EPHX gene was carried out in a volume of 20µl. Restriction digestion for exon 3 and exon 4 was carried out by enzymes EcoRV and RsaI respectively. The digested products were loaded on 2.5% agarose gel and subjected to gel electrophoresis and visualized under UV (Fig. 3 & 4). 134 lung cancer cases and 185 controls were included in the study. EPHX1 exon 3 genotype frequency of Tyr/Tyr, His/Tyr and His/His were 41.7%, 25.3% and 32.8% in cases. For controls, the distribution of the same was 30.8%, 44.8% and 24.3%. The prevalence of the His113/Tyr113 genotype was significantly lower (p=0.002) in the lung cancer cases (25.8%) than in the controls (44.8%). The frequencies of His/Arg and Arg/Arg were higher in cases as compared to controls. Lung cancer patients with exon 3 His113Tyr genotypes, were at lower risk and those harboring the exon 4 Arg139Arg genotype, were at higher risk of lung cancer (OR 0.417, 95% CI=0.243-0.717, p=0.002 and OR 11.785, 95% CI=1.842-74.452, p=0.006). These results indicate that the mEH genotype (His113Tyr) which confers low enzyme activity decreased the risk of lung cancer whereas the fast activity genotype (Arg139Arg) increased the risk. This suggests that although the products of hydrolysis are generally less reactive than the parent epoxide, some resulting intermediates are precursors of highly reactive and carcinogenic compounds. This metabolic pathway could account for the increasing risks of lung cancer observed with increasing levels of mEH activity.

2. EFFECT OF PESTICIDE EXPOSURE IN CAUSATION OF CANCER IN NORTH EAST INDIA

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Duration	:	**2005-2010**

Aims, Objectives & Background:

High incidence of various cancers in NE states of India is attributed to genetic as well as environmental factors. The major environmental factors include widespread use of pesticides in the tea gardens and consumption of smoked food and tobacco in various forms. Exposure to pesticides has been reported to be associated with breast carcinogenesis and Non-Hodgkin's lymphomas. A multicentric study had been designed to find out possibility of gene-environment interaction in the occurrence of breast cancer and lymphoma in NE India.

Work done during the year:

Breast Cancer

To investigate the contribution of genetic and environmental factors for high incidence of breast cancer in NE region of India, we selected genes related to catabolism and detoxification of xenobiotics (GSTM1, GSTT1, and GSTP1), tumour suppressor gene (TP53) and oestrogen biosynthesis (CYP17), to explore their contribution in breast cancer.

Genotyping for GSTT1, GSTM1, GSTP1, TP53 and CYP17 polymorphisms in 117 northeast Indian breast cancer cases and 174 matched controls was performed. Genotyping of GSTM1 and GSTT1 deletion polymorphisms was carried out by multiplex polymerase chain reaction with three pairs of primers. GSTP1 Ile105 Val, CYP17 T>C and TP53 codon 72

polymorphism was analysed by PCR-restriction fragment length polymorphism (PCR-RFLP). In addition, about 10% random samples were rechecked by the same method (multiplex PCR or PCR-RFLP).

Women with GSTT1 null polymorphism were 41 percent less susceptible [0.59, (0.34-1.03), 0.06] for having breast cancer. Women with GSTM1 null polymorphism were also 55 per cent less susceptible [0.55 (0.30-1.02), 0.05] for having breast cancer. Marginally significant risk was observed among women having G/G genotype of GSTP1 gene [3.04 (0.85 - 0.82), 0.08], but when adjusted for the exposure variables, significance was lost. Allele frequencies of different alleles of GSTP1, CYP17 and TP53 genes were also compared. The G allele of the GSTP1 gene was found to be over represented in cases as compared to the controls indicating that G allele might be a risk factor for breast cancer [1.43 (0.96 - 2.11), 0.07]. The frequencies of alleles of other genes did not differ significantly. MDR analysis revealed betel quid chewing to be the single factor imparting the main effect [testing accuracy of 0.6851 and cross validation consistency 10/10, p =0.05]. Figure 1 depicts the interactions between nine attributes from the MDR analysis via a graphical representation of a 'dendrogram'. It shows betelquid chewing, GSTT1 and GSTM1 on a separate branch imparting their independent effects to breast cancer risk.

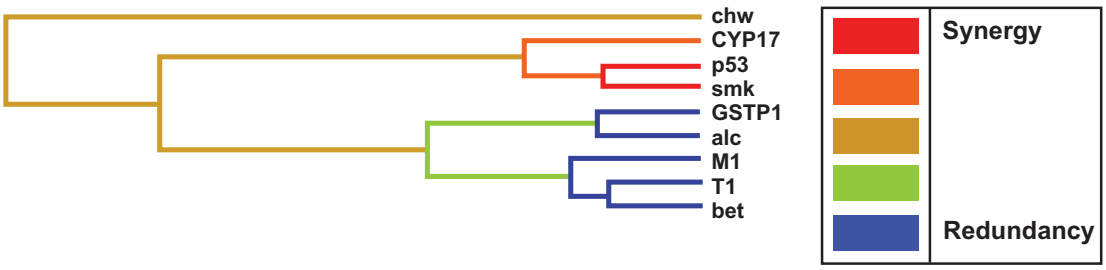


Fig. 1: Interaction dendrogram for the breast cancer dataset: graphical representation of interactions between nine attributes (GST1 (T1), GSTM1 (M1), GSTP1, CYP17, TP53 (p53), tobacco smoking (smk), tobacco chewing (chw), betel quid chewers (bet) and alcohol consumption (alc)) from the multifactor dimensionality reduction analysis using an 'interaction dendrogram'. Synergy, the interaction between two attributes provides more information than the sum of the individual attributes; redundancy, the interaction between attributes provides redundant information.

BRCA2 –Tumor suppressor gene

The BRCA2 gene is located on chromosome 13q. The complete sequence of the BRCA2 gene was reported in 1996. The gene contains 27 exons distributed over the 84 Kb of genomic DNA and encodes a large protein of 3418 amino acids. A segment of exon 11 contained 8 repetitive units composed of 30-80 amino acids termed the BRC repeats and these are the more conserved regions of BRCA2 gene. Carriers of mutations in BRCA1 and BRCA2 are relatively infrequent in the general population and BRCA mutations are thought to be involved in at most 5% to 10% of all breast cancers.

Mutations were seen in Exon27 (Fig. 2), Exon 2 (Fig. 3) and Exon 18 (Fig. 4) in the BRCA2 gene.

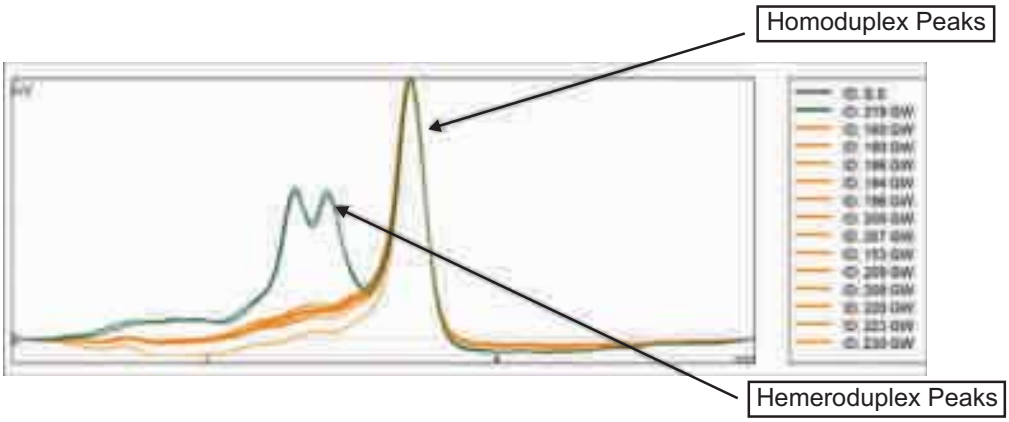


Fig. 2 : Exon27C- T>C polymorphison by DHPLC Analysis

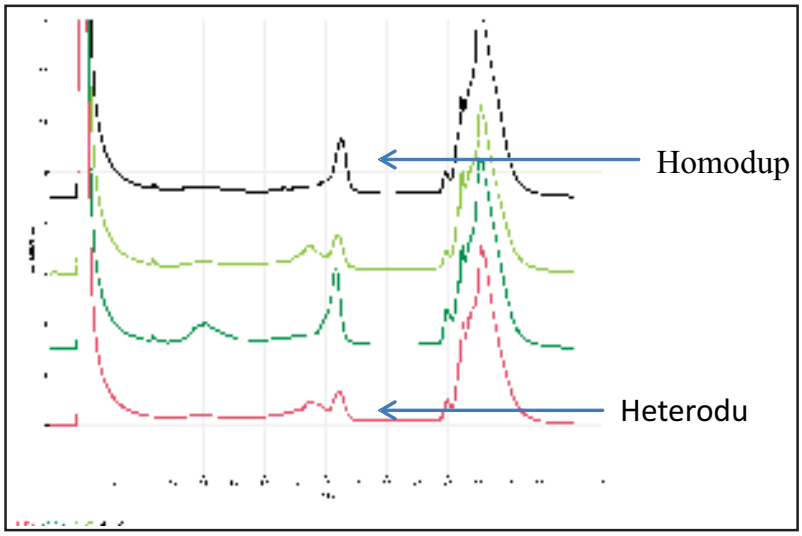


Fig. 3: DHPLC analysis of Exon 2 -26 G>A 5'UTR polymorphism

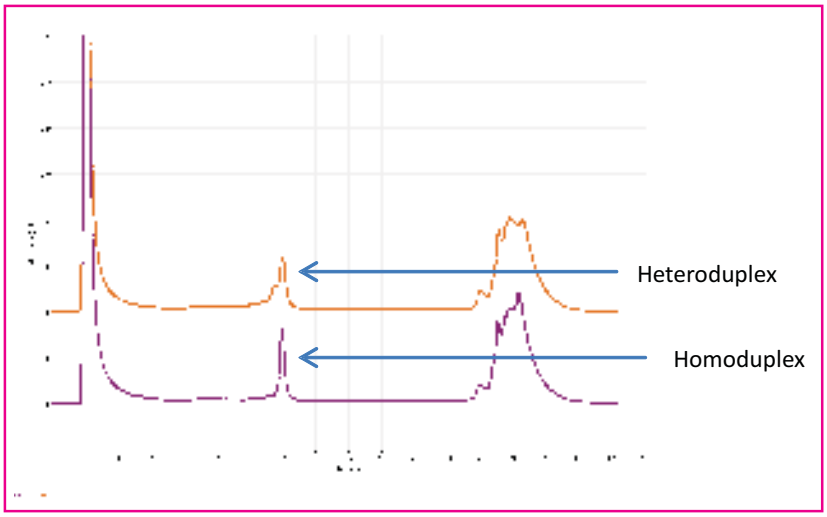


Fig 4: DHPLC analysis of Exon 18

Copy Number Analysis

Copy number analyses of 46 breast tumors was done using SNP 10K array. DNA from thirty tumor samples and ten blood samples was extracted. 250 ng of genomic DNA digested with XbaI and ligated to XbaI adaptor before subsequent PCR amplification was fragmented with DNaseI and visualized on a 4% Tris-borate EDTA agarose gel to confirm DNA fragmentation. Fragmented PCR products were biotin labeled. Hybridization and detection was done with an Affymetrix Fluidics Station 450 and GeneChip Scanner 3000. Genotype information was extracted from the Mapping 10K Arrays using the Genechip DNA Analysis Software (GDAS) from Affymetrix. Genotypes derived from germline and cancer DNA was loaded into the software package dChip which was used for copy number analysis.

DNA copy number gains were obtained as determined by dChip using analysis of signal intensity values based on the Hidden Markov Model. Arrays with >93% call rates were included in the analysis as per Affymetrix manual. Copy number data were obtained for chromosome 1 using CytoBand information files from the dChip website. Both the raw copy number and log2 ratio (Signal/mean signal of normal samples at each SNP) were computed to estimate copy number changes in chromosome view. For each SNP, the signal values of all of the normal cell lines were averaged to obtain the mean signal of 2 copy (male X chromosomes are multiplied by 2 before averaging), and the observed copy number is defined as (observed signal/mean signal of two copy) * 2, and visualized either log 2 ratio. The detailed analysis is undergoing.

V. BRAIN TUMORS

1. IDENTIFICATION OF DIAGNOSTIC BIOMARKERS THAT DIFFERENTIATE FIBROBLASTIC MENINGIOMA FROM SCHWANNOMA

Scientific staff	:	**Dr. Avninder Singh**
Collaborators	:	**Dr. KC Sharma, Neurosurgery Department, Safdarjung Hospital, Delhi Dr. Stephen M Hewitt, TARP Lab, National Cancer Institute, NIH, Bethesda, USA**
Duration	:	**2008-2010**

Aims, Objectives & Background:

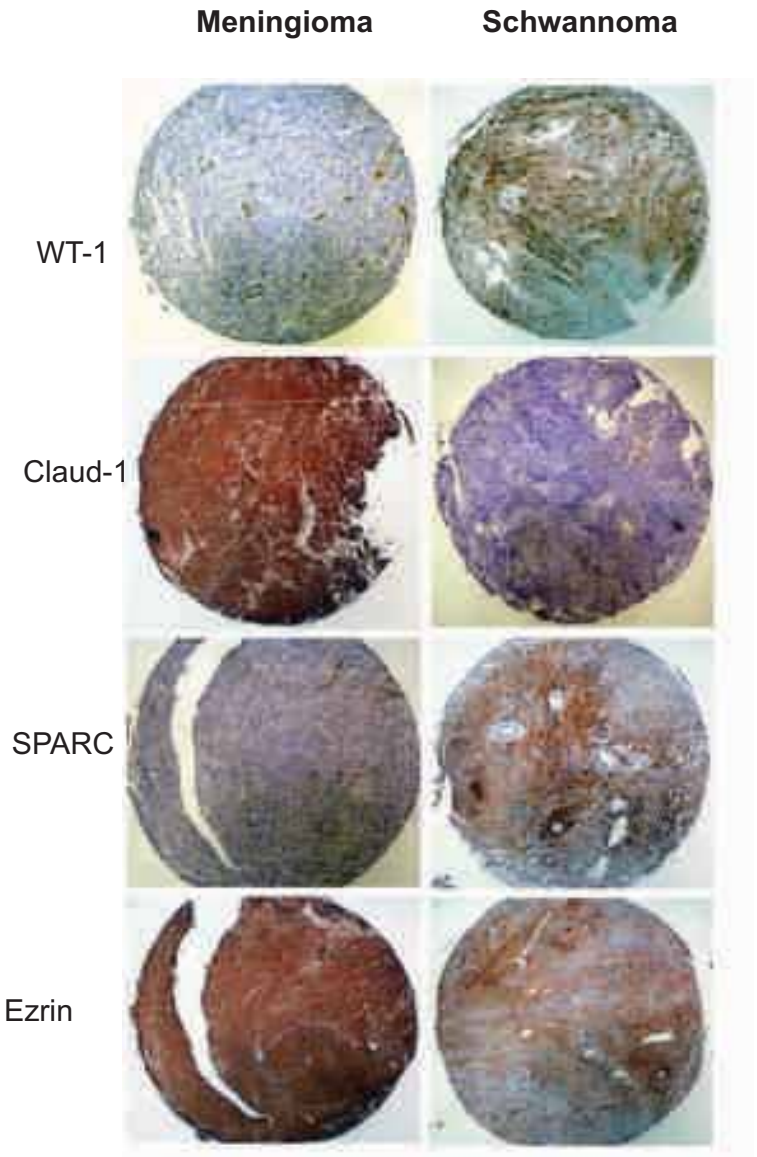
The aim of this study is to identify immunohistochemical (IHC) markers that can reliably separate Fibroblastic Meningioma (FM) and Schwannoma (SCHW). FM and SCHW are usually distinguishable by their histomorphological pattern and cellular composition on routine H&E staining. With smaller size of the biopsies being made available to the pathologist, occasionally distinction between these two can cause a diagnostic dilemma. Adjuvant IHC markers routinely used to support the diagnosis of meningioma (MEN) and SCHW has been Epithelial Membrane Antigen (EMA) and S100, respectively. However, these two markers cannot reliably distinguish FM from SCHW and there is a need to identify new diagnostic markers that aid in differentiating these two neoplasms.

Work done during the year:

During the period, 106 archival blocks of cases of FM (n=50) and SCHW (n=56) were selected and a tissue microarray (TMA) of core diameter of 1.5 mm was constructed. A TMA-IHC was performed using 17 antibodies. After IHC staining, 100 cores were found suitable for evaluation. Distribution of positivity (X) was scored as 0 (no, <5%), 1 (<50%), 2 (>50%) stain and the intensity (Y) was scored as 0 (no stain), 1 (weak), 2 (strong). A dichotomized score (X x Y) had a range of 0-4. Immunoexpression was classified as 1=weak, 2=moderate and 4=strong expression. A discriminant analysis (DA) (Wilks'Lambda test) was performed to assess the

relative importance of these biomarkers in classifying the two groups FM and SCHW. A p-value of 0.05 was considered statistically significant. Results of DA and determination of p-value showed for these were WT1 (0.074, $p<0.001$), EMA (0.267,$p<0.001$), SPARC (0.286, $p<0.001$), Claudin-1 (0.407,$p<0.001$), Ezrin (0.556, $p<0.001$) and S100 (0.597, $p<0.001$) were the most useful IHC markers that differentiated FM from SCHW.

Conclusion: WT-1, SPARC, Ezrin, claudin-1 and EMA are useful markers that reliably differentiate FM from SCHW.



PATHOLOGY OF INFECTIOUS DISEASES

INFECTIOUS DISEASES

CHLAMYDIASIS

1. ROLE OF CHLAMYDIAL HEAT SHOCK PROTEINS IN PATHOGENESIS OF GENITAL TRACT INFECTION IN WOMEN

Scientific staff	:	Dr. Aruna Singh, Mr. Rajneesh Jha
In collaboration with	:	Dr. Sudha Salhan, Safdarjung Hospital, New Delhi
Technical Staff	:	Mrs. Madhu Badhwar, Mrs. Asha Rani
Duration	:	2006-2010

Aims, Objectives & Background:

There are number of studies that have been devoted to cell-mediated and humoral immune responses to cHSP60 and cHSP10, however, no study has been dedicated to their potential role in apoptosis of primary cervical epithelial cells that are privilege target for chlamydial infection. We have recently reported that there are higher levels of cHSP60 and cHSP10 mRNA and proteins in cervical epithelial cells of CT infected infertile women than in fertile women which may be due to different metabolic state of *Chlamydia* in infertile and fertile women and also to different levels of apoptosis of endocervical epithelial cells. Thus we further investigated the potential role of chlamydial heat shock proteins (cHSP) 60 and cHSP10 in apoptosis of primary cervical epithelial cells.

Work done during the year:

Primary cervical epithelial cells were stimulated with cHSP60 and cHSP10 for 4 hours. Quantitative measurement of apoptosis was performed by cytofluorometry. An apoptosis pathway-specific cDNA microarray analysis was used to examine the expression of epithelial cell genes that might be affected by cHSP60 and cHSP10. The up-regulation of genes of interest was confirmed in real-time RT-PCR analysis.

cHSP60 and cHSP10 stimulation of endocervical epithelial cells induces apoptosis

After 4 h -incubation in the presence of recombinant cHSP60 or cHSP10, the number of cells exhibiting annexin V binding activity increased 6-and 5-fold respectively ($p < 0.05$) (Fig 1).

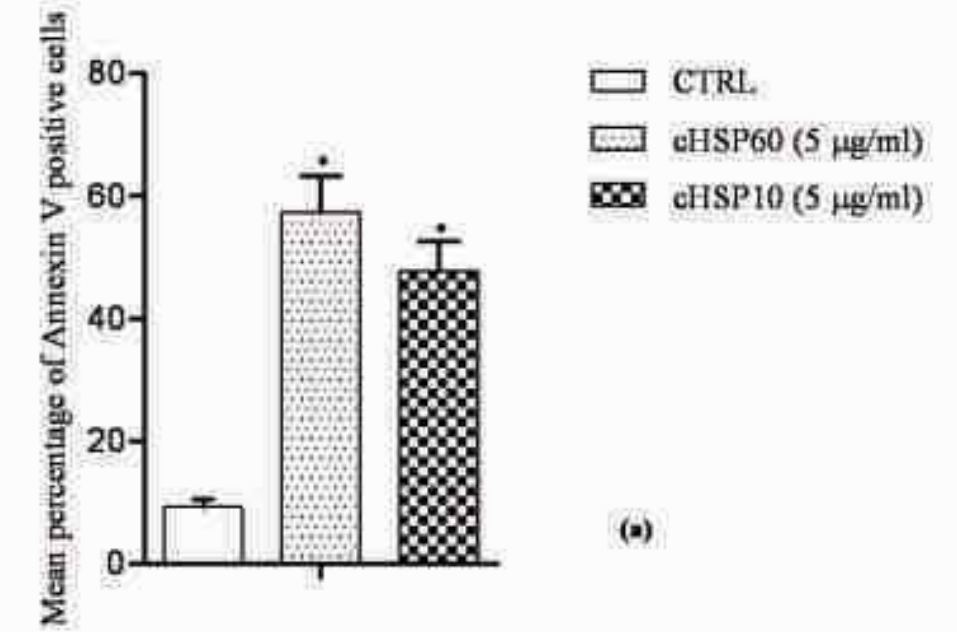
Analysis of epithelial cell mRNA expression by cDNA microa rray and real-time RT-PCR in response to cHSP60 and cHSP10 stimulation

To study the changes in mRNA expression of genes related to apoptosis in these epithelial cells, we employed a cDNA microarray approach using the Clontech atlas human apoptosis expression array 205 different human genes related to apoptosis. In cHSP60 stimulated cells, 10 genes were significantly ($p < 0.05$) upregulated and 6 genes were significantly ($p < 0.05$) downregulated. Similarly, in cHSP10 stimulated cells, 11 genes were significantly ($p < 0.05$) upregulated and 4 genes were significantly ($p < 0.05$) downregulated. The mean fold changes expression of genes and their accession numbers are listed in Table 1. To confirm the data obtained using the human apoptotic cDNA microarray and to further characterize mRNA expression profile, few genes of interest were subsequently quantified by real-time RT-PCR. We observed that interleukin (IL)-1 β-convertase, caspase-3, -8 and -9 which were upregulated in cDNA microarray were also upregulated in real-time RT-P CR upon stimulation with cHSP60 and cHSP10. The mean fold changes expression of all genes and their accession numbers after stimulation of cHSP60 and cHSP10 are listed in Table 2.

Protein expression in response to cHSP60 and cHSP10 stimulation

To examine whether protein correlated with mRNA expression, western blot assay was performed to quantify caspase-3, -8, and -9 in the same experimental conditions. Expression of caspase-3 was significantly higher in both cHSP60 (7742 ± 1114.5 arbitrary units, $p < 0.01$) and cHSP10 (4662 ± 776.4 arbitrary units, $p < 0.05$) stimulated cells than in control cells (869 ± 66.7 arbitrary units). Furthermore, expression of both caspase-8 (cHSP60; 5877 $\pm$ 398.01 arbitrary units, $p < 0.01$) (cHSP10; 6915 $\pm$ 415.5 arbitrary units, $p < 0.01$) and -9 (cHSP60; 1300 $\pm$ 257.8 arbitrary units, $p < 0.05$) (cHSP10; 1392 $\pm$ 257.4 arbitrary units, $p < 0.05$) were also significantly

higher in cHSP60 and cHSP10 stimulated cells than in control cells (caspase-8; 458 ± 169.7 arbitrary units) (caspase-9; 409 ± 182.7 arbitrary units) (Fig. 2).



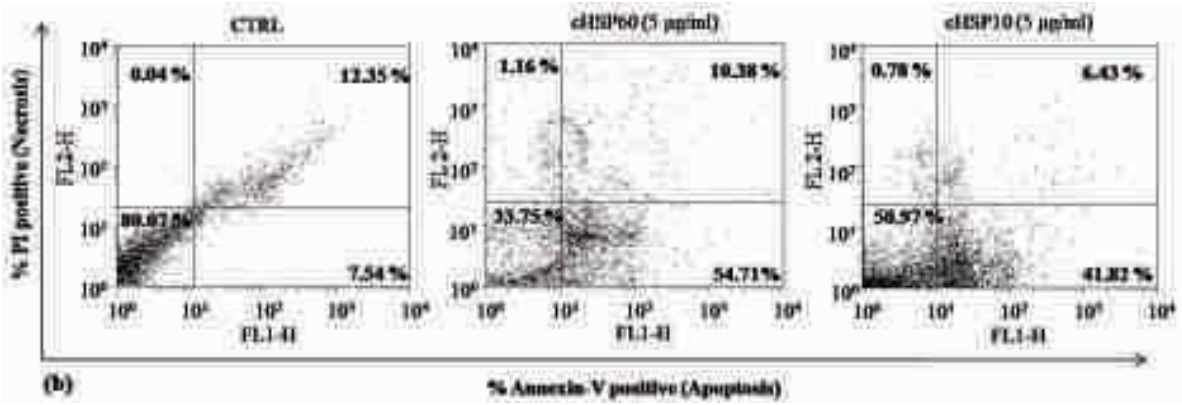


Fig. 1: Detection of apoptosis by flow cytometry in vitro stimulated epithelial cells.

caspase-9.

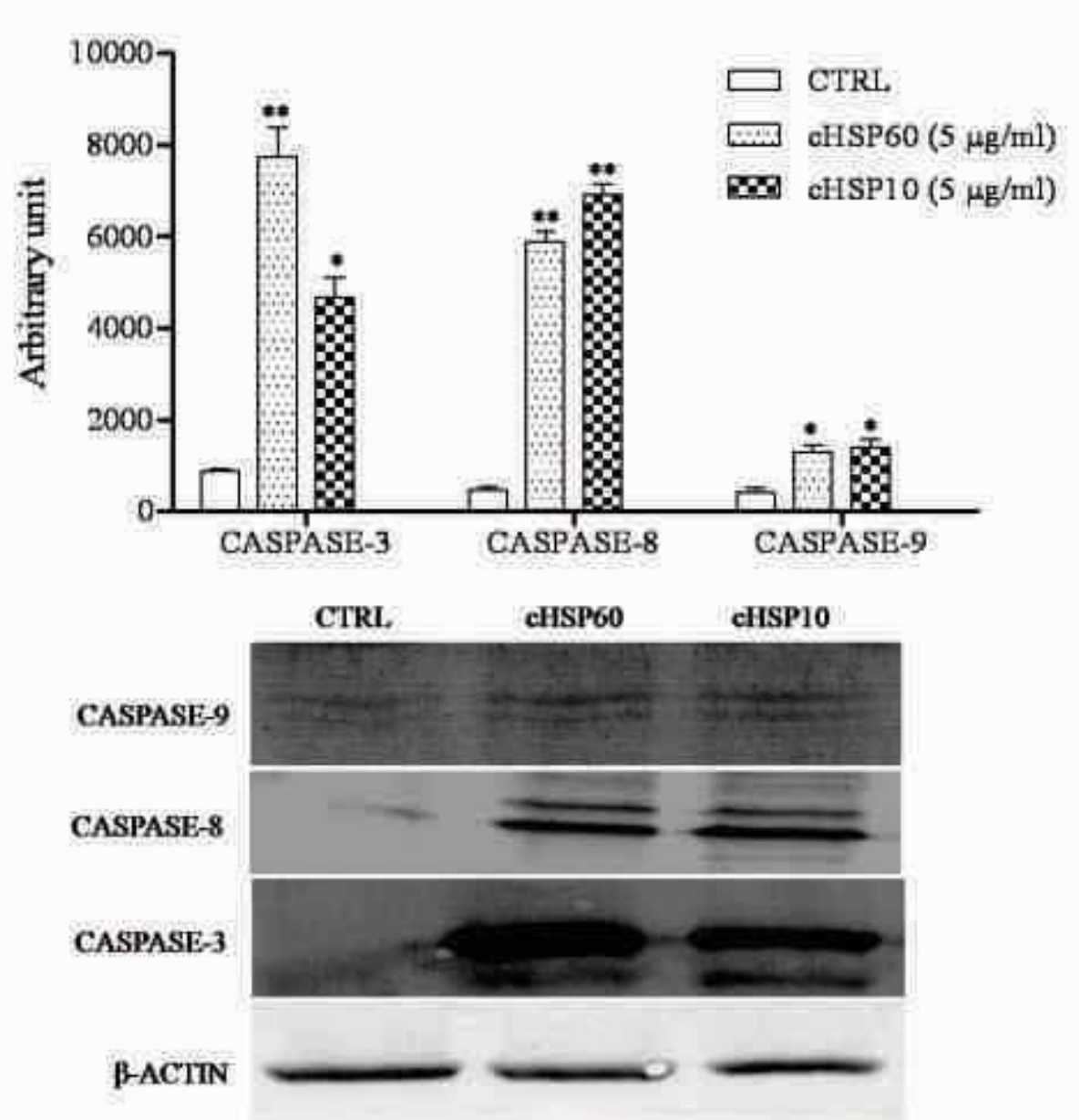


Fig. 2: cHSP60 and cHSP10 induces increased expression of caspase-3, caspase-8 and caspase-9

S. No.	Gene Description	Accession No.	cHSP60	cHSP10
1	Cyclin dependent kinsae 4	M14505	(-)8.3+ _0.32	(-)5.2+ 0.46
2	Cyclin-B1	M25753	(-)17.1+ _0.19	(-)4.6+ 0.24
3	Phospholipase D1	U38545	3.8+ _0.62	9.2+ 0.57
4	ERK-1	X60188	4.6+ _0.44	5.4+ 0.19
5	MAPK Kinase kinase 3	U78876	9.3+ _1.2	3.2+ 0.41
6	PCNA	M15796	(-)2.4+ _0.22	Unchanged
7	E2F transcription factor	M96577	(-)3.9+ _0.61	Unchanged
8	P53 induced protein	AF010315	4.1+ _0.2	Unchanged
9	Bcl-2 antagonist of cell death	U66879	Unchanged	3.1+ _0.12
10	IL-1 beta convertase	U13699	11.8+ _1.1	8.3+ 1.3
11	Caspase-3	U13737	4.5+ _0.64	5.9+ 0.22
12	Caspase-8	U60520	3.3+ _0.23	6.4+ 0.67
13	Caspase-9	U56390	7.1+ _0.19	4.6+ 0.32
14	TNF-receptor associated factor-6	U78798	3.1+ _0.91	5.7+ 0.81
15	FAAD-like a poptosis regulator	AF010127	2.2+ _0.33	9.7+ 0.41
16	TNF superfamily member 6	Z70519	Unchanged	1.8+ _0.11
17	TNF receptor family member 1B	M32315	(-)14.3 ± 1.3	(-)7.2 ± 0.82
18	TNF receptor family member 10B	AF016268	(-)2.8+ _0.41	(-)6.6 ± 0.36

The expression of genes was analyzed in epithelial cells of 3 women using an apoptosis cDNA expression array from Clontech containing 205 immobilized human cDNAs. Values in columns entitled cHSP60 and cHSP10 are representing normalized mean fold change ± standard deviation after stimulation as compared to unstimulated control, (-) indicates fold down regulation and rest are fold upregulated genes.

S. No.	Gene Description	Gene Bank ID	cHSP60	cHSP10
1	Apoptosis peptidase activating factor 1	NM_001160	13.6±0.75	6.2±0.86
2	BH3 interacting domain death antagonist	NM_001196	(-)5.5±1.16	Unchanged
3	NLR family apoptosis inhibitory protein	NM_004536	(-)7.1±0.38	(-)9.6±2.41
4	Interleukin β convertase	NM_033292	7.3±1.22	6.2±0.44
5	Caspase-3	NM_004346	9.1± 1.46	3.6±0.74
6	Caspase-8	NM_001228	4.7±0.54	10.2±1.41
7	Caspase-9	NM_001229	7.2±0.69	7.9±0.36
8	Fas-associated death domain	NM_003824	(-)4.5±1.44	(-)6.1±2.17
9	Fas-ligand	NM_000639	12.4±1.11	Unchanged
10	Bcl-2 interacting domain	NM_003806	5.6±1.91	5.9±0.74
11	TNFRSF1A associated death domain	NM_003789	Unchanged	4.6±1.22
12	TNF receptor family member 1B	NM_001065	(-)9.4±0.58	Unchanged

Table 2: Modulation of gene expression in epithelial cells after stimulation with cHSP60 and cHSP10 for 4 h.

The expression of mRNA was analyzed in epithelial cells of 15 women using real-time RT-PCR. Values in columns entitled cHSP60 a nd cHSP10 are representing normalized mean fold change ± standard deviation after stimulation as compared to unstimulated control, (-) indicates fold down regulation and rest are fold upregulated genes.

2. EFFECT OF SEX HORMONES ON INDUCTION OF IMMUNITY BY DENDRITIC CELLS IN FEMALE REPRODUCTIVE TRACT DURING *CHLAMYDIA TRACHOMATIS* INFECTION

Scientific staff	:	**Dr. Aruna Singh, Ms. Tanvi Agrawal, Mr. Vikas Vats**
In collaboration with	:	**Dr. Sudha Salhan, Safdarjung Hospital, New Delhi Dr. Paul Wallace, Roswell Park Cancer Institute, Buffalo, USA**
Technical Staff	:	**Mrs. Madhu Badhwar, Mrs. Asha Rani**
Duration	:	**2005-2010**

Aims, Objectives & Background:

Dendritic cells (DCs) at the mucosal surfaces are central to the generation of immune protection against pathogens. Understanding the mechanism of DC antigen presentation and the role of sex hormones in modulating the immune responses during chlamydial infection will help in understanding of immunopathogenesis of *Chlamydia trachomatis*.

Work done during the year:

Pulsing of immature MDDCs with chlamydial EBs and estradiol treatment

Peripheral blood mononuclear cells (PBMCs) from control age matched women were prepared by ficoll-Hypaque density gradient centrifugation and $CD14^+$ cells (monocytes) were separated from other leukocytes using a magnetic cell sorter. The $CD14^+$ cells were cultured in the presence of 50 ng/ml Granulocyte Macrophage Colony Stimulating factor (GM-CSF) and 20 ng/ml IL-4 for 6-7 days and were then washed and infected with live chlamydial EBs at an MOI of 2: 1 (DC: EB) for 6 hrs. In parallel experiments DCs were pretreated with estradiol (Sigma) at concentrations of 10µg/mL, 1µg/mL and 0.1µg/ml for 24 hrs and were then pulsed with chlamydial EBs. After exposure, total RNA was extracted from the cells and TLR signalling pathway genes are studied using Real Time PCR based arrays. Quantification of cytokines (IL-6, IL-12, IL-4, IL-10, TNF-α and IFN-γ) in culture supernatants was done by using commercially available ELISA kits.

Supernatants were harvested 24 hrs post-infection and tested for secreted cytokines by ELISA (Figure 1). Estradiol treatment significantly reduced the release of proinflammatory cytokines IL-12 and TNF-α at the highest concentration, however, a non-significant decrease in levels of IL-6 was observed. A significant increase in secretion of IL-10 was observed with highest E2 concentration. The expression of a number of Toll like receptor signalling genes such as IRAK4, MyD88 and nF-κB were upregulated following chlamydial infection. Gene expression of significantly expressed genes in estradiol treated and untreated conditions are shown in Figure 2. Estradiol pretreatment significantly reduced expression of TLR4 as compared to untreated cells. Gene expression of TLR4 associated downstream signalling molecules as

IRAK4 and nF-κB was also found to be downregulated. Gene expression of Th1 associated cytokines IL-12, IL-6, TNF-α and IFN-γ was also reduced, however, expression of IL-10 by dendritic cells increased significantly after estradiol pretreatment.

