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Autoimmune Hepatitis: Diagnosis and management

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Abstract

Waldenstrom had first described in 1950 a chronic inflammatory liver disease, now termed as autoimmune hepatitis (AIH). AIH is an unresolving inflammation of the liver of unknown cause, characterized by the presence of interface hepatitis, periportal hepatitis, hypergammaglobulinemia, circulating autoantibodies and response to immunosuppressive treatment in majority of the patients. Prednisone alone or at a reduced dose combined with azathioprine is the conventional treatment. Newer therapies with cyclosporine or tacrolimus have been used as salvage therapy in patients with steroid resistance. Lifetime maintenance therapy may be required, especially for patients with type 2 AIH and those who have cirrhosis at presentation. Liver transplantation has been successful in patients who are refractory or intolerant to immunosuppressive therapy and in whom end stage liver disease has developed.

Key words: autoimmune hepatitis, immunosuppressive therapy, cirrhosis, auto-antibodies, portal plasma cells.

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Introduction

Epidemiology and Natural History

AIH is a rare disorder and its annual prevalence is between 0.1 and 1.2 cases/100,000 in Western Europe and North America. AIH accounts for 6% of liver transplantations in US and 2.6% of liver transplantations in Europe. AIH affects females more than males in the ratio of 3:1; all ages and ethnic groups are susceptible (1).

The natural history of AIH is varied and depends on host issues, which are poorly understood. Forty percent of patients with untreated severe disease die within 6 months of diagnosis (2); at least 40% of survivors develop cirrhosis; 54% develop esophageal varices within 2 years after cirrhosis and 20% patients who develop esophageal varices die from hemorrhage(1). Factors identifying patients with early mortality are serum aminotransferase levels more than 10 fold normal or more than 5 fold normal in conjunction with serum ϒ globulin concentration of at least 2 fold normal(2). Immuno-suppressive therapy has changed the course of AIH; only a small number of patients develop cirrhosis during therapy and even fibrosis score tend to improve in up to 70% of patients (3). In children, almost 50% have cirrhosis at the time of presentation and only a few are steroid free until they reach adulthood (4). The elderly patients aged = 60 years have a higher frequency of cirrhosis at presentation than adults aged = 30 years (5). These elderly patients may have an indolent progressive disease that is asymptomatic or masked by other concurrent rheumatic conditions. An acute, even fulminant, presentation may reflect de novo disease or a spontaneous exacerbation of a pre-existent chronic process (5). Elderly patients require treatment, which has a favorable outcome.

Pathogenesis

The cause for liver injury in AIH is probably autoimmunity, which implies the existence of misdirected immune response against self(6). The mechanism for this autoreactivity is probably due to molecular mimicry in which foreign antigens that resemble self-antigens activate lymphocytes. The liver cell injuries in AIH are due to cell-mediated immunity, which is directed against genetically predisposed hepatocytes. Abnormal HLA class II present on the surface of hepatocytes facilitates the presentation of normal liver cell membrane constituents to antigen presenting cells. The cells in turn stimulate the clonal expansion of autoantiantigen-sensitized cytotoxic T

lymphocytes, which in turn infiltrate the liver tissue and destroy liver cells. The abnormal HLA expression may be triggered by genetic factors, viral infections such as Ebstein Barr, cytomegalo, hepatitis A, B, C and D viruses, and drugs such as oxyphenistain, minocycline, etc. These viral and pharmacological agents trigger autoantibody response to various cytochrome P450 (CYP) enzymes expressed in liver and kidney(7). The most convincing evidence is related to hepatitis viruses e.g., hepatitis C virus elicits anti-liver kidney and microsomal (LKM)-1 against CYP2D6 in a subset of individuals during long standing chronic infection; hepatitis D virus elicits anti-LKM-3 against UDP glucouronoyl transferase 1A, etc.

Clinical features and Diagnosis

The clinical spectrum of presentation ranges from no symptoms to severe symptoms and even as acute or fulminant liver failure. The symptoms are non-specific of varying severity and include fatigue, malaise, anorexia, nausea, abdominal pain, arthralgia, jaundice and pruritis. Physical examination may be entirely normal or may reveal hepatomegaly, splenomegaly, jaundice and signs of liver failure. AIH may present for the first time during pregnancy or in the early post partum period.

The diagnosis of AIH is based on various clinical and biochemical findings along with circulating autoantibodies, raised immunoglobulin levels and characteristic histological picture (Table 1) (8). Interface hepatitis and portal plasma cells are the characteristic histological hallmarks of AIH.

Classification of AIH

AIH has been further classified into three types on the basis of presence of autoantibodies (Table 2). These autoantibodies in AIH can exhibit mutual exclusivity, and they have been the basis for designating two types of AIH. They however do not have distinctive etiologies or responses to corticosteroid therapy. International Autoimmune Hepatitis Group (IAHG) has not endorsed them as valid clinical entities but the designations have been useful as clinical and research descriptors.

Type 1 AIH is the most common form of the disease worldwide and is associated with ANA and/or SMA (9). It constitutes 80% of all the cases of AIH. In 48% of cases, AIH type 1 is associated with extrahepatic autoimmune syndromes. It has a strong

Table 1 : Diagnostic criteria (8)

Requisites	Definite	Probable
No viral infection	No markers of hepatitis A, B, and C viruses	No markers of hepatitis A, B, and C viruses
No alcohol injury	Daily alcohol <25 g/d	Daily alcohol <50 g/d
No toxic injury	No recent use of hepatotoxic drugs	No recent use of hepatotoxic drugs
No genetic liver disease	Normal á1 anti-trypsin phenotype, normal ceruloplasmin, Cu, Fe ferritin levels	Partial á1 antitrypsin deficiency, nonspecific ceruloplasmin, Cu, Fe, ferritin abnormalities
Laboratory features	↑↑ serum ALT/AST, Globulin, ã-globulin or immunoglobulin G level ≥ 1.5x normal	↑↑ serum ALT/AST, Hypergammaglobulinemia of any degree
Autoantibodies	ANA, SMA, or anti-LKM-1 ≥ 1:80 in adults and ≥ 1:20 in children; no AMA	ANA, SMA, or anti-LKM-1 ≥ 1:40 in adults or other autoantibodies
Histologic findings	Interface hepatitis, No biliary lesions, granulomas	Interface hepatitis, No biliary lesions, granulomas

Abbreviation: AMA, antimitochondrial antibodies; ANA, antinuclear antibody; AST, aspartate aminotranferase; ALT alanine aminotranferase; anti-LKM, anti-liver kidney and microsomal; SMA, smooth muscle antibody.

Based on recommendations of the International Autoimmune Hepatitis Group (J Hepatol 1999;31:929-938) (8).

association with HLA DR3 and HLA DR4 haplotype. Type 1 AIH has a mild but acute onset and about 25% of patients can have cirrhosis as the initial presentation. It has high titers of ANA and/or SMA. ANA is the most common antibody in AIH. These antibodies do not correlate with disease activity,

progression, and requirement of treatment. SMA frequently occurs in association with ANA. In pediatric population it may be the only autoantibody in cases of AIH and that too in low titer (1:20).

Type 2 AIH is characterized by anti-LKM 1 positivity and the absence of ANA and SMA (9). It is generally considered to have a poor prognosis, but recent reports do not corroborate this premise. Both type 1 and type 2 AIH respond well to corticosteroids.

The existence of type 3 AIH is the most controversial one and is not recognized by IAHG and is the least common of the three subtypes. It is characterized by presence of anti SLA/LP in serum. The clinical and laboratory features are undistinguishable from type 1 AIH and these cases also respond well to immunosuppressive therapy.

Our own experience at Chandigarh (10) and experience from Lucknow (11) confirms the global distribution of AIH by indicating its occurrence in India. The disease, albeit uncommon among chronic liver disorders in India (frequency, ~2%), is similar to that

Table 2 : Classification of AIH

Variable	**Type 1**	**Type 2**
Characteristic autoantibodies	ANA , SMA	Anti-LKM1
Geographic variation	Worldwide	Worldwide; rare in North America
Age at presentation	Any age	Pediatric 2-14 years; rare in adults
Gender	75% women	90% women
Clinical severity	Wide range	Generally severe
Histopathologic features at presentation	Wide range	Generally advanced
Treatment failure	Infrequent	Frequent
Relapse after drug withdrawal	Variable	Common
Need for long term maintenance therapy	Variable	Approximately 100%

found in North America and Northern Europe. Indian patients did not have antibodies to LKM1. Among 54 adult patients [42 women, mean age 43.5 years(range 30-70)] with autoimmune liver disease studied over a period of 10 years (1994 to 2003) 31 had AIH (all type 1 AIH), 13 had primary biliary cirrhosis (PBC) 5 had AIH + PBC overlap, and 5 had AIH + primary sclerosing cholangitis (PSC) overlap. None of the patients with AIH showed presence of anti-LKM 1. The clinical presentations of patients with AIH included jaundice in 17, generalized weakness in 11, pruritus in 9, dryness of eyes in 3. Of the 19 patients with AIH in whom treatment was given only 14 patients achieved remission. Only one of the five patients with AIH + PBC overlap improved on prednisolone, azathioprine and ursodeoxycholic acid. Two patients with AIH+PSC showed partial response to prednisolone, azathioprine and UDCA.

Treatment

AIH is generally responsive to steroid therapy. It was the first chronic liver disease in which the medical treatment was shown to prolong the survival. Three landmark studies in 1970s have shown that prednisone, prednisolone, or prednisone and azathioprine combination therapy but not azathioprine monotherapy have reduced mortality and morbidity in patents with AIH (12-14). These trials have also shown improvement in clinical as well as histological features. The 20-year life expectancy of all treated patients with severe disease exceeds 80% and survival is similar in age and sex- matched patients from same geographical region (15). Corticosteroid therapy also reduces hepatic fibrosis. Fibrosis scores improved in 56% of patients followed for 55 ± 9 months and did not progress in 33% of patients followed for 62 ± 14 months (3). Histological activity indices decreased concurrently, and patients in whom histological activity indices improved had a higher frequency of improvement in fibrosis scores (80% vs 25%, respectively; $p = 0.002$) (3). These findings suggest that improvement in hepatic fibrosis occurs in conjunction with reductions in liver inflammation and that corticosteroid therapy facilitates the disappearance of fibrosis by suppressing inflammatory activity. Patients with histological features of cirrhosis respond well to steroid treatment as do patients without cirrhosis.

Therapeutic trials have not been done in patients with mild disease and the indications for treatment remain uncertain and have to be individualized. The symptoms, disease behavior and potential for drug related

side effects must be balanced against each other. Clinical judgment remains the basis of treatment. Patients with poor outcome are those with advanced inactive cirrhosis, post menopause, osteopenia or vertebral compression, emotional liability or psychosis, poorly controlled hypertension and brittle diabetes.

Indications of treatment

The clear indications of treatment are – relentless progressive disease with disabling symptoms, fulminant presentation, more than ten fold elevation of aminotransferase, patients with aminotransferase levels that are five fold the upper limit of normal in conjunction with a serum ϒ globulin levels at least twice the upper limit of normal and last but not the least is histological features, such as, bridging necrosis or multiacinar necrosis (Table 3) (16). The indications of treatment in children are similar to those in adults. The disease is more severe in children

Table 3 : Indications for treatment

Parameters	Absolute	Relative	No indication
Clinical	Disabling symptoms Relentless clinical progression Fulminant presentation	Mild symptoms	Asymptomatic
Laboratory	Serum AST ≥ 10-fold upper limit of normal Serum AST ≥ 5-fold upper limit of normal and gamma-globulin level ≥ twice normal	Serum AST level 3 to 9 times normal values Serum AST ≥ 5-fold upper limit of normal and gamma-globulin level < twice normal	Serum AST level < 3 times normal values, Severe cytopenia
Histologic	Bridging necrosis or multiacinar necrosis, severe and confluent interface hepatitis	Mild and moderate interface hepatitis	Inactive cirrhosis Focal interface hepatitis Portal hepatitis Decompensated inactive cirrhosis with variceal bleeding or encephalopathy

at the initial presentation probably due to a delay in diagnosis.

Treatment Regimens

There are two treatment regimens which are generally accepted and have similar efficacy. Prednisolone alone or lower dose of prednisolone in conjunction with azathioprine induces clinical laboratory and histological remission with similar frequency (Table 4).

Table 4 : Treatment regimens for adults

	Prednisone only (mg/d)	Combination	
		Prednisone (mg/d)	Azathioprine (mg/d)
Week 1	60	30	50
Week 2	40	20	50
Week 3	30	15	50
Week 4	30	15	50
Maintenance until end point	20	10	50
Reasons for Preference	Cytopenia, thiopurine methytransferase deficiency, pregnancy, malignancy, short course (=6mo)	Postmenopausal state osteoporosis, brittle diabetes, obesity, acne, emotional lability, hypertension	

The combination therapy is associated with lower incidence of side effects than the higher dose of prednisolone regimen (10% versus 44%) and is the preferred treatment (14). Only 13% of patients develop complication to therapy and necessitate dose reduction or discontinuation (14). The common side effects of azathioprine include cholestatic hepatitis, veno-occlusive disease, pancreatitis, nausea, rash and bone marrow depression. These side effects can be minimized by decreasing dose of the drug. Theoretical possibility of malignancy is a long-term complication. The frequency of extrahepatic malignancy is 5% after 42 months of treatment (17). The risk of

malignancy is 14 fold that of age sex matched population (17).

The role of corticosteroids in the treatment of severe acute and fulminant AIH has not been established, but limited experience suggests that the prompt initiation of corticosteroid therapy can be beneficial in 36–100% of such patients (18).

Prednisone alone is given in patients with severe cytopenia, those undergoing short term treatment trial, women who are pregnant or contemplating pregnancy, patients with active malignancy and individuals with thiopurine methyltransferase deficiency (1). Combination therapy is usually recommended for patients who require more than six months of treatment, postmenopausal women, individuals with emotional instability, osteoporosis, brittle diabetics labile hypertension and obesity (1).

Therapy is continued until remission, treatment failure, incomplete response or drug toxicity. There are no predictions for an early or later remission and hence no prescribed minimum or maximum duration of treatment. Remission is indicated by disappearance of symptoms, improvement of serum aminotransferase levels to less than twice normal, restoration of serum bilirubin and γ globulins levels to normal and improvement of the liver tissue to normal, or portal hepatitis or cirrhosis with minimal or no activity (19). Histological improvement lags behind clinical and biochemical improvement by three to six months and treatment should be continued for at least this period. Sixty-five percent of patients enter remission within eighteen months and 80% achieve remission within three years (13).

Appearance of disease activity after remission and termination of therapy indicates relapse. It is characterized by increase in serum aminotransferase levels to more than three times the upper limit and/or increase in serum gamma globulin level to more than 2 g/dL. Patients who relapse have a greater frequency of progression to cirrhosis (40% versus 18%) development of esophageal varices (25% versus 15%) and death from hepatic failure (15% versus 4%) than patients who sustain remission after drug withdrawal (20). Re-treatment with the original regimen typically induces another remission, but relapse recurs in 79% within 6 months after drug withdrawal (21). With each re-treatment and relapse, the frequency of drug-related adverse effects increases and this risk outweighs the low probability of sustained remission with repeated

conventional therapy. Those patients who relapse can be put either on low dose prednisone (22) or on azathioprine to prevent symptoms and maintain serum aminotransferase at low levels.

Treatment failures have been managed with higher dose of prednisone alone (60 mg/day) or prednisone 30 mg/day in conjugation with azathioprine (150 mg/day). The regimen is continued for at least one month and then dose of prednisone is reduced by 10 mg and dose of azathioprine is reduced by 50 mg after each month of clinical and laboratory improvement Dose reduction is continued till maintenance levels of medications are again achieved (1).

Pregnancy

Pregnancy is not a contraindication to treatment. These pregnant females have slightly higher than normal frequency of prematurity, lower birth weight and fetal anomalies. However, concerns continue regarding the use of azathioprine in pregnancy and the behavior of autoimmune hepatitis during this period. Recently reported cases indicate an unpredictable behavior during pregnancy. Fetal mortality of 19% (usually before the twentieth week); perinatal mortality of 4%; and maternal mortality of 3% has recently been reported (23). Hence, prospective collaborative studies are needed to define the consequences of the liver disease and its treatment on the mother and fetus and to formulate universal management strategies.

Emerging treatment strategies

Treatment with current corticosteroids and azathioprine works in most patients but issues of intolerance and incomplete response arise. These situations led to the investigation of newer immunosupressants including mycophenolate mofetil, budesonide cyclosporine, tacrolimus and ursodeoxycholic acid. These agents promise greater and more site-specific immune suppression than prednisone or azathioprine. These newer agents have been studied in small patient numbers so they are not first-line treatment yet but do have a clear role in those patients with intolerance of incomplete response to standard corticosteroids and azathioprine. Clinical trials are needed to establish their efficacy, safety and target population.

Cyclosporine has been used as salvage therapy in patients with steroid resistance (24,25). Tacrolimus is an empiric therapy for the refractory patient, and it may allow corticosteroid withdrawal (26). Treatment with

mycophenolate mofetil has shown its benefit in individuals refractory to or intolerant of conventional therapy (27). However, these experiences have been counterbalanced by others who have shown treatment failure or inadequate improvement during administration of this expensive medication (28). Mycophenolate mofetil thus may be useful in corticosteroid-responsive patients who require a nonsteroidal maintenance regimen. Other alternative drugs like ursodeoxycholic acid, budesonide, 6-mercaptopurine, methotrexate, and cyclophosphamide have also been used in various small studies. Larger studies need to be done on these drugs to confirm their role in treatment of AIH.

