CYTOKINES AS BIOMARKERS IN ASTHMA

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ABSTRACT

Asthma is a lung disease characterized by wide variations in airflow over short periods of time. Exacerbations of asthma can be accompanied by symptoms of chest tightness, shortness of breath and wheezing; airway inflammation characterized by an influx of eosinophils and/or neutrophils; and the expression of pro-inflammatory cytokines in the airway. There is strong evidence supporting a central role for the T cell in asthma. In atopic asthma, T cells are documented components of the late-phase response to inhaled allergen, driving airway inflammation, mucus hypersecretion, and bronchoconstriction through the release of cytokines and other mediators. T cells have also been shown to produce inflammatory cytokines in response to allergen in nonatopic asthmatics, indicating a potential role in mediating disease in this phenotype. In both atopic and nonatopic asthma, aberrant T cell responses to allergen may drive the infiltration of neutrophils and eosinophils into the airway through the production of pro-inflammatory cytokines, leading to exacerbations of disease. This project has investigated the role of several T cell cytokines in driving disease and acting as biomarkers in asthma: interleukin-5, interleukin-17A, interleukin-23, interleukin-10, and interferon-γ. We have measured allergen-induced cytokine production by peripheral blood mononuclear cells (PBMCs) and examined its ability to distinguish between different asthma phenotypes: asthma vs normal, atopic vs nonatopic asthma, eosinophilic bronchitis vs noneosinophilic bronchitis, and neutrophilic vs nonneutrophilic bronchitis. Our data shows that allergen-induced peripheral blood mononuclear cell responses to allergen are not good biomarkers of disease in asthma. No differences in PBMC cytokine production are seen in patients with asthma, compared with normal controls, or between patients with different asthmatic phenotypes. It is not possible to determine a patient's disease state, atopic status, or type of bronchitis by examining their PBMC cytokine responses to allergen.

DEDICATION

I would like to dedicate my thesis to Dr. Frederick Hargreave, a physician and researcher at the Firestone Institute for Respiratory Health at St. Joseph's Hospital in Hamilton. Dr. Hargreave's contributions to the field of asthma research have shaped the understanding and treatment of this disease and have made my project possible. He has been an invaluable member of my supervisory committee, and without his knowledge and encouragement this project could not have been successfully completed. Dr. Hargreave passed away suddenly just three weeks before the writing of this thesis. His dedication to his patients and his passion for his work continue to inspire me.

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INTRODUCTION

ASTHMA

Asthma is most commonly defined as a unique disease entity characterized by airway inflammation, reversible airway obstruction, and airway hyperresponsiveness leading to symptoms of wheezing, difficulty breathing, coughing, and chest tightness. This airway disease is estimated to affect 300 million people worldwide, causing 250 000 deaths annually[5]. It results in a decreased quality of life for the affected individual, and places a burden on society as a whole due to elevated health care costs and decreased productivity of asthmatic individuals.

Despite its widespread use, the above definition of asthma is a controversial one. A single genetic or environmental cause of asthma has not been identified, and this descriptive definition fails to differentiate asthma from other lung diseases and accurately represent the disease heterogeneity seen between and within asthmatic patients[26].

For the purposes of this project, asthma refers to an airway abnormality that is characterized by wide variations in airflow over short periods of time. These variations in airflow may occur spontaneously, in response to a stimulus (i.e. bronchoconstriction following the inhalation of an allergen) or as a result of treatment (i.e. the reversal of bronchoconstriction after inhalation of a rapid-acting β-agonist)[71]. Patients with asthma may also express other components of airway disease, including airway inflammation, obstruction, and remodeling[26]. The presence, nature, and severity of these disease components can vary between and within asthmatic patients over time.

Airway inflammation occurs when an individual's immune system reacts to stimuli including allergens, pollutants, microbes, and viruses, leading to an influx of inflammatory cells into the airway. Airway obstruction is the result of mucus hypersecretion that blocks airflow and/or the constriction of airway smooth muscle. Remodeling refers to characteristic structural changes of the airway, involving collagen deposition under the epithelium, thickening of the airway smooth muscle, and an increased number of mucus-secreting goblet cells[30]. Remodeling in asthma occurs as a consequence of repeated damage to the airway, followed by inadequate repair. This damage may be the result of allergic reactions, poor clearance of viral infections[79], and reduced antioxidant defense mechanisms[13]. It is coupled with an altered repair process, in which the airway responds to damage by activating pro-inflammatory pathways, as opposed to appropriate repair pathways[9].

Asthma is most commonly treated using rapid-acting and long-acting β-agonists, and inhaled corticosteroids. β-agonists act to reverse bronchoconstriction by relaxing airway smooth muscle and inhaled corticosteroids are anti-inflammatory drugs used to reduce chronic airway inflammation. In cases of severe asthma exacerbations, oral steroids such as prednisone may also be employed[5].

ATOPIC AND NONATOPIC ASTHMA

Asthma has traditionally been classified into three categories: atopic, nonatopic, and occupational. Atopic asthma occurs in individuals whose disease is mediated by IgE antibodies produced against allergens the individual has been sensitized to. Nonatopic asthma is non-IgE mediated and is considered to occur in individuals who are nonallergic. Occupational asthma occurs after individuals have been exposed to specific proteins or small molecular weight chemicals in their workplace. The importance of IgE in this category of asthma has not yet been established[14].

In approximately half of asthmatic patients, symptoms are triggered by an allergic response to an identifiable antigen[14]. These patients are classified as having *atopic* asthma, and the allergens that trigger their asthma are identified using a skin-prick test. A positive skin-prick test indicates that the B-lymphocytes of the patient are producing IgE antibodies against a particular allergen, and the patient is therefore classified as being allergic to that material[34]. Inhalation of this allergen results in the cross-linking of allergen-specific IgE antibodies bound to FcεRI receptors on the surface of mast cells. Receptor activation signals mast cells to degranulate, releasing inflammatory mediators such as histamine, leukotrienes, prostaglandins and inflammatory cytokines like IL-4, IL-5 and IL-13. This release of pro-inflammatory granules leads to vasodilation, smooth muscle contraction, and an influx of inflammatory cells into the airway[39]. Once a patient is aware of his or her allergies, steps can be taken to avoid or treat the allergy, resulting in a reduction of asthma symptoms.

Asthmatic patients who do not test positive for any allergies when the skin-prick test is administered, and have no clinical history of allergic disease, are classified as *nonatopic* asthmatics. It is assumed that their asthma is triggered by something other than a typical allergic response mediated by allergen-specific IgE antibodies and mast cell degranulation[34].

Asthma in nonatopic patients is remarkably similar to asthma in patients who have typical allergic asthma. The two patient groups experience wide variations in airflow over short periods of time and exhibit many of the same components of airway disease: similar symptoms of chest tightness, shortness of breath and wheezing; airway inflammation characterized by an influx of eosinophils and/or neutrophils; and expression of pro-inflammatory cytokines in the airway, such as IL-4 and IL-5[34]. These are interesting similarities between what have traditionally been classified as two separate disorders: *atopic* asthma and *nonatopic* asthma.

These striking similarities between the two types of asthma have prompted us to ask the question: could asthma in nonatopic patients actually be triggered by exposure to allergens? These sensitivities have not been identified by a conventional skin-prick test, and may not be IgE-mediated, but may be driven by other immune pathways. Potential allergen-associated mechanisms of disease at work in nonatopic asthma will be discussed further in a later section of this report.

EOSINOPHILS AND NEUTROPHILS IN ASTHMA

Airway inflammation in asthma can be classified according to the predominant inflammatory cell type present in a patient's sputum: neutrophils, eosinophils, both, or neither. This inflammatory infiltrate will vary over time with changes in the patient's environment and/or treatment[26].

Eosinophils

Eosinophils are granular leukocytes that play an important role in the defense against parasitic infections. They have the ability to bind IgE-coated parasitic worms, via Fcε receptors, and release toxic granule proteins and free radicals that neutralize the parasite. Eosinophils also play an important role in the pathogenesis of allergy and asthma. When these cells are recruited and activated inappropriately, the release of their toxic granules and cytokines leads to tissue damage and inflammation[39].

Eosinophils originate in the bone marrow as $CD34^+$ precursors. Though several cytokines, including IL-3 and GM-CSF, are important in the early stages of $CD34^+$ commitment to eosinophil lineage, IL-5 is the main cytokine driving eosinophil maturation and recruitment to the airway[70].

Once in the airway, eosinophils secrete a host of harmful granules and cytokines. Eosinophil collagenase, eosinophil peroxidase, and major basic protein (MBP) are toxic to mammalian cells. MBP also triggers histamine release from mast cells, inducing vasodilation and smooth muscle contraction[14]. Leukotrienes instigate smooth muscle contraction, increase vascular permeability and increase mucus secretion. IL-8, IL-3, IL-5, and GM-CSF attract neutrophils, macrophages, and other eosinophils, resulting in a cascade of pro-inflammatory signals[39].

Though the significance of IL-5 and eosinophils in asthma has been debated[21], two studies in particular have demonstrated their importance in a subset of asthmatic patients. Unlike previous reports[46], these studies focused on patients whose disease is driven by persistent airway eosinophilia.

A 2009 study published *The New England Journal of Medicine* examined a rare group of asthmatic patients who have sputum eosinophilia and airway symptoms despite continued treatment with prednisone and high-dose corticosteroids[60]. Patients were treated with mepolizumab, a monoclonal antibody against IL-5, and prednisone sparing, asthma symptoms, blood and sputum eosinophil levels, FEV_1 and frequency of asthma exacerbations were assessed. Among the 10 patients who were treated with a placebo, there were 12 asthma exacerbations, 9 with sputum eosinophilia at the time of exacerbation. Patients in this group were able to reduce their prednisone dose by approximately 47.7% of the maximum possible reduction (from a mean of 10.7 mg to a mean of 6.4 mg), though this reduction was accompanied by an increase in blood and sputum eosinophil levels. Among the 9 patients treated with mepolizumab, there was 1 exacerbation, which was not accompanied by sputum eosinophilia (instead it was accompanied by sputum neutrophilia). These patients were able to reduce their prednisone dose by approximately 83.8% of the maximum possible reduction (from a mean of 11.9 mg to a mean of 3.9 mg), and this was not accompanied by a significant increase in

blood and sputum eosinophil levels. Patients in the mepolizumab group also saw improvements in quality of life and FEV_1 values, which were maintained for up to 8 weeks after treatment with no increase in prednisone. It was concluded that mepolizumab treatment did provide clinical benefit in this group of asthmatic patients, allowing for a reduction in prednisone dose and a decreased risk of asthma exacerbations.

A similar article was published in the same issue of *The New England Journal of Medicine*[23]. This study examined the effect of mepolizumab on the frequency of asthma exacerbations in patients with refractory eosinophilic asthma and a history of recurrent severe exacerbations. Patients who received mepolizumab treatment experienced significantly fewer exacerbations than those in the placebo group and reported an increased quality of life. Decreased airway wall area and thickness were also seen the mepolizumab group. A similar effect has been seen in a mouse model of allergic asthma – eosinophil-deficient mice showed a significant protection from collagen deposition in the airway and increases in airway smooth muscle[35].

These studies demonstrate the importance of IL-5 and eosinophils in asthma pathogenesis, and underscore the need to understand and treat asthma as a heterogeneous disease. It is necessary to recognize the impact that the eosinophil has on a subset of asthmatics prone to eosinophilic bronchitis. Targeting this cell type can help improve disease in these patients, and it has been demonstrated that using sputum cell counts to guide corticosteroid treatment can be an effective method for controlling asthma exacerbations[40].

Neutrophils

Neutrophils are granulocytes that play an important role in the immune response to bacterial infection. They act as phagocytes to engulf and destroy microbes with toxic granules, and also release a host of bactericidal agents that are effective at killing bacteria, but are harmful to host cells. Neutrophils are recruited to the airway through chemotactic interactions of IL-8 and Gro-α with their CXCR2 receptor[39].

In asthma, IL-17A secreted by Th17 cells stimulates airway epithelial cells to produce the pro-neutrophilic chemokines IL-8 and Gro-α[78]. Once recruited to the airway, neutrophils produce a number of compounds with damaging potential. Neutrophil elastase degrades elastin (a structural component of the airway) and induces mucus hypersecretion from goblet cells. Matrix metalloproteinases (MMP-8 and MMP-9) degrade extracellular matrix components, and reactive oxygen species have been shown to induce nonspecific airway hyperresponsiveness[3].

Neutrophils have come to be associated with severe asthma and asthma-related death. One study found that patients who suffered short duration fatal attacks of asthma (dying within two hours of attack onset) had increased levels of neutrophils in the large and small airways, compared to patients who suffered long duration fatal attacks of asthma (dying within five hours of attack onset)[12]. Another reported that patients intubated for acute severe asthma displayed neutrophil counts in tracheal aspirate 10x higher than normal, and neutrophil number correlated with duration of intubation. The pro-neutrophilic cytokine IL-8 was present at levels 19x higher than normal, and correlated with the number of

neutrophils and the duration of mechanical ventilation of the patient[65]. Sputum neutrophil counts are significantly increased in severe asthma when compared to mild asthma and normal controls, and asthmatic patients with severe disease exhibit the highest levels of IL-8 and neutrophil myeloperoxidase[38]. A higher sputum total neutrophil count has been associated with lower postbronchodilator FEV_1, suggesting a role for neutrophils in persistent airflow limitation[72]. None of these studies found eosinophils and their associated cytokines to be similarly implicated, and thus have prompted speculation that neutrophilic asthma may be a distinct asthma phenotype, associated with more severe disease.

It is important to note that none of these studies have accounted for the possibility of undetected respiratory infection. Since neutrophils play a key role in the response to pathogenic microbes and associated asthma exacerbations[19], lung infection could account for the influx of neutrophils seen in patients with neutrophil-associated exacerbations of asthma and asthma-related death. Another important aspect to consider when discussing neutrophilic asthma exacerbations is inhaled corticosteroid treatment. Corticosteroids effectively suppress eosinophilia in asthma, but can increase neutrophils and neutrophil-associated cytokines in the airway[15]. These medications also serve to suppress the immune system of the patient[5], potentially resulting in a greater susceptibility to microbial infection. Thus, it is possible that some neutrophilic-associated asthma exacerbations are a consequence of the effects of corticosteroid treatment.

Mechanism of Disease

Since asthma is characterized by variations in airflow over time, asthmatic patients often experience periods where their disease is well controlled, followed by exacerbations involving bronchoconstriction and airway symptoms. Exacerbations of asthma are often triggered by exposure to environmental allergens or pathogens, but they can also occur spontaneously or in response to other stimuli such as exercise or cold air.

During a typical allergic exacerbation, effector cells of the innate and adaptive immune systems, as well as airway structural cells, play an integral role in the pathogenesis of asthma. These exacerbations can be broken down into an acute response and a late-phase response, both of which are mediated by complex interactions between antibodies, immune cells, airway epithelium, and airway smooth muscle.

Acute Response

The acute response is an immediate reaction that occurs within minutes of allergen inhalation[8]. It is initiated by inhaled allergen crossing the airway epithelial barrier and directly activating mast cells by cross-linking allergen-specific IgE antibodies bound to FcεRI cell-surface receptors. In asthmatic patients, the airway epithelial barrier displays increased permeability, characterized by a loss of columnar cells and disruption of tight junctions between cells[6]. This decreased epithelial integrity allows for greater infiltration of allergens and other irritants into asthmatic airways.

Mast cells are the primary cells involved in the acute response[62]. They are granulocytes that are recruited to the airway surface by stem-cell factor secreted by airway epithelial cells as well as IL-8 and CXCL-10 secreted by airway smooth muscle cells. Cross-linking of allergen-specific IgE bound to FcεRI cell-surface receptors stimulates mast cell degranulation, releasing histamine, heparin, proteases, cytokines, matrix metalloproteinases, eicosanoids, and leukotrienes[30]. These inflammatory mediators stimulate smooth muscle contraction, microvascular permeability, chemotaxis of other inflammatory cells, and airway remodeling[11,17,30,42,64,67].

Late-phase Response

The late-phase response occurs within 3-4 hours after initial exposure to allergen, and may persist for several days. It is characterized by the infiltration of Th2 cells, neutrophils, and eosinophils into the airway[62].

The process begins when immature dendritic cells (DCs) take up inhaled allergen by extending cellular projections through the airway epithelium and into the lumen[25]. Thymic stromal lymphopoietin (TSLP) is produced by airway epithelial cells in response to allergen, and stimulates these immature DCs to mature and migrate to the draining lymph nodes[36]. This migration to the lymph nodes is guided by the chemotactic interaction of the CCR7 receptor of DCs with its ligands CCL19 and CCL21[77]. DCs upregulate the co-stimulatory molecule OX40L and MHC class II, onto which they load processed allergen in preparation for presentation to T cells. In the draining lymph nodes, DCs present antigen to naïve $CD4^+$ T cells on MHC class II and deliver a co-stimulatory activation signal through interactions between OX40L expressed on DC cell membranes and OX40 expressed on T cell membranes[9]. Delivered together, these two signals stimulate T cells to start producing interleukin-4 (IL-4), polarizing them to become allergen-specific inflammatory Th2 cells[36] by inducing the transcription factor STAT6 to activate the expression of the GATA3 transcription factor[4]. These newly primed Th2 cells receive chemotactic signals through their CCR4 receptors to migrate to the airway. They respond to a number of chemokines, including CCL17 (TARC) and CCL22 (MDC) produced by TSLP-stimulated DCs[52].

Once in the airway, Th2 cells secrete a host of inflammatory cytokines important in asthma. IL-4 and IL-13 stimulate B cells to produce allergen-specific IgE antibodies. IL-3, IL-4, IL-9, and IL-13 recruit mast cells and basophils. IL-5 stimulates the maturation and recruitment of eosinophils. IL-13 induces mucus hypersecretion from goblet cells and airway smooth muscle contraction, leading to the airway obstruction and subsequent reduction in airflow characteristic of asthma[69].

The T cell-mediated infiltration of eosinophils and neutrophils into the airway plays a key role in asthma exacerbations. As mentioned above, eosinophils and neutrophils produce a host of inflammatory mediators and other molecules that promote airway narrowing, tissue damage and airway remodeling.

Other Important Contributors to Asthma Pathogenesis

Aside from producing TSLP, which plays an important role in DC-mediated activation of Th2 cells, airway epithelial cells contribute to asthma pathogenesis in other ways. In response to allergen exposure, the airway epithelium produces granulocyte-monocyte colony-stimulating factor (GM-CSF)[9], which stimulates the proliferation and differentiation of precursors of neutrophils, eosinophils, and monocytes. This cytokine also enhances cell-surface adhesion proteins on mature eosinophils and neutrophils that could lead to their accumulation in the airway, and increases their generation of reactive oxygen intermediates which cause tissue damage and remodeling[30].

Airway smooth muscle cells and mucus-producing goblet cells are increased in asthma and are both important contributors to reduced airflow. Constriction of airway smooth muscle narrows the airway, while mucus hypersecretion by goblet cells obstructs the airway[30]. Airway smooth muscle cells can also secrete pro-inflammatory mediators that promote mast cell recruitment and proliferation[59] and neutrophil recruitment[78].

Th17 cells are a $CD4^+$ T cell subset that produces IL-17A, a cytokine known to stimulate airway epithelium, bronchial fibroblasts, and airway smooth muscle cells to produce the pro-neutrophilic cytokines IL-8 and Gro-α[78]. Th17 cells develop and mature in response to IL-6 and IL-23 produced by activated dendritic cells, and play a role in driving exacerbations of asthma associated with an influx of neutrophils into the airway[61].

Alternate Mechanisms of Disease

The mechanisms described above are known to drive disease in atopic asthma, while those at work in nonatopic asthma remain unclear. Since nonatopic asthma may account for up to half of all asthma cases[14], it is important to discover the underlying driving forces of disease in these patients. The following alternate mechanisms have been proposed to help explain the pathogenesis of nonatopic asthma.

Local IgE Production in the Lung

Although patients with nonatopic asthma are classified as such based on the absence of detectable IgE production, studies have shown that IgE may in fact play a role in driving their airway disease locally in the lung.

Gould *et al* have demonstrated that class switching from IgM/IgG/IgA to IgE in the bronchial mucosa occurs in both atopic asthmatics *and* nonatopic asthmatics. Both groups of patients express mRNA coding for: 1) ε circle transcripts (Iε-Cμ CT and Iε-Cγ CT), which indicate DNA recombination is occurring at the heavy-chain locus to produce the ε heavy-chain characteristic of IgE and 2) the ε heavy-chain of IgE itself. The presence of both of these mRNA transcripts indicates that class switching to IgE does occur in the airways of nonatopic asthmatic patients[73].

Burney and colleagues have demonstrated that nonatopic asthmatic patients can in fact produce IgE in response to airborne allergen[10]. The study asked 297 patients using bronchodilators, half of which were nonatopic, to report any acute

respiratory events. Over the coming months, small particles were collected on the roof of their clinic using a high volume sampler. These were assumed to be representative of small particles and aeroallergens present in the air around the clinic. When a patient reported an exacerbation, blood was drawn. Particles collected the weekend before a patient's exacerbation were assessed for their ability to bind IgE in the patient's serum. That same ability was assessed for particles collected on a control weekend (2-3 weeks before or after the reported exacerbation). Exacerbations were associated with a 25% increase in IgE binding to particles collected on the weekend before the exacerbation compared to the control weekend. This was seen in patients with and without positive skin-prick tests to grass or tree pollens, suggesting that airborne allergen may be an important trigger of IgE-mediated asthma exacerbations, even in nonatopic patients. This mechanism may be local and specific to the lung or the inhalation route, as no reaction was seen with the skin-prick test[10]. IL-4 and IL-13 mRNA have been found to be elevated in the bronchial biopsies of nonatopic asthmatic patients[34], indicative of a cytokine milieu in nonatopic asthmatic airways that is conducive to IgE production by B cells.

IgE-independent T cell-mediated Response to Allergen

Evidence of T cell mediated responses to allergen, in the absence IgE involvement, has been reported in the literature. This suggests that the late phase of an 'allergic' response may occur in the absence of the acute response and without the corresponding IgE production traditionally used to identify trigger allergens in asthmatic patients.

Haselden *et al* have demonstrated that intradermal administration of peptides derived from cat allergen can elicit late phase asthmatic responses in cat-allergic asthmatics. These peptides did not cross-link IgE when tested *in vitro,* or elicit the early wheal and flare cutaneous response that is usually associated with an allergic response to antigen. However, 2-3 hours following peptide administration, 9 of 40 subjects experienced a decline in FEV_1, as well as chest tightness and wheezing[28].

Mori *et al* have demonstrated that peripheral blood mononuclear cells from nonatopic asthmatic patients can produce IL-5 when incubated with *Candida albicans* extract[57]. When these patients were given an inhalation challenge of *Candida albicans* acid protease they experienced a late phase asthmatic response characterized by a 45% drop in FEV_1 6 hours after challenge. A skin-prick challenge also elicited a late phase skin response 24 hours after the test was administered[56].

PROJECT RATIONALE

There is strong evidence supporting a central role for the T cell in asthma. In atopic asthma, T cells are documented components of the late-phase response to inhaled allergen, driving airway inflammation, mucus hypersecretion, and bronchoconstriction through the release of cytokines and other mediators. T cells have also been shown to produce inflammatory cytokines in response to allergen in nonatopic asthmatics, indicating a potential role in mediating disease in this phenotype. In both atopic and nonatopic asthma, aberrant T cell responses to

allergen may drive the infiltration of neutrophils and eosinophils into the airway through the production of pro-inflammatory cytokines, leading to exacerbations of disease.

This project will investigate allergen-induced T cell cytokines as biomarkers of asthma, both atopic and nonatopic. For this investigation, it will be important to consider the type of airway inflammation characteristic of each patient. We hypothesize that pro-eosinophilic cytokines will characterize the allergen-induced T cell responses of patients with a history of eosinophilic bronchitis, and that pro-neutrophilic cytokines will characterize the allergen-induced T cell responses of patients with a history of neutrophilic bronchitis.

Cytokine Selection

T cell cytokines were chosen based on an association with asthma and their potential ability to either drive disease by instigating airway inflammation (IL-5, IL-17A, and IL-23) or protect against disease by countering inappropriate immune responses (IL-10 and IFN-γ).

Interleukin-5

IL-5 is predominantly produced by Th2 cells[14] and is the main cytokine responsible for driving the maturation and recruitment of eosinophils to the airway[70]. IL-5 protein and mRNA has been detected in the bronchial mucosa of both atopic and nonatopic asthmatic patients[33].

Animal models have demonstrated that transgenic overexpression of IL-5 results in systemic eosinophilia[18], and that constitutive IL-5 expression in the lung causes the accumulation of eosinophils in the airways and airway hyperresponsiveness to methacholine[47]. They have also shown that using an anti-IL-5 monoclonal antibody to neutralize IL-5 inhibits antigen-induced airway eosinophilia and airway hyperresponsiveness[2,75].

IL-5 mRNA expression is increased in bronchial biopsies from asthmatic patients in comparison to normal controls. In addition, asthmatic patients expressing IL-5 mRNA in the airway show a significant increase in the number of activated T cells and activated eosinophils when compared to IL-5 mRNA- asthmatic patients[24]. IL-5 mRNA expression in bronchial biopsies has also been shown to correlate with symptom severity and airway hyperresponsiveness in atopic asthma, indicating that it may play an important role in driving disease[32]. In comparison to normal controls, IL-5 production from $CD4^+$ T cells in response to non-specific stimulation is enhanced in both atopic and nonatopic asthmatics. This cytokine also plays a role in the asthmatic response to allergen, as allergen-specific T cells isolated from mite-allergic atopic asthmatic patients produce IL-5 in response to dust mite[58].

Interleukin-17A

IL-17A is principally produced by $CD4^+$ Th17 cells[3,61], but other cell types, such as eosinophils, have been shown to produce this cytokine as well[55]. Th17 cells are important in host defense against bacteria and fungi[63], and have also been implicated in inflammatory disease. Transfer of Th17 cells into mice has been

shown to result in experimental autoimmune encephalitis, which worsens with increasing numbers of Th17 cells. Treatment with an IL-17A neutralizing antibody affords partial protection against this inflammation[45].

In the lung, IL-17A acts on bronchial epithelial cells to induce the production of IL-8 and Gro-α, resulting in the recruitment of neutrophils to the airway[78]. Animal models have demonstrated that intratracheal administration of IL-17A in rats induced neutrophil recruitment and activation in the airway[31]. In OVA-challenged mice, the adoptive transfer of Th17 cells resulted in neutrophil accumulation in the lung and airway hyperresponsiveness to methacholine that was not reversible with steroid administration. This response was not seen in IL-17RA knockout mice, indicating that IL-17A was the driving force[54].

Increased levels of IL-17A are present in the sputum and bronchoalveolar lavage of asthmatic patients[55], and levels of IL-17A in sputum correlates with airway hyperresponsiveness to methacholine[7]. Allergic asthmatics display elevated plasma IL-17 in comparison to normal controls[80], and their T cells produce IL-17A following allergen challenge[29]. Activated peripheral blood mononuclear cells from atopic asthmatics display higher levels of RORγt, the key transcription factor in controlling the differentiation of Th17 cells[82].

INTERLEUKIN-23

IL-23 is a cytokine produced by dendritic cells and is considered to act "upstream" to IL-17A in the process of neutrophil recruitment, as it is integral for the maintenance and survival of Th17 cells[45]. IL-23 is a member of the IL-12 cytokine family, sharing a p40 subunit with IL-12 in addition to having its own unique p19 subunit[8].

Animal models have demonstrated a role for IL-23 in asthma. IL-23 and IL-23R mRNA are upregulated in the lung following allergen challenge[76], and production of IL-23 following allergen uptake by dendritic cells is linked to airway hyperresponsiveness in mice[49].

IL-23 has also been associated with increased airway eosinophilia in animal models, though the mechanism behind this phenomenon is currently unknown. In a mouse model of allergic asthma, transgenic overexpression of IL-23R resulted in increased eosinophil infiltration of the airway and production of IL-4, IL-5, and IL-13[66]. Another group demonstrated that forced overexpression of IL-23 in mice leads to recruitment of *both* neutrophils and eosinophils to the lung, followed by the production of Th2 cytokines (IL-5 and IL-13), IL-17A, TNF-α, goblet cell hyperplasia, and airway hyperresponsiveness[76]. Silencing IL-23 expression in the lung has been shown to significantly reduce the influx of neutrophils and eosinophils into the lungs of mice following allergen challenge[50].

Compared with normal controls, IL-23 has been found to be elevated in the peripheral blood plasma of atopic asthmatics and correlates positively with plasma IL-17A levels. Activated peripheral blood mononuclear cells from these patients produced significantly higher levels of IL-23 than controls[81,82].

