CHAPTER – 4

COMPREHENSIVE DISCUSSION
4. COMPREHENSIVE DISCUSSION

Asthma is a chronic inflammatory disorder of the airways, in which many cells and cellular elements play a role, in particular mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible persons, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, especially at night and in the early morning hours (NHLBI, 1997). The inflammation results in airflow obstruction, bronchial hyperresponsiveness to a variety of stimuli, and mucus hypersecretion. Irreversible structural remodeling may then occur in some patients, contributing to persistent abnormalities in lung function. Asthma usually begins in childhood. Determinants commonly associated with persistent childhood asthma include viral respiratory infections, history of allergy, family history of allergy, and atopy. Asthma exacerbations commonly result from respiratory viral infections, especially rhinovirus infection (Johnston et al., 1995). Childhood asthma is frequently associated with atopy, which involves a genetic susceptibility to produce IgE in response to common environmental allergens, particularly house dust mites, pet dander, and fungi (Koren, 1997). Many airborne allergens can cause asthma, but these airborne allergens are most commonly associated with asthma onset. Atopy is probably the strongest predisposing factor in the development of asthma.

The etiology of asthma is complex and multifactorial. It involves the interaction between genetic factors and environmental stimuli. The strong familial clustering of asthma has encouraged an increasing volume of research into the genetic predisposition to disease. Although identification of all asthma genes is incomplete, genetic findings are already changing the prevailing view of asthma pathogenesis. Candidate-gene and linkage studies followed by positional cloning have already provided a large number of genes accountable for the susceptibility to asthma (Vercelli, 2008a). In addition, many genome-wide association studies (GWASs) published in recent decade have identified several genetic loci to be associated with asthma and/or its related phenotypes in different populations (Mathias et al., 2010; Moffatt et al., 2010; Li et al., 2010).

In asthma, the development of immune response depends on a repertoire of cytokines produced by numerous cells, including CD4+ helper T cells. These lymphocytes can be
divided into two subsets, T helper type 1 (Th-1) and T helper type 2 (Th-2), based on their cytokine profiles (Romagnani, 1992). Effector Th1 cells are involved in delayed-type hypersensitivity through their production of IFN-γ and IL-2, whereas Th2 cells secrete IL-4, IL-5, IL-9 and IL-13, and promote antibody-mediated humoral immune responses (Brown and Ennis, 2005). It has been suggested that an alteration in cytokine milieu, with excess Th-2 products (IL-4, IL-5, and IL-13) in concert with decreased Th-1 products (IFN-γ and IL-2), is predicted to drive the asthma phenotype (Castro et al., 2000). A hypersensitivity reaction initiated by immunologic mechanisms mediated by IgE antibodies occurs in allergic asthma. IgE plays a central role in the initiation and propagation of the inflammatory cascade and thus the allergic response (Buhl, 2005). Indeed, recent studies reveal that IgE, through its high affinity IgE receptor (FcεRI), is a critical regulator of Th2 responses (Peng, 2009). There are clear parallels between human allergic disease and the “Th-1/Th-2 paradigm” originally described in rodents (Romagnani, 1991; Mosmann and Sad, 1996). A substantial body of evidence implicates Th-2 type cytokines (IL-4, IL-5, IL-9, and IL-13) in the development and expression of allergy and airway inflammation (Chang et al., 1996; Gabrielsson et al., 1997; Kimura et al., 2000; Jenmalm et al., 2001).

Asthma and allergic diseases remain the most common diseases in the modern world, making it essential to determine the causal pathways and underlying mechanisms, with the hope that this may lead to more definitive treatment and prevention strategies. A more complete understanding of the processes involved in local and systemic immune development is a crucial part of this process. Therefore, the objectives of the study were to estimate the prevalence of asthma in children aged between 3-12 years and to investigate the associated risk factors, to determine the serum C-reactive protein (CRP) concentration in asthmatic children to understand the inflammatory process(es) in asthma and to study the effect of corticosteroid on serum CRP level, to estimate the levels of total serum IgE in asthmatic and control subjects and to investigate the relationship of various demographic and clinical characteristics with the level of total serum IgE in asthmatics, to determine the serum levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines in order to investigate the alteration
in Th1/Th2 balance in asthma, if any and to determine the frequency of some of the selected HLA class I and class II allelic groups in asthmatic and control groups.