Liver transplantation is indicated in patients who are refractory or intolerant to immunosuppressive therapy and in whom end stage liver

Table 5 : Alternative immunosuppressive agents in the treatment of autoimmune hepatitis not responding to standard treatment

Drug	**Dose (Therapeutic goal)**	**Side Effects**
Cyclosporine A	5 to 6 mg/kg/BW/ od (induction /maintenance of remission)	Nausea/diarrhea, hypertension, renal insufficiency
Tacrolimus	3-4 mg bd (induction / maintenance of remission)	Nausea/diarrhea, hypertension renal insufficiency pancytopenia
Mycophenolate mofetil	1 g bd (induction / maintenance of remission)	Nausea/diarrhea, leucopenia, CMV- infections
Budesonide	3-mg tid (induction / maintenance of remission)	Rare and resembles those of systemic steroids
Cyclophosphamide	1 to 1.5 mg/kg/BW/ od (induction /maintenance of remission)	Pancytopenia, cystitis
Ursodeoxycholic acid	13–15 mg/kg body weight daily in 2 divided doses (maintenance of remission)	Rare, diarrhea

disease develops. Patient and graft survival after liver transplantation range from 83% to 92%, and the actuarial 10-year survival after liver transplantation is 75% (29). Recurrence is recognized in at least 17% of patients after 5 ± 1 year, especially in individuals receiving inadequate immunosuppression (30).

References

1. Czaja AJ, Freese DK (2002). AASLD practice guidelines: Diagnosis and treatment of autoimmune hepatitis. *Hepatology* **36**:479-97.
2. Soloway RD, Summerskill WHJ, Baggenstoss AH, Geall MG, Gitnick GL, Elveback LR, Schoenfield LJ (1972). Clinical, biochemical, and histological remission of severe chronic active liver disease: a controlled study of treatment and early prognosis, *Gastroenterology* **63**:820-33.
3. Czaja AJ, Carpenter HA (2004). Decreased fibrosis during corticosteroid therapy of autoimmune hepatitis. *J Hepatol* **40**:646-52.
4. Gregorio GV, Portman B, Reid F, Donaldson PT, Doherty DG, McCartney M, *et al* (1997). Autoimmune hepatitis in childhood: a 20-year experience. *Hepatology* **25**:541-7.
5. Czaja AJ (2008). Clinical features, differential diagnosis and treatment of autoimmune hepatitis in the elderly. *Drugs Aging*. **25**:219-39.
6. Czaja AJ (2005). Current concepts in autoimmune hepatitis. *Ann Hepatol* **4**:6–24.
7. Strassburg CP, Mans MP (2002). Autoantibodies and autontigens in autoimmune hepatitis, *Semin Liver Dis.* **22**:339-52.
8. Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, *et al* (1999). International Autoimmune Hepatitis Group report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol.* **31**:929-38.
9. Krawitt EL. Autoimmune Hepatitis. *New Engl J Med* 2006;354:54-66.
10. Dhiman RK, Jain P, Duseja A, Das A, Walker R, Singh V, Chawla Y (2004). Autoimmune liver disease:

Indian experience at a tertiary care centre. Presented at Falk Symposium 142 on Autoimmune Liver diseases, held at Frieburg, Germany in October 2004 (Abstract Page 62).

11. Choudhuri G, Somani SK, Baba CS, Alexander G (2005). Autoimmune hepatitis in India: profile of an uncommon disease. *BMC Gastroenterol.* **5**:27–34.

12. Murray-Lyon IM, Stern RB, Williams R (1973). Controlled trial of prednisone and azathioprine in active chronic hepatitis, *Lancet* **1**:735-7.

13. Soloway RD Summerskil WH, Baggenstoss AH, Geall MG, Gitniæk GL, Elveback IR, Schoenfield LJ (1972). Clinical, biochemical, and histological remission of severe chronic active liver disease: a controlled study of treatments and early prognosis. *Gastroenterology* **63**:820-33.

14. Summerskill WH, Korman MG, Ammon HV, Baggenstoss AH (1975). Prednisone for chronic active liver disease: dose titration, standard dose, and combination with azathioprine compared. *Gut* **16**:876-83.

15. Roberts SK, Therneau T, Czaja AJ (1996). Prognosis of histological cirrhosis in type 1 autoimmune hepatitis, *Gastroenterology* **110**:848-57.

16. Montano Loza AJ, Czaja AJ (2007). Current therapy for autoimmune hepatitis. *Nature Clin Pract Gastroenterol Hepatol.* **4**:202-14.

17. Czaja AJ, Wolf AM, Baggenstoss AH (1981). Laboratory assessment of severe chronic active liver disease (CALD): correlation of serum transaminase and gamma globulin levels with histological features. *Gastroenterology* **80**:687-692

18. Ichai P, Duclos-Vallée JC, Guettier C, Hamida SB, Antonini T, Delvart V, *et al* (2007). Usefulness of corticosteroids for the treatment of severe and fulminant forms of autoimmune hepatitis. *Liver Transpl.* **13**:996-1003.

19. Montano-Loza AJ, Carpenter HA, Czaja AJ (2007). Improving the end point of corticosteroid therapy in type 1 autoimmune hepatitis to reduce the frequency of relapse. *Am J Gastroenterol.* **102**:1005-12.

20. Czaja AJ, Davis GL, Ludwig J, Baggenstoss AH, Taswell HF (1983). Autoimmune features as determinants of prognosis in steroid-treated chronic active

hepatitis of uncertain etiology. *Gastroenterology* **85**:713-17.

21. Czaja AJ, Ammon HV, Summerskill WH (1980). Clinical features and prognosis of severe chronic active liver disease (CALD)after corticosteroid-induced remission. *Gastroenterology.* **78**:518-23.

22. Cazja AJ (1990). Low dose corticosteroid therapy after multiple relapses of severe HbsAg-negative chronic active hepatitis. *Hepatology* **11**:1044-9.

23. Candia L, Marquez J, Espinoza LR (2005). Autoimmune hepatitis and pregnancy: a rheumatologist's dilemma. *Semin Arthritis Rheum.* **35**:49–56.

24. Malekzadeh R, Nasseri-Moghaddam S, Kaviani MJ, Taheri H, Kamalian N, Sotoudeh M (2001). Cyclosporin A is a promising alternative to corticosteroids in autoimmune hepatitis. *Dig Dis Sci.* **46**:1321-7.

25. Fernandes NF, Redeker AG, Vierling JM, Villamil FG, Fong TL (1999). Cyclosporine therapy in patients with steroid resistant autoimmune hepatitis. *Am J Gastroenterol.* **94**:241-8.

26. Aqel BA, Machicao V, Rosser B, Satyanarayana R, Harnois DM, Dickson RC (2004). Efficacy of tacrolimus in the treatment of steroid refractory autoimmune hepatitis. *J Clin Gastroenterol.* **38**:805-9.

27. Chatur N, Ramji A, Bain VG, Ma MM, Marotta PJ, Ghent CN, *et al* (2005). Transplant immuno-suppressive agents in non-transplant chronic autoimmune hepatitis: the Canadian association for the study of liver (CASL) experience with mycophenolate mofetil and tacrolimus. *Liver Int.* **25**:723-7.

28. Czaja AJ, Carpenter HA (2005). Empiric therapy of autoimmune hepatitis with mycophenolate mofetil: comparison with conventional treatment for refractory disease. *J Clin Gastroenterol.* **39**:819–25.

29. Ratziu V, Samuel D, Sebagh M, Farges O, Saliba F, Ichai P, *et al* (1999). Long-term follow-up after liver transplantation for autoimmune hepatitis: evidence of recurrence of primary disease. *J Hepatol.* **30**:131-41.

30. González-Koch A, Czaja AJ, Carpenter HA, Roberts SK, Charlton MR, Porayko MK, *et al* (2001). Recurrent autoimmune hepatitis after orthotopic liver transplantation. *Liver Transpl.* **7**:302-10.

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Liver Pathology in Non-alcoholic Fatty Liver Disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a common condition worldwide, but only a small number of patients with NAFLD develop liver related complications. Liver biopsy is useful in confirming the diagnosis and excluding other liver diseases. It provides prognostic information by staging and grading the disease. Standardized and validated pathologic criteria to grade and stage liver disease related to NAFLD is important, not only for epidemiologic studies of NAFLD, but also to design more effective therapies for NAFLD patients, who are at risk to develop progressive liver disease.

Key words: Non-alcoholic fatty liver disease, liver biopsy, steatohepatitis, fibrosis.

Introduction

NAFLD is one of the most common forms of liver disease and is considered to be a hepatic manifestation of metabolic syndrome. It represents a spectrum of clinico-pathological entities ranging from simple steatosis to cryptogenic cirrhosis and even hepatocellular carcinoma, in patients without a history of significant alcohol intake. NAFLD accounts for approximately 70% to 80% of patients who have elevated ALT after other liver diseases have been ruled out (1). The chances of having NAFLD are

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increased if the patient has risk factors like metabolic syndrome.

Hepatic involvement in diabetes was reported in 1950s (2) and later in obesity in 1979 by Adler and Schaffner (3). In both these conditions there was steatosis, pericentral fibrosis, Mallory hyaline and ballooning degeneration, a pathologic picture that mimicked alcoholic liver disease (3). Ludwig in 1980 coined the term NASH in patients who morphologically had features indistinguishable from alcoholic hepatitis without any history of excessive consumption of alcohol (4).

The gold standard for the diagnosis of NAFLD is liver biopsy, which also clearly differentiates relatively benign simple steatosis from potentially progressive NASH. The decision for doing a liver biopsy should take into account how such information would affect the management of such patients. The liver biopsy assesses the extent of necroinflammatory activity, fibrosis, and architectural alterations. It documents lesions of steatosis or steatohepatitis in lean individuals, individuals with hepatomegaly but normal liver function tests and those taking certain medications known to produce steatosis.

Liver biopsy gives significant information on the natural history of NAFLD. Lee *et al* has done a follow up study in 13 obese and diabetic women with NASH by doing a liver biopsy after a mean of 3.5 yrs. Progression of fibrosis was seen in 5, and cirrhosis in 2 (5). Yet another autopsy study on 200 obese individuals by Wanless *et al* showed severity of steatosis proportional to the degree of obesity ranging from 7% in lean subjects to 29.2% in patients who were morbidly obese. They found a higher prevalence of steatohepatitis with increasing obesity reaching 18.5% in morbidity obese (6).

Histological Grading and Staging in NAFLD

Despite increased recognition of NAFLD, uniform defining criteria has been elusive till now. The current staging systems are usually intended for diagnostic purposes and might not be the best for detecting changes in fibrosis because of its heterogeneity which can lead to sampling error. A pathologic protocol for NAFLD was developed in 1998 and its reproducibility was evaluated in 19 diabetics for pathological features which included steatosis, inflammation, liver cell injury and fibrosis. Interobserver and intraobserver agreement on the extent of steatosis, sinusoidal and pericellular locations of fibrosis, presence of vacuolated nuclei

and ballooning degeneration was good amongst the group of hepatopathologists, but was less so with grading of inflammation (7). Long term outcome was assessed in 132 patients of NAFLD with liver biopsy. They were divided into 4 different pathologic groups of **Type 1**- fatty liver alone, **Type 2** - fatty liver plus lobular inflammation, **Type 3** -steatosis and ballooning degeneration, **Type 4** - type 3 with Mallory hyaline or fibrosis (Fig.1 (a) – (d)). A benign course was observed in Type 1 & 2 NAFLD, while a progressive course was seen in Type 3 and 4 who had NASH (8).

Subsequently the grading and staging of NASH was proposed by Brunt *et al* (9). They proposed 10 histological variables to determine the

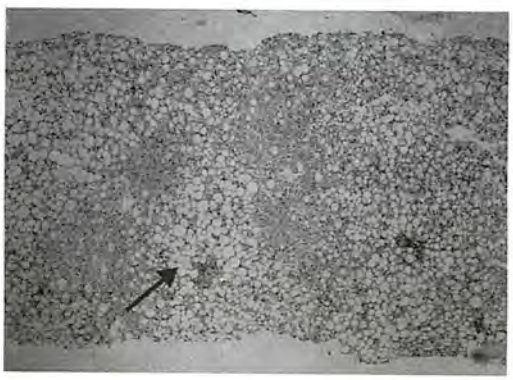

Figure 1(a) : Liver biopsy showing Type I NAFLD with > 66 % steatosis (H&E)

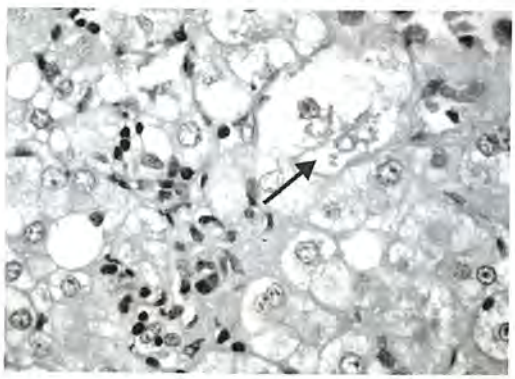

Figure 1(b) : Liver biopsy showing Type II NAFLD with ballooned hepatocytes with Mallory hyaline (H&E)

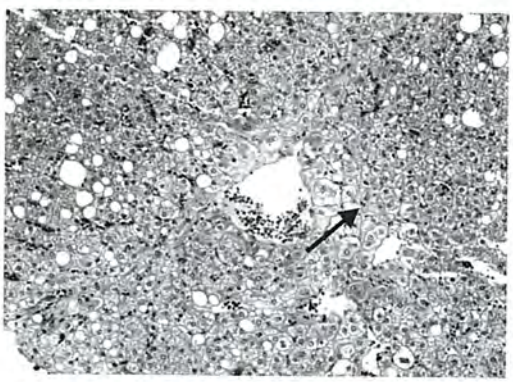

Figure 1(c) : Liver biopsy showing Type III NAFLD with pericellular fibrosis (Massons)

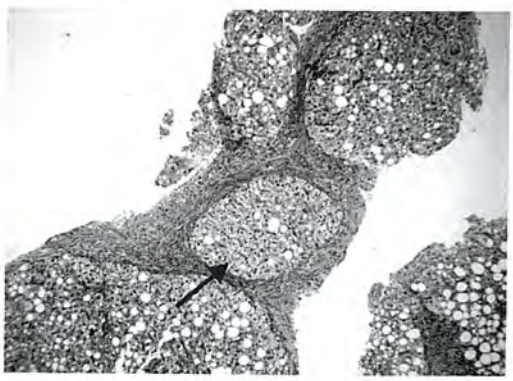

Figure 1(d) : Liver biopsy showing Type IV NAFLD with cirrhosis

activity or grade of the disease. It included **Grade 1** - hepatocellular steatosis typically in zone 3 with occasional ballooning and possible scattered intra acinar inflammation, **Grade 2** - moderate steatohepatitis, ballooning, disarray and intra acinar inflammation predominantly in zone 3 with perisinusoidal fibrosis and mild to moderate portal inflammation, **Grade 3** - Panacinar steatosis, severe inflammation, ballooning and disarray and mild or moderate portal inflammation. Staging of NASH included **Stage 1** - zone 3 perisinusoidal fibrosis, **Stage 2** - focal or extensive periportal fibrosis, **Stage 3** - bridging fibrosis and **Stage 4** - cirrhosis. It is widely used now.

The Nonalcoholic Steatohepatitis Clinical Research Network developed and validated another histologic scoring system applicable to the entire spectrum of NAFLD since Brunt scoring was meant for only NASH (10). They proposed a NAFLD activity score (NAS) based on 3 items and its scoring system included steatosis (0-3) lobular inflammation (0-3) and ballooning degeneration (0-2). Most patients with a diagnosis of NASH had an NAS of =5 while those with a score of =2 did not have NASH. Those with scores of 3 or 4 were split between above 2 categories. Agreement on pathological scoring was however less strong in pediatric patients than in adults.

The above scoring system provided a tool for stratifying patients and systematically recording disease features and assessing changes objectively. They would also be helpful to design more effective therapeutic options and assessing their response histologically.

Does everyone require a liver biopsy?

Even though a definite diagnosis of NAFLD/NASH can be made only on histology, there is reluctance to proceed with a liver biopsy due to the slowly progressive nature of the disease and lack of specific treatment. We could do a liver biopsy in only 43 out of 127 patients of NAFLD, who presented with persistently raised ALT. Approximately 80% of individuals who have hepatic steatosis on sonography in the general population have aminotransferases in normal range (11). Arguments in favour of biopsy include: (i) exclusion of alternative causes of liver disease, (ii) distinguish simple steatosis from NASH, (iii) estimate prognosis based on degree of fibrosis, and (iv) determination of progression of fibrosis over time. Arguments against biopsy include: (i) generally good prognosis of

NAFLD, (ii) lack of effective therapy, (iii) risks and costs associated with biopsy.(iv) and sampling error on assessing fibrosis stage with inter and intra observer variations on histopathology interpretation. Predictors of severe liver fibrosis (bridging fibrosis/cirrhosis) on liver biopsy in patients with NAFLD include old age, presence of diabetes mellitus, obesity, AST: ALT more than 1, ALT ≥ 2 times normal and triglycerides ≥ 1.7 mmol/L (12,13). This is the subgroup of patients with NAFLD who would be expected to derive the most benefit from having a liver biopsy and for considering therapy. Liver biopsy helps in prognosticating the liver disease by the presence and severity of fibrosis rather than severity of steatosis, inflammation or hepatocyte ballooning. Liver related mortality in patients with NAFLD has exclusively been shown to occur in patients of NAFLD with advanced fibrosis and cirrhosis (14). Fibrosis progresses slowly in patients with NAFLD at an average of approximately 0.1 stage per year (15). Hence an elderly person with minimal fibrosis is unlikely to develop cirrhosis, but a young individual would be at a greater risk, and this needs active intervention for further progression of fibrosis. In fact young patients with advanced fibrosis should undergo screening for esophageal varices and early detection of HCC (16).