INTERLEUKIN-10

In atopy and asthma, the balance between inflammatory T cells and regulatory T cells is skewed towards the pro-inflammatory type[69]. IL-10 is produced by a population of $CD4^+$ T regulatory cells, some of which express the surface protein CD25 and/or the transcription factor FOXP3. These cells are responsible for suppressing the inflammatory response and maintaining tolerance to self-antigens and harmless environmental antigens. IL-10 acts on antigen presenting cells to dampen their activation of T cells, inhibits mast cells and eosinophils, and promotes IgG4 production, an antibody thought to counter the effects of IgE[41,53].

In a mouse model of allergic asthma, the adoptive transfer of allergen-specific $CD4^+CD25^{hi}$ T cells increased expression of IL-10 and reduced airway hyperresponsiveness and Th2 inflammation. These effects were ameliorated by the administration of an anti-IL10 mAb[43]. The depletion of $CD4^+CD25^+$ regulatory T cells in a dust mite-allergic mouse model increased airway hyperresponsiveness, airway eosinophilia, IgE, and IL-5 and IL-13 production by Th2 cells. Dendritic cells from these mice demonstrated elevated expression of MHC II, CD80, and CD86, accompanied by an increased ability to stimulate T cell proliferation and Th2 cytokine production[48]. An allergic model of IL-10 knockout mice exhibited increased IL-5 in bronchoalveolar lavage and eosinophilic inflammation of the airway[22].

IL-10 was found to be the predominant cytokine produced by allergen-specific T cells from healthy volunteers, while allergen-specific T cells from atopic individuals produced high IL-4[1]. $CD4^+CD25^+$ T cells from atopic donors have an impaired ability to suppress allergen-specific T cell proliferation and IL-5 production[51], and $CD4^+CD25^+$ T cell numbers and function are reduced in the bronchoalveolar lavage of pediatric patients with asthma[27].

INTERFERON-γ

IFN-γ, the principal cytokine involved in the Th1 response, is produced by $CD4^+$ and $CD8^+$ T cells[69]. Several studies have demonstrated its importance in the resolution of airway inflammation.

Animal models have shown that mice lacking the IFN-γ receptor experience prolonged airway eosinophilia following allergen inhalation[16]. Mice without T-bet, the Th1 transcription factor, display spontaneous features of airway disease, including airway hyperresponsiveness and an infiltration of Th2 cells and eosinophils into the airway[20]. In another model, administration of IFN-γ prevented the accumulation of T cells and eosinophils in the airway following antigen challenge in sensitized mice, while pretreatment with an anti-IFN-γ mAb had the opposite effect, increasing the infiltration of these cells into the airway[37].

Expression of the Th1 transcription factor T-bet is reduced in the airways of asthmatic patients, while expression of the Th2 transcription factor GATA3 is increased[69]. Serum levels of IFN-γ are significantly decreased in children with asthma[44], and a two-year study of asthmatic children demonstrated that a decline in lung function, assessed by FEV_1 values, correlated with a progressive decrease in

IFN-γ production by peripheral blood mononuclear cells[74]. The frequency of IFN-γ^+CD4^+ T cells is decreased in the peripheral blood of allergic asthmatics, while the frequency of IL-4^+CD4^+ and IL-17^+CD4^+ T cells is increased[83].

HYPOTHESIS

Allergen-induced T cell cytokines will act as biomarkers of disease in asthma. In response to allergen, both atopic and nonatopic asthmatic patients will exhibit PBMC activation and cytokine production characteristic of Th2 and Th17 inflammation.

PROJECT OBJECTIVES

1. To investigate differences in PBMC responses to allergen between asthmatic patients and normal controls:
    a) Do PBMCs from asthmatic patients show greater activation in response to allergen in comparison to normal controls?
    b) Do PBMCs from asthmatic patients show greater Th2 or Th17 cytokine production in response to allergen in comparison to normal controls?
    c) Do PBMCs from normal controls show greater Th1 or T regulatory cytokine production in response to allergen in comparison to asthmatic patients?

2. To investigate the relationship between PBMC responses to allergen and clinical asthma symptoms:
    a) Does PBMC activation and inflammatory cytokine production correspond with atopic asthmatic patients' known asthma triggers?
        i. Do known asthma triggers stimulate Th2 or Th17 cytokine production by a patient's PBMCs?
        ii. Do non-trigger allergens stimulate the production of Th1 or T regulatory cytokines?

3. To investigate PBMC responses of nonatopic asthmatic patients to allergen:
    a) Do PBMCs from nonatopic asthmatic patients become activated in response to allergen?
    b) Do PBMCs from nonatopic asthmatic patients produce inflammatory cytokines in response to allergen?

4. To investigate differences in PBMC responses to allergen between different groups of asthmatic patients:
    a) Is allergen-stimulated PBMC activation able to discriminate:
        i. Atopic asthma vs nonatopic asthma
        ii. Eosinophilic bronchitis vs noneosinophilic bronchitis
    b) Is cytokine production by allergen-stimulated PBMCs able to discriminate:
        i. Atopic asthma vs nonatopic asthma
        ii. Eosinophilic bronchitis vs noneosinophilic bronchitis
        iii. Neutrophilic vs nonneutrophilic bronchitis

5. To investigate the effects of steroid treatment on allergen-induced PBMC responses. Is there a difference in response to allergen between the following treatment groups:
   a) 0-500 mg inhaled corticosteroids (ICS)
   b) 500-1000 mg ICS
   c) > 1000 mg ICS
   d) 0-10 mg Prednisone
   e) > 10 mg Prednisone

EXPERIMENTAL METHODS

ETHICS

This study was approved by the Research Ethics Board at St. Joseph's Hospital in Hamilton, Ontario.

PATIENT CHARACTERISTICS

The patients in this study are followed at the Firestone Institute for Respiratory Health at St. Joseph's Hospital in Hamilton, Ontario. They have been diagnosed with asthma based on a ≥ 12% improvement in FEV_1 after inhalation of salbutamol and a PC_{20} of < 8 mg/mL. Both of these measurements demonstrate that the patients are experiencing a wide variation in airflow over a short period of time. Salbutamol is a bronchodilator and FEV_1 refers to forced expiratory volume in one second, or the volume of air that an individual can forcibly breathe out in one second. PC_{20} refers to the provocative concentration of inhaled methacholine (a bronchoconstrictor) required to cause a 20% fall in FEV_1.

Asthmatic patients have also been sub-classified as atopic or nonatopic and eosinophilic or noneosinophilic. Atopy has been defined as a positive skin-prick test to one or more allergens. Eosinophilic asthmatic patients have been defined as having > %3 sputum eosinophils on > 2 occasions. It has also been noted if a patient has experienced a neutrophilic asthma exacerbation, defined as a sputum total cell count > 10×10^6 of which ≥ 65% are neutrophils at the time of exacerbation.

Normal controls have been identified on the basis of PC_{20} > 16 mg/mL, a negative skin-prick test, total serum IgE < 120 units/mL, and no history of asthma or allergic disease. They are not patients followed at the Firestone.
See Appendix 1: Patient Characteristics

SKIN PRICK TEST – ASSESSMENT OF ATOPY

Liquid preparations from 19 common allergens were placed on the patient's skin which was then pricked with a lancet. Histamine was used as a positive control and saline as a negative control. After 20 minutes, the skin reactions of the patients were assessed. A wheal reaction > 3 mm was considered a positive result, indicating atopy.

Sputum Induction and Processing – Assessment of Type of Bronchitis

Samples were obtained by having the patients breathe in nebulized saline for 7 minutes and then cough up sputum. This process was repeated 3-4 times until an appropriate size sample was obtained (approximately half the size of a pea). Patients' FEV_1 was assessed prior to sample induction and between saline inhalations. Sputum induction was stopped if a patient's FEV_1 fell by 200 mL and by 20% or more from baseline, and the patient was treated with salbutamol.

For processing, sputum samples were sent to certified medical laboratory technologists who identified total and differential cell counts using the ACCUFILTER sputum processing kit.

Allergen Selection

Prior to blood collection, allergens were plated in a 96-well plate at a concentration of 200 ug/mL in AIM-V media and frozen. Since the patient cohort under investigation displays year-round asthma, we selected allergens that the patients would most likely have continuous exposure to, such as animal dander or molds that can grow indoors. All allergens were sourced from Greer Laboratories. Four microbial antigens implicated in lung infection have also been included: three surface proteins from *Streptococcus pneumoniae* and one surface protein from *Haemophilus influenzae*. These microbial proteins were kindly provided by the lab of Dr. Wayne Thomas at the University of Western Australia. Media alone was used as a negative control and the superantigen Staphylococcal enterotoxin B (SEB) was used as a positive control.

MITES	
• House Dust Mite - *Dermatophagoides farinae*	• House Dust Mite - *Dermatophagoides pteronyssinus*
• House Dust Mite - *Euroglyphus maynei*	• Food/Storage Mite - *Acarus siro*
• Food/Storage Mite - *Lepidoglyphus destructor*	

INSECTS	
• Cockroach, American - *Periplaneta Americana*	• Cockroach, German - *Blatella germanica*

EPITHELIA AND DANDER	
• Cat Dander/Antigen - *Felis catus (domesticus)*	• Cat Epithelia - *Felis catus (domesticus)*
• Dog Dander, Mixed-Breed - *Canis familiaris*	• Hamster Epithelia - *Mesocricetus auratus*
• Mouse Epithelia - *Mus musculus*	• Rat Epithelia - *Rattus norvegicus*

FUNGI	
• Alternaria alternaria - *Alternaria tenuis*	• Aspergillus fumigatus
• Aureobasidium pullulans - *Pullularia pullans*	• Botrytis cinerea
• Candida albicans	• Cladosporum herbarum
• Cladosporium sphaerospermum - *Hormodendrum hordei*	• Epidermophyton floccosum
• Fusarium solani	• Helminthosporium solani- *Spondylocladium atrovirens*
• Microsporum canis	• Mucor circinelloides f. lusitanicus - *Mucor racemosus*
• Penicillium notatum	• Phoma betae
• Saccharomyces cerevisiae	• Trichophyton rubrum

GRASS	
• Timothy - *Phleum pratense*	

WEEDS	
• Mugwort, Common - *Artemisia vulgaris*	• Ragweed, Short - *Ambrosia artemisiifolia*

MICROBIAL ANTIGENS	
• *Streptococcus pneumoniae* – PspA1	• *Streptococcus pneumoniae* – PspA2
• *Streptococcus pneumoniae* – PspC	• *Haemophilus influenzae* - Omp p6

CELL CULTURE

At *DAY 0*, 30 mL of blood was collected from each patient into heparin-coated green top tubes (to prevent coagulation). Peripheral blood mononuclear cells were then separated from whole blood (T cells, monocyetes, B cells, and NK cells). To isolate PBMCs, 25 mL of blood was layered onto 15 mL of Histopaque 1077 and centrifuged at 400 RCF for 30 minutes at 25^{o}C with slow acceleration and deceleration. The buffy coat was removed and washed in 40 mL of serum-free AIM-V media at 250 RCF for 10 minutes at 25^{o}C with maximum acceleration and deceleration. The cells were resuspended in 3 mL of AIM-V media, counted using a Countess automatic cell counter, and brought up to a final concentration of 2.5 x 10^{6} cells/mL. 100 µL of cells (2.5 x 10^{5} cells) were added to each well of premade plates and incubated for 5 days at 37^{o}C.

CYTOKINE MEASUREMENT

At *DAY 5*, 100 µL of supernatant was removed from each well for cytokine analysis by ELISA (eBioscience). 100 µL of AIM-V media was added to each well as a replacement. ELISA results are displayed in pg/mL of cytokine produced under

each condition. ELISA sensitivities are as follows: IL-5: 4-500 pg/mL, IL-17A: 4-500 pg/mL, IL-23: 15-2000 pg/mL, IL-10: 2-300 pg/mL, IFN-γ: 4-500 pg/mL.

PROLIFERATION ASSAY

At *DAY* 6, the PBMCs were pulsed with radioactive ^{3}H-thymidine (0.5 μCi per well) and incubated for 16 hours. Since thymidine is incorporated into the DNA of new cells during cell division, the amount of ^{3}H-thymidine taken up by cells in culture indicates their degree of proliferation.

At *DAY* 7, the PBMCs were harvested onto glass fibre filter mats and assessed for proliferation (by way of ^{3}H-thymidine incorporation) using a MicroBeta counter. The MicroBeta counter detects luminescence from the ^{3}H label and expresses this in the units counts per minute (CCPM). Proliferation results are displayed as a delta measurement, which indicates the degree of PBMC proliferation seen under each condition in comparison to that seen with media alone. To generate delta measurements, proliferation under media alone has been subtracted from proliferation under each experimental stimulus. This delta measurement can be used as an index for T cell activation.

STATISTICS

Differences between patient groups were analyzed using unpaired Mann-Whitney t tests. p values < 0.05 are considered significant. Data was graphed showing the standard error of the mean (SEM) and statistical significance (*).

RESULTS

All consolidated patient results are presented in Appendix II and all individual patient results are presented in Appendix III. Both PBMC proliferation (expressed as a delta measurement) and cytokine data are shown for each individual antigen as well as for each antigen class: Mites and Insects, Animal Dander and Epithelia, Fungi, Microbial Proteins, and Grass and Weeds.

OBJECTIVE 1: *Investigating Differences in PBMC Responses to Allergen Between Asthmatic Patients and Normal Controls*

a) PBMCs from asthmatic patients did not display higher proliferation in response to allergen when compared with normal controls. This suggests that there is a comparable level of T cell activation in response to these antigens between the two groups.
   See Figures 1.1 and 1.2.

Figure 1.1 Delta: Asthma vs Normal

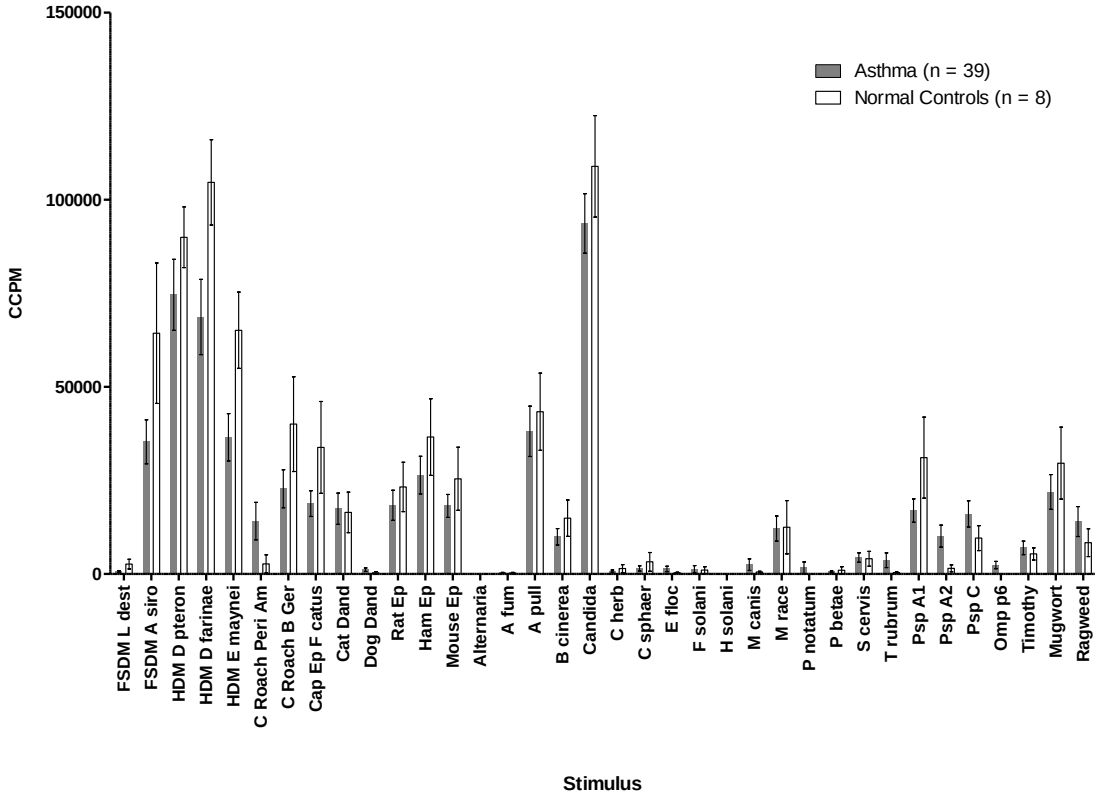
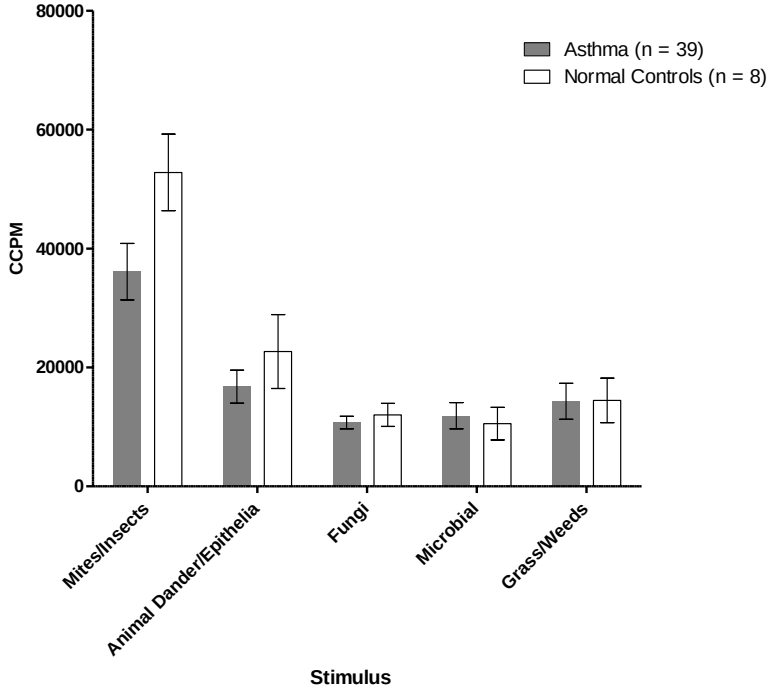


Figure 1.2 Delta Grouped: Asthma vs Normal



b) PBMCs cells from asthmatic patients did not display significantly greater levels of Th2 or Th17 cytokine production in response to allergen. Although it appears that asthmatic patients produce more IL-5 in response to allergen than normal controls, this trend did not reach statistical significance.
See Figures 2.1 and 2.2 for IL-5 data
See Figures 3.1 and 3.2 for IL-17A data.
See Figures 4.1 and 4.2 for IL-23 data.

