V. DISCUSSION

COPD has been known for many years both in industrialized and non-industrialized countries as a disease of high prevalence with rising mortality (Murray and Lopez, 1997; Pauwells 2001). The mortality rates of COPD are tremendously increasing worldwide due to the lack of awareness of this disease. The close association between tobacco smoking and the development of emphysema, chronic bronchitis and a full spectrum of COPD has been known for many years. Smoking attributes for 80 to 90% COPD cases (Stang et al., 2000; Dowson et al., 2001; Ward and Casaburi, 2001). But only 25% of smokers develop COPD and > 15% of COPD related mortality occurs in non-smokers, clearly indicating the involvement of genetic and other environmental factors (Burrows et al., 1987; Silverman et al., 1998; Mannino, 2002).

Alpha-1-antitrypsin deficiency is the only proven genetic risk factor and accounts for approximately 3% of the COPD resulting in early onset emphysema (Lieberman et al., 1969; Cox et al., 1976; WHO, 1997; Silverman et al., 2001). Alpha-1-antitrypsin has been studied extensively both at DNA and protein level because of the prevalence of mutants or the polymorphic forms of the DNA/protein which can be associated with emphysema. It is also responsible for one of the most common single gene defects resulting neonatal hepatitis in about 1 in 2000 to 7000 of the newborns of European origin (Povey, 1990; Mahadeva and Lomas, 1998).
A number of variants with a broad range of molecular mechanisms have been reported to associate with the development of alpha-1-antitrypsin deficiency related emphysema. But severe deficient alleles like PiZ, PiS and the Pi null alleles are the highest risk factors for developing COPD (Graver, 1986; Cox and Levison, 1988). With the small number of null-null subjects identified, it is unclear whether they have an increased risk for lung disease when compared with PiZZ individuals. Similarly it is not clear whether PiZZ and PiZ Null individuals have differential risks for lung diseases. Hence in any population or case control studies the gene frequencies for the normal M1 and deficient \textit{aat} alleles PiZ and PiS are calculated to find the "at risk" population (Luisetti and Seersholm, 2004).

Genetic epidemiological survey data on the general population in countries worldwide have been used to determine the number of carriers and those homozygous or heterozygous for \alpha\textsubscript{1}-AT deficient alleles. It has been estimated that there are approximately 100,000 severely deficient individuals in the USA and approximately 25 million carriers of at least one deficient gene for \textit{aat}. Similar numbers have been suggested for the European population and only less than 6\% of severely deficient individuals have been currently identified (de Serres \textit{et al.}, 2003; Sandhaus, 2004).

Based on the data obtained from various epidemiological studies on \alpha\textsubscript{1}-AT deficiency world wide, we investigated the prevalence of \textit{aat} normal variant PiM1 (Val/Ala), deficient variants PiZ and PiS in COPD patients from south India. For the present study we recruited COPD patients who were
undergoing treatment at the Government Hospital for Thoracic Medicine (GHTM), Tambaram Sanatorium, Chennai, Tamilnadu, India.

The diagnosis of COPD was established on the basis of a history of smoking, cough with expectoration occurring for at least three months in a year for two years or more accompanied by progressive dyspnea on exertion. Individuals completed a self-administered questionnaire, which included questions on respiratory symptoms and tobacco smoking. The presence of emphysema was documented by physical examination showing a hyper resonant chest, flattened hemi diaphragm and distant breath sounds. Chest film (Fig. 12, Page No. 61) showed hyperinflation, flattened diaphragms and a marked loss of vascularity at both bases as reported by previous studies.

In order to find the effect of age on the prevalence of COPD the study was conducted on different age group ranging from 5 to 80 years. The percentage of distribution of COPD patients according to the age group was calculated. On analysis, we could observe an increased prevalence of chronic obstruction in the age group of 50-65 years followed by 35-50 years (Fig. 13, Page No.62). The study showed that COPD is manifested in the third to fifth decades of life. Nearly similar results were obtained in a population based epidemiological study carried out in the general population of Spain. Approximately half of the cases were observed in patients aged 60 to 69 years with the other half divided relatively equally between patients aged 50 to 59 years and 40 to 49 years (Masa, 1999).
COPD is characterized by persistent cough, excessive sputum production and dyspnea. In the present study the most frequent symptom elicited was dyspnea in 69% of the COPD patients (Fig. 15, Page No. 64). Self-reported wheezing was seen in 33% of patients. A chronic cough with sputum production was observed in 44% of COPD patients (Table 7, Page No. 65).