4.1 Assessment of prevalence and associated risk factors of asthma

Asthma and other allergic disorders are common health problems especially in children. A high proportion of infants with early symptoms of atopic dermatitis and food allergy frequently develop more persistent, recurrent airway pathology. Asthma has a negative impact on quality of life. It impairs the child’s social interaction and academic achievement (von Mutius, 2000). The burden of asthma and/or allergic disorders has been steadily rising in Western countries, with almost 40% of these populations showing evidence of allergic sensitization. There is now mounting concern that billions of people will be affected as developing countries begin to show the same trends (Lewis, 1998).

The prevalence of asthma has been reported to increase in many places around the world during the last decades (Manning et al., 2007). Many factors have been reported that contribute to this increase. These include genetic factors as well as environmental factors such as lifestyle, infections and diet. In our preliminary hospital based study, the mean annual prevalence of asthma in children, aged 3 to 12 years, was observed to be 3.06%. The assessment of the risk factors revealed the association of family history of asthma/atopy with asthma.

Rising prevalence and morbidity of childhood asthma and allergic diseases have been observed globally (Pearce et al., 2007; Eder et al., 2006). Although some recent reports suggest the declining trend of prevalence of asthma but no overall global declining trend in the prevalence of asthma was shown in a recent review of epidemiological studies conducted to examine the international trends in asthma prevalence in children and adults for the period 1990-2008 (Anandan et al., 2010). Many factors have been reported that contribute to the increased prevalence of asthma which include genetic factors as well as environmental factors such as lifestyle, infections and diet (Kiadeh et al., 2013). Numerous epidemiologic studies have reported the varied rates of asthma prevalence in Indian children (Singh et al., 2002; Awasthi et al., 2004; Jain et al., 2010; Ganesh et al., 2012;
Cheraghi et al., 2012). The variations in the prevalence of asthma in children as reported by various authors in Indian children could be due to regional variation, differences in diagnostic criteria, and most importantly the sample size.

Family history of asthma/atopy was found to be associated with development of asthma ($\chi^2 = 8.89$, $p=0.003^{**}$). Previous studies from different parts of the world have also reported a strong association between family history of atopy and asthma with reported prevalence of asthma (Jenkins et al., 1993; Christie et al., 1999; Lee et al., 2003; Rönmark et al., 1999). The finding of the association between family history of asthma/atopy and development of asthma in children in our study highlights the fact that family history of asthma/atopy is indeed an important risk factor for asthma.

4.2 Serum C-reactive protein level

C-reactive protein was discovered in humans in 1930 as a serum component that binds the C polysaccharide of Streptococcus pneumoniae (hence CRP). Structurally, CRP is usually composed of five identical subunits (hence pentraxin), each of 23 kDa in mass, which are linked noncovalently to form a disc-like pentagonal ring (Anderson, 2006). It has long been used clinically to evaluate the presence and degree of inflammation because CRP blood levels increase as much as 1,000-fold within 24 hours after the onset of inflammation (Kushner et al., 1981). It has been demonstrated that CRP levels are associated with age, sex, race (African-American), body mass index (BMI), smoking, serum lipids, blood pressure, presence of diabetes mellitus, 2-h post-challenge glucose, frequency of exercise, and cardio-respiratory fitness (Folsom et al., 2002; Hashimoto et al., 2004).