Figure 2.1 IL-5: Asthma vs Normal

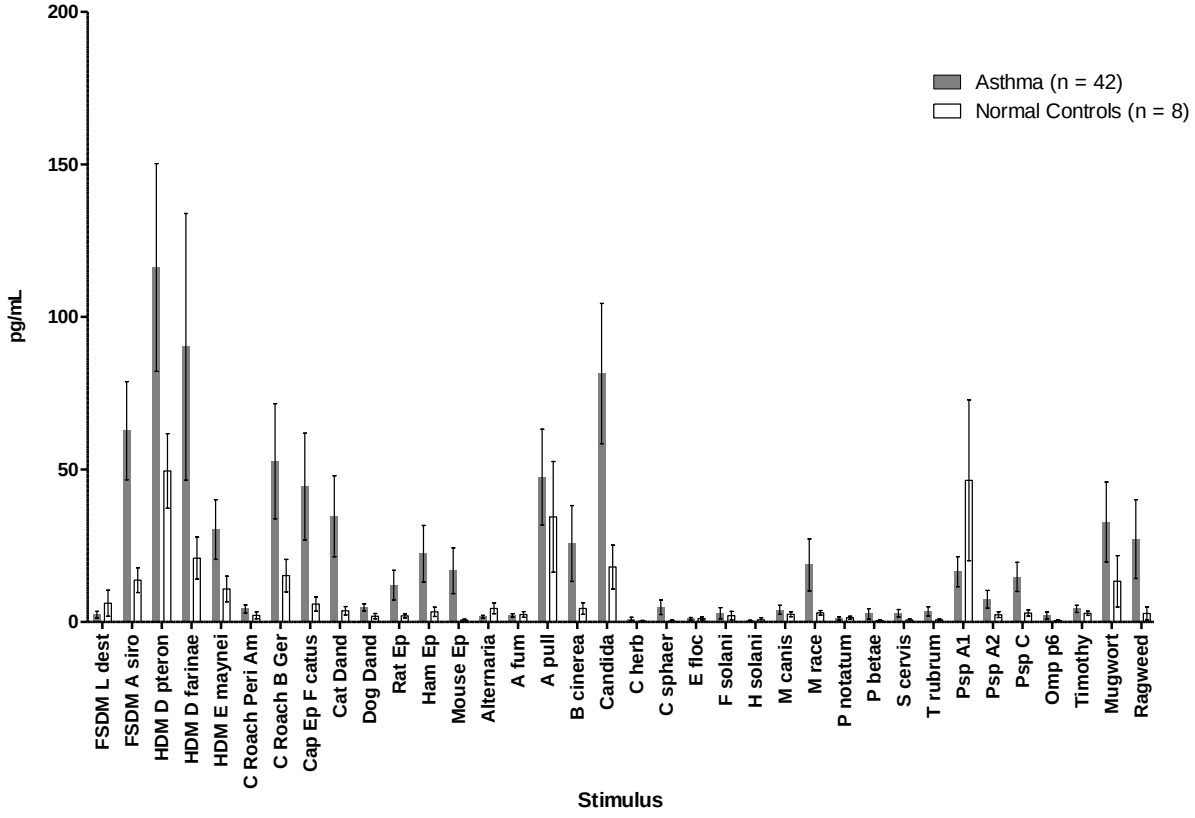


Figure 2.2 IL-5 Grouped: Asthma vs Normal

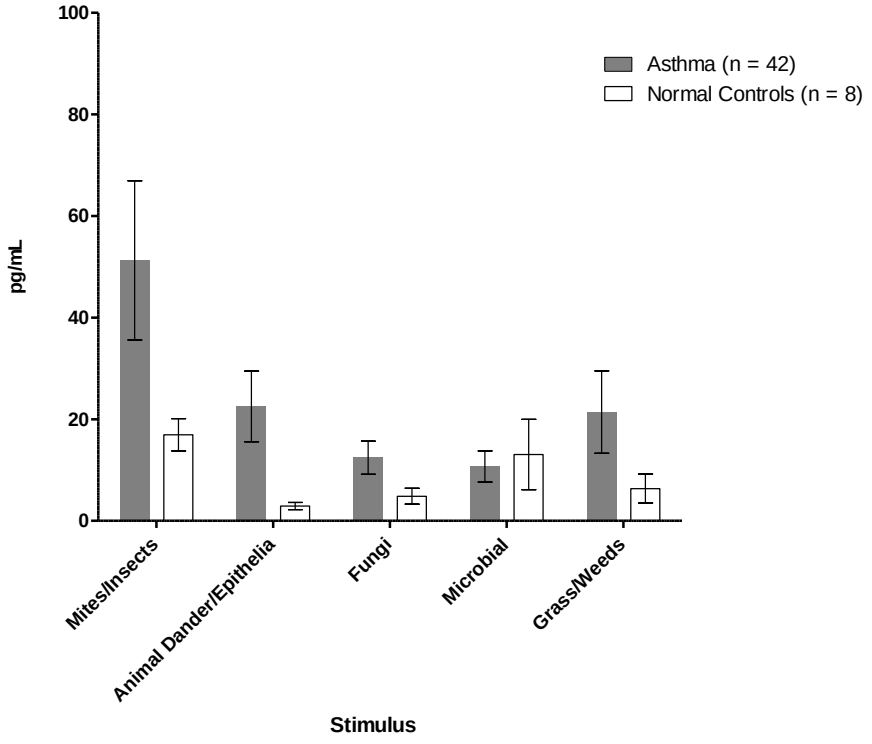


Figure 3.1 IL-17A: Asthma vs Normal

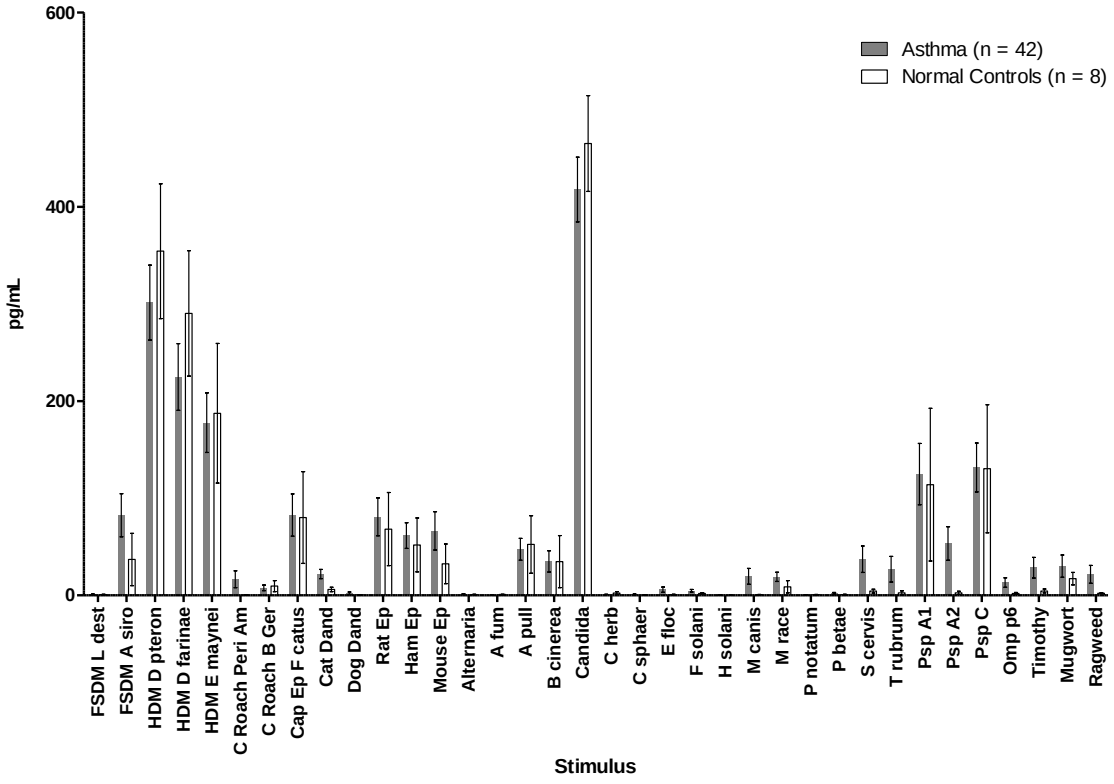


Figure 3.2 IL-17A Grouped: Asthma vs Normal

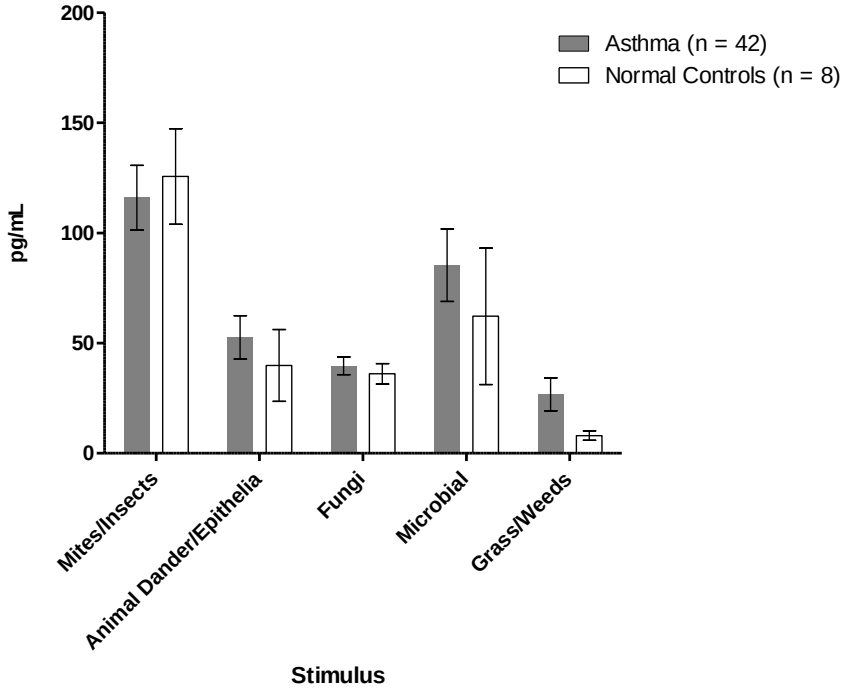


Figure 4.1 IL-23: Asthma vs Normal

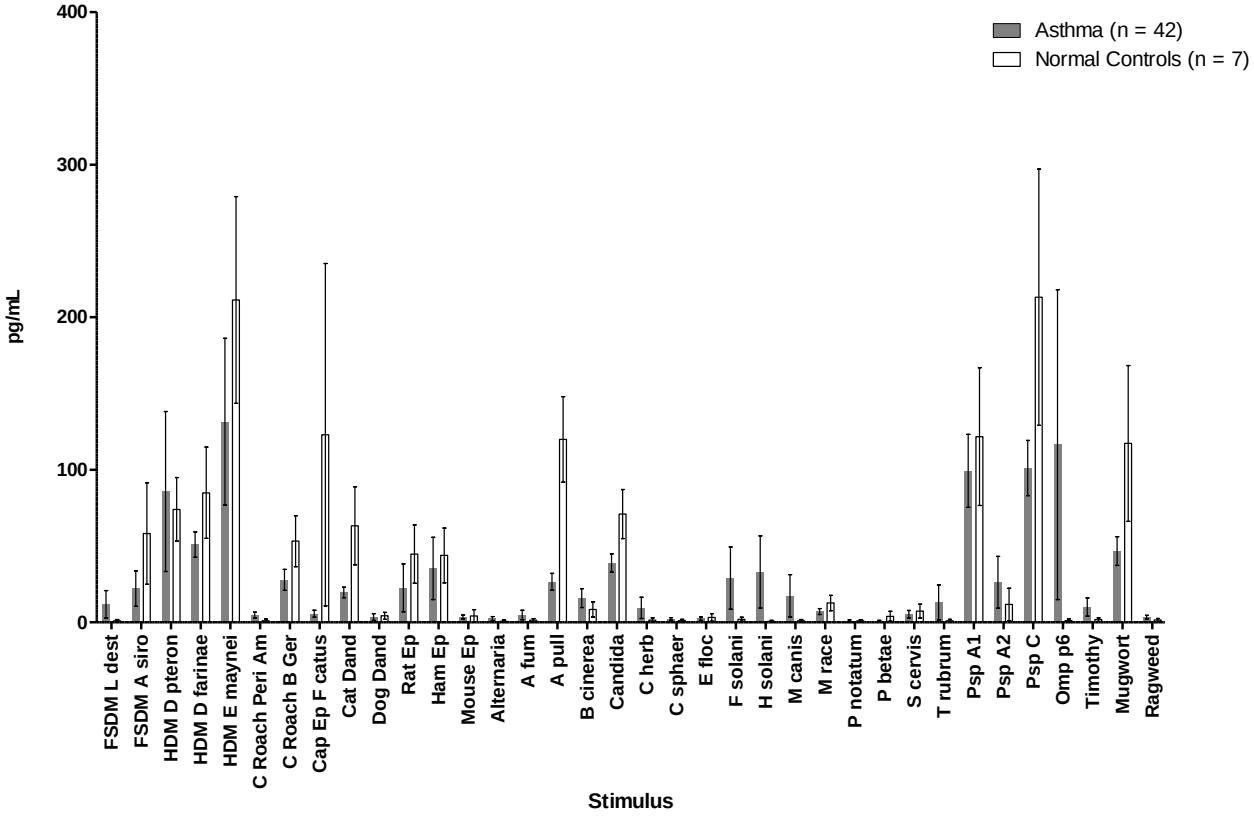
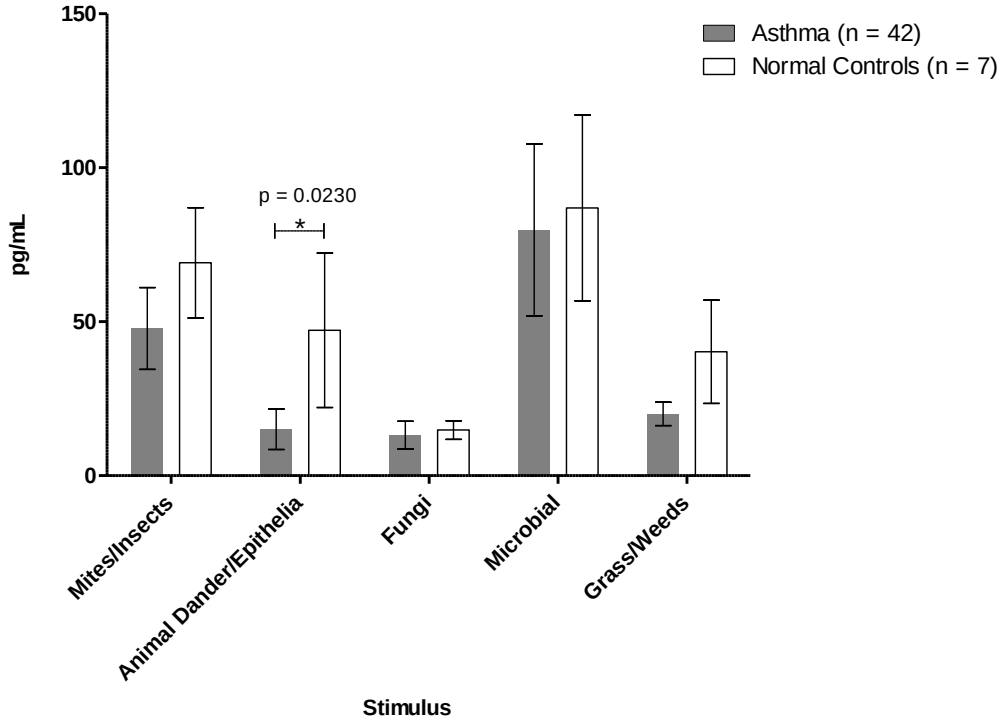


Figure 4.2 IL-23 Grouped: Asthma vs Normal



c) PBMCs cells from normal controls did not display greater levels of Th1 or T regulatory cytokine production in response to allergen.
The difference between the IL-10 responses of asthmatic patients and normal controls to the "Grass and Weeds" antigen class was statistically significant. This was due to the response to mugwort, and was not representative of either ragweed or timothy grass, or any of the other allergens on the panel.
See Figures 5.1 and 5.2 for IL-10 data.
There was a trend for higher IFN-γ responses in the normal controls that reaches statistical significance only in the "Grass and Weeds" antigen class, again due to the response to mugwort.
See Figures 6.1 and 6.2 for IFN-γ data.

Figure 5.1 IL-10: Asthma vs Normal

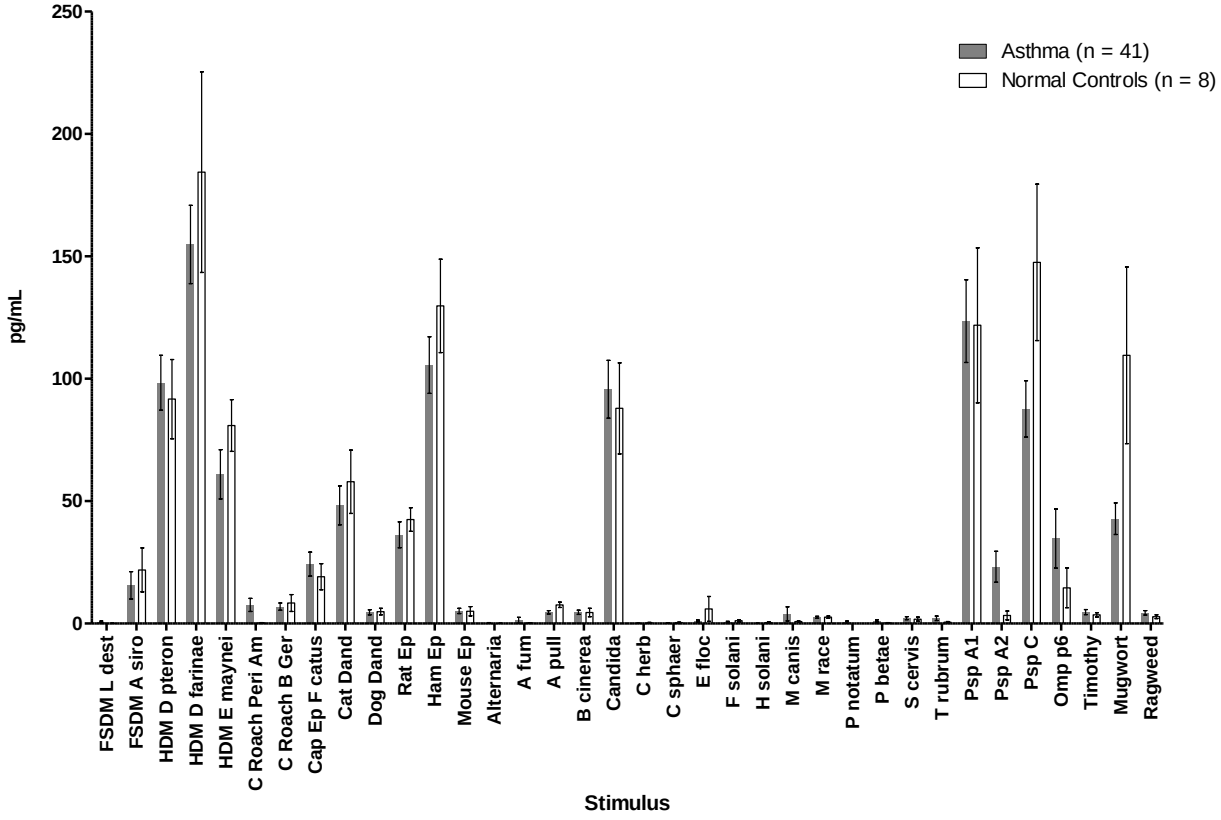


Figure 5.2 IL-10 Grouped: Asthma vs Normal

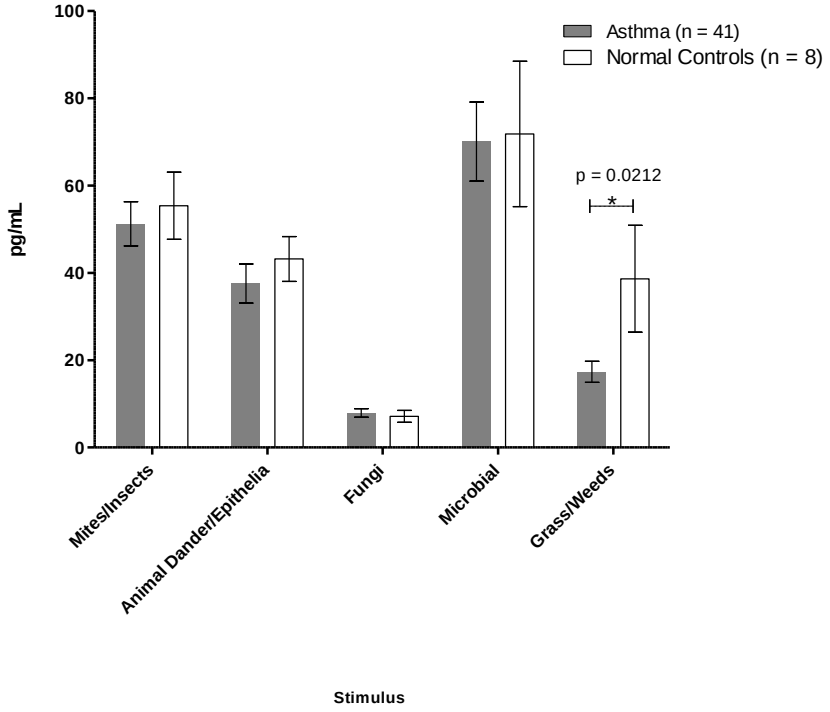


Figure 6.1 IFN-gamma: Asthma vs Normal

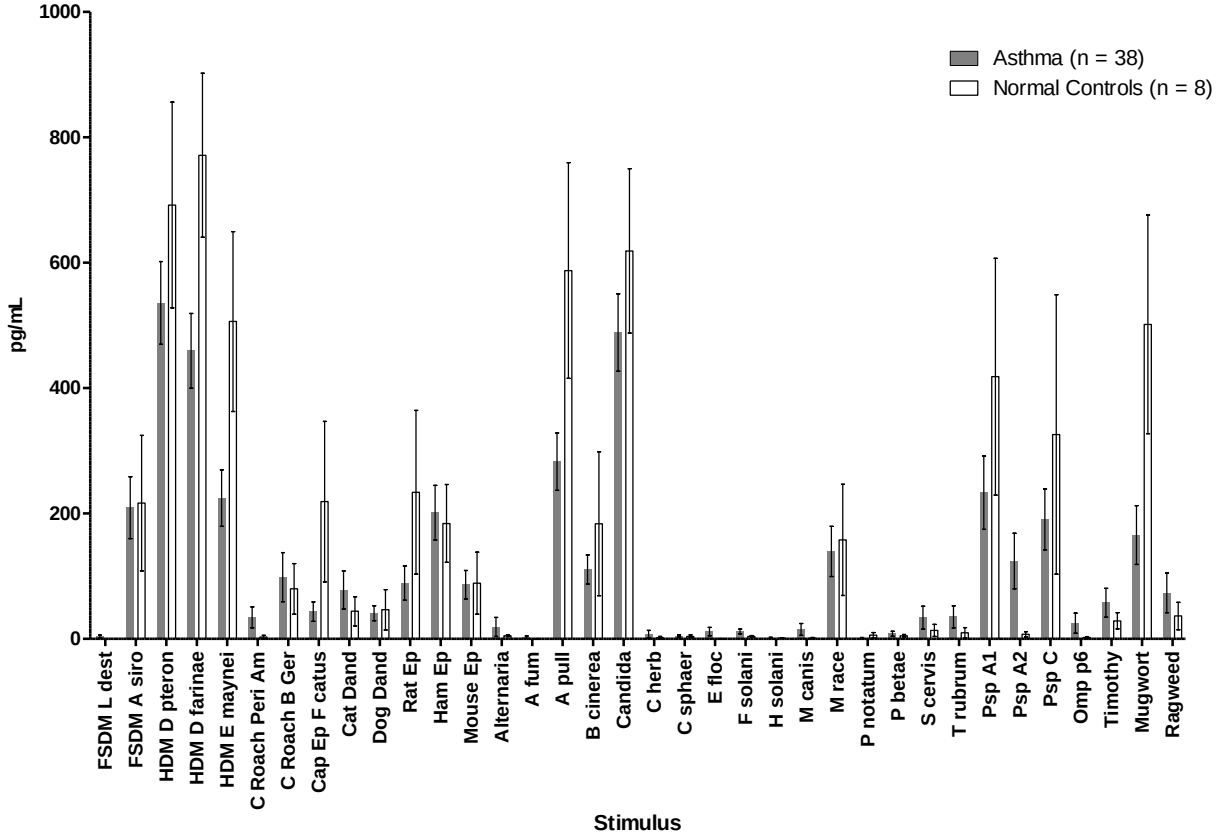
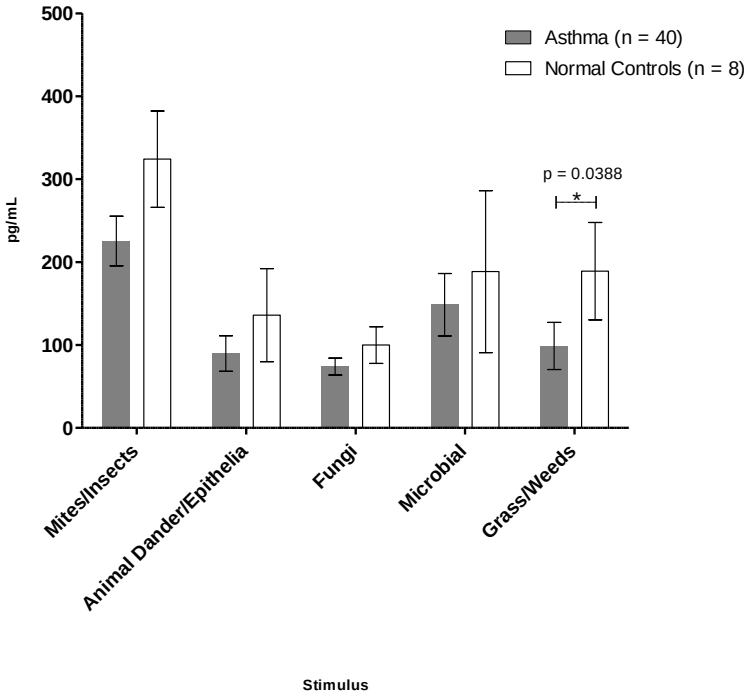


Figure 6.2 IFN-gamma Grouped: Asthma vs Normal



OBJECTIVE 2: *Investigating The Relationship Between PBMC Responses to Allergen and Clinical Asthma Symptoms*

a) PBMC proliferation and inflammatory cytokine production did not necessarily correspond with known asthma triggers in atopic asthmatic patients. Atopic patients produced cytokines to a variety of allergens, some of which are known to trigger their asthma, and some of which are not. They also failed to produce cytokines in response to some of their known asthma triggers.

As an example, we can consider atopic asthmatic patient 7, who is known to be sensitive to dust mite. This patient showed dominant IL-5 and IL-17A production to dust mite, but also to cat dander, mouse epithelium, and grass. *See Figures 11.11 and 11.12 for this patient's cytokine data.*

Another interesting example is atopic asthmatic patient 10, who is sensitive to the mold *Alternaria alternaria*, cat, horse, feathers, and grass. This patient showed high IL-5 in response to cat epithelia and cat dander, but no cytokine production to *Alternaria* or grass.

See Figure 11.17 and 11.18 for this patient's cytokine data.

See Figures 11.1 – 11.32 for all individual atopic asthmatic patients' cytokine data.

Figure 11.11 Atopic Asthmatic Patient 7 - Eosinophilic
Allergies: dust mite

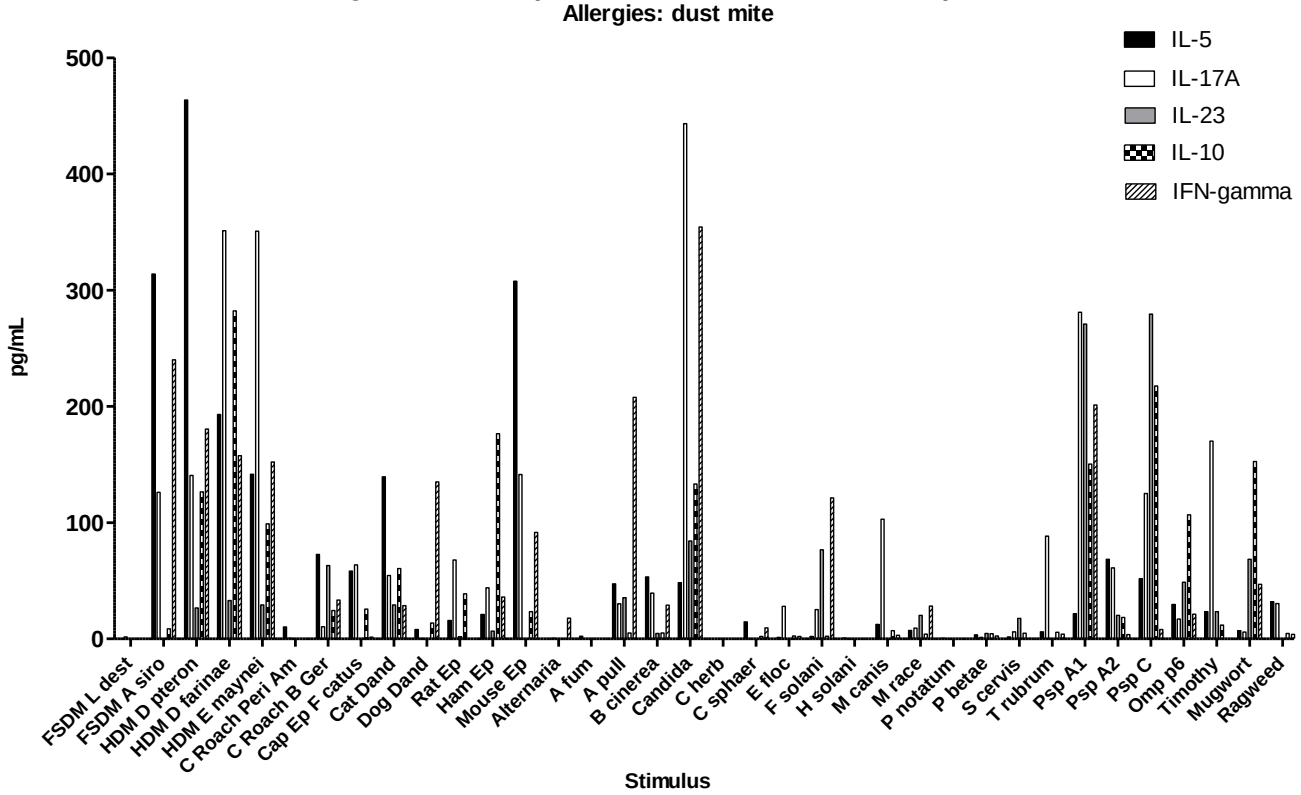


Figure 11.12 Atopic Asthmatic Patient 7 - Eosinophilic
Allergies: dust mite

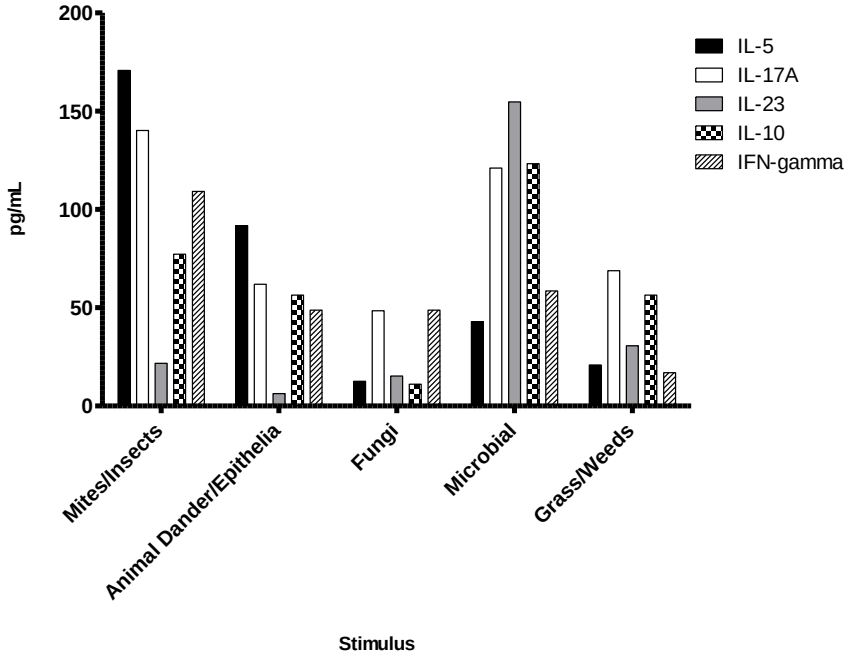


Figure 11.17 Atopic Asthmatic Patient 10 - Eosinophilic
Allergies: Alternaria, cat, horse, feathers, grass

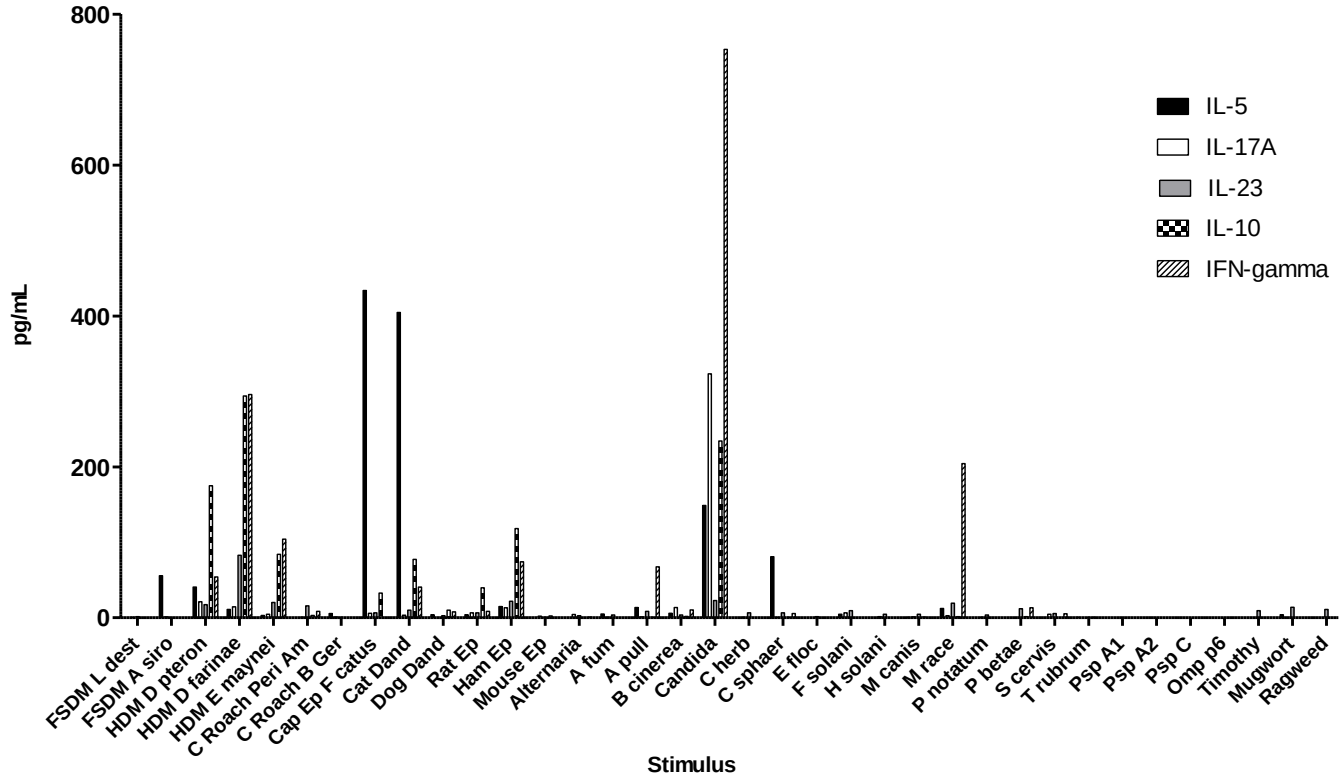
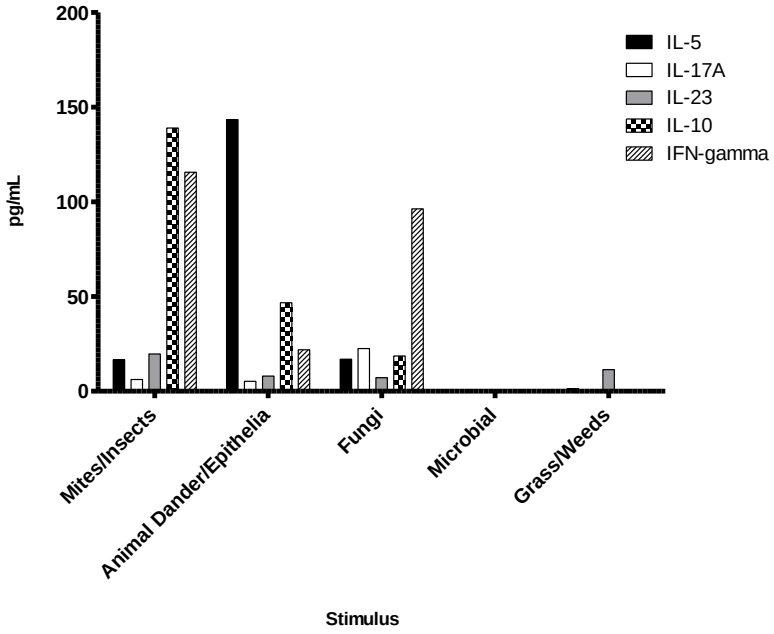


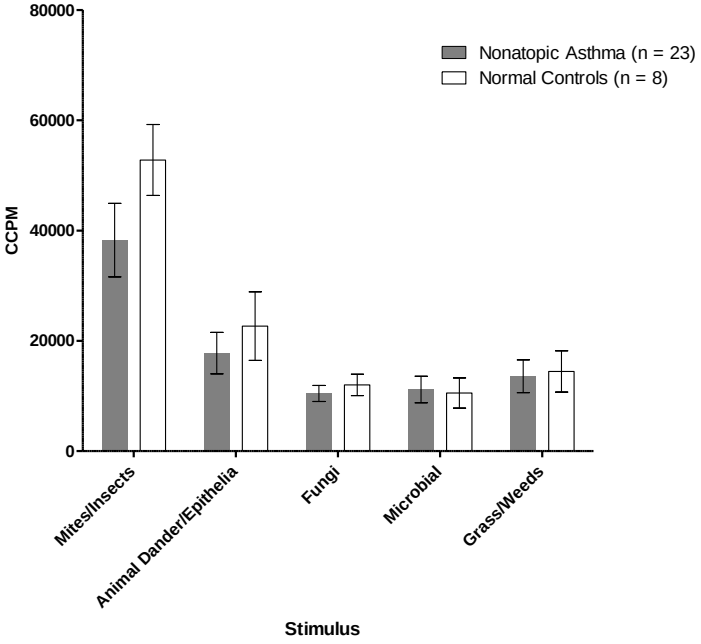
Figure 11.18 Atopic Asthmatic Patient 10 - Eosinophilic
Allergies: Alternaria, cat, horse, feathers, grass



OBJECTIVE 3: *PBMC Responses of Nonatopic Asthmatic Patients to Allergen*

a) Nonatopic asthmatic patients did not display increased PBMC proliferation in response to allergen when compared to normal controls.
See Figure 1.7.