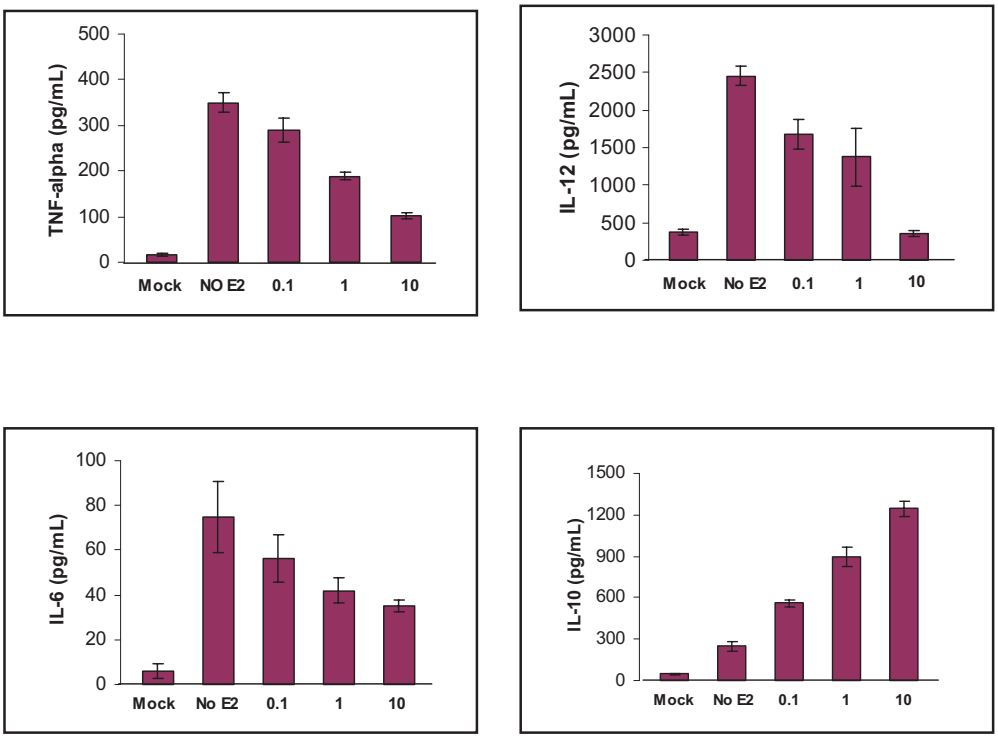


Fig. 1:. Cytokine levels in supernatant of dendritic cells pulsed with Chlamydia. Significant reduction in secretion of TNF-α and IL-12 was observed. IL-10 levels increased upon increasing estradiol concentrations. No E2: estradiol untreated dendritic cells

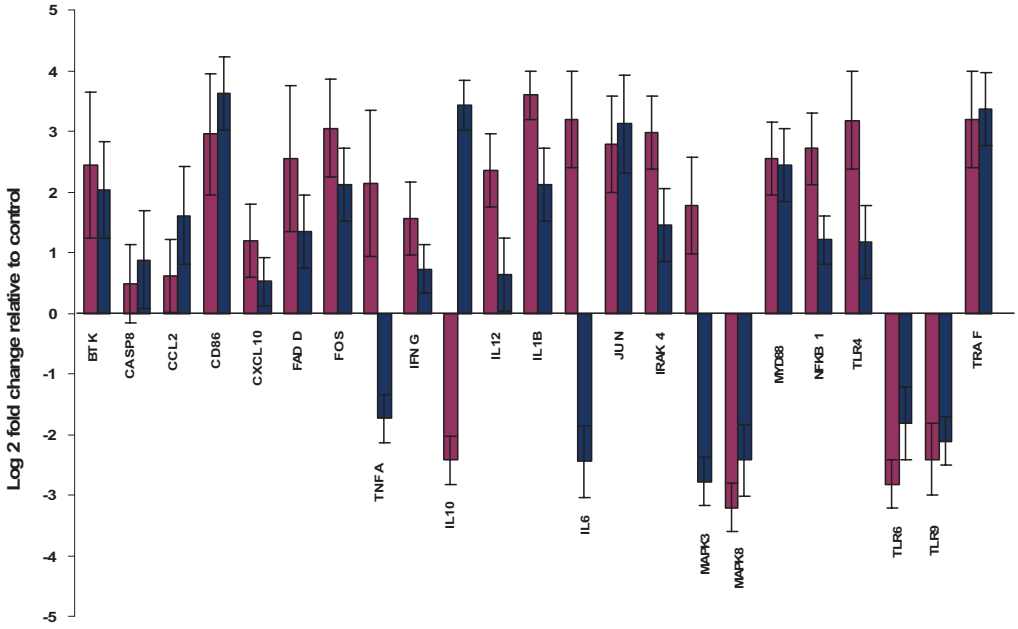


Fig. 2: Effect of estradiol pretreatment on Toll like receptor signalling pathway. Results are shown as log 2 – transformed means of three experiments.
Dark columns indicate gene expression in dendritic cells untreated with estradiol. Light coloured columns show gene expression in estradiol pretreated dendritic cells. Estradiol pretreated cells show significant decrease in gene expression of TLR4.

Western blot analysis

Dendritic cells were grown and infected with chlamydial EBs, lysed and protein concentration were determined. Twenty micrograms of lysate protein per lane was boiled and separated by SDS-PAGE under reducing conditions and blotted on to the nitrocellulose membranes. Membranes were then incubated overnight at 4^0C with primary antibodies specific for phosphorylated or total signaling pathway members (IκBα monoclonal antibody and nF-κB antibody) along with and β-actin antibody (internal control). Membranes were then incubated with 0.2µg/ml peroxidase-conjugated anti-rabbit immunoglobulin G and were then visualized on X-ray film using enhanced chemiluminescence. Estradiol pretreatment significantly reduced nuclear nF-κB p65 production, thereby, reducing the secretion of proinflammatory cytokines (Figure 3). However, IκBα degradation was reduced during E2 treatment but not significantly. In

E2 treated dendritic cells pulsed with *E. coli* LPS, no significant change in IκBα and nF-κB activity was seen compared to untreated cells.

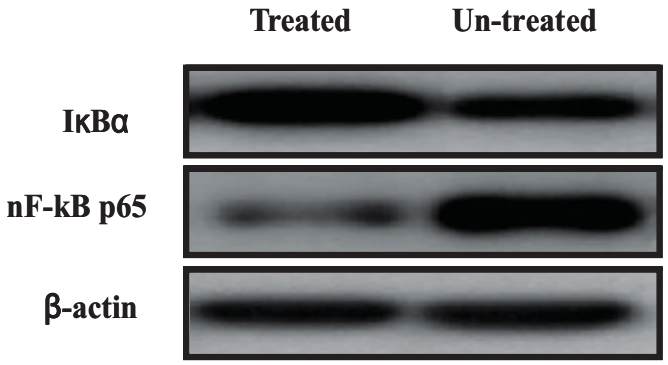


Fig. 3: Effect of estradiol treatment on IκBα and nf-kB activation. A representative Western Blot of treated and untreated cytosolic and nuclear extracts of dendritic cells showing IκBα and nF-κB.

Co-culture of Dendritic cells with autologous CD4 T lymphocytes

Immature MDDCs were infected with live *C. trachomatis* EB's and cultured in RPMI 1640 for 24 hrs at 37^0C. Autologous $CD4^+$ T cells were separated from PBMCs using a magnetic cell sorter and were then co-cultured with the EB pulsed dendritic cells for further 4 days. The culture supernatants were harvested and analyzed for production of various cytokines. In parallel experiments culture of dendritic cells with autologous T cells was performed in presence of estradiol. The mean levels of IL-1β, IL-2, IL-6, IL-10, IL-12 and IFN-γ in culture supernatants of DC activated autologous $CD4^+$ T cells along with controls are shown in Fig. 4. IL-2 and IL-12 levels were found to be significantly higher ($P<0.05$) in supernatants of activated autologous $CD4^+$ T cells compared to other conditions. Pretreatment of dendritic cells with estradiol significantly reduces secretion of IL-6 and IL-12 ($P<0.05$) by CD4 T cells, however, estradiol increases secretion of IL-10 from activated CD4 T cells.

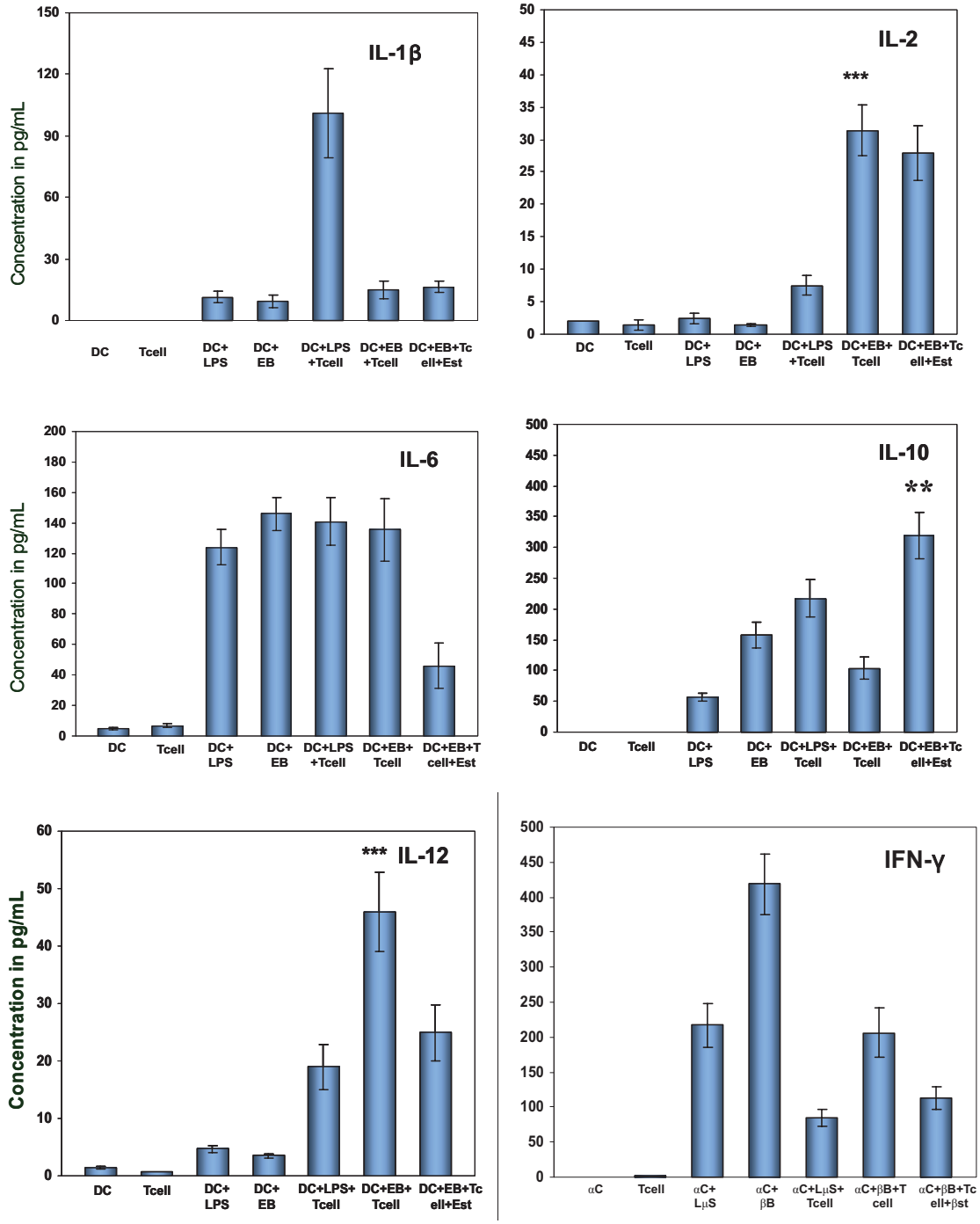


Fig. 4. Cytokine levels in supernatants of CD4$^+$ T cells activated in presence of dendritic cells pulsed with Chlamydia elmentary bodies

3. MODULATORY ROLE OF ANTICHLAMYDIAL AGENTS IN *CHLAMYDIA TRACHOMATIS* INFECTION AND THEIR THERAPEUTIC POTENTIAL

Scientific staff	:	**Dr. Aruna Singh, Ms. Pragya Srivastava, Ms. Apurb Rashmi Bhengraj**
In collaboration with	:	**Dr. Sudha Salhan, Safdarjung Hospital, New Delhi**
Technical Staff	:	**Mrs Madhu Badhwar, Mrs Asha Rani**
Duration	:	**2007-2010**

Aims, Objectives & Background:

Chlamydia trachomatis infection induces a wide array of inflammatory cytokines, which may contribute to chlamydia-induced pathologies. To date studies of reproductive sequelae following azithromycin and doxycycline treatment for *C. trachomatis* infection and their effect on immune molecules like cytokines in humans are lacking. Hence in this study we have evaluated the effect of azithromycin and doxycycline *in vitro* on cytokines in cells stimulated with chlamydial elementary bodies (EB's). Further, we have evaluated the efficacy of these antibiotics in treatment of *C. trachomatis* infection in fertile and infertile women.

Work done during the year:

Patients attending the gynecology out patient department, Safdarjung Hospital, New Delhi, India for gynecological complaints (cervical discharge, cervicitis and infertility) were enrolled in the study.

Efficacy of treatment of *C. trachomatis* infection with Azithromycin and Doxycycline in women with primary and recurrent infection

In women with primary *C. trachomatis* infection the treatment efficacies for both the antibiotics were similar: Azithromycin: In fertile women (n=21) one was found positive after treatment. In infertile women (n=19) one was found positive after treatment. Doxycycline- In

fertile women (n=25) one was found positive after treatment. In infertile women (n=25) two were found positive after treatment.

In women with recurrent *C. trachomatis* infection the treatment efficacies for both the antibiotics were found to be different: Azithromycin- In fertile women (n=4) three were treated in a month and one was treated after repeating the treatment after one month. In infertile women (n=4) one was treated after repeating the treatment after one month and three were treated after repeating the treatment for another two consequent months. Doxycycline- In fertile women (n=4) one was treated in a month and three were treated after continuous treatment for two months. In infertile women (n=4) one was treated after repeating the treatment for another two consequent months and three were not treated even after continuous three month treatment.

Quantification of cytokines by ELISA

A significant decrease in the IL-1β, IL-6, IL-8 and TNF-α was observed in Group I and Group II in presence of azithromycin in *C. trachomatis* stimulated cells (Table 1). In presence of doxycycline a significant decrease in the levels of IL-1β and IL-6 was observed in Group I whereas no significant decrease in the levels of cytokines was observed in Group II (Table 1).

Women with recurrent infection were divided into: Group I (n=17) comprised of *Chlamydia* positive fertile women; Group II (n=14) comprised of *Chlamydia* positive infertile women.

A significant decrease in the IL-1β, IL-6, IL-8, IL-10 and TNF-α was observed in Group I and Group II in presence of azithromycin in *C. trachomatis* stimulated cells (Table 2). In presence of doxycycline no significant decrease in the levels of cytokines was observed in both the groups (Table 2).

	Group I (n=52)					Group II (n=44)				
	CT	CT + Azithromycin	P value [a]	CT + Doxycycline	P value [b]	CT	CT + Azithromycin	P value [c]	CT + Doxycycline	P value [d]
IL-1β (pg/ml)	143 (22-490)	76 (15-396)	0.002*	73 (32-365)	0.01*	240 (52-657)	120 (25-381)	0.006*	175 (25-606)	0.1
IL-2 (pg/ml)	5 (UDL-38)	5 (UDL-26)	0.6	6 (UDL-37)	0.4	6 (UDL-42)	5 (UDL-36)	0.9	5 (UDL-20)	0.8
IL-6 (pg/ml)	77 (42-688)	48 (7-354)	0.002*	53 (20-428)	0.007*	217 (20-537)	114 (15-374)	0.001*	172 (22-383)	0.08
IL-8 (pg/ml)	193 (78-717)	63 (8-258)	<0.0001*	180 (30-584)	0.1	255 (110-966)	117 (30-448)	<0.0001*	244 (57-627)	0.1
IL-10 (pg/ml)	138 (24-692)	101 (23-512)	0.2	136 (28-448)	0.5	246 (48-819)	197 (37-739)	0.09	219 (56-654)	0.5
IL-13 (pg/ml)	5 (UDL-28)	6 (UDL-20)	0.9	6 (UDL-33)	0.9	7 (UDL-24)	5 (UDL-18)	0.2	6 (UDL-40)	0.9
IFN-γ (pg/ml)	144 (21-611)	91 (14-488)	0.07	115 (11-437)	0.2	88 (20-574)	61 (22-524)	0.3	73 (15-446)	0.4
TNF-α (pg/ml)	185 (27-582)	94 (30-439)	0.01*	127 (12-522)	0.08	115 (18-389)	76 (15-277)	0.01*	87 (21-392)	0.2

Group I comprised of *Chlamydia* positive fertile women; Group II comprised of *Chlamydia* positive infertile women; CT represents *Chlamydia trachomatis*; Cytokine concentration is denoted by median and range in parenthesis; UDL-Under detection limit

[a] denotes significance level between CT+Azithromycin and CT in Group I; [b] denotes significance level between CT+Doxycycline and CT in Group I; [c] denotes significance level between CT+Azithromycin and CT in Group II; [d] denotes significance level between CT+Doxycycline and CT in Group II; * Denotes significance level

Table 1 **Secreted cytokines concentration after *C. trachomatis* EBs stimulation in the presence and absence of azithromycin and doxycycline in cells obtained from women with primary infection**

	Group I (n=17)					Group II (n=14)				
	CT	CT + azithromycin	P value [a]	CT + Doxycycline	P value [b]	CT	CT + azithromycin	P value [c]	CT + Doxycycline	P value [d]
IL-1β (pg/ml)	177 (15-544)	74 (20-385)	0.03*	162 (28-414)	0.7	389 (36-1240)	189 (14-422)	0.02*	259 (28-440)	0.1
IL-2 (pg/ml)	4 (UDL-16)	6 (UDL-30)	0.5	6 (UDL-16)	0.2	5 (UDL-12)	5 (UDL-13)	0.6	6 (UDL-32)	0.6
IL-6 (pg/ml)	260 (37-377)	107 (14-361)	0.04*	190 (19-443)	0.5	538 (116-865)	233 (33-454)	0.01*	371 (32-676)	0.1
IL-8 (pg/ml)	218 (34-614)	90 (29-487)	0.04*	163 (30-625)	0.7	666 (150-1195)	339 (55-552)	0.001*	525 (50-882)	0.2
IL-10 (pg/ml)	240 (60-864)	100 (24-550)	0.04*	227 (54-537)	0.8	751 (74-1680)	319 (25-570)	0.01*	613 (80-1180)	0.3
IL-13 (pg/ml)	6 (UDL-16)	6 (UDL-18)	0.9	6 (UDL-14)	0.9	7 (UDL-30)	5 (UDL-16)	0.5	8 (UDL-20)	0.5
IFN-γ (pg/ml)	217 (30-785)	160 (17-490)	0.2	162 (25-564)	0.4	167 (42- 644)	91 (22-300)	0.1	131 (23-392)	0.7
TNF-α (pg/ml)	316 (20-655)	88 (16-475)	0.009*	254 (20-477)	0.2	214 (36-560)	76 (36-315)	0.04*	184 (26-455)	0.6

Group I comprised of *Chlamydia* positive fertile women; Group II comprised of *Chlamydia* positive infertile women; CT represents *Chlamydia trachomatis*; Cytokine concentration is denoted by median and range in parenthesis; UDL-Under detection limit

[a] denotes significance level between CT+Azithromycin and CT in Group I; [b] denotes significance level between CT+Doxycycline and CT in Group I; [c] denotes significance level between CT+Azithromycin and CT in Group II; [d] denotes significance level between CT+Doxycycline and CT in Group II; * Denotes significance level

Table 2 **Secreted cytokines concentration after *C. trachomatis* EBs stimulation in the presence and absence of azithromycin and doxycycline in cells obtained from women with recurrent infection**

4. ANTICHLAMYDIAL DRUGS: SENSITIVITY AND EMERGENCE OF RESISTANCE IN TREATMENT FAILURES

Scientific staff	:	**Dr. Aruna Singh, Ms. Apurb Rashmi Bhengraj, Ms. Pragya Srivastava**
In collaboration with	:	**Dr. Sudha Salhan, Safdarjung Hospital, New Delhi**
Technical Staff	:	**Mrs Madhu Badhwar, Mrs Asha Rani**
Duration	:	**2007-2010**

Aim, Objectives & Background :

Tetracycline and its derivatives, (e.g., doxycycline) has been the mainstay and erythromycin and azithromycin as alternatives of antichlamydial therapy in adults for many years and continue to be recommended by the Centre for Disease Control, Atlanta, USA. *C.trachomatis* has been historically sensitive to the tetracyclines, macrolides, and fluoroquinolones. Recent reports have noted increasing in vitro resistance. The clinical significance of these findings is unknown. Hence, it would be valuable to understand why recurrent or persistent *C. trachomatis* infection occurs in 10%–15% of women treated for *C.trachomatis* infection**.** Recurrent genital *Chlamydia trachomatis* infection often results in serious sequelae and it has major impact on reproductive health. Further study is needed to support or refute the hypothesis that heterotypic resistance of *C. trachomatis* is emerging and is related to increase in clinical treatment failures. So, in this study our objective is to determine the drug sensitivity profile of *C.trachomatis* isolates from patients with treatment failure and recurrent infection.

Work done during the year:

Recurrent infections were determined in symptomatic female patients. *In vitro* susceptibility assay was performed for azithromycin and doxycycline by using cell culture

technique against 21 clinical isolates obtained from *C. trachomatis* positive patients including those who were recurrently infected.

Drug sensitivity assay:

Cytotoxicity assay (MTT) was done on the azithromycin and doxycycline treated HeLa cell line, cytotoxic effect was not observed on the HeLa cell line after which *C.trachomatis* drug sensitivity assay was performed.

The doxycycline and azithromycin drug sensitivity assay was performed on the C. trachomatis isolates using cell culture method for minimum inhibitory concentration (MIC) determination. The HeLa cells infected with C. trachomatis isolate were incubated with different concentrations of doxycycline and azithromycin. Subsquently Inclusions were counted after staining with *C. trachomatis* MOMP specific monoclonal antibody. Percent of infections were calculated for each isolate at different drug concentrations. These isolates showed different sensitivity patterns against doxycycline and azithromycin.

Results: Thirteen isolates (61.9%) were found to be susceptible towards azithromycin and doxycycline with the minimum inhibitory concentration (MIC) values of ⩽ 0.125µg/ml, ⩽ 0.25µg/ml respectively. Eight isolates (38%) were found less susceptible to the drugs. Two of them had MICs of 8µg/ml for both the drugs and could not be completely eradicated as observed by minimum bactericidal concentration (MBC) assay.

Decreased antibiotic susceptibility was observed in isolates obtained from recurrently infected patients towards the current first line drugs (azithromycin and doxycycline) for chlamydial infection treatment. The MIC and MBC values of two isolates was 8µg/ml and it was seen that its complete eradication could not occur at this higher concentration. However, the number and size of inclusion bodies were found to be decreased with increasing concentration of antibiotics as also observed in other studies. This small percentage of organisms may reflect the presence of heterotypic resistance.

S. No.	Isolate number	Age	Diagnosis	Previous treatment	MIC(µg/ml)		MBC			
					Azithromycin	Doxycycline	Azithromycin		Doxycycline	
							4µg/ml	8µg/ml	4µg/ml	8µg/ml
1	CT222	38	Chronic cervicitis	Doxycycline	0.12	8.0	-	-	-	-
2	CT227	30	Chronic cervicitis	Azithromycin	8.0	8.0	+	+	+	+
3	CT231	40	PID	Doxycycline	1.0	2.0	-	-	-	-
4	CT232	29	Cervicitis	Doxycycline	4.0	4.0	-	-	-	-
5	CT233	32	Cervicitis	*	0.5	0.025	-	-	-	-
6	CT235	24	Infertility	Doxycycline	2.0	4.0	-	-	+	+
7	CT244	20	PID	Doxycycline	8.0	8.0	+	+	+	+
8	CT247	32	Infertility	*	2.0	2.0	+	-	-	-

MIC Minimum inhibitory concentration
MBC Minimum bactericidal concentration
+ Inclusion bodies detected at the given concentration of antibiotic.
- Inclusion bodies were not detected at the given concentration of antibiotic.

- Status of treatment taken not clear from the history.

Table: Susceptibility profile of C. trachomatis against doxycycline and azithromycin.

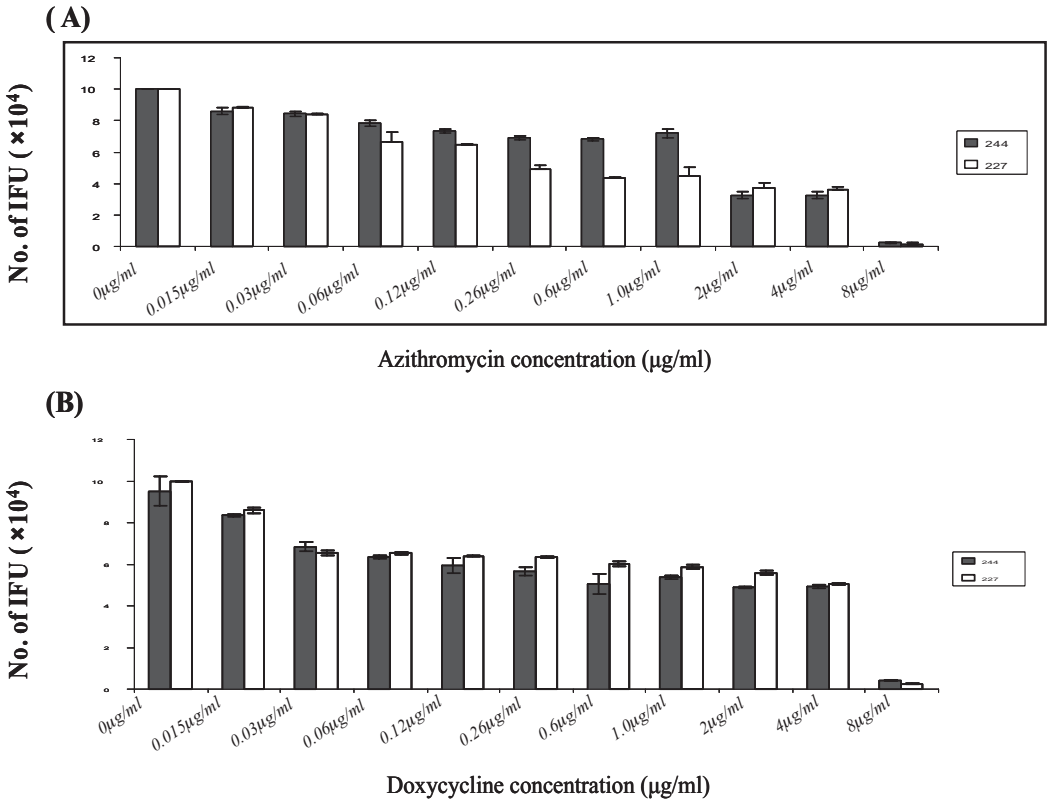


Fig. : Inclusion bodies Counts of the two less su sceptible C. trachomatis isolates (244 and 227).

Resistance Marker Selection

Macrolide resistance usually arises by (i) mutation of the 23S rRNA at the macrolide contact site, (ii) methylation of this site by adenine N-methyltransferase enzymes encoded by the erm gene family, (iii) mutation of the L4 or L22 ribosomal protein that is expected to contact the macrolides, or (iv) a drug efflux pump mechanism. The first mechanism occurs among organisms with single copies of the rRNA in their genome, as is the case with *C. pneumoniae*; usually leads to high-level resistance; and has been demonstrated to occur in *C. trachomatis*. Mutations in ribosomal proteins L4 and L22 have been implicated in the development of various degrees (as little as fourfold) of macrolide resistance in Escherichia coli, Bacillus subtilis, and Streptococcus pneumoniae. Some L22 mutants of *C. trachomatis* were also associated with clinical failure.

Work done: DNA was extracted and purified from the *C. trachomatis* isolates. Primers were selected for PCR to amplify the predicted macrolide resistance regions of domain of the 23S rRNA and the genes encoding the L4 and L22 proteins. Sequencing of the target genes are in the progress.

5. ROLE OF IRON IN PATHOGENESIS OF *CHLAMYDIA TRACHOMATIS*

Scientific staff	:	**Dr Aruna Singh, Dr. Harsh Vardhan**
Duration	:	**2006-2010**

Aim, Objectives & Background:

Chlamydia trachomatis (CT) is a leading cause of sexually transmitted infection worldwide. It resides intracellular and is dependent on the host to complete its developmental cycle. Chlamydia diverts host resources to benefit its own development and modulate innate host defense for successful completion of productive infection cycle. Such a long developmental cycle creates numerous interactions between host and chlamydia. These interactions decide the

fate of pathological consequences through various intracellular and secretory proteins. Chlamydial modulatory effect on host for survival is majorly conferred to intracellular factors, however extracellular or secretory factors needs to be analysed for understanding complete sequence of events leading to pathogenesis. In this direction changes in the expression of multiple proteins using two-dimensional electrophoresis (2-DE) promise to provide a powerful strategy for characterizing complete pathophysiologic processes and designing novel drug therapies.

Work done during the year:

Analysis of lower molecular weight and highly abundant proteins in supernatant and their identification: When CT infected HeLa Cells were exposed to DFO, changes were observed in the expression of secretory proteins as compared to control in 2-DE gel analysis. In these experiments, all proteins were within the 1.3–1.5-fold expression range, typical of the inherent noise in the proteomics experiment. However, based on the statistical evaluation of the variation in the baseline, we chose a 2-fold cutoff for data analysis of regulated protein expression. CT infection regulate 19 proteins spots, there were 4 proteins that were down-regulated and 15 protein spots that were up-regulated more than 2-fold when cells were exposed to DFO in selected part (10-45 KDa/ 4-6) of profile. Highly abundant proteins, specifically lying between pI 4-6/below 15 kDa molecular co-ordinates were chosen for MALDI-TOF identification. Only protein spots that were down-regulated in response to the DFO treatment were excised from the gel and digested for mass spectrometry analysis. The protein within the detection range of the 12.5% polyacrylamide gels was identified as Trx-1 (pI 5.4/12.1 kDa) (Fig 1).

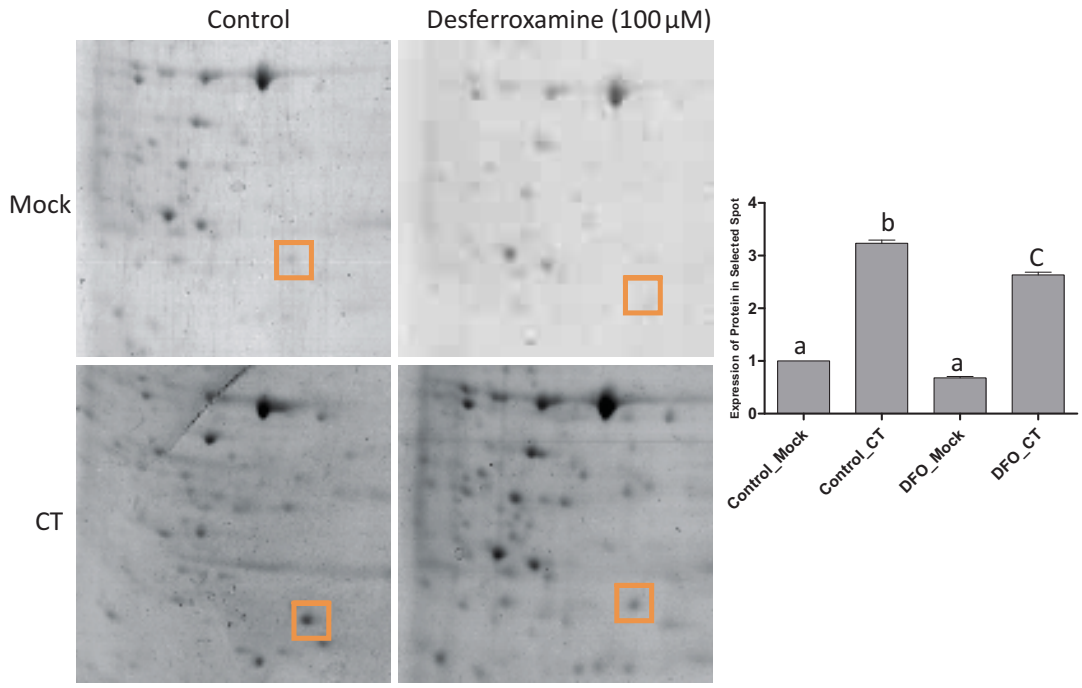


Fig. 1: Secretory protein profile of CT infected and DFO treated CT infected cells:

Contribution of Trx-1 in establishment of chlamydial infection

Finding emerged from 2-DE protein map guided this study to ascertain the role played by Trx-1 in establishment of chlamydial infection. Here siRNA specific to Trx-1 was used to inhibit the protein synthesis of thioredoxin-1. Inhibition of Trx-1 was resulted in decrease ($p < 0.001$) of CT inclusion (Fig 2A); however there was non-significant change was observed in cells transfected with scrambled siRNA in comparison to control. Moreover, similar change was observed on addition of DFO as in Trx-1 inhibited condition. In CT infected cells, western blotting results confirmed the significant down regulation of Trx-1 in presence of Trx-1 SiRNA and DFO in comparison to control (Fig 2B).

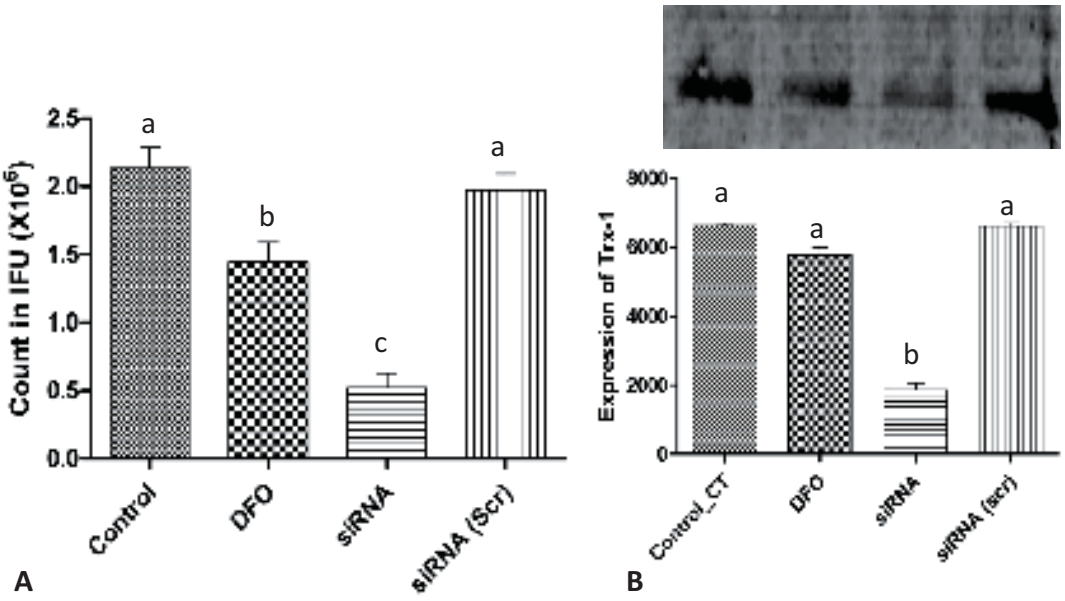


Fig. 2: Trx-1 inhibition and chlamydial survival

Level of Trx-1 and viability of CT infected cells

The effect of secreted Trx-1 on viability of CT infected cells was ascertained by using MTT cell viability assay. In presence of DFO (100uM) and Trx-1 Si RNA, CT infected cells showed significant ($p < 0.001$) decline in cellular viability in comparison to control (Fig 3). However, there was a non-significant ($p < 0.01$) changes were detected in CT infected cells treated with Si RNA experimental control (Scramble Si RNA). Therefore, these results confirm important role of Trx-1 in cellular viability of CT infected HeLa 229 cells.

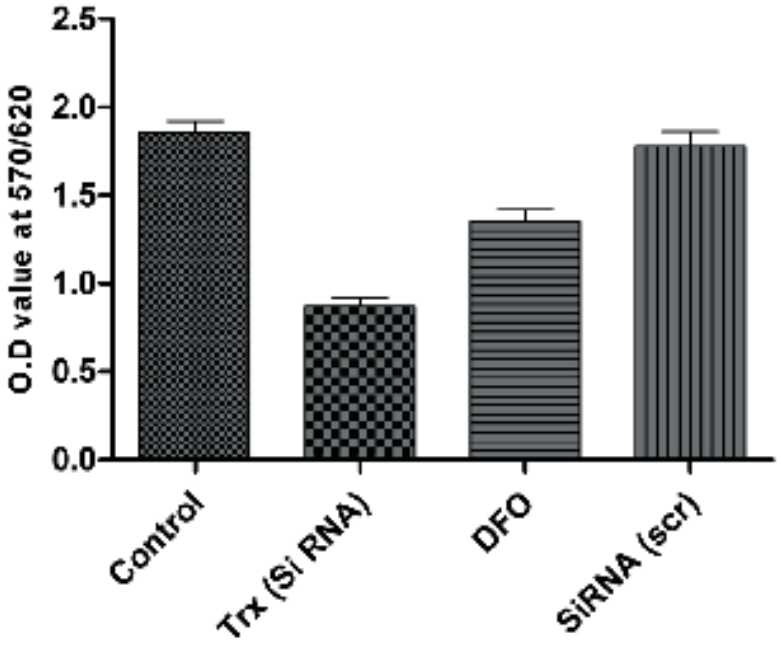


Fig. 3: Trx-1 and viability of CT infected cells.

6. INCLUSION MEMBRANE PROTEINS AND THEIR ROLE IN CHLAMYDIAL PATHOGENESIS

Scientific staff	:	**Dr. Aruna Singh, Dr. Rishein Gupta**
In collaboration with	:	**Dr. Sudha Salhan, Safdarjung Hospital, New Delhi**
Technical Staff	:	**Mrs Madhu Badhwar, Mrs Asha Rani**
Duration	:	**2006-2009**

Aim, Objectives & Background :

Chlamydial Inclusion membrane proteins (Incs), are involved in biochemical interactions with host cells and infecting Chlamydiae. We have previously reported the role of two Chlamydia trachomatis (CT) Incs, namely IncB and IncC in generating host immunity in CT infected women. Emerging data shows involvement of Inc stimulated CD4 positive T cells in aiding host immunity in infected fertile and infertile women through the secretion of interferon

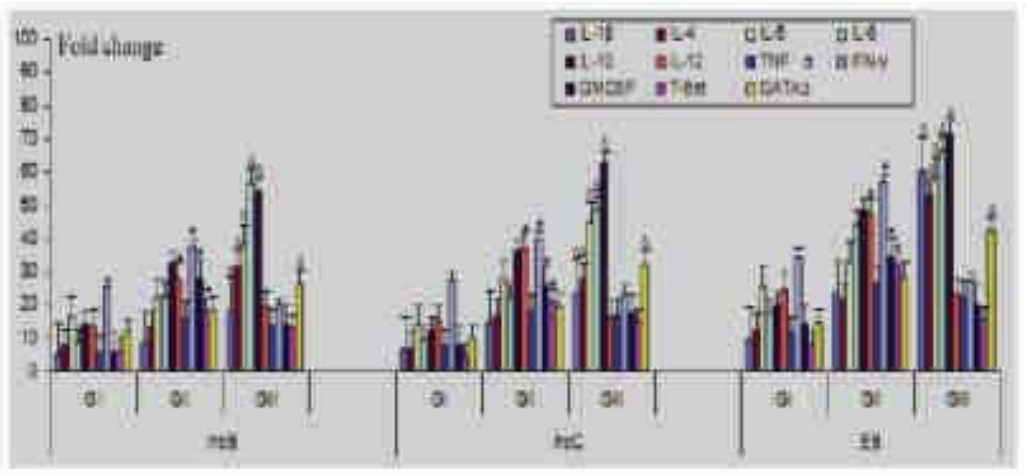
gamma. However the lack of data on the intra-cytokine interplay to these Incs in infected cell milieu prompted us to investigate further.