Histological severity in Indian patients with NAFLD

Overall liver histology is mild at presentation in Indian patients presenting with raised transaminases and only half of them have evidence of histological NASH (Table 1) (17,18). On histological analysis, only 22 of our 43 (51%) patients qualified for histological NASH, others having type I or II disease. Most patients with histological NASH also had grade 1 or 2 inflammation and stage 1 or 2 fibrosis;

Table 1 : Liver histology in 43 patients with nonalcoholic fatty liver disease (NAFLD)[10]

	(n (%)
Type I	2 (5)
Type II	19 (44)
Type III	8 (19)
Type IV	14 (32)
NASH (Type III + IV) on histology (n =22)	
Grade 1	10 (41)
2	12 (50)
3	0
Stage 0	6 (27)
1	7 (32)
2	5 (23)
3	4 (18)
4	0

only four patients had bridging fibrosis but none had cirrhosis as defined by Brunt *et al* (9). Among these patients, 61% patients were obese, 33% had metabolic syndrome and 81% were insulin resistance based on HOMA IR. The pathology was lobulocentric with characteristic steatosis, inflammation, ballooning and pericellular fibrosis. Well formed Mallory hyaline was seen in only 3 cases. Steatosis did not significantly correlate with grade and stage of the disease in cases of NASH.

In comparison, among patients with chronic hepatitis C, steatosis was seen in 75.6% cases which was macrovesicular and predominantly non-zonal. Steatosis significantly correlated with fibrosis and with genotype 3 infections but not with BMI. There was no significant difference in age, BMI, waist circumference, ALT, AST and metabolic syndrome between cases of chronic hepatitis C with less than 5% steatosis and those with more than 5% steatosis or NASH. (unpublished observation).

Similar data was recently published by Madan *et al* from AIIMS. Twenty-eight (55%) out of 51 patients with NAFLD who presented with abnormal transaminases had histological evidence of NASH. Majority of patients had grade 1 [32(63%)] or grade 2 [16(31%)] inflammation and either had no [23(45%)] fibrosis or stage I [19(37%)] fibrosis. None of the patients had cirrhosis (19).

These studies highlight that at least in a cohort of Indian NAFLD patients who present with incidental detection of raised transaminases, the disease is mild at presentation. If we look at a different subset of NAFLD patients, the severity of disease may be different especially in those with major risk factors like diabetes mellitus and those presenting late in the course of disease.

Role of Iron in Indian patients with NAFLD

Role of iron and HFE gene mutations in the pathogenesis of NAFLD is controversial. We studied iron parameters in our 60 patients and HFE gene mutations in 30 patients with NAFLD and showed that serum iron does not contribute to the injury in these patients. In addition we showed that HFE gene mutation mainly (C282Y) is ethnicity specific and does not contribute to pathogenesis in our patients of NAFLD/NASH (18,20,21).

Majority of patients (67%) had negative Perls staining for iron on liver biopsy and only 20% and 13% of

patients had 1+ or 2+ iron staining , further proving the point that iron probably had a little role to play in our patients with NASH. (Table 1)

Electron Microscopy in Indian patients with NAFLD

A total of eleven cases with NAFLD were also subjected to ultrastructural examination. The mitochondria were found to be swollen and round with loss of cristae and a few showed concentric lamellations. Constrictions were also noted in the mitochondria giving them a "dumb bell" shape. In addition, "tram track" paracrystalline inclusions were demonstrable in 5 cases. These inclusions were seen in swollen and enlarged mitochondria. Their diameter varied between 12 and 13 nm. No correlation was found between the presence of morphological changes in the mitochondria and the grade and stage of NAFLD. No correlation was also noted between metabolic variables and mitochondrial Ultrastructure. Two patients with clinically diagnosed cases of NAFLD in which the biopsies failed to demonstrate fat also showed mitochondrial changes. Similar findings have also been reported earlier (22,23). These abnormalities are likely to be due to an adaptive response to oxidative injury (24). These may also be associated with functional changes like decreased respiratory enzyme activity (25).

In conclusion liver biopsy is essential for a diagnosis of NASH. Grading and Staging of the liver biopsy helps in prognosticating the NAFLD so that effective therapeutic measures could be given.

Acknowledgement

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References

1. Sorbi D, Mcgill DB, Thistle JL *et al* (2000). Assessment of the role of liver biopsies in asymptomatic patients with chronic liver disease abnormalities. *Am J Gastroenterology* **95**:3206-10
2. Jaques WE (1953). The incidence of portal cirrhosis and fatty metamorphosis in patients dying with diabetes mellitus. *N Eng J Med.* **249**:442-5
3. Adler M, Schaffner F (1979). Fatty liver hepatitis and cirrhosis in obese patients. *Am J Med* **67**:811-6
4. Ludwig J, Viggiano TR, Mcgill DB *et al* (1980). Nonalcoholic steatohepatitis. Mayo Clinic

experience with a hitherto unnamed disease. *Mayo Clin Proc* **55**:434-8

5. Lee RG (1989). Non alcoholic steatohepatitis: a study of 49 patients. *Hum Pathol* **20**:594-8
6. Wanless IR, Lentz JS (1990). Fatty liver hepatitis and obesity. An autopsy study with analysis of risk factors *Hepatology* **12**:1106-10
7. Younossi ZM, Gramlich T, Liu YC, Matteoni C, Petrelli M (1998). Nonalcoholic fatty liver disease: assessment of variability in pathologic interpretations. *Mod Pathol.* **11**:560-5
8. Matteoni CA, Younossi 27 Gramlich T *et al* (1999). Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gasteroenterology* **116**;413-9
9. Brunt EM, Janey CG, Di Bisceglie AM, Nender-terri BA, Bacon BR (1999). Nonalcoholic steatohepatitis. Proposal for grading and staging the histological lesions. *Am J Gastroenterology* **94**:2467-74
10. Leiner DE, Brunt EM, Van Natta *et al* (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41**:1313-21
11. Browning JD, Szczepaniak LS, Dobbins R *et al* (2004). Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology.* **40**:1387-95
12. Angulo P, Keach JC, Batts KP, Lindor KD (1999). Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* **30**:1356-62.
13. Dixon JB, Bhathal PS, O' Brien PE (2001). Nonalcoholic fatty liver disease: Predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology* **121**:91-100
14. Adams LA, Lymp JF, St Sauver J *et al* (2005)The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology*. **129**:113-21
15. Adam LA, Sanderson S, Lindor KD *et al* (2005). The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J. Hepatol.* **42**:132-8
16. Adams PC, Arthur MJ, Boyer TD *et al* (2004). Screening in liver disease: report of an AASLD Clinical Workshop Hepatology **39**:1204-12

17. Duseja A, Das A, Dhiman RK, Chawla YK, Das R, Bhadada S (2007). Indian patients with nonalcoholic fatty liver disease presenting with raised transaminases are different at presentation. *World J Gastroenterol* **13**:649-50

18. Duseja A, Das A, Das R, Dhiman RK, Chawla Y, Bhansali A, Kalra N (2007). Clinicopathological profile of Indian patients with nonalcoholic fatty liver disease is different from that in the west. *Dig Dis Sci.* **52**:2368-74

19. Madan K, Batra Y, Gupta SD *et al* (2006). Non-alcoholic fatty liver disease may not be a severe disease at presentation among Asian Indians. *World J Gastroenterol.* **12**:3400-5

20. Duseja A, Das R, Nanda M, Das A, Garewal G, Chawla Y (2005). Nonalcoholic steatohepatitis in Asian Indians is neither associated with iron overload nor with HFE gene mutations. *World J Gastroenterol* 21:**11**:393-5.

21. Duseja A, Das R, Das A, Dhiman RK, Chawla YK, Garewal G (2006). Serum iron levels and hepatic iron overload in patients with nonalcoholic steatohepatitis. *Dig Dis Sci.* **51**:1730-1

22. Sanyal AJ, Campbell-Sargent C, Mirshahi F *et al* (2001). Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology*. **120**:1183-92

23. Caldwell SH, Chang CY, Nakamoto RK, Krugner-Higby L (2004). Mitochondria in nonalcoholic fatty liver disease. *Clin Liver Dis.* **8**:595-617

24. Cortez-Pinto H, Chatham J, Chacko VP, Arnold C, Rashid A, Diehl AM (1999). Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *JAMA* **282**:1659-64

25. Pérez-Carreras M, Del Hoyo P, Martín MA *et al* (2003). Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* **38**:999-1007

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Computer-Aided Design of a New Class of Potent and Selective Inhibitors of Cyclooxygenase 2(COX2), a Key Enzyme in the Inflammatory Pathway

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have been the therapeutic agents of choice for more than a century. However, their adverse gastrointestinal side-effects led to the advent of COX2 selective inhibitors, the coxibs. Recent reports on the harmful cardiovascular and renal side-effects of the conventional NSAIDs as well as the COX2 selective inhibitors valdecoxib and rofecoxib have once again led to the quest for a novel class of inhibitors. Using a structure-based approach, a small, potent and selective peptide inhibitor of COX2 has been identified. The designed peptide is predicted to have a higher activity than the most potent, known inhibitor (celecoxib), and a 800-fold selectivity for COX2 over COX1. It is thus a promising lead compound for the development of a new class of COX2 selective inhibitors.

Key words: Structure-based drug design, Cyclooxygenase, Docking, Peptide inhibitor, Anti-Inflammatory drugs.

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Introduction

There are a variety of enzymes which can function as potential targets in the inflammatory pathway. Also there are a few receptors at the target site which, if scientifically analyzed, can function as potential targets for drug design. Some of the important targets for designing drugs are:

1. Phospholipase A
2. Cyclooxygenases
3. Lipoxygenases
4. Thromboxane A Synthase
5. PGF Synthase
6. PGE Synthases
7. PGD synthase
8. Receptor – PGE_2 receptor, $PGF_{2\alpha}$ receptor, etc.

Cyclooxygenase Pathway and Inflammation

One of the most important mediators of inflammation, prostaglandins (PG), belong to the group of compounds called *eicosanoids*. Prostaglandins are a group of derivatives from C_{20} polyunsaturated fatty acids in the cyclooxygenase pathway. Eicosanoids can mediate virtually every step of inflammation (1). Eicosanoids (arachidonic acid [AA] metabolites) are synthesized by two major classes of enzymes: cyclooxygenases and lipoxygenases. The cyclooxygenase (COX) pathway (Fig. 1) generates eicosanoids with a ring structure known as prostanoids (PGs, TXs, Prostacyclins) while lipoxygenase (LOX) produces open chain compounds (Leukotrienes). Further course in a particular tissue depends on the type of isoenzymes or other enzymes present in it (2). The synthesis of prostaglandin group of eicosanoids is mediated mainly by COX1 and COX2 enzymes. COX1 is a constitutive enzyme expressed all the time, whereas COX2 is inducible and is over expressed at sites of inflammation. COX1 regulates a number of house keeping functions, including gastroprotection, vascular homeostasis, renal blood flow and maintenance of glomerular function (3,4). COX2, in addition to maintenance of cardiovascular, renovascular physiological functions and platelet aggregation, is responsible for the expression of pro-inflammatory prostaglandins (5-7). Prostaglandins will in turn cause vasodilatation thus furthering the inflammatory process. The PGs are also involved in the pathogenesis of fever (temperature regulation in the hypothalamus) (1). Further, PGE_2 is hyperalgesic in that

it makes the skin hypersensitive to pain stimuli (8).

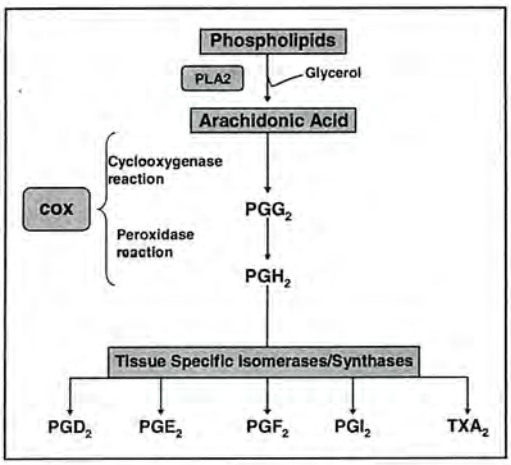

Figure 1: Cyclooxygenase (COX) pathway

Although there are clear differences in function and the DNA and mRNA structures of COX1 and COX2, there is much less difference between the protein structure and function of these enzymes. The core sequences of both enzymes, as well as their crystal structures, are 60% identical (9). Both enzymes have similar kinetics for AA. Despite these similarities, evidence indicates that COX1 and COX2 function as separate enzyme systems. COX1 is localized in the endoplasmic reticulum, whereas COX2 is localized in the endoplasmic reticulum and the nuclear membrane. In addition, COX1 and COX2 use different pools of arachidonate that are mobilized in response to different cellular stimuli for prostaglandin synthesis. PGD_2 is the major metabolite of cyclooxygenase pathway in mast cells; along with PGE_2 and $PGF_{2\alpha}$ (which is more widely distributed), it causes vasodilatation and potentiates edema. Vasodilatory function is also carried out by PGI_2 and PGE_2. PGI_2 also markedly potentiates the permeability-increasing and chemotactic effects of other mediators of inflammation.

There are many possible ways to control inflammation. The narcotic agents like morphine, are one of the most potent analgesic drugs available; but they have very serious side effects - drug abuse and dependency. The steroids are one of the most effective anti-inflammatory drugs currently available for a patient. But the large number of unwanted side effects has led to a focus on non-steroidal drugs with anti-inflammatory properties. Thus the medical field saw the advent of non-steroidal anti-inflammatory drugs (NSAIDs), which function as COX inhibitors. These drugs are free not only from the abuse potential of narcotic analgesics, but also from the steroidal side effects. The production of PGs responsible for the inflammatory process could be sufficiently controlled with conventional NSAIDs. However, these drugs, which inhibited both COX1 and COX2 were found to have adverse gastrointestinal side-effects and, therefore, drugs which selectively

inhibit COX2, such as coxibs were developed. Recent reports on the harmful cardiovascular and renal side-effects of the conventional NSAIDs as well as the COX2 selective inhibitors valdecoxib and rofecoxib have once again led to the quest for a novel class of COX2 selective inhibitors (10-12).

The coxibs (COX2 selective NSAIDs) are now finding new therapeutic applications in the field of colorectal cancer prevention as well as the prevention of progression of Alzheimer's disease (13-18). Celecoxib (19) has been approved for patients with Familial Adenomatous Polyposis (FAP) who have not had their colons removed. Coxibs have also found newer therapeutic application in lung chemoprevention (20).

The current classification of NSAIDs (Table 1) is based on the selectivity of these drugs to the two major isoforms of the COX. NSAIDs are classified as Nonselective Inhibitors [Conventional NSAIDs] (e.g., Aspirin, Indomethacin, Diclofenac etc), Preferential Inhibitors (e.g., Nimesulide, Meloxicam, Nabumetone, Etodolac), Selective Inhibitors (e.g., Celecoxib, Lumiracoxib) and Analgesic-antipyretics with poor anti-inflammatory action (e.g., Paracetamol, Nefopam) (21).

The concept of selective COX2 inhibitor drugs emerged from the need to overcome the unwanted gastrointestinal side effects of the NSAIDs currently available in the market. By definition selectivity is the degree of preference of a drug for a target with respect to another target molecule. In case of COX2 selective inhibitors, popularly known as Coxibs, biochemical assays usually reveal more than a 100-fold difference in the concentration that inhibits recombinant COX2 as compared with COX1 (23).

Table 1 : Classification of NSAIDs (2, 22)

Highly COX2 Selective	**Preferential COX2 Selective**	**COX2 Non-Selective**	**NSAIDs Different Mechanism**
Etoricoxib	Nimesulide	Aspirin	Acetaminophen
Lumiracoxib	Meloxicam	Diclofenac	
Parecoxib	Etodolac	Ibuprofen	
Rofecoxib		Indomethacin	
Celecoxib		Ketorolac (8)	
Valdecoxib			

Diclofenac is considered as one of the safest and has a better pharmacological profile when compared to other conventional NSAIDs (24). The lower the IC_{50} value the drug possesses, the higher is its affinity for the target enzyme. The IC_{50} values and the COX2 selectivity of various NSAIDs with human COX are given in the Table 2.