Figure 1.7 Delta Grouped: Nonatopic Asthma vs Normal



b) PBMCs from nonatopic asthmatic patients did produce inflammatory cytokines in response to allergen, although cytokine levels were not statistically different from those produced by normal controls.
Although it appears that nonatopic asthmatics produced more IL-5 in response to allergen than normal controls, this trend did not reach statistical significance.
See Figure 2.9 for IL-5 data.
See Figure 3.9 for IL-17A data.
There was a trend for more IL-23 production in normal controls than in nonatopic asthmatics.
See Figure 4.9 for IL-23 data.

Figure 2.9 IL-5 Grouped: Nonatopic Asthma vs Normal

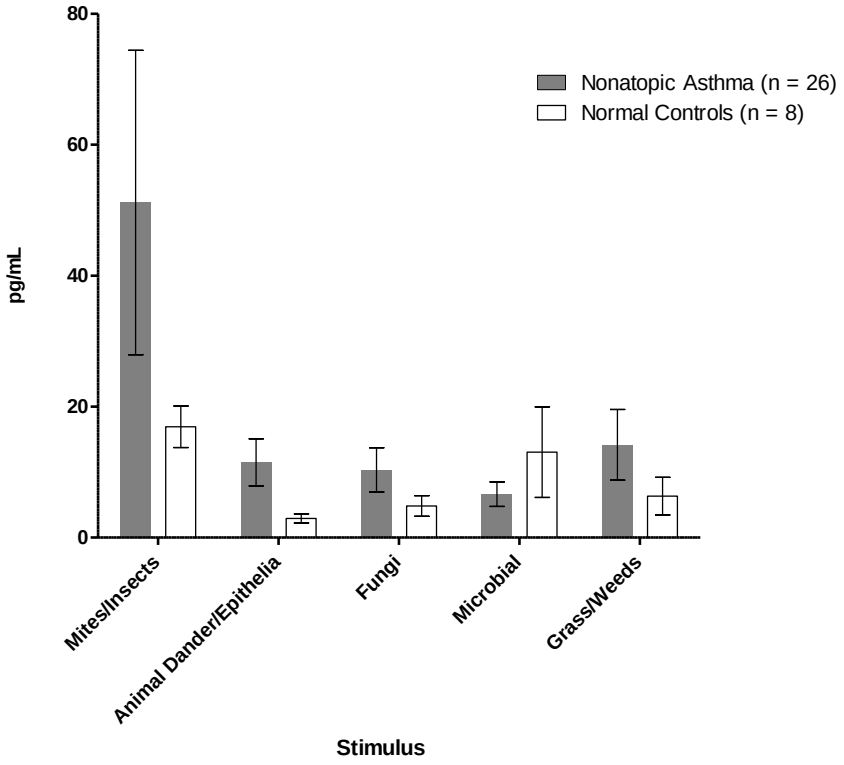


Figure 3.9 IL-17A Grouped: Nonatopic Asthma vs Normal

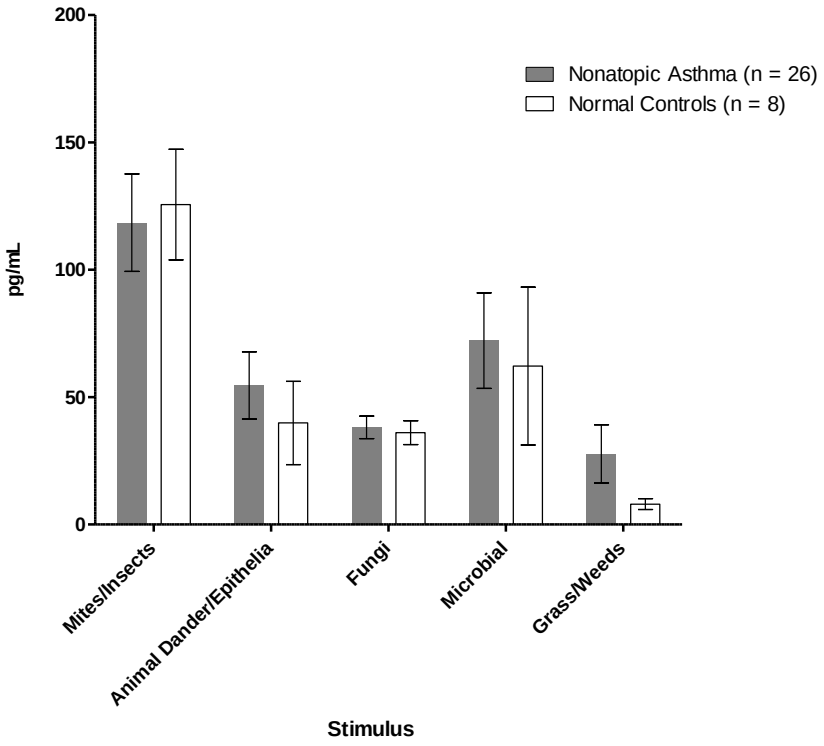
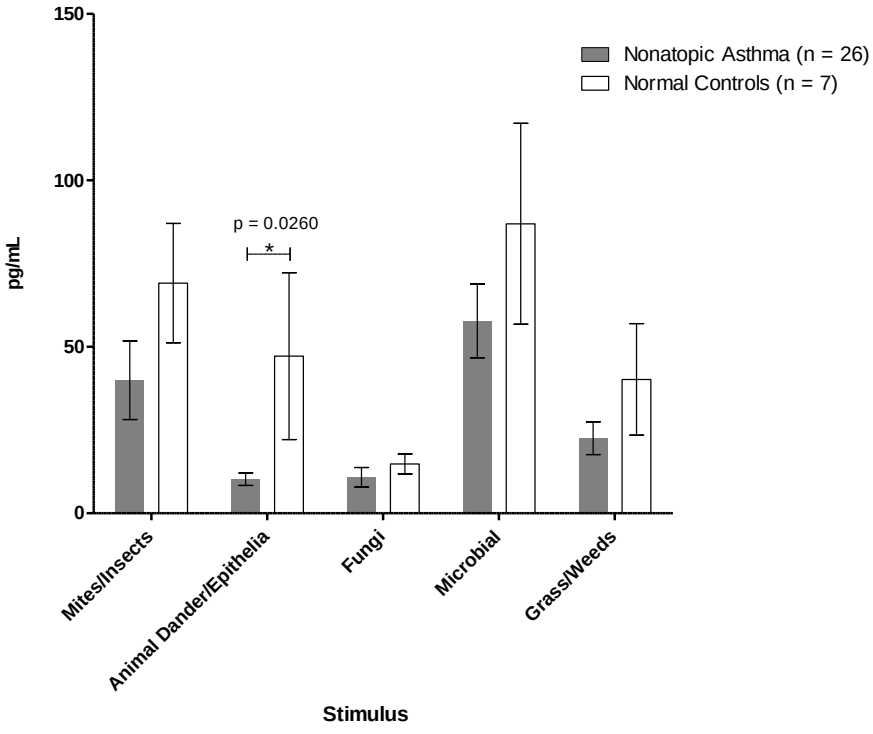


Figure 4.9 IL-23 Grouped: Nonatopic Asthma vs Normal



OBJECTIVE 4: *Differences in PBMC Responses to Allergen Between Different Groups of Asthmatic Patients*

a) Allergen-stimulated PBMC proliferation was unable to distinguish different patient groups.
   i. No difference in PBMC proliferation was seen between atopic and nonatopic patient groups.
   See Figures 1.3 and 1.4.
   ii. No difference in PBMC proliferation was seen between eosinophilic and noneosinophilic patient groups.
   See Figures 1.5 and 1.6.

Figure 1.3 Delta: Nonatopic vs Atopic Asthma

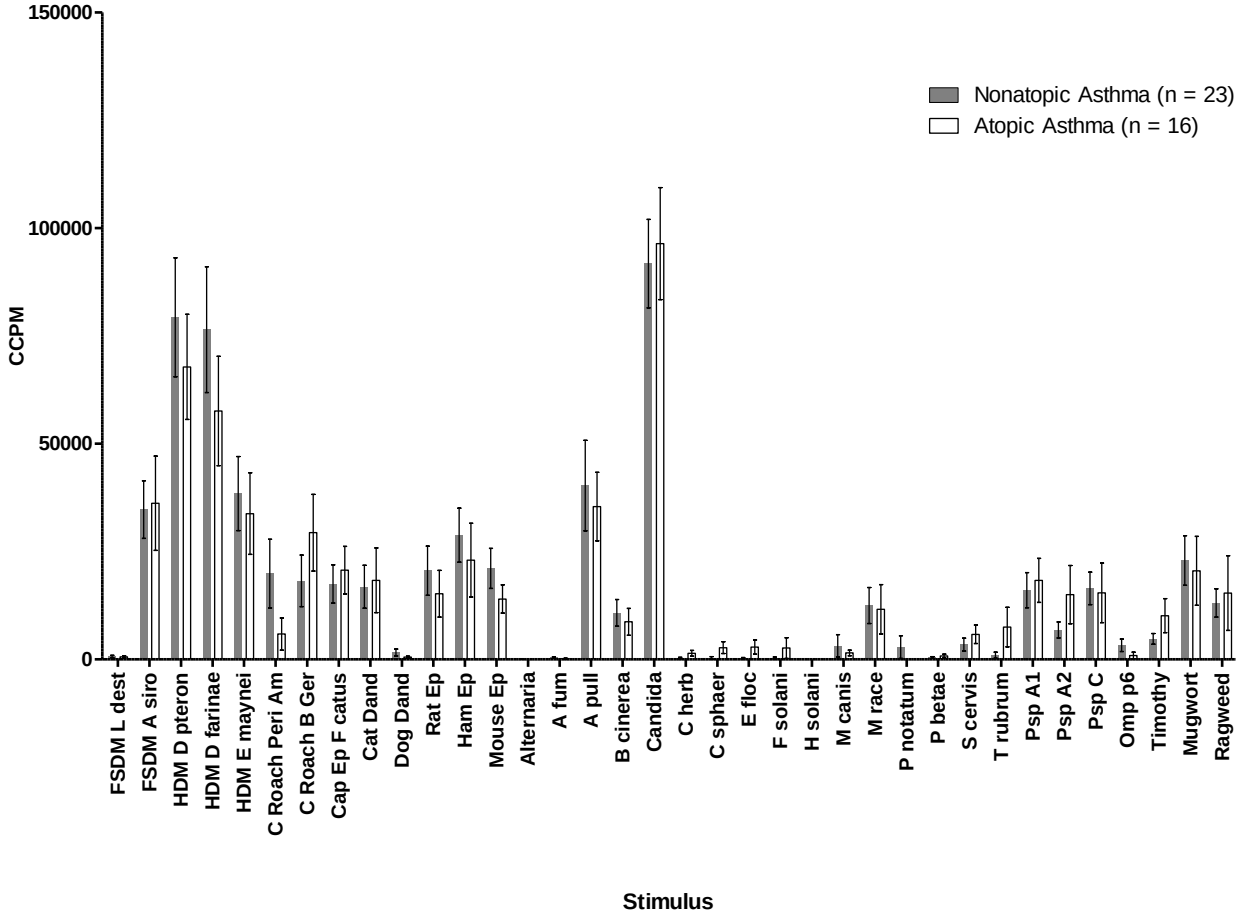


Figure 1.4 Delta Grouped: Nonatopic vs Atopic Asthma

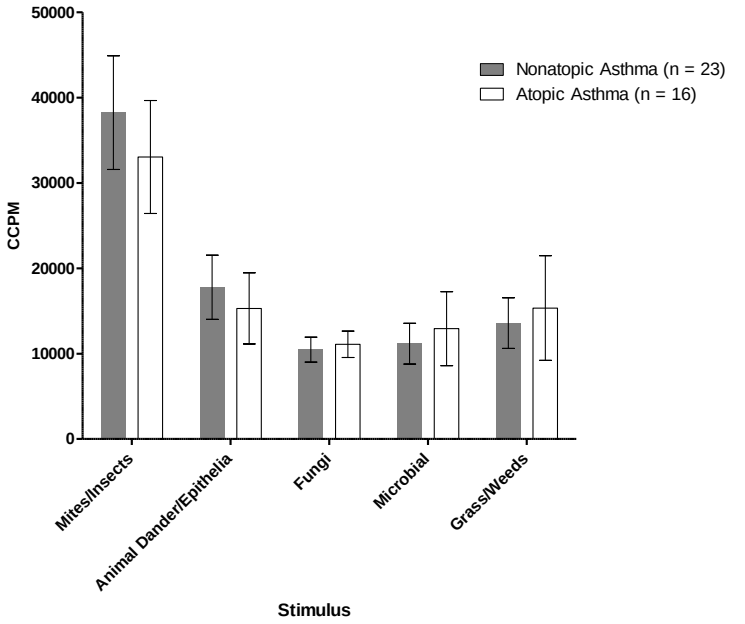


Figure 1.5 Delta: Eosinophilic vs Noneosinophilic

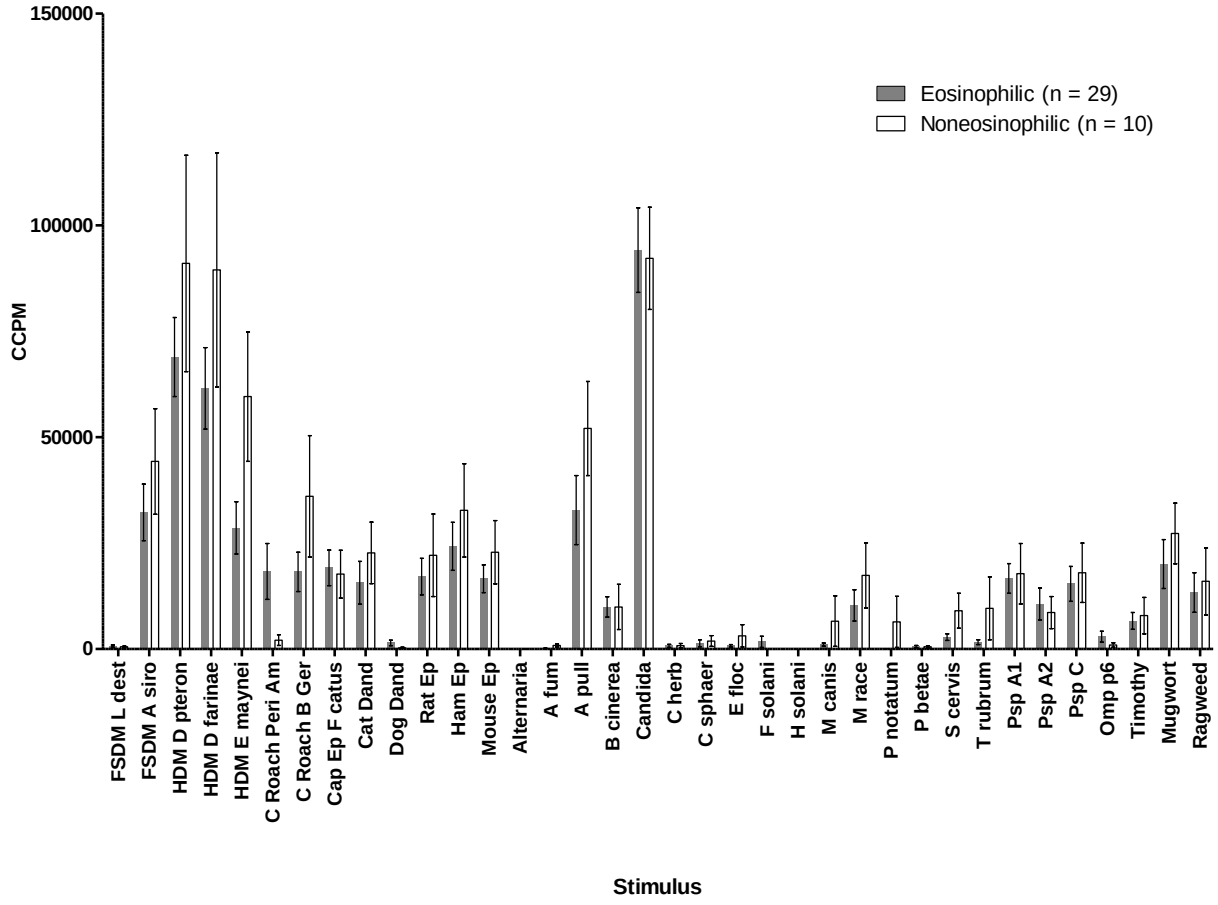
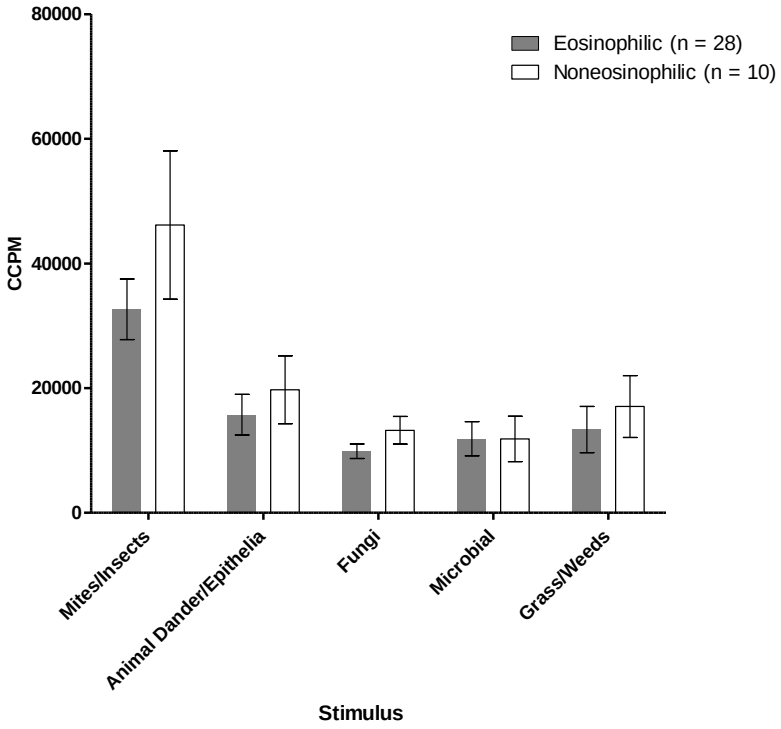


Figure 1.6 Delta Grouped: Eosinophilic vs Noneosinophilic



b) Cytokine production by allergen-stimulated PBMCs was unable to definitively distinguish different patient groups.
   i. Atopic asthma vs nonatopic asthma. Although there were trends for higher IL-5, IL-23, and IFN-γ production by atopic asthmatic patients, these were not statistically significant.
   See Figures 2.3 and 2.4 for IL-5 data.
   See Figures 3.3 and 3.4 for IL-17A data.
   See Figures 4.3 and 4.4 for IL-23 data.
   See Figures 5.3 and 5.4 for IL-10 data.
   See Figures 6.3 and 6.4 for IFN-γ data.