A few studies have described a chronic production of cough for months in at least two successive years consistent with chronic bronchitis in α1-AT deficient patients. A high level of respiratory symptoms was observed in the European Respiratory Society study on chronic obstructive disease population, which evaluated patients with mild or pre-clinical COPD. The respiratory symptoms included cough, expectoration, wheezing and dyspnea (Pauwels et al., 2000).

In the registry prepared by National Heart, Lung and Blood Institute (NHLBI), 84% of the participants had dyspnea on exertion. Self-reported wheezing during infections was prominent (76%), although wheezing independent of infection was also common in 65% of the participants (McElvaney et al., 1997). In the European Respiratory Society study on COPD population, 78% of the study population with pre-clinical COPD reported coughs and phlegm, 47% reported dyspnea and 56% reported wheezing (Pauwells et al., 2000).

The prevalence of exertional dyspnea, cough and sputum were found to increase with age (Fig. 16, Page No. 66.). In the present study, the prevalence
of the clinical symptoms was observed high in the age group of 50 to 65 years followed by 35-50 years. Similar results were obtained in a random sample analysis of COPD in middle aged and older adults (Abramson et al., 2002). The commonest symptoms reported were exertional dyspnea (27.2%) and wheezing (20.5%).

After analyzing the clinical presentations of COPD patients, mutational analyses were carried out in these patients and the results were compared with that of healthy controls. A number of sensitive and convenient methods are available for the detection of substitutional changes in DNA samples. The present work made use of SSCP and RFLP techniques.

When the 110bp PCR product obtained from exon V were denatured and electrophoresed, all the samples exhibited same mobility pattern. There was no variation in the mobility of single strands in patients and control samples (Fig. 22, Page No. 74). Similar results were seen in the case of 98bp PCR products amplified from exon III (Fig. 23, Page No. 76).

In the case of 360bp PCR products amplified from exon III, except one sample, all others showed same mobility pattern (Fig. 24, Page No. 77). A slight variation in the mobility of single strands was seen in the case of the PCR product obtained from patient no: 176 when compared with the mobility pattern of single strands obtained from other PCR products (Fig. 25, Page No. 78). From SSCP analysis it was concluded that all the samples, except one carried normal sequences at the exon III and exon V regions of aat gene.
SSCP is only a scanning method for the identification of mutations and differ widely in their power, accuracy, speed and cost, and each can detect only a subset of mutations. At present, therefore, detection and localization of point mutations often require the sequential application of two or more scanning methods, followed by DNA sequencing to specify the mutation. Hence to further confirm the SSCP gel patterns and the genotypes, restriction digestion analysis (RFLP) was also carried out in the present study.

Our procedure for the restriction digestion analysis was PCR-mediated with the primers for the Z and S alleles labeled as RG 11 and 12 and RG 13 and 14 respectively. Primer mediated artificial Taq I restriction sites were introduced. On restriction digestion, the 110bp PCR products amplified from exon V will yield 89bp and 21bp fragments for the M allele and 110bp fragment for the Z allele, as they remain uncut by Taq I. In case of the 98bp PCR products amplified from exon III, 78bp and 20bp fragments for the M allele and an uncut 98bp fragment for the S allele will be obtained.

As observed by Taq I restricted DNA gel pattern, the 110bp products were cleaved into 89bp and 21bp products indicating the absence of PiZ allele among the samples analyzed (Fig. 26, Page No. 79). Similar results were obtained upon restriction digestion of 98bp PCR products (Fig. 27, Page No. 81). The samples were cleaved into 78bp and 20bp products indicating the absence of PiS allele as well. No 110bp and 98bp bands were seen in both reactions.
The PiM1 allele is the normal allele resulting in normal physiological function. At genomic level there are two forms of M1 allele, i.e., M1Val213 and M1Ala213. Sequence change in the 213th codon, Ala 213(GCG) to Val213 (GTG) alters the restriction site for BstE II enzyme (Nukiwa et al., 1986; Rieger et al., 1999). In case of M1 Val213, digestion of the PCR product with BstE II cleaves the 360bp into fragments of 228, 83 and 49bp. In the presence of M1 Ala213, the BstE II recognition site at codon 213 is lost, and only two bands of 311 and 49bp are generated.