The serum CRP concentration was determined in asthmatic children with and without ICS treatment in order to understand if serum CRP concentration could be taken as a marker for asthmatic inflammation. The result showed that the CRP concentration was significantly elevated in the serum of ICS-naïve group of asthmatic children compared to ICS-inhaling asthmatic children ($p < 0.001$). Recent publications suggest that CRP could be taken into
consideration as a simple, cheap and reliable marker for monitoring asthmatic inflammation (Kony et al., 2004; Olafsdottir et al., 2005).

The CRP is predominantly synthesized in the Liver in response to inflammation and tissue damage. Monocytes, lymphocytes and neutrophils are also able to produce CRP (Baumann and Gauldie, 1994). CRP is regulated by pro-inflammatory cytokines, with a recognized important role in the pathophysiology of asthma, primarily the TNF-α, NF-kappa B, IL-6 and IL-1β (Voleti and Agrawal, 2005). Although its function is still unclear, the CRP may serve as a general scavenger protein and play an important role to recognize bacteria and damaged human cells and to mediate their elimination through opsonization, phagocytosis, and cell-mediated cytotoxicity. The CRP can also activate the classical complement cascade by binding directly to the complement fragment C1q (Pepys and Hirshfield, 2003). A correlation of peripheral blood CRP levels with severity, extent, and progression of inflammatory pathologies has been well established. Recent publications suggested that hs-CRP has a contributing role in the pathogenesis of disease in addition to its being merely an inflammatory marker (Devaraj et al., 2005).

Many studies have focused on the possible role of inhaled steroids in the attenuation of CRP levels in asthma and chronic obstructive pulmonary disease (COPD). Pinto-Plata et al. (2006) have reported that CRP levels were lower in COPD patients treated with inhaled steroids. Similarly, Karthikeyan et al. (2014) have reported significantly higher CRP levels in steroid naïve asthmatics compared to control subjects. While the serum CRP levels in steroid inhaling asthmatics were comparable with control group.

4.3 Total serum IgE level

Total serum immunoglobulin E (IgE) is known to be elevated in various allergic disorders such as allergic asthma, allergic rhinitis (AR), eczema, atopic dermatitis, bronchial hyper-responsiveness, and sometimes forms the basis of allergic diseases (Yunginger, 1988). A number of epidemiological studies have shown a strong association between total serum
IgE levels, skin test reactivity to aeroallergens, and asthma phenotype (Friedhoff and Marsh, 1993; Sherrill et al., 1999). It has also been shown that noncognate production of IgE (i.e. production of IgE that is not driven by one or few specific allergens) is a significant inherited risk factor for the development of asthma (Suayer et al., 1996).

The level of total serum IgE was measured in asthmatic and control subjects using the ELISA kits (AccuBind, Monobind Inc., USA). The mean total serum IgE level was significantly higher in asthmatic subjects compared to that of the control subjects (269.21 ± 150.97IU/ml versus 146.89 ± 77.32IU/ml; p<0.001***). Increased serum IgE levels in asthma may be due to increases in IgE-dependent processes and cellular components of the immune system. The secretion of IgE by lymphocytes defines the allergic state of an individual. The cellular events associated with IgE-dependent processes are very much important in asthma (Tracey et al., 1995). Higher IgE levels indicate some types of inherent susceptibility and/or presence of a disease process involving airway inflammation (Chowdary et al., 2003; Sherril et al., 1995).