Figure 2.3 IL-5: Atopic vs Nonatopic Asthma

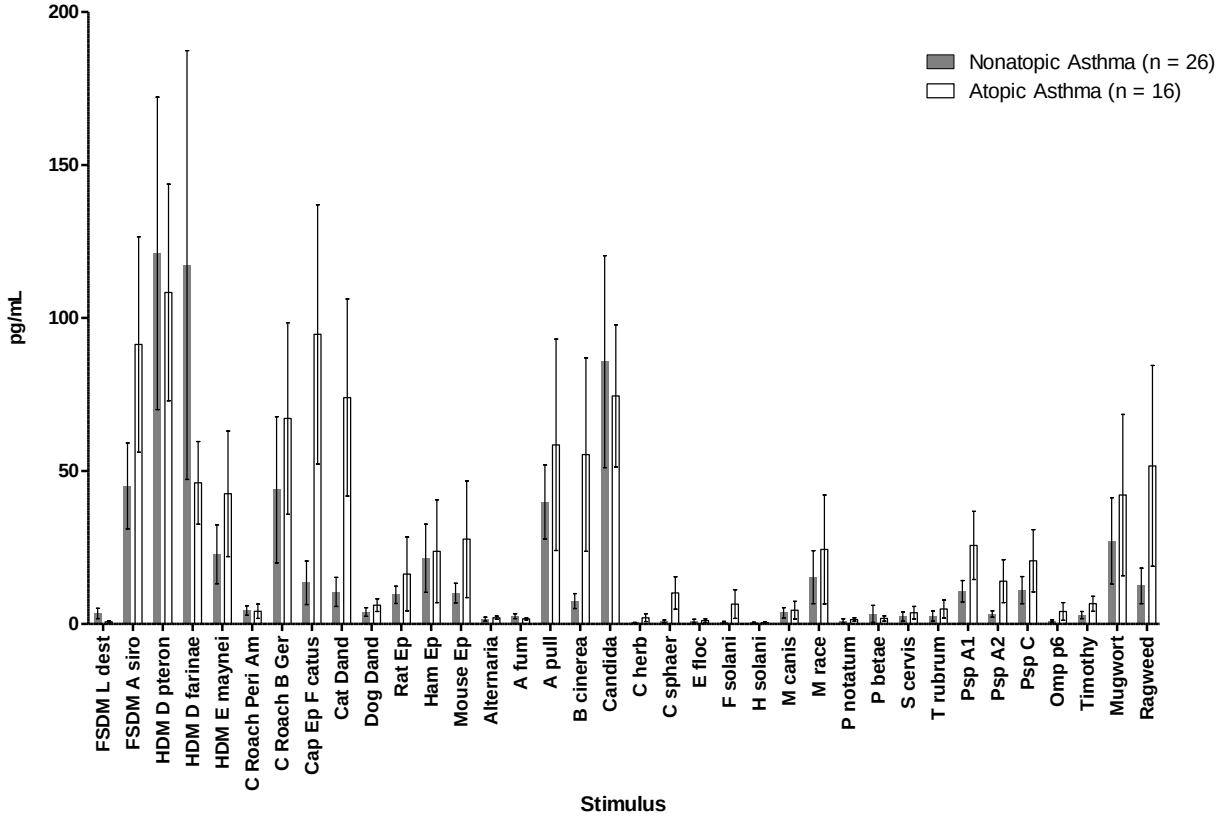


Figure 2.4 IL-5 Grouped: Atopic vs Nonatopic Asthma

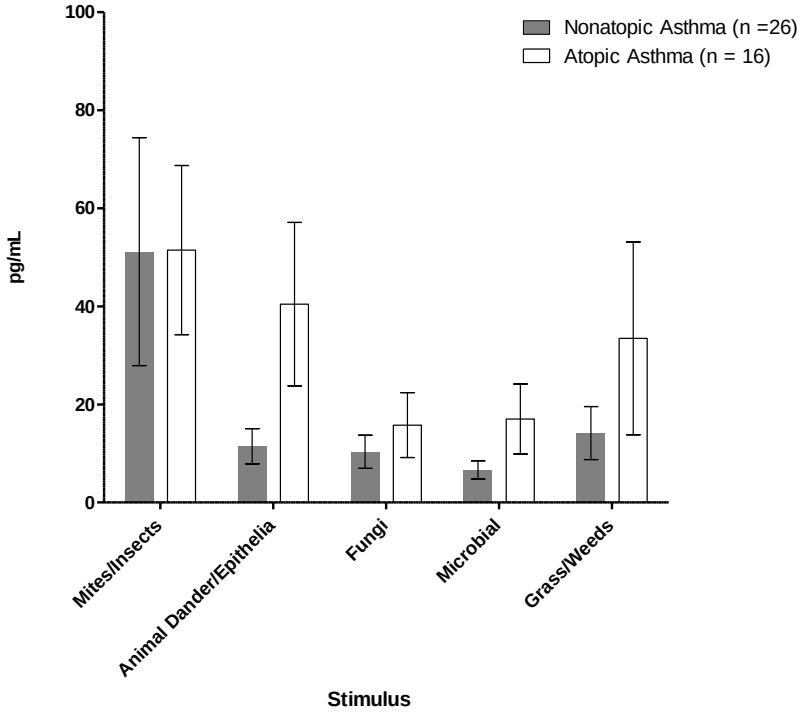


Figure 3.3 IL-17A: Atopic vs Nonatopic Asthma

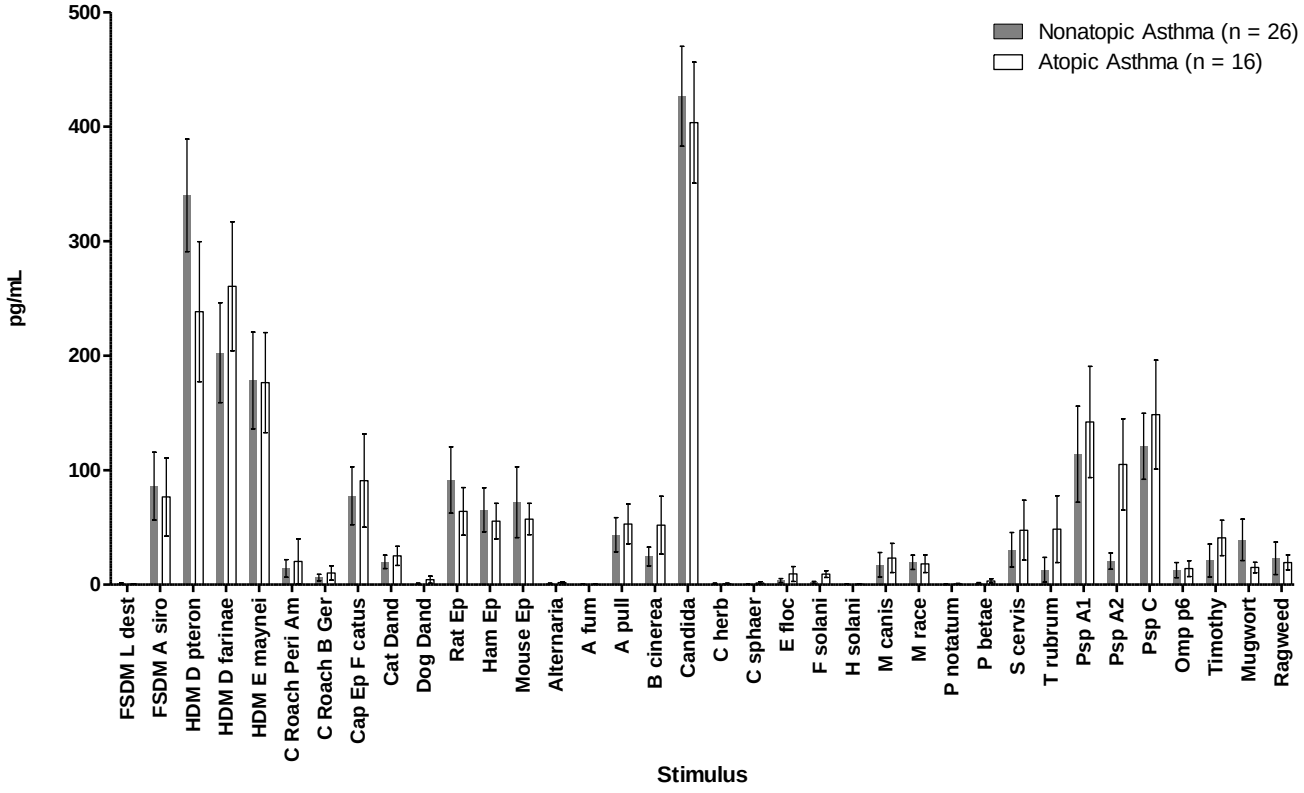


Figure 3.4 IL-17A Grouped: Atopic vs Nonatopic Asthma

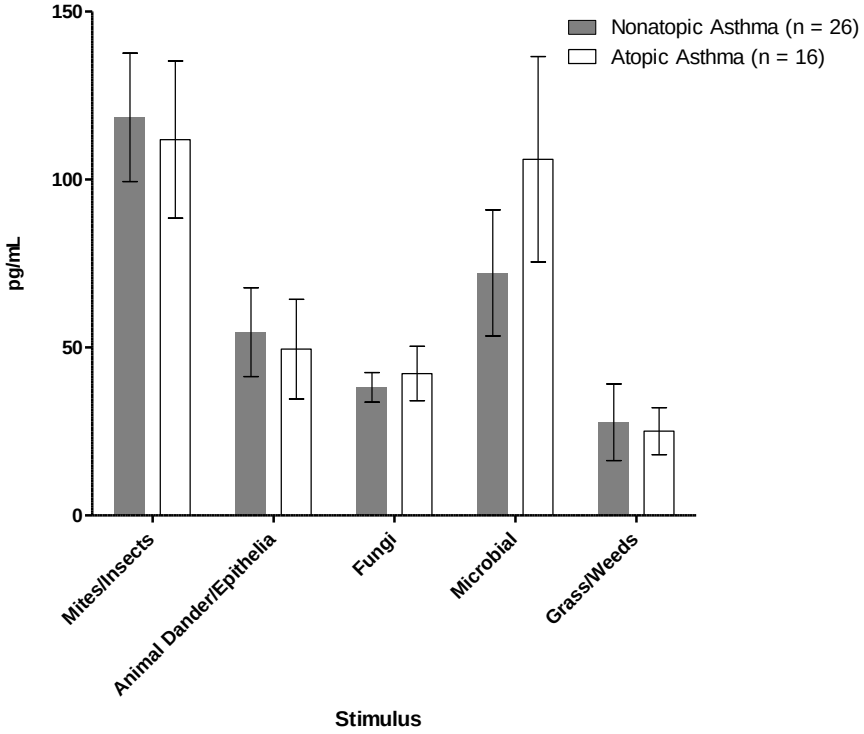


Figure 4.3 IL-23: Atopic vs Nonatopic Asthma

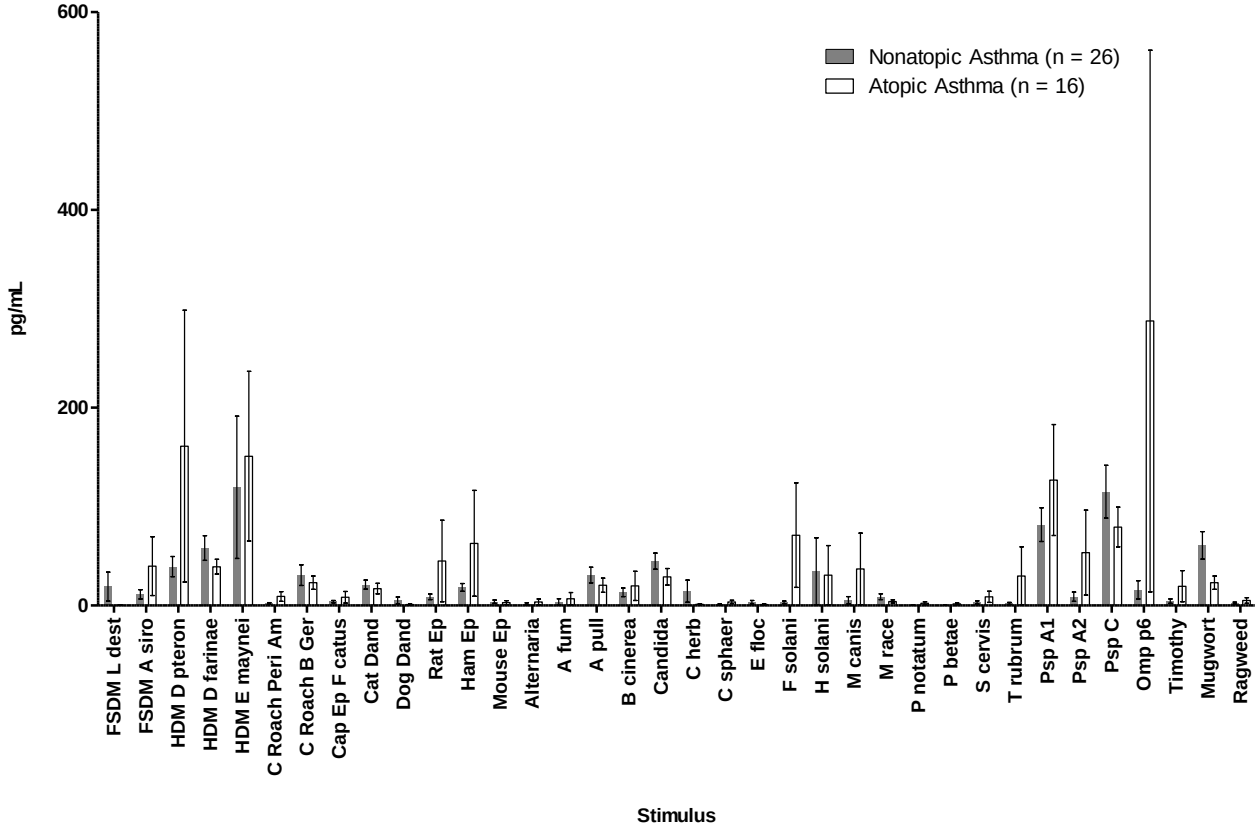


Figure 4.4 IL-23 Grouped: Atopic vs Nonatopic Asthma

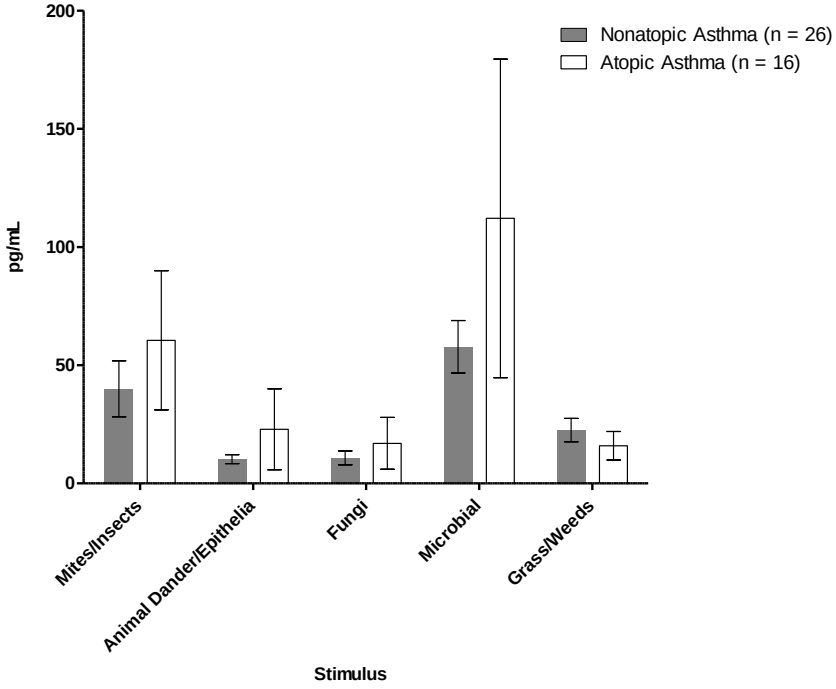


Figure 5.3 IL-10: Atopic vs Nonatopic Asthma

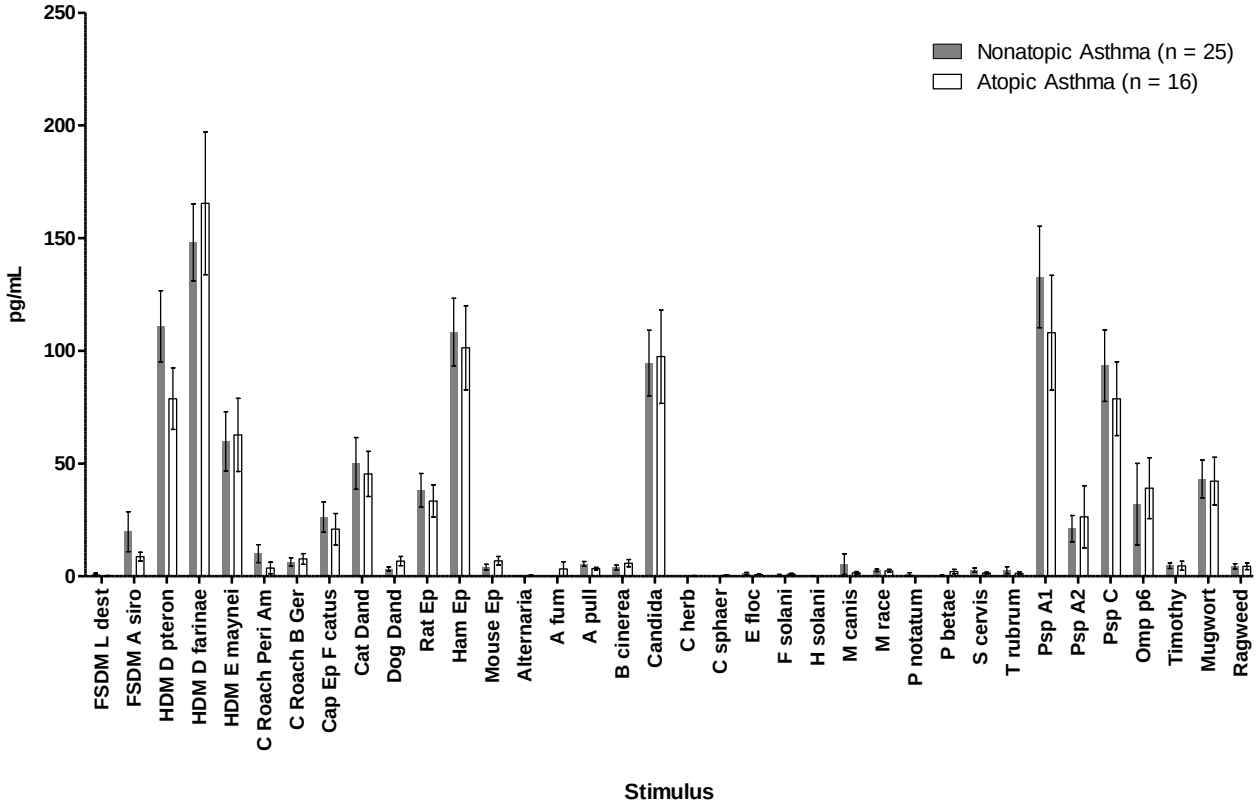


Figure 5.4 IL-10 Grouped: Atopic vs Nonatopic Asthma

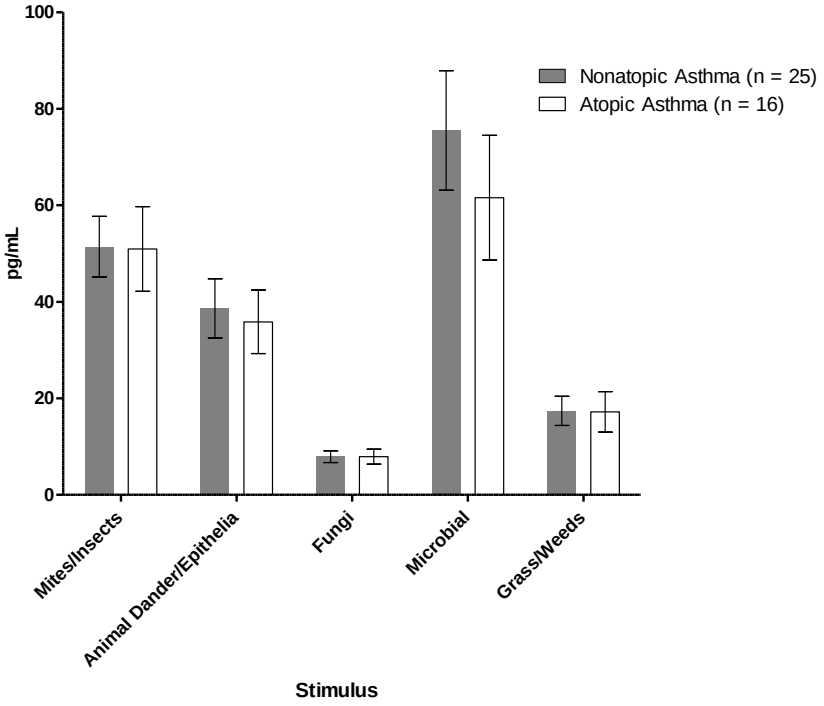


Figure 6.3 IFN-gamma: Atopic vs Nonatopic Asthma

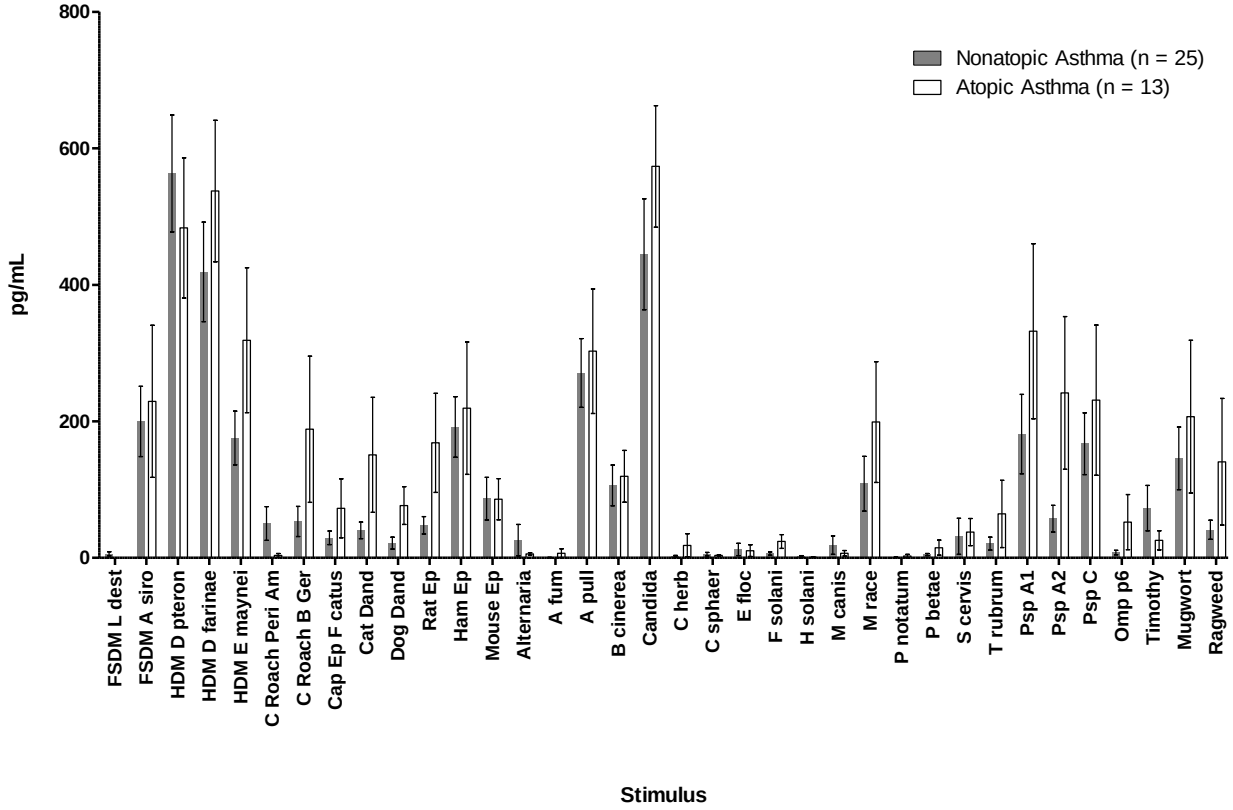
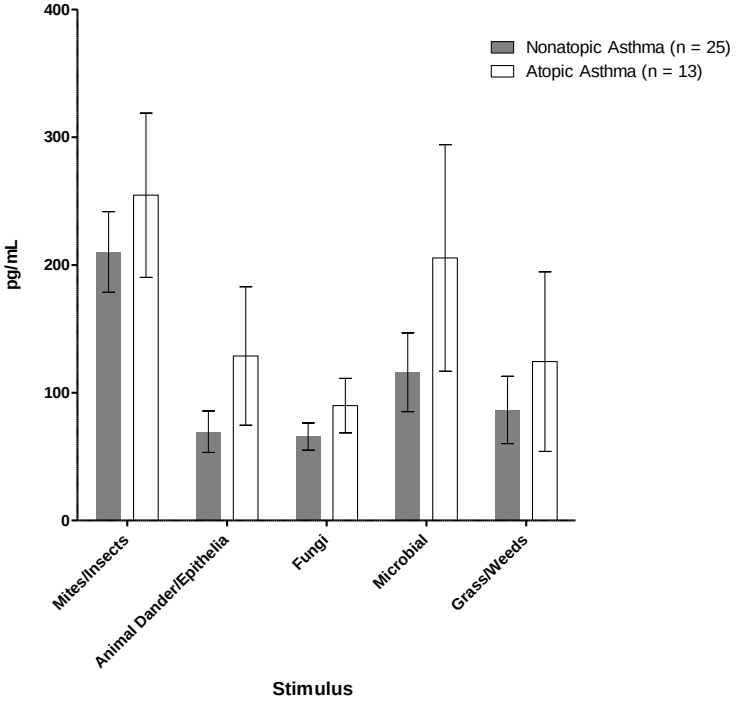


Figure 6.4 IFN-gamma Grouped: Atopic vs Nonatopic Asthma



ii. Eosinophilic bronchitis vs noneosinophilic bronchitis. There were trends for increased IL-5, IL-17A, and IFN-γ production by patients with noneosinophilic bronchitis. Interestingly, the noneosinophilic group produced significantly more IL-5 in response to the "Mites and Insects" and "Animal Dander and Epithelia" allergen classes. This group also produced significantly more IL-17A to the "Animal Dander and Epithelia" class.
See Figures 2.5 and 2.6 for IL-5 data.
See Figures 3.5 and 3.6 for IL-17A data.
See Figures 4.5 and 4.6 for IL-23 data.
See Figures 5.5 and 5.6 for IL-10 data.
See Figures 6.5 and 6.6 for IFN-γ data.