Work done during the year:

CD8 depleted, CD4 enriched cervical cells were isolated and upon stimulation with IncB and IncC, modulation of cytokines and T cell lineage regulating transcription factors T-Bet and GATA3 was determined by real-time reverse-transcriptase (RT)-PCR and ELISA.

Significant higher expression ($P < 0.05$) of Interferon-gamma, IL-12, IL-23 and GM-CSF were found in Inc-stimulated CD4 enriched cervical cells of CT-positive fertile women and contrastingly high IL-1 Beta, IL-4, IL-5, IL-6 and IL-10 levels were found in CT-positive infertile women. Positive correlation ($P < 0.05$) was found between Interferon-gamma and T-Bet levels in CT-positive fertile women and IL-4 mRNA and GATA3 levels in CT-positive infertile patients upon IncB and IncC stimulation.

Our study also suggests that Incs are able to modulate expression of T cell lineage determinants indicating their involvement in regulation of immune cells.



mRNA expression of IL-1β, IL-4, IL-5, IL-6, IL-10, I *in vitro* stimulation with IncB, IncC and CT EB CSF, T-Bet and GATA3 in CD4+ cervical cells in GI,

Fig. 1: mRNA expression of IL-1 , IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, IFN- , GM-CSF, T-Bet and GATA3 in CD4+ cervical cells in GI, GII and GIII after in vitro stimulation with IncB, IncC and CT EB. Real-time RT-PCR analysis of mRNA levels was done at 12 hours post infection where, Group I (GI) comprised of healthy women with no CT infection, Group II (GII) comprised of CT-positive fertile women, Group III (GIII) comprised of CT-positive infertile women. * P < 0.05 Expression of cytokine mRNA in GII compared to corresponding levels in GI and GIII by Kruskal Wallis test. P < 0.05 Expression of cytokine mRNA in GIII compared to corresponding levels in GI and GII by Kruskal Wallis test.

7. ROLE OF *C. PNEUMONIAE* IN CORONARY ARTERY DISEASE (CAD) PATIENTS:

Scientific staff	:	**Dr. Aruna Singh, Mr. Hem Chandra Jha**
In collaboration with	:	**Dr. Sudha Salhan, Safdarjung Hospital, New Delhi**
Duration	:	**2006-2010**

Aims, Objectives & Background:

Chlamydia pneumoniae heat shock protein (HSP) 60 is abundantly produced during chronic chlamydial infection and may stimulate and activate innate immune and inflammatory responses thereby contributing to atherogenesis. However, to date there is no study in which signaling cascade have been delineated in human atheromatous plaques of *C. pneumoniae* positive coronary artery disease (CAD) patients which is required for understanding *C. pneumoniae* mediated immuno-pathogenesis. Hence our aim was to study cHSP60 mediated signaling cascade in CAD patients and for this we performed experiments at RNA and protein level in cHSP60 positive and negative group of CAD patients.

Work done during the year:

Forty patients (28- men, 12- women) attending Department of Cardio Thoracic & Vascular Surgery, Safdarjung Hospital, New Delhi from September 2007 to April 2008 were enrolled in the study. Atheromatous plaques of CAD patients (cHSP60 positive and negative) were used in all experiments. Real time PCR experiments were performed for gene expression studies- i) individual gene expression (11genes) and ii) pathway-focused gene expression profiling (signal transduction pathway - 84 genes and MAPKinase signaling pathway - 84 genes) at RNA level.

Gene expression in cHSP60 positive and negative CAD patients

Significantly higher expression ($p<0.001$) was found for IL-4, IL-6, TLR-2, TLR-4, and TGF-β whereas IL-10 was found to be significantly lower ($p<0.001$) in cHSP60 positive CAD patients compared to cHSP60 negative. Additionally, expression of GM-CSF was higher and IFN-γ was lower in cHSP60 CAD patients (Figure 1).

Focused Gene Expression Profiling using PCR Array for human

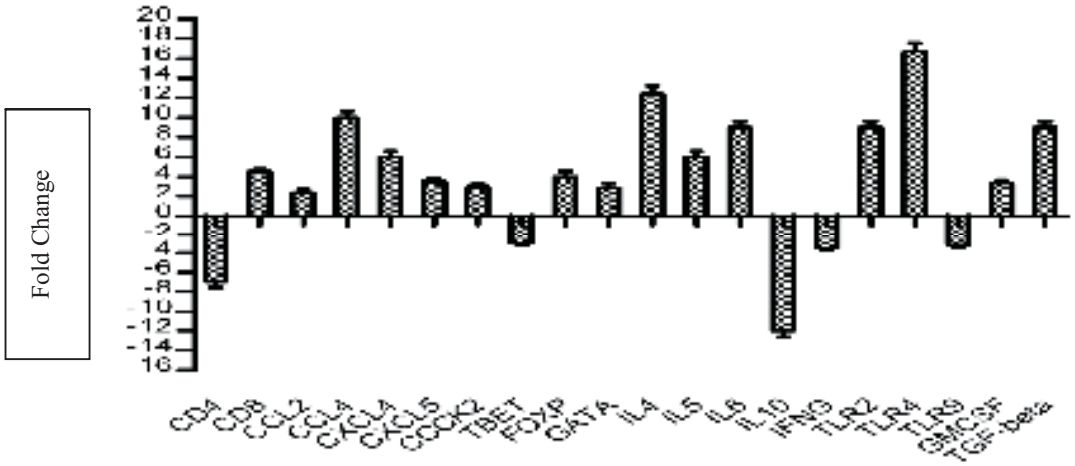
Two constitutive genes (B2-microtubulin and beta Actin) were used in all experiments as uniform expression pattern was observed in both the genes (out of 5 constitutive genes given in PCR array plate) in cHSP60 positive and negative CAD patients.

a) Signal transduction related genes in cHSP60 positive and negative CAD patients

In our study VCAM1, NF-kB1, IL-8, ICAM1, FASLG, EGR1, BAX, and MMP7 were significantly ($p<0.001$) up-regulated while c-JUN, IkkB, BIRC1 were significantly ($p<0.001$) down regulated in cHSP60 positive CAD patients compared to cHSP60 negative CAD patients. Moreover, expression of MDM2, IL-4, and FAS were also higher
but not significant (Figure 2).

b) APKinase related genes in cHSP60 positive and negative CAD patients

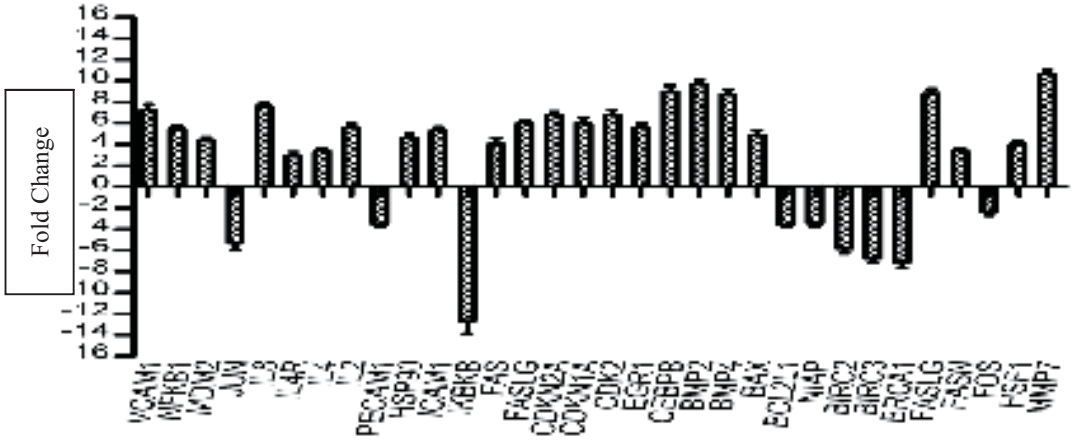
CREBP, CDK2, CDK4, MAPK1, MAPK8IP2, EGR1, MAPK2K6, RAC1, and EGFR were significantly ($p<0.001$) up regulated, however, SMAD4, MAPK9, CDKN20, MAPK10, MAPK11, ETS1, and BRAF were significantly down regulated ($p<0.001$) in cHSP60 positive CAD patients compared to cHSP60 negative CAD patients. Additionally, most of the ERK precursors were up regulated while; precursors of JNK and p38 were down regulated (Figure 3).



CD8, CCL4, CXCL4, IL4, IL-6, TLR2, TLR4, TGFβ

CD4, IL10, IFNG

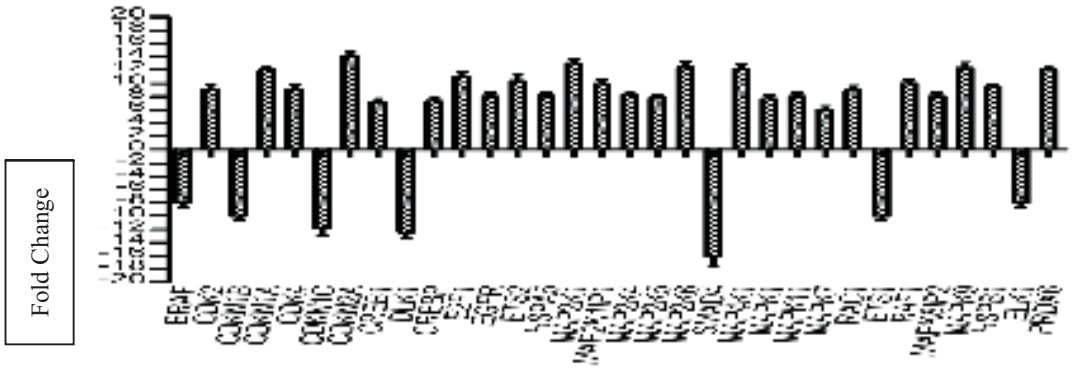
Fig. 1: Individual gene expression in cHSP60 positive and negative CAD patients



IL-8, NF-KB1, ICAM1, VCAM1, BAX, FASLG, IL-4, IL-2, HSF1
JUN, BCL21, BIRC2, FOXA2, SELE, FOS, BRCA1, IKBKB

Fig. 2: Signal Transduction Pathway genes in cHSP60 positive and negative coronary artery

NF-kB=Nuclear factor of kappa light polypeptide gene enhancer in B cells, ICAM=Intercellular adhesion molecule, BAX=BCL2 associated x protein, FASLG=Fas ligand (TNF superfamily, member 6), JNU=Jun oncogene, BCL_2= B-cell CLL/lymphoma 2, BIRC2=Baculoviral IAP repeat containing 2, FOS=V-fos FBJ murine osteosarcoma viral Oncogene homolog, IkkB=Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta



CREBP, CDK2, CDK4, MAPK1, MAPK8IP2, EGR1, MAPK2K6, RAC1, EGFR
SMAD4, MAPK9, CDKN20, MAPK10, MAPK11, ETS1, BRAF, ELK-1

Fig. 3: MAPKinase Signaling Pathway genes in cHSP60 positive and negative coronary artery disease patients

CREBP=CREB binding protein, CDK=Cyclin dependent kinase, MAPK=Mitogen activated protein kinase, RAC=Ras related C3 boyulinum toxin substrate, EGFR=Epidermal growth factor receptor, EGR=Early growth response, SMAD4= SMAD family member 4, ELK=Member of ETS oncogene family, ETS=V-ets erythroblastosis virus E26 oncogene homolog, BRAF=V-raf murine sarcoma viral oncogene homolog B1

8. ROLE OF OXIDATIVE STRESS IN CHLAMYDIA TRACHOMATIS INFECTED FIRST-TRIMESTER SPONTANEOUS ABORTERS

Scientific staff	:	**Dr. Sangita Rastogi, Ms. Namita Singh, Ms. Priya Prasad**
In collaboration with	:	**Dr. Sudha Salhan, Dr. Banashree Das, Safdarjung hospital**
Duration	:	**2007-2010**

Aims, Objectives & Background:

In miscarriage, the development of the placento-decidual interface is severely impaired leading to an early and widespread onset of maternal blood flow and major oxidative degeneration. The placental syncytiotrophoblast is particularly sensitive because it is the outermost tissue of the conceptus and is exposed to the highest concentration of oxygen coming from the mother and also, because it contains low concentration of the principal anti-oxidant enzymes, particularly during the early pregnancy. Also, reactive oxygen species stimulate the synthesis of prostaglandin $F_2\alpha$ leading to uterine contraction. Although bacterial infection with *C. trachomatis* is a recognized cause of fetal loss in humans, however, the underlying mechanism of either expulsion of the uterine content to terminate pregnancy *(accompanied by vaginal bleeding and uterine contraction)* or else, retention of dead products of conception in the uterus without bleeding for several weeks *(missed abortion)* is not completely understood and it is hypothesized that increased free radical activity/ oxidative stress in the decidua of failed pregnancy may possibly play a role. Our study is focused on the identification of biomarkers of oxidative stress in the first trimester spontaneous aborters infected with *C. trachomatis*.

Work done during the year:

The results of our study showed that the level of superoxide dismutase was significantly downregulated while lipid peroxidation (malondialdehyde) was found to be upregulated in *C. trachomatis* infected spontaneous aborters presenting with bleeding per vagina in comparison to spontaneous aborters without any vaginal bleeding. However, there was no statistically significant difference in the generation of nitric oxide between *C. trachomatis* infected spontaneous aborters presenting with vaginal bleeding versus women with missed abortion. The increased lipid peroxides/ malondialdehyde might be responsible for inducing uterine contraction and expulsion of

the uterine content in *C. trachomatis* infected spontaneous aborters presenting with vaginal bleeding.

9. IMMUNOMOLECULAR EXPRESSION OF CYCLOOXYGENASES AND PROSTAGLANDIN RECEPTORS IN ENDOMETRIAL CURETTAGE TISSUE OF CHLAMYDIA TRACHOMATIS INFECTED WOMEN DURING FAILED PREGNANCY

Scientific staff	:	**Dr. Sangita Rastogi, Ms. Namita Singh, Ms. Priya Prasad**
In collaboration with	:	**Dr. Sudha Salhan, Dr. Banashree Das, Safdarjung hospital**
Duration	:	**2008-2011**

Aims, Objectives & Background:

The molecular changes underlying the complex transition from uterine quiescence to labour are not fully elucidated. In this regard, the differential expression of the prostaglandin receptors might be important for regulating uterine activity throughout pregnancy and also, during spontaneous abortion in women. However, there is lack of information regarding expression of EP receptor subtypes/ isoforms in human pregnancy and in preterm labour in *C. trachomatis* infected women. This study was undertaken to characterize the expression/ distribution of prostaglandin receptors/ cyclooxygenases/ cytokines in the endometrial curettage tissue of *C. trachomatis* infected women undergoing spontaneous abortion. Spontaneous aborters without evidence of *C. trachomatis* infection served as controls for the study.

Work done during the year:

In comparison to controls, our results showed that the expression of cox-2 and tumor necrosis factor alpha were increased (RT-PCR/ IHC) in *C. trachomatis*-infected women undergoing spontaneous abortion. However, it was observed that there was no differential expression of EP1 and EP2 prostaglandin receptor isoforms in *C. trachomatis*-positive spontaneous aborters when compared with the control group. It appears that both cox-2 and tumor necrosis factor alpha are important mediators of *C. trachomatis*-induced pregnancy loss in women.

10. IMMUNOPATHOGENESIS OF REACTIVE ARTHRITIS/ UNDIFFERENTIATED SPONDYLOARTHROPATHY INDUCED BY *CHLAMYDIA TRACHOMATIS*

Scientific Staff	:	**Dr. Sangita Rastogi, Mr. Praveen Kumar**
In collaboration with	:	**Dr. VK Sharma, Dr. G. Khanna, Dr. S. Batra, Safdarjung hospital**
Duration	:	**2009-2012**

Aims, Objectives & Background:

Reactive arthritis (ReA) is triggered by pathogens, of which *C. trachomatis* is prominent as several researchers have detected *C. trachomatis* components, *i.e.* MOMP/ rRNA/ DNA in the affected inflamed synovial joints of ReA/ Undifferentiated Spondyloarthropathy (uSpA) patients. Urogenital infection with *C. trachomatis* can lead to the development of an acute inflammatory arthritis and this disease may become chronic in some individuals. Moreover, *C. trachomatis* has been reported to exist in a persistent state in ReA patients. For clinical and therapeutic reasons, these are critical issues. Hence, the focus of our research is to prospectively determine the association of *C. trachomatis* infection with ReA in our patients. While ReA induced due to enteric pathogens has been reported from India, yet, to the best of our knowledge, studies on ReA patients with symptoms suggestive of genitourinary infection (gReA) due to *C. trachomatis* are scanty. This is despite a high prevalence of *C. trachomatis* genital infection reported by various researchers from our country. The available literature so far on *C. trachomatis* induced ReA is largely from other countries. Although *C. trachomatis* could not be cultured from the synovial fluid from patients with *C. trachomatis* induced ReA, direct fluorescence *(MicroTrak; Syva)* was initially the most widely used method for chlamydial antigen diagnosis in the synovial fluid. Molecular methods of analysis including PCR to detect *C. trachomatis* rRNA/ DNA in affected joints have been gaining importance over the last few years. Although molecular tests are now considered to be the most sensitive and specific tools for chlamydial diagnosis using joint samples, however, recently the specificity of *C. trachomatis* nucleic acid testing in arthritic joints has been questioned. Also various commercial testing systems for molecular diagnosis are available. Hence, standardization of methodology to allow comparisons to be made between studies is needed.

Work done during the year :

During the reporting year, the research study was initiated subsequent to obtaining ethical clearance for the project wherein nine patients with gReA/ uSpA attending CIO at Safdarjung hospital were enrolled for collecting samples from the synovial compartment, *viz.:* synovial fluid/ biopsy. Cell deposits of synovial fluid were stained with fluorescein conjugated monoclonal antibodies against the MOMP of *C. trachomatis (MicroTrak; Syva)* and examined by fluorescence microscopy. Four patients showed the presence of chlamydial EBs by this method. Conditions are being optimized for PCR amplification of *C. trachomatis* in joint samples for confirmation of DFA-positives wherein detection is targeted at the *C. trachomatis* 16s rRNA, plasmid and MOMP gene. An increased intra-articular production of IgA anti-*C.trachomatis* antibodies (*IBL International, Germany*) was also observed in the synovial fluid of ReA/ uSpA patients.

LEISHMANIASIS

1. DRUG RESISTANCE IN VISCERAL LEISHMANIASIS

Scientific staff	:	**Dr. Poonam Salotra, Dr. Dhiraj Kumar, Ms Arpita Kulshrestha, Ms Vasundhra Bhandari**
In collaboration with	:	**Dr. N. S. Negi, Dr. V. Ramesh**
Duration	:	**2006-2012**

Aims, Objectives & Background:

Chemotherapy of Visceral Leishmaniasis (VL) in India is challenged by unresponsiveness towards traditional antimonial therapy in more than 60% cases from endemic areas. Moreover, prolonged half life and widespread misuse of the new oral drug Miltefosine (MIL) in healthcare systems in India threatens the development of resistance to this valuable class of drugs. Hence, it is essential to understand the mechanism of resistance towards these drugs for effective control of VL. During the period under study, we have developed and characterized the lab generated MIL resistant *L.donovani* parasite (adapted to 30µg/ml MIL) based on its growth kinetics, sensitivity and expression of MIL transporters at transcript level. In addition to the existing drugs, it is essential to determine the response of prevailing parasite population towards new treatment options such as paromomycin (PMM) and sitamaquine (SIT) to develop effective control measures to prevent resistance to these drugs. Our previous studies revealed that *L.donovani* isolates from high SAG resistance zone exhibited lower susceptibility for MIL, AmphotericinB and SIT in comparison to those from low SAG resistance zone, while isolates from different zones exhibited similar susceptibility to PMM. As infected host macrophages generate nitric oxide (NO), the free radical effector molecule to kill the intracellular parasites, we investigated how PMM/SIT mount their cytotoxic effect on the intracellular parasites by evaluating their effect on the release of NO from *L. donovani* infected macrophages.

Work done during the year:

Generation and Characterization of experimental *L. donovani* resistant to Miltefosine Generation of MIL resistant *L.donovani*.

Based upon the MIL sensitivity profile of *L. donovani* parasites, one isolate with low MIL susceptibility was selected and stepwise exposed to increasing MIL concentration (2.5, 5, 7.5,10, 20 and 30µg/ml). At each step, parasites were cultured for at least 5-8 passages to attain steady and optimal cell growth comparable to the wild type.

Growth kinetics of MIL adapted parasite LdM30- The growth kinetics of the resistant parasite was compared to the wild type parasite The resistant parasites exhibited growth kinetics similar to the wild type parasite with negligible effect on the infectivity in macrophages (Fig 1).

(i) ***MIL sensitivity of LdM30*:**

*Ld*M30 displayed mean ED_{50} of >15µg/ml towards MIL at intracellular amastigote stage which was more than 7.5 fold higher in comparison to its wild type (WT) counterpart. MIL concentration above 15 µg/ml could not be tested as the drug is cytotoxic to macrophages. Mean ED_{50} of LdM30 promastigotes was 64.38 ± 2.17µg/ml which was 9 fold higher compared to WT parasite (Mean ED_{50} WT: 7.02 ± 0.18 µg/ml).

(ii) Evaluation of Cross-resistance to other antileishmanial drugs towards MIL resistant parasite:

In addition to paromomycin and sitamaquine, we also evaluated the susceptibility of LdM30 towards SAG and amphotericinB to determine if there is any alteration in the susceptibility of resistant parasite to these drugs. No cross-resistance was found towards SAG and AmphotericinB in the MIL resistant parasite population (Table 1).

(iii) Gene expression analysis of LdMT and LdRos3 in MIL resistant parasite:

For further characterization at molecular level, the mRNA expression of P type ATPase drug transporters, LdMT and LdRos3, reported markers for MIL resistance, were analysed in the resistant parasites.

(a) mRNA expression of LdMT in miltefosine resistant parasite *Ld*M30: The gene expression levels of LdMT as marker of MIL resistance was determined. Initially, primer efficiency of each primer was determined using absolute quantification and slope of each primer was observed to be ⩾-3. The resistant parasite exhibited down regulation of LdMT (more than 7 fold) gene in comparison its wild type counterpart .With respect to reference *L donovani* isolate LdAG83, the relative down regulation of gene expression was more than 25 fold (fig 2, table 2).

(b) mRNA expression of LdRos3 in miltefosine resistant parasite LdM30: The resistant parasite exhibited down regulation of LdRos3 (more than 2.5 fold) gene in comparison to its wild type counterpart. With respect to reference *L donovani* isolate LdAG83, the relative down regulation of gene expression was more than 6 fold (fig 3, table 2).

Drug susceptibility test using resazurin based high throughput assay

In an effort to develop a high throughput drug screening method, a fluorimetric bioassay was developed and standardized in the lab to establish the relative cytotoxicity of antileishmanial agents simultaneously in a large number of parasite samples. This assay is based on non fluorescent redox indicator resazurin, which gets converted to fluorescent reporter molecule resorufin by the reducing capability of metabolically active cells. This method has immense advantage over the direct counting method as it is rapid, convenient and can analyze a large number of samples at a time.

L.donovani parasites along with MIL resistant parasite were tested for sensitivity towards MIL. The representative graph depicting the percent parasite killing in response to different MIL concentrations is shown in fig 4.The assay is now well standardized in our lab for high throughput screening of antileishmanial drugs.

Determination of Cross-resistance towards PMM and SIT in antimony/MIL resistant parasites

During this year, we further tested the efficacy of PMM and SIT on lab generated antimony and miltefosine resistant *L. donovani* parasites to determine their efficacy in resistant parasite populations that may prevail in future. Lab generated MIL resistant parasites lines K417MIL30 and K59MIL30 (resistant to 30 µg/ml MIL) and SbIII resistant *L. donovani*

K80SbIII (resistant to125 µg/ml potassium antimony tartarate) were utilized for drug susceptibility assays to determine any cross resistance towards other antileishmanial drugs at both promastigote and amastigote parasite stages. Susceptibility to paromomycin remained comparable in wild type and antimony resistant parasite. Likewise, development of miltefosine resistance had marginal effect on the susceptibility to paromomycin. Lab generated antimony resistant parasites displayed 2.9 fold higher ED_{50} for sitamaquine compared to its wild type while miltefosine resistant parasites retained their sensitivity to this drug. Overall, based on 5 fold cut off value, no cross-resistance was found towards the two drugs in antimony/miltefosine resistant parasites at either parasite stage (Table 3).

Effects of paromomycin and sitamaquine on production of nitric oxide (NO) by infected macrophages

We observed an increase in NO levels upon paromomycin or sitamaquine treatment in both uninfected and infected macrophages, the increase being higher in infected cells compared to uninfected cells (Fig 5A).

Paromomycin

Despite inducing a small increase in the NO levels, paromomycin treatment resulted in a marked decrease in the number of amastigotes in infected macrophages. We observed that the paromomycin activated macrophages produced NO in a dose dependent manner and percentage parasite killing correlated significantly with the NO release ($r=0.70$, $p<0.05$) (Fig 1A). Further, upon addition of N LMMA (NOS inhibitor), decrease in NO production comparable to the control infected cells was observed, while percent parasite killing did not alter significantly ($p=0.186$) suggesting that activity of paromomycin against *L. donovani* may not be solely dependent on NO (Fig 5B).

Sitamaquine

We observed that the sitamaquine treatment induced NO production in a dose dependent manner in infected macrophages. Percentage parasite killing correlated positively with the nitric oxide release ($r=0.58$, $p<0.05$). Addition of N LMMA, resulted in a decrease in NO production comparable to the control infected cells in sitamaquine treated macrophages with significant

decrease in the parasite killing (p= 0.019) suggesting that the cytotoxic activity of sitamaquine against *L. donovani* involves NO pathway (Fig 5B). The data suggests that sitamaquine promotes macrophage activation resulting in toxicity against the parasite.

Future work

Mechanism of Miltefosine and paromomycin resistance will be explored in experimentally resistant *L.donovani* parasites. Genes upregulated in miltefosine resistance in well characterized lab generated miltefosine resistant parasite will be identified through genomic microarray analysis.

Table 1: Cross resistance indices of the MIL adapted parasite towards other antileishmanial drugs

Antileishmanial drug	Wild Type	MIL resistant (LdM30)	Cross resistance Index (CRI)
ED_{50} SAG (µg/ml)	**14.65 ± 0.67**	**17.76±1.38**	**1.21**
ED_{50} MIL (µg/ml)	**1.90 ±0.04**	**> 15**	**7.89**
ED_{50} AmB (µg/ml)	**0.44 ± 0.01**	**0.52±0.14**	**1.18**

ED_{50} represents the mean (± SD) from 3 separate assays.

Table 2: Expression indices of Miltefosine transporters LdMT and LdRos3 in MIL resistant parasite

Parasite	Relative fold decrease in Gene Expression	
	LdMT	LdRos3
LdAG83	**1**	**1**
Wild Type	**3.53±1.17**	**2.51±0.28**
MIL adapted (30 µg/ml)	**25.5±2.25**	**6.05±0.24**

Mean ±SEM of the results from two separate assays. Expression levels indicative of decreases relative to those with strain LdAG83.

Table 3: Sensitivities of antimony and miltefosine resistant *L. donovani* parasites towards sitamaquine and paromomycin as determined by cross resistance assays

		Paromomycin		Sitamaquine	
	Parasite	ED_{50} (μM)[b]	Cross resistance Index[c]	ED_{50} (μM)[b]	Cross resistance Index[c]
Prom[a]	**K417 WT[a]**	23.4±0.63	**1.79**	12.05±1.89	**1.535**
	K417MIL30[a]	41.91±2.83		18.5 ±2.4	
Amas[a]	**K417 WT[a]**	5.46±0.2	**1.4**	3.18± 0.38	**0.83**
	K417MIL30[a]	7.64±0.15		2.65±0.45	
Prom[a]	**K59 WT[a]**	50.67± 0.56	**0.84**	18.90±1.55	**1.14**
	K59 MIL30[a]	42.67±2.24		21.68±1.8	
Amas[a]	**K59 WT[a]**	4.48 ±0.06	**1.45**	3.74 ±0.78	**0.6**
	K59 MIL30[a]	6.54±0.43		2.24±0.32	
Prom[a]	**K80 WT[a]**	52.73±3.51	**0.423**	16.89±1.22	**1.8**
	K80 SbIIIR[a]	22.32±2.2		30.5±3.24	
Amas[a]	**K80 WT[a]**	3.97 ± 0.31	**1.17**	0.91 ± 0.07	**2.9**
	K80 SbIIIR[a]	4.66±0.36		2.64±0.12	

[a]Amast: amastigotes; Prom: promastigotes. WT: wild type parasite, MIL30: parasite adapted to grow at 30 µg/ml miltefosine, SbIII R: parasite adapted to grow under potassium antimony tartarate at 125 µg/ml

[b] Mean ED_{50} ± SEM of the results from three separate assays.

[c] Cross-resistance indices were determined as the ratios of ED_{50} values of resistant lines to the wild type. A drug is considered cross-resistant when an index over 5 is consistently determined.

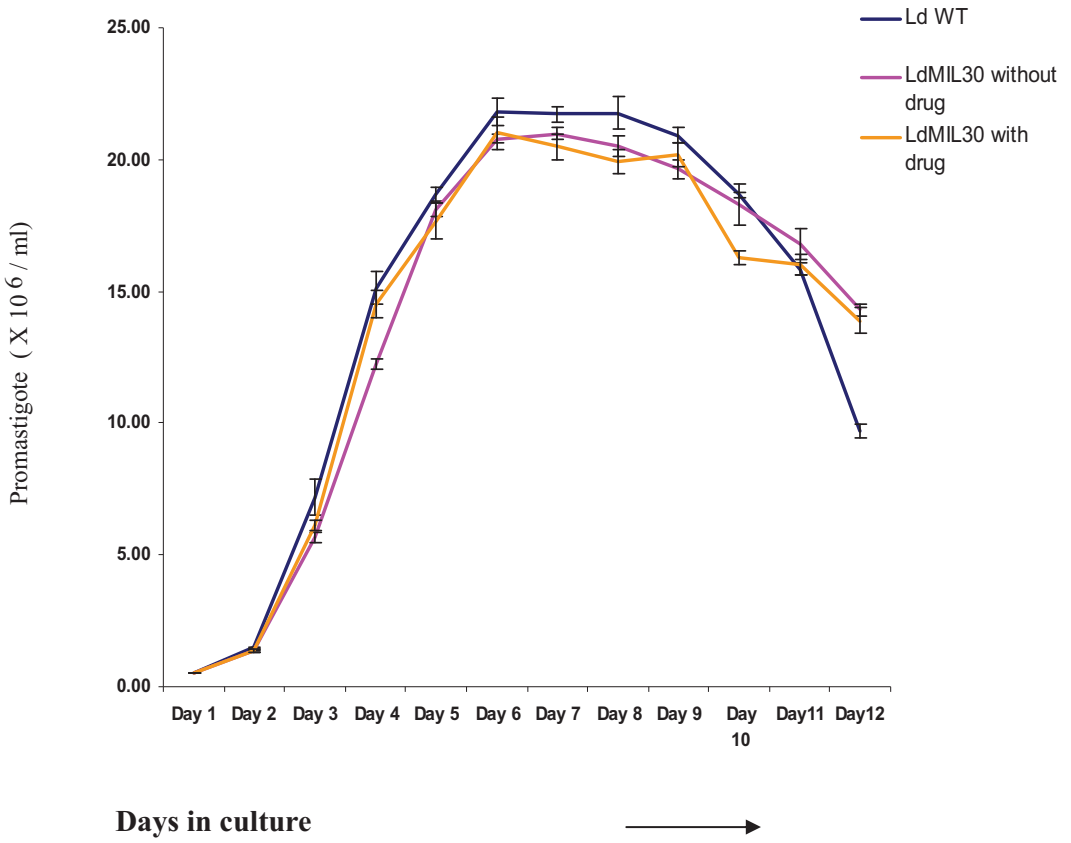


Fig. 1: Growth kinetics of the MIL resist ant parasite compared to the Wild type

L.donovani. Growth of Miltefosine resistant *L.donovani* promastigote (LdM30), wild type (LdWT) was monitored. *L. donovani* promastigotes were cultured *in vitro* at an initial concentration of $5x10^5$ cells/ml. The cells were kept in culture for 12 days and counted at indicated days. Results are the mean of three independent experiments. Error bars indicate the standard deviation.

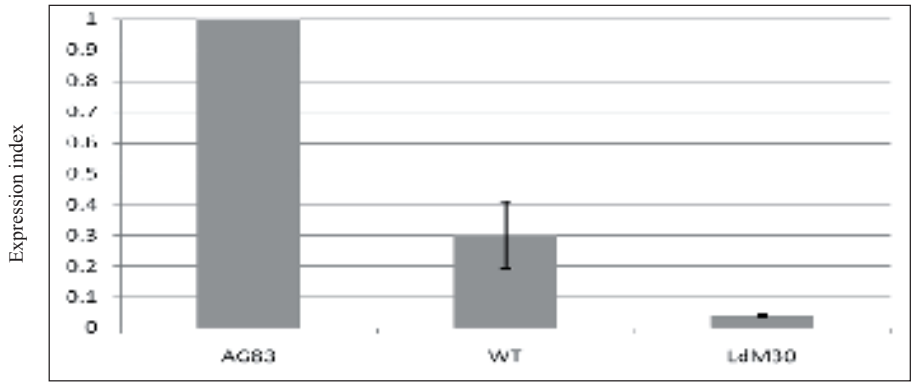


Fig. 2 : mRNA expression of LdMT in miltefosine resistant parasite by Real time PCR . The expression pattern of LdRos3 in MIL resistant parasite. Gene Expression was analysed using Real Time PCR. Expression index of gene in these isolates is indicated with respect to LdAG83.Values given are mean ± SEM of two different experiments performed in triplicate.

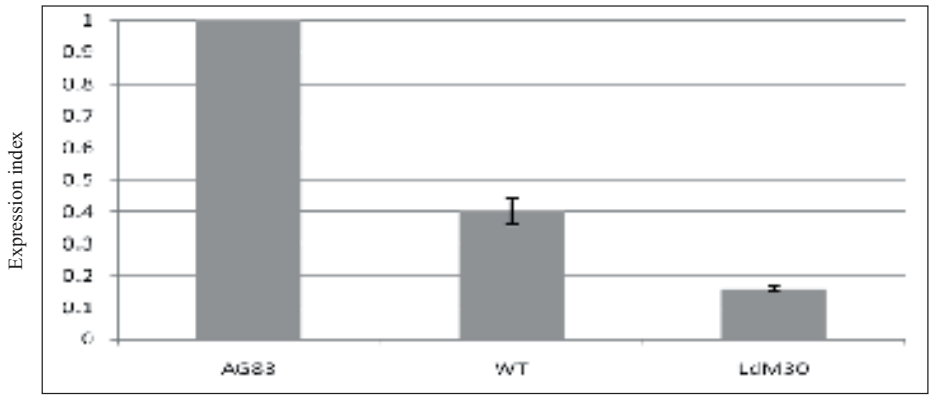


Fig. 3: mRNA expression of LdRos3 in miltefosine resistant parasite by Real time PCR.

The expression pattern of LdRos3 in MIL resistant parasite. Gene Expression was analysed using Real Time PCR. Expression index of gene in these isolates is indicated with respect to LdAG83.Values given are mean ± SEM of two different experiments performed in triplicate.

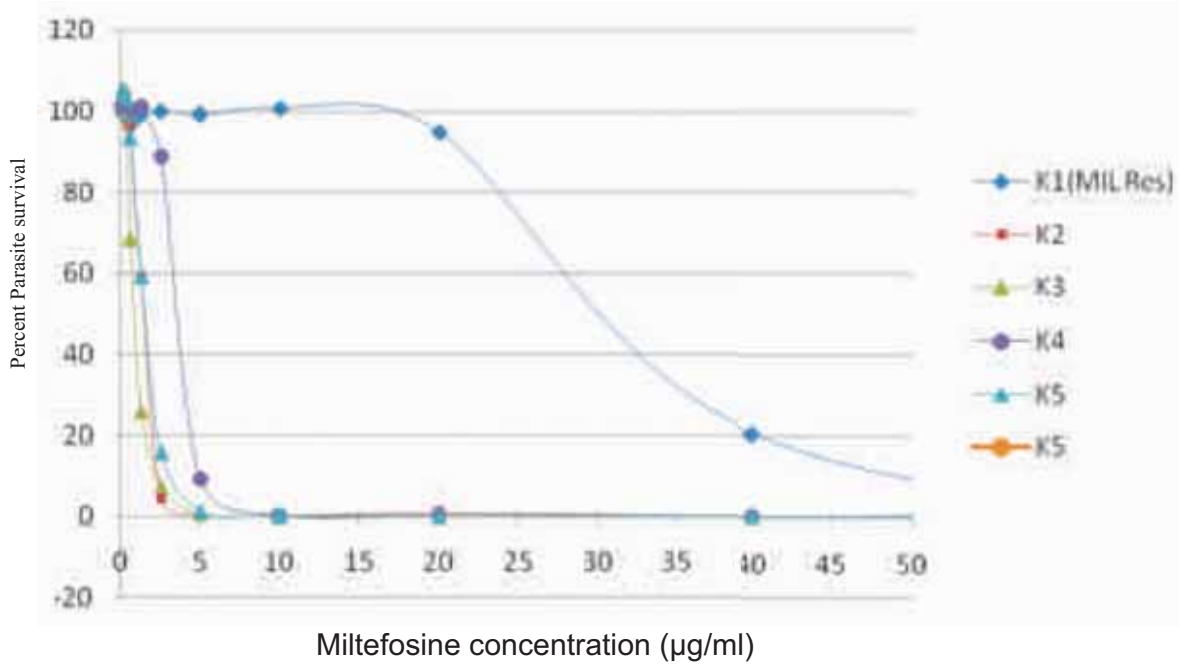


Fig. 4: Percent parasite killing calculated by Resazurin based promastigote viability assay.