Due to the similarity of the COX isoenzymes (9), all non-selective NSAIDs also inhibit COX1 thereby blocking its constitutive function and causing various side-effects (10). So it is desirable to design a new class of selective inhibitors on the basis of the structures of COX1 and COX2. Keeping this in mind, we have used the available

Table 2 : Activity and Selectivity of NSAIDs

Drug	IC_{50} for COX1 [ì M]	IC_{50} for COX2 [ì M]	COX2 Selectivity
Aspirin	1.67	278	0.006
Sulindac	1.12	112	0.010
Indomethacin	0.03	1.68	0.017
Naproxen	9.56	5.65	1.692
Flurbiprofen (25)	0.08	0.90	0.089
Ibuprofen	0.03	1.68	0.167
Piroxicam	0.002	0.60	0.004
Diclofenac (26)	0.61	0.63	0.970
Nimesulide	70	1.27	55.118
Celecoxib (23,27)	15	0.04	375
Rofecoxib (23)	15	0.02	833.333
Nabumetone (28)	149	230	0.648

X-ray crystal structures of the complexes of COX1 and COX2 with the known inhibitors to carry out a structure-based, rational, molecular modeling approach to design small peptide inhibitors, which are both potent and selective for COX2.

Structural Details

COX is a heme containing dioxygenase bound to endoplasmic reticulum membranes. Both COX1 and COX2 catalyze two different reactions: a dioxygenase (cyclooxygenase) reaction

and a peroxidase reaction. Structural studies indicate that the active sites are spatially distinct from each other and separated by a heme group (29). COX1 and COX2 have very similar structures characterized by a membrane-binding domain comprised of amphipathic helices forming the entrance to a long hydrophobic channel. This channel leads deep inside the protein, and at its upper end comprises the cyclooxygenase active site.

The important binding residues in the active site (substrate binding) of COX (30) are as follows:

- ARG-120
- VAL-349
- SER-353
- LEU-384
- TYP-387
- VAL-523
- ALA-527
- LEU-531
- TYR-348
- LEU-352
- TYR-355
- TYR-385
- MET-522
- GLY-526
- SER-530

The active site contains mainly hydrophobic residues (9). The known inhibitor, diclofenac, makes hydrogen bonds with tyrosine 385 and serine 530 (31) in the crystal structure complex with COX2 (Fig. 2a). These hydrogen bond interactions are different from the other NSAIDs like flurbiprofen, which have hydrogen bonding to the residues R120 and Y355 as shown in Fig. 2b (found near the lower part of the defined active site of COX2) (32). Hence

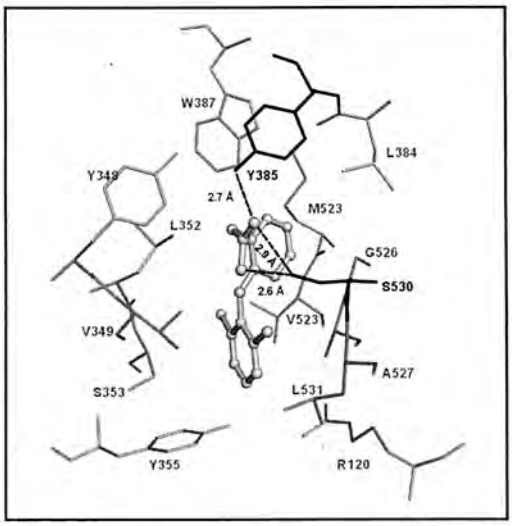

Figure 2a: Crystal structure of COX2 complex with Diclofenac (in light-grey, ball-and-stick rendering) showing the active site residues (stick rendering). Hydrogen bonds are shown as black, dashed lines together with distances. All figures were produced using PyMol v0.99 (35).

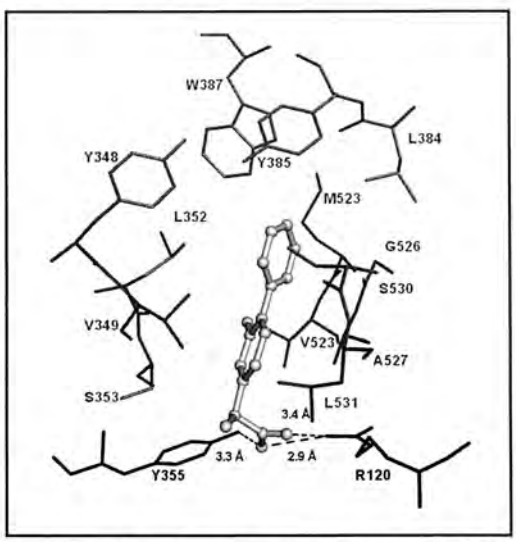

Figure 2b: Crystal structure of COX2 complex with Flurbiprofen. Coloring and rendering are the same as in Fig. 2a.

it would be challenging to design an inhibitor which has hydrogen bonding to residues both in the upper part as well as in the lower part of the active site. Aspirin binds irreversibly to serine 530 by acetylation, whereas most other NSAIDs sterically and reversibly bind to tyrosine 385 or arginine 120, thus blocking the cyclooxygenase action of the enzyme (33, 34). The ligand binding sites of COX1 and COX2 are almost identical except for the amino acid residue at position 523, which is Ile in COX1 and Val in COX2. This leads to a smaller binding site in the case of COX1 (Fig. 3), and the difference can be exploited for the design of COX2 specific inhibitors.

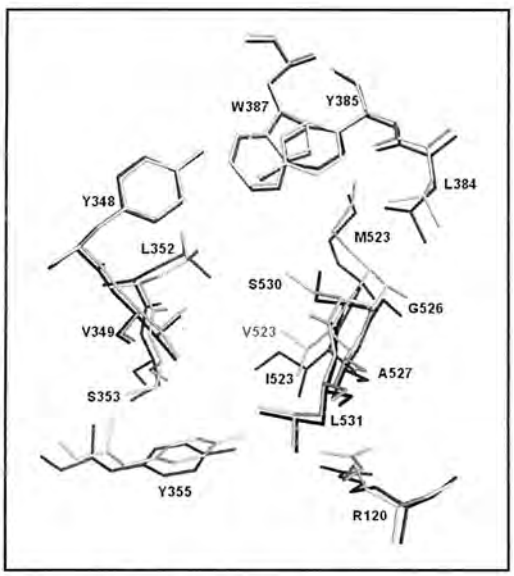

Figure 3: Superposition of COX1 (in grey) and COX2 (in black) active sites showing the difference.

The crystal structures of COX1 and COX2 complexed with several inhibitors have been determined. Our aim in this work is to design a small peptide inhibitor which has

(i) interactions with all the important active site residues,

(ii) potency higher than that of the known inhibitors, and

(iii) high selectivity for COX2

Materials and Methods

The starting point for the computational studies was the X-ray crystal structure of COX2 complexed with the ligand diclofenac [PDB code: 1PXX] (31, 36).

All computations were done using the molecular modeling software package SYBYL 6.81 (Tripos Inc.) running on a Silicon Graphics O2 Workstation. Docking of ligands to PTP1B was done with the version 3.0 of the program AutoDock (37).

Results and Discussion

Validation of the docking methodology

The AutoDock 3.0 methodology was first tested on two known inhibitors (diclofenac and flurbiprofen), which were docked in the active site of COX2, after extracting the ligand from the crystal structure. These two inhibitors

are known to bind to different regions in the active site of COX2.

Figure 4 shows the superimposition of the docked diclofenac (in grey-white) with the crystal structure position (in black). The two structures are seen to overlap very well with a positional root mean square deviation (rmsd) of 2.0 Å. The free energy of binding, ÄG was -9.44 kcal/mole, and the corresponding K_d value at 298K was 0.11µM, which agrees well with the observed value of 0.63µM (26).

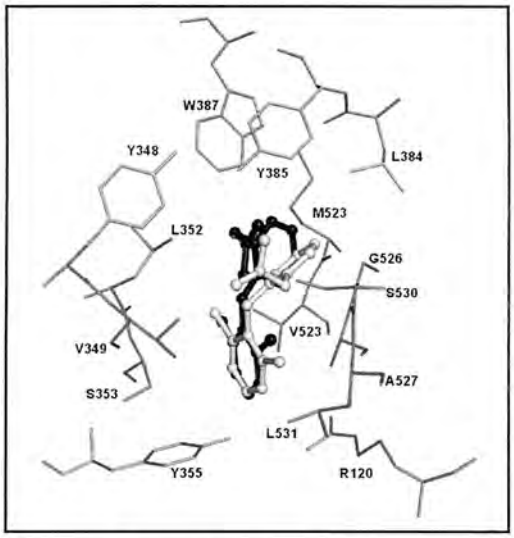

Figure 4: Docked position of diclofenac (in grey-white, ball-and-stick rendering) superimposed on the crystal structure position (in black, ball-and-stick rendering).

Figure 5 shows the superimposition of the docked flurbiprofen (in grey-white) with the crystal structure position (in black) [PDB ID: 1HT8]. It can be seen that the two structures overlap very well with a positional root mean square deviation (rmsd) of 0.6 Å. The free energy of binding, ÄG was -9.46 kcal/mole, and the corresponding K_d value was 0.16 µM, which agrees well with observed value of 0.9 +/- 0.25 µM (25).

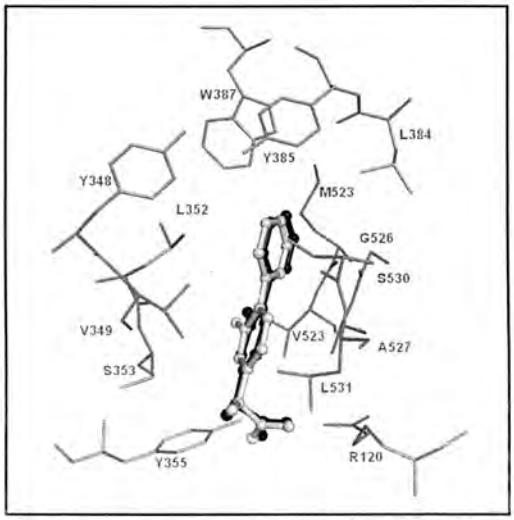

Figure 5: Docked position of flurbiprofen (in grey-white, ball-and-stick rendering) superimposed on the crystal structure position (in black, ball-and-stick rendering).

Thus the docking methodology gives results that are consistent with the observations.

Design of the Peptide Inhibitor and Modeling of the COX2 inhibitor complex

The known potent inhibitors of COX2, such as nimesulide and celecoxib (Fig. 6) have two aromatic rings and a negatively charged group.

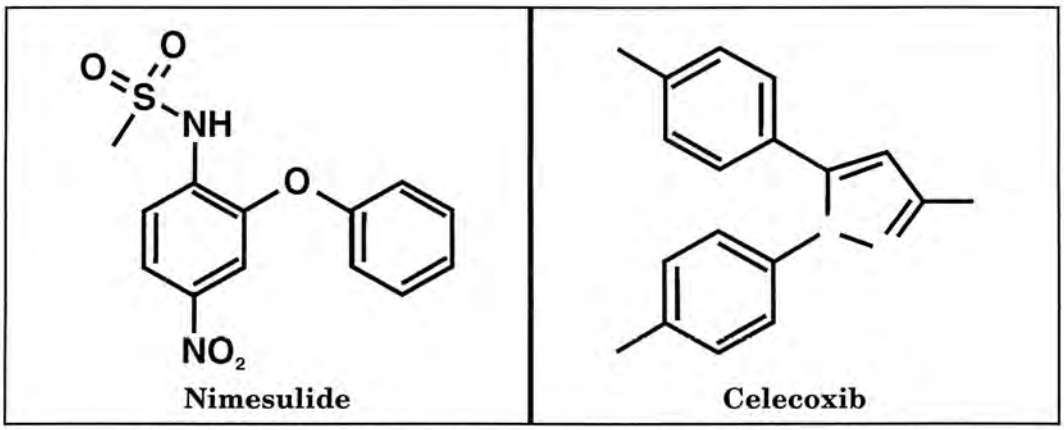

Figure 6: Chemical structure of known, potent inhibitor of COX2.

Using this information, we designed several tripeptide sequences containing two aromatic rings, and a charged residue at the C-terminal end. Each of these tripeptides was docked after a conformational energy search.

One of the most suitable ones (in terms of the docking energy and the interactions) was found to have the following sequence.

$NH3^{+}$ - YWG - COO^{-}

The final docked position of the designed inhibitor is shown in Figures 7 and 8, and the list of contacting residues (up to 4Å) is given in Table 3. It can be seen from the figures and the table that the tripeptide inhibitor occupies a position similar to that of diclofenac and flurbiprofen, but with a larger set of interacting residues. The inhibitor is seen to have hydrogen bonding with the residues Ser 530, Ala 527, Tyr 385, Arg 120 and His 90. The AutoDock3.0 results gave a free energy of binding ÄG = -13.15 kcal/mole and a corresponding K_d value of 2.28 × 10^{-4}ì M, which is about 21 times compared to the most potent known inhibitor (4.8 × 10^{-3}ì M for celecoxib (27)).

Conclusion

Using a computer modeling approach we have identified a small peptide inhibitor of COX2 which has a higher activity than that of the most potent, known inhibitor (celecoxib). In addition the designed peptide has an 800-fold selectivity for COX2 over COX1. Thus the designed inhibitor is a highly suitable lead compound for a new class of potent and selective COX2 inhibitors. Another promising potent and COX2 selective tripeptide inhibitor (WYD) has recently been identified by us (38).

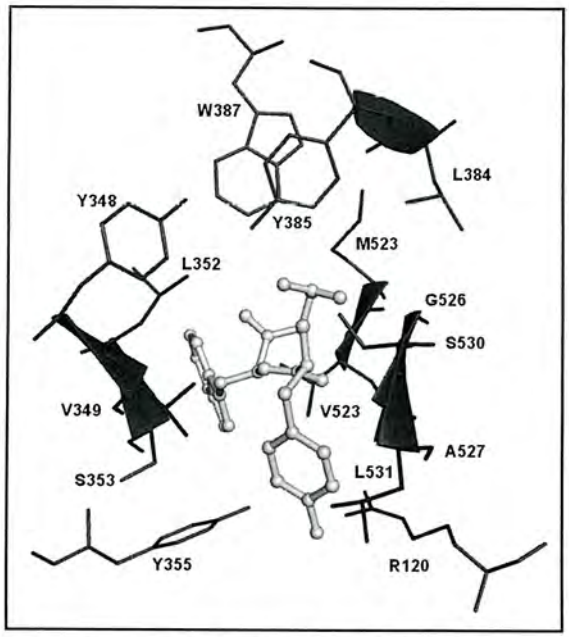

Figure 7: Final docked position of the designed peptide inhibitor, YWG (grey-white, ball-and-stick rendering) with COX2.

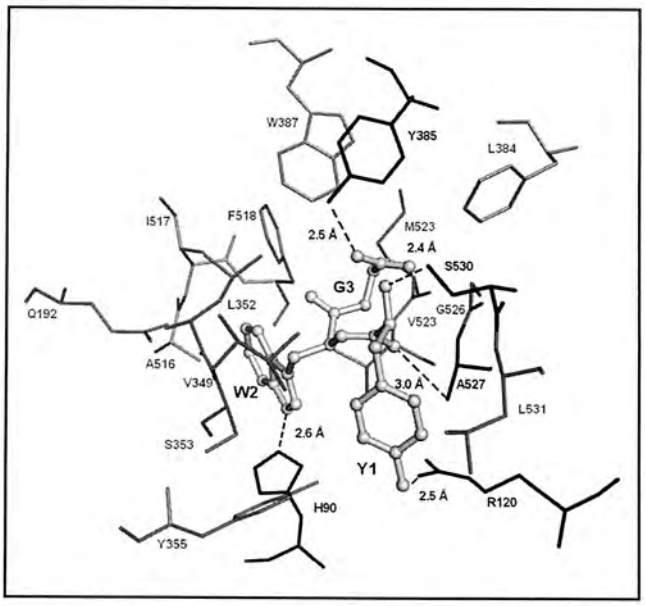

Figure 8: Final docked complex of COX2 with the designed inhibitor (grey-white, ball-and-stick rendering) showing the interacting residues (stick rendering). Hydrogen bonds are shown as black, dashed lines together with distances.

Table 3 : SYBYL6.81 Docking Results

Ligand	Docked Energy(kcal/mol)			Contacting residues (upto 4Å) in the final docked position (hydrogen bonded residues are highlighted in bold)
	Steric	Electrostatic	Total	
Diclofenac	-23.0	-0.4	-23.4	Y348, V349, L352, S353, Y355, L384, **Y385,** W387, F518, M522,V523, G526, A527, **S530**, L531.
Flurbiprofen	-20.0	-4.1	-24.1	**R120**, V349, **Y355**, L384, Y385, W387, M522, V523, G526, A527, S530, L531.
YWG	-59.9	-30.7	-90.6	**H90**, **R120**, Q192, V349, L352, S353, Y355, F381, **Y385**, W387, A516, I517, F518, M522, V523, G526, **A527**, **S530**, L531

References

1. Tucker C (2000). Acute and Chronic Inflammation. *Robbins Pathologic Basis of Diseases* **6th edition**: 50-88.
2. Tripathi KD (2003). Prostaglandins, Leukotrienes (Eicosanoids) and Platelet activating factor. *Essentials of Medical Pharmacology* **5th edition**: 156-166.
3. DeWitt DL and Smith WL (1988). Primary Structure of Prostaglandin G/H Synthase from sheep vesicular gland determined from the Complementary DNA Sequence. *Proc. Natl. Acad. Sci. USA.* **85:** 1412–1416.
4. Yang T, Schnermann JB and Josephine P (1999). Regulation of cyclooxygenase-2 expression in renal medulla by tonicity in vivo and in vitro. *Am J Physiol Renal Physiol.* **277**: F1-F9.
5. Hershman HR (1996). Prostaglandin synthase 2. *Biochim. Biophys.Acta.* **1299**: 125–140.
6. Masferrer JL, Seibert K, Zhang Y, Leahy K, Hauser S, Perkins W, Lee L, Isakson P (1994). Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci USA.* **91**: 3228-3232.
7. Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W,

Lee L and Isakson P (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci USA.* **91**: 12013-12017.