Figure 2.5 IL-5: Eosinophilic vs Noneosinophilic

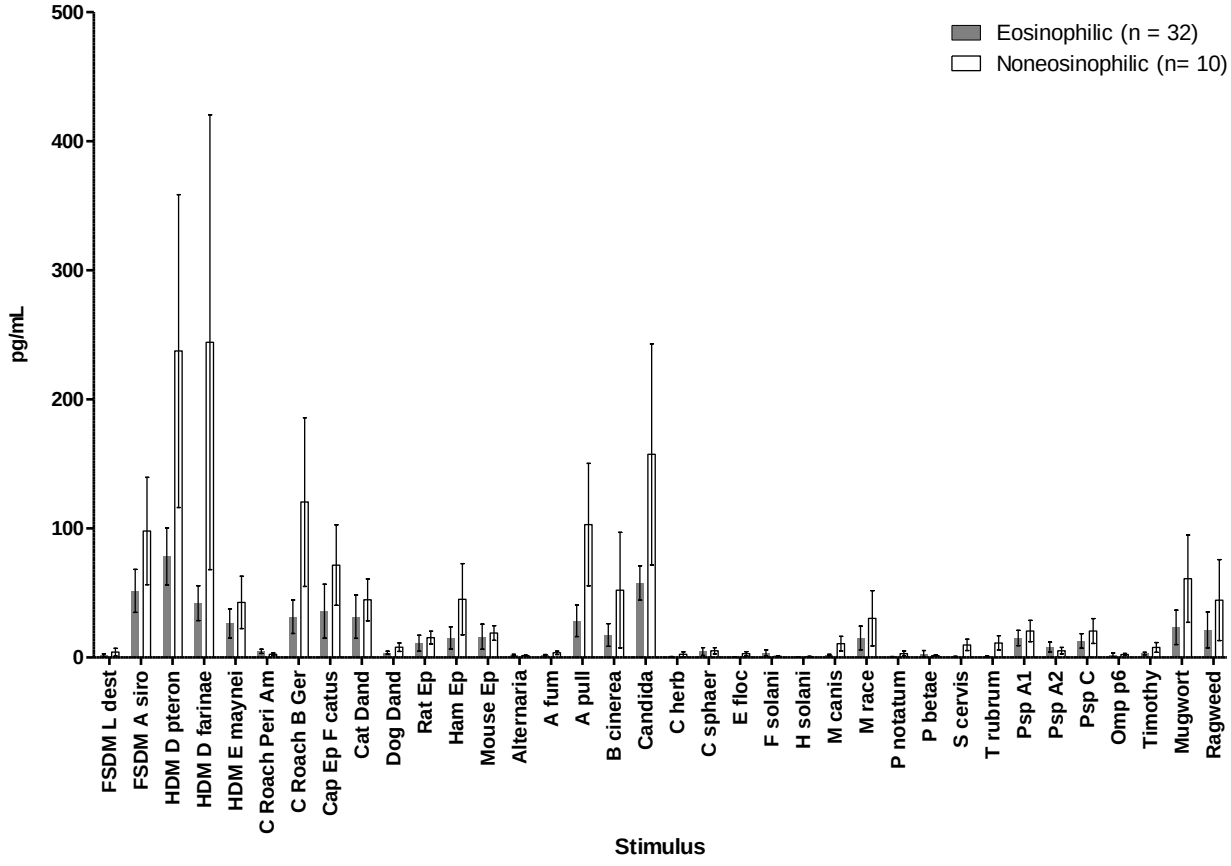


Figure 2.6 IL-5 Grouped: Eosinophilic vs Noneosinophilic

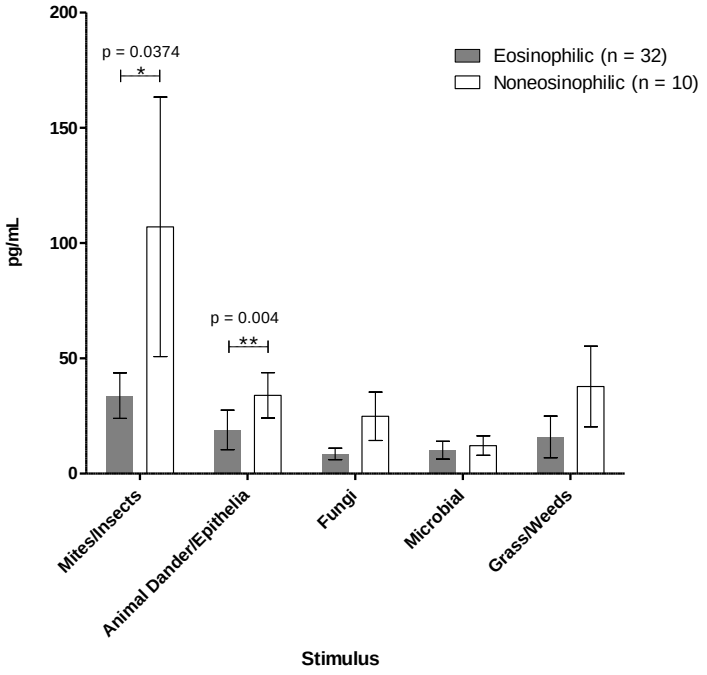


Figure 3.5 IL-17A: Eosinophilic vs Noneosinophilic

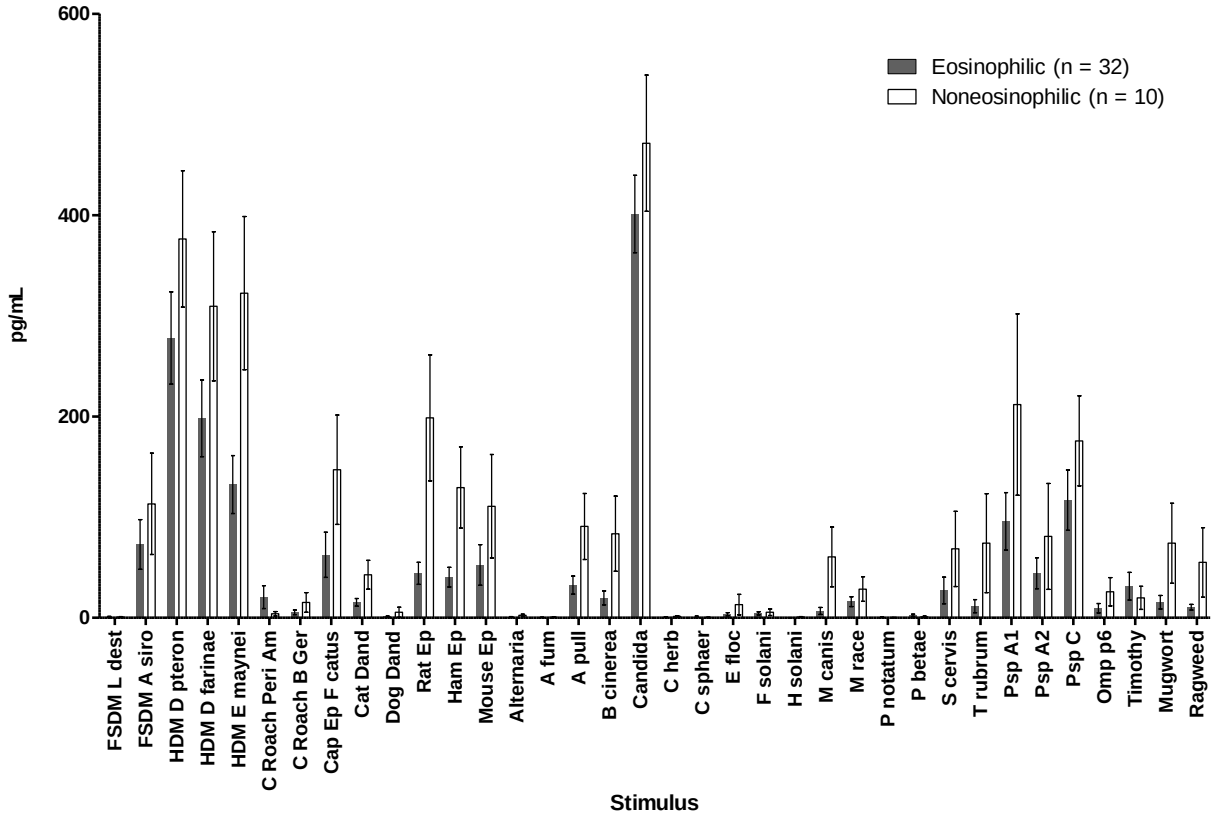


Figure 3.6 IL-17A Grouped: Eosinophilic vs Noneosinophilic

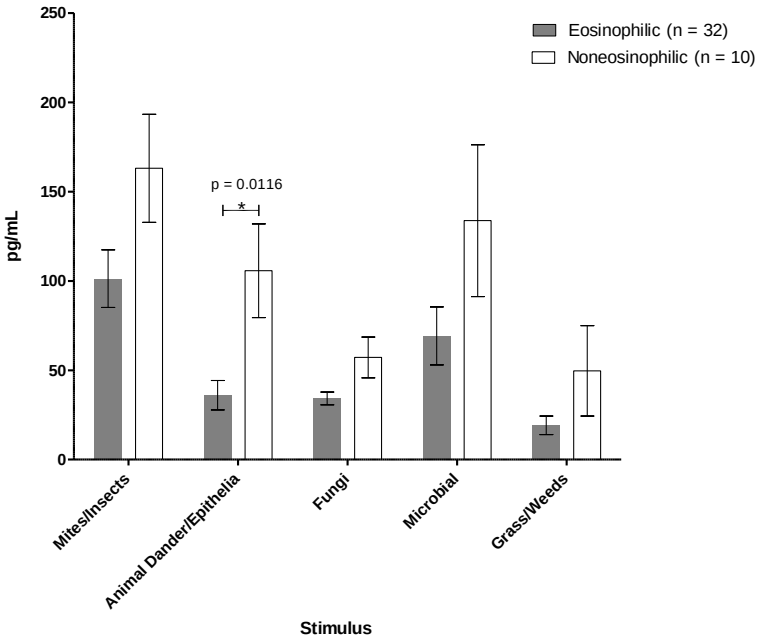


Figure 4.5 IL-23: Eosinophilic vs Noneosinophilic

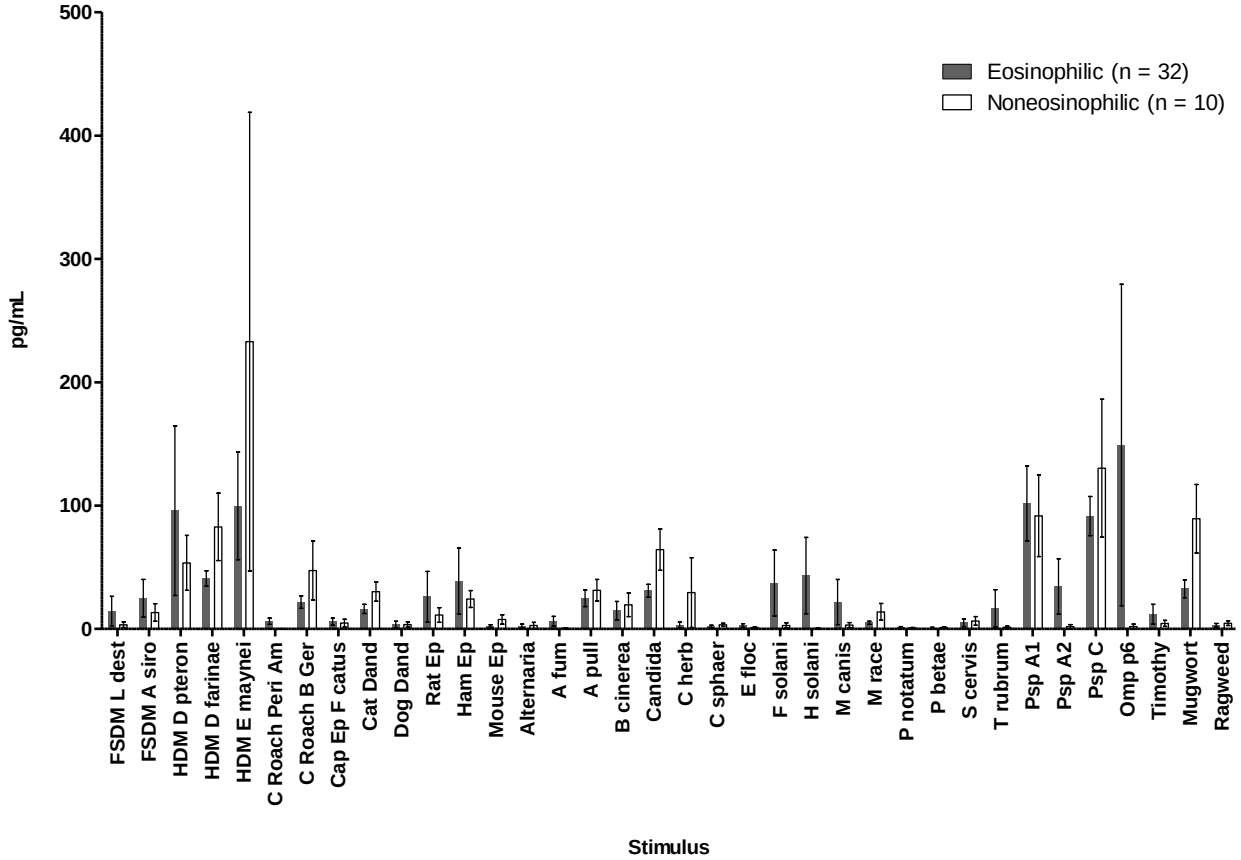


Figure 4.6 IL-23 Grouped: Eosinophilic vs Noneosinophilic

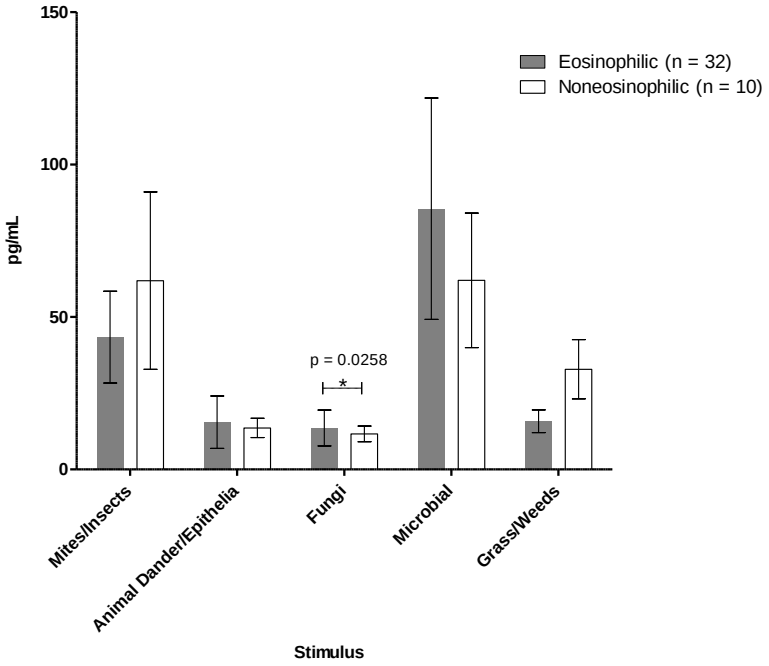


Figure 5.5 IL-10: Eosinophilic vs Noneosinophilic

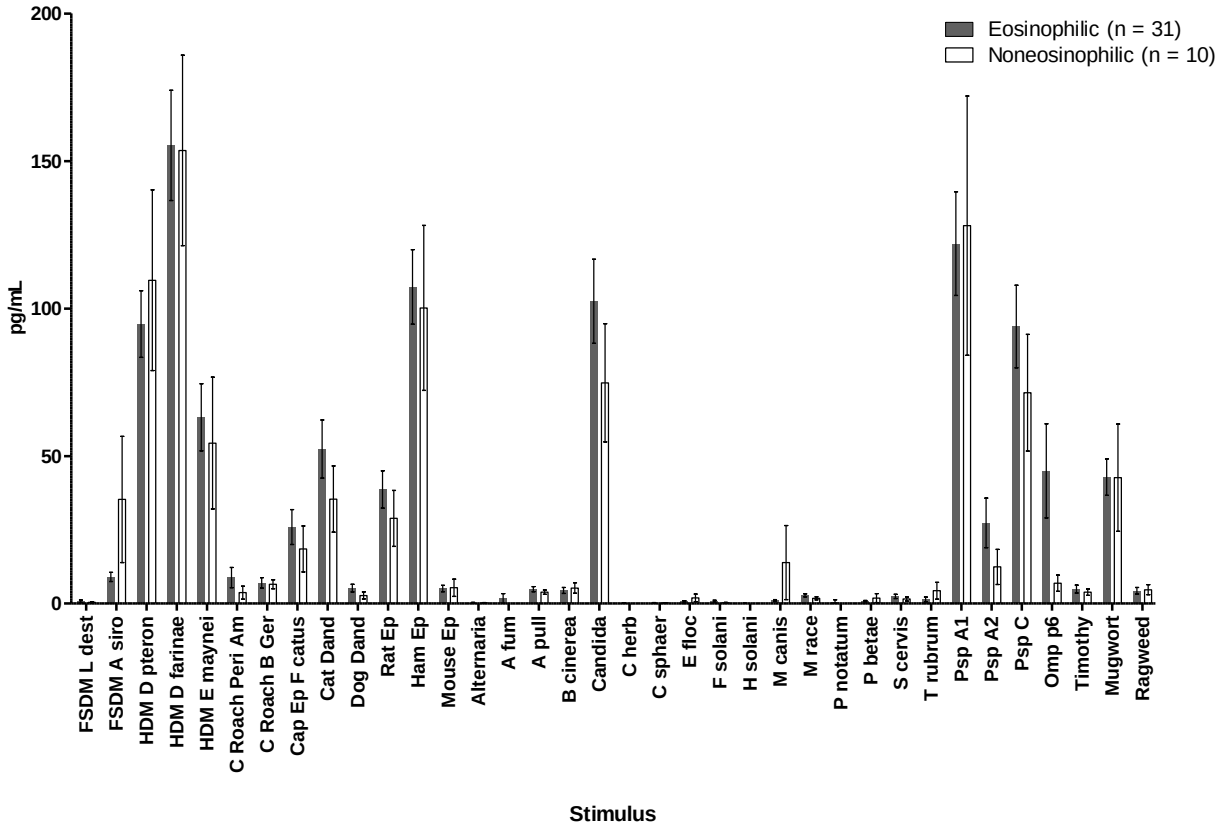


Figure 5.6 IL-10 Grouped: Eosinophilic vs Noneosinophilic

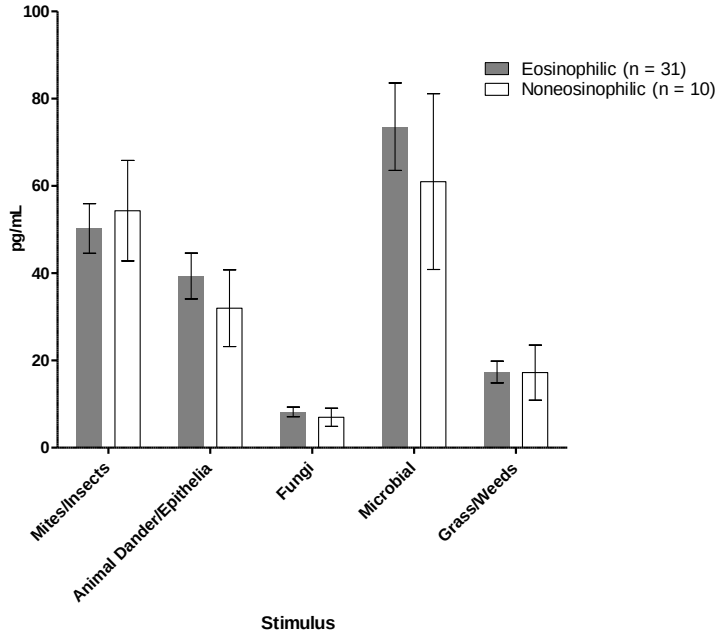


Figure 6.5 IFN-gamma: Eosinophilic vs Noneosinophilic

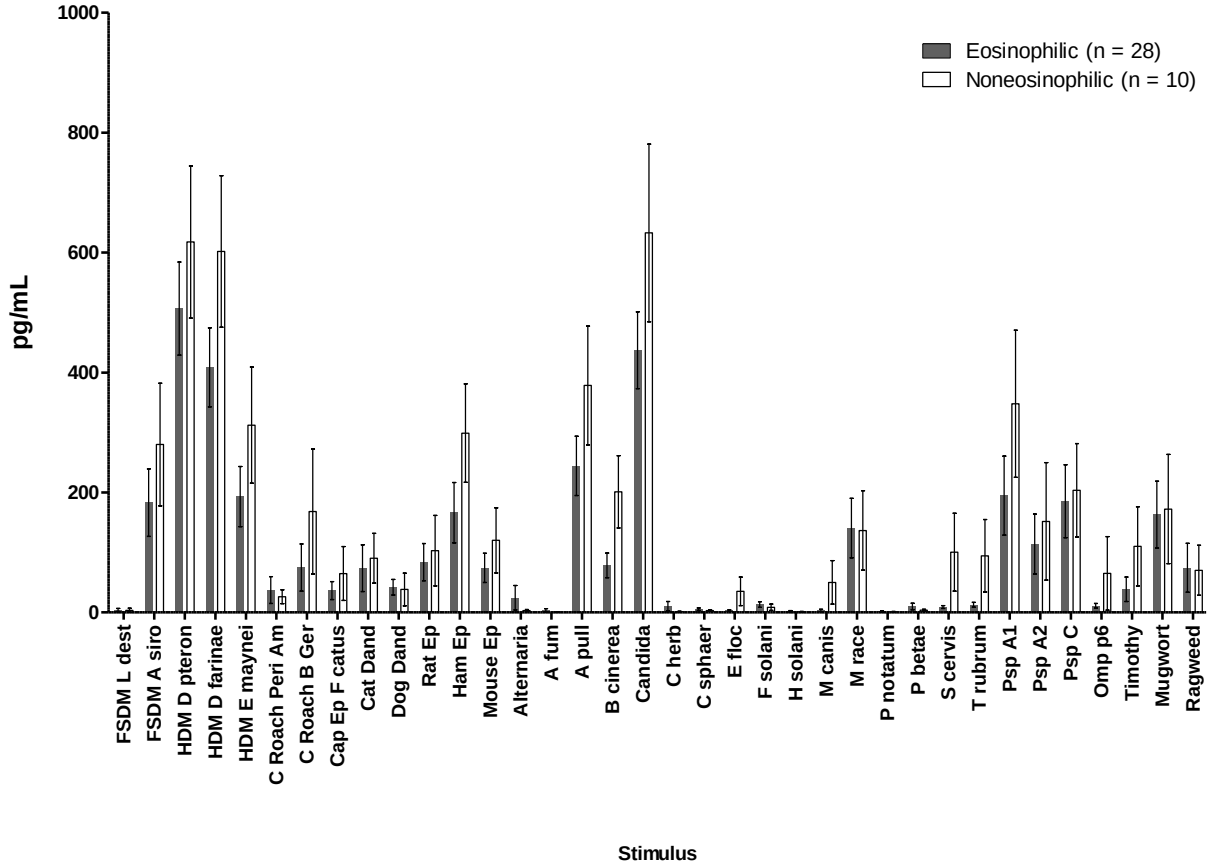
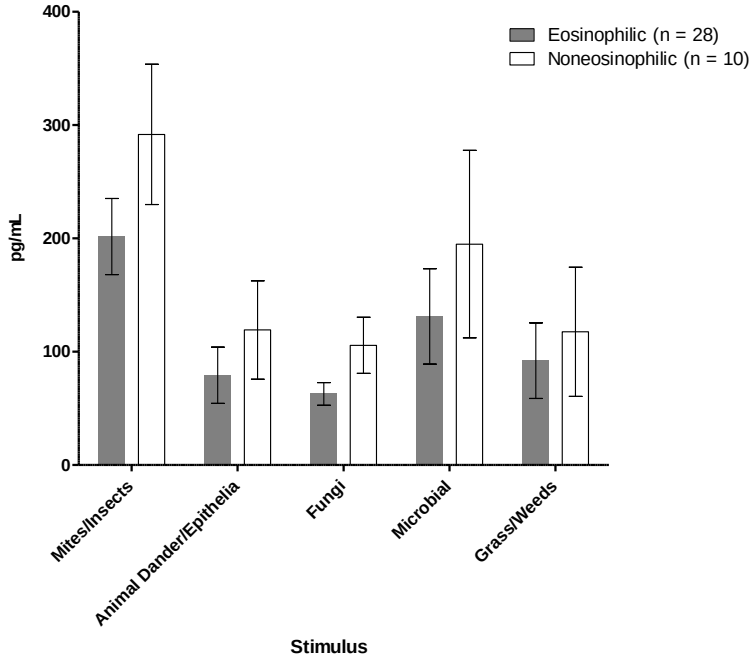


Figure 6.6 IFN-gamma Grouped: Eosinophilic vs Noneosinophilic



iii. Neutrophilic vs nonneutrophilic bronchitis. No significant differences were seen between these two groups.
See Figures 2.7 and 2.8 for IL-5 data.
See Figures 3.7 and 3.8 for IL-17A data.
See Figures 4.7 and 4.8 for IL-23 data.
See Figures 5.7 and 5.8 for IL-10 data.
See Figures 6.7 and 6.8 for IFN-γ data.