The percent promastigote survival on exposure to increasing miltefosine concentrations as determined by high throughput resazurin bioassay is depicted in the plot. Each value represents the mean of four independent value. K1-Miltefosine resistant parasite.K2-K5 are the *L.donovani* field isolates.

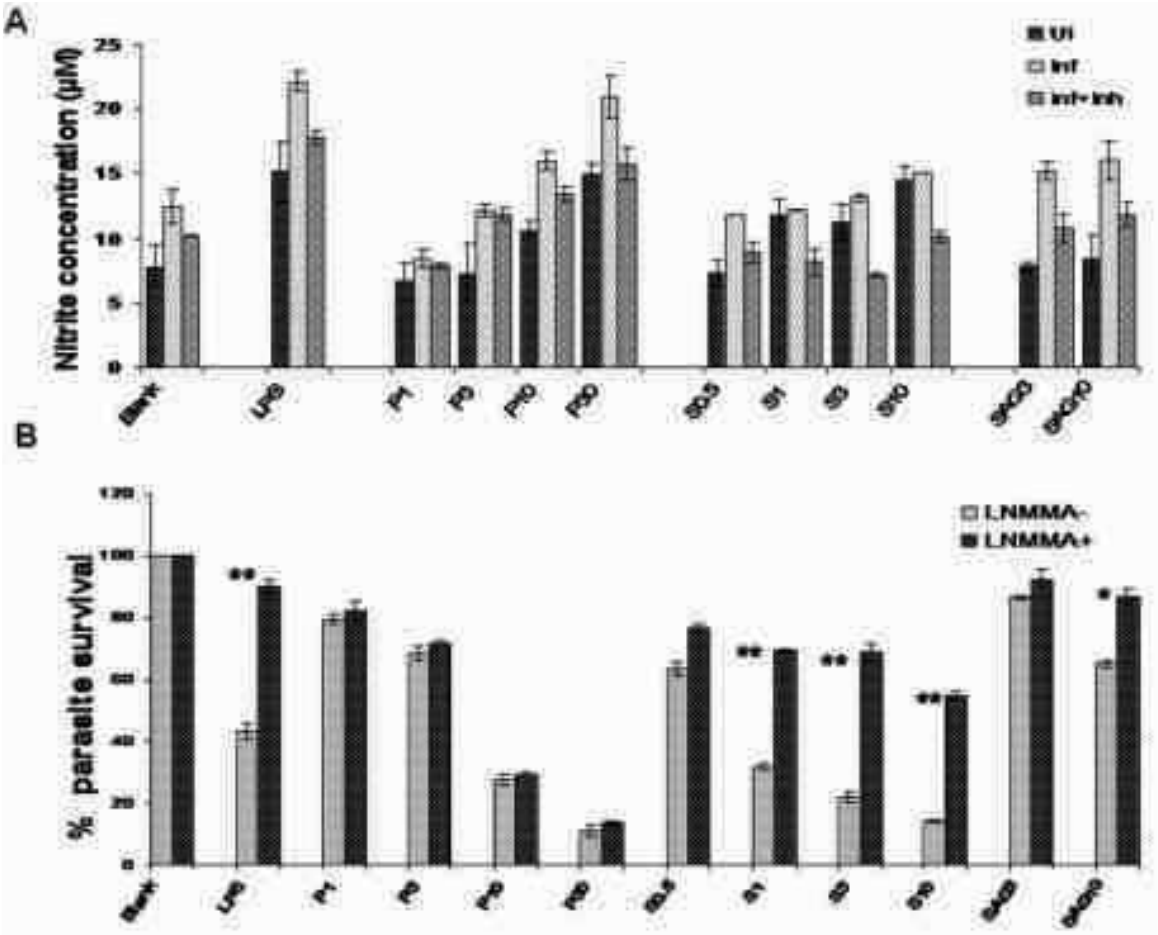


Fig. 5: Effects of paromomycin and sitamaquine on production of nitric oxide by macrophages and parasite survival

(A) **J774.A.1 Murine macrophages uninfected (UI), infected (Inf) with L.donovani LdAG83 (1:10 infection ratio) or infected macrophages treated with L-NMMA 1 hr prior to drug exposure (inf+L-NMMA) were incubated with medium alone or medium containing increasing concentrations of paromomycin (P1, P3, P10, P30 denoting 1, 3, 10 and 30 µM respectively) or sitamaquine (S0.5, S1, S3, S10 denoting 0.5, 1, 3 and 10 µM respectively). SAG3 and SAG10 denoting 3 and 10 µg/ml respectively. Lipopolysaccharide (LPS, 1µg/ml). The graphs represent the mean of triplicates from two independent experiments. * ($p<0.05$); ** ($p<0.001$) as determined by t- test.**

(B) **Effect of nitric oxide inhibitor on parasite survival in L.donovani infected macrophages treated with paromomycin/sitamaquine. Percent parasite survival in infected macrophages upon treatment with different drugs in presence (L-NMMA+) or absence of inhibitor (L-NMMA_) is shown.**

2. STUDIES ON CHARACTERIZATION OF E1-LIKE UBIQUITIN-FOLD MODIFIER ACTIVATING ENZYME (LDUBA5) IN LEISHMANIA

Scientific staff	:	**Dr. Poonam Salotra, Dr. Paresh Sharma, Ms Vanila Sharma**
In collaboration with	:	**Dr. Hira Nakhasi**
Duration	:	**2008-2010**

Aims, Objectives & Background:

It is well known that ubiquitin (Ub) and ubiquitin-like protein-conjugating enzymes play central roles in post-translational modification processes. The ubiquitin-fold modifier 1 (Ufm1), one of a variety of ubiquitin-like modifiers, covalently attaches to target proteins via Uba5 and Ufm1-conjugating enzyme 1 (Ufc1), which are analogous to the E1 and E2 ubiquitylation enzymes, reported in humans. Ufm1-related proteins are conserved in metazoa and plants but not in yeast. While the majority of ubiquitin and ubiquitin-like (UBL) post-translational modifiers pathway in mammalian and yeast cells have been studied and characterized, relatively little is known about how these systems are used by parasites. The regulation of gene expression and protein turnover is clearly critical for both life cycle and disease progression in medically important protozoa, the mechanisms regulating these processes are not well understood. Given the known functions of ubiquitin and Ubls in other organisms, a better understanding of these posttranslational modifiers is likely to be critical to understanding how parasites control many basic biological processes. UBLs (for example SUMO, RUB1/NEDD8, APG12) do not seem to promote proteasomal degradation, but regulate a variety of cellular functions as critical regulators of many cellular processes, such as transcription, DNA repair, signal transduction, autophagy and cell cycle.

In the previous report we characterized *Ld*Uba5 gene and here we report the characterization of other components of Uba5-Ufm 1 based pathway in *Leishmania.*

Work done during the year:

We investigated for the presence of an Ubiquitin like modifier 1 (Ufm1) and E2-like conjugating enzyme (Ufc1) in *L.donovani* and established the complete pathway in Leishmania.

Identification and Sequence analysis of a novel Ubiquitin-fold molecule, Ufm1

In work reported last year we iden tified the protein of ~12kDa that forms an intermediate complex with Δ*Ld*Uba5^{C217S} leading to an additional band of ~57kDa in Western blot. Since human Uba5 has been known to interact with Ufm1 (Komatsu et al, 2004), its homolog was searched in the *L. infantum* GeneDB. An approximately 115 amino acid protein with a predicted molecular mass of ~12.4kDa showed high percentage of similarity with accession no. Linj16.V31100 (Conserved hypothetical protein). Primers were designed for ORF region of this *L.infantum* gene and amplified with *L. donovani* DNA, sequencing of which showed 100% homology with the *L.infantum* gene. Clustal W analysis of Ufm1 showed conserved glycine residue similar to human Ufm1 and Ubiquitin in *Leishmania* and *Tyrpanosoma* (Fig 6 A, B). Ufm1 has a single Gly residue conserved across species at the C-terminal region, however the length and sequences of amino acids extending from this Gly residue vary among species. In case of *Leishmania* (*L.donovani*, *L.major and L.infantum*) the C terminal Glycine residue is followed by 17 AA in comparison with only two AA in humans and *Trypanosoma*. An interesting difference in the size of Ufm1 was found in different *Leishmania* species; *L.donovani* and *L.infantum* contained 115 AA while in *L.major* 249 AA were present.

***Ld*Ufm1 undergoes post translational cleavage at its C-terminus conserved Glycine residue**

Human Ufm1 undergoes C-terminal cleavage to expose the Gly residue as part of maturation process before it can be activated by the activating enzyme *Ld*Uba5. To investigate if the *Leishmania* Ufm1 undergoes enzymatic processing prior to the activation by *Ld*Uba5, *Leishmania* transfectants were prepared that overexpress either the full length LdUfm1 (Ufm1WT) (Fig. 7A) or a mutant in which the C-terminal Gly is changed to Ala (Ufm1G98A) or a variant of LdUfm1 in which 18 amino acid residues at the C-terminus were removed including Gly 98 and therefore will not undergo processing (Ufm1$^{\Delta C}$). These transfectants carried an N-terminal HA-epitope tag. Immunoblotting with total lysates from these transfectants indicated

that the exogenously expressed wild type Ufm1 indeed undergoes processing as revealed by its faster migration on the SDS-PAGE gel compared to Ufm1G98A . The slower migration of Ufm1G98A likely reflects failure of cleavage of the C-terminal extension as has been observed in previous G>A mutant of human Ufm1(Fig 7B) . The ($Ufm1^{\Delta C}$) showed the fastest migration as can be expected and the wild type endogenously expressed Ufm1 (WT) did not show any reactivity with α-HA antibodies . These results indicated that LdUfm1 undergoes enzymatic processing prior to activation and suggested the existence of an Ufm1 processing C-hydrolase activity in *L. donovani*.

Uba5 is an Ufm1-activating enzyme

*Ld*Uba5 acts as an Ufm1-activating enzyme for *Ld*Ufm1 and forms an intermediate complex with it. Lysate from *L. donovani* cells expressing *Ld*Uba5 or Δ*Ld*$Uba5^{C217S}$ with HA & 6XHis-tagged Ufm1was subjected to SDS PAGE and immunoblotted with anti His antibody, a band of 60 kDa was obtained in case of Δ*Ld*$Uba5^{C217S}$ but not with *Ld*Uba5 or in wild type *L.donovani* cells taken as control (Fig 8A). This indicated that *Ld*Uba5 forms an intermediate with *Ld*Ufm1and hence may function as an activating enzyme for it.

Identification and sequence analysis of a novel protein-conjugating enzyme, Ufc1

Existence of a functional LdUfm1and its activating enzyme LdUba5 that has E1-like activity indicated that other components such as conjugating enzymes might also be present in the trypanosomatid parasites. Bioinformatic analysis using human Ufc1 protein that has been previously shown to be the conjugating enzyme of human Ufm1 allowed us to identify genes encoding putative Ufc1 proteins in the genomes of *Leishmania infantum* (LinJ15_V3.1270), *Trypanosoma brucei* (Tb09.160.4150) and *Trypanosoma cruzi* (Tc00.1047053506445.100). A comparison of the putative trypanosomatid Ufc1 aminoacid sequences with the human ortholog showed several blocks of conserved aminoacid residues including the Cys115 residue which has been shown to be the active site in human Ufc1 (Fig. 9). Overall, primary amino acid sequence comparison revealed that *Leishmania* and *Trypanosoma* Ufc1 proteins display ~60% identify with human Ufc1.

*Ld*Ufc1 is an *Ld*Ufm1 conjugating enzyme and interacts with *Ld*Uba5 and *Ld*Ufm1

To ascertain if the putative LdUfc1 can interact with LdUfm1 for conjugation to potential protein targets, the putative Ufc1 coding sequence was amplified from the *L. donovani* genome and cloned into an expression vector. *Leishmania* transfectants that overexpress full length LdUfc1 alone or coexpress LdUfc1 and LdUfm1 proteins were prepared. The full length LdUfc1 carried a C-terminal FLAG-epitope tag (fig10 A) where as the LdUfm1 carried an N-terminal HA-epitope tag. To detect the molecular interaction between these proteins, we performed coimmunoprecipitation experiments. Immunoprecipitation reactions followed by immunoblotting using lysates from the transfectants showed that the putative LdUfc1 interacts with LdUfm1 as revealed by the presence of the nearly ~26 kDa LdUfc1 protein only in the transfectants that express both LdUfm1 and LdUfc1 and not when either of the epitope tagged proteins is expressed alone (fig 10B). This result showed that the LdUfc1 can interact with LdUfm1 and potentially carry out the conjugation reaction. To test whether a molecular interaction could be detected between the endogenous LdUba5 and transfected LdUfc1, similar to human Uba5 and Ufc1, lysates from transfectants expressing LdUfm1, LdUfc1 alone or expressing both proteins were used in a co immunoprecipitation reaction using α-LdUba5 antibodies followed by immunoblotting with α-FLAG antibody which recognizes LdUfc1. The result showed that FLAG tagged LdUfc1 can be detected in an immunoblot both in LdUfc1 transfectants and those expressing the combination of LdUfc1 and LdUfm1 but not in the untransfected *L. donovani* lysates (Figure 10C). On the other hand, when α-FLAG antibodies were used in the immunoprecipitation reaction, LdUfc1 could pull down endogenous LdUba5 protein as shown by the immuno-reactive ~43 kDa band on the immunoblot using LdUba5 antibodies (Figure 10D, indicated by arrow head). Together, these results demonstrate that LdUba5 and LdUfc1 can independently interact with LdUfm1 and also can be found in a molecular complex with all three components.

*Ld*Ufm1 mutants have a Dominant negative Effect on the Growth of the Parasite.

We have already established that *Ld*Uba5 mutants have a dominant negative effect on the growth of the parasite almost 10 fold reduction in the growth. Similar results were obtained when *Ld*Ufm1 or *Ld*Ufm1^{G83A} were overexpressed. A significant growth reduction as compared with the control cells (Fig 11) was seen indicating role of *Ld*Ufm1 in parasite growth.

Future Plan

The present study establishes the identification and characterization of the components of the a novel LdUfm1 pathway in Leishmania.

(A)

```
LdUfm1  ------------------------------------------------------------
LiUfm1  ------------------------------------------------------------
LmUfm1  MTLCRYFVFAPLSRCASFRYLPVCCAQVPAPLLPSLTTYTHTHTPTHTHASTVLPAVIPS
TcUfm1  ------------------------------------------------------------
TbUfm1  ------------------------------------------------------------
HsUfm1  ------------------------------------------------------------

LdUfm1  ------------------------------------------------------------
LiUfm1  ------------------------------------------------------------
LmUfm1  AFRWHSFSQHFYFFRHSTSLSPRARTIDTDRPGRTDGHLVQNLFDRRSGDEWRERGEGEP
TcUfm1  ------------------------------------------------------------
TbUfm1  ------------------------------------------------------------
HsUfm1  ------------------------------------------------------------

LdUfm1  ---------------MSAANDSGAPAAPSGNKVTFRVILTSERSQPFRVISIAEEAPLTA
LiUfm1  ---------------MSAANDSGAPAAPSGNKVTFRVILTSERSQPFRVISIAEEAPLTA
LmUfm1  QRKQKKTFTSVVRRTMSAANQSGAPAAPSGNKVTFRVILTSERSQPFRVISIAEEAPLTA
TcUfm1  ---------------MSQEEQT-APAA-SGGKVTFRVILTSERTQPFRVISIAEEAPLTA
TbUfm1  ---------------MSQ--NEEAPAR-SGGKVTFRIILTSERSQPFRVISIAEEAPLTA
HsUfm1  --------------------------------MSKVSFKITLTSDPRLPYKVLSVPESTPFTA
                                         .**:*:: ***:   *::*:*:.*.:*:**
LdUfm1  VLRFAAEEFGVASVDSMAATTKDGTGINPAQTAGTVFMKYGQEIRLIPRDRVGAAPCVRL
LiUfm1  VLRFAAEEFGVASVDSMAATTKDGTGINPAQTAGTVFMKYGQEIRLIPRDRVGAAPCVRL
LmUfm1  VLRFAAEEFGIASVDSMAATTKDGTGINPAQTAGTVFMKYGQEIRLIPRDRVGAAPCVRL
TcUfm1  VLRFAAEEFGIASVDSMAATTKDGTGINPAQTAGSVFMKYGQEIRLIPRDRVGAA-----
TbUfm1  ALRFAAEEFGIASVDSMAATTKDGTGINPAQTAGNVFMKYGQEIRLIPRDRVGRV-----
HsUfm1  VLKFAAEEFKVPAATS-AIITNDGIGINPAQTAGNVFLKHGSELRIIPRDRVGSC-----
        .*:****** :.:. * *  *:** *********.**:*:*.*:*:*******
LdUfm1  LRSRWRWWRG
LiUfm1  LRSRWRWWRG
LmUfm1  LRSRW-WCRG
TcUfm1  ----------
TbUfm1  ----------
HsUfm1  ----------
```

(B)

```
HsUfm1 MS--------------KVSFKITLTSDPRLPYKVLSVPESTPFTAVLKFAAEEFKVPAAT 46
LdUfm1 MSAANDSGAPAAPSGNKVTFRVILTSERSQPFRVISIAEEAPLTAVLRFAAEEFGVASVD 60
Ub     -------------------MQIFVKTLTGKTIT-LEVEPSDTIENVKAKIQDKEGIPPDQ 40
                           ::: :.:    .   :.:  . .:  *     ::  :..

HsUfm1 S-AIITNDGIGINPAQTAGNVFLKHGSELRIIPRDRVGSC--------------- 85
LdUfm1 SMAATTKDGTGINPAQTAGTVFMKYGQEIRLIPRDRVGAAPCVRLLRSRWRWWRG 115
Ub     Q--RLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG----------------- 76
               *  :: .:* .   ::  . :::: * * *
```

Fig. 6: Clustal W analysis of Ufm1, a Ubiquitin-fold molecule.

(A) Sequence alignment of *Ld*Ufm1 with its homologs in *Homo sapiens*, *L.major* and trypanosomes. The sequence of hsUfm1 is available from GenBanK under the accession number BC005193. The sequence of *L.donovani* Ufm1; *L.major* (LmjF16.1065); *T.brucei* (Tb927.8.5380); *T.cruzi* (Tc00.1047053507491.59); are from GeneDB . The C-terminal conserved Gly residue is boxed in red.

(B) Sequence alignment of hsUbiquitin with hsUfm1 and *Ld*Ufm1. The C-terminal conserved Gly resiue is boxed in red.

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* :: .:* .  :: . :::: * * *
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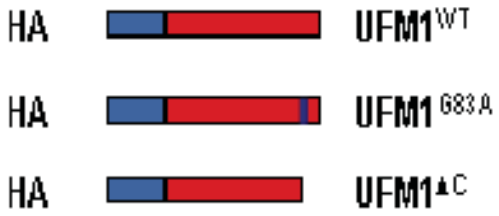
(A)



(B)

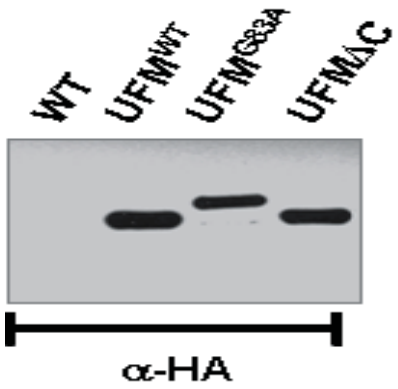


Fig. 7: Post translational processing of *Ld*Ufm1

(A) Schematic representation of *Leishmania* expression plasmids for Ufm1 and the derivative mutants. *Ld*Ufm1 was constructed with HA tagged at the N-terminus (*Ld*Ufm1WT). To construct *Ld*Ufm1ΔC, residues from C-terminal Gly^{98} of mature Ufm1 were deleted by PCR. To construct *Ld*Ufm1^{G98A}, Gly^{98} of HA-*Ld*Ufm1 was mutated to Ala by site-directed mutagenesis. The *Ld*Ufm1ΔC and *Ld*Ufm1^{G98A} mutants were tagged with HA at the N-terminus.

***(B)* *L.donovani* cells were transfected with *Ld*Ufm1WT, *Ld*Ufm1ΔC and *Ld*Ufm1^{G98A} using wild type cells as control. The cell lysates were subjected to SDS–PAGE and analyzed by immunoblots with anti-HA antibodies. Cell lysates from wild type (WT) *Leishmania* cells are used as control which showed no band with anti-HAAb (lane 1).**