In the case of BstE II restriction digestion, all the samples were cleaved into 228, 89 and 49bp products indicating the M1 Val213 as the only allele present in the present study (Fig. 28, Page No. 82). No 311bp bands were seen in the reactions indicating the absence of M1 Ala213 allele in the study samples.

One SSCP variant was identified in the present study. On restriction digestion it showed a band pattern specific to M1Val213 allele. DNA sequencing was carried out for this SSCP variant (COPD sample no.176) and two control samples (No. 85 and 152). In the sense strand of the SSCP variant at the 7489\textsuperscript{th} base sequence 'Guanine' was substituted by 'Adenine' (Fig. 29, Page No. 84). The codon change is from AAG to AAA with no amino acid change, Lys201Lys i.e., a silent variation. The significance of such silent variations is yet to be fully understood. Gaillard et al (1999) observed such silent mutations in PiM1Ala213 homozygous and PiAla/PiV heterozygous individuals and suggested that it may represent evolutionary intermediates between the PiM1 (Ala213) and PiM1 (Val213) subtypes.
Absence of the severe deficient alleles in this study shows that alpha-1-antitrypsin deficiency is a rare phenomenon among south Indian. Other studies from India suggest the prevalence of heterozygous state and low occurrence of severe $\alpha_1$-AT deficiency. Saha (1990) studied the distribution of serum alpha-1-antitrypsin subtypes in six mongoloid populations of east Asia and Dravidian Indians. The populations consisted of Chinese, Malays, Filipinos, Thais, Koreans and Dravidian Indians. The frequency of PiM1 allele was reported to vary from 0.65 in the Thais to 0.81 in the Chinese. The highest frequency of PiM2 was found in the Dravidian Indians followed by the Thais. The frequency of PiM3 was found to vary from 0.03 to 0.07 in these populations. A low frequency of PiF (0.01 to 0.02) and PiS (0.01 to 0.04) were observed in the Mongoloid populations but were absent in Dravidian Indians. The PiZ allele was reported to be absent in all the populations studied.

From studies based on serum $\alpha_1$-AT levels, no severe $\alpha_1$-AT deficiency has so far been reported from India (Ray and Sri Krishna., 1993). In another study on the distribution of genetic variants of serum $\alpha_1$-AT in Baigas of Madhya Pradesh, central India, allelic frequencies of PiM1 = 0.7179, PiM2 = 0.1750, PiM3 = 0.0893, PiS = 0.0107 and PiF = 0.0071 were observed. No PiZ was detected (Reddy and Mastana, 1995). Gupta et al (2005) reported a high frequency ($P < 0.0001$) of the PiM3 allele in COPD patients in the Indian population and suggested an association of PiM3 with the pathogenesis of COPD.
Various studies report the origin of PiZ in the northern Europe approximately 2000 years ago and PiS in the Iberian Peninsula around 15,000 to 10,000 years ago which subsequently spread to other European countries (Carrell, 1984; Lomas and Parfrey, 2004). The population migration and admixture during the 9th, 10th and 11th centuries and probably till the 16th century by the Vikings, and also from England and France could have played an important role in the spread of PiS allele from the Iberian peninsula to other European and middle eastern and north African populations. However recent reports on the presence of these PiS and PiZ deficient alleles in central and southern African and Asian countries has not been accounted for by any evidence of population migration and admixture.

Studies on the distribution of the Pi types S and Z in Europe demonstrated that these deficient alleles occur mainly among those of European stock. The population prevalence for the MM, MS and MZ genotypes calculated among whites is found to be 86%, 9% and 3% respectively (Hutchison, 1990; 1998). The highest prevalence (gene frequency of >0.0200) of the PiZ variant was observed in northern and western European countries specifically in southern Scandinavia, Denmark, the Netherlands and northern France (Fagerhol, 1967; Sesboeue et al, 1978).

The first population-based analysis on the distribution of alpha-1-antitrypsin alleles suggested the existence of differing \textit{aat} allelic frequencies among European, American and Asiatic populations (Fagerhol and Tenjord, 1968). Other population studies also showed a low frequency of deficient variants in Finns, Lapps and Asians and a high frequency in European
populations especially the Spanish and Portuguese. PiM1 was the most frequent allele found in all the populations studied, PiM2 the next most frequent and PiM3 relatively uncommon (Fagerhol and Laurell, 1970; Kellermann and Walter, 1970).

Lieberman et al (1976) observed a racial distribution of aat variants in a survey on α1-AT phenotypes and serum trypsin inhibitory capacities in 1,841 seventh grade junior school students. Alpha-1-antitrypsin deficiency was detected only in white subjects and not in any other races.

In an analysis of serum protein polymorphisms in β1 Aka Pygmy group, PiM1, PiM2 and PiM3 frequencies were found to be similar to those of black Americans. The study did not find PiS, PiZ and other variants (Constans et al, 1981).

Hitzeroth et al (1987) studied aat genetic polymorphism in a south African population consisting of 144 whites, 100 coloured, 190 Indian and 127 black healthy individuals and compared with the worldwide data available from other population groups. They observed a combined frequency of 0.3-0.4% for the deficient Pi phenotypes S, Z and SZ in coloureds and whites and very low frequency in blacks and Indians.

DeCroo et al (1991) studied the frequency of aat alleles in US whites, US blacks and Nigerian blacks. The PiS allele was present at polymorphic (p = <1) levels in US whites, sporadic in US blacks and completely absent in African blacks.
In contrast to the common occurrence of α1-AT deficiency in Caucasians, among Orientals the deficient variants have been recognized to be extremely rare and are considered to be a low prevalence area for the occurrence of α1-AT deficiency. The deficiency reported is mostly associated with non-Z alleles. For example the Siiyama variant (Ser 53-TCC to Phe 53-TTC) characterized by polymerization and intracellular aggregation of α1-AT results in reduced serum α1-AT levels and is the most common cause of severe α1-AT deficiency in Japan (Siiyama et al., 1991; Yuasa et al., 2001).

The deficient alleles were reported at very low frequency (>0.01) in the Korean and Chinese populations. PiM1 allele was the predominant aat phenotype occurring at a frequency of 0.65 and 0.66 respectively, followed by PiM2 = 0.22 and 0.25, and PiM3 = 0.06 and 0.09 respectively (Lee et al., 1981). Determination of allelic frequencies of plasma alpha-1-antitrypsin in a Chinese population of 1049 unrelated individuals showed absence of the severe deficient allele PiZ and the less severe deficient allele PiS (Ying and Liang, 1984). In another study on the geographic variability within the Chinese population, the major variants observed were M1, M2, M3 and M4. No deficient alleles were observed. New alleles Etokyo, Pweiishi and Pyasugi were reported (Ying et al., 1985).

Lam et al (1997) analyzed 2005 unrelated Chinese subjects free of primary lung and liver disease for α1-AT gene polymorphism. In this study only 2 individuals were found to be heterozygous for PiZ and PiS variants (MZ and MS). No SS, SZ, and ZZ were found. The predominant phenotype identified was PiMM with a frequency of 99.8%. In another study on the
phenotypic distribution of α1-AT in southern Chinese population by Lee et al (2002), M1 or M2 homozygosity was 66.1% and heterozygosity was found to be 32.6%. The frequency of allelic variants was only 0.007 and the deficiency variants were absent. In Thailand the prevalence of α1-AT deficient phenotypes in pediatric patients was found to be negligible (Chongsrisawat et al, 1998). Recently Kwok et al (2004) reported the absence of α1-AT deficient phenotypes from Chinese patients with COPD in Hong Kong and suggested that the recommendations of the WHO that all COPD patients to be screened for deficient alleles, may not be applicable to such populations.

Alpha-1-antitrypsin deficiency due to PiZ allele is rare in Japan. In a population study of 965 healthy Japanese individuals and 183 Japanese with pulmonary diseases no PiZ and M1 (Ala213) variant were detected. Since the Z variant has developed on the M1 (Ala213) base allele, this may account for the extreme rarity of the PiZ gene in the Japanese and other Asian populations (Kawakami et al., 1981).

Among Japanese new variants like Pi Etokyo, Pi Nnagato, Pi M5 Gunma and Pi Pyongo, which exhibit normal function were also identified (Yuasa et al., 1984; Seyama et al., 1991; Gaillard et al., 1994; Seyama et al., 1995; Saito et al., 2004).

In an analysis on the distribution of α1-AT deficient alleles (Z and S) in Japanese and Korean patients with Aneurysmal Subarachnoid Hemorrhage, Yoneyama et al (2004) could not detect any of the deficient alleles. This
shows that the *aat* deficient alleles in far east Asian populations are absent in other disease conditions also.

Population study from Pakistan shows a low frequency for *aat* phenotypes associated with total and intermediate deficiency (Shahid *et al.*, 2000). The PiMM was the most predominant phenotype identified indicating a very low total and intermediate deficiency in their population.

These population studies show that risk factors other than α1-AT deficiency may have a predisposing effect on the development of COPD. In our present study we observed increased environmental determinants like cigarette smoking, occupational exposure and a low socio-economic status. On genotype analysis, they were found to have normal *aat* alleles at the Pi locus. Hence it can be presumed that COPD in these persons would have resulted not from α1-AT deficiency but due to above mentioned factors and that the development of COPD in these patients can be non-genetic in nature.

In the present study cigarette smoking is the major risk factor identified and majority of the patients started smoking at a very early age (Fig. 17, Page No. 67). Other studies supporting our results indicate that subjects who start smoking regularly during the adolescent years may become disabled between their thirties and fifties (Piitulainen *et al.*, 1997; 1998). This could have resulted in the severity of clinical symptoms in the age group of 50 to 65 years followed by 35-50 years (Fig. 18, Page No. 69). This implicates that COPD should be screened among smokers to counsel strongly against the use of tobacco.
Agents and occupations associated with an increased risk of COPD are minerals, coal (mining), man-made vitreous fibres, oil mist, cement (construction), silica, chemicals, cadmium, vanadium, welding fumes, isocyanates, vinyl chloride, polycyclic aromatic hydrocarbons, organic dusts, cotton (textile industry), grain and wood (paper milling) (Viegi, 2001; Balmes, 2003).

It has been reported that farm workers can be exposed to a variety of gases e.g. ammonia, methane, and hydrogen sulphide from animal manure and organic dusts and may effect the development of respiratory diseases (Zock et al., 2001). In the present study majority of the patients were identified as agricultural farmers and about 15% of the patients were involved in goods loading works. This, along with smoking, may account for the development of COPD in the present study group.

Studies comparing urban and rural populations have found increased frequency of respiratory symptoms and reduced levels of lung function in areas with high pollution (Detels et al., 1991; Tashkin et al., 1994; Silverman and Speizer, 1996; Peters et al., 1999; Gauderman et al., 2000; Anto et al., 2001). In the present study all the COPD patients were from semi-urban environment and suffered from a low lung function.

In developing countries, development of COPD was inversely related to socio-economic status (Behera and Jindal, 1991). An analysis by Prescott et al (1999) showed that socio-economic status based on income and education was associated with lung function, independent of smoking. In the
present study majority of the patients diagnosed as having COPD came from a low socioeconomic class who earned their livelihood on a daily wages basis. It may be presumed that the poor lung function and increased risk of hospitalization in these individuals are due to lower socioeconomic status, occupational exposure, poor housing conditions and smoking.

It is also reported that the contribution of homozygous affected individuals to the total population at risk for chronic lung disease is too small (Wencker et al., 2002; Hersh et al, 2004). Severe deficiency of α1-AT is a proven genetic risk factor, but only 1-2% of all cases of COPD are estimated to be due to severe α1-AT deficiency suggesting that most COPD in the general population probably represents a complex disease with multiple genetic and environmental contributions (DeMeo and Silverman, 2004).