8. Dray A (1995). Inflammatory mediators of pain. *Br J Anaesth.* **75:** 125-131.

9. Picot D, Loll PJ and Garavito RM (1994). The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* **367**: 243-249.

10. Hinz B, Renner B and Brune K (2007). Drug Insight: cyclo-oxygenase-2 inhibitors - a critical appraisal. *Nat Clin Pract Rheumatol* **3**: 552-560.

11. Puhlmann U, Schafer D and Ziemann C (2006). Update on COX-2 inhibitor patents with a focus on optimized formulation and therapeutic scope of drug combinations making use of COX-2 inhibitors. *Expert Opinion on Therapeutic Patents* **16**: 403-430.

12. Solomon DH, Avorn J, Sturmer T, Glynn RJ, Mogun H, Schneeweiss S (2006). Cardiovascular outcomes in new users of Coxibs and nonsteroidal antiinflammatory drugs: high risk subgroups and time course of risk. *Arthritis and Rheumatism* **54**: 1378-1389.

13. Xiao Z, Luke BT, Izmirlian G, Umar A, Lynch PM, Phillips RKS, Patterson S, Conrads TP, Veenstra TD, Greenwald P, Hawk ET and Ali IU (2004). Serum proteomic profiles suggest Celecoxib-Modulated targets and response predictors. *Cancer Research* **64**: 2904–2909.

14. ADAPT Research Group (2007). Naproxen and celecoxib do not prevent AD in early results from a randomized controlled trial. *Neurology*. **68(21)**: 1800-1808.

15. Altman R (2003). Acute coronary disease Athero-Inflammation: Therapeutic approach. *Thrombosis Journal* **1**, 2.

16. Altman R, Luciardi HL, Muntaner J, Del Rio F, Berman SG, Lopez R and Gonzalez C (2002). Efficacy assessment of meloxicam, a preferential cyclooxygenase-2 inhibitor, in acute coronary syndromes without ST-segment elevation: the Nonsteroidal Anti-Inflammatory Drugs in Unstable Angina Treatment-2 (NUT-2) pilot study. *Circulation* **106**: 191-195.

17. Malmstrom K, Kotey P, Cichanowitz N, Daniels S and Desjardins PJ (2003). Analgesic efficacy of Etoricoxib in primary dysmenorrhea: Results of a randomized, controlled trial. *Gynecologic and Obstetric Investigation* **56**: 65-69.

18. Prescott WA and Johnson CE (2005). Antiinflammatory therapies for cystic fibrosis: past, present, and future. *Pharmacotherapy* **25(4)**, 555-573.

19. Henney JE (2000). New indication for Fluoxetine. *JAMA* **283**, 1131

20. Hernández-Díaz S and Rodríguez LAG (2007). Nonsteroidal anti-inflammatory drugs and risk of lung cancer. *International Journal of Cancer* **120(7)**: 1565-1572.

21. Tripathi KD (2003). Prostaglandins, Leukotrienes (Eicosanoids) and Platelet activating Factor. *Essentials of Medical Pharmacology*. **5th edition**, 167-184.

22. Davies NM and Jamali F (2004). COX-2 selective inhibitors cardiac toxicity: getting to the heart of the matter. *J Pharm Pharmaceut Sci.* **7(3)**: 332-336.

23. Rehman Q and Sack KE (1999). When to try COX-2-specific inhibitors. Safer than standard NSAIDs in some situations. *Postgraduate Medicine*. **106(4)**: 95-106.

24. Wyatt PJ, Illingworth RN, Clancy MJ, Munro PT and Robertson CE (2005). *Oxford Handbook of Accident and Emergency Medicine*. **2nd edition**, 263-310.

25. Santini G, Sciulli MG, Panara MR, Padovano R, di Giamberardino M, Rotondo MT, Del Soldato P, Patrignani P (1996). Effects of flurbiprofen and flurbinitroxy-butylester on prostaglandin endoperoxide synthases. *European Journal of Pharmacology* **316 (1)**: 65-72.

26. Blanco FJ, Guitian R, Moreno J, de Toro FJ and Galdo F (1999). Effect of antiinflammatory drugs on COX-1 and COX-2 activity in human articular chondrocytes. *J Rheumatol.* **26(6)**: 1366-1373.

27. Lu ZH, Xiong XY, Zhang BL, Lin GC, Shi YX, Liu ZG, Meng JR, Zhou YM and Mei QB (2005). Evaluation of 2 celecoxib derivatives: analgesic effect and selectivity to cyclooxygenase-2/1. *Acta Pharmacol Sin.* **26(12)**: 1505-1511.

28. Kato M, Nishida S, Kitasato H, Sakata N and Kawai S (2001). Cyclooxygenase-1 and cyclooxygenase-2 selectivity of non-steroidal anti-inflammatory drugs: investigation using human peripheral monocytes. *Journal of Pharmacy and Pharmacology* **53(12)**, 1679-1685(7).

29. Malkowski MG, Ginell SL, William LS and Garavito RM (2000). The productive conformation of arachidonic acid bound to

prostaglandin synthase. *Science* **289**: 1933-1937.

30. Bathon JM, Chilton FH, Hubbard WC, Towns MC, Solan NJ and Proud D (1996). Mechanisms of prostanoid synthesis in human synovial cells: cytokine-peptide synergism. *Inflammation*. **20:** 537-554.

31. Rowlinson SW, James RK, Prusakiewicz JJ, Pawlitz JL, Kozak KR, Kalgutkar AS, Stallings WC, Kurumbail RG and Marnett LJ (2003). A novel mechanism of cyclooxygenase-2 inhibition involving interactions with Ser-530 and Tyr-385. *J Biol Chem*. **278:** 45763–45769.

32. Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, W. C. Stallings *et al*(1996). Structural basis for selective inhibition of cyclo-oxygenase-2 by anti-inflammatory agents. *Nature* **384:** 644-648.

33. Loll PJ, Picot D and Garavito RM (1995). The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. *Nat Struct Bio*. **2:** 637-645.

34. DeWitt DL, El-Harith EA, Kraemer SA, Andrews MJ, Yao EF, Armstrong RL and Smith WL (1990). The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J Biol Chem*. **265:** 5192-5198.

35. DeLano WL (2002). The PyMOL Molecular Graphics System (2002) on World Wide Web http://www.pymol.org.

36. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN and Bourne PE (2000). The Protein Data Bank. *Nucleic Acids Research*. **28**, 235-242.

37. Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Beleu RK and Olson AJ (1998). Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem*. **19**: 1639-1662.

38. Vijayakrishnan R, Ramachandran MV and Subba Rao G (2008). Computer-aided, rational design of a potent and selective small peptide inhibitor of cyclooxygenase 2 (COX2). *J. Biomol. Struct. & Dynamics* **25(5)**: 535-542.

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Craniospinal Irradiation and Carboplatin and Etoposide combination chemotherapy in Medulloblastoma : AIIMS experience

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Abstract

The management of medulloblastoma includes surgery and craniospinal irradiation (CSI). Additionally, different chemotherapy schedules have been tried with an aim to improve survival. The outcome in patients with medulloblastoma treated with surgery, CSI and chemotherapy with carboplatin and etoposide was studied. The records of 50 patients with medulloblastoma treated at AIIMS from May 2001 to May 2005 were reviewed. After surgery, CSI was given to all patients. All patients were planned for 6 cycles of chemotherapy with carboplatin and etoposide after completing CSI, except in patients under 5 years of age who were given chemotherapy upfront. Patients were followed with clinical examination every 3 months and MRI of brain and spine every 6 months. At last follow up, 34 (68%) patients were disease-free, 3 (6%) had local progression, 1 (2%) had residual disease. Disease status was not available for 12 (24%) patients. No grade 3 or 4 chemotherapy or radiotherapy induced toxicities were seen. Chemotherapy with carboplatin and etoposide is an effective combination to be used in the adjuvant treatment of medulloblastoma following surgery and CSI.

Key words: Medulloblastoma, craniospinal irradiation, chemotherapy, carboplatin, etoposide

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Introduction

Medulloblastoma is the most common childhood malignant brain tumor, accounting for 16% of all pediatric brain tumors (1). The peak incidence of medulloblastoma is between 3 and 4 years of age, and the tumor is rare in adults. For adult medulloblastoma the peak age is between 20 and 35 years, after which the incidence steadily declines (2).

Medulloblastoma was first identified in Bailey and Cushing's classification of CNS tumors in 1925(3). The efficacy of radiation therapy was reported within a decade of Cushing's initial report of the tumor. The seminal report documenting the cure of medulloblastoma with CSI was published by Bloom in 1969, citing 32% survival at 5 years and 25% disease free survival at 10 years, with CSI(4).

The first randomized trials incorporating the role of chemotherapy in medulloblastoma were by SIOP (5) and CCG (6) in 1990, which established the role of chemotherapy in high risk patients. A range of alkylating agents and platinum-based regimens has been used in various studies. These have been used with reduced doses of radiotherapy in standard risk patients and with standard doses of radiotherapy in high risk patients.

We present here the outcome of 50 patients with medulloblastoma who were treated with surgery followed by adjuvant CSI and 6 cycles of chemotherapy with carboplatin and etoposide.

Materials and methods

We retrospectively reviewed the records of 50 patients with histopathologically proven medulloblastoma who were treated at our institution from May 2001 to May 2005. Staging work up included MRI of brain and spine and CSF analysis. All patients underwent a maximal safe resection with a histopathological analysis of the operative specimen. Adjuvant management comprised of CSI and chemotherapy.

All patients received CSI with a dose of 36 Gray in 20 fractions to whole brain and 30 Gray in 20 fractions to spine over 4 weeks, followed by a posterior fossa boost of 20 Gray in 10 fractions over 2 weeks (total dose 56 Gray). This was followed 3-4 weeks later by 6 cycles of adjuvant chemotherapy, which consisted of a combination of Carboplatin (AUC 5 iv D1) and Etoposide (100 mg/m^2 iv D1-D3) given every 3 weeks.

In children less than 5 years of age chemotherapy was started upfront after surgery in order to delay initiation of radiotherapy, which was instituted after completion of chemotherapy.

Blood counts were monitored twice weekly during radiotherapy. Additionally, complete blood counts, electrolyte levels, renal and liver function tests were performed before each cycle of chemotherapy. Chemotherapy was initiated only if these were within normal limits. Common toxicity criteria (CTCAE v 3.0) were used for recording treatment related toxicity.

After completion of the planned treatment regimen, patients were followed up longitudinally with a complete clinical evaluation including neurologic examination every 3 months and surveillance magnetic resonance imaging scans of the brain and spine every 6 months.

Results

Patient and disease characteristics

50 patients with medulloblastoma were treated at our institution between May 2001 and May 2005. Mean age was16.22± 11.83 years with a median age of 11.5 years (range 3-55 years). The main characteristics of patients and presenting features are described in Table 1.

Treatment

All patients were staged with MRI of brain and spine and CSF analysis. Surgery comprised maximal safe resection. The extent of resection and postoperative histology are described in Table 2.

Table 1

Age group (years)	Number (%)
0-10	23 (46)
10-20	14 (28)
20-30	6 (12)
30-40	5 (10)
40-50	1 (2)
50-60	1 (2)
Gender	
Male	35 (70)
Female	15 (30)
Presenting features	
Headache	38 (76)
Vomiting	6 (12)
Ataxia	6 (12)
Tumor location	
Midline	33 (66)
Lateralized	17 (34)

Table 2

Parameter	Number (%)
Extent of resection	
Gross total excision	30 (60)
Near total excision	12 (24)
Tumor decompression	8 (16)
Histology	
Classic	41 (82)
Desmoplastic	7 (14)
Anaplastic	2 (14)

All patients completed CSI as per the planned protocol. A total of 242 cycles of chemotherapy were given, the

median number of cycles per patient being 6 (range 0-6). Thirty nine (78%) patients completed 6 cycles of prescribed chemotherapy. Eight (16%) patients did not receive any chemotherapy. No grade 3 or 4 toxicities were observed with either radiotherapy or chemotherapy.

Follow up

Patients were followed up for a median duration of 59 weeks (range 13-230 weeks). At last follow up, 34 (68%) patients showed no evidence of disease, 1 (2%) showed residual local disease on imaging, and 3 (6%) patients showed local progression. Twelve (24%) patients were lost to follow up.

Discussion

Surgery followed by CSI is an accepted standard of care for management of medulloblastoma. Chemotherapy has been used in various studies with an improvement in outcome. With the identification of risk factors, i.e., age, extent of resection and presence or absence of metastases, a number of investigators are now using a risk-adapted approach for medulloblastoma (7) (Table 3) .

Table 3

Factor	Standard risk	High risk
Age at diagnosis	>3 years	=3 years
Extent of surgical resection	<1.5 cm^2 residual	≥1.5 cm^2 residual
Stage	M0	=M1

In standard risk cases, chemotherapy has been tried with the intention of reducing the craniospinal radiotherapy dose and thus the attendant neurocognitive and endocrine effects, while preserving or improving the survival rates. Packer *et al* first proved the efficacy of this concept in 65 patients in a CCG study in 1999(8). They reduced the craniospinal radiotherapy dose from the standard 36 Gray to 23.4 Gray, while maintaining the posterior fossa dose of 55.8 Gray, and added adjuvant chemotherapy comprising of vincristine, cisplatin and lomustine. A progression-free survival (PFS) of 79% at 5 years was observed, which compared favourably with historical controls.

A larger study randomized 421 patients aged 3 to 21 years to treatment with reduced dose CSI (23.4 Gray) and posterior fossa boost of 55.8 Gray, followed by adjuvant chemotherapy with either of two combination regimens; vincristine, cisplatin and lomustine or vincristine, cisplatin and cyclophoshamide(9). 379 patients were available for analysis. Five-year event free survival (EFS) and overall survival (OS) rates were 81% and 86% respectively for the entire cohort. EFS was unaffected by sex, race, age, treatment regimen, brainstem involvement or anaplasia. Patients in the lomustine arm experienced electrolyte imbalances more frequently while those in the cyclophosphamide arm had higher infection rates.

European studies such as the SIOP III study(10) have compared pre-radiation chemotherapy with vincristine, carboplatin and cyclophosphamide followed by standard dose CSI with CSI alone, and showed significant improvement in event free survival at 5 years with pre-radiation chemotherapy (74% vs 60%; p=0.036).

Reduced dose CSI in combination with chemotherapy, as opposed to conventional CSI schedules, has also shown some degree of intellectual preservation in prospective studies (11).

High risk medulloblastoma, especially if metastatic at diagnosis, has a particularly poor prognosis, despite use of standard CSI and chemotherapy. Addition of chemotherapy improves survival by 15-20% compared with historical controls administering radiotherapy alone (12). Pre-radiation chemotherapy has been seen to be inferior to post-radiation regimens in high risk patients, possibly by delaying radiotherapy, which might be responsible for the worse outcome (13).

Chemotherapy has not been able to improve outcomes for adult medulloblastoma patients, and its role in treatment of adult medulloblastoma in first remission is uncertain at present (14).

Our study included all age groups of patients treated for medulloblastoma at our institution from 2001 to 2005. The extent of disease and completeness of resection were noted but were not used for risk stratification of varying treatment regimens. All patients were offered surgery followed by standard dose CSI, the dose to whole brain being 36 Gray and the posterior fossa boosted to 56 Gray. The spine was treated to a dose of 30 Gray. The timing of chemotherapy was determined by the age of the patient. Patients older than 5 years were given CSI first followed by chemotherapy. In younger patients, where it was desirable to delay radiotherapy, chemotherapy followed surgery.

Both carboplatin and etoposide have been seen to be effective chemotherapeutic agents in medulloblastoma. A combination of the two has not been used earlier for the treatment of newly diagnosed medulloblastoma. A phase II study by SFOP evaluated the antitumor efficacy of this combination in 26 patients with measurable disease (residual or recurrent) following surgery, chemotherapy and/or radiotherapy (15). Two courses of carboplatin (160 mg/m^2/day D1-5) and etoposide (100 mg/m^2/day D1-5) were given. 36 courses were evaluated for efficacy and toxicity. Response rate, as assessed by CT, MRI, or myelogram and CSF, was 72%, with 6 patients developing progressive disease. Median duration of grade 4 neutropenia was 8 days. 6 patients developed life-threatening septicemias. Severe toxicity was encountered mainly in heavily pretreated patients.

We used the carboplatin-etoposide combination at lower doses compared with the SFOP study, and found this schedule to be both feasible and effective. No grade 3 or 4 adverse events were noted. Our follow up duration was short, the median duration being 59 weeks. 68% patients were disease-free at last follow up. It remains to be seen how these patients fare on long term follow up, in order to be able to compare our results with those of other studies.