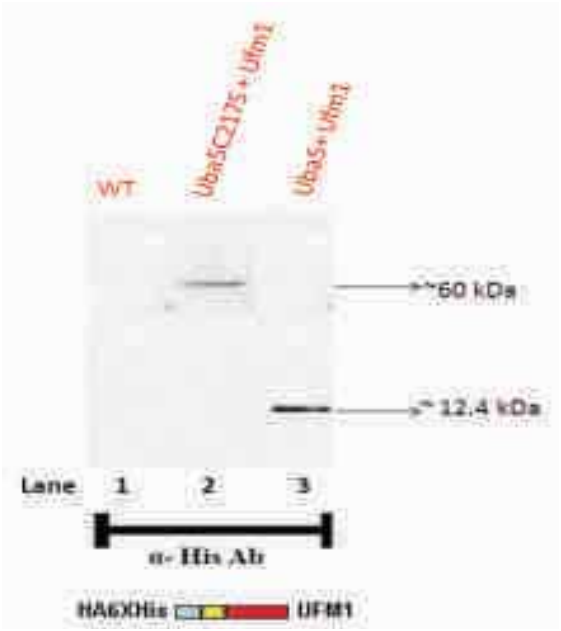


Fig. 8: Demonstration that Uba5 is an Ufm1-activating enzyme.
(A) Each *Ld*Uba5/*Ld*Uba5^{C217S} and *Ld*Ufm1 were co expressed (lanes 2 and 3, respectively) in *Leishmania* cells using wild type (WT) cells as control (lane 1). The cell lysates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-His antibody. The bands corresponding to *Ld*Uba5 and *Ld*Uba5–*Ld*Ufm1 intermediates are indicated on the right.

```
LiUfc1 -MEPSVKESVSRIPLLKTKAGPRDGDKWTARLKEEYASLITYVEHNKASDSHWFHLESNP
LdUfc1 -MEPSVKESVSRIPLLKTKAGPRDGDKWTARLKEEYASLITYVEHNKASDSHWFHLESNP
LmUfc1 -MEPSVKESVSRIPLLKTKAGPRDGDKWTARLKEEYASLITYVEHNKASDSHWFHLESNP
TbUfc1 -MDPAVRESVSRIPLLKTKAGPRDGEQWTQRLKEEYTSLIQFVENNKASDNHWFKLESNE
HsUfc1 MADEATRRVVSEIPVLKTNAGPRDRELWVQRLKEEYQSLIRYVENNKNADNDWFRLESNK
         : :.:. **.**:***:***** : *. ****** *** :**:** :*..**:****

LiUfc1 QGTRWYGTCWTYYKNEKYEFEMNFDIPVTYPQAPPEIALPELEGKTVKMYRGGKICMTTH
LdUfc1 QGTRWYGTCWTYYKNEKYEFEMNFDIPVTYPQAPPEIALPELEGKTVKMYRGGKICMTTH
LmUfc1 QGTRWYGTCWTYYKNEKYEFEMNFDIPVTYPQAPPEIALPELEGKTVKMYRGGKICMTTH
TbUfc1 AGTRWYGTCWTYYKNERYEFNMNFDLAVTYPQAPPEIALPELEGKTVKMYRGGKICMTTH
HsUfc1 EGTRWFGKCWYIHDLLKYEFDIEFDIPITYPTTAPEIAVPELDGKTAKMYRGGKICLTDH
        ****:*.**  :.  :***:::**:.:*** :.****:***:***.*********:* *

LiUfc1 FFPLWARNVPYFGISHVLALGLGPWLSIEVPAIVEEGYLKPASAATVPTTAE
LdUfc1 FFPLWARNVPYFGISHVLALGLGPWLSIEVPAIVEEGYLKPASAATVPTTAE
LmUfc1 FFPLWARNVPYFGISHVLALGLGPWLSIEVPAIVEEGYLKPASAATVPTTGE
TbUfc1 FFPLWARNVPYFGISHALALGLGPWLSIEVPAMVEDGVLKPKKVES------
HsUfc1 FKPLWARNVPKFGLAHLMALGLGPWLAVEIPDLIQKGVIQHKEKCNQ-----
       * ******** **::* :********::*:* :::.* ::  .  .
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Fig. 9: Clustal W analysis of Ufc1, a Ufm1 conjugating enzyme.
Sequence alignment of *Ld*Ufc1 with its homologs in *Homo sapiens*, *L.major* and trypanosomes. The sequence of hsUfm1 is available from *GenBanK* under the accession number BC005187. The sequence of *L. donovani* Ufc1 (No. to be obtained); *L.major* (LmjF15.1250); *T.brucei* (Tb09.160.4150); *T.cruzi* (Tc00.1047053506445.100); are from GeneDB . The putative active site Cys residue is boxed in red.

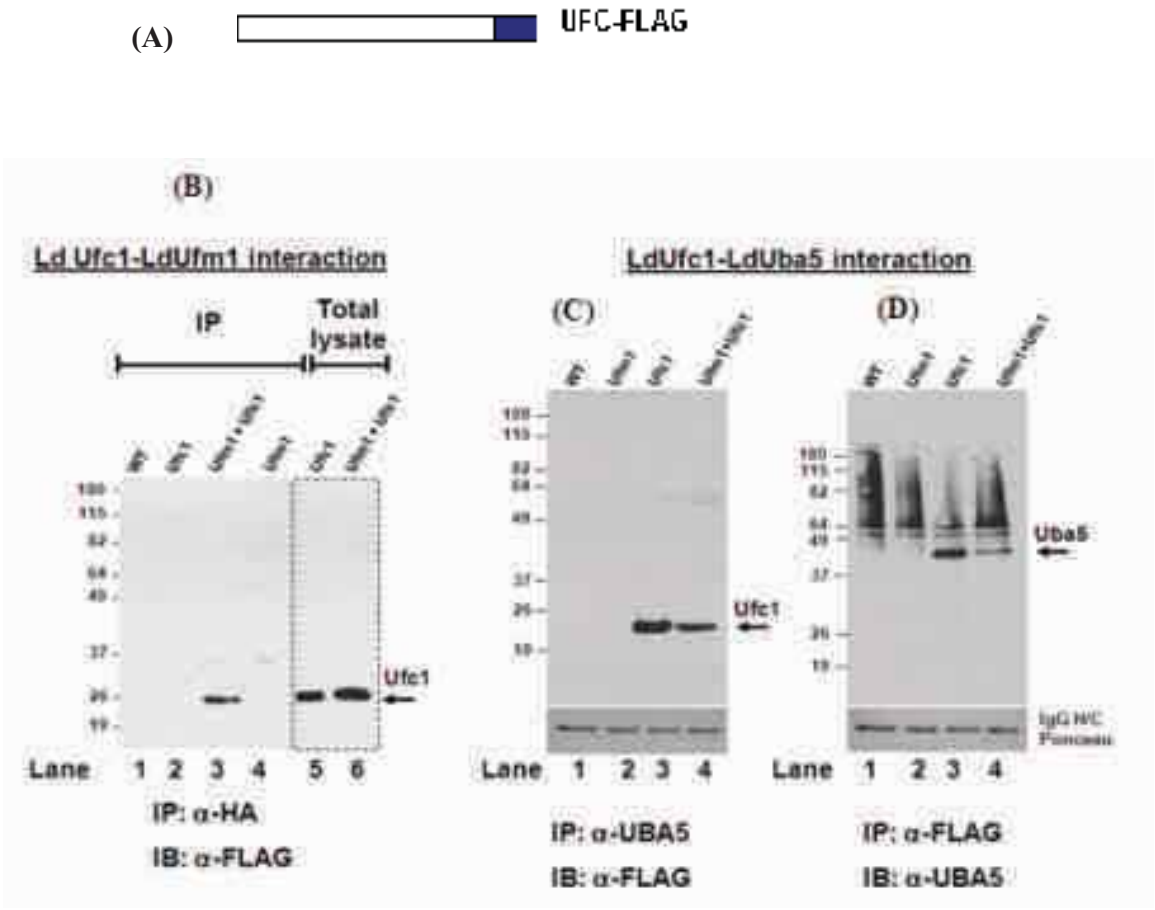


Fig. 10: Characterization of Ufc1, a novel E2-like enzyme.

(A) Schematic representation of *Leishmania* expression plasmids for *Ld*Ufc1. *Ld*Ufc1 was constructed with flag tagged at the C-terminus (Flag-*Ld*Ufc1).

(B) Immunoblotting analysis after immunoprecipitation showing *Ld*Ufc-1 and *Ld*Ufm-1 interaction. Cell lysates of WT (lane 1), Flag-*Ld*Ufc1(lane 2) Flag-*Ld*Ufc1 + HA *Ld*Ufm1(lane 3) and HA-*Ld*Ufm1(lane 4) were immunoprecipitated with anti-HA antibody, resulting immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag antibodies. The bands corresponding to *Ld*Ufc-1 are indicated.

(C) Immunoblotting analysis after immunoprecipitation showing *Ld*Uba5 and *Ld*Ufc-1 interaction. Cell lysates of WT (lane 1), HA*Ld*Ufm1(lane 2) Flag-*Ld*Ufc1 + HA *Ld*Ufm1(lane 3) and Flag*Ld*Ufc1(lane 4) were immunoprecipitated with anti-*Ld*Uba5 antibody, resulting immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag antibodies. The bands corresponding to *Ld*Ufc-1 are indicated.

(D) Immunoblotting analysis after immunoprecipitation showing *Ld*Ufc-1 and *Ld*Uba5 interaction. Cell lysates of WT (lane 1), HA-*Ld*Ufm1 (lane 2), Flag*Ld*Ufc1 (lane 3) and Flag-*Ld*Ufc1 + HA *Ld*Ufm1 (lane 4) were immunoprecipitated with anti-Flag antibodies, resulting immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Uba5 antibody. The bands corresponding to *Ld*Uba5 are indicated.

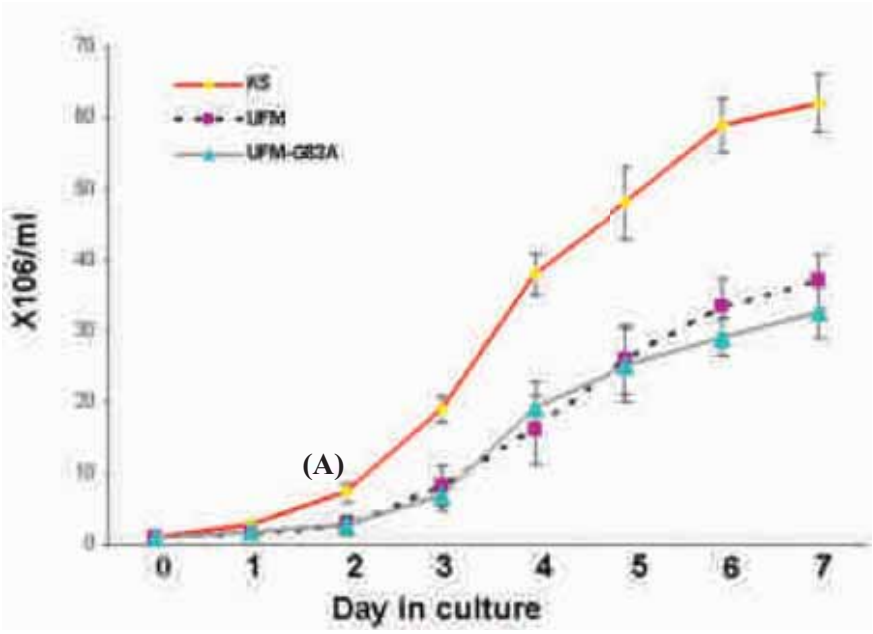


Fig. 11: Effect of the over expression of *Ld*Ufm1 and its mutants on the growth of the parasite. Growth of *L.donovani* promastigote cultures-plasmid control pKSNeo (WT), wild type *Ld*Ufm1 and mutant *Ld*Ufm1^{G83A}(G>A) was monitored. *L. donovani* promastigotes were cultured in vitro at an initial concentration of 1x10^{6} cells/ml. The cells were kept in culture for 7 days and counted at indicated days. Results are the mean of three independent experiments. Error bars indicate the standard deviation.

3. ANALYSIS OF HOST IMMUNO-DETERMINANTS INVOLVED IN PATHOGENESIS OF KALA-AZAR AND POST-KALA-AZAR DERMAL LEISHMANIASIS

Scientific staff	**:**	**Dr. Poonam Salotra, Mr. Gajendra Kumar Katara, Mr. Sandeep Verma, Mr. Himanshu Kaushal**
In collaboration with	**:**	**Dr. V. Ramesh, Dr Sumita Saluja, SJH**
Duration	**:**	**2007-2011**

Aims, Objectives & Background:

This study was designed to capture the picture of host immune-determinants modulated during the active disease of KA and PKDL. In the previous year, based on cDNA array, we have documented a number of immunodeterminants involved in pathogenesis of PKDL. The major immuno-determinants identified included chemokines (MCP-1, MIP1-a), Apoptotic molecules

(IRF-1, TRAIL, Fas), growth factors (M-CSF, GM-CSF), Kinases (TYRO3, AXL, PTK) and various receptors of different host defense mechanisms PKDL. We also identified distinct Th17 type of cell response based on high expression of IL 17 in PKDL. The validation of key molecules at message level was reported. In the year under report, we attempted to understand the host immune responses in localized tissue BMA of KA patients using cDNA array. Results of cDNA array were validated at message and protein level in individual KA samples. In addition, JAK/STAT pathway was analyzed in PKDL us ing real time PCR based signaling array. The apoptotic molecules (IRF-1 and Caspase-3) identified in PKDL cDNA array as well as signaling array were validated at protein level using IHC in individual samples. This study was carried out under a project funded by LSRB, DRDO.

Work done during the year:

Analysis of host immuno-determnants in bone marrow aspirates of KA patients

We compared gene expression in bone marrow aspirates (BMA) of KA patients with normal BMA from healthy individuals (HBM) using cDNA array.

Microarray Analysis: hybridization, autoradiograph and analysis of data:

Details of experiments have already been given in previous year report. In brief HBM RNA procured from Clontech and RNA from KA BMA was used to synthesize radio labeled probe. cDNA was prepared in the presence of 50 µCi of α-^{33}P dATP and 268 gene specific primers for each gene represented on the array. Using Scintillation Counter $5x10^6$ cpm of each cDNA probe was used for hybridization. Atlas arra y membranes were prehybridized at 68°C for 1 h with ExpressHyb prehybridization solution containing 100 µg of sheared salmon testis DNA/ml. Probes were added to separate nylon arrays and allowed to hybridize overnight at 68°C under identical hybridization conditions. After washing, membranes were exposed to phosphor screen and images were captured with phosphoimager Typhoon. Imagequant software was used for analysis of images. The ratio of gene expression levels was determined by dividing the mean signal intensity (average of 3 array experiments) of KA with Control. Differential gene expression was considered significant when the average ratio was greater than 2.0 on comparison

between disease and control. A comparative assessment of gene expression in lesions tissue revealed that 59 (22.2%) genes in KA showed 2.0 fold higher expressions compared to control, as shown in Table 4. Genes were categorized in three groups based on gene characteristics viz. group 1 (cytokines and chemokines), group 2 (receptors) and group 3 (others, comprising of signaling molecule, transcription factor, apoptotic genes etc).

Validation of Microarray results in KA using real time PCR

2µg RNA was reverse transcribed using High capacity cDNA preparation kit. To validate the results of cDNA array quantitative real-time PCR was used in individual samples to verify the changes in expression of selected genes including IFN-γ, TNF-a, IL-10, and TGF-β. These genes were selected on the basis of their expression pattern in the KA as revealed by microarray analysis and their perceived biological interest. Real time analysis of gene expression in KA versus control strongly supported the results obtained with the gene array. Further analysis revealed that the expression of all mentioned gene increased significantly (P=0.01 IFN-γ, P=0.003 TNF-a, P=0.03 IL-10, P=0.03 TGF-β) in KA compared to the control (Fig. 12).

Analysis of JAK/STAT signaling pathway in PKDL

Previously we have shown down regulation of IFN-γR in *Leishmania* infection and JAK2/STAT1 signaling is the principal pathway down-stream of IFN-γR. Study of this pathway may provide understanding of *Leishmania* subversion mechanism by which this pathogen modulate host signaling. In this study we utilized real time PCR based array (Clontech) for analyzing JAK/STAT pathway in PKDL. This array contains 84 genes of JAK/STAT pathway and 8 housekeeping genes, human genomic controls and negative controls. In brief, cDNA synthesized from same 2ug RNA pooled from same 6 PKDL patients and controls used earlier for Nylon array studies. PCR assay was carried out using ABI 7000 Prism real time PCR and the relative expression was determined with $\Delta\Delta C_t$ method. Each experiment was performed in triplicate in order to get technical replicates.

Analysis of results showed 47 genes of this pathway had 2 fold up/down regulation in PKDL compared to control (up=14, down=33, Table 5). Results of PCR array showed upregulation in mRNA expression of IFN-γ (80 fold) and IRF-1 (3.16 fold) compared to control

which also confirmed results of cDNA array. In addition STAT-1 was found upregulated (3.16 fold) and BCL-2 was downregulated (4 fold).

Validation of expression of selected genes at protein level by Western blotting and IHC in KA and PKDL

Assaying intralesional cytokine is important, largely because the *in situ* cytokine profile might be relevant to the ongoing physiological or pathological process. Western blotting (WB) was used to document the presence of TNF-α, at protein level. The expression of TNF-α were found elevated in tissue lesion lysates of KA (n=3) and PKDL (n=3) compared to control (n=3) using WB (Fig 13). α-tubulin was used as endogenous control for normalization.

IHC was used to document the presence of IL-8 at protein level in individual samples of PKDL. IL-8 was evident in the cytoplasm of lymphomononuclear cells in the dermis of all the 3 PKDL cases compared to control tissue (Fig 14). Presence of IL-8 at protein level was confirmed in PKDL.

Future work: Based on high expression of IL17 in microarray results, role of Th17 pathway in PKDL pathogenesis will be investigated. Validation of JAK/STAT pathway molecules will be taken up. In addition, involvement of natural T regulatory cells in PKDL pathogenesis and association with parasite burden in lesion tissues will be investigated.

Table 4: Genes showing higher expression in bone marrow aspirates of KA patients compared to human normal bone marrow RNA (HBM)

Gene Accession No	Gene description	Fold Change KA/HBM
Cytokines & Chemokines		
J04130	Small inducible cytokine A4 (MIP-1β)	12.10
M24545	Small inducible cytokine A2 (MCP-1)	8.69
X53799	GRO2 oncogene	4.51
L09753	Tumor necrosis factor (ligand) superfamily, member 8	4.22
X01394	Tumor necrosis factor alpha (TNF-a)	3.78
L08096	Tumor necrosis factor (ligand) superfamily, member 7	3.71
M28622	Interferon, beta 1	3.64
M23452	Small inducible cytokine A3 (MIP-1a)	3.25
X04688	Interleukin 5	3.24
L07414	Tumor necrosis factor (ligand) superfamily, member 5	3.23
X01992	IK cytokine, down-regulator of HLA II	3.18

X79929	Tumor necrosis factor (ligand) superfamily, member 4	3.03
M65290	Interleukin 12B (p40)	2.92
M65291	Interleukin 12A (p35)	2.88
U57059	Tumor necrosis factor (ligand) superfamily, member 10	2.83
L15344	Interleukin 14	2.82
U14407	Interleukin 15	2.6
M57627	Interleukin 10	2.50
J03241	Transforming growth factor, beta	2.46
U32659	Interleukin 17	2.39
J04156	Interleukin 7	2.35
J00209	Interferon, alpha 10	2.34
M28622	Interferon, beta 1	2.25
A14844	Interleukin 2	2.17
Receptors		
M26062	Interleukin 2 receptor, beta	5.48
AF016268	Tumor necrosis factor receptor superfamily, member 10b	5.14
U90875	Tumor necrosis factor receptor superfamily, member 10a	4.46
M21574	Platelet-derived growth factor receptor	4.21
Z70519	Tumor necrosis factor receptor superfamily, member 6 (Fas)	3.19
X60592	Tumor necrosis factor receptor superfamily, member 5 (CD40)	3.18
D10923	Putative chemokine receptor; GTP-binding protein	2.89
X66945	Fibroblast growth factor receptor 1	2.80
J03171	Interferon (alpha, beta and omega) receptor 1	2.80
M27492	Interleukin 1 receptor, type I	2.79
U03187	Interleukin 12 receptor, beta 1	2.69
Y09392	Tumor necrosis factor receptor superfamily, member 12	2.65
M97675	Receptor tyrosine kinase-like orphan receptor 1	2.35
M87290	Angiotensin receptor 1	2.24
U03882	Chemokine (C-C motif) receptor 2	2.23
M60459	Erythropoietin receptor	2.21
U11814	Fibroblast growth factor receptor 2	2.20
Others		
D38122	Tumor necrosis factor (ligand), member 6 (FASL)	9.45
X14454	Interferon regulatory factor 1 (IRF-1)	6.25
X68203	fms-related tyrosine kinase 4	5.59
M11220	Colony stimulating factor 2 (granulocyte-macrophage)	5.50
X63454	Fibroblast growth factor 6	4.46
M29645	Insulin-like growth factor 2 (somatomedin C)	3.39
X51602	fms-related tyrosine kinase 1	3.14
U02687	fms-related tyrosine kinase 3	3.03
M59964	KIT ligand	2.91
X02492	Interferon, alpha-inducible protein	2.91
U66197	Fibroblast growth factor 12	2.79
AF022385	Programmed cell death 1	2.71
D13365	Metallothionein 3	2.57

M37825	Fibroblast growth factor 5	2.52
U43142	Vascular endothelial growth factor C	2.41
X03438	Colony stimulating factor 3 (granulocyte)	2.34
U41745	PDGFA associated protein 1	2.25
M27968	Fibroblast growth factor 2	2.02

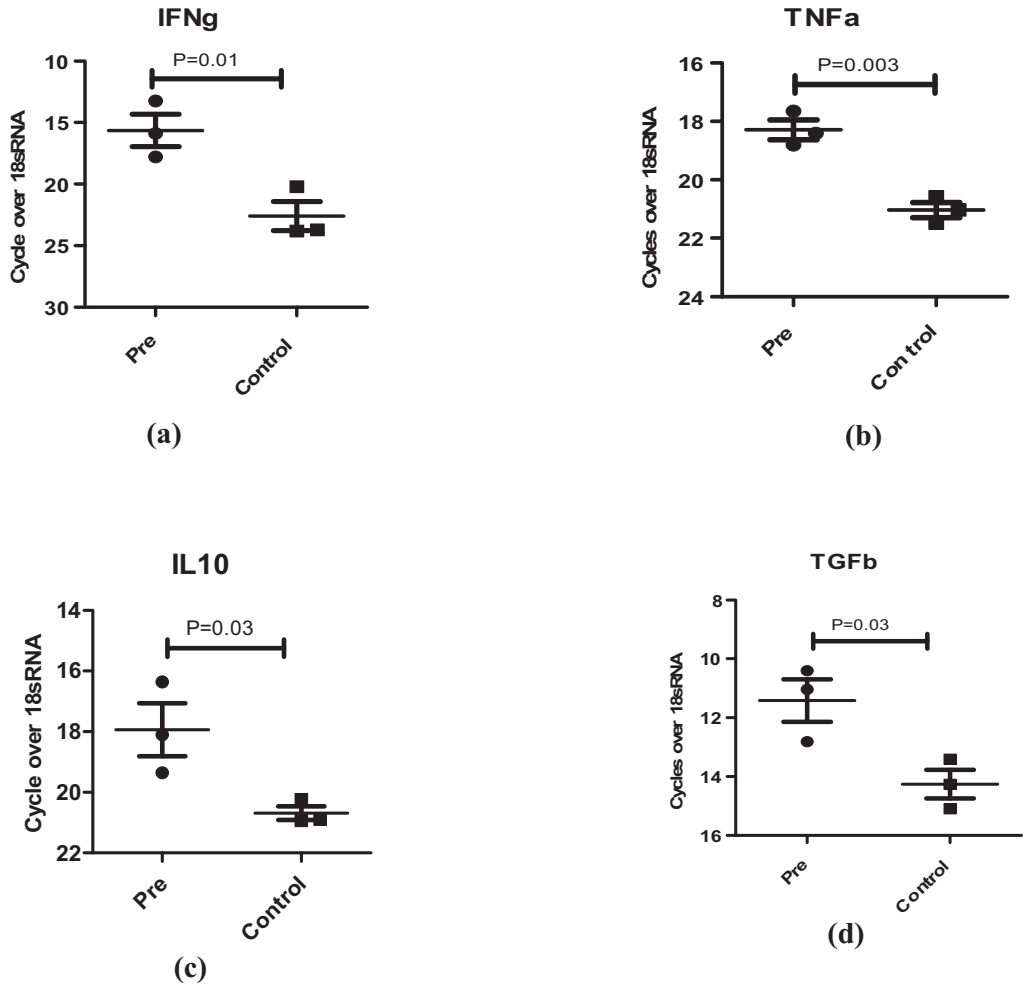


Fig. 12: mRNA levels of IFN-g (a), TNF-a (b), IL-10 (c), TGF-b (d), in bone marrow aspirates of KA patients and normal bone marrow RNA from healthy individuals. Gene expressions were quantified using real time RT-PCR. Values were normalized to 18S rRNA expression.

Table 5: Genes of JAK/STAT pathway showing modulated expression in dermal lesion tissue of PKDL patients compared to normal skin (HC) of healthy individuals

Up-regulated genes Gene discription	Fold change PKDL/HC
Chemokine (C-X-C motif) ligand 9	160.90
Interferon, gamma	80.44
Suppressor of cytokine signaling 1 (SOCS1)	26.72
Fc fragment of IgG, high affinity Ia, receptor (CD64)	8.0
Guanylate binding protein 1, interferon-inducible	6.32
Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	5.02
SH2B adaptor protein 1	3.17
Interleukin 10 receptor, alpha	3.16
Interferon regulatory factor 1	3.16
Signal transducer and activator of transcription 1 (STAT-1)	3.16
Colony stimulating factor 2 receptor, beta	2.51
Src-like-adaptor 2	2.51
coagulation factor II (thrombin) receptor	2.0
Interleukin 2 receptor, alpha	2.0
Spleen focus forming virus (SFFV) proviral integration oncogene	2.0
Down-regulated genes	
Nitric oxide synthase 2, inducible	64.0
Prolactin receptor	16.0
JUN oncogene	12.65
Suppressor of cytokine signaling 2 (SOCS2)	6.32
Myeloproliferative leukemia virus oncogene	6.06
Growth hormone receptor	5.0
Suppressor of cytokine signaling 5 (SOCS5)	4.92
BCL2-like 1	4.0
Erythropoietin receptor	4.0
Interleukin 6 signal transducer	4.0
Suppressor of cytokine signaling 4 (SOCS4)	4.0
Signal transducing adaptor molecule	4.0
High mobility group AT-hook 1	3.16
Nuclear receptor subfamily 3, group C, member 1	3.16
Protein inhibitor of activated STAT, 2	3.16
SMAD family member 1	3.16
SMAD family member 4	3.16
Alpha-2-macroglobulin	3.03
SH2B adaptor protein 2	3.03
v-crk sarcoma virus CT10 oncogene homolog	2.51
C-reactive protein, pentraxin-related	2.51
Platelet-derived growth factor receptor, alpha polypeptide	2.51

Protein inhibitor of activated STAT, 1	2.51
SMAD family member 5	2.51
Signal transducer and activator of transcription 5B	2.51
YY1 transcription factor	2.51
CCAAT/enhancer binding protein (C/EBP), beta	2.0
Epidermal growth factor receptor	2.0
Insulin receptor	2.0
Janus kinase 1	2.0
Janus kinase 3	2.0
Myc protein	2.0
Protein phosphatase 2	2.0
Suppressor of cytokine signaling 3 (SOCS3)	2.0

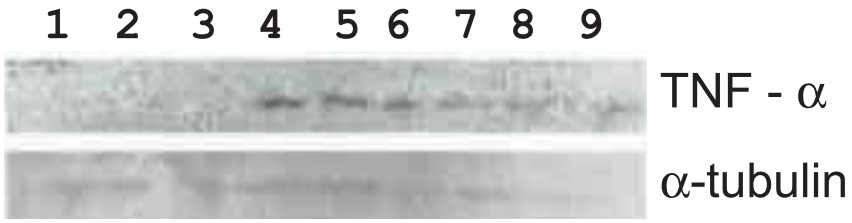


Fig. 13: Western blot analysis for protein level of TNF-a in lesion tissue lysate from patients with μKαL (4-6), KA (7-9)), compared with that in control tissue (1- 3). The blot was stripped and reprobed with anti-a-tubulin antibody (Santa Cruz Biotechnology), to control for protein loading. The experiment was performed with tissue from 3 different patients with μKαL, KA and 3 control tissues.

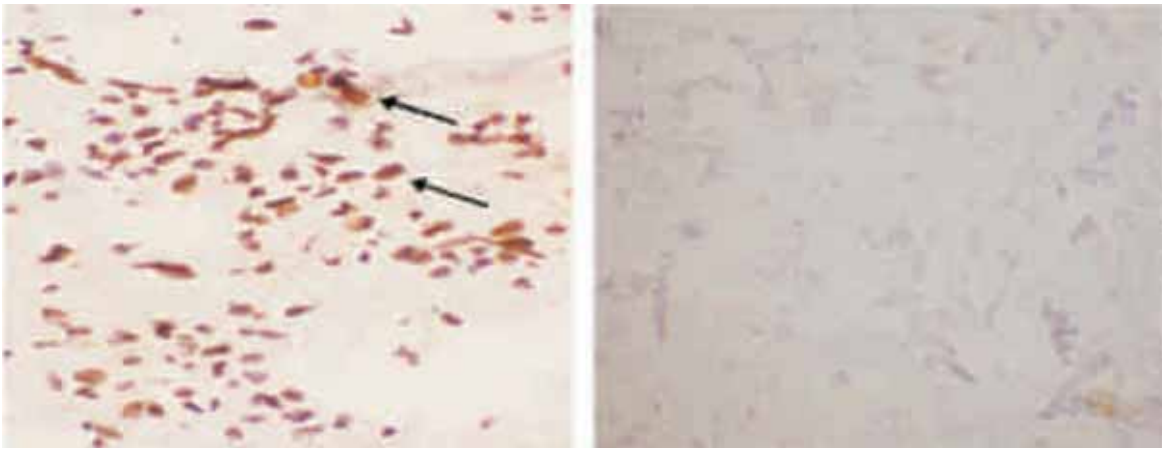


Fig.14: Immunohistochemical analysis in tissue biopsies of PKDL patients for IL-8 (A) and control (B). Immunostaining in tissue sections was visualized at a magnification of X40.

4. ANALYSIS OF LOCALIZED AND CIRCULATING IMMUNE RESPONSES IN PATIENTS OF CUTANEOUS LEISHMANIASIS CAUSED BY LEISHMANIA TROPICA

Scientific staff	:	**Dr. Poonam Salotra, Dr. Gajendra Kumar Katara, Mr. Anand Raj**
In collaboration with	:	**Dr. R. A. Bumb**
Duration	:	**2008-2011**

Aims, Objectives & Background:

As most of the studies in host immune response have been carried out in CL patients infected with *L. major*, the immune status of Indian CL patients, infected predominantly with *L. tropica*, remains to be characterized. In the previous year, we reported the circulating and localized immune responses in Indian CL patients. We proposed that IL-8 is an effector immune-determinant in the disease progression, while NO facilitates the parasite killing by macrophages via MCP-1 mediated stimulation.

The primary objective of the present study was to capture global picture of cytokine gene expression in localized tissue lesions of Indian CL patients using cDNA array. In addition, correlation of IL-4 expression with parasite burden was also established in CL patients.

Work done during the year:

We utilized cDNA array technology and compared gene expression in lesion tissues of CL patients with normal skin from healthy individuals. Real time PCR was used to validate the array results at message level. In addition, IHC was utilized to confirm expression of IL-8, iNOS and myeloperoxidase in lesion tissues reported previously at message level. Parasite load were measured by quantitative real time PCR in lesion tissues of CL patients.

Analysis of localized mRNA expression of immune parameters in lesion tissue by cDNA array

Seven patients of similar age group, with average age of 42.5 ± 3.93 yrs (mean ± S.E.) and erythematous ulcerated type of dermal lesions and four endemic controls were included in

this study. 10µg RNA from each sample were pooled and purified using RNeasy mini kit (*Qiagen, Germany*). cDNA probe prepared from pooled total RNA (3µg) of lesion tissue and controls were hybridized in parallel to identical pairs of cDNA array membranes under identical hybridization conditions. Subsequent wash steps, image capture, and densitometric analysis of data were performed in parallel. This approach facilitates the direct comparison of mRNA levels between infected tissue and uninfected tissue. Each experiment was performed 3 times in order to have technical repeats.

Array data was analyzed by summing the duplicated intensity signals for each gene in individual experiments and finally the average intensity of 3 experiments was used for comparison. Analysis was performed using Image Quant software (Amersham Biosciences). The ratio of gene expression levels was determined and differentially expressed genes were identified between CL and Endemic Control. A comparative assessment of gene expression in infected tissue lesions revealed that 113 of 268 (42%) genes showed 2.0 fold or higher expression in CL compared to Endemic control, as shown in Table 6.

Validation of Nylon array data by Real Time PCR

2 µg total RNA from CL (n=10) and Control (n=7) was reverse transcribed using High capacity cDNA preparation kit (*Applied Biosystems*). cDNA synthesis was performed in a DNA thermal cycler (*Perkin Elmer*), and 50 ng of cDNA was used for real time PCR amplification. PCR was performed in an ABI 7000 Sequence Detection System (*Applied Biosystems*) using TaqMan Universal PCR Master Mix and cDNA specific FAM-MGB labeled primers sets for IFN-γ, TNF-α, IL-10, IL-17, CD40 and MCP-1 (*Applied Biosystems*), with18S rRNA as control for the relative amount of mRNA in each sample. The relative quantification of products was determined by the number of cycles over 18S rRNA endogenous control required to estimate the expression of gene of interest. The results convincingly validated the nylon array data and provided evidence for both inflammatory, non inflammatory and co-stimulating response, as reflected by elevated CD40, TNF-a, IL-10, MCP-1, IL-17 and IFNg (Fig 15) in CL lesion tissue compared with control normal tissue ($p<0.0001$). Statistical significances were analyzed using PRISM4 (*GraphPad Software*). Differences between groups were compared by student t-test.

Categorization of upregulated genes into different pathways

Out of 162 genes, that were upregulated in nylon array analysis, 28 genes were categorized based on their involvement in different pathways as mentioned in the Table 7. Pathways most affected include JAK/STAT and IL17, 13 genes out of 28 are directly involved in JAK/STAT pathway.

In vivo evaluation of MPO, IL-8 and iNOS by IHC.

In vivo analysis of MPO (PMN marker), IL-8 and iNOS was carried out by IHC in CL lesions (n=5) and healthy controls (n=2). IHC revealed the presence of an inflammatory infiltrate consisting of predominantly neutrophils, which presented a heterogeneous pattern of distribution. A difference in cell morphology was also observed: in sections with fewer neutrophils these cells were well compacted, whereas in sections presenting larger numbers this cell type was characterized by a larger size and cytoplasmic content (Fig. 16A). An intense expression of IL-8 (figure 16C) and moderate expression of iNOS (Fig. 16E) was evident in all the lesions. Infiltrate neutrophils, IL-8 and iNOS were not detected in controls (Figure 16B, 16D, and 16F).

Measurement of parasite load in clinical samples for CL patients

Linearity of the PCR assay

Serial dilution of *L. tropica* with final concentration ranging from 10^5 parasites to 0.1 parasite was performed; DNA extracted at each dilution was subjected to real-time PCR to establish the standard curve for determining the absolute quantification of parasites. The reproducibility of the assay was tested in triplicate samples with standard curve dilutions and controls on the same plate and on different days. The mean standard curve was linear over 6-log range of DNA concentrations with a correlation coefficient (r^2) of 0.991. A non-template negative control (water instead of template DNA) with each PCR assay was included for stringent measures to control contamination. Table 8 summarizes the results obtained with amplification of DNA from serially diluted parasite stocks.

Association of parasitic load with Interleukin-4 response in patients with cutaneous leishmaniasis due to *Leishmania tropica*

We have established the association between parasite burden and localized immune response in patients of Cutaneous Leishmaniasis (CL) caused by *Leishmania tropica*. Real-time PCR was employed to measure parasitemia in tissue lesions of CL patients at pre-treatment (n=26) and post-treatment stage (n=10). *L. tropica* was detected in all CL lesions with a mean value of 1,18,357 parasite/g of dermal tissue (Table 9). Following treatment, only 1/10 patient showed residual parasite (100 parasite/g tissue). Parasite load was high (mean, 3,06,000 parasite/g tissue) in acute infections (early lesions) and low (mean, 1081 parasite/g tissue) in chronic infections (late lesions). Intra-lesional transcripts of interferon-γ, tumor necrosis factor-α, interleukin (IL)-1β, IL-8, IL-10 and IL-4 were investigated in early lesions (≤2 months, n=14) and late lesions (>2 months, n=15) by reverse transcriptase-PCR, where IL-4 was found significantly up-regulated in early lesions ($p<0.02$). Further more, levels of parasite burden and IL-4 were distinctly correlated in various clinical forms of CL (Fig 17). Other cytokines were at comparable levels in early/ late lesions and in different clinical forms. Up-regulation of IL-4 was correlated with higher parasite burden in early lesions of CL, which may be involved in pathogenesis of CL by inhibiting protective immune response.

Future scope of the study

On the basis categorization of cDNA array results, JAK/STAT pathwa y will be investigated in clinical samples of CL patients and the defect in signaling mechanism upon infection will be evaluated exploiting signaling pathway based on Real-time PCR assay followed by investigation of key molecules at protein level. In addition, based on reports on *L. major* infection, involvement of T regulatory cells and Th17 pathway in Indian CL will be investigated.

Table 6: Genes showing higher expression (>2 fold) in CL compared to control, categorized into Cytokines, Chemokines, Receptors & Others.

Genes CL/EC	Accession no	Major Function	Expression Cytokines
interleukin 8	Y00787	Interleukins & Interferons	22.51
interferon, gamma	X01992	Interleukins & Interferons	15.32
interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	M65291	Interleukins & Interferons	14.87
tumor necrosis factor (TNF superfamily, member 2)	X01394	Death receptors Ligands	10.81
fibroblast growth factor 1 (acidic)	X65778	Growth Factors, Cytokines & Chemokines	6.27
tumor necrosis factor (ligand) superfamily, member 6	D38122	Death receptor ligand	6.20
colony stimulating factor 2 (granulocyte-macrophage)	M11220	Growth Factors, Cytokines & Chemokines	6.16
interleukin 13	L06801	Interleukins & Interferons	5.50
tumor necrosis factor, alpha-induced protein 6	M31165	Cell Surface Antigens	5.03
oncostatin M	M27288	Growth Factors, Cytokines & Chemokines	4.99
fibroblast growth factor 5	M37825	Growth Factors, Cytokines & Chemokines	4.73
lymphotoxin alpha (TNF superfamily, member 1	D12614	Death receptors Ligands	4.24
transforming growth factor, alpha	K03222	Growth Factors, Cytokines & Chemokines	4.15
interleukin 10	M57627	Interleukins & Interferons	3.92
interleukin 14	L15344	Interleukins & Interferons	3.79
interleukin 17 (cytotoxic T-lymphocyte-associated	U32659	Interleukins & Interferons	3.77
interleukin 1, alpha	X02851	Interleukins & Interferons	3.75
lymphotoxin beta (TNF superfamily, member 3)	L11015	Death receptors Ligands	3.72
interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40	M65290	Interleukins & Interferons	3.65
interleukin 9	X17543	Interleukins & Interferons	3.59
tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kD)	X79929	Growth Factors, Cytokines & Chemokines	2.97
tumor necrosis factor (ligand) superfamily, member 7	L08096	Death receptors Ligands	2.92
interleukin 7	J04156	Interleukins & Interferons	2.64
interleukin 4	M13982	Interleukins & Interferons	2.62
colony stimulating factor 3 (granulocyte)	X03438	Growth Factors, Cytokines & Chemokines	2.47
interferon, alpha 10	J00209	Interleukins & Interferons	2.05
interleukin 11	M57765	Interleukins & Interferons	2.05
transforming growth factor beta (TGF-beta2; TGFB)	X02812	Growth Factors, Cytokines & Chemokines	2.01
Chemokines			
small inducible cytokine A4	J04130	Growth Factors, Cytokines & Chemokines	9.65
small inducible cytokine A1, I-309	M57502	Growth Factors, Cytokines & Chemokines	5.30
small inducible cytokine A3	M23452	Growth Factors, Cytokines	4.38

		& Chemokines	
small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	U10117	Growth Factors, Cytokines & Chemokines	3.87
small inducible cytokine subfamily B (Cys-X-Cys), member 5	X78686	Growth Factors, Cytokines & Chemokines	3.81
GRO2 oncogene	X53799	Growth Factors, Cytokines & Chemokines	3.23
small inducible cytokine A2 (monocyte chemotactic protein 1)	M24545	Growth Factors, Cytokines & Chemokines	2.31
Receptors			
calcitonin receptor	L00587	Hormones receptors	34.25
platelet-activating factor receptor	D10202	Growth Factors & Chemokine receptors	12.58
endothelin receptor type A	L06622	Growth Factors & Chemokine receptors	11.66
corticotropin releasing hormone receptor 1	X72304	Growth Factors & Chemokine receptors	10.43
chemokine (C-C motif) receptor 2	U03905	Growth Factors & Chemokine receptors	10.40
tumor necrosis factor receptor superfamily, member 12 (translocating chain-association membrane protein)	Y09392	Death receptors	9.01
interleukin 10 receptor, alpha	U00672	Interleukins & Interferons receptors	8.63
putative chemokine receptor; GTP-binding protein	D10923	Intracellular Transducers, Effectors & Modulators	8.48