Analyses of the demographic and clinical characteristics in association with total serum IgE revealed that higher age group (8-12 years), raised eosinophil count and exposure to cigarette smoke were significantly associated with the elevated level of total serum IgE in asthmatic subjects. In a study, a significant relation between age and IgE levels was reported in the allergic patients (Sharma et al., 2002). In this study, the authors have observed the highest mean IgE levels in 8-12 years age group (642IU/ml). Similarly, in an American population, it was reported that in asthmatic patients, IgE increases until the age of 9 years and after reaching a peak, the IgE levels decrease in the teenage years (Grundbacher and Massie, 1985). According to Halonen et al. (1982), a significant relationship exists between serum IgE levels and eosinophilia in populations presumed to be free of parasites where IgE levels presumably provide a better clue to atopy than do skin tests. Various earlier studies have also identified exposure to cigarette smoke (passive smoking) as an important risk factors for the elevated level of total serum IgE in asthmatics (Satwani et al., 2009; Kartasamita et al., 1994). The mechanism of modulation of IgE levels by tobacco smoke is not well understood (Sherrill et al., 1994, Sapigni et al., 1998). There could be indirect and direct actions of tobacco smoke on IgE levels (Villar and
Holgate, 1995). An indirect action could increase the likelihood of developing sensitivities to inhaled allergens. In fact, smoke increases permeability in the lungs possibly facilitating and enhancing penetration of allergens. Tobacco smoke could have a direct action on IgE levels through immune system cellular regulation changing the function of T lymphocytes (Holt, 1987). Th2 lymphocytes regulate IgE production. Thus, newborns with smoking parents have higher cord IgE levels, regardless of parental atopy, than newborns born to non-smokers (Magnusson, 1986).

Elevated serum levels of specific IgE towards common environmental allergens are a key component in the pathogenesis of allergic asthma. IgE antibodies cause chronic airway inflammation through effector cells such as mast cells, basophils, etc., activated via high-affinity (FcεRI) or low-affinity (FcεRII) IgE receptors. IgE has been viewed as a target for immunological drug development in asthma. Despite an increase in the availability of drugs for asthma, a number of strategies aimed at inhibiting the proinflammatory action of IgE have been developed in recent years (D’Amato et al., 2014).

4.4 Serum levels of IL-4 and IFN-γ

Asthma is characterized by chronic inflammation in the airways and the presence of a predominance of CD4+ T-helper 2 (Th2) cells that secrete IL-4, IL-5, and IL-13 cytokines (Ober, 2005). Th2 cells contribute to the immunopathogenesis of asthma by recruiting eosinophils and mast cells to the airways (Ober, 2005; Romagnani, 1994; de Vries et al., 2000) and by inducing B-cells to produce immunoglobulin E antibodies (Kuo et al., 2001). Allergic and asthmatic subjects are more likely to have elevated levels of the Th2 cytokines and reduced levels of the Th1 cytokines (IFN-γ and TNF-β).

Serum levels of IL-4 and IFN-γ were determined among 48 asthmatic children (18 steroid-naïve and 30 steroid-treated) and 32 control subjects using Enzyme linked immunosorbent assay (ELISA) method. It was observed that serum level of IL-4 was significantly higher in steroid-naïve group as compared to control subjects but it was lower in steroid-treated group. In contrast, serum levels of IFN-γ were significantly lower in both steroid-naïve as
well as steroid-treated groups of asthmatic children compared to control subjects. It has been suggested that an alteration in cytokine milieu, with excess Th-2 products (IL-4, IL-5, and IL-13) in concert with decreased Th-1 products (IFN-γ and IL-2), is predicted to drive the asthma phenotype (Castro et al., 2000). Elevated levels of IL-4, an essential cofactor for IgE production, and IL-5, responsible for the final differentiation, activation and recruitment of eosinophils (Kay, 1991), have been found in serum of patients with asthma (Matsumoto et al., 1991; Hashimoto et al., 1993; Matsumoto et al., 1994; Tang et al., 1995). On the other hand, IFN-γ is thought to protect against the development of asthma by regulating Th-2 cytokine production, although a mixed Th-1/Th-2 pattern has also been reported (Heaton et al., 2005). In a recent study, Figueiredo et al. (2012) have shown that non-atopic asthma was associated with IFN-γ and elevated monocytes in blood and suggested that IFN-γ and monocytes might play a role in immunopathology of non-atopic asthma in Latin American children.