Figure 2.7 IL-5: Neutrophilic vs Nonneutrophilic

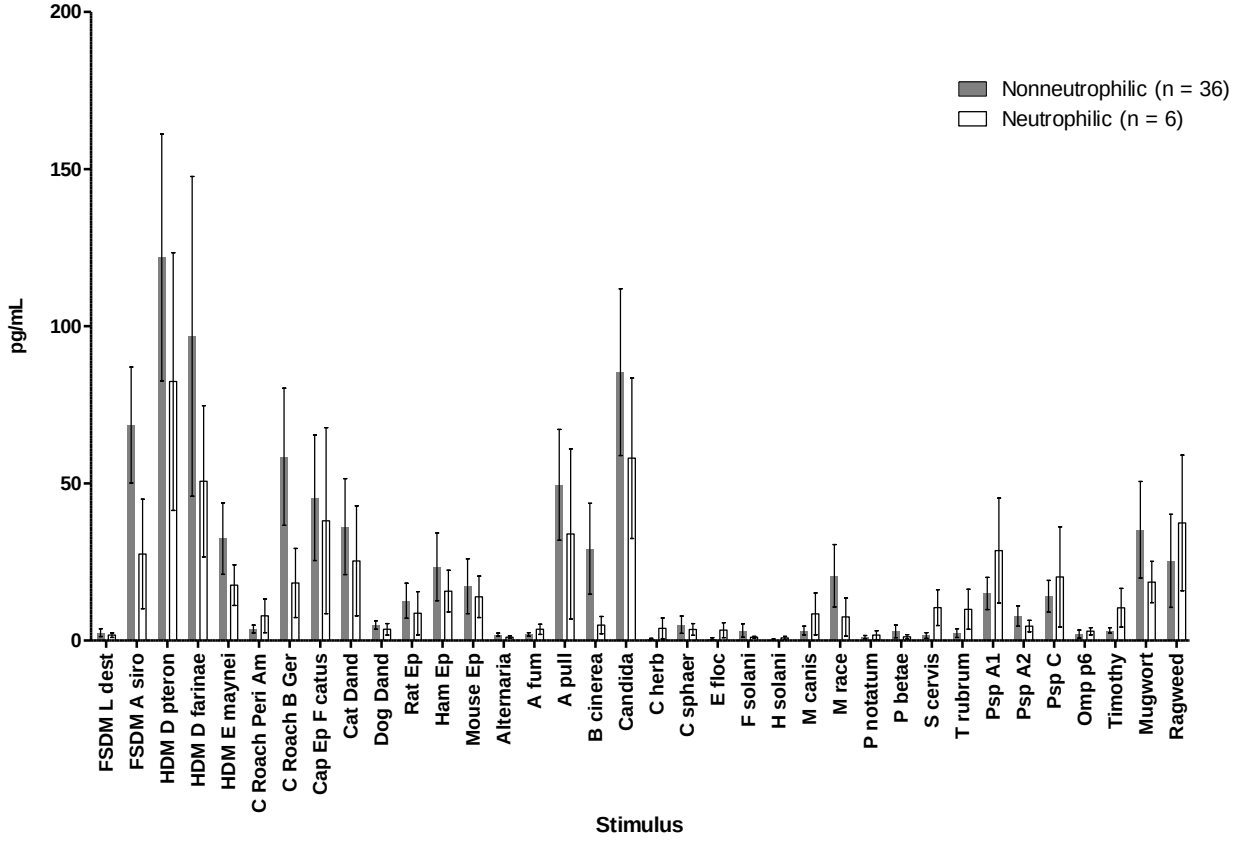


Figure 2.8 IL-5 Grouped: Neutrophilic vs Nonneutrophilic

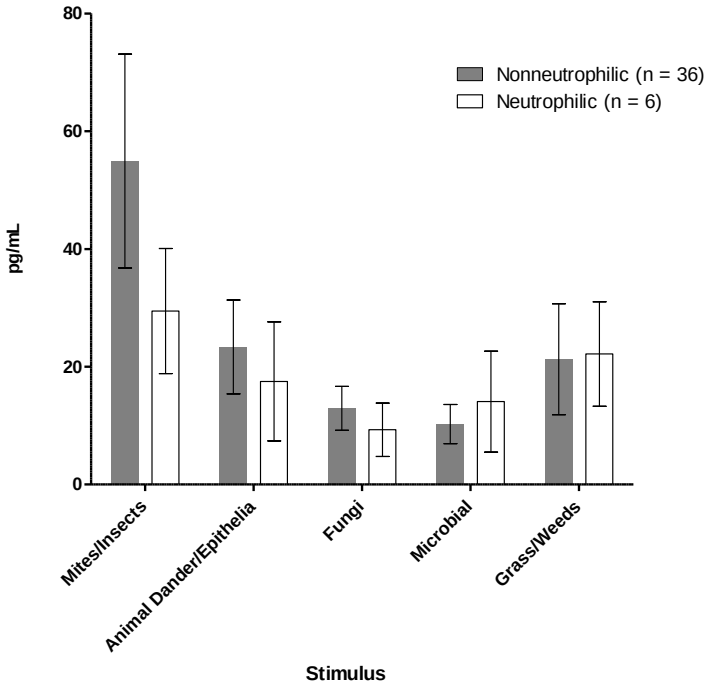


Figure 3.7 IL-17A: Neutrophilic vs Nonneutrophilic

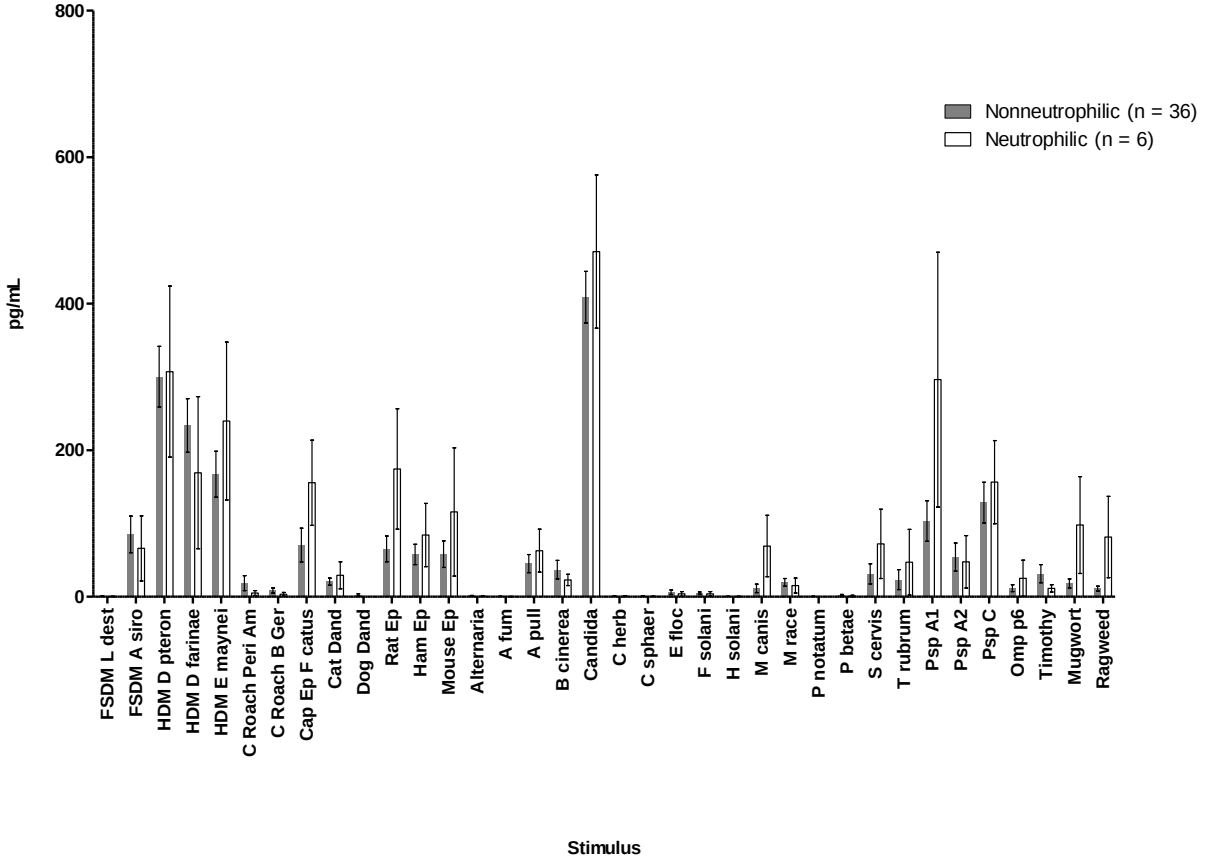


Figure 3.8 IL-17A Grouped: Neutrophilic vs Nonneutrophilic

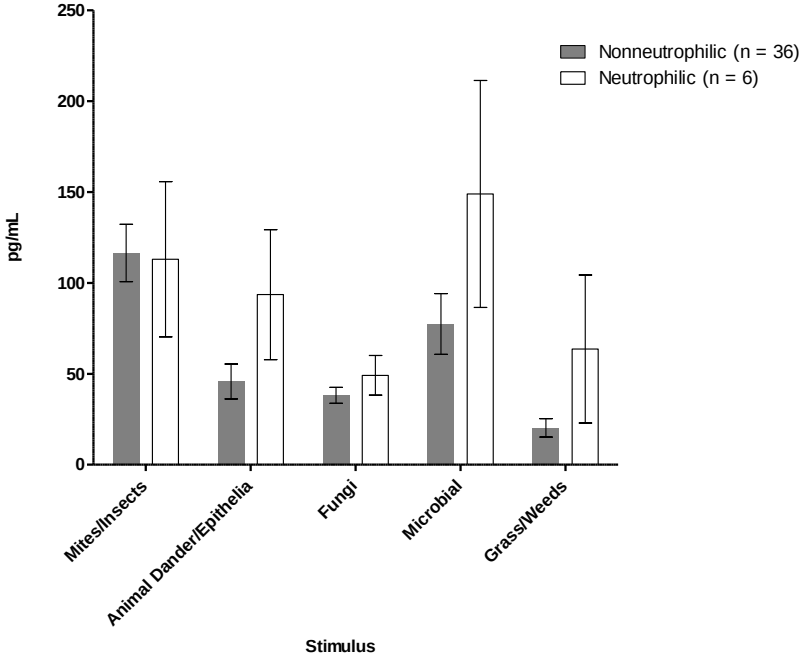


Figure 4.7 IL-23: Neutrophilic vs Nonneutrophilic

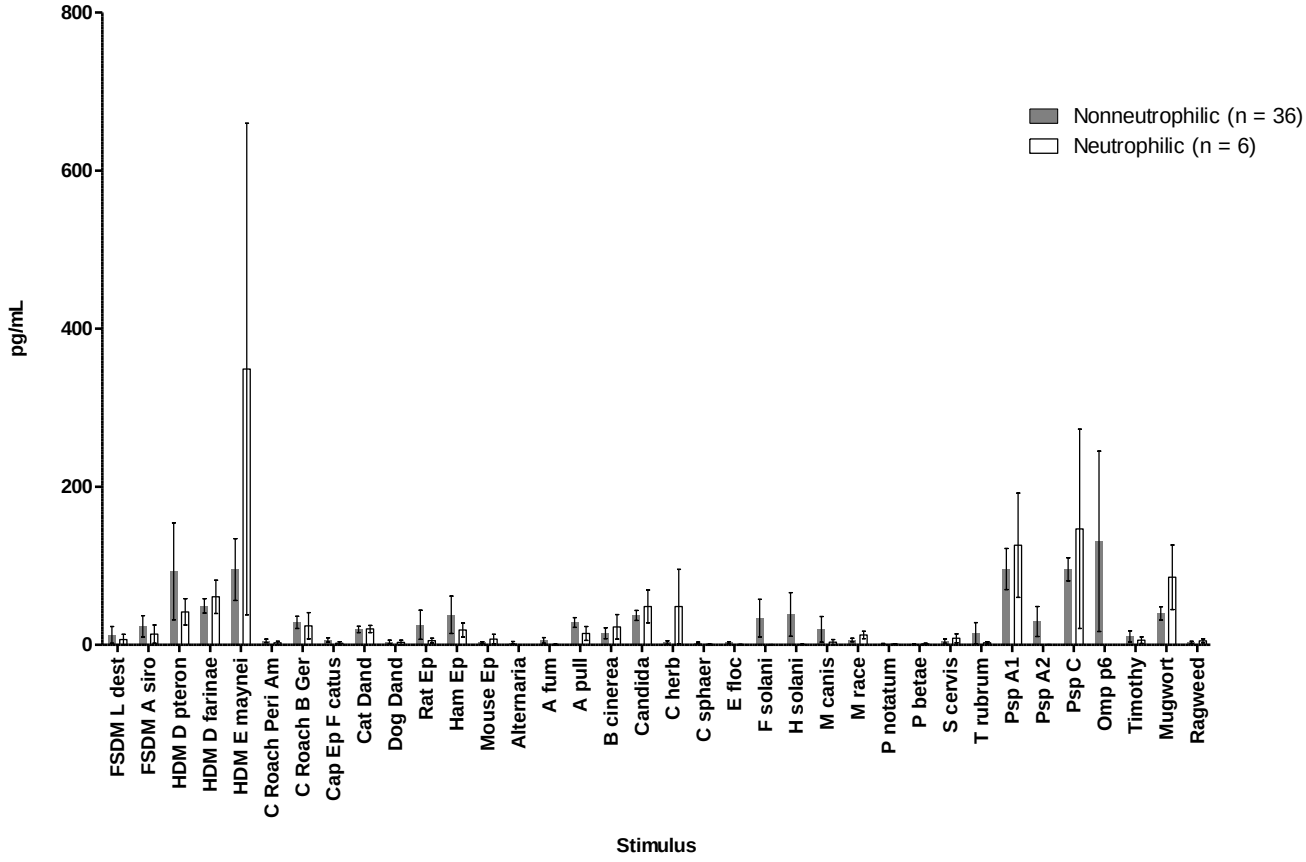


Figure 4.8 IL-23 Grouped: Neutrophilic vs Nonneutrophilic

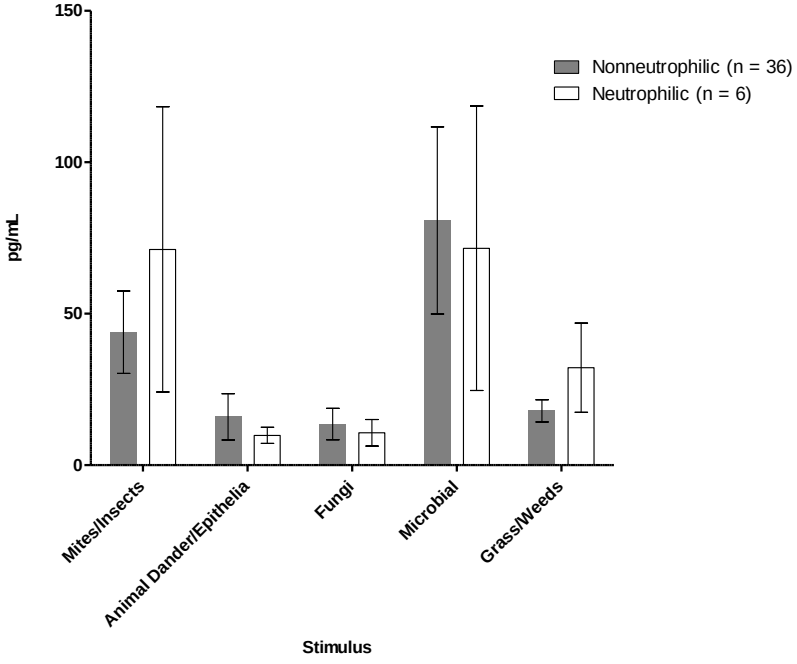


Figure 5.7 IL-10: Neutrophilic vs Nonneutrophilic

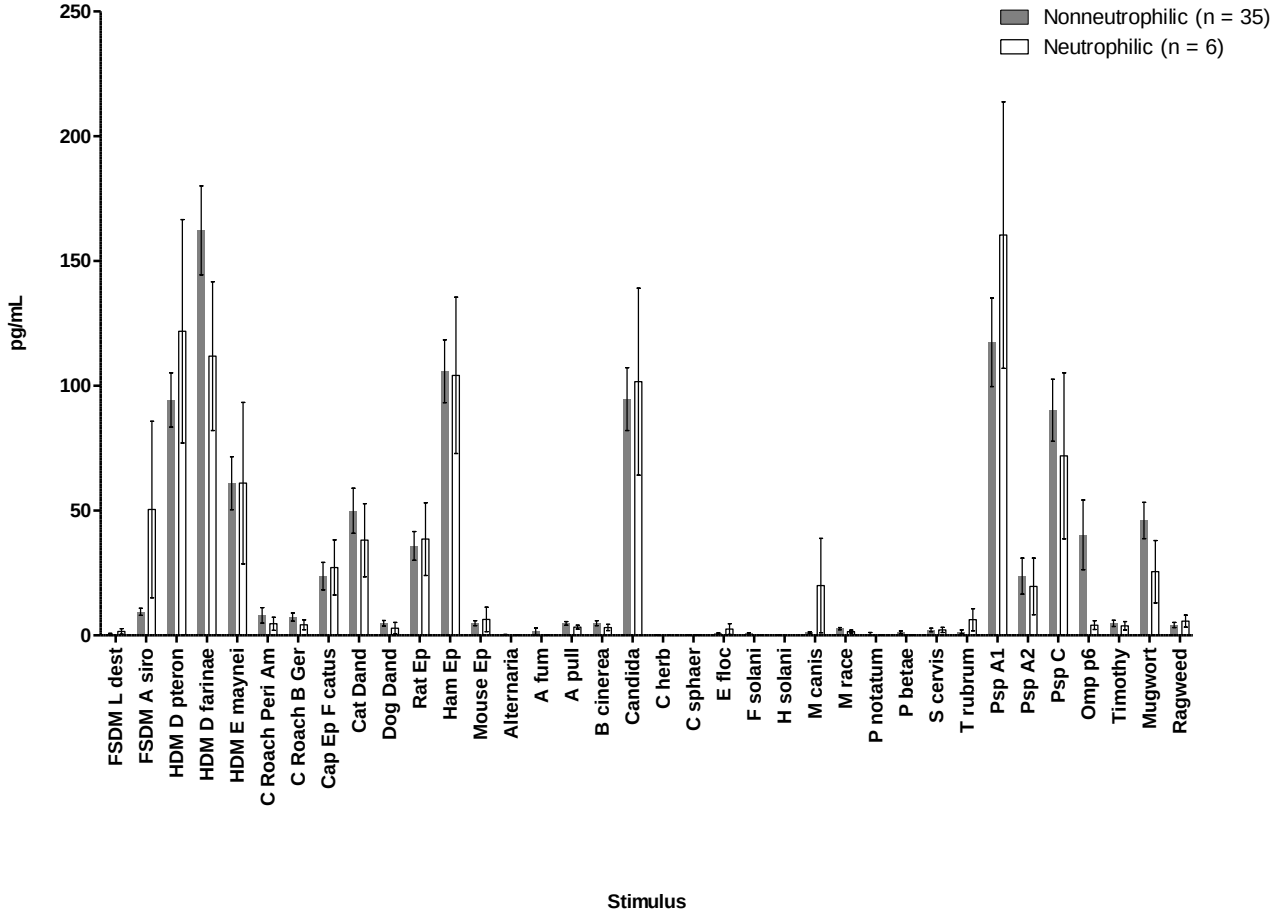


Figure 5.8 IL-10 Grouped: Neutrophilic vs Nonneutrophilic

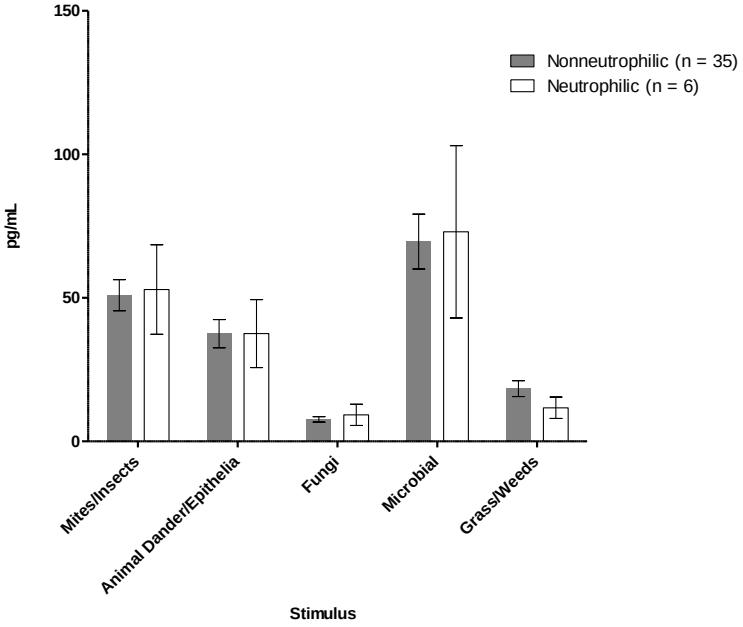


Figure 6.7 IFN-gamma: Neutrophilic vs Nonneutrophilic

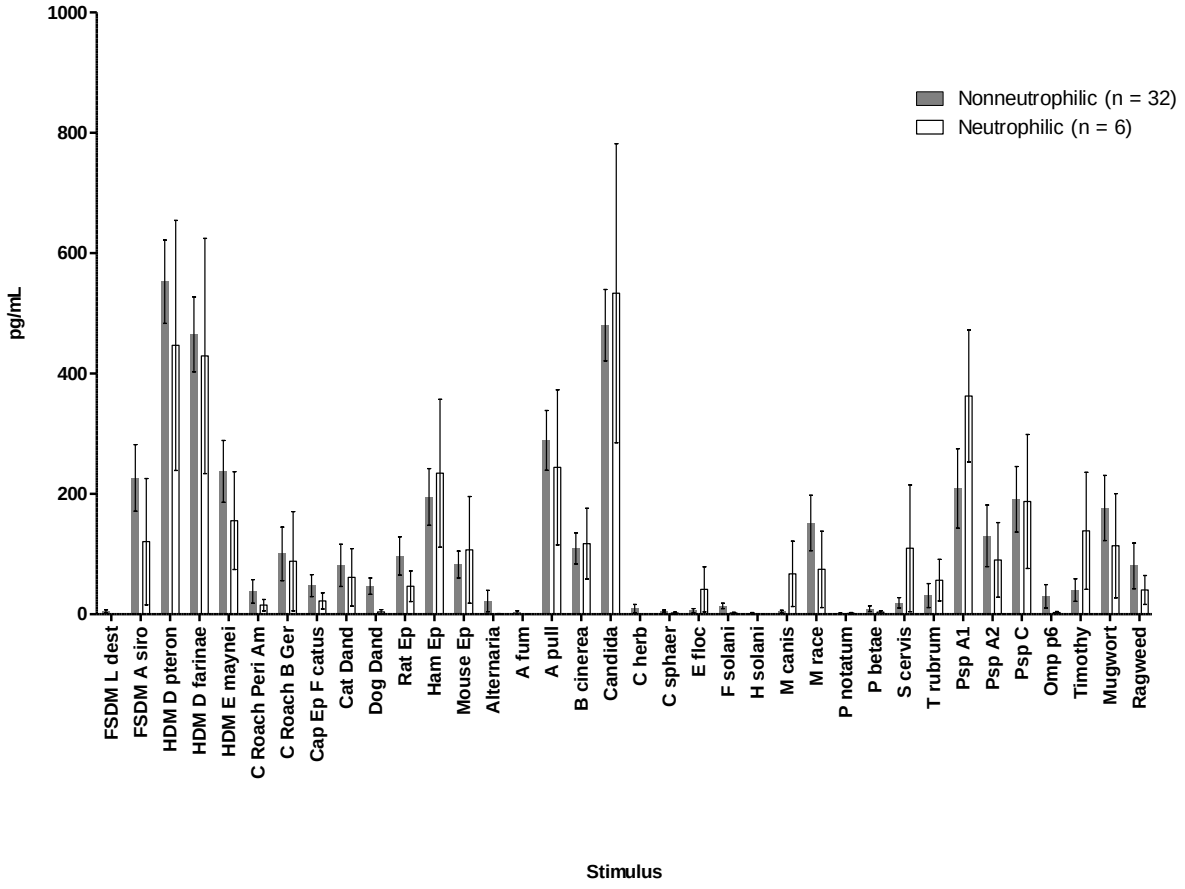
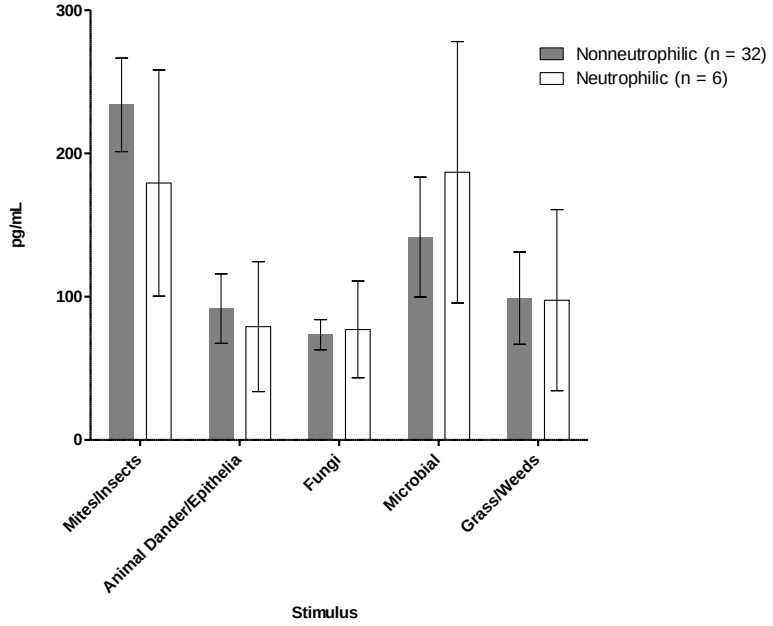


Figure 6.8 IFN-gamma Grouped: Neutrophilic vs Nonneutrophilic



OBJECTIVE 5: *Effects of Steroids on PBMC Responses to Allergen*

Average IL-5 production declined significantly with increasing steroid dosage. This effect was not seen with PBMC proliferation or production of other cytokines.

See Figures 9.2 and 9.7 for IL-5 data.

See Figure 9.1 for PBMC proliferation data.

See Figures 9.3-9.6 for IL-17A, IL-23, IL-10 and IFN-γ data.

Figure 9.2 IL-5 Grouped: Steroids

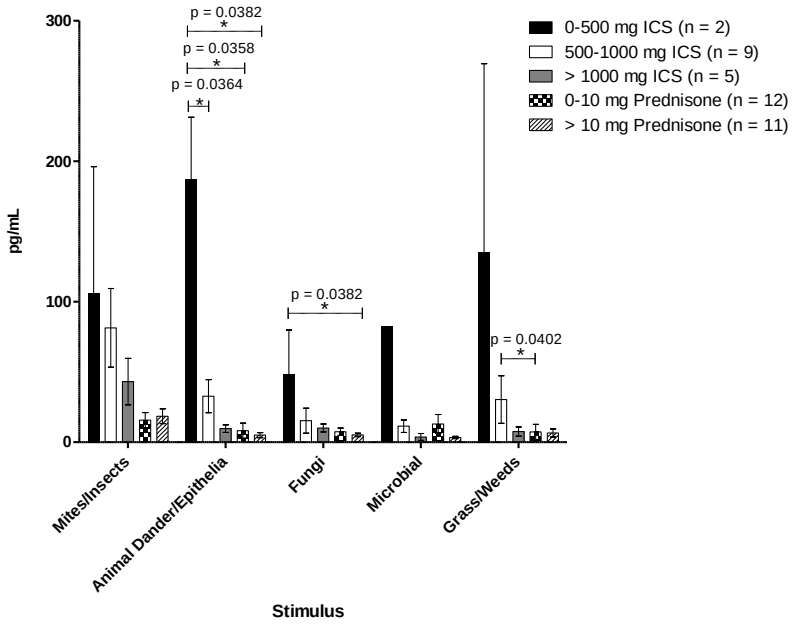
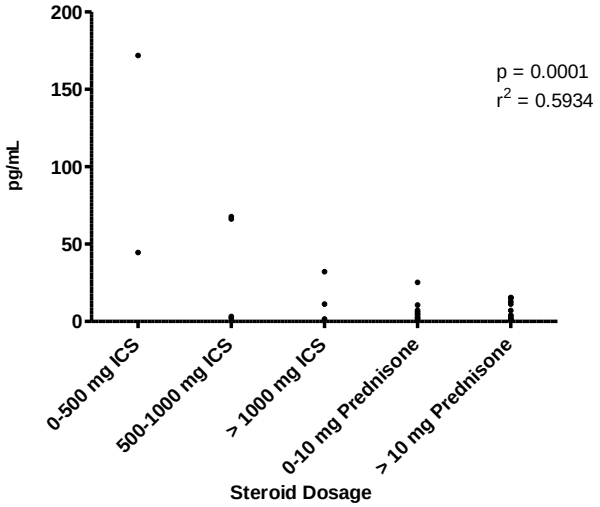


Figure 9.7 IL-5: Steroid Dosage



Average allergen-induced IL-5 production by PBMCs from eosinophilic asthmatic patients compared across 5 steroid treatment groups and analyzed by one-way ANOVA.

DISCUSSION

PBMC PROLIFERATION

PBMC proliferation in response to allergen did not differ between any of the patient groups in this study: asthma vs normal, atopic vs nonatopic asthma, or eosinophilic bronchitis vs noneosinophilic bronchitis. This suggests that allergen-induced PBMC proliferation, and possibly T cell activation, in peripheral blood is not a good marker of clinical disease in asthma. It cannot distinguish between the presence or absence of disease, or between different phenotypes of asthma.

CYTOKINES

The results from this project indicate that peripherally produced cytokines, or at least the cytokines selected for this study, do *not* act as effective, specific biomarkers that can distinguish 1) asthma vs normal controls 2) atopic asthma vs nonatopic asthma 3) eosinophilic bronchitis vs noneosinophilic bronchitis or neutrophilic bronchitis vs nonneutrophilic bronchitis. All patient groupings in this study exhibited fairly similar patterns of cytokine production, regardless of phenotype. *See Figures 7.1-7.8*. This observation was not altered when we focused in on allergens deemed to be especially clinically relevant – those featured in the common skin-prick test. *See Figures 8.1-8.8.*

In some cases, wide patient variability, indicated by large SEM values, contributed to a lack of statistical significance of the cytokine data. Though this variability in cytokine production makes it difficult to draw solid conclusions based on statistics, it does support the idea of asthma as a heterogeneous disease in which individual patients' asthmatic responses to stimuli are highly varied. Such variability in cytokine responses has been documented in other studies. Mentioned previously in this report, Mori's group found IL-5 production in response to *Candia*

albicans from nonatopic asthmatic patients ranged from less than 50 pg/mL to greater than 250 pg/mL[57].

Asthma vs Normal

We predicted that asthmatic patients would display higher levels of Th2 and Th17 associated cytokines in response to allergen, and that normal controls would display higher levels of Th1 or T regulatory associated cytokines. This prediction was not confirmed by the data.

If we look at the consolidated patient data as a whole, no statistically significant differences in cytokine production between the two groups exist and no clear trends emerge.

If we examine each patient individually, the response to allergen by normal controls is dominated by IL-10 and IFN-γ production. For example, if we consider normal control 14 (*see Figures 12.13 and 12.14*): this individual produces IL-5, IL-17A, and IL-23 in response to allergen, but the dominant cytokines expressed in response to each class of allergens are IL-10 and IFN-γ. This finding makes it tempting to conclude that the expression of Th2 and Th17 cytokines may not lead to asthma or other allergic disease in a person if they are "balanced" by the expression of Th1 and T regulatory cytokines. However, if we examine individual asthmatic patients, the same trend in cytokine expression can be observed. For example, the cytokine response to allergen is dominated by IL-10 and IFN-γ in nonatopic asthmatic patient 7 (*see Figures 10.13 and 10.14*) and atopic asthmatic patient 17 (*see Figures 11.31 and 11.32*), among others. Not all asthmatic patients follow this trend, as is seen in nonatopic asthmatic patient 13 (*see Figures 10.25 and 10.26*), who displays predominantly high IL-17A levels. Overall, the main message illustrated by this comparison is that we cannot look at a patient's cytokine data and determine whether that patient has asthma or is a normal control. The amount of cytokine and the balance of cytokine types produced in response to allergen is not an effective way to distinguish disease from non-disease in asthma.

Figure 12.13 Normal Control 14

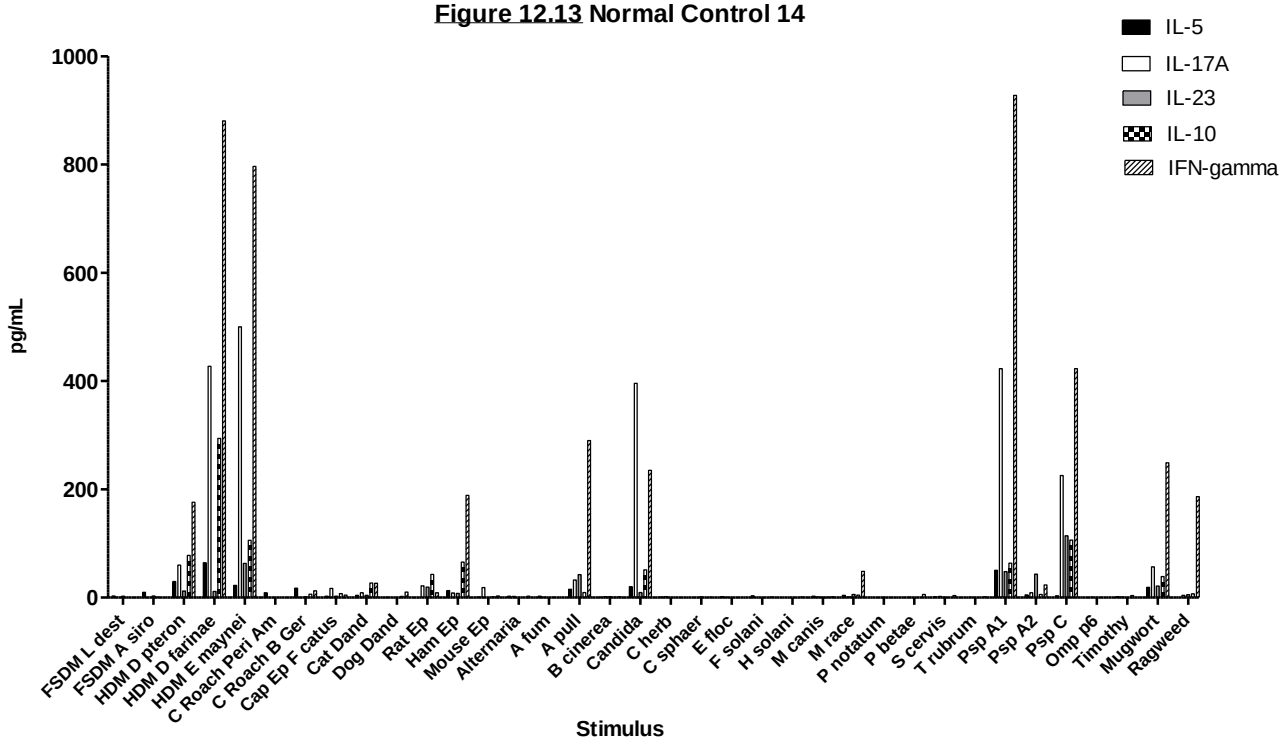


Figure 12.14 Normal Control 14

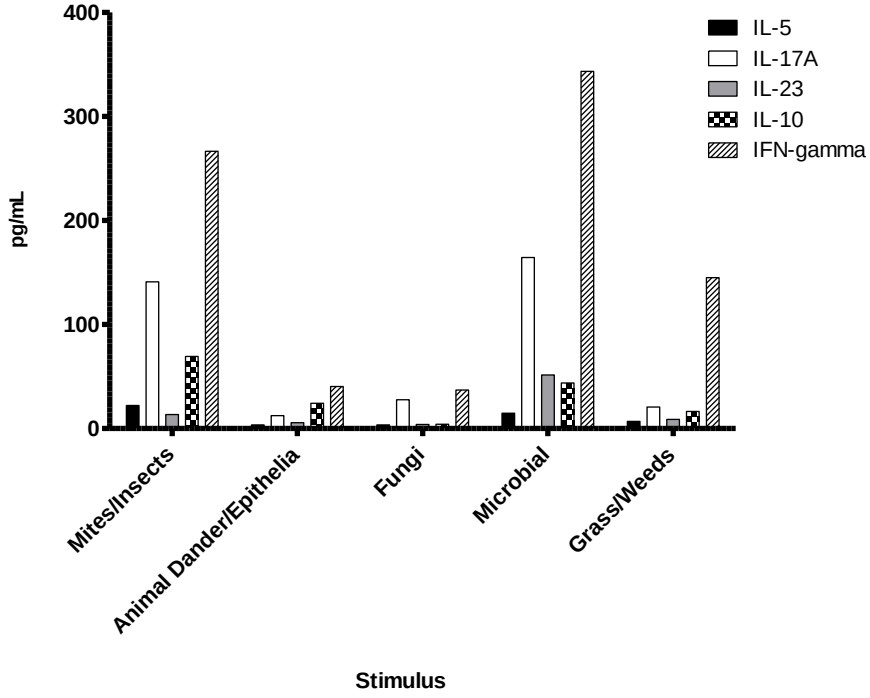


Figure 10.13 Nonatopic Asthmatic Patient 7 - Eosinophilic

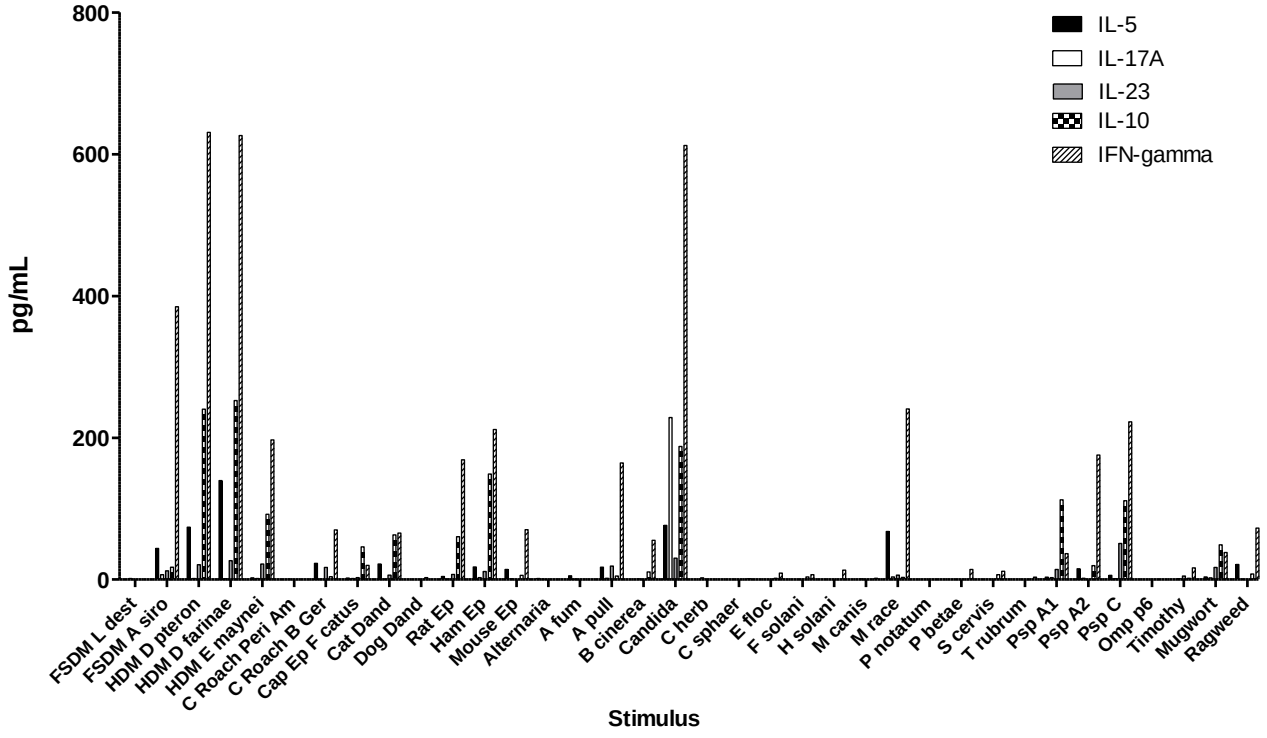


Figure 10.14 Nonatopic Asthmatic Patient 7 - Eosinophilic

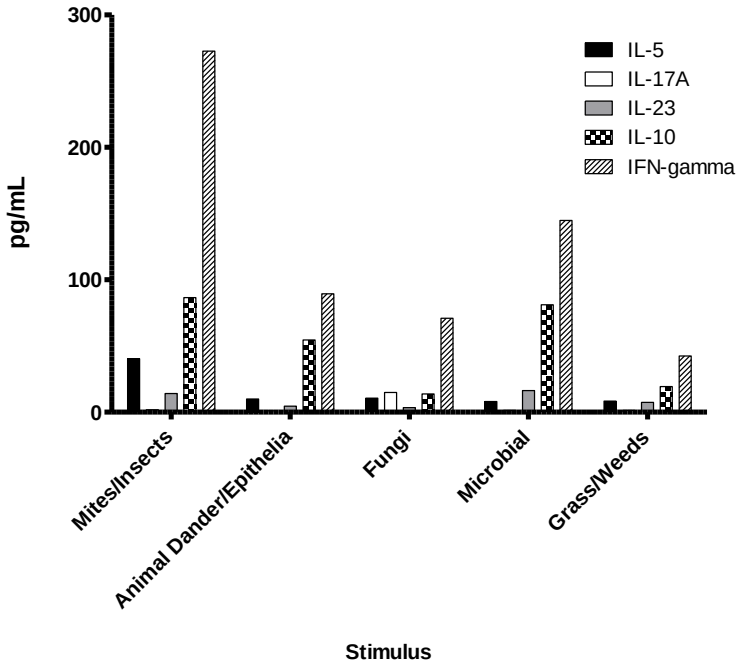


Figure 11.31 Atopic Asthmatic Patient 17 - Eosinophilic
Allergies: cat, horse, feathers, dust mite