interleukin 12 receptor, beta 1	U03187	Interleukins & Interferons receptors	6.17
interleukin 1 receptor antagonist	M63099	Growth Factors, Cytokines & Chemokines	6.03
interleukin 7 receptor	M29696	Interleukins & Interferon receptors	5.13
colony stimulating factor 2 receptor, alpha, low-affinity	X17648	Growth Factors & Chemokine receptors	5.07
tumor necrosis factor receptor superfamily, member 10a	U90875	Death receptors	4.91
ephrin type-A receptor 5 precursor; tyrosine-protein kinase receptor EHK1; EPH homology kinase-1; HEK7	X95425	Other receptors (by Ligand)	4.86
interferon-gamma receptor (IFNGR)	A09781	Interleukins & Interferons receptors	4.75
interleukin 9 receptor	M84747	Interleukins & Interferons receptors	4.71
tumor necrosis factor receptor superfamily, member 5	X60592	Growth Factors & Chemokine receptors	4.58
tumor necrosis factor receptor superfamily, member 6	M67454	Death receptors	4.21
interleukin 6 receptor	X12830	Interleukins & Interferons receptors	4.16
interleukin 4 receptor	X52425	Interleukins & Interferons receptors	3.91
interferon (alpha, beta and omega) receptor 2	X77722	Interleukins & Interferons receptors	3.87

erythropoietin receptor	M60459	Growth Factors, Cytokines & Chemokines receptors	3.64
CD27-binding (Siva) protein	U82938	Death receptors-associated proteins	3.46
interleukin 8 receptor, alpha	M68932	Interleukins & Interferons receptors	3.33
chemokine (C-X-C motif), receptor 4 (fusin)	D10924	Intracellular Transducers, Effectors & Modulators	3.28
endothelin receptor type B	L06623	Growth Factors & Chemokine receptors	3.17
interleukin 2 receptor, beta	M26062	Interleukins & Interferons receptors	3.00
neurotrophic tyrosine kinase, receptor, type 1	X03541	Oncogenes & Tumor Suppressors	2.96
interleukin 1 receptor, type II	X59770	Interleukins & Interferons receptors	2.95
platelet-derived growth factor receptor, beta polypeptide	M21616	Growth Factors & Chemokine receptors	2.90
chemokine (C-C motif) receptor 2	U03882	Growth Factors & Chemokine receptors	2.86
interferon (alpha, beta and omega) receptor 1	J03171	Interleukins & Interferons receptors	2.64
interferon gamma receptor 2 (interferon gamma transducer 1)	U05875	Intracellular Transducers, Effectors & Modulators	2.53
interleukin 3 receptor, alpha (low affinity)	M74782	Interleukins & Interferons receptors	2.46
interleukin 2 receptor, gamma (severe combined immunodeficiency)	D11086	Interleukins & Interferons receptors	2.38
interferon gamma receptor 1	J03143	Interleukins & Interferons receptors	2.29
tumor necrosis factor receptor superfamily, member 1B	M32315	Death receptors	2.11
Others			
bone morphogenetic protein 3 (osteogenic)	M22491	Growth Factors, Cytokines & Chemokines	12.77
monokine induced by gamma interferon	X72755	Growth Factors, Cytokines & Chemokines	12.47
thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)	L36052	Growth Factors, Cytokines & Chemokines	12.33
connective tissue growth factor	M92934	Growth Factors, Cytokines & Chemokines	10.36
epidermal growth factor (beta-urogastrone)	X04571	Growth Factors, Cytokines & Chemokines	9.45
S100 calcium binding protein A8 (calgranulin A)	X06234	Growth Factors, Cytokines & Chemokines	7.18
KIT ligand	M59964	Growth Factors, Cytokines & Chemokines	7.07
vascular endothelial growth factor B	U48801	Growth Factors, Cytokines & Chemokines	6.36
ciliary neurotrophic factor	S72921	Growth Factors, Cytokines & Chemokines	6.32
interferon consensus sequence binding protein 1	M91196	Transcription Activators &	6.16

		Repressors	
LAG1 longevity assurance homolog 1 (S. cerevisiae)	M62302	Growth Factors, Cytokines & Chemokines	5.61
growth differentiation factor 10	D49493	Growth Factors, Cytokines & Chemokines	5.37
fms-related tyrosine kinase 3 ligand	U04806	Other Apoptosis-Associated Proteins	4.80
prohibitin	S85655	Oncogenes & Tumor Suppressors	4.67
golgi apparatus protein 1	U28811	Growth Factors & Chemokine receptors	4.65
neurotrophin 6, gamma pseudogene	M86528	Cell Signalling & Extracellular Communication Proteins	4.46
S100 calcium binding protein A9 (calgranulin B)	X06233	Growth Factors, Cytokines & Chemokines	4.28
nerve growth factor, beta polypeptide	X52599	Neuropeptides	4.17
vascular endothelial growth factor C	U43142	Growth Factors, Cytokines & Chemokines	4.01
PTK7 protein tyrosine kinase 7	U33635	Intracellular Transducers, Effectors & Modulators	3.92
hyaluronan binding protein 2	D49742	Serine Proteases	3.56
endothelin 3	J05081	Growth Factors, Cytokines & Chemokines	3.34
neurotrophin 3	X53655	Cell Signalling & Extracellular Communication Proteins	3.32
growth associated protein 43	M25667	Cell Signalling & Extracellular Communication Proteins	3.29
insulin-like growth factor binding protein 2 (36kD)	M35410	Oncogenes & Tumor Suppressors	3.21
vascular endothelial growth factor	M32977	Growth Factors, Cytokines & Chemokines	3.12
interferon regulatory factor 1	X14454	Transcription Activators & Repressors	3.09
macrophage stimulating 1 (hepatocyte growth factor-like)	M74178	Growth Factors, Cytokines & Chemokines	3.09
TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	L06139	Intracellular Transducers, Effectors & Modulators	2.78
bone morphogenetic protein 8 (osteogenic protein 2)	M97016	Growth Factors, Cytokines & Chemokines	2.61
fibroblast growth factor 2 (basic)	M27968	Growth Factors, Cytokines & Chemokines	2.60
transforming growth factor, beta-induced, 68kD	M77349	Microfilament Proteins	2.56
AXL receptor tyrosine kinase	M76125	Oncogenes & Tumor Suppressors	2.53
fibroblast growth factor 6	X63454	Growth Factors, Cytokines & Chemokines	2.50
fibroblast growth factor 8 (androgen-induced)	U36223	Growth Factors, Cytokines & Chemokines	2.42
interferon, alpha-inducible protein (clone IFI-6-16)	X02492	Functionally unclassified Proteins	2.32

RYK receptor-like tyrosine kinase	S59184	Intracellular Transducers, Effectors & Modulators	2.29
fibroblast growth factor 9 (glia-activating factor)	D14838	Growth Factors, Cytokines & Chemokines	2.27
EphB1	L40636	Other receptors (by Ligand)	2.26
platelet-derived growth factor alpha polypeptide	X06374	Growth Factors, Cytokines & Chemokines	2.24
metallothionein 3 (growth inhibitory factor (neurotrophic))	D13365	Other cell cycle Proteins	2.15

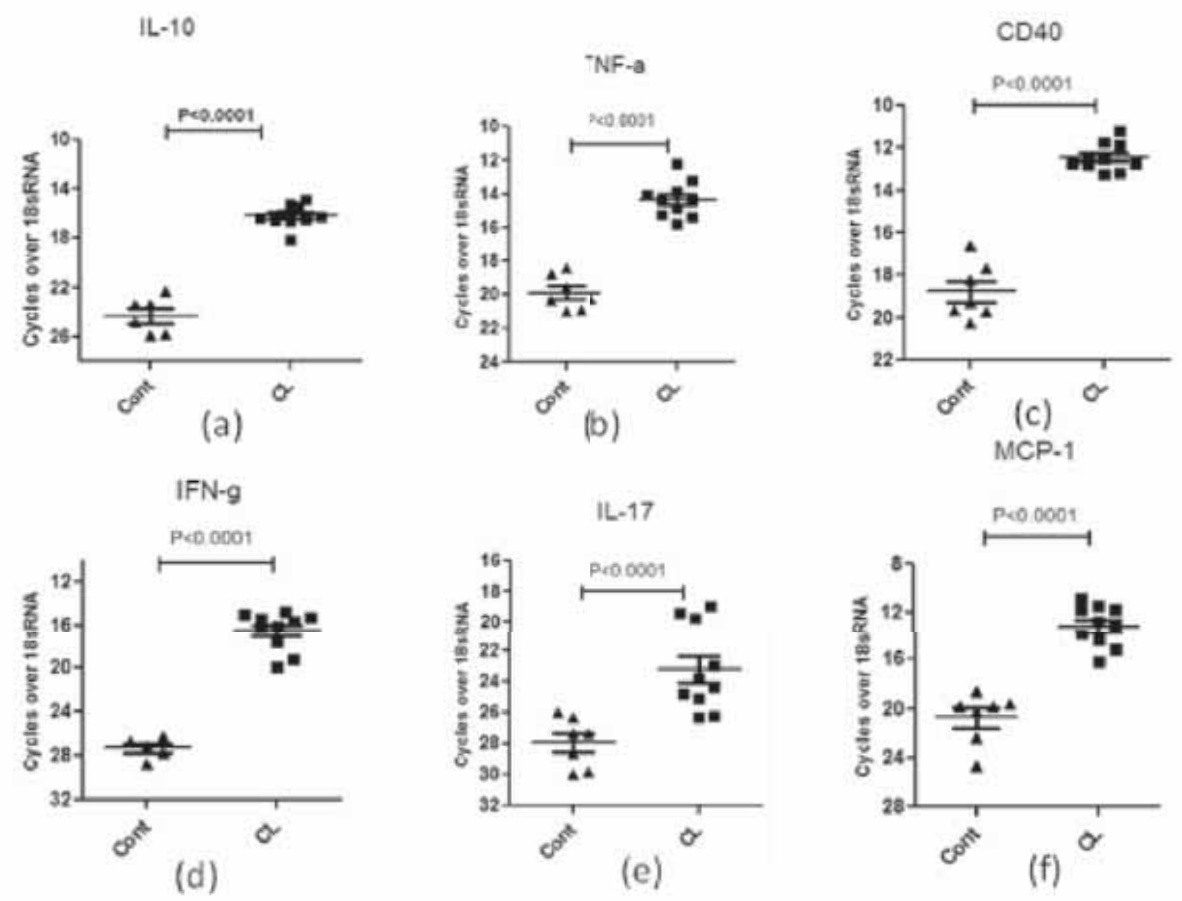


Fig. 15: Relative m RNA levels of (a) IL-10, (b) TNFa, (c) CD40, (d) IFN-g, (e) IL-17 (f) MCP in lesion tissue of CL(n=10) and control tissue individuals(n=7). Gene expression were quantified using real time PCR. Values were normalized to 18s rRNA expression

Table 7: Categorization of 28 Genes upregulated in different pathways in CL.

Nylon array genes	CL/Control	TLR	JAK/STAT	Apoptosis	Th17	Dendritic & APC	Inflammatory Response and Autoimmunity
interleukin 8	22.51	√			√	√	√
myeloproliferative leukemia virus oncogene	22.16		√				
interferon, gamma	15.32	√	√				√
interleukin 12A	14.87	√				√	
TNFa	10.81	√		√	√	√	
interleukin 10 receptor, alpha	8.63		√				
Colony stimulating factor 2 (granulocyte-macrophage)	6.16	√					
IL 13	5.50				√		
oncostatin M	4.99		√				
IL 10	3.92	√			√		√
IL 4 receptor	3.91		√				
Insulin receptor	3.80		√				
IL 17	3.77				√		
IL 1, alpha	3.75	√					√
IL 12B	3.65				√	√	
erythropoietin receptor	3.64		√				
interferon regulatory factor 1	3.09	√	√				
coagulation factor II (thrombin) receptor	2.63		√				
IL 4	2.62		√		√		
colony stimulating factor 3 (granulocyte)	2.47	√					

IL 2 receptor, gamma	2.38		√				
interferon gamma receptor 1	2.29		√		√		
TGF beta 1	2.01				√		
IL 15	1.92				√		
IL 6	1.86	√			√		√
TNF	1.64			√			
IL 2	1.49	√				√	
IL 2 receptor, alpha	1.48		√				
Total no. of Genes		**11**	**13**	**2**	**11**	**5**	**5**

No. of parasite/ reaction	MeanCt value ± SD	Intra-assay coefficient of variations
100000	9.73 ± 0.17	1.74
10000	11.6 ± 0.03	0.25
1000	14.27 ± 0.08	0.56
100	18.39 ± 0.01	0.05
10	22.17 ± 0.26	1.19
1	25.15 ± 0.18	0.71
0.1	28.1 ± 0.1	0.35

Table 8: Mean Ct values, intra-assay standard deviations (SDs) and coefficient of variations of dilution series of promastigote DNAs from L. tropica

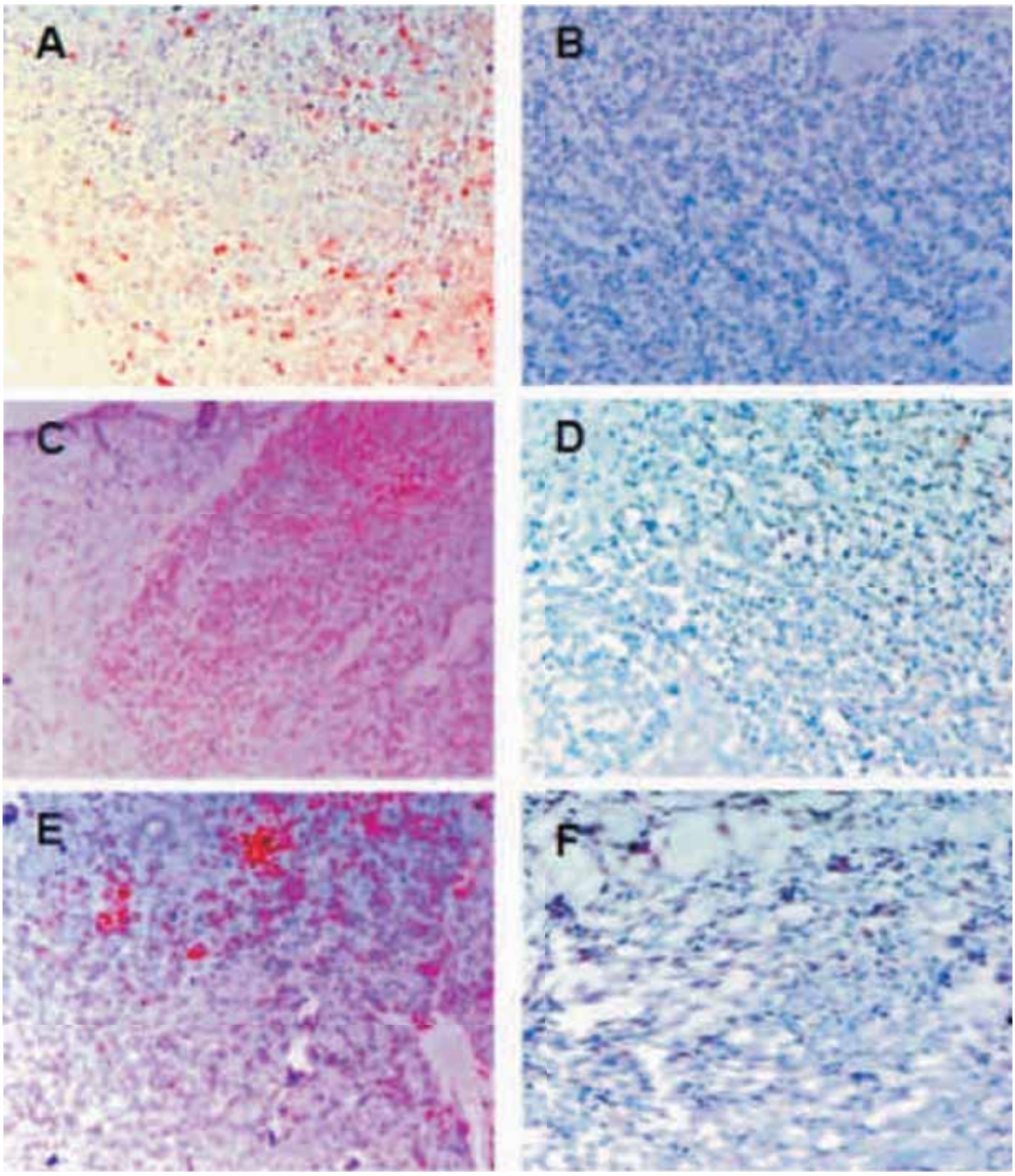


Fig. 16: Immunohistochemical analysis in tissue biopsies of CL patients for myeloperoxidase (A), IL-8 (C), inducible nitric oxide synthase (E), and in respective controls (B, D, F). Immunostaining in tissue sections was visualized at a magnification of X20.

Patients samples	Age (yrs)	Type of lesions	No. of Lesions	Duration of illness (Months)	C_T value (Mean ± Standard deviation)	Parasite/ g of dermal tissue
1	13	Erythematous plaque	1	0.5	23.1 ± 0.008	6,000
2	40	Granulomatous	1	1.5	22 ± 0.03	10,000
3	12	Ulcerated crusted plaque	4	1.5	23.2 ± 0.06	10,000
4	12	Erythematous nodule	4	2	24.3 ± 0.18	3,000
5	24	Ulcerated plaque	1	2	19 ± 0.17	90,000
6	22	Granulomatous nodule	2	2	16.4 ± 0.04	900,000
7	19	Erythematous nodule	3	2	25 ± 0.09	2,000
8	35	Erythematous plaque	4	2	22.2 ± 0.04	40,000
9	18	Ulcerated crusted plaque	5	2	15.3 ± 0.01	1,000,000
10	15	Ulcerated plaque	2	2	15.97 ± 0.02	1,000,000
11	15	Erythematous nodule	1	3	24.9 ± 0.05	1,000
12	24	Ulcerated plaque	2	3	26 ± 0.29	1,000
13	76	Erythematous ulcerated	4	3	27.3 ± 0.28	500
14	5	Erythematous nodule	1	3	27.4 ± 0.21	500
15	27	Erythematous plaque	4	4	27.4 ± 0.11	500
16	52	Erythematous ulcerated	1	4	24.6 ± 0.16	2,000
17	13	Granulomatous	1	5	25.7 ± 0.2	2,000
18	70	Granulomatous	3	5	27.8 ± 0.19	200
19	18	Granulomatous	1	6	27.2 ± 0.1	500
20	25	Erythematous ulcerated	1	6	24.1 ± 0.02	3,000
21	25	Erythematous ulcerated	1	6	24 ± 0.05	3,000
22	18	Erythematous plaque	1	6	26.9 ± 0.24	400
23	18	Erythematous ulcerated	2	7	26.9 ± 0.14	400
24	5	Erythematous plaque	1	7	26.2 ± 0.12	1,000
25	18	Erythematous nodule	7	9	26.4 ± 0.13	800
26	5	Erythematous plaque	2	12	27.4 ± 0.33	500

Table 9. Results of real-time PCR assay for parasite load in various dermal lesions of CL patients

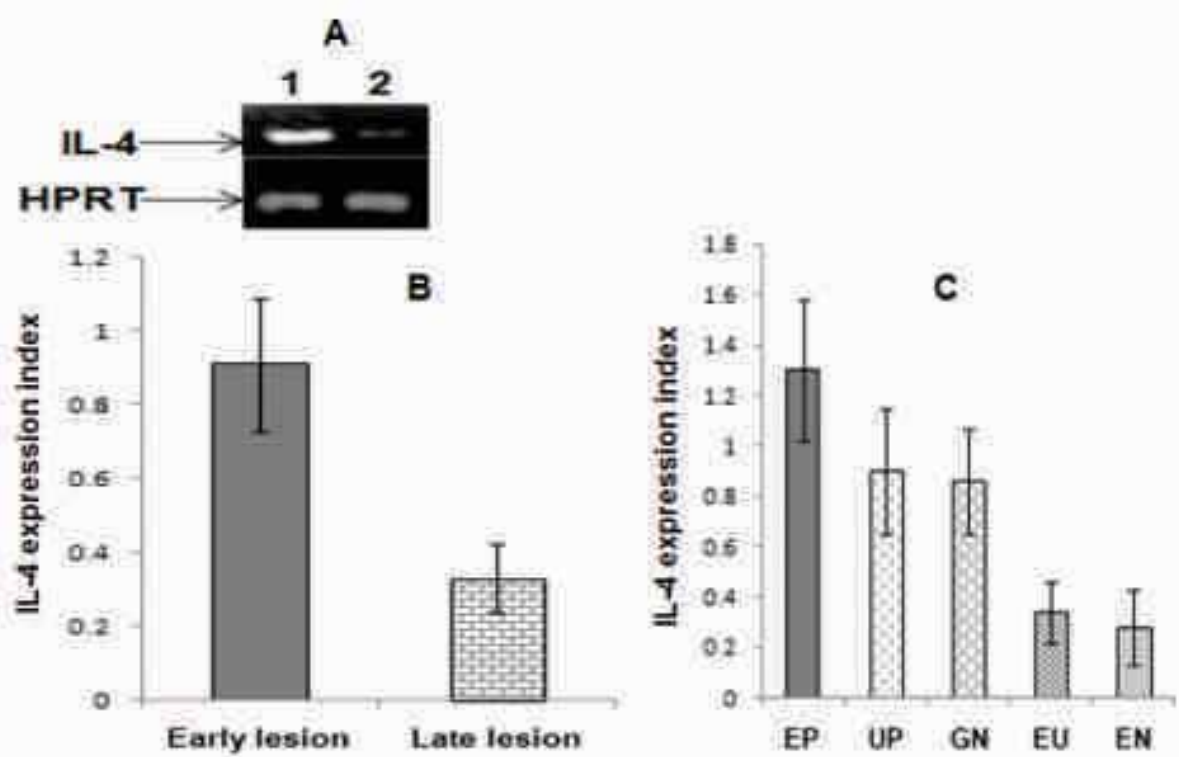


Fig. 17. Comparative assessment of intra-lesional interleukin [IL]-4 mRNA expression in patients with CL.
A. cDNA from early (lane 1) and late (lane 2) lesions was amplified with IL-4 and HPRT primers and electrophoresed on 1% agarose**.**
B. Level of IL-4 in early lesions **(<2 months**, n=14) and in late lesions (**>2 months**, n=15, ***P*<0.02**).
C. Level of IL-4 in various polymorphic forms of CL; erythematous plaque (EP, n=5), ulcerated plaque (UP, n=3) and granulomatous nodule (GN, n=3) erythematous ulcerated (EU, n=15), erythematous nodule (EN, n=5); ***P*<0.04 for EP, UP, GN compared to EU or EN**. The intensity of signal was determined by densitometry; The graph shows results as an expression index, defined as the ratio of the intensity of cytokine with respect to the HPRT gene. The bars indicate mean ± **SEs**.

5. DEVELOPMENT OF MOLECULAR TESTS FOR NONINVASIVE DIAGNOSIS AND QUANTITATIVE ASSESSMENT OF PARASITE LOAD IN KALA AZAR AND PKDL

Scientific staff	:	**Dr. Poonam Salotra, Mr. Sandeep Verma Mr. Gajendra Katara, Mr. Kumar Avishek**
In collaboration with	:	**Dr. V. Ramesh, Dr. N. S. Negi, SJH**
Duration	:	**2009-2012**

Aims, Objectives & Background:

Current diagnostic methods for KA and PKDL based on parasite detection (stained smears, culture or histopathology) are invasive and have poor sensitivity, while immunological methods (Direct Agglutination Test, enzyme-linked immunosorbent assay etc.) have limited specificity fail to distinguish between past and present infections because of persistence of antibodies and are not reliable in immune-compromised patients. Real-time PCR is reported as a promising tool for detection and quantification of various parasites including *Toxoplasma gondii*, *Trypanosoma cruzi* and *Plasmodium* spp. We report here the development of Real-time PCR assay for diagnosis as well as accurate quantification of *L. donovani* parasite in clinical samples of KA and PKDL patients.

Work done during the year:

Standardization of Real-time PCR assay: Real-time PCR based on SYBR Green I was applied for accurate quantification of the target sequence. Genus specific primers based on kDNA were designed by using Primer Express software 2.0 (*Applied Biosystems, USA*) and consisted of 5'-CTTTTCTGGTCCTCCGGGTAGG-3' (forward), and 5'-CCACCCGGCCCTATTTTACACCAA-3' (reverse). A standard curve was constructed using 10-fold serially diluted *L. donovani* parasite DNA corresponding to 10^4 to 0.1 parasite per reaction. Amplification and detection were performed using an ABI Prism 7000 sequence detection system (*Applied Biosystems, USA*). Standards, samples, and negative controls were

analyzed in triplicate for each run. A 10 µl of the PCR reaction was performed, consisting of 1X SYBR Green I PCR Master mix (Applied Biosystems, USA), 5 pmol forward primer, 5 pmol reverse primer, and 1 µl volume of DNA from the blood, BMA and tissue sample. Cycling parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. A standard curve was obtained by plotting the Ct values against each standard of known concentration parasite DNA. Each real time PCR reaction was carried out in triplicate (Fig. 18 A).

Quantification of the human albumin gene in BMA samples. In order to allow a comparison of parasite loads between the different BMA samples, we quantified the number of nucleated human cells using housekeeping gene (Albumin). We used primers, forward- 5' GCT GTC ATC TCT TGT GGG CTG T 3' (100 pmol) and reverse- 5'GGA GAG ATT TGT GTG GGC ATG ACA 3' (100 pmol) and thermal profile was identical to those of kinetoplast DNA amplification. The standard curve was established from DNA extracted from the THP-1 human monocytic cell line (Fig 18 B).

Analysis of parasite load in Blood and BMA of KA patients: At the time of diagnosis, the mean parasite load in KA blood (n=30) was 8,372 parasites/ml with a wide range of 39 parasites/ml to 2.1 x 10^5 parasites/ml (Table 10). Parasite load in BMA (n=12) indicated a mean value of 194,962 parasites/million nucleated cells with a range of 106 parasites to 10.9 x 10^5 parasites/million nucleated cells (Table 11). The microscopically positive cases had significantly higher (P=0.0152) parasite load in blood (mean=14,490 parasites/ml) in comparison with negative cases (mean=1,010 parasites/ml).

Correlation of immune response with parasite load: Expression levels of IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 were analyzed in sera of VL patients (n=29) by CBA. The level of IL-10 was significantly higher (P<0.0001) in cases with high parasite load with correlation coefficient r=0.82 (Fig. 19 A). No correlation with parasite load was observed for other cytokines examined. The levels of two major cytokines implicated in VL, IFN-γ and IL-6, with respect to parasite load are shown in Fig. 19 B.

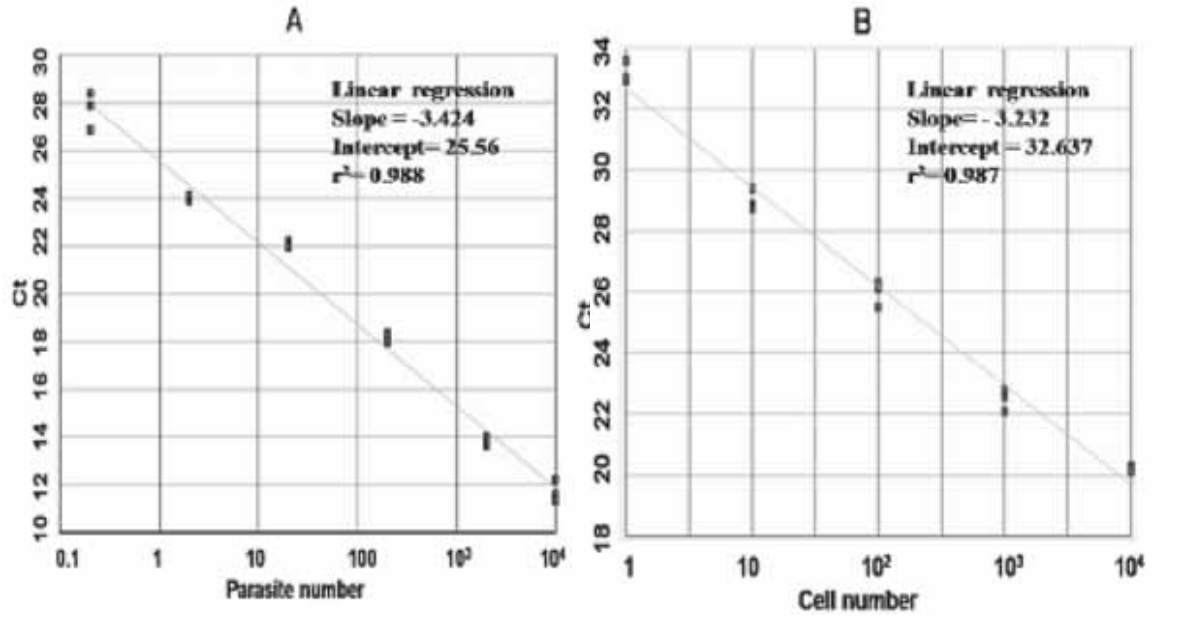


Fig. 18: Standard curves obtained with SYBR Green 1 based real time PCR.
(A) Amplification of *Leishmania* DNA expressed as the number of parasites per reaction. (B) Amplification of THP-1 cell line DNA expressed as the number of human nucleated cells per reaction.

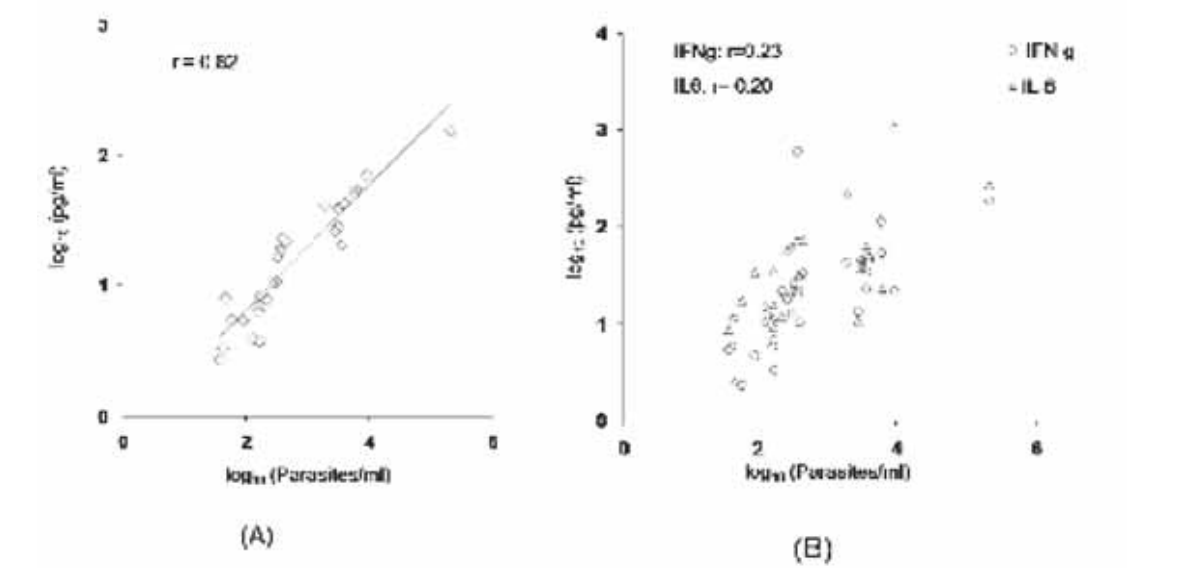


Fig. 19: Comparative assessment of IL-10, IFN-γ, IL-6 and parasitic load in blood samples of VL patients.
The serum levels (pg/ml) of IL10 (Panel A) and IFN-γ and IL-6 (Panel B) were measured by CBA and parasite load (Parasites/ml) determined by real-time PCR. Diagonal line represents linear regression.

Patient no.	Age/Sex	Microscopy with BMA	Region	Ct ±S.D.	Parasite load/ml Blood
1	24/M	N	H	29.70±0.24	39
2	42/M	N	L	29.47±0.36	39
3	23/F	N	H	29.31±0.3	43
4	6/M	N	L	29.17±0.49	48
5	18/F	ND	L	28.81±0.28	60
6	42/F	P	H	28.17±0.36	92
7	16/M	N	H	27.65±0.37	131
8	11/M	N	H	27.31±0.67	164
9	7/M	N	L	27.24±0.18	171
10	13/M	P	H	27.23±0.31	172
11	25/F	P	L	27.22±0.23	173
12	12/M	P	H	26.83±0.15	225
13	48/M	P	L	26.55±0.16	270
14	17/F	N	H	26.50±1.09	281
15	63/M	P	H	26.34±0.43	312
16	17/M	N	L	26.23±1.0	336
17	12/M	P	L	26.07±0.66	372
18	4/F	N	H	26.05±0.14	377
19	15/M	N	L	25.97±0.59	399
20	4/M	P	H	25.79±0.77	450
21	11/M	N	L	23.59±0.33	1939
22	8/F	P	H	23.04±0.87	2801
23	6/F	P	L	22.87±0.50	3130
24	7/M	P	H	22.85±0.38	3165
25	12/M	P	H	22.63±0.70	3663
26	35/M	P	H	22.61±0.31	3729
27	7/M	P	L	22.49±0.8	4021
28	40/F	P	H	21.91±0.30	5939
29	19/M	P	L	21.84±0.45	6195
30	9/F	N	H	21.25±0.19	9171
31	14/F	P	H	16.53±0.48	211613

Table 10: Parasite load in blood samples of VL patients from low and high endemicity regions.

M- Male, F-Female, P- Positive, N- Negative, ND- Not Determined, H- region of High endemicity, L- region of Low endemicity, Ct- Threshold cycle , S.D.- Standard deviation

S. No.	Patient no.	Microscopy with BMA	Region	Parasite load per million nucleated cells
1	2	N	L	431
2	3	N	H	715
3	4	N	L	106
4	7	N	H	332
5	10	P	H	2849
6	13	P	L	53818
7	14	N	H	444518
8	15	P	H	1091315
9	16	N	L	23772
10	17	P	L	31857
11	19	N	L	6720
12	26	P	H	683106

Table 11: Parasite load in BMA samples of VL patients from low and high endemicity regions. N-Negative, P-Positive, H- region of High endemicity, L- region of Low endemicity

Parasite load in PKDL tissues: *Leishmania* parasite was detected in lesion tissue of the 25 patients of PKDL. The mean value of parasite load was 9,502 parasites/µg tissue DNA with range of 2 to 144,586 parasites/µg tissue DNA (Table 12). Comparative assessment of parasite load in various clinical polymorphic forms of PKDL revealed significantly higher (P<0.0001) parasite load in nodular lesions (n=12) with a mean of 19,586 parasites/µg tissue DNA as compared to parasite load in papular/macular lesions (n=13), which had a mean of 193 parasites/µg tissue DNA.

Patient no.	Age/Sex	Region	Clinical presentation	Duration of chronicity	Slit Smear	Ct ± S.D.	Parasite Load/µg tissue DNA
1	45/M	H	Papular	2 Months	N	25.02 ± 0.12	39
2	10/M	H	Papular	6 Months	N	22.44 ± 0.07	82
3	25/M	H	Papular	7 Months	N	21.96 ± 0.38	50
4	12/M	H	Macular	1.5 Year	P	22.69 ± 0.64	82
5	20/F	H	Papular	2 Years	P	22.25 ± 0.23	105
6	16/M	L	Macular	3 Years	N	25.61 ± 0.45	14
7	65/M	H	Papular	3 Years	N	25.15 ± 0.96	2
8	18/M	H	Papular	3 Years	P	23.82 ± 1.29	39
9	60/M	H	Papular	3 Years	N	19.55 ± 0.76	603
10	21/M	H	Papular	4 Years	N	25.71 ± 1.24	11
11	19/M	L	Papular	4 Years	P	21.79 ± 0.29	461
12	18/M	H	Papular	6 Years	P	21.64 ± 0.53	1022
13	22/M	H	Papular	10 Years	N	28.73 ± 0.59	2
14	19/M	L	Nodular	1.5 Years	P	19.58 ± 0.04	522
15	30/F	H	Nodular	2 Years	N	16.43 ± 0.23	3022
16	14/M	H	Nodular	2.5 Years	P	19.48 ± 0.98	719
17	17/M	H	Nodular	4 Years	P	20.13 ± 0.07	966
18	27/M	L	Nodular	4 Years	P	13.43 ± 0.20	144586
19	35/M	H	Nodular	6 Years	P	19.15 ± 0.40	499
20	35/M	H	Nodular	6 Years	P	16.30 ± 0.82	5180
21	28/M	H	Nodular	6 Years	N	16.62 ± 0.52	3926
22	28/M	L	Nodular	7 Years	N	16.57 ± 1.26	1512
23	52/M	H	Nodular	7 Years	ND	16.18 ± 0.60	14870
24	19/M	H	Nodular	7 Years	N	15.36 ± 0.37	12945
25	26/F	H	Nodular	10 Years	P	11.61 ± 0.75	46286

M- Male, F-Female, P- Positive, N- Negative, ND- Not Determined, H- region of High endemicity, L- region of low endemicity

Table 12: Parasite load in dermal lesions in different clinical presentations of PKDL

Future work

We plan to extend the study the Real time PCR diagnostic method for diagnosis of PKDL by using slit aspirate as sample, which is less invasive and will be preferable particularly in paedriatic cases. Further, we propose to evaluate the assay as a prognostic tool by monitoring the parasite load after therapy.

Adult Stem Cell Biology

Adult Stem Cell Biology

1. OPTIMAL ATTENUATION CONDITIONS FOR 3T3 FIBROBLASTS FOR USE AS FEEDER CELLS

Scientific staff	:	**Dr. L. K. Yerneni, Mr. Ashok Kumar**
Technical staff	:	**Mr. Bijender**
Duration	:	**2006-2009**

Aims, Objectives & Background:

The adult and embryonic stem cells are effectively established *in vitro* using feeder cells that are growth arrested by either gamma irradiation or exposure to mitomycin C. However, an abrupt overgrowth of feeders as a consequence of failed attenuation while using mitomycin C is a serious setback. Since mitomycin C approach is convenient and does not require high establishment and operational costs as with gamma-irradiation, the same has been pursued to grow Cultured Epithelial Autografts (CEA). Earlier it was shown that mitomycin C was effective as an attenuating agent on 3T3 fibroblasts in a 'numerical' dose-dependent manner. Subsequent experiments carried out indicated that such differentially growth arrested feeders proportionately stimulated the proliferation of human epidermal keratinocyte stem cells. The aim was to study the effectiveness of various numerical doses of mitomycin C in the medium on 3T3 fibroblast in stimulating human epidermal keratinocyte stem cells.

Work done during the year:

The successful experimental evidences in the direction of identifying a fool-proof method of growing human epidermal keratinocyte stem cells were filed as a **patent**. In this patent description, it was shown that differentially attenuated feeders produced by titrating with arithmetically derived numerical doses of mitomycin C brought about irreversible attenuation and increase in keratinocyte cell proliferation in a dose dependant manner as revealed by differential counts in a co-culture system performed during 9-days period.

The BrdU incorporation studies undertaken in continuation, revealed a significantly ($P<0.01$) higher proliferation index in those keratinocyte cultures grown in presence of effective & irreversible feeders compared to less effective or reversible feeders (Fig. 1). The reversal of growth arrest finally caused contamination of keratinocytes consequently ending in stunted growth of keratinoctytes with proliferating 3T3 cells.

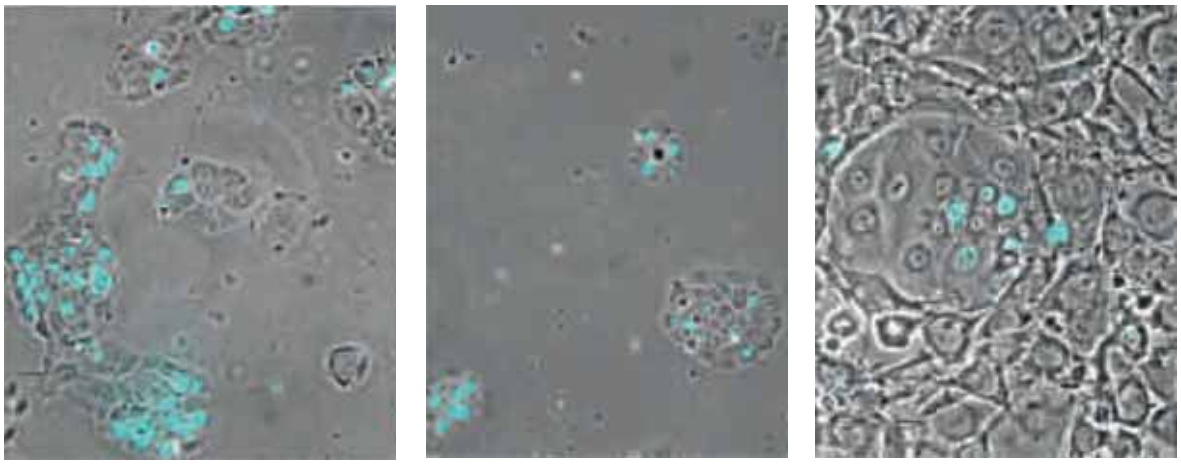


Fig. 1: Human epidermal keratinocytes stained with anti-BrdU antibody after they were grown in presence of feeders from effective & irreversible feeders (left), less effective & irreversible feeders (middle) and reversible feeders (right) in which feeder cells are persistent

The results are concomitant with keratinocyte-3T3 differential cell count experiments. In continuation, research work towards characterization of CEA to be produced by employing the innovative method on more stable feeder cell lines will be initiated as part of the newly sanctioned project entitled *"A novel arithmetic approach for fool-proof production of growth arrest in 3T3 cells suitable for human epidermal culture."*

2. A NOVEL ARITHMETIC APPROACH FOR FOOL-PROOF PRODUCTION OF GROWTH ARREST IN 3T3 CELLS SUITABLE FOR HUMAN EPIDERMAL CULTURE

Scientific staff	:	**Dr. L. K. Yerneni, Mr. Rishi Man Chug**
Technical staff	:	**Mr. Bijender**
Duration	:	**2010-2013**

Aims, Objectives & Background:

The clinical application of ex-vivo expanded human keratinocytes using the basic feeder cell based technique with our own in-house innovative research inputs to form Cultured Epidermal Autografts (CEA), requires development and practice of safer application protocol. There is a requirement for large-scale sophisticated facilities and services like a custom designed cGMP and cGTP compliant culture facility and formulation of validation protocols, SOPs for clean operations; comprehensive culture technique involving feeders and epidermal keratinocytes; histological; cytological; microbiological; serological and biochemical QC protocols; waste disposal, grafting procedure; etc.

Work done during the year:

Further development of CEA prototype into a workable model using the 'unique' process (patent filed) of attenuation on Swiss albino 3T3 cells, which are ideal for large-scale clinical application, is initiated through the recently sanctioned extramural project from ICMR. The research work involves estimation of a range of values for effective numerical dosing by our unique 'Density Gradation' technique as verified by the rate of cellular extinction. This will be followed by pinpointing a range of numerical dose values through arithmetic conversion employing our unique 'Volumetric Titration' method. This will lead to co-culture of keratinocytes with variedly generated batches of feeders and comparative characterization of keratinocytes > colonies > epidermis (CEA). So far Density Gradation method is partly accomplished.

Looking at the complexity of clinical trial with CEA, it is preferable to involve a commercial organization for further development of the prototype and the grafting with the inventor's technical collaboration. The developed technique is a prototype requiring further development of large-scale expansion of epithelia followed by phased autologous grafting in burns patients. Simultaneously, this activity is presently taken up by IPR division of ICMR headquarters through National Research Development Corporation (NRDC), which is also pursuing efforts to find a suitable Pharma partner towards continuation of work on further development of prototype as well as clinical trials. In the meantime, specifications to call 'Expression Of Interest' for setting up a GMP facility as part of our internal endeavor towards clinical trial are being framed with permission from ICMR.

3. INVESTIGATION INTO THE UTILITY OF A PATENTED SYNTHETIC THERMO-REVERSIBLE HYDROGEL POLYMER AS SUPPORTIVE MATRIX TOWARDS THE DEVELOPMENT OF 3- D COMPOSITE SKIN FOR APPLICATION IN WOUND HEALING AND OTHER DERMATOLOGICAL DISORDERS

Scientific staff	:	**Dr. L.K . Yerneni, Mr. Rishi Man Chug**
Collaborators	:	**Dr. Abraham Samuel, Prof. Yuichi Mori, Dr. Hiroshi Yoshioka**
Duration	:	**2007-2010**

Aims, Objectives & Background:

Tissue engineering involving *in vitro* construction of 3-dimensional cellular structures using various matrix materials is one of the recent approaches for wound healing. A synthetic non-toxic thermoreversible gelation polymer hydrogel (TGP), Mebiol gel, has been proven to support hepatic progenietor cells, corneal limbal cells and several other cell lines. Mebiol gel is being tested to support the growth and differentiation of epidermal keratinocyte stem cells which could pave way to their large scale expansion under xenogeneic-free culture conditions.

This study is undertaken to verify the potential of Mebiol gel (TGP) to support without the animal sourced feeder cells the growth and differentiation of primary human epidermal keratinocyte stem cells.

Work done during the year:

Evidence for keratinocyte proliferation in Gel-Sandwich :

Keratinocytes 2.5 X 10^4 from a running P4 culture were suspended in 10 µl 1X medium and laid on a 200 µl pre-cast gel prepared in 2X in a culture insert (with 1 µm pore size, *Greiner*) that became a disc of about 25 mm diameter followed by entombing in 150 µl similarly prepared gel. The culture plate was observed every 3 days. The primary cast gel was about 10 mm in diameter; the cells could be comfortably placed at the center followed by incubation for 2-hour period and finally entombing in 150 µl gel that spread out into a top disc of about 5 mm diameter (Fig. 2).

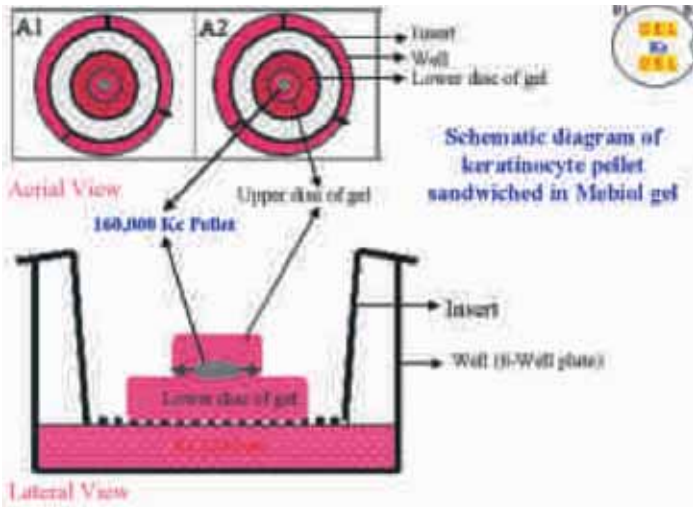


Fig. 2: Aerial and lateral views of schematic diagram of keratinocytes pellet sandwich discs of TGP in a culture insert.

The attenuated NIH 3T3 cells were similarly sandwiched. These 'sandwich' experiments revealed marked keratinocyte migrations towards the periphery of the discs (Fig. 3), while no such movements were noticed with 3T3 cells (Fig. 4).These migrations were predominantly by the small-sized keratinocytes measured to be ≅ 5µm by using manual measurements tool of Image Pro Express software (*Media Cybernetics*).

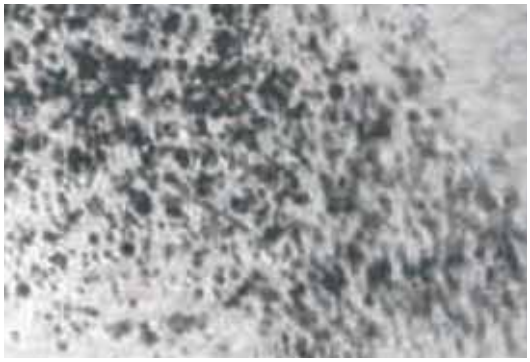


Fig. 3: A stream of keratinocytes migrating towards the periphery of discs of TGP in which 2.5 x 10^3 cells were embedded.

Fig. 4: An even margin of 3T3 cell-pellet sandwiched in between 2 discs of gelated TGP without cell migrations.

In order to resolve the peak cell proliferation time point, an additional experiment using the 'Sandwich in culture insert model' was performed with 3-day cell counts over a period of 12 days. Kertinocytes and normal 3T3 cells at a density of 2.5 x 10^4 per well were separately sandwiched in TGP in Culture Inserts (1uM pore) and viable (trypan exlusion method) as well as total Kc / 3T3 were counted periodically.

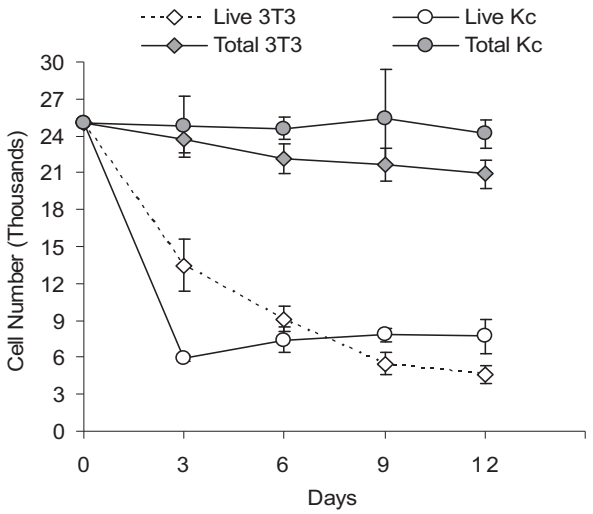


Fig. 5: Line Graph showing live and total (live + dead) number of keratinocytes and 3T3 embedded in between 2 discs of gelated TGP over a period of 12 days.

It was found that viable 3T3 cell number gradually decreased while their total cell number showed a significant ($P<0.05$) fall in the total cell count by day 12 suggesting

degeneration of 3T3 by apoptosis (Fig. 5). On the other hand, keratinocytes exhibited rapid fall in their number by day 3 followed by a significant ($P < 0.01$ in comparison to day-3 cell number) transient elevation in viable cell number with a peak on day 9, while the total cell count remained more or less consistent. The viable cells were predominantly (70%) small-sized keratinocytes that measured less than 5µm in diameter while the dead cells comparatively constituted large-sized cells measuring more than 7 µm in diameter. The results are indicative of selective proliferation of keratinocytes of smaller size by TGP after an initial elimination of large sized cells.