References

1. Central Brain Tumor Registry of the United States (2002). 1995–1999 Statistical Report: Primary Brain Tumors in the United States. Chicago: Central Brain Tumor Registry of the United States.
2. Chan AW, Tarbell NJ, Black PM *et al* (2000). Adult medulloblastoma: prognostic factors and patterns of relapse. *Neurosurgery* **47(3)**: 623-631, discussion 631-632.
3. Bailey J, Cushing H (1925). Medulloblastoma cerebelli, common type of mid-cerebellar glioma of childhood. *Arch Neurol Psychiatry* **14**: 192-224.
4. Bloom HJ, Wallace EN, Henk JM (1969). The treatment and prognosis of medulloblastoma in children. A study of 82 verified cases. *Am J Roentgenol Radium Ther Nucl Med* **105(1)**: 43-62.
5. Tait DM, Thornton-Jones H, Bloom HJ, Lemerle J, Morris-Jones P (1990). Adjuvant chemotherapy for medulloblastoma: the first multicentre control trial of the International Society of Pediatric Oncology (SIOP I). *Eur J Cancer* **26(4)**: 464-469.

6. Evans AE, Jenkin RD, Sposto R *et al* (1990). The treatment of medulloblastoma. Results of a prospective randomized trial of radiation therapy with and without CCNU, vincristine and prednisone. *J Neurosurg* **72(4)**: 572-582.

7. Packer RJ, Cogen P, Vezina G, Rorke LB (1999). Medulloblastoma: clinical and biologic aspects. *Neuro-oncol* **1(3)**: 232-250.

8. Packer RJ, Goldwein J, Nicholson HS *et al* (1999). Treatment of children with medulloblastoma with reduced-dose craniospinal radiation therapy and adjuvant chemotherapy: A Children's Cancer Group Study. *J Clin Oncol* **17(7)**:2127-2136.

9. Packer RJ, Gajjar A, Vezina G *et al* (2006). Phase III study of craniospinal radiation therapy followed by adjuvant chemotherapy for newly diagnosed average-risk medulloblastoma. *J Clin Oncol* **24(25)**:4202-4208.

10. Taylor RE, Bailey CC, Robinson K *et al* (2003). Results of a randomized study of preradiation chemotherapy versus radiotherapy alone for nonmetastatic medulloblastoma: The International Society of Paediatric Oncology/United Kingdom Children's Cancer Study Group PNET-3 Study. *J Clin Oncol* **21(8)**: 1581-1591.

11. Ris MD, Packer RJ, Goldwein J, Jones-Wallace D, Boyett JM (2001). Intellectual outcome after reduced-dose radiation therapy plus adjuvant chemotherapy for medulloblastoma: a Children's Cancer Group Study. *J Clin Oncol* **19(15)**:3470-3476.

12. Tarbell NJ, Friedman HS, Kepner J, Barnes P, Burger P, Kun LE (2000). Outcome of children with high stage medulloblastoma: Results of the Pediatric Oncology Group 9031. *Int J Radiat Oncol Biol Phys* **48(3) (Suppl 1)**:179.

13. Zeltzer PM, Boyett JM, Finlay JL *et al* (1999). Metastasis stage, adjuvant treatment, and residual tumor are prognostic factors for medulloblastoma in children: conclusions from the Children's Cancer Group 921 randomized phase III study. *J Clin Oncol* **17(3)**: 832-845.

14. Greenberg HS, Chamberlain MC, Glantz MJ, Wang S (2001). Adult medulloblastoma: multiagent chemotherapy. *Neuro-oncol* **3(1)**: 29-34.

15. Gentet JC, Doz F, Bouffet E *et al* (1994). Carboplatin and VP 16 in medulloblastoma: a phase II Study of the French Society of Pediatric Oncology (SFOP). *Med Pediatr Oncol* **23(5)**:422-427.

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Early Detection and Molecular Targeting of Human Papillomavirus for Control of Cervical Cancer

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Abstract

Infection with specific types of high risk human Papillomaviruses (HR-HPVs), particularly the HPV type 16 and HPV 18 are essential for the development of cervical cancer. Since it takes 10-15 years to develop invasive cancer, early detection of HPV and treatment of precancerous lesions can reduce the incidence of cervical cancer significantly. Several new technologies have been developed for HPV screening tests. Early detection of HPV infection provides an opportunity to interfere with the viral persistence, propagation and progression of lesions. Several studies revealed that HPV uses several host cellular protein factors for expression of two viral oncogenes, E6 and E7. Targeting these key host cellular regulatory factors is important to silence the expression of oncogenic HPVs. We have therefore screened a few potent anticancer herbal derivatives such as curcumin, *Emblica Officinalis* and B*ryophyllum Pinnata* which are known to have no or negligible toxic/ adverse effects on human systems in order to discover an effective anti-HPV molecule. All the three herbal products are found to have strong suppression

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activity on HPV oncogene expression. Thus early detection of HPV and its targeting by nontoxic herbal drugs can be an effective approach for controlling and reducing cervical cancer.

Key words: Cervical cancer, Human Papillomavirus (HPV), VIA, VILI, Multiplex PCR, Curcumin, Transcription factor.

Introduction

Cervical cancer is the most common cancer amongst women in developing countries including India. The worldwide annual incidence rate of cervical cancer was estimated to be 493,000 and mortality of 274,000 per year and more than two thirds of them are from developing countries (1). The areas of the highest incidence are sub-Saharan Africa, Latin America, the Caribbean, south central Asia, and Southeast Asia (1). In India more than 130,000 cases, comprising one fourth of total world annual incidence, are diagnosed and about 74,000 deaths occur every year. It constitutes 24% of the total cancer incidence; much ahead of breast cancer in India.

It is now well established that the specific types of oncogenic human papillomaviruses (HPVs) are the major etiological agents associated with the development of cervical cancer (2). In addition, various cofactors such as early marriage, high parity, promiscuity, smoking, and other reproductive tract infections may modify the risk of developing cancer in women following infection with HPV. Of more than 100 HPV types known, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 71 and 82 have been found to be associated with invasive cervical cancer and hence classified as high risk types (3).

The major reason for high mortality is due to the cervical cancer insidious latent period of 10-15 years when clinical symptoms are not clear for the diagnosis. In the past two and half decades research has produced enormous amount of evidence that indicates nearly all cervical cancer cases are caused by certain specific types of human Papillomavirus (HPV), making it perhaps the first cancer to be recognized as virally induced. This viral association with disease progression provides a unique opportunity to detect HPV infection and associated cellular changes, low or high grade lesions at an early stage of the disease. With the development of simple, reliable and cost effective

detection procedure of HPV and with early and regular screening programs for disease precursors, most of the cervical cancer can be prevented and/or successfully treated.

Organized screening for cervical pre-cancer and cancer lesions become the most effective way of prevention and treatment of this disease as is evidenced by the experience in developed countries like USA, where screening of women through Pap-Smear had vast impact on reducing (~70%) the incidence and mortality caused by this disease (4). In developing countries like India where cervical cancer is the most serious public health problem among women, such screening programs are not implemented at national level because of large population, high cost, complex procedure and lack of trained manpower and of expertise.

Early detection of high-risk types of HPV is of great importance for identification of clinically latent papillomavirus infections which are highly prevalent in the general population. Reliable diagnosis of HPV would facilitate early identification of high risk population. The techniques available at present for the early detection of cervical pre-cancer and cancerous lesions can be broadly classified in two categories:

1) Conventional Cytological Diagnostics Approaches
2) Molecular Diagnostic Approaches

Conventional Cytological Diagnostics Approaches:

Currently the cervical cancer screening programs are based on conventional cytological and histopathological examinations.

Although *cytology based Pap test* (Papanicolaou-stained smear method) is the universal cervical cancer screening method and has been shown to be effective in reducing cervical cancer incidence in US and other developed countries, in India screening is highly inadequate or non-existing in major part of the country. Recent improvements in liquid based cytology have made the testing more reliable but the tests are still prone to error and not being practiced commonly. *Cervicography* is another technique which uses a photograph of cervical lesion (cervigram), which is then highly magnified and examined. Though this technique is painless, easy to use and provides documentation of area, but it is not sensitive for HPV detection and is limited to only abnormal cellular changes. Another important technique for detection of cervical abnormalities is *colposcopy* which magnifies the cervix

opportunity to interfere with the viral persistence, propagation and progression of lesions. The most common treatment available is the removal of tumorous part by surgery, excision by laser technique and cryosurgery, followed by chemo or radiotherapy. But all these measures are effective against the diseased part of the tissue, not against the HPV that causes the disease. Since the only way to prevent and cure cervical cancer is through effective intervention of viral life cycle within the host cell, different strategies are being developed employed worldwide to restrict and remove HPV infection from the host cell. Molecular studies suggest that HPV uses several host cells signaling pathways for their replication and oncogenes expression.

Host Cell Transcription Factor: The Molecular Target

The tumorigenicity of HPV is dependent upon the aberrant expression of its two early genes E6 and E7(37), the products of which interact and silence the two principal tumor suppressor genes p53 and Rb respectively and interfere with the host cell cycle control, which leads to uncontrolled cell cycle proliferation and neoplasm. The expression of HPV E6 and E7 genes is mainly depended on the availability of host cell transcription factor, Activator Protein-1(AP-1). AP-1 is a very crucial factor for the life cycle of HPV as is evidenced by the several studies where site directed mutagenesis of the AP-1 binding sites on the upstream regulatory region of the HPV genome completely abolishes transcription of URR-driven reporter constructs, either under transient transfection conditions or in stable differentiation-dependent infection assays in organotypic raft cultures (38, 39). Numerous studies have shown that AP-1 is indispensable for HPV transcription regulation and oncogene expression.

What is Activator Protein-1 (AP-1)?

AP-1 is a one transcriptional factor, which is involved in cellular growth, proliferation, differentiation and neoplastic transformation. It is formed by either homodimerization of two jun proteins(c-Jun, JunB, JunD) or heterodimerization of jun and fos proteins(c-Fos, FosB, Fra-1, fra-2) through the "leucine zipper" (40). The upstream regulatory region (URR) of HPV 16 genome contains three crucial binding sites for the AP-1, whereas the HPV 18 URR contains only two binding sites (Fig. 2). Since DNA binding is a necessary prerequisite of transactivation, the expression of different proteins of the jun and fos family is crucial for the activation of

downstream genes regulated by AP-1. Earlier we demonstrated a higher binding activity of AP-1 in both HPV positive and HPV negative cervical cancer cell lines namely HeLa and C33a, respectively, as well as in HPV16 transformed human keratinocytes HPK1a cells (41).

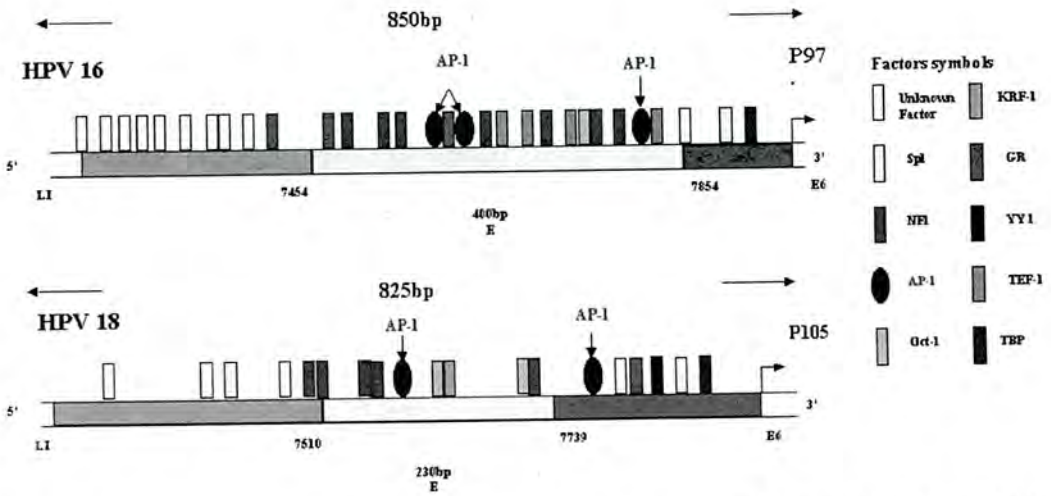

Figure 2: Schematic diagram of upstream regulatory region (URR) of HPV 18 and HPV 16. Transcription factors binding sites in URR of HPV.

In a previous study, we had demonstrated that the transactivation and DNA binding affinity of AP-1 can be modulated by alterations of the intracellular redox status if the cells are treated with a potent antioxidative agent, pyrrolidine dithiocarbamate (PDTC) (41), which blocked not only the transcription of endogenous cellular NFkB–responsive genes such as the monocyte chemoattractant protein 1 (MCP-1) gene, (42) but also selectively suppressed HPV16 expression. Although PDTC induced elevated binding of the AP-1 transcription factor to its cognate recognition sites within the viral regulatory region, it was revealed that the blockage of HPV transcription was mainly due to antioxidant-induced reconstitution of the AP-1 transcription complex (41).

Since the therapeutic utility of synthetic antioxidants such as PDTC is limited due to their high toxicity and other side effects and since there is no

chemotherapeutics available for the treatment of HPV, we have investigated the role of a potential antioxidative agent Curcumin (diferuloylmethane), an active component of the perennial herb turmeric in transcription regulation of HPV. The cell line used for the experiment is HPV18-positive cervical carcinoma cells, HeLa. Treatment was done, in different concentrations of curcumin (50 μM, 100 μM and 200μM) for a fixed duration of 1 hr as well as for different time periods (15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 12 hr and 24 hr).

Curcumin selectively suppresses HPV18 transcription in cervical cancer cells, HeLa

The results revealed a time and concentration-dependent decrease in HPV18 expression (Fig. 3). A nontoxic dose of 100μM curcumin was finalized for further experiments as verified by MTT 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium bromide assay, which showed no cell death, indicating nontoxicity of the dose. A slight decline in HPV18-specific mRNA expression was observed after incubation of HeLa

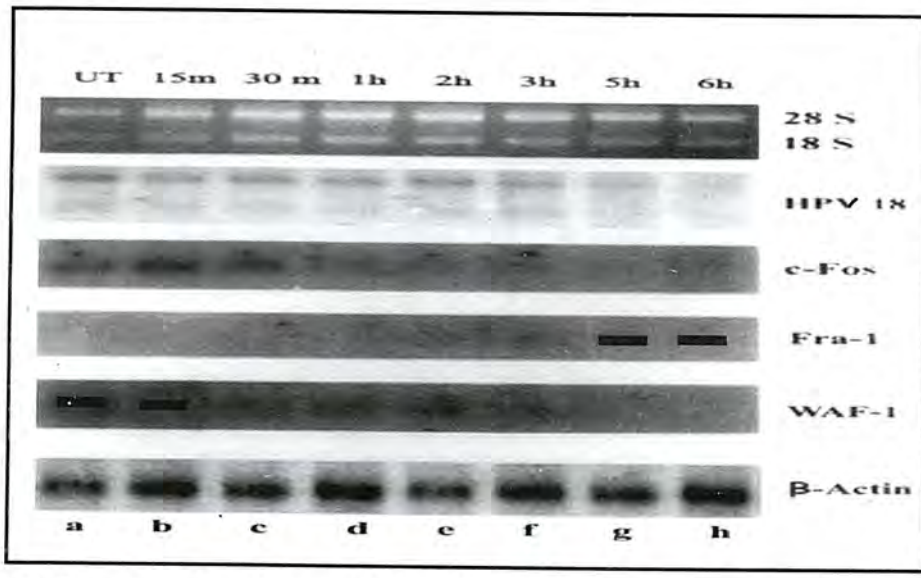

Figure 3: Time-dependent down regulation of HPV 18 transcription in presence of Curcumin. Northern blot analysis showing selective down regulation of HPV18, c-Fos and WAF-1 mRNA expression and up-regulation of Fra-1 mRNA expression after time dependent treatment of 100μm Curcumin in HeLa cells. Lane a, untreated cells; lane b to h, 15min, 30 min, 1h, 2h, 3h, 5h and 6h of incubation in the presence of 100μm Curcumin respectively. To confirm equal RNA loading, the filters were reincubated with β-actin probes. The positions of the 28S and 18S rRNAs are indicated.

cells with 100 μM curcumin for 2.5 hr, and by 4–5 hr the HPV18 expression was completely abolished (Fig. 3). This abolition of HPV transcription was found to be selective and virus-specific and not the consequence of a general transcriptional blockage, since curcumin has no effect on the expression of the endogenous control gene β-actin (Fig. 3). Since the disappearance of HPV18 mRNA follows roughly the same kinetics as observed for HPV16, with the half life of its mRNA being 2.5 hr (41), curcumin interference seems to act at the level of initiation of transcription. This is also borne out by our findings that the disappearance of viral mRNA follows similar kinetics (approximately 2.5 hr) as observed after treatment of HeLa cells with a nonspecific transcription inhibitor, actinomycin D. The viral mRNA, however, started reappearing by 8–9 hr and regained its normal expression by 12 hr (data not shown) possibly because of complete metabolization of the drug.

Curcumin downregulates the AP-1, but not the SP-1, transcription factor

Incubation of HeLa cells with 100 μM curcumin for different durations indicated time-dependent down-regulation of AP-1 binding activity. The AP-1 binding activity started declining after about 2 hr, by which time HPV18 transcription also showed a decline, and was completely abolished by 4–5 hr (Fig. 4). To investigate the specificity of the effect of curcumin on AP-1, we analyzed its effect on other transcription factors such as SP-1. But we could not find any inhibitory effect of curcumin on the SP-1 transcription factor.