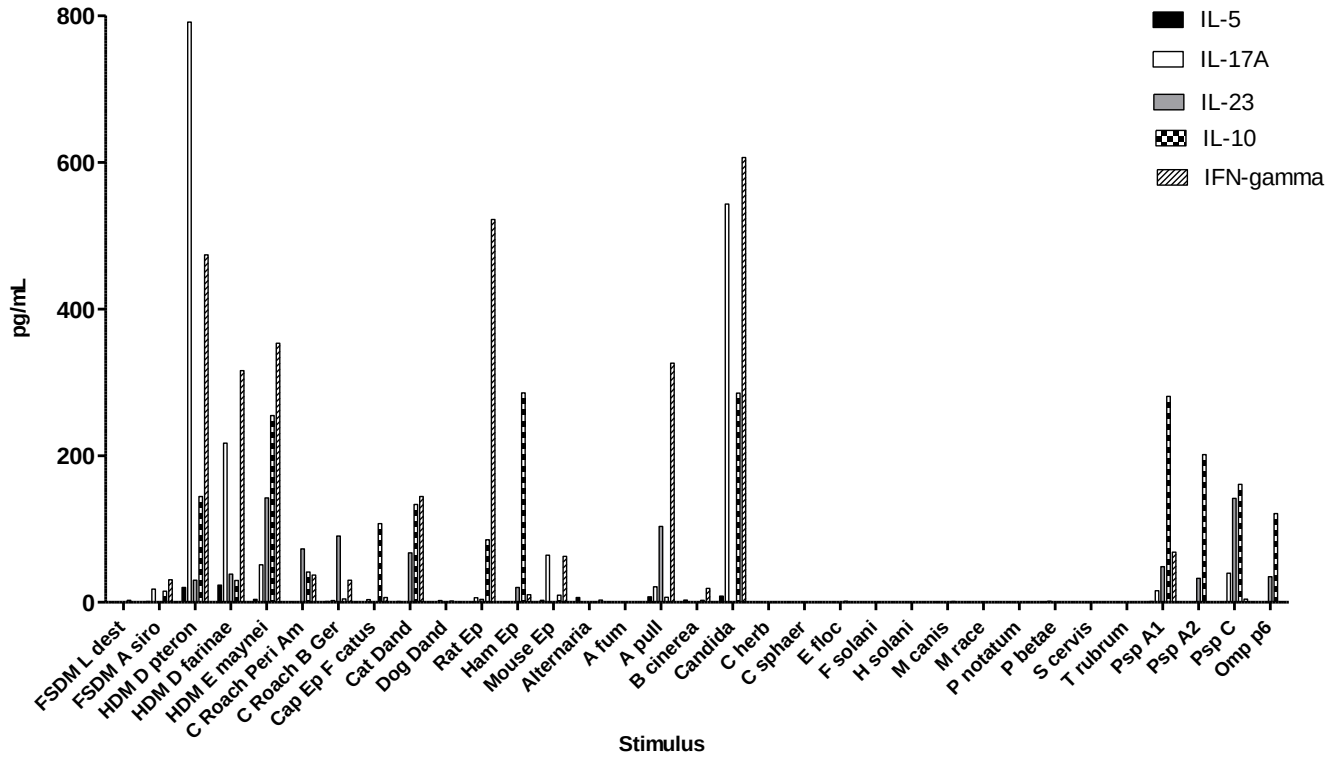


Figure 11.32 Atopic Asthmatic Patient 17 - Eosinophilic
Allergies: cat, horse, feathers, dust mite

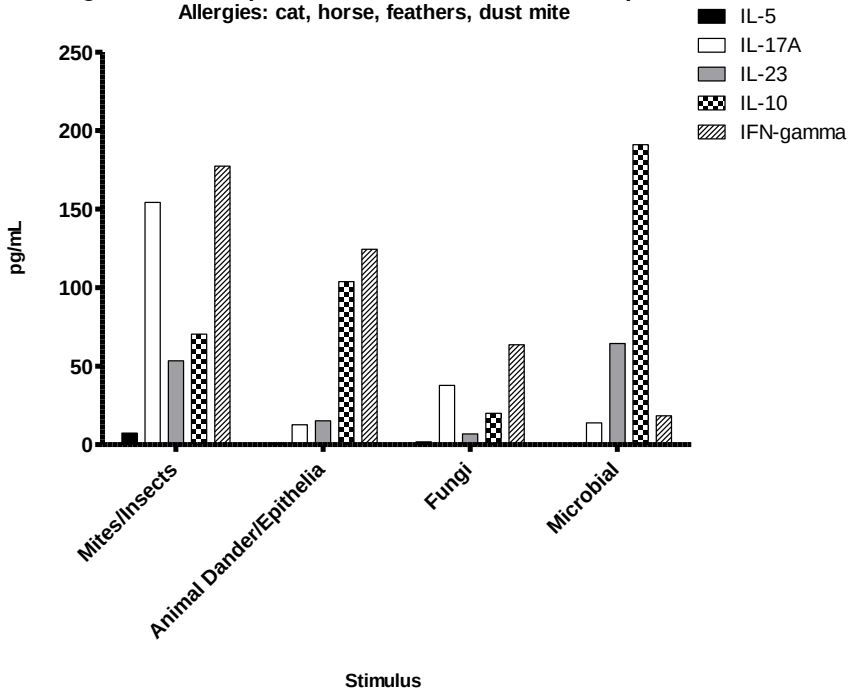


Figure 10.25 Nonatopic Asthmatic Patient 13 - Neutrophilic

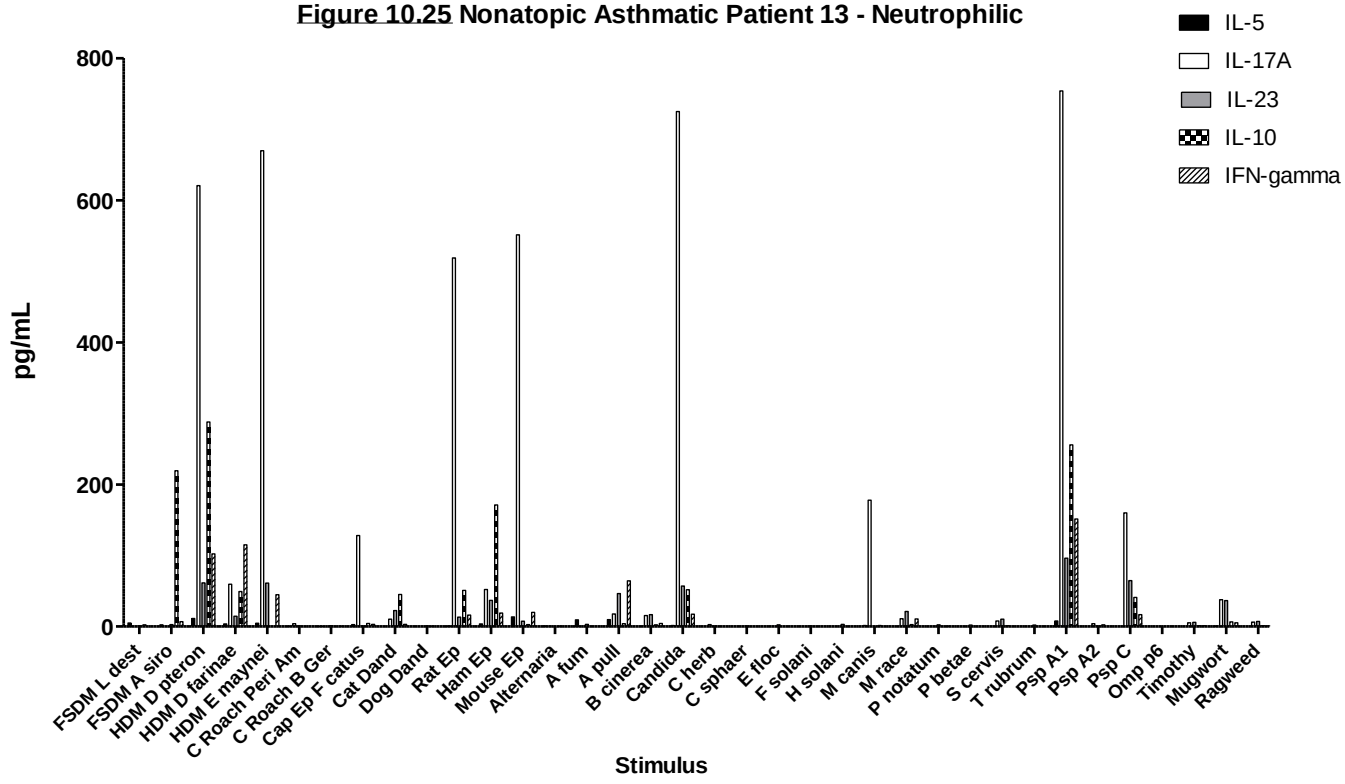
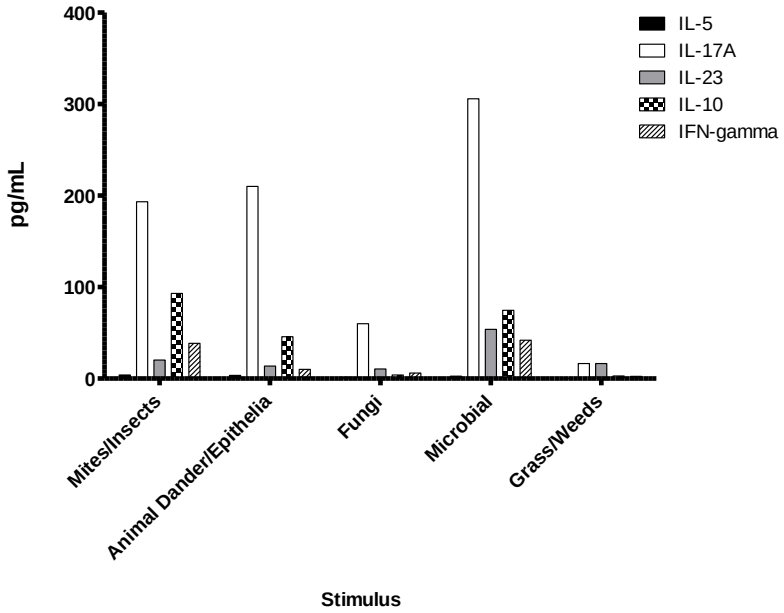


Figure 10.26 Nonatopic Asthmatic Patient 13 - Neutrophilic



We expected cytokine levels to be higher in atopic asthmatic patients than nonatopic asthmatic patients. Atopic asthmatic patients have an IgE-mediated mechanism of hypersensitivity at work in addition to a T cell-mediated mechanism, while our prediction was that nonatopic asthmatics only displayed the T cell-mediated mechanism. Therefore, we predicted that the combined effect of both pathways would lead to greater levels of cytokine production. No significant differences were found between the groups for any of the five cytokines, despite a trend in higher amounts of IL-5 in atopic asthmatics.

We anticipated that atopic asthmatic patients would produce pro-inflammatory cytokines in response to asthma triggers they have known sensitivities to. We also expected a response to non-trigger allergens characterized by the absence of Th2 and/or Th17 inflammation or balanced by a Th1 and/or T regulatory response. Instead, atopic asthmatic patients produced pro-inflammatory cytokines to a number of different allergens on our allergen panel, and this cytokine profile did not necessarily match their known sensitivities.

The results from atopic asthmatic patients indicate that pro-inflammatory cytokine production, at least by PBMCs, does not necessarily follow exposure to allergens a person is sensitized to. Individuals may exhibit inflammatory cytokine responses to allergens that do not cause them to have any clinical symptoms of asthma or allergic disease. They may also lack inflammatory cytokine responses to allergens they have known sensitivities to. In addition, IL-5, IL-17A and IL-23 responses are not consistent across our allergen panel for each patient.

Cytokine profile clearly does not reflect sensitivity profile. From this, we can conclude that the skin-prick test is not necessarily indicative of a patient's cellular response, as measured by inflammatory cytokine production by PBMCs, to aeroallergen. This leads to the question: what is important in driving asthmatic response - IgE or inflammatory cytokines? Or are both required for a meaningful allergic or asthmatic response to occur? If this is true, local IgE production in the lung may play an important role in asthma pathogenesis. Also, despite their strong association with asthma, the cytokines investigated in this project may not be integral to driving disease in asthma patients.

We predicted that nonatopic asthmatic patients would display distinct pro-inflammatory cytokine responses to some allergens on our panel. This could indicate a previously undetected T cell-mediated sensitivity to allergen in these patients that could be driving their asthma. If this was observed, we planned to challenge nonatopic patients with allergens they showed strong pro-inflammatory cytokine responses to and look for a late-phase asthmatic response. Our work did show that nonatopic asthmatic patients produce IL-5, IL-17A, and IL-23 to various allergens. However, the data from the atopic asthmatic group showed that pro-inflammatory cytokine production is not indicative of sensitivity to allergen. Therefore, it is difficult to make any predictions about clinically relevant sensitivity to allergen in our nonatopic asthmatic group based on their cytokine responses.

Type of Bronchitis

We expected to see greater IL-5 responses in asthma patients with a history of eosinophilic bronchitis, as well as greater IL-17A and/or IL-23 responses in

patients with a history of neutrophilic bronchitis. Neither of these predictions was supported by the data.

The most difficult data set to explain is IL-5 production from eosinophilic vs noneosinophilic asthma patients. In two categories of allergic stimuli, "Mites and Insects" and "Animal Dander and Epithelia", noneosinophilic patients produced statistically significant higher quantities of IL-5. This was an unexpected result, since it has been shown that levels of IL-5 mRNA+ cells in the airway correlate with the total number of infiltrating eosinophils and activated eosinophils[24] and that the administration of anti-IL-5 decreases eosinophil recruitment to the lung and subsequently improves asthma[60]. Our results seem to be in contradiction with these findings, indicating that peripheral blood IL-5 responses to allergen may not play a clinically relevant role in airway eosinophilia. It is possible that a significantly stronger IL-5 response to allergen is occurring locally in the lungs of eosinophilic asthmatics, and we are just unable to detect this in the periphery. Alternatively, this discrepancy may be due to differences in steroid use between the two groups. The patients in our study with eosinophilic bronchitis are taking higher doses of corticosteroids, which have been shown to suppress IL-5 production by PBMCs. It is possible that in the absence of this steroid treatment, eosinophilic asthmatics would produce significantly higher levels of IL-5 in response to allergen than noneosinophilic asthmatics.

Though not statistically significant, our data reveals an elevated IL-17A response to microbial antigens by asthmatic patients with a history of neutrophilic asthma exacerbations, which may have interesting implications for the idea of "neutrophilic asthma". It could indicate that neutrophilic asthma is not in fact a distinct phenotype seen in patients with more severe disease, but is actually the product of a strong IL-17A (neutrophil-recruiting) recall response to microbial antigens in certain individuals. Therefore, exposure to exogenous microbes is still the trigger for this strong neutrophilic response. Based on our limited data, we should be cautious about how much emphasis is placed on this observation. Nonetheless, it would be interesting to investigate whether this elevated IL-17A response to microbial antigens in the blood is also seen locally in the lung.

If we consider individual patients, it is clear that allergen-induced PBMC cytokine profiles do not necessarily match what we would expect based on known clinical characteristics. In some patients, such as nonatopic patient 13 (*see Figure 10.26*), cytokine responses to allergen match what we would predict based on phenotype. This patient has a history of neutrophilic asthma exacerbations and has responses to allergen characterized by dominant IL-17A production. However, nonatopic patient 8 is a noneosinophilic patient who displays strong IL-5 responses (*see Figure 10.16*). As another example, we can consider atopic asthmatic patient 5 (*see Figure 11.12*) who is known to be sensitive to dust mite and mold and has a history of airway eosinophilia. The patient's cytokine responses to the "Mites and Insects" and "Fungi" classes of allergen are dominated by IL-17A and IFN-γ production, as opposed to IL-5. Again, the message here is that we cannot look at a patient's PBMC cytokine responses to allergen and predict their phenotype.

Figure 10.26 Nonatopic Asthmatic Patient 13 - Neutrophilic

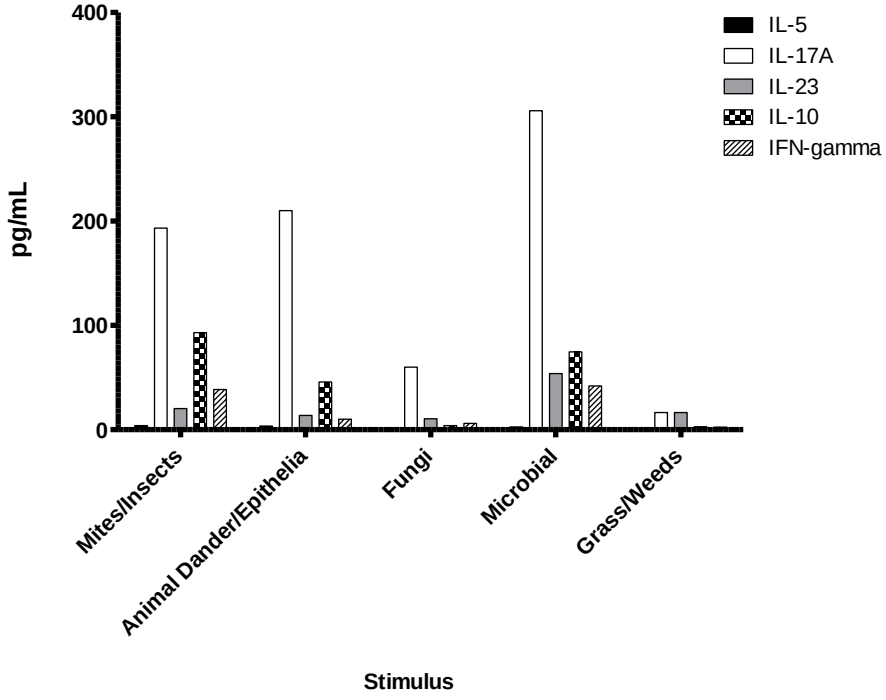
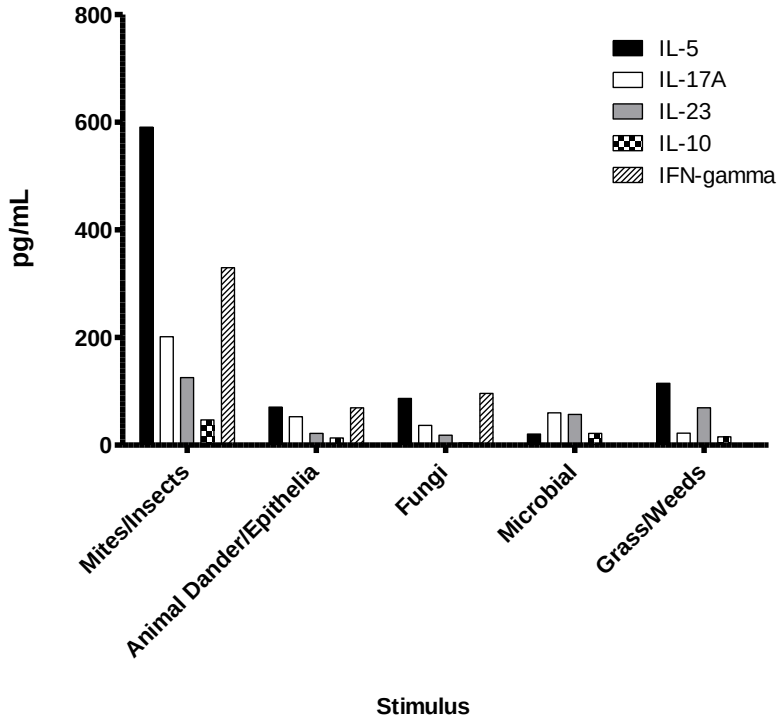
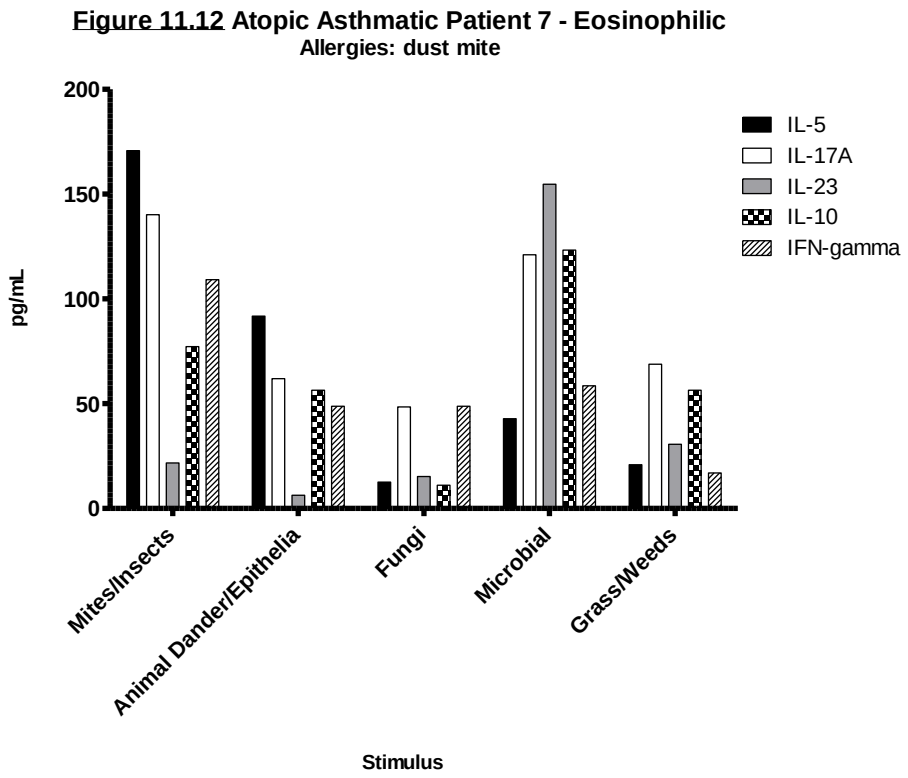


Figure 10.16 Nonatopic Asthmatic Patient 8 - Noneosinophilic





Steroid Dosage

It is important to acknowledge the effect that steroid treatment may have had on the outcomes of this study, as our results show a significant decrease in IL-5 production with increasing steroid dosage. Had the eosinophilic patients in our study not been on high doses of steroids, a more significant role for PBMC-produced IL-5 as a biomarker of their disease may have emerged.

CONCLUDING REMARKS

The main findings of this project are as follows:

1. Antigen-induced PBMC proliferation does not distinguish asthma from normal controls, atopic asthma from nonatopic asthma, or differentiate between different types of bronchitis.
2. The cytokines IL-5, IL-17A, IL-23, IL-10, and IFN-γ produced by PBMCs in response to allergen are not good biomarkers of disease in asthma.
    a. They do not distinguish asthma from normal controls, atopic asthma from nonatopic asthma, or differentiate between different types of bronchitis.
3. Pro-inflammatory cytokine production by PBMCs does not necessarily match sensitization profile. The skin prick test, indicative of IgE production, does not always predict a patient's cellular response to allergen.

The cytokine data from this project reveals an apparent disconnect between PBMC activation/pro-inflammatory cytokine production and clinically relevant disease. There are two ways in which our findings can be interpreted.

The first is to assume that responses to allergen measured in patients' peripheral blood are representative of the cellular activity in their airways. Several studies mentioned previously in this report have suggested that cytokines present in serum or produced by peripheral blood cells accurately represent the environment of the airways and can act as biomarkers of lung disease. In this case, we would have to conclude that the cytokines investigated in this study (as produced by T cells, macrophages, dendritic cells, B cells, or NK cells) do not play a significant role in driving disease in asthma and cannot be used as biomarkers for asthmatic phenotypes. This conclusion still leaves room for significant cytokine production from other sources, such as mast cell production of IL-5, but calls into question a central role for the T cell in asthma.

However, it is important to recognize the limitations of using peripheral blood cells to study a disease of the lungs. Therefore, the second interpretation of our data is that allergen-induced peripheral blood mononuclear cell responses to allergen are not good biomarkers of disease in asthma. PBMC activity may or may not be representative of what is actually going on in patient airways, but it does not appear to drive disease in asthma. No differences in PBMC activity were seen in patients with asthma, compared with normal controls, or between patients with different asthmatic phenotypes. It is not possible to determine a patient's disease state, atopic status, or type of bronchitis by looking at their PBMC responses to allergen. We believe this is the most correct conclusion to draw from the results of our study. This opens the door for an interesting follow-up project: investigating the cellular responses to allergen by samples taken from our patients' airways and compare these with their PBMC data. This would determine whether or not PBMC responses to allergen of asthma patients are representative of their airway environment and could clarify the role of T cell cytokines in driving asthma.

This project has highlighted both the importance and the uniqueness of the local environment in patients with asthma. Factors driving asthma may be unique to the airways and not manifest systemically in a patient. For example, this suggests a central role for IL-5 production by mast cells and T cells in the lung, potentially mediated by local IgE production, in patients with airway eosinophilia. We may not be able to detect this IL-5 and IgE production using PBMC assays or the skin-prick test, but it certainly holds clinical significance for the patient. Future studies may shed light on the ability of locally produced T cell cytokines to act as biomarkers in asthma, but our data indicates that these cytokines produced in the periphery are unable act as such.

APPENDIX I: PATIENT CHARACTERISTICS

Patient ID	Age	Gender	Asthma Status	Atopic Status	Allergies	Sputum Eos	Sputum Neuts	FEV1	FEV1 %	FEV1/VC	< 500 mg ICS	> 500 mg ICS	Prednisone	Diagnosis
ES001NAA-0	41	F	Yes	Yes	None	No	No	1.73	53%	0.83		a1000	0	11% reversibility (historical)
ES002NAA-E	68	M	Yes	Yes	None	Yes	No	1.85	62%	0.69		2000		24% reversibility historical data
ES003NAA-E	48	M	Yes	Yes	None	Yes	No	4.2	113%	0.77		1000	0	Pc20>16 mg/ml
ES004NAA-E	63	M	Yes	Yes	None	Yes	No	2.61	87%	0.79		2000	12.5	9% reversibility
ES005NAA-N	37	M	Yes	Yes	None	No	Yes	2.53	58%	0.49		s1200	0	PC20 0.094 mg/ml
ES006NAA-E	66	F	Yes	Yes	None	Yes	No	2.61	98%			F1000	7.5	28% reversibility (historical data)
ES007NAA-E	23	F	Yes	Yes	None	Yes	No	3.21	91%	0.78		1000	25	cannot find either in her chart, she has confirmed Hypereosinophilic syndrome
ES008NAA-0	41	F	Yes	Yes	None	No	No	2.64	90%	0.81	0	0	0	PC20 1.95 mg/ml
ES009NAA-E	46	F	Yes	Yes	None	Yes	No	2.56	83%	0.78		1000	20	reversibility 15.6%
ES010NAA-E	55	F	Yes	Yes	None	Yes	No	1.63	60%	0.67		f1500 a1000	25	reversibility,
ES011NAA-N	64	M	Yes	Yes	None	No	Yes	1.45	41%	0.53		a1000 f1000	5	14% reversibility
ES012NAA-E	20	M	Yes	Yes	None	Yes	No	5.05	107%	0.84		a1000 f1000	20	11.2% reversibility, hypereosinophilic syndrome
ES013NAA-N	44	F	Yes	Yes	None	No	Yes	2.27	78%	79		a1000 f1000	10	PC20 > 16 mg/ml
ES015NAA-E/N	70	F	Yes	Yes	None	Yes	Yes	1.85	92%	74		s800 p1200	12.5	history of 15% reversibility from a physician in Ottawa
ES016NAA-E	51	M	Yes	Yes	None	Yes	No	3.08	80%	60		f2000	10	EB, PC20>16 mg/ml

ES017NAA-E	52	M	Yes	Yes	None	Yes	No	4.34	104%	87		a1000 al400	10	unable to provide suitable flow-volume curves for MIT
ES018NAA-E	50	M	Yes	Yes	None	Yes	No	3.18	80%	82		f1000	7.5	PC20 >16
ES020NAA-E	57	M	Yes	Yes	None	Yes	No	1.27	47%	0.57		p1600 s800	20	14% reversibility
ES021NAA-E	15	F	Yes	Yes	None	Yes	No	2.25	76%	68		f1000 a1000	18	21% historical data
ES022NAA-0	53	F	Yes	Yes	None	No	No	1.91	72%	78		s1200	0	PC20 4.09 mg/ml
ES023NAA-E	68	M	Yes	Yes	None	Yes	No	1.49	44%	61				
ES024NAA-N	78	F	Yes	Yes	None	No	Yes	0.9	44%	43		a1000	0	24% reversibility from PFT in 2005
ES025NAA-E	54	M	Yes	Yes	None	Yes	No	3.2	83%	58		s800	7.5	17% reversibility, no MIT done
ES026NAA-E		F	Yes	Yes	None	Yes	No							
ES027NAA-E	50	M	Yes	Yes	None	Yes	No	1.86	45%	49		s800		reversibility 14%
ES028NAA-E	56	M	Yes	Yes	None	Yes	No	1.49	44%	49		f1000 a1000	5	EB, no evidence of reversibility and no MIT done,
ES001AA-N	51	M	Yes	Yes	Ragweed, mold, dust mite, peanut, birch tree	