One of the first studies measuring cytokine concentrations in children with allergic disease, revealed a significant increase in the level of IL-4 in serum from atopic asthmatics compared to controls, which correlated with IgE (Matsumoto et al., 1991). Other subsequent studies in serum and blood supported the importance of IL-4 in childhood asthma (Akacakaya et al., 1994; Daher et al., 1995; Krogulska et al., 2009). IL-4 demonstrates a broad range of biological activities. It is a main cytokine involved in the pathogenesis of allergic responses and at the same time it can also down-regulate acute inflammatory changes (Chung and Barnes, 1999). IL-4 has also got additional effects on asthma pathogenesis which include stimulation of mucus producing cells and fibroblasts leading to airway remodeling (Dabbagh et al., 1999; Trautmann et al., 1998; Doucet et al., 1998). It has also been confirmed that the crucial role of IL-4 lies in its effect on Th-2 development, rather than on the induction of IgE synthesis and subsequent mast cell degranulation (Coyle et al., 1995). Several other invasive studies involving bronchoalveolar lavage (BAL) fluid and lung biopsies have confirmed that a Th-2-like mediated immune response is seen in asthma (Robinson et al., 1992; Umetsu et al., 1997; Walker et al., 1992). Gemou-Engesaeth et al. (1997) and Krouwels et al. (1996) have reported the imbalance in the production of IL-4 and IFN-γ in children with atopic asthma, and corticosteroids appear to correct it.
4.5 Typing of HLA allelic groups

Allergic asthma is considered a multifactor disease, the possibility of increasing understanding of the mechanisms by which inherited factors influence disease has stimulated the study of human leukocyte antigen (HLA). HLA is controlled by genes within the major histocompatibility complex which is associated with other aspects of immune response, some complement components, and susceptibility to certain diseases (Svejgaard et al., 1975). The products of major histocompatibility complex play a fundamental role in regulating immune responses since they encode the molecules that represent the linkage elements between environmental allergens and the immune system. HLA genes have been implicated in the development of asthma and atopy, but the importance of associations between HLA genes and asthma remains unclear. Different HLA genes may represent factors conferring risk or protection for the development of allergic diseases. It is assumed that HLA genes as genetic markers have influence on the atopy and asthma as well as on sensitization against specific inhalant allergens.

According to the published data, it seems that individual antigens do not have a significant influence on the intensity of specific IgE immunological response. It is more probable that environmental factors or other loci (e.g. genes for T-cell receptor or TNF-α) are important in determining the individual sensitization to a specific allergen (Li et al., 1995). The class I genes of MHC may have important effects on atopic responses, but these have not yet been adequately investigated. Similarly, the class III complement genes contain polymorphisms which may be of relevance to inflammatory or immune diseases. Polymorphism in the HLA class II molecules may lead to allelic forms that are more effective in binding allergenic peptides (i.e. epitopes) on the membranes of antigen-presenting cells, thus leading to allelic disequilibrium of HLA class II alleles among sensitized individuals (Howell and Holgate, 1995; Tomlinson and Bodmer, 1995). The most replicated and possibly the strongest association between the HLA system and allergic disease is that between increased IgE production in response to the ragweed Artemisia artemisiifolia pollen allergens Amb aV, Amb tV, AmbpV, and Amb aVI in individuals expressing the HLA-DR5 allele (Marsh et al., 1987).
In the present study, we studied the selected HLA class I and class II allelic groups which have been reported to be associated with childhood asthma in various populations. The result of our study did not show the significant association of HLA class I allelic groups with asthma. The HLA A-B haplotype analysis revealed the higher frequencies of A*01-B*37, A*24-B*08, A*26-B*37 haplotypes and lower frequencies of A*11-B*44 and A*25-B*52 haplotypes in asthmatic subjects than in controls. As only few selected class II allelic groups were included in the study, haplotypes of HLA class I and class II loci were not undertaken. Several studies have investigated the association of HLA class I alleles with the asthma. The work of Thorsby et al. (1997) has been cited showing an increased frequency of A1/B8 in asthmatic children, but the figures were not significant. Morris et al. (1980) reported that HLA- B*12 (B44 and B45) was increased in the allergic asthmatics compared to controls (46% vs. 29%) and it is suggested that B*12 is associated with the ability to produce the IgE antibodies. A3/B7/DRw2 (which are in linkage disequilibrium) all show a decreased frequency in intrinsic asthmatic patients compared to controls (24%, 12% and 9% vs. 32%, 26% and 24% respectively). Besides, HLA-B*8 and DRw3, which showed a moderate increase in frequency in all three groups of asthmatics, were found in five of seven patients with low atopy but persisting antibodies to A. fumigates. Wang et al. (1988) reported the much higher frequencies of HLA-A*9, -A*10, -BW*61 and -BW*62 and much lower frequency of HLA-A*03 in the asthmatic subjects than in the normal controls. However, after the p-values were corrected, the significant difference only existed in HLA-Bw61.