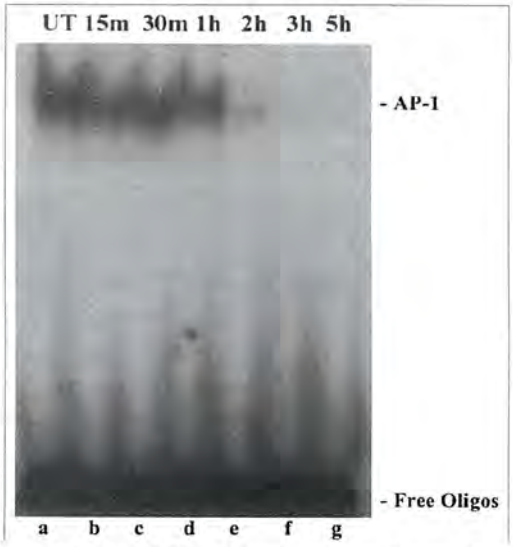

Figure 4: EMSA showing time dependent down-regulation in binding activity in HPV-18 positive HeLa cell extracts following curcumin treatment for different time periods. Lane a, untreated cells; lane b to g 15min, 30min, 1h, 2h, 3h and 5h of incubation in the presence of 100 mM Curcumin respectively. The positions of the specific retarded bands are indicated.

Curcumin downregulates c-fos, but upregulates fra-1 expression

When we analyzed the protein expression pattern of c-fos following

100μM curcumin treatment for different durations (15 min, 30 min, 1 hr, 2 hr, 3 hr and 5 hr), it first showed a decrease in c-fos expression by 2 hr and further decreased gradually in the following hours until it was completely abolished by 4–5 hr. In contrast, fra-1, which was almost nil in untreated HeLa cells, showed its clear appearance by the same time period of 2 hr (when c-fos had started declining) and by 4–5 hr, the fra-1 expression reached its peak (Fig. 5). It is very interesting to note that even though the c-fos mRNA level became almost nil after about 2 hr of curcumin treatment the c-fos protein remains there up to about 3–4 hr. This may be because the pre-existing c-fos protein pool remains for a few hours and is then gradually metabolized, although the transcriptional machinery is blocked by 2 hr of curcumin treatment. Additional experiments were also carried out to detect variation in other AP-1 components after curcumin treatment but no major change in AP-1 composition could be discerned. We also checked the change in composition of the AP-1 binding activity after 100μM curcumin treatment. Since AP-1 binding activity starts decreasing by 2 hr and becomes nil after around 4–5 hr, we could analyze the AP-1 composition only at 1 hr of curcumin treatment. We found that JunB remains the major component, but involvement of c-fos becomes almost nil. A very small amount of Fra1 and Fra2 reappears, but the rest of the components showed no change in their involvement. The results revealed a time dependent down regulation of c-fos expression matching the down regulation of AP-1 activity, but more interestingly, curcumin up-regulated Fra-1 expression to a level generally observed in normal cervical cells. This further strongly strengthens the possible role of Fra-1 in tumor suppression. Thus the inverse correlation of expression dynamics between c-fos and jun B within the AP-1 complex appears to play a crucial role during development and tumorigenic progression of cervical cancer.

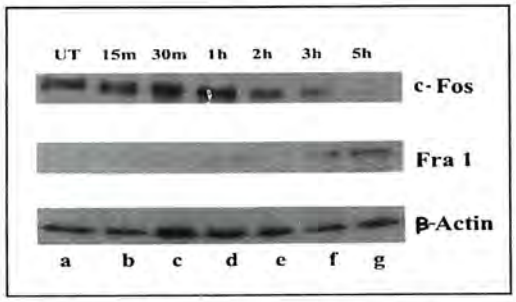

Figure 5: Expression of c-Fos and Fra-1 proteins after time dependent treatment of 100μm curcumin in HeLa cells. Lane a, untreated cells; lane b to g, 15 min, 30 min, 1h, 2h, 3h and 5h of incubation in the presence of 100μm curcumin respectively.

The most significant finding of our study is the selective downregulation of HPV18 transcription in HeLa cells following curcumin treatment. This is

the first time it has been demonstrated that expression of HPV can be specifically suppressed by an antioxidant of herbal origin. It follows almost the same kinetics as revealed by treatment with a transcription inhibitor actinomycin D; therefore it is clear that curcumin acts at the level of initiation of transcription, since downregulation of HPV transcription begins by 2.5 hr, which is also estimated to be approximately the half-life of the HPV18-specific mRNA.

The observation that curcumin-induced suppression of HPV18 expression is paralleled by concomitant abolition of AP-1 binding indicates that AP-1 is indispensable for efficient viral gene expression. This is in sharp contrast to our earlier observation with a synthetic antioxidant, PDTC, which did suppress HPV expression but increased the binding activity of the AP-1 complex. This has been shown to be caused either by changing the composition of AP-1 and/or by posttranslational modifications (41). But curcumin appears to follow a different pathway; it does not depend much on the alteration in AP-1 composition, but it acts at the transcriptional level and downregulates the expression of its components. Although the therapeutic potential of curcumin is implicit, the mechanism(s) of curcumin-induced inhibition of AP-1 binding is not yet clearly understood. It is known that DNA binding of the Jun/Fos complex could be modulated by redox regulation of a single conserved cysteine residue located in the DNA binding domain (43). But it is also possible that curcumin may work in other ways than just as an antioxidant that brings about changes in the redox status of the cell. Whether there exists a curcumin responsive receptor/ element or whether curcumin can activate/inhibit some cellular protein(s) or protein kinases that in turn can interact with Jun/Fos binding, is not known. It is possible that curcumin can act through inhibition of JNK; a c-Jun N-terminal kinase needed for AP-1 activation (44). Another possibility is that curcumin may directly inhibit formation of the jun-fos DNA complex. Similarly, we have investigated anti HPV activity of two other herbal extracts, *Bryophyllum Pinnata* and *Emblica officinalis* on HPV-18 positive cells, HeLa.

Emblica Officinalis suppress HPV18 transcription in HeLa cells.

To analyze the effect of *Emblica Officinalis* on HPV 18 transcription we incubated HPV 18-positive cervical carcinoma cells, HeLa with different concentration of *Emblica Officinalis*

(2002). A Beginner's Manual. Leon, France. *IARC Press.*

10. Carr KC, Sellors JW (2004). Cervical cancer screening in low resource settings using visual inspection with acetic acid. *Journal of Midwifery and women's health* **49**:329-337.

11. Denny L, Quinn M, Sankaranarayanan R. Screening for cervical cancer in developing countries (2006). *Vaccine* **(Suppl 3)** S71-S77.

12. Saraian LO, Derchain SF, Naud P *et al.* (2005).Evaluation of visual inspection with acetic acid (VIA), Lugol's iodine(VILI), cervical cytology and HPV testing as cervical cancer screening tools in Latin America. *Journal of medical screening* **12(3)**:142-149.

13. Hubbard RA (2003). Human Papillomavirus Testing Methods. Arch *Pathol Lab Med* **127**:August 2003

14. Southern EM (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**:503-517.

15. Schiffman MH, Bauer HM, Lorincz AT *et al.* (1991).Comparison of Southern Blot hybridization and polymerase chain reaction methods for the detection of human papillomavirus DNA. *J Clin Microbiol.* **29**:573-577.

16. Das BC, Sharma JK, Gopalkrishna V, Das DK, Singh V, Gissmann L, zur Hausen H, Luthra UK (1992). A high frequency of human papillomavirus DNA sequences in cervical carcinoma of Indian women as revealed by Southern blot hybridization and polymerase chain reaction. *J Med Virol.***36**:239-45.

17. Das BC, Sehgal A, Murthy NS, Gopalkrishna V, Sharma JK, Das DK, Singh V, Luthra UK (1989). Human papillomavirus and cervical cancer in Indian women. *Lancet* **2**:1271.

18. Huang CC, Qiu JT, Kashima ML, Kurman RJ, Wu TC (1998). Generation of type-specific probes for the detection of single-copy human papillomavirus by a novel in situ hybridization method. *Mod Pathol.* **11**: 971-977.

19. Sankaranarayanan R, Gaffikin L, Jacob M, Sellors J, Robles S (2005). A critical assessment of screening methods for cervical neoplasia. *Int J Gynae. & Obstet.* **89(suppl 2)**:S4-S12.

20. Howard M, Sellors J, Kaczorowski J (2002). Optimizing the hybrid capture II human papillomavirus test to detect cervical intraepithelial neoplasia. *Obstetrics & Gynecology* **100**:982-980.

21. Monk BJ, Cook N, Ahn C, Vasilev SA, Berman ML, Wilczynski SP (1994). Comparison of the polymerase chain reaction and Southern blot analysis in detecting and typing human papillomavirus deoxyribonucleic acid in tumors of the lower female genital tract. *Diagn Mol Pathol.* **3**:283-291.

22. Meyer T, Arndt R, Stockfleth E, Flammann HT, Wolf H, Reischl U (1995). Strategy for typing human papillomavirus by RFLP analysis of PCR products and subsequent hybridization with a generic probe. *Biotechniques* **19**:632-639.

23. Rady PL, Arany I, Hughes TK, Tyring SK (1995). Type-specific primer mediated direct sequencing of consensus primer-generated PCR amplicons of human papilloma viruses: a new approach for the simultaneous detection of multiple viral type infections. *J Virol Methods* **53**:245-254.

24. Gravitt PE, Peyton CL, Alessi TQ *et al.* (2000). Improved amplification of genital human papillomaviruses. *J Clin Microbiol.* **38**:357-361.

25. Coutlee F, Gravitt P, Kornegay J *et al.* (2002). Use of PGMY primers in L1 consensus PCR improves detection of human papillomavirus DNA in genital samples. *J Clin Microbiol.* **40**:902-907.

26. Clavel C, Richet S, Masure M *et al* (1998). DNA-EIA to detect high and low risk HPV genotypes in cervical lesions with E6/E7 primer mediated multiplex PCR. *J Clin Pathol.* **51**:38-43.

27. Noffsinger AE, Suzuk L, Hui YZ, Gal AA, Fenoglio-Preiser CM (1995). Differential sensitivities of E6 type-specific and L1 consensus primers in the detection of human papillomavirus in anal carcinoma. *Mol Pathol.* **8**:509-514.

28. Laconi S, Greco M, Pellegrini-Bettoli P, Rais M, Laconi E, Pani P (2001). One-step detection and genotyping of human papillomavirus in cervical samples by reverse hybridization. *Diagn Mol Pathol* **10**:200-206.

29. Perrons C, Kleter B, Jelley R, Jalal H, Quint W, Tedder R (2002). Detection and genotyping of human papillomavirus DNA by SPF10 and

MY09/11 primers in cervical cells taken from women attending a colposcopy clinic. *J Med Virol.* **67**:246-252.

30. Vernon SD, Unger ER, Williams D (2000). Comparison of human papillomavirus detecting and typing by cycle sequencing, line blotting, and hybrid capture. *J Clin Microbiol.* **38**:651-655.

31. Nelson JH, Hawkins GA, Edlund K *et al.* (2000). A novel and rapid PCR-based method for genotyping human papillomaviruses in clinical samples. *J Clin Microbiol.* **38**:688-695.

32. Hart KW, Williams OM, Thelwell N *et al.* (2001). Novel method for detection, typing, and quantification of human papillomaviruses in clinical samples. *J Clin Microbiol.* **39**:3204-3212.

33. Sun CA, Liu JF, Wu DM, Neih S, Yu CP, Chu TY (2002). Viral load of high-risk human papillomavirus in cervical squamous intraepithelial lesions. *Int J Gynaecol Obstet.* **76**:41-47.

34. Kailash U, Hedau S, Gopalkrishna V, Katiyar S, Das BC (2002). A simple 'Paper Smear' method for dry collection, transport, and storage of cervical cytological specimens for rapid screening of HPV infection by PCR. *J. Med Microbiol.* **51**:606-610.

35. Prusty BK, Kumar A, Arora R, Batra S, Das BC (2005). Human papillomavirus (HPV) DNA detection in self-collected urine. *Int J Gynaecol and Obstet.* **90**:223-227

36. Sellors J (2005). HPV in screening and triage: towards an affordable test. *HPV Today* **8**:4-5.

37. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R (1989). The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol.* **63**:4417–21.

38. Offord EA, Beard P (1990). A member of the activator protein 1 family found in keratinocytes but not in fibroblasts required for transcription from a human papillomavirus type 18 promoter. *J Virol.* **64**:4792– 8.

39. Cripe TP, Alderborn A, Anderson RD, Parkkinen S, Bergman P, Haugen TH, Pettersson U, Turek LP (1990). Transcriptional activation of the human papillomavirus-16 P97 promoter by an 88-nucleotide enhancer

containing distinct cell-dependent and AP-1-responsive modules. *New Biol.* **2**:450–63.

40. Angel P, Karin M (1991).The role of jun, fos and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys.Acta.* **1072**:129-157.

41. Rosl F, Das BC, Lengert M, Geletneky K, zur Hausen H (1997). Antioxidant induced changes of the AP-1 transcription complex are paralleled by a selective suppression of human papillomavirus transcription. *J Virol.* **71**:362–70.

42. Rosl F, Lengert M, Albrecht J, Kleine K, Zawatzky R, Schraven B, zur Hausen H (1994). Differential regulation of the JE gene encoding the monocyte chemoattractant protein (MCP-1) in cervical carcinoma cells and derived hybrids. *J Virol.* **68**:2142–50.

43. Abate C, Patel L, Rauscher FJ, III, Curran T (1990). Redox regulation of fos and jun DNA-binding activity *in vitro*. *Science* **249**:1157– 61.

44. Chen YR, Zhou G, Tan TH (1999). C-Jun N terminal kinase mediates apoptotic signaling induced by N-(4-hydroxyphenyl) retinamide. *Mol Pharmacol* **56**:1271-9.

45. Kyo S, Tam A, Laminis LA (1995). Transcriptional activity of human papillomavirus type 31b enhancer is regulated through synergistic interaction of AP-1 with two novel cellular factors. *Virology*. **211**: 184-97.

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Parkinson's Disease: Not just TRAP

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Abstract

James Parkinson described Parkinson's Disease (PD) as a purely motor disorder of progressive stoop, shuffling of gait, tremors and slowness. Over the years some motor and non-motor problems faced by patients PD are being recognized. The main motor features of PD include resting tremor (T), cogwheel rigidity (R), akinesia or bradykinesia (A) and postural instability with gait disturbance (P). The acronym for these motor features can therefore be conveniently called as TRAP. The major problems faced by patients of PD are sleep, problems, neuropsychiatric problems, cognitive impairment, dysautonomia, sensory symptoms, motor fluctuations and dyskinesia and maintenance of balance. A systematic study of some of these problems provides an insight into the morbidity of PD patients which are beyond the symptoms encompassed by TRAP and affect quality of life.

Keywords: Parkinson's disease, resting tremor, cogwheel rigidity, akinesia, postural instability

Introduction

Parkinson's disease is the second common neurodegenerative disorder. The prevalence varies widely in different ethnic groups. On an average it varies from $100/10^6$ to $300/10^6$

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population. Since its first description about two centuries ago by James Parkinson, new symptoms are being increasingly identified which bother the patients (1). James Parkinson described it as a purely motor disorder of progressive stoop, shuffling of gait, tremors and slowness. Over the years some motor and non motor problems faced by patients of Parkinson's disease (PD) are being recognized. The cardinal motor features of PD include resting tremor (T), cogwheel rigidity (R), akinesia or bradykinesia (A) and postural instability with gait disturbance (P). The acronym for these motor features can therefore be conveniently called as TRAP. This also describes very aptly the condition of the patients who seem to be trapped in an immobile rigid body. The standard therapy for PD is based on dopaminergic replacement using dopamine precursor levodopa or dopamine agonists. Typically these provide satisfactory control over motor symptoms in the early stage of the disease. Many patients, however, experience disability despite dopaminergic therapy because of drug related motor complications and emergence of non motor features such as autonomic disturbance, speech and sleep disturbance, gait disturbance and falls, psychosis and dementia. These non motor disturbances are believed to be result of involvement of non dopaminergic neurons in the progressive neurodegeneration which characterizes PD. Further, dopaminergic therapy does not prevent progression and natural course of the disease.

In a very elegant study Braak and colleagues studied brains of PD patients in different stages of disease and individuals who did not have PD. They were able to recognize six stages in which PD could be classified (2). According to Braak's staging, stage 1, is characterized by involvement of lower brainstem and the olfactory area, stage 2 with spread of pathology to the more rostral area into the pons especially locus coeruleus, raphe nucleus and reticular formation. In these two stages the motor symptoms do not appear, but non motor symptoms such as impaired olfaction, constipation and sleep disturbance occur in this stage. In stage 3, the pathological process spreads further rostrally to involve mid brain (subtantia nigra (SN), amygdala, central sub nucleus of forebrain and nucleus of Meynert). It is in this stage when SN pars compacta is affected that the motor symptoms appear. In stages 4, 5 and 6 there is progressive involvement of mesocortex of temporal lobes, Ammon's horns, cingulate and

insular cortex, entero-rhinal cortex and autonomic, limbic and somatomotor areas. This forms the basis of symptoms and signs pertaining to sense of smell, autonomic nervous system, cognition, sleep, depression, anxiety, psychosis and sensory system. The problems of postural instability and falls appear later on in the illness and affect quality of life and adversely affect morbidity and mortality. Some of these problems faced by the PD patients were systematically studied by us providing an insight into the morbidity of PD patients which are beyond the symptoms encompassed by TRAP and affect quality of life.