Failure in isolating newly formed keratinocytes:

Attempts were made to isolate this population by collagen type IV adhesiveness and by clonal propagation using feeder-based cell culture system for further characterization. Keratinocytes were plated at densities of 1 x 10^5 and 2 x 10^5 per sandwich in a loose configuration in order to maximize the output of selectively proliferating cells and allowed to grow for 12 days. At the end of 6 days, new cells appeared around the loose clusters of plated keratinocytes in well-circumscribed areas (Fig. 6 left) that further enlarged by day 12 (Fig. 6 right). However, the standard method of collecting these cells by the addition of cold medium to the solidified TGP in order to bring the gel to 'sol' state followed by centrifugation did not result in the isolation of these cells in large number as indicated by the poor colony formation on feeder layer. The well circumscribed areas were found to collapse soon after the addition of cold medium due to reasons not yet clear and no such phenomenon was observed while recovering the 3T3 cells or low initial seeding density of 25,000 keratinocytes per sandwich.

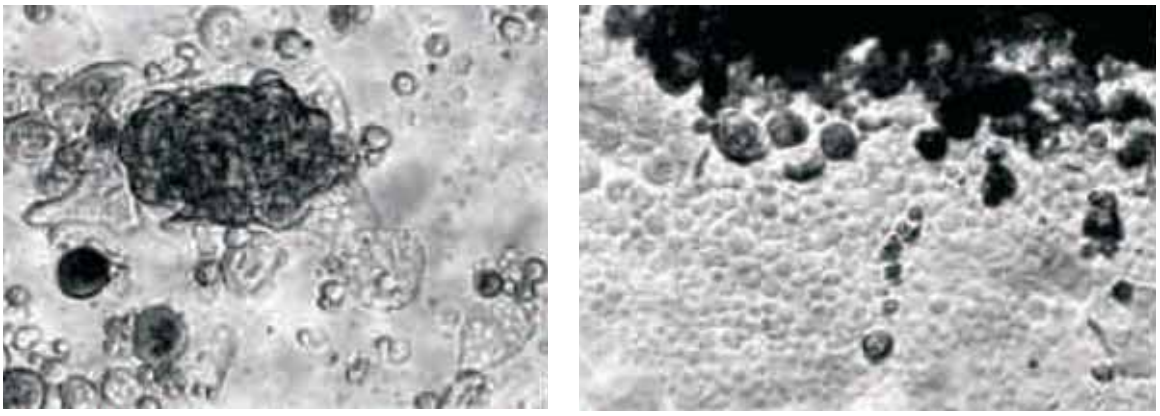


Fig. 6: Appearance of newly formed keratinocytes (light cells) in well-circumscribed regions arising from underside of the plated cell clusters (dark clusters) at the end of 6 days (Left) and 12 days (Right) post-plating of 1 x 10^5 cells in between 2 discs of Gelating TGP.

Looking at the failure of extracting the newly formed keratinocytes, it is now planned to first isolate the feeder-grown keratinocytes into three clonally distinct populations, namely the Rapidly Adhering (RA) cells, Transiently Adhering (TA) cells and Never Adhering (NA) cells by using type IV collagen-adherence assay (*Juxue et al. 2008, Cell Research 18:360–371*) and sandwich these populations in Mebiol gel to identify the class of cells proliferating in Mebiol gel.

Environmental Toxicology

Environment Toxicology

1. HEALTH HAZARDS OF PHTHALATES VIS-À-VIS IDIOPATHIC MALE INFERTILITY

Scientific staff	:	**Dr. Arun Kumar Jain, Ms. Rashmi Tomar**
In collaboration with	:	**Dr. Sunil Kumar, NIOH, Ahmedabad Dr. N. K. Mohanty, SJH, New Delhi**
Technical Staff	:	**Mrs. Asha Rani Srivastava, Mrs. Anita Bhatia, Mr. Suresh, Mr. Manoj**
Duration	:	**2006-2011**

Aims, Objectives & Background:

Phthalates are family of industrial compounds employed as plastic softeners in many different consumer products, including children's toys and medical devices. In 2002, the non-profit Environmental Working Group launched campaign to raise awareness about the dangers of phthalates also used as solvents in many cosmetics, viz.: hair sprays, deodorants, nail polishes and perfumes. Phthalates can be absorbed through the skin or inhaled and several animal studies have shown that these can cause damage to the liver, kidneys, lungs and reproductive systems. A recent study by the U.S. Centers for Disease Control and Prevention (CDC) found that five percent of women between ages 20 and 40 had upto 45 times more phthalates in their bodies than researchers initially hypothesized. CDC found phthalates in virtually every person tested, but the largest concentrations - 20 times higher than the rest of the population -- were found in women of child-bearing age.

Endocrine disruptive nature of the some of the phthalate compounds may pose possible threat to the endocrine and male reproductive system of animals as well as human beings. The relative binding affinity of DBP for estrogen receptor was reported to be (1/36,000 that of 17 β-

estradiol (E2)) in a binding assay using uterine homogenate from immature female SD rats and 1/28,000 that of E2 in a binding assay using human estrogen receptors expressed in Sf9/Baculovirus. In the NTP-CERHR (Center for Evaluation of Risks to Human Reproduction) Expert Panel Report on DBP, it was described that various malformations seen in male F1 animals from the pregnant rats given DBP orally were not mediated by androgen receptors but were due to inhibition of testosterone biosynthesis. The obvious relationship of endocrine and reproductive toxicities in humans with the exposure to DBP has not been described in the literature. Thus, this study has been taken up with a view to study correlation between morphological changes in the sperm and levels of marker testicular enzymes in blood on one hand and concentration of phthalates and their metabolites in urine of infertile males on the other hand.

Work done during the year:

A total of 146 patients, who came for infertility treatment to Urology OPD of Safdarjang Hospital, New Delhi were prospectively enrolled for this study. Twenty one healthy fertile men whose partners had a time-to pregnancy (TTP) of ⩽ 12 months were enrolled as controls.

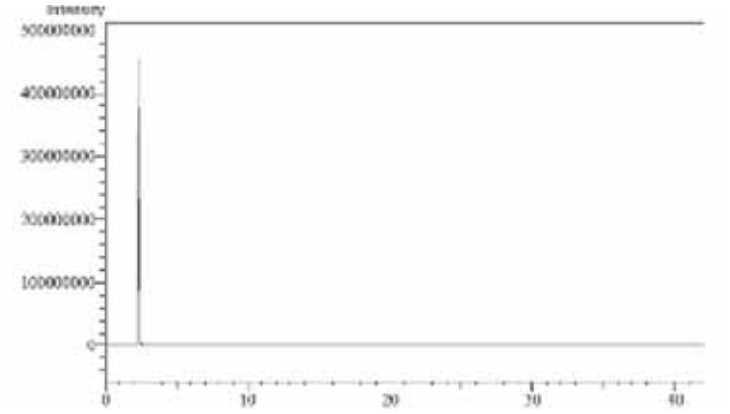
Based on reported occupation and environmental conditions, the patients as well as controls were initially divided into 3 groups viz.: High, medium and low risk. Subsequently, based on analysis of blood serum by Gas Chromatography, all the subjects were re-grouped into Phthalate positive and Phthalate Negative Groups.

	Phthalate Negative		Phthalate Positive	
	Infertile	Fertile	Infertile	Fertile
Normozoospermia	3	11	11	10
Azoospermia	16	0	31	0
Oligospermia	5	0	22	0
Asthenozoospermia	0	0	12	0
Teratozoospermia	4	0	7	0
Oligoasthenozoospermia	5	0	14	0
Oligoteratozoospermia	0	0	5	0
Asthenoteratozoospermia	0	0	2	0
Oligoasthenoteratozoospermia	5	0	4	0
Total	**38**	**11**	**108**	**10**

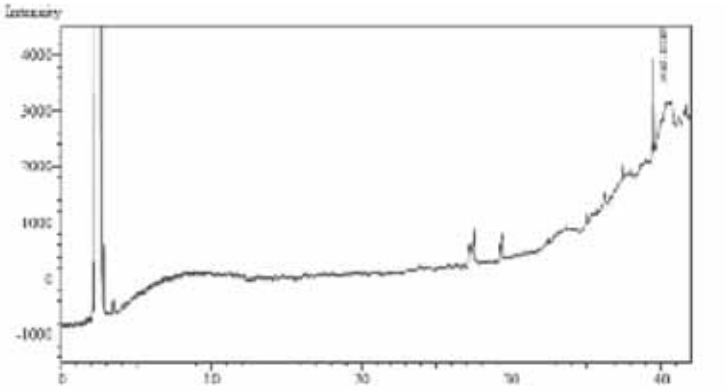
The sperm count was more than 30 million per millilitre of semen in all the controls in comparison to average sperm count of less than 20 million per millilitre in infertile patients. While all the control samples showed normal motility and morphology, the semen quantity, sperm motility and vitality was significantly reduced in infertile group. However, these parameters showed only minor differences between phthalate positive and phthalate negative groups. Mean testosterone levels were lower in patient group (5.8±2.2 ng/ml) as compared to controls (6.0±1.66 ng/ml). The decrease was more significant in phthalate positive groups (mean 4.9 ng/ml). In contrast, the estradiol levels were elevated in patients (mean 14.6 pg/ml) in comparison to healthy controls (mean 6.2 pg/ml).

The ultrastructural studies revealed the altered micro-tubular arrangement in the tail of the sperms, presence of vacuoles in head cap, abnormalities in the acrosome, head shape and tail membranes, attachments of cytoplasmic body on sperm body and head of the infertile patients in comparison with fertile male.

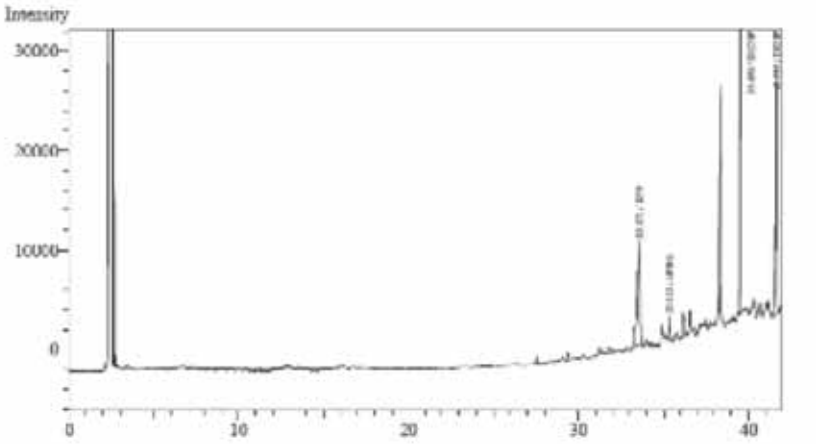
Estimation of phthalates by Gas Chromatography revealed presence of one or more phthalates in 74% of infertile patients as compared to 47.6% of control subjects. Number of phthalate peaks observed in phthalate positive patients ranged from 2 to 14 phthalates. Most common phthalate was DICHP which was present in 65.5% of infertile patients and 38.15 of fertile controls. DBP, DEHP and BEHIP were present in 47.3%, 30.4% and 27.7% of infertile patients respectively while in fertile men, next three most common phthalates were BIOP (19%), DEHP & DPP (14.3%). While DIHP and DMP were not recorded in fertile men, DDIP, DIOP, IBCHP, DINP and BEHIP were observed in only 4.8% samples. Amongst these phthalates, BEHIP (27.7%) followed by DMP (13.5%) and DIOP (12.2%) were most prominent. Concentration-wise, average concentration of DIBP, DIOP and BPBG was significantly higher in infertile men compared to fertile men. The study is being continued.



Control with no phthalate peak



Control with one phthalate peak



Infertile Patient with multiple phthalate peaks

2. ASSESSMENT OF PESTICIDE EXPOSURE IN TEA GARDEN WORKERS OF NORTH-EASTERN STATE OF INDIA (HEBM)

Scientific staff	:	**Dr. Arun Kumar Jain, Dr. S. Sriramachari, Dr. Madhu Bhatnagar, Mr. Shashi N. Kumar**
In collaboration with	:	**Dr. S. K. Sharma & Dr. A. M. Khan, RMRC, Dr. Deepa Borgohain, Assam Medical College, Dibrugarh, Dr. Sudha Salhan, SJH, New Delhi**
Technical Staff	:	**Mr. Manoj**
Duration	:	**2008-2011**

Aims, Objectives & Background:

India is one of the major tea producing countries with north eastern states accounting for more than 50% of total tea production. It is reported that over 300 species of arthropods and 58 species of fungi infest tea cultivation in India alone necessitating the continuous application of heavy doses of several pesticides. Poisoning from pesticides affects more than 0.07 million farmers and workers every day. Annually, an estimated 25 million workers suffer from pesticide poisoning throughout the world. Farmers and agricultural workers are exposed to pesticides directly when they are mixing and spraying these pesticides, especially so in developing countries such as Asia. Every year, about 3 million people are poisoned around the world and 200,000 die from pesticide. More than 50% of over 1.1 million workers in the labour intensive tea industry are women. The workers involved in activities such as pesticides spray, mixing and storing, rarely use any safeguard, take food without washing of hands and may even use jerry cans and bags (emptied after usage of pesticide) for storage of household food grain. Present study has been designed to assess the pesticide exposure of tea garden workers in North Eastern States of India using placenta and blood of women.

Work done during the year:

Screening of the pregnant women.

Study Population:

The study population comprised of the following groups.

1. **Exposed Individuals**:
   a. Women involved in tea farming activities in Dibrugarh.
   b. Women involved in agricultural activities other than tea farming in Dibrugarh.
2. **Unexposed Individuals**:
   a. Women in Dibrugarh region not involved in any agricultural or tea farming activities. These included pregnant women admitted / coming for delivery in Assam Medical College, Dibrugarh and
   b. Women from urban Delhi region not involved in any agricultural or tea farming activities. These included pregnant women from Delhi NCR admitted / coming for delivery in Safdarjung hospital, New Delhi.

The sample population was screened for inclusion as well as exclusion criterion and relevant portion of proforma questionnaire were filled. Samples of placenta and blood (maternal and cord) were collected at the time of delivery

	Group	No. of Subjects Recruited	Samples Collected	Location
Exposed Individuals	Women involved in Tea farming activities in Dibrugarh	89	Placenta Maternal Blood Cord Blood	Assam Medical College, Dibrugarh
	Women involved in Agricultural activities other than tea farming in Dibrugarh	19	Placenta Maternal Blood Cord Blood	Assam Medical College, Dibrugarh
Total		**108**		

Unexposed Individuals	Women in Dibrugarh Region not involved in any agricultural or tea farming activities	59	Placenta Maternal Blood Cord Blood	Assam Medical College, Dibrugarh
	Women from urban Delhi region not involved in any agricultural or tea farming activities	50	Placenta Maternal Blood Cord Blood	Safdarjang Hospital, New Delhi
Total		**109**		

Table 1: Details of sample collected

Analysis:

Several hundred pesticides of different chemical nature are used for agriculture and non - agriculture purpose throughout the world today. Because of their widespread use coupled with unknown exposure conditions, multi-residue analytical methods are needed for analyzing pesticide residues in tissue sample extracts.

In the first year, a cocktail of commonly used pesticides, namely deltametherin. fenverate, flufenacet, endosulphan, malathion, tebuconazole, dimethoate were selected for multi-residue analysis and the analytical conditions for HPLC were optimized and standardized so as to distinctly separate the selected pollutants.

During the year under report, the list of pesticides and organic pollutants was elaborated to sixty pollutants consisting of organochlorine, organophosphorus, pyrethroid, herbicide, fungicide and carbamate pesticides as well as polyaromatic hydrocarbons (Table 2).

S. No	Pesticides		
1	Endosulfan	31	Flufenacet
2	Endosulfan Sulphate	32	Profenphos
3	Dieldrine	33	Glufosinate
4	Deet	34	Hexaconazole
5	Tiram	35	Mancozeb
6	Ziram	36	Parathion methyl
7	Dichlorvos	37	Tebuconazole

8	Ethion	38	Acetamipride
9	Lindane	39	Aterazine
10	Heptchloar	40	Propargite
11	Dicofol	41	Chlordane
12	DDT	42	Acetachloar
13	Propoxur	43	Fenzaquin
14	Monochrotophos	44	Oxyflurofen
15	Chlorpyriphos	45	Phosphamidon
16	Malathion	46	Trizophos
17	Primiphos methyl	47	Acetamipride
18	Permethrin	48	Napthalene
19	Phosalone	49	Acenapthaylene
20	Dimethoate	50	Anthracene
21	Deltamethrin	51	Benzo(a) Anthracene
22	Cypermetharin	53	Benzo(a) Pyrene
23	Fenvalrate	53	Benzo(b) fluanthracene
24	Ethion	54	Benzo(ghi) perylene
25	Glyphosate	55	Benzo (k) fluanthracene
26	Paraquat	56	Fluorene
27	Acephate	57	Phenathrene
28	Propaconizole	58	Pyrene
29	Endrine	59	Fluoranthene
30	Lamda cyhalothrin	60	Dibenz (ah)anthracene

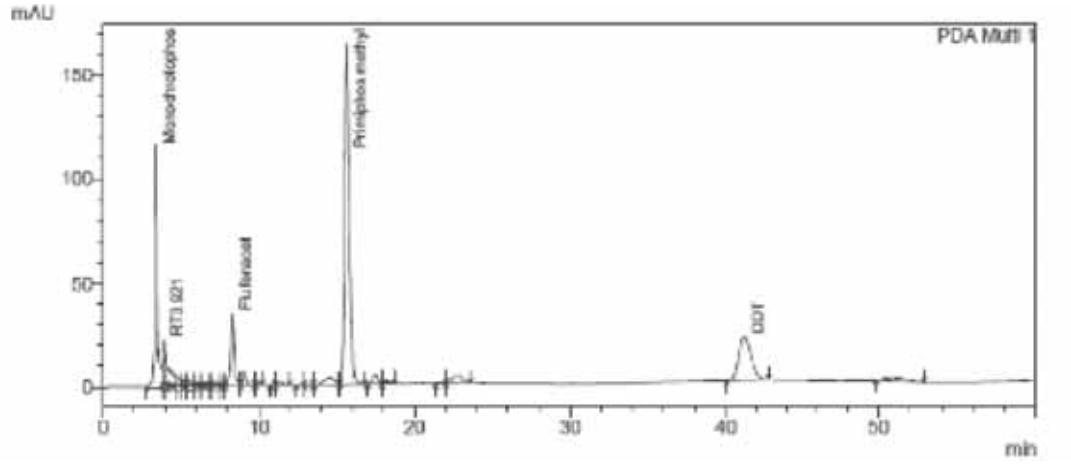
Table 2: List of Pesticides and Organic Pollutants

Chromatography:

The extracts obtained from placental tissue as per the standardized protocol were separated and analyzed for presence of the pesticide residues by reversed phase HPLC using a Shimadzu high performance liquid chromatography System Model LC-20AD , equipped with a variable wavelength UV-Visible detector (S PD-20A) and a Luna C18 (250 x 4.6 mm, 5 µm) analytical column from Phenomenex (USA). Shimadzu SPD-M20A Diode Array Detector spectrophotometer was employed for the measurement of the UV-Visible spectra at 190-800 nm. The analyte solution was directly injected in the HPLC system using a 70:30 acetonitrile: water mobile phase and a 0.8mL /min flow rate. The column temperature was kept at 40°C.

Pesticides detected: A total of 50 extracts of placental tissue from Delhi and 60 extracts from Dibrugarh have been analysed by HPLC. The pesticides recognized in Delhi regions and Dibrugarh are as follows:

	Pesticides Detected				
S. No	Delhi			Dibrugarh	
1	Malathion	14	Tebuconazole	1	Dimethoate
2	Monocrotophos	15	Paraquat	2	Tebuconazole
3	Glyphosate	16	Cypermetharin	3	Flufenacet
4	Chlorpyriphos		Flufenacet	4	Malathion
5	Deltametharin	17	Naphthalene	5	Pyrene
6	Atrazine	18	Ethion	6	DDT
7	Chlorpyriphos	19	Phosalone	7	Fenzaquine
8	Fenvalerate	20	Quinalphos	8	Benzo(a) Pyrene
9	Endosulfan sulphate	21	Primiphos methyl	9	Glyphosate
10	DDT	22	Pyrene	10	DEET
11	Dicofol	23	Dibenzo anthracene	11	Glufosinate
12	Dimethoate			12	Phosalone
13	Delta BHC				



HPLC Chromatogram of Placental extract showing pesticide peaks

3. DYNAMICS OF ULTRASTRUCTURAL AND IMMUNOLOGICAL EVENTS IN RESPONSE TO TREATMENT IN DIFFERENT FORMS OF PSORIASIS : AN INVESTIGATION INTO THE ROLE OF OXIDATIVE STRESS IN PSORIASIS

Scientific staff	:	**Dr. Arun Kumar Jain, Dr. Usha Agrawal, Dr. Avninder Pal Singh**
In collaboration with	:	**Dr. V. Ramesh, SJH, New Delhi**
Technical Staff	:	**Mrs. Asha Rani Srivastava, Mrs. Anita Bhatia, Mr. Suresh**
Duration	:	**2006-2009**

Aims, Objectives & Background:

Psoriasis is a chronic, non-contagious, autoimmune disease, characterized by red scaly patches on the skin. The exact molecular pathogenic mechanism of psoriasis is largely unknown, however, several factors have been shown to play a role in pathogenesis of psoriasis including T-cells, keratinocytes, langerhan's cells, macrophages, natural killer cells and an array of Th1 type cytokines.

Recently oxidative stress has also been implicated in the etiopathogenesis of psoriasis due to reactive oxygen species (ROS) that originate in the environment and in the skin itself. ROS are generated during normal metabolism, are an integral part of normal cellular function, and are usually of little harm because of intracellular mechanism that decreases their damaging effects. Antioxidants attenuate the damaging effect of ROS and can impair and/or reverse many of the events that contribute to epidermal toxicity and diseases. Increased or prolonged free radical action can overwhelm ROS defense mechanisms, contributing to development of cutaneous diseases and disorders. Although ROS may play a role in diseases such as psoriasis and skin cancers, their biological targets and pathogenic mode of action are still not fully understood. In addition, strategies useful in therapeutic management of ROS action in human skin are still lacking.

This study was undertaken to unravel the role of oxidative stress in psoriasis, and to find out possible molecular mechanisms in oxidative stress which may be used as a candidate for therapeutic intervention.

Work done during the year:

Thirty patients attending the skin OPD of Safdarjung hospital, New Delhi were enrolled for this study. Patients were thoroughly questioned for the history of the disease, past treatment taken, familial involvement, diet and a questionnaire including name, age, sex, address, type of lesion, duration of disease, sites involved, morphology, number of lesions, h/o concomitant disease. P.A.S.I (Psoriasis area severity index) scoring was done for each patient and 3 mm punch biopsies were taken and processed for light microscopy followed by immunohistochemistry and electron microscopy.

Twenty five patients belonged to Delhi while remaining 5 came from UP, Bihar, Rajasthan and Uttranchal. All 10 healthy volunteers were from Delhi. There were 25 males and 5 females along with 4 female and 6 male healthy volunteers. The duration of psoriatic symptoms varied from 4 to 131 months. B M I (Basal Metabolic Index) of the patients varied from 17.11 to 26.23 with a median of 22.85. Two patients had high B.M.I (>25). P.A.S.I. (Psoriasis Area Severity Index) score of the patients varied from 6 to 20.4 with an average of 12.82.

Important electron microscopic findings seen were - presence of neutrophils and macrophages in the epidermis; split in basal layer; melanosomes higher up in supra basal layer; binucleolated keratinocytes; langerhans cells with characteristic birbeck's granules and reduced tonofilaments. Clustering of blood vessels (which are generally present deeper in the dermis) was seen in sub-epidermal region (Fig. 1), along with inflammatory cells like neutrophils and lymphocytes and oedema in dermis (Fig. 2).

Immunohistochemistry of skin sections was done using polyHRP kit (*Biovision, USA*). Primary monoclonal antibodies against 8-OHdG (8-hydroxy, 2-deoxy guanosine), 4-HNE (4-hydroxy-2-nonenal), dibrY (Di bromo tyrosine) were used. 8-OHdG was overexpressed in all layers of epidermis except stratum corneum. Positive staining was also observed in inflammatory cells and endothelial cells (Fig. 3). Positive staining of 4-HNE was observed in epidermis and sub-epidermal

region (Fig. 4) but not in deep dermis. A positive immunoreaction of Dibry was seen in stratum corneum, dermis and weak staining in other epidermal layers (Fig. 5). A positive DiBrY reaction is indicative of production of HClO and HBrO mediated through myeloperoxidase and eosinophil-peroxidase by neutrophils and eosinophils. Thus, this study has revealed oxidative damage of phospholipids moiety in cell membranes and DNA injury may be the triggering factor initiating the formation of psoriatic lesions. Further detailed studies from this aspect are needed to solve the mystery behind psoriasis.

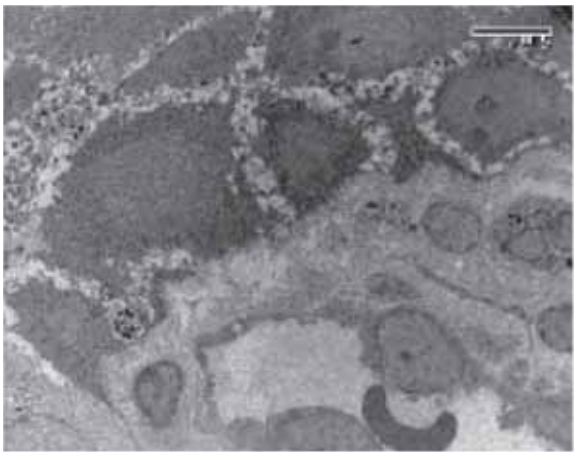


Fig. 1 : Electron micrograph of blood vessel with inflammatory cells (3500x)

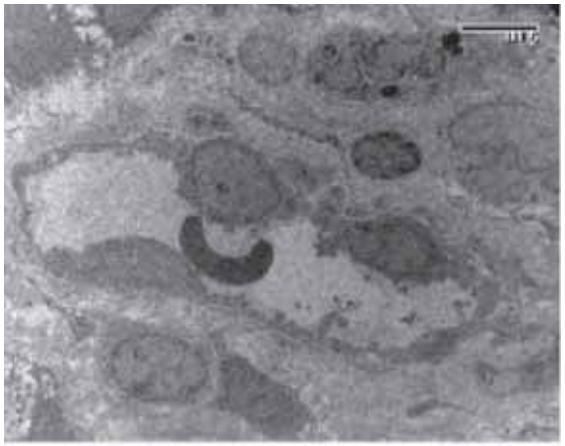


Fig. 2 : Epidermis with blood vessel in sub-epidermal region and inflammatory cells nearby (3500x)

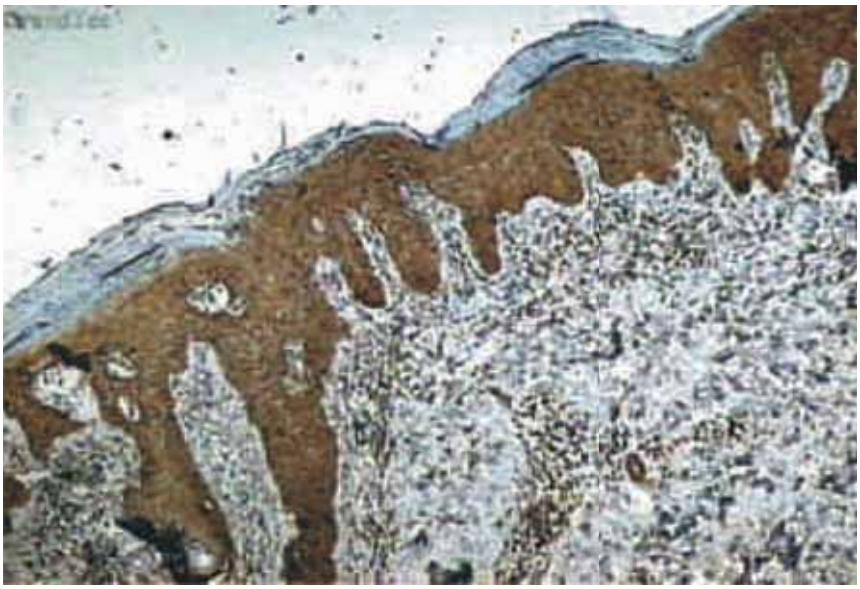


Fig. 3 : Micrograph showing expression of 8-OHdG in psoriasis skin; positive staining is seen in all layers of skin except stratum corneum.

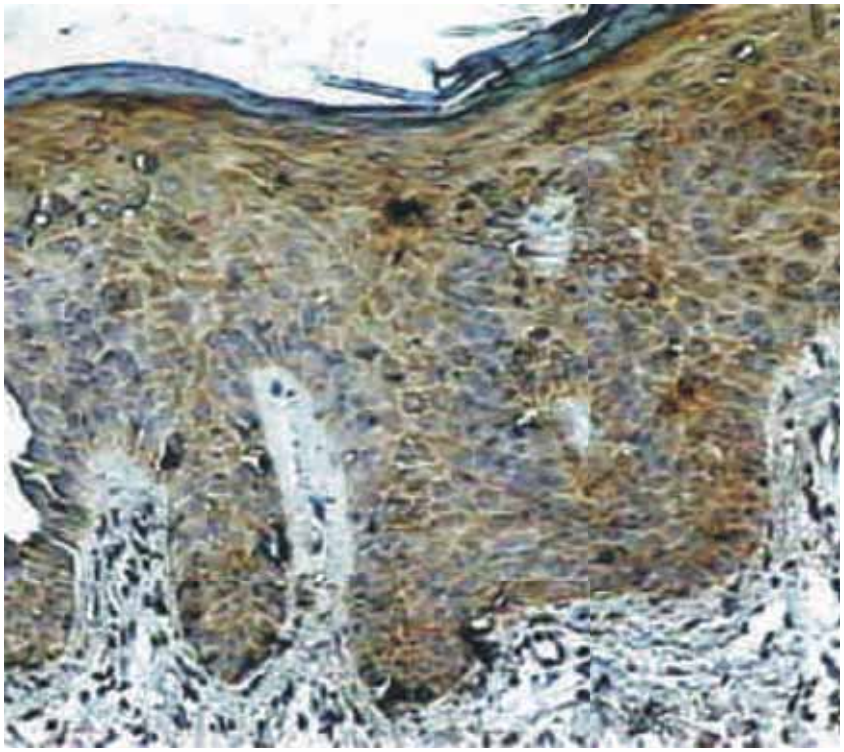


Fig. 4 : Micrograph of psoriasis skin shows positive staining of G-HNE in epidermal & sub-epidermal regions.

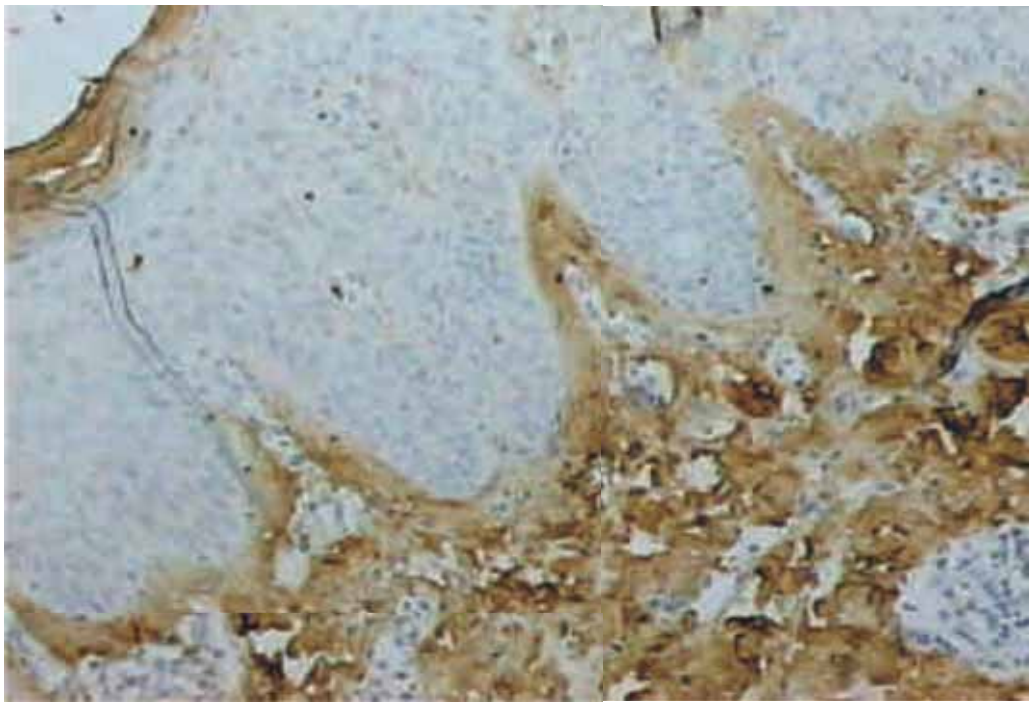


Fig. 5 : Micrograph of skin from psoriasis patient stained for DiBry; immunoreaction shows staining in stratum corneum and dermis.

Academic Activities

MAJOR ACTIVITIES AT INSTITUTE OF PATHOLOGY

✶ **Dr. VM Katoch**, Secretary, Department of Health Research & Director General (ICMR) delivered the **9th Smt. Pushpa Sriramachari Founder's Day Oration** on **'Understanding of granulomas with special reference to mycobacterial diseases'** on 18th January 2010.

✶ **Professor Indira Nath,** Ramanna Fellow & **Dr. Sunita Saxena,** Director, IOP, New Delhi organized **'International Workshop on Molecular & GIS Based Epidemiology of Leprosy'** at Institute of Pathology (ICMR) from 4th – 9th March 2010.

✶ On the eve of 15th August 2009 and 26th January 2010, flag hoisting was done at IOP.


✶ IOP Library conducted **Workshop and Training Programme on JCCC@ICMR ERMED Consortia** through Informatics India on 4th September 2009.



✶ **Scientific Advisory Committee** Meeting was held at IOP on 13th October 2009.



✶ Organized quarterly meeting of Delhi chapter of Indian Association of Pathologists and Microbiologists on 5th December 2009. **Dr. Avninder Pal Singh** , Scientist 'C', IOP gave a talk on "**Tissue microarray as a tool in validation and discovery of tumor markers**".

✶ **Dr. Anavaj Sakuntabai** , Institute Pasteur, Paris gave a seminar on "Genetics of Human Response to Infection" at IOP in the framework of Bonjour-India, Festival of France on 16th December 2009.

✶ Guest lecture by **Dr. Prakash Hrideyesh,** German Cancer Research Centre, Heidelberg, Germany on " **The Macrophages : Potential target for translational Medicine**" on 7th January 2010.

AWARDS AND HONOURS

1. **Dr. Sunita Saxena**, Director, IOP was awarded the **Novartis Oration Award** for research in the field of Cancer by ICMR for the year 2006 on 18th Sept. 2009.

2. **Dr. Aruna Mittal**, Dy. Director (Sr. Grade) was awarded **ICMR Lala Ram Chand Kandhari** Award for research in the field of *Chlamydia trachomatis* by ICMR for the year 2007 on 18th Sept. 2009.

Patent

Yerneni LK and Kumar A: A culture system for the growth of stem cells. Indian Patent File No. 2086/DEL/2009.

EXTRAMURAL PROJECTS

New Projects

1. Immunogenetic profile of nasopharyngeal cancer.
   Dr. Sujala Kapur, Dr. Sunita Saxena - DBT Project (2010-13).

2. A novel arithmetic approach for fool-proof production of growth arrest in 3T3 cells suitable for human epidermal culture.
   Dr. LK Yerneni – ICMR (2010 – 13).

Ongoing Projects

1. Characterisation of host immune factors associated with progression of superficial TCC of bladder by microarray analysis.
   Dr. Sunita Saxena - ICMR (2009-12).

2. Establishment of breast cancer cell lines from primary breast tumours.
   Dr. Sunita Saxena, Dr. Sujala Kapur, Dr. Usha Agrawal – DBT (2008-11).

3. Study on gene expression and hypermethylation profiles in early onset breast cancer.
   Dr. Sunita Saxena, Dr. Sujala Kapur, Dr. BSA Raju - ICMR Multicentric Task Force Project (2007-10).

4. Role of tobacco use in causation of cancer in north-east India.
   Dr. Sunita Saxena, Dr. Sujala Kapur, Dr. Usha Agrawal - ICMR Multicentric Task Force Project (2005-10).

5. Effect of pesticide exposure in causation of cancer in north-east India.
   Dr. Sunita Saxena, Dr. Sujala Kapur, Dr. Usha Agrawal - ICMR Multicentric Task Force Project (2005-10).

6. New tools for monitoring drug resistance and treatment response in visceral leishmaniasis in the Indian subcontinent.
   Dr. Poonam Salotra - European Commission (2009-12).

7. Pre-clinical studies of a PSA based human vaccine candidate targeting visceral , cutaneous and muco-cutaneous leishmaniasis and development of the associated procedures for further clinical trials.
   Dr. Poonam Salotra - European Commission (2009-12).

8. Parasite surface antigen-2 (PSA-2) of *Leishmania donovani*: Studies on its role in parasite virulence, drug resistance and modulation of host macrophage function.
Dr. Poonam Salotra - DST (2008-11).

9. Transcriptome profiling for identification and characterization of miltefosine resistance associated genes of *Leishmania donovani*.
Dr. Poonam Salotra - ICMR (2009-12).

10. Analysis of host immuno-determinants involved in the pathogenesis of Indian cutaneous leishmaniasis exploiting cDNA microarray.
Dr. Poonam Salotra - ICMR (2007-10).

11. Assessment of pesticide exposure in tea garden workers of north-eastern state of India (HEBM).
Dr. AK Jain - ICMR (2008-10).

12. Investigation into the utility of a patented synthetic thermo-reversible hydrogel polymer as supportive matrix towards the development of 3-D composite skin for application in wound healing and other dermatological disorders.
Dr. LK Yerneni - ICMR (2007-10).

Completed Projects

1. Microsatelite instability in androgen receptor gene, p53 gene polymorphisms/mutations and expression profile of mismatch repair genes in prostate cancer.
Dr. Sunita Saxena - DST (2005-08).

2. Comprehensive study of carcinoma oesophagus at northeast India - Multidiscipline approach.
Dr. Sunita Saxena, Dr. Sujala Kapur - ICMR Multicentric Task Force Project (2004-08).

3. Evaluation of host immunodeterminants involved in the pathogenesis of kala-azar and post kala-azar dermal leishmaniasis using cDNA array.
Dr. Poonam Salotra - DRDO (2006-09).

PUBLICATIONS

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2. Chattopadhyay I, Singh A, Phukan R, Purkayastha J, Kataki A, Mahanta J, Saxena S, Kapur S. Genome-wide analysis of chromosomal alterations in patients with esophageal squamous cell carcinoma exposed to tobacco and betel quid from high-risk area in India. **Mutation Research - Genetic Toxicology and Environmental Mutagenesis,** 696: 130-138, 2010. DOI information: 10.1016/j.mrgentox.2010.01.001.

3. Chattopadhyay I, Phukan R, Singh A, Vasudeva n M, Purkayastha J, Hewitt S, Kataki A, Mahanta J, Kapur S, Saxena S. Molecular profiling to identify molecular mechanism in esophageal cancer with familial clustering. **Oncol Rep**, 21(5): 1135-1146, 2009. PMID: 19360286.

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cytokine secretion profile of cervical mucosal cells. **Clinical Immunology**, 130: 347-354**,** 2009.

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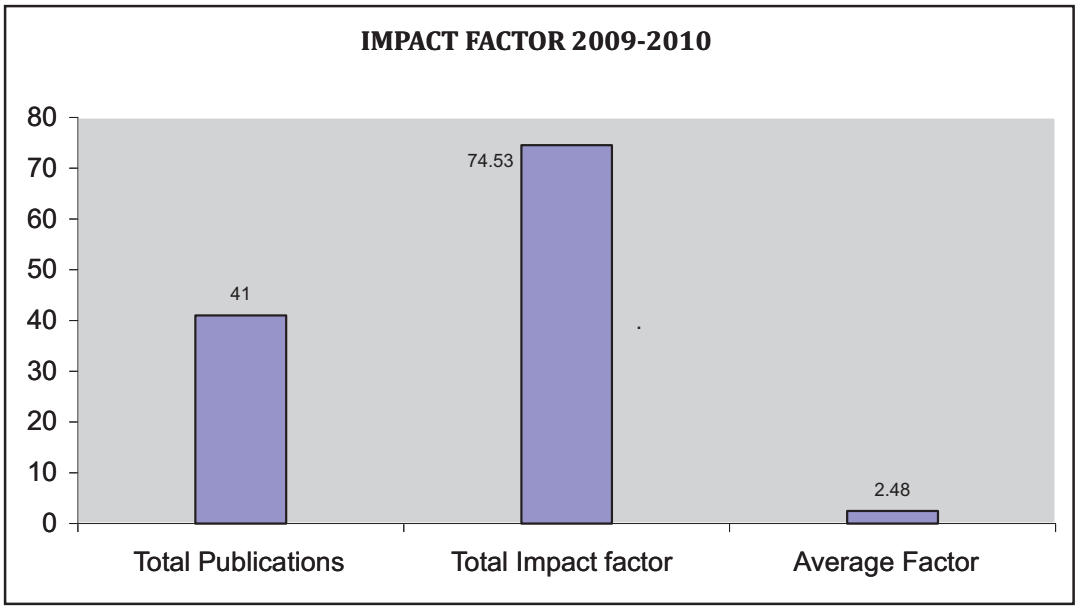
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80
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Total Impact factor
Average Factor

ACCEPTED FOR PUBLICATION

1. Yadav Dhirendra Singh, Thoudam Regina Devi, Ihsan Rakhshan, Mishra AK, Kaushal Mishi, Chattopadhyay Indranil, Chauhan Pradeep Singh , Sharma Jagannath, Zomawia Eric, Verma Yogesh, Nandkumar A, Mahanta Jagadish, Phukan Rupkumar, Saxena Sunita, Kapur Sujala. Polymorphisms of Glutathione-S-transferase (GST) genes and the risk of aerodigestive cancers in northeast Indian population. **Genetic Testing and Molecular Biomarker**.

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9. Soni Simmi, Rath Gayatri, Prasad Chandra Prakash, Salhan Sudha, Saxena Sunita, Jain Arun Kumar. Apoptosis and Bcl-2 protein expression in human placenta over the course of normal pregnancy. **Anatomia Histologia Embryologia.**

10. Prasad N, Ghiya B, Kaushal H, Satoskar A A, Davilia Caludio L, Salotra P, Bumb R A, Satoskar AR. Tackling treatment refractory cutaneous leishmaniasis in HIV injected patient. **The Lancet** (in press)

11. Rath G, Jain AK, Mukherjee A, Bastia B, Shrivastava P, Raghunandan C. Placental vasculosyncytial membrane in tobacco exposed Indian mothers: A quantitative and ultrastructural study. **J Int Med Sci Acad.**

12. Agrawal Usha, Rai Himanshu, Jain Arun K. Morphological and ultrastructural characteristics of Extracellular Matrix (ECM) changes in Oral Squamous Cell Carcinoma (OSCC). **Ind J of Dental Res.**

13. Aggarwal A, Agarwal U, Verma S, Mohanty NK, Saxena S. Serum Th1 and Th2 cytokines balance in patients of superficial transitional cell carcinoma of bladder pre and post intravesicle combination therapy. **Immunopharmacol Immunotoxicol.**

ABSTRACTS/PROCEEDINGS

1. Kapur Sujala, Chattopadhyay Indranil, Phukan Rupkumar, Purkayastha Joydeep, Marshal Vikki, Kataki Amal, Mohanta Jagdish, Bowtell David, Saxena Sunita. Genome-wide analysis of genetic alterations in esophageal squamous cell carcinoma by snp array. 20th Asia Specific Cancer Conference, 12th –14th November, 2009, Japan.