Among class II allelic groups, HLA-DRB1*03 was found to be associated with childhood asthma (OR=3.78, 95% CI=1.61-8.85, p=0.0025, pcorr < 0.05). Further analysis of HLA allelic groups in asthmatics with high and low total serum IgE levels did not show the significant association. Various studies have reported the association of HLA class II alleles and haplotypes with asthma in different populations. In a study, HLA-DQA1*0104 and -DQB1*0201 were reported to be positively associated while HLA-DQA1*0301 and -DQB1*0301 alleles were negatively associated with asthma (Gao et al., 2003). Another study in Greek children with allergic asthma revealed that DRB1*04 and DQA1*0301 might be important factors in susceptibility to asthma with sensitivity to mites (Parapanissiou et al., 2005). Horne et al. (2000) have shown that HLA-DRB1*0401-
$DQB1^{*}0302$ haplotype as the most susceptible haplotype in development of asthma due to red cedar. While the presence of the $DRB1^{*}0101$-$DQB1^{*}0501$ haplotype appeared to confer protection. Similarly, Wosczek et al. (2002) showed a significant association between $HLA-DRB1^{*}02, B5^{*}$ haplotype and asthma phenotype in patients with grass-pollen allergy. They also showed the association between $HLA-DRB1^{*}01$ alleles and higher total serum IgE levels in the patients with grass pollen allergy. HLA class II genes relate to non-specific modulation of inflammation. $HLA-DRB1$ and $DQB1$ SNPs and haplotypes have been associated with a higher risk of toluene diisocyanate-induced occupational asthma (Choi et al., 2009), total serum IgE in Iranian subjects (Movahedi et al., 2008), atopy in Northern Chinese (Gao et al., 2003), Dermatophagoides spp.-sensitive asthma in Venuezuelan individuals (Lara-Marquez et al., 1999), and asthma severity in whites in the United States (Juhn et al., 2007), suggesting a broad role for these genes in asthma pathogenesis across ethnic groups.

Among genetic factors contributing to the development of atopic diseases, HLA genes have been implicated in triggering an allergen specific IgE response. HLA-DR alleles were found to be associated with the development of specific IgE reactions to seasonal (Marsh et al., 1982) and perennial allergens (O’Hehir et al., 1990) and to some drugs (Kowalski et al., 1998). A different role for HLA gene polymorphisms has been suggested by a study of Blumenthal et al. (1992), who found that in pollen allergy the asthma phenotype may be associated with MHC extended haplotype $HLA-B7/SC31/DR2$ and patients with rhinitis alone have increased frequency of $HLA-B8/SC01/DR3$ haplotype. It has been reported that a potent pro-inflammatory cytokine TNF-α gene polymorphism, which is in linkage disequilibrium with the HLA loci, may affect cytokine generation and also the severity of the disease (Brinkman et al., 1997). Interestingly, an association of extended TNF-α haplotype $LTa Ncol*1/TNF-308*2/HLA-DRB1^{*}02$ and asthma has also been reported (Moffatt et al., 1999), suggesting that it is the combination of different polymorphic loci localized to chromosome 6 (particular extended haplotype) that influences asthma phenotype.