Sleep problems

Virtually all patients with Parkinson's disease have sleep problems which usually starts early on in the disease (3). The causes are multifactorial including pathological degeneration of central sleep regulation centres in the brainstem and thalamo-cortical pathways (4), motor problems, pain, frequent urination etc. Several sleep problems are reported in patients with Parkinson's disease. These are: sleep fragmentation, excessive day time sleepiness, REM sleep behaviour disorder, paroxysmal limb movement of sleep, and restless leg syndrome. In a prospective study we investigated sleep problems in 149 PD patients and compared it with age matched controls. Sleep problems were reported by 42% of patients compared to 12% among controls (5). Common problems were insomnia (32%), nightmares (32%), excessive day time sleepiness (15%) and RLS (14%). Sleep problems were seen more often in patients with advanced disease, higher disability, higher levodopa dose, and higher rigidity score. Restless leg syndrome, characterized by desire to move legs occurring in late evenings and night, improving with movement of legs and walking is characteristically described as a disorder of dopamine deficiency and responds to dopaminergic agents. These were shown to be 10 times more frequently reported by PD patients as compared to age and gender matched healthy controls (6). REM sleep behaviour disorder (RBD) occurs in about a third of PD patients. It is a parasomnia characterized by loss of normal skeletal muscle atonia during REM sleep resulting in patient physically enacting their dreams. Patient may vocalize, move their limbs or even fall out of bed. It may precede motor symptoms in about 40% of PD patients (7). It is speculated that RDB arises due to degeneration of lower brainstem nuclei including peduculo-pontine and sub-coeruleal nucleus.

Excessive day time sleepiness (EDS) affects up to 50% of PD patients (8). It could be due to poor quality nocturnal sleep or dopaminergic drugs. It rarely may cause sudden onset sleep ("sleep attacks") with motor vehicular accident. This is associated with short sleep latency (<5min) and transition from wakefulness to sleep stage 2 occurring within seconds, similar to narcolepsy. In a study involving 149 PD patients in different stages of illness we observed EDS in 26% of the PD population. It was not a special feature of any drug (9). The suprachasmatic nucleus regulates sleep-wakeful cycle, but its involvement in Parkinson's disease is not proven.

Neuropsychiatric problems

The neuropsychiatric disturbances form important non motor symptoms in PD and range from anxiety, depression and apathy to psychosis. Psychosis is the main reason prompting placement in nursing home in western countries.

Anxiety and Apathy:

Anxiety disorders are common in PD and occur in all stages. When seen in early stage it can be an important preclinical risk factor. In late stages it is usually related to drug induced motor fluctuations. It varies in severity from very mild to severe panic attacks. Apathy has been identified as a distinctive symptom in PD and is indicative of neuro-degenerative process (10). Involvement of frontal subcortical areas and areas between ventral tegmentum and nucleus accumbens is implicated in development of apathy. These symptoms do not respond so well to dopamine replacement therapy indicating involvement of more extensive neurotransmitter pathways.

Depression:

Depending on criteria used depression can affect 10-45% of PD patients (11). Serotonergic as well as limbic non adrenergic and dopaminergic pathways are implicated in its causation. Low levels of 5 HIAA and $5HT1^{A}$ levels are observed in CSF of depressed PD patients as compared to non depressed patients, supporting the role of serotonergic involvement (12). Characteristic features of depression in PD are low self esteem, feeling of guilt, sadness and remorse. Depression can precede motor symptoms of PD supporting Braak's hypothesis. Suicides are rare though suicidal ideations are common. Depression in PD is a significant determinant of quality of life.

Psychosis and hallucinations:

Up to 40% of PD patients have hallucinations especially in advanced

stage. In most of these, the hallucinations are benign. More sinister symptoms such as delusions, paranoid ideation and delirium are not so common. Though often the hallucinations are visual, occasionally auditory hallucinations may also occur. Visual hallucinations are usually due to the drugs but degeneration of pedunculopontine nucleus, locus coeruleus and dopaminergic raphe nucleus are also implicated (13). Risk factors for hallucinations are use of dopa agonists, cognitive impairment and old age. According to one hypothesis, sleep fragmentation leads to vivid dreams, hallucinations and delirium. Some studies have shown polymorphism in cholecytokinin gene in PD patients with hallucinations (14). Psychosis and hallucinations are an important cause of placement in nursing home in western countries and are an important cause of care giver burden.

Cognitive impairment:

Though frank dementia occurs in only about 40% people with PD, subtle cognitive impairment in terms of neuropsychological examination is observed in almost all patients (15). Dementia is progressive and is characterized by dysexecutive syndrome with impaired visuo-spatial ability and memory impairment. The causative factors are degeneration of nigral cells, presence of cortical lewy body and loss of cholinergic cells in nucleus basalis of Myenert. In addition, other co-morbidity like Alzheimer's disease, vascular pathology and APoE genotype may also be contributory factors.

Dysautonomia:

Though prominent dysautonomia is a feature of multiple system atrophy (MSA), early and subtle involvement of autonomic functions are observed in PD and are due to involvement of dorsal nucleus of vagus, nucleus ambiguus, and other medullary centers (2). Additionally, degeneration of cholinergic mono-aminergic and serotonergic nuclei leads to abnormalities in modulation within central autonomic system. In a study almost 50% of PD patients reported their symptoms of autonomic disturbance as highly disabling (16). Cardiac MIBG imaging shows early cardiac sympathetic denervation in PD and not MSA. Whereas, postural hypotension, sweating disturbance etc. occur late in PD, constipation is a prominent symptom seen even before motor symptoms appear. Although there is severe loss of dopaminergic neurons in the colon, constipation does

not respond to dopaminergic treatment suggesting role of non-dopaminergic mechanism. Additionally sexual dysfunction is also reported. Aberrant sexual behaviour and drive are especially features of dopamine dysregulation syndrome (17). Disturbance of sweating is also a major problem in PD especially during off phase. This problem was studied by us using electrophysiological methods of sympathetic response. We observed a large number of patients with abnormal sympathetic skin response suggesting peripheral involvement of sympathetic fibres (18).

Sensory symptoms:

Sensory symptoms such as pain are especially prominent symptoms in patients with PD who develop off periods and dystonias (19). "Frozen shoulder" due to painful restriction of shoulder movements is often the first symptom in PD especially if it starts in an upper limb. Impaired olfactory function is being recognized as a preclinical marker of PD. It gains support from Braaks' hypothesis according to which in stages 1 and 2 the olfactory bulb and anterior olfactory nucleus are predominantly affected. In a study involving 361 asymptomatic relatives of PD patients, 40 were identified to have hyposomnia of which 10% developed PD in 2 years and another 12% showed presynaptic abnormality on SPECT scan. None of the relatives with normal smell sensation developed PD or abnormal SPECT scan (20). It is also seen that hyposomnia like RBD is a marker of synucleopathy (such as PD, diffuse Lewy body disease and multiple system atrophy) and is not seen in other disorders in which parkinsonism is a feature such as progressive supra-nuclear palsy and cortico-basal ganglion degeneration.

Motor fluctuations and dyskinesia:

Early in the course of PD, levodopa controls the primary motor symptoms and provides smooth relief for almost all patients. Initially the effect of a single dose of levodopa lasts for more than 4 hours. Over the years, the drug fails to provide these relatively long symptom free period and patients experience "wearing off", "on" and "off" phenomenon. In some patients in advanced stage there is "delayed on" or "on-on" as well. In addition some patients develop periods of involuntary choreic movements (levodopa induced dyskinesia) characterized by twisting and turning. These may occur when the peak effect is on or during "off" phase. In advanced PD patients these motor phenomenon may be more disabling

than the symptoms of PD itself. Though levodopa is the goid standard in the treatment of PD more than half the patients develop dyskinesia after 5 years of disease. The patients also experience a shorter duration of symptom relief. The literature suggests that motor complications in PD are related to progression of disease and pulsatile non-physiological stimulation of dopamine receptors (21). These result in molecular and physiological changes in receptors resulting in motor complication. In ELLDOPA and CALM-PD trials it was observed that patients initially started on dopamine agonists with longer duration of action and non-pulsatile stimulation of dopaminergic receptors developed dyskinesia much later as compared to patients started on levodopa right in the beginning (22,23). Table 1 shows incidence of motor fluctuations as observed by some authors. Obesso and colleagues presented a classification scheme of PD in which an early stage (3-5 years) is characterized by good response to levodopa, an intermediate stage (5-10 year) in which approximately 50-70% of patients suffer from some drug related motor symptoms and an advanced stage (more than 10 year) when motor complication impair quality of life (29).

Table 1 : Levodopa induced dyskinesia as observed by various authors.

Author	Fluctuations (Wearing off)	Dyskinesia	Duration Years
Rajput *et al* (1984)(24)	10%	25%	5
Poewe *et al* (1986)(25)	52%	54%	6
PSG (1996)(26)	50%	30%	2
Denny and Behari (1999)(28)	27%	25%	—
PSG (2000)(23)	—	30%	2
Rascol *et al* (2000)(27)	45%	—	5

Maintenance of Balance and Falls:

Falls are major problems in patients with Parkinson's and are responsible for major morbidity and mortality. As compared to age matched healthy individuals, patients with PD fall about twice as often (30% vs. 60%) (30). Several causes of falls are identified, such as muscle weakness, sensory abnormality, extrapyramidal

dysfunction and postural instability. Though PD patients complain of weakness, objective isometric muscle strength testing is not performed in many studies. Isometric, isokinetic and torque testing have shown impaired velocity and strength of muscles (31). Impaired righting reflexes are also important in falls resulting in fracture neck of femur in contrast to wrist fracture in healthy controls. Somatosensory functions especially proprioception is also impaired in PD patients resulting in misjudgment of extent of movements (32).

In a comprehensive study we assessed the motor and sensory organization of PD patients during "on" and "off phase" in a quantitative manner to study the role of each of these factors and the effect of drugs (33). The sensory organization test done with eyes open, eyes dosed, altering proprioception, and sway vision showed significant difference between the controls and patients, both during "off" phase and "on" phase, especially on test of proprioception, sway vision, and eyes open-sway support indicating that though vestibular function is not impaired proprioception is impaired in PD patients. Tests of limits of stability showed significant difference on maximum velocity, directional control, maximum excursion and end point excursion between controls and PD patients.

The dynamometer assessment of peak torque also showed significant difference between PD patients and controls in both "on" and "off" periods. The test of gait which measured, base, cadence, stride length, step length and velocity also showed highly significant difference between the PD patients and controls. We also observed that antiparkinsonism drugs have little effect on all these functions except dynamic motor functions. It showed that both motor and sensory functions are impaired in PD patients. The basal ganglion functions as central integration of motor and sensory functions. The supplementary motor area, well connected with the basal ganglion plays a major role in motor planning. Studies on MPTP treated parkinsonian monkeys suggest impairment of cortical processing of sensory information (34). In another study using clinical tests, among 102 PD patients we observed that 35% of PD patients were fallers, and predictors of falls were longer duration of PD, presence of motor blocks, freezing of gait, dyskinesia, duration of "off" period and fear of falls.

References

1. Parkinson J (2002). An essay on the shaking palsy (reprint of monograph published by Sherwood, Neely and Jones. London 1817). *J.Neuropsychiatry Clin Neurosci* **14**:223-36.
2. Braak H, Del Tredici K, Rub U, de Vos RAI, Jansen Steur ENH, Braak E (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* **24**:197-210.
3. Chaudhuri KR (2003). Nocturnal symptom complex in PD and its management. *Neurology* **61(Supp 3)**: S17-S23.
4. Shouse MN, Siegel JM (1992). Pontine regulation of REM sleep components in cats: integrity of the pedunculopontine tegmentum (PPT) is important for phasic events but unnecessary for atonia during REM sleep. *Brain Res* **571**:50-63.
5. Kumar S, Bhatia M, Behari M (2002). Sleep disorders in Parkinson's disease. *Movement Disorders* **17 (4)**: 775-781.
6. Krishnan PR, Bhatia M, Behari M (2003). Restless legs syndrome in Parkinson's disease: A case controlled study. *Movement Disorders* **18(2)**:181-185.
7. Schenck CH. Bundle SR, Mahowald MW (1996). Delayed emergence of a parkinsonian disorder in 38% of 29 older men initially diagnosed with idiopathic rapid eye movement sleep behavior disorder. *Neurology* **46**:388-93.
8. Abbott RD, Ross GW, White IR *et al* (2005). Execessive daytime sleepiness and the future risk of Parkisnon's disease. *Mov. Disord* **20(Suppl 10)**:S101.
9. Kumar S, Bhatia M. Behari M (2003). Excessive daytime sleepiness in Parkinson's disease as assessed by Epworth Sleepiness Scale (ESS). *Sleep Medicine.* **4(4)**: 339-342.
10. Weisskopf MG, Chen H, Schwarzchild MA, Kawachi I, Ascherio A (2003). Prospective study of phobic anxiety and risk of Parkinson's disease. *Mov Disord* **18**:646-651.
11. Burn DJ (2002). Beyond the iron mask: towards better recognition and treatment of depression associated with Parkinson's disease. *Mov Disord* **17**:445-454.
12. Doder M, Rabiner EA, Turjanski N, Lees AJ, Brooks DJ (2000). Brain serotonin 5HTA receptors in Parkinson's disease with and

without depression measured by positron emission tomography with 11CWAY 10635. *Mov.Disord* **15**:213.

13. Diederich NJ, Goetz CG, Stebbins GT (2005). Repeated visual hallucinations in Parkinson's disease as disturbed external/ internal perceptions: focused review and a new integrative model. *Mov.Disord* **20**:130-140.
14. Goldman JG, Goetz CG, Berry-Kravis E. Keurgans S, Zhou L (2004). Genetic polymorphisms in Parkinson disease subjects with and without hallucinations: an analysis of the cholecystokinin system. *Arch Neurol* **61**:1280-84.
15. Emre M (2003). Dementia associated with Parkinson's disease. *Lancet Neurol* **2**:229-37.
16. Magerkurth C, Schnitzer R, Braune S (2005). Symptoms of autonomic failure in Parkinson's disease: prevalence and impact on daily life. *Clin Auton Res* **15**:76-82.
17. Pezzella FR, Colosimo C, Vanacore N *et al* (2005). Prevalence and clinical features of hedonistic homeostatic dysregulation in Parkinson's disease. *Mov Disor* **20**:77-81.
18. Srivastava S, Behari M, Bhatia M (1998). Sympathetic skin response in early Parkinson's disease. *Neurology India* **46** (Suppl 1):
19. Waseem S, Gwinn-Hardy K (2001). Pain in Parkinson's disease: Common yet seldom recognized symptom is treatable. *Postgrad Med* **110**:33-40.
20. Montgomery EB Jr., Baker KB, Lyons K *et al* (1999). Abnormal performance on the PD test battery by asymptomatic first degree relatives. *Neurology* **52**:757-62.
21. Pahwa R, Lyon KE (2004). Options in the treatment of motor fluctuations and dyskinesias in Parkinson's disease: a brief review. *Neurol Clin* **22**:S35-S52.
22. Barone P (2003). Clinical strategies to prevent and delay motor complications. *Neurology* **61(Suppl 3)**: S12-16.
23. Parkinson Study Group (2000). A randomized controlled trial comparing pramipexole with levodopa in early Parkinson's disease: design and methods of CALM-PD study. *Clin Neuropharmacol* **23**:34-44.
24. Rajput AH. Stern W, Laverty WH (1984). Chronic low-dose levodopa therapy in Parkinson's disease: an argument for delaying levodopa therapy. *Neurology* **34**:991-6.

25. Poewe WH, Lees AJ, Stern GM (1986). Low-dose L-dopa therapy in Parkinson's disease: a 6-year follow-up study. *Neurology* **36**:1528-30.

26. Parkinson study group (1996). Impact of deprenyl and tocopherol treatment on Parkinson's disease in DATATOP patients requiring levodopa. *Ann Neurol* **39**:37-45.

27. Rascol O, Brooks DJ, Korczyn AD, De Deyn PP, Clarke CE, Lang AE (2000). A five year study of incidence of dyskinesia in Patients with early Parkinson's disease who were treated with ropinirole or levodopa. *N Engl J Med* **342**:1484-91.

28. Denny AP, Behari M (1999). Motor fluctuations in Parkinson's disease. *J Neurol Sc* **165**:18-23.

29. Obeso JA, Rodriguez-Oroz MC, Chana P, Lera G, Rodriguez M, Olanow CW (2000). The evolution and origin of motor complications in Parkinson's disease. *Neurology* **55(Suppl 4)**:S13-S20; discussion S21-23.

30. Bloem BR, Beckley DJ, Van Dijk JG (1999). Are automatic postural responses in patients with PD abnormal due to their stooped posture? *Exp Brain Res* **124**: 481-8.

31. Pederson SW, Oberg B, Larsson LE *et al* (1997). Gait analysis, isokinetic muscle strength measurement in patients with Parkinson's disease. *Scan J Rehabil Med* **29**:67-74.

32. Schnieder JS, Diamond SG, Markham CH (1987). Parkinson's disease: Sensory and motor problems in arms and hands. *Neurology* **37**:951-6

33. Nallegowda M, Singh U, Handa G, Khanna M, Wadhwa S, Yadav SL, Kumar G, Behari M (2004). Role of sensory input and muscle strength in maintenance of balance, gait and posture in Parkinson's disease: a pilot study. *Am J* Phys *Med Rehabil* **83**: 898-906.

34. Escola L, Michelet T, Douillard G *et al* (2002). Disruption of the proprioceptive mapping in the medial wall of parkinsonian monkeys. *Ann Neurol* **52**:581-7.