2. Saxena Sunita, Wasson Mishi Kaushal, Chatterjee Indranil, Bhatnagar A, Chintamani, Bhatnagar D, Kapur Sujala. Genome-wide analysis of DNA copy number variations in Indian breast cancer patients using high-density SNP arrays 20th Asia Specific Cancer Conference, November 12-14, 2009, Japan (Poster).

3. Saxena Sunita, Kaushal Mishi, Chatterjee Indranil, Bhatnagar A, Chintamani, Bhatnagar D, Kapur Sujala. Genome-wide analysis of genetic alterations in breast cancer patients from Northeast India using 10k SNP arrays. Organisation for Oncology and Translational Research, 26th-27th Feb. 2010, Kyoto (Poster).

4. Chauhan Pradeep Singh, Ihsan Rakhshan, Mishra Ashwani Kumar, Bhushan Bharat, Kaushal Mishi, Yadav Dhirendra Singh, Regina Thoudam Devi, Soni Abha, Chattopadhyay Indranil, Saxena Sunita, Kapur Sujala. Glutathione S-transferase and Microsomal Epoxide Hydrolase Gene Polymorphisms and Risk of Acute myeloid leukemia. First international conference on Hematologic Malignancies: Bridging the Gap 2010 Feb 5-7, 2010, Singapore City, Singapore (Poster).

5. Chattopadhyay Indranil, Singh Avninder, Kapur Sujala, Saxena Sunita. Genome-wide mRNA profiling of familial and non-familial esophageal squamous cell carcinoma for identification of molecular mechanism of esophageal cancer in high-risk area of India. 2009 NCRI Cancer Conference, Birmingham, UK (Poster No. B148).

6. Agarwal Shweta, Saluja Sumita, Paliwal Purnima, Jairajpuri Zeeba S, Kapur Sujala. Angiogenesis and the evidence of endothelial mesenchymal transition (EndMT) in bone

marrow disorders showing fibrosis. 6th Asia Pacific Indian Association of Pathologists (IAP) Congress, 20th – 23rd August, 2009, Kochi.

7. Singh Avninder, Kapur Sujala, Saxena Sunita. Tissue Microarray: A high throughput chip for the pathologist. 58th Annual Conference of IAPM, Science City, Kolkata. 18th -23rd December 2009.

8. DNA Microarray in Cancer: Principle, Methodology and Implications. RGCON-2010 CME, New Delhi, March 2010.

9. Rastogi S, Yadav C, Misro MM, Das B, Salhan S, Mittal A. *Chlamydia trachomatis* infection in spontaneous aborters. Proceedings of International Conference on Reproductive Health, Jaipur, 2010.

10. Chugh Rishi Man, Mori Yuichi, Yoshioka Hiroshi, Abraham Samuel, Yerneni LK Growth of human epidermal keratinocytes in Mebiol gel. Proceedings of Symposium on Regenerative Medicine, Nichi-in Centre of Regenerative Medicine (NCRM), Chennai, 24th October 2009.

SCIENTIFIC ACTIVITIES

Dr. Sunita Saxena

1. Attended seminar on Next Generation Sequencing held at JNU, New Delhi on 9th April 2009.

2. Attended IAPM (Delhi Chapter) meeting held at University of Delhi organized by U.C.M.S., Delhi on 11th April 2009.

3. Attended 4th Medical Development Congress on Stem Cell Research & Therapy held at Assocham House, New Delhi on 13th April 2009.

4. Attended SAC meeting held at Institute of Cytology & Preventive Oncology, Noida on 17th April 2009.

5. Attended Screening Committee meeting for the post of Scientist 'C' held in ICMR on 22nd April 2009.

6. Attended PRC Meeting to consider proposals under Cellular and Molecular Biology held at ICMR H.Q. on 22nd April 2009.

7. Attended Selection Committee Meeting for the selection for the post of SRF at Dept. of Microbiology, S.J. Hospital, New Delhi on 24th April 2009.

8. Invited to attend the first DSMB meeting on "Curcumin Clinical Trial in CaCx Cancer" on 4th May 2009 at NII, New Delhi.

9. Attended Selection Committee Meeting, walk-in interview for the project at Dept. of Microbiology, S.J. hospital, New Delhi on 5th May 2009.

10. Chaired Ethical Committee Meeting held at Safdarjang hospital on 5th May 2009.

11. Invited to attend Ph.D. viva as a reviewer held at Dr. B.R. Ambedkar University, Agra on 8th May 2009.

12. Attended viva for registration for Ph.D. as a guide of Ms. P. Shanti Latha held at BITS, Pilani on 15th May 2009.

13. Attended Brain Storming Session called by Secretary, Department of Health Research & Director General, ICMR, New Delhi held at ICMR on 19th May 2009.

14. Inspected Western Common Hospital, Chandimandir, Haryana on 20th May 2009 for renew of accreditation for conducting DNB course of NBE, New Delhi.

15. Invited to attend Ph.D. viva as a reviewer held at PGIMR, Chandigarh on 20th May 2009.

16. Attended First Symposium on HPV Vaccination in the Asia Pacific and Middle East Region held at Seoul, Korea during 1st - 3rd June 2009.

17. Attended Task Force Project Meeting at ICMR H.Q. on 18th June 2009.

18. Attended Project Review Committee meeting of NCD Division, ICMR held at ICMR on 25th June 2009.

19. Chaired Ethical Committee meeting of Safdarjang hospital, New Delhi on 30th June 2009.

20. Attended Project Review Committee meeting on North-East held at ICMR on 6th July 2009.

21. Attended Selection Committee meeting for the selection of Scientist 'C' held at ICMR, New Delhi on 21st July 2009.

22. Chaired the session on "Renal Pathology" (talk given by Dr. Vinita Batra) at Delhi Chapter of IAPM held at Maulana Azad Medical College, New Delhi on 1st Aug. 2009.

23. Chaired Ethical Committee meeting of Safdarjang hospital, New Delhi on 6th August 2009.

24. Nominated to visit as an expert, Cell Biology Division, Gujrat Cancer and Research Institute, MP Shah Cancer Hospital, NCH Campus, Asarwa, Ahmedabad by NCD- ICMR on 10th August 2009.

25. Attended the meeting of Directors held at National Board of Examinations, New Delhi on 18th August 2009.

26. Nominated by ICMR to attend ICMR – University of Minnesota Workshop on Cancer & Diabetes held during 26th - 28th August 2009 at Minneapolis, University of Minnesota.

27. Attended Scientific Advisory Committee meeting of Institute of Pathology held at IOP on 13th October 2009.

28. Attended Project Review Committee meeting on Cellular and Molecular Biology of BMS Division, ICMR, New Delhi held on 27th October 2009.

29. Delivered a lecture on "Molecular Biology of Cancer by Genome Wide Approach" at CME in Pathology at Maulana Azad Medical College on 18th Nov. 2009.

30. Attended meeting on Research Data Repository & Business Intellegence (BI) held at ICMR, New Delhi on 8th December 2009.

31. Chaired Ethical Committee meeting of Safdarjang hospital, New Delhi on 15th December 2009.

32. Attended Project Review Committee meeting of NCD Division held at ICMR, New Delhi on 17th December 2009.

33. Conducted inspection of B.R. Singh Hospital & Centre for Medical Education & Research, Eastern Railway, Kolkata, on 18th December 2009 for renewal of accreditation for conducting DNB course of NBE, New Delhi.

34. Attended APCON-2009 organized by IAPM in Kolkata during 18th – 20th December 2009.

35. Conducted interview for the selection of DNB Trainee for DNB Training Programme 2010 at Institute of Pathology on 4th January 2010.

36. Invited to present a talk on "Understanding molecular biology of cancer using genomic approach" in National Symposium on Current Trends in Genomics & Proteomics organized by Deptt. of Biotechnology, Deshbandhu Gupta college, University of Delhi, New Delhi on 4th –5th February 2010.

37. Attended ICMR-European Union (EU) Workshop in areas of cancer and neurosciences held at ICMR on 18th – 19th February 2010.

38. Invited to give talk on "Esophageal cancer in north east India – Contribution of genetic vis-à-vis environmental factors" held during 20th – 23rd February 2010 at Amrita Institute of Medical Sciences and Research Centre, Cochin.

39. Attended 5th AOHUPO Congress held at Centre for Cellular and Molecular Biology (CCMB), Hyderabad as joint event with 14th DNAT convention and 1st Conference of the Proteomic Society of India (PSI) held on 24th February 2010.

40. Attended Technical Committee meeting held at ICMR on 26th February 2010.

41. Attended Screening Committee meeting for the Selection of Scientist 'E' held in ICMR on 26th February 2010.

42. Attended Technical Committee meeting held at ICMR on 16th March, 2010.

43. Invited to attend Selection Committee meeting for award of PDF of ICMR held at ICMR, New Delhi during 25th - 27th March 2010.

Dr. Aruna Singh

1. Invited to attend symposium on "Commercialization of biotechnology" at India International Centre, New Delhi on 6th March 2009 organized by Biotechnology, Consortium of India Ltd (BCIL).

2. Invited to give key note address at " National conference on emerging trends in life sciences research" held at Birla Institute of Science and Technology, Pilani on 6th –7th March 2009.

3. Nominated to attend meeting on "Sustainable national vaccine policy" at NISTADS, CSIR from 4th - 5th June 2009 to give a lecture on 'Need for new vaccine –*Chlamydia trachomatis'*.

4. Member Selection Committee for Integrated Program (M.Sc., Ph.D) at Dr. B. R. Ambedkar Center, University of Delhi on 7th June 2009.

Dr. Sujala Kapur

1. Attended 6th Asia Pacific Indian Association of Pathologists (IAP) Congress, 20th -23rd August 2009, Kochi.

2. Attended 58th Annual Conference of IAPM, Science City, Kolkata during 18th –23rd December 2009.

3. Invited to deliver a talk on "Tobacco and pesticide related cancers in north-east" India during National Institute of Advanced Studies (NIAS)-DST Programme on Gender Issues in Indian Science, Bangalore.

4. Invited to deliver a talk on "Immunogenetic profile of nasopharyngeal cancer". BBCI, Guwahati, 2009.

5. Attended PRC for review of projects on cancer, Division of NCD, ICMR Headquarters, New Delhi, 2009.

6. Attended PRC for review of projects on Gastroenterology, ICMR Headquarters, New Delhi, 2009.

7. Attended Brainstorming session on prevalence and prevention of NPC in north-east India. Organized by DBT and BBCI, Guwahati, 2009.

8. Attended Chapter and Annual meetings of IAPM, New Delhi.

9. Attended Conference of Organisation for Oncology and Translational Research (OOTR): 6th Annual Conference on 26th and 27th February 2010 at Kyoto, Japan.

10. Attended First NIAS-DST Programme on Gender Issues in Indian Science on Transcending Barriers to Performance: The Indian woman in Science. Bangalore, March 22 to 26, 2010.

11. Invited to deliver a talk on "DNA microarray in cancer: Principles, methodologies and implications" in Workshop on Molecular Biology Techniques in Cancer Diagnosis and Treatment at Rajiv Gandhi Cancer Research Institute and Research Centre, New Delhi in March 2010.

Dr. Sangita Rastogi

1. Deputed to participate in Training Workshop on *'Applications and importance of laboratory animals in biomedical research'* at NIRRH, Mumbai during 22nd-25th April 2009.

2. Assisted in compilation of Performance Budget of the Institute for onward submission to ICMR headquarters (2009).

3. Attended ICMR Prize Distribution ceremony for award to popular medical books in Hindi at ICMR headquarters, New Delhi on 5th May 2009.

4. Participated in seminar on *'Workflow solutions for identifying genetic variation'* organized by M/S Genetix Biotech Asia (P) Ltd. at Indian Habitat Centre, New Delhi on 3rd September 2009.

5. Attended Meeting on *'Advances in Rheumatology- Concepts to practice'* at AIIMS, New Delhi on 11th October 2009.

6. External examiner for Ph.D. (Zoology) thesis evaluation by CCSU (2009).

7. Participated in international symposium on *'Molecular pathology and applied genomics'* organized by Religare SRL Diagnostics at Indian Habitat Centre, New Delhi during 6th-7th November 2009.

8. Attended national symposium on *'Translational research in health sciences'* at AIIMS, New Delhi on 24th November 2009.

9. Examiner for M.Sc. (Toxicology) Part I examination, CCS University (2009).

10. Faculty for Ph.D. students registered with BITS, Pilani at Institute of Pathology, New Delhi.

11. Chaired Screening Committee Meeting for the post of Scientist 'B' held at Institute of Pathology (2009).

12. Attended series of lectures on *'Emerging technologies in life sciences'* during Research Solutions Seminar organized by ILS-Lonza at ITC Sheraton, New Delhi on 15th January 2010.

13. Invited talk titled, '*Chlamydia trachomatis infection in spontaneous aborters'* delivered at ICRH-2010 organized by ISSRF at Jaipur during 8th-10th February 2010.

14. Assisted in compilation of write-up of the Institute's research achievements for onward inclusion in Annual Report of DHR (2010).

15. Reviewer for international project proposal submitted to ICMR (2010).

16. Attended Selection Committee Meeting for the selection of Senior Research Fellow at Dept. of Microbiology, Safdarjung hospital, New Delhi (2010).

Dr. Poonam Salotra

1. Participated in the meeting of Molecular Immunology Forum held at Mumbai in March 2009.

2. Attended ICMR 4th Medical Development Congress on Stem Cell Research and Therapy at ASSOCHAM House, New Delhi on 13th - 14th April 2009.

3. Participated in Symposium on "Self reliance through Innovation in Science & Technology" at NIPGR, New Delhi on 2nd May 2009.

4. Participated in the Steering Committee Meeting *of Leishmania* Vaccine project at Madrid, Spain in June 2009.

5. Participated in the Steering Committee Meeting of *Leishmania* Drug project at Antwerp, Belgium in October 2009.

6. Participated in workshop conducted by Voluntary Health Association of India, Program on Policy Issues in Kala-Azar in South-East Asia, Qutab Institutional Area, New Delhi on 27th November 2009.

7. Invited as a member of PRC meeting on Malaria, *Leishmania* and Filariasis held at ICMR, New Delhi on 8th December 2009.

8. Invited speaker at 97th Indian Science Congress held at Kerala University, Thiruvananthapuram in January 2010.

9. Appointed as a reviewer for several project proposals submitted to DBT, DST, ICMR, etc.

10. Appointed as a reviewer for several manuscripts submitted to international journals.

Dr. Usha Agrawal

1. Deputed to attend the "Brainstorming Session" on "Data Repository and Management" in ICMR Hqs.

2. Served on the organizing committee of IAPM, Delhi Chapter Meet held at Institute of Pathology on 5th December 2009.

3. Attended IAPM Delhi Annual Chapter meet at Lady Hardinge Medical College, New Delhi, 2009.

4. Attended Guest Lecture given by Dr. S. Sriramachari to commemorate the 25th Anniversary of the Bhopal Gas Disaster given at Patel Chest Institute on 3rd December 2009.

Dr. LK Yerneni

1. Invited to deliver a talk entitled "Burn Injury: A Challenge For Tissue Engineers" at a Plenary Session on Regenerative Medicine held at Nichi-in Centre of Regenerative Medicine (NCRM), Chennai, on 24th October 2009.

Dr. Avninder Pal Singh

1. Presented an oral paper in 5th International Dermatopathology Conference at India Habitat Center from 6th - 8th November 2009.

2. Participated in "Hands-on Training Course in Quantitative Proteomics" at Center for Cellular and Molecular Biology, Hyderabad during 26th Feb. – 12th March 2010.

Dr. Ashwani K. Mishra

1. Attended "31st Annual Conference of the Indian Association for the Study of Population (IASP)" held at Tirupati, October 2009 and presented paper entitled "Regression approaches in understanding the role of vitamin D receptor gene polymorphism(s) in breast cancer risk in north Indian population".

2. Attended "27th Annual National Conference of Indian Society for Medical Statistics", held at Banaras Hindu University, Varanasi (UP), November 2009 and presented paper entitled "Vitamin D receptor gene polymorphism(s) and its association with breast cancer risk in north Indian population-

   A multinomial logistic regression approach".

3. Involved as Co-Investigator in the extramural research project entitled "Immunogenetic profile of NPC–Nasopharygeal cancer in a high prevalence region of northeast India", funded by Department of Biotechnology Government of India, Ministry of Science of Technology, Government of India.

4. Involved as Co-Guide for the thesis dissertation work entitled "Role of epithelial mesenchymal transition in progression of bladder-

   Immunohistochemical analysis" as part of the DNB training programme at the Institute of Pathology, New Delhi.

5. Faculty for the course no. BIO-G532 'Biostatistics and Biomodelling' under the off-distance campus Ph.D programme in collaboration with BITS, Pilani for the session 2009-10.

6. Attended Project Review Meeting on project entitled "Comparative study on HIV/AIDS with ART with add-on homoeopathic drugs" approved by Centre Council for Research in Homoeopathic and Siddha, AYUSH, Ministry of Health and Family Welfare, New Delhi, India, February 2010.

7. Delivered lecture on 'Techniques of Data Integration" at the International Workshop on "Molecular and GIS based Epidemiology of Leprosy", at Institute of Pathology, New Delhi, March 2010.

DNB/ Ph.D PROGRAMME

DNB Programme

The Post-Graduate Level Training Programme in the speciality of Pathology continued during 2009-10. During the year, two students were admitted in the DNB course at Institute of Pathology:

1. **Dr. Sherry Khanna**
2. **Dr. Manav Sawhney**

The following two students who appeared for the DNB Theory Exam held in December 2009 have come out with flying colours:

1. **Dr. Shweta Aggrwal**
2. **Dr. Binita Sinha**
3. **Dr. Sonal Agarwal**

As per guidelines of the National Board of Examination, the Institute conducted Review Examinations of the DNB students in July 2009. Dr. Medha Tatke, Professor, Department of Pathology, GB Pant Hospital, New Delhi came as reviewer.

Ph.D Programme

1. **Mr. Bharat Bhushan** was conferred with the degree of Doctorate of Philosophy for his work on Acute Leukaemia by Jamia Hamdard in Dec. 2009 .

2. **Mr. Rishein Gupta** was conferred with the degree of Doctorate of Philosophy for his work on Chlamydiasis by BITS-Pilani in Dec. 2009 .

3. **Mr. Indranil Chattopadhyay** was conferred with the degree of Doctorate of Philosophy for his work on Esophageal Cancer by BITS-Pilani in 2009.

4. **Ms. Anurupa Chakroborty** was conferred with the degree of Doctorate of Philosophy for her work on Breast Cancer by Guru Gobind Singh Indraprastha University in 2009.

5. **Mr. Harsh Vardhan** was conferred with the degree of Doctorate of Philosophy for his work on Chlamydiasis by BITS-Pilani in 2010.

6. **Mr. Hem Chandra Jha** submitted his PhD thesis on Chlamydiasis to BITS-Pilani in Feb. 2010.

7. 2 students are currently registered for the Off-Campus Ph.D. programme of BITS-Pilani.

During 2008-09, Institute of Pathology further attracted young researchers- Research scholars with CSIR Junior Research Fellowships (2), ICMR Senior/Junior Research Fellowships (14) and UGC Fellowships (2) joined the Institute during this period.

OTHER ACADEMIC ACTIVITIES

As part of academic activities, the Institute organized journal clubs, slide seminars and seminars by various experts from both within and outside the country.

Scientific activities of Ph.D./DNB students

Pradeep Singh Chauhan

1. Chauhan Pradeep Singh, Ihsan Rakshan, Mishra Ashwani Kumar, Bhushan Bharat, Kaushal Mishi, Yadav Dhirendra Singh, Devi Thoudam Regina, Soni Abha, Chattopadhyay Indranil, Saxena Sunita, Kapur Sujala. Poster presented on "Glutathione S-transferase and Microsomal Epoxide Hydrolase Gene Polymorphisms and Risk of Acute myeloid leukemia" in the first international conference "Hematologic Malignancies: Bridging the Gap 2010" Feb. 5-7, 2010 Singapore City, Singapore.

2. Attended the "National Workshop of CCamp Hands-on Basic Flow Cytometry Course" organized by National Centre for Biological Sciences from 17th – 21st December 2009 at TIFR, Bangalore.

3. Attended the National workshop on "Molecular Cytogenetics: Cancer Cytogenetics (solid tissue) by FISH" organized by Department of Reproductive Biology at AIIMS from 24th - 29th August 2009.

Mishi Kaushal

1. Saxena Sunita, Kaushal Wasson Mishi, Chatterjee Indranil, Bhatnagar A, Chintamani, Bhatnagar D, Kapur Sujala. Genome-wide analysis of DNA copy number variations in Indian breast cancer patients using high-density SNP arrays. 20th Asia Specific Cancer Conference, Japan (Poster), 12th –14th November 2009.

2. Saxena Sunita, Kaushal Mishi, Chatterjee Indranil, Bhatnagar A, Chintamani, Bhatnagar D, Kapur Sujala. Genome-wide analysis of genetic alterations in breast cancer patients from Northeast India using 10k SNP arrays. Organisation for Oncology and Translational Research, 26th -27th February 2010, Kyoto (Poster).

Pragya Srivastava

1. Attended 'International Symposium on Molecular Pathology and Applied Genomics' on 6th -7th Nov. 2009 at India Habitat Centre, New Delhi, India.

Scientific Advisory Committee

SCIENTIFIC ADVISORY COMMITTEE

1	**Dr. Indira Nath,** **Raja Ramanna Fellow**, 707, SP Apartments, Sarvapriya Vihar, New Delhi - 110016	Chairperson
2	**Dr. Rajiv Sarin, Director**, Tata Memorial Centre, Advance Center for Treatment, Research & Education in Cancer, Kharghar, Navi Mumbai – 410 210.	Member
3	**Dr. Kusum Joshi, Professor & Head** Department of Pathology Post Graduate Institute of Medical Sciences, Chandigarh	Member
4	**Dr. Chitra Sarkar, Professor,** Department of Pathology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi -29	Member
5	**Dr. Subhroto Sinha, Professor**, Department of Biochemistry, All India Institute of Medical Sciences, Ansari Nagar, New Delhi – 110 029.	Member
6	**Dr. N.K. Mehra,** **Professor & Head** Deptt. of Transplant Immunology & Immunogenetics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi – 110 029.	Member
7	**Dr. R.R. Bhonde,** **Technical Director,** Stempeutics Research Malaysia, Kuala Lumpur, Malaysia	Member
8	**Dr. Ravi Sirdeshmukh, Scientist,** Center for Cellular & Molecular Biology, (Council of Scientific & Industrial Research), Uppal Road, Hyderabad – 500 007.	Member
9	**Dr. Sudha Bhattacharya,** **Professor & Dean,** School of Environmental Sciences, Jawahar Lal Nehru University, New Delhi.	Member
10	**Dr. Dhananjaya Saranath, Director Research** Reliance Life Sciences (Pvt.) Ltd., Dhirubhai Ambani Life Sciences Centre, R-282, TTC Area of MIDC., Tale Hane Lanapure Road, Navi Mumbai - 400 701.	Member

11	**Dr. Kiran Katoch,** **Director-in-Charge** National Jalma Institute of Leprosy & Other Mycobacterial Diseases, Taj Ganj, Agra, UP	Member
12	**Dr. Chandrima Saha,** **Deputy Director,** National Institute of Immunology Aruna Assaf Ali Marg, New Delhi - 67	Member
13	**Dr. Shyamal Roy**, Indian Institute of Chemical Biology, Council of Scientific & Industrial Research, 4, Raja SC Mullick Road, Kolkata – 700 032., West Bengal.	Member
14	**Dr. Ashok Sehgal,** **Director, In-charge,** Institute of Cytology & Preventive Oncology (ICMR), 17, Sector-39, Near Degree College, Noida-201301, UP	Member
15	**Dr. Ashwini Kumar**, Industrial Toxicology Research Centre, Post Box No. 80, Mahatma Gandhi Marg, Lucknow – 226 001.	Member
16	**Dr. NK Mohanty** Addl. DG & Medical Superintendent, Safdarjang Hospital, New Delhi	Member
17	**Dr. Lalit Kant,** **Scientist G,** Head, BMS, Indian Council of Medical Research, New Delhi	Member
18	**Dr. Vijay Kumar,** **Scientist F,** BMS, Indian Council of Medical Research, New Delhi	Member
19	**Dr. Sunita Saxena,** **Director,** Institute of Pathology, New Delhi	Member Secretary

Animal Ethical Committee

INSTITUTIONAL ANIMAL ETHICAL COMMITTEE (IAEC) OF IOP

IOP Members:

- Dr. Sangita Rastogi, Scientist 'E' (Member Secretary)
- Dr. Poonam Salotra, Scientist 'E'
- Dr. Usha Agrawal, Scientist 'D'
- Dr. Laxman Kumar Yerneni, Scientist 'D'

External Members:

Scientist Member

Dr. Harmeet Singh Rehan, Professor & Head, Department of Pharmacology, LHMC, N. Delhi

Social Scientist Member

Dr. B B Batra , CMO, NFSG, New Delhi

Veterinary consultant

Dr. P K Yadav, Sr. Veterinary Officer, Experimental Animal Facility, AIIMS, New Delhi

CPCSEA Nominee

Dr. D N Rao, Professor, Department of Biochemistry, AIIMS, New Delhi

Link CPCSEA Nominee

Dr. Smriti Rekha Dutta (Link CPCSEA Nominee)

Institutional Committees

INSTITUTIONAL COMMITTEES

Purchase Committee	
Dr. A. Mittal Dr. A. K. Jain Dr. L K. Yerneni	Mrs. R. Saratha Mr. V.K. Khanduja
Tender Committee	
Dr. Sangita Rastogi Dr. Ranvir Singh Dr. Saurabh Varma	Mrs. R Saratha Mr. VK Khanduja
Fire Fighting Committee	
Dr. Ranvir Singh Mr. AK Bagga	Mr. Raja Ram
Transport Committee	
Dr. Sangita Rastogi Dr. Purnima Paliwal Dr. BS Raju	Ms. R. Saratha Mr. Raja Ram
Library Committee	
Dr. Sujala Kapur Dr. Saurabh Varma Dr. Usha Agrawal	Dr. Avninder P Singh Mrs. R Saratha Ms. Anita Sharma
Condemnation Committee	
Dr. Ranvir Singh Dr. Saurabh Varma Dr. BS Raju	Mrs. R. Saratha Mr. V.K. Khanduja
Technical Committee	
Dr. Sayed Akhtar Hussain Dr. SN Das Dr. Chandrima Saha Dr. Aruna Mittal	Dr. A.K. Jain Dr. L.K. Yerneni Mrs. R. Saratha Mr. V.K. Khanduja
Maintenance/Security & Sanitation Committee	
Dr. Poonam Salotra Dr. Ranvir Singh Dr. AP Singh	Dr. Ashwani Kr. Mishra Mrs. R. Saratha Mr. Yogender Kumar

Canteen Committee	
Dr. Sangita Rastogi Dr. Anju Bansal Dr. Ashwani Kr. Mishra Mrs. R Saratha	Mr. Ravi Kapoor Mr. Yogender Kumar Ms. Madhu Badhwar
Monitoring/Building Committee	
Mr. B. Bose, Consultant, NII, New Delhi Mr. M. M. Prasad, Consultant, ICPO, New Delhi Dr. Sunita Saxena, Director, IOP Dr. Sujala Kapur Dr. Sangita Rastogi	Mr. AK Srivastava, Executing Eng. ICMR Dr. AK Jain Dr. Poonam Salotra Dr. LK Yerneni Mrs. R Saratha Mr. V K Khanduja

STAFF LIST

STAFF LIST

SCIENTIFIC STAFF

1.	Dr. Sunita Saxena, MBBS, DCP, MD (Path)	Director
2.	Dr. Aruna Singh, M.Sc., Ph.D.	Scientist 'F'
3.	Dr. Sujala Kapur, MBBS, MD (Path & Microbiology)	Scientist 'E'
4.	Dr. Sangita Rastogi, M. Sc., M.Phil., Ph.D. (Zoology)	Scientist 'E'
5.	Dr. Poonam Salotra, M.Sc. (Biochem), Ph.D. (Biochem)	Scientist 'E'
6.	Dr. AK Jain, M.Sc. (Dairy Bacteriology), Ph.D.	Scientist 'E'
7.	Dr. Ranvir Singh, MBBS	Scientist 'D'
8.	Dr. AK Bagga, MBBS	Scientist 'D'
9.	Dr. LK Yerneni, M.Sc., Ph.D.	Scientist 'D'
10.	Dr. Usha Agrawal,MBBS, MD (Path)	Scientist 'D'
11.	Dr. Anju Bansal, MBBS, MD (Path)	Scientist 'C'
12.	Dr. Saurabh Verma, M.Sc., Ph.D.	Scientist 'C'
13.	Dr. Purnima Malhotra, MBBS, MD (Path)	Scientist 'C'
14.	Dr. Avninder Pal Singh, MBBS, MD (Path)	Scientist 'C'
15.	Dr. S. Appala Raju Bagadi, , M.Sc., Ph.D.	Scientist 'B'
16.	Dr. AK Misra, Ph.D. (Statistics)	Scientist 'B'

TECHNICAL STAFF

1.	Mrs. Asha Rani Srivastava, T.O
2.	Dr. L.C. Singh, T.O.

3. Mr. Moti Lal, Technical Officer
4. Mrs. Madhu Badhwar, Technical Officer
5. Mr. Shiv Prakash, Data Processing Assistant (Gr. 'B')
6. Mrs. Seema Sharma. Data Processing Assistant (Gr. 'A')
7. Mr. Pushp Raj, Dark Room Assistant
8. Mrs. Asha Rani, Technical Assistant
9. Mr. Chandi Prasad, Technical Assistant
10. Mrs. Anita Bhatia, Technical Assistant
11. Mrs. Karuna, Technical Assistant
12. Mrs. Krishna, Technical Assistant
13. Mr. Kuldeep Kumar Sharma, Lab. Technician
14. Mrs. Valsamma Mathew, Lab. Technician
15. Mr. Ishwar Singh, Lab. Technician
16. Mr. Jagdish Pant, Lab. Technician
17. Mr. P.D. Sharma, Lab. Technician
18. Mr. Rameshwar, Lab. Assistant
19. Mr. Suresh Bhimrao Kamble, Lab. Technician
20. Mr. Satyapal Singh, Lab. Technician
21. Mr. Madan Lal, Lab. Assistant
22. Mr. Shiv Bahadur, Lab. Assistant
23. Mr. Dharampal, Driver-cum Mechanic
24. Mr. Sushil Kumar, Driver
25. Mr. Manwar Singh, Driver

26. Mr. Puran Singh, Messenger-cum-Driver
27. Mr. Shyam Sunder, Lab. Attendant
28. Mr. Ajit Singh Lehra, Lab. Attendant
29. Mr. Daya Sagar, Lab. Attendant
30. Mr. Bala Dutt, Lab. Attendant
31. Mr. Jagdish Ram, Lab. Attendant

ADMINISTRATIVE STAFF

1. Mrs. R.Saratha, Administrative Officer
2. Mr. V.K. Khanduja, Accounts Officer
3. Mr. Raja Ram, S.O.
4. Mr. Ravi C. Kapoor, S.O.
5. Mr. Yogendra Kumar, S.O.
6. Mrs. Ganga Misra, P.S.
7. Mr. Jagdish Prashad, P.A.
8. Ms. Rekha Rani, P.A.
9. Mrs. Anita Sharma, Assistant (Librarian)
10. Mrs. Sunita Ahuja, Assistant
11. Mr. V. S. Rawat, Assistant
12. Mr. Dashrath G. Khambadkar, Assistant
13. Mr. Subhash Babu, Assistant
14. Mr. Mangey Ram, Assistant
15. Mrs. E. Sharda, Stenographer
16. Mrs. Sangeeta Batra, Jr. Librarian

17. Mrs. Sushma Ralhan, U.D.C.
18. Mrs. Sharmila Kamra, U.D.C.
19. Mr. Ajay Joshi, U.D.C.
20. Mrs. Sonia Khattar, U.D.C.
21. Mr. Rajesh Kumar, U.D.C.
22. Mrs. Santosh Deora, Receptionist-Cum-Tele.Operator
23. Mr. Brijender Singh, LDC
24. Mrs. Archana, LDC
25. Mr. Rajender Kumar, Sr. Gestetner Operator
26. Mr. Anil Kumar Verma, Gestetner Operator
27. Mr. Jagdish, Lab. Assistant
28. Sh.Bijendra Kumar, Lab.Assistant
29. Ms.Jyoti, LDC
30. Sh.Kamal Dev, Lab.Assistant
31. Mr. Dharmendar Singh, Attender
32. Mr. Ram Chander Das, Attender
33. Mr. Puran Chand, Attender
34. Mr. Rajendra Kumar, Attender
35. Mr. Manoj Kumar, Attender
36. Mr. Anish Kumar Saxena, Attender
37. Mr. Raj Singh, Attender
38. Mr. Sajid Hussain, Lab. Attendant
39. Mr. Chandrika Prasad, Attender
40. Mr. Sanjay Dutt Upreti, Attender
41. Mrs.Charanjit Kaur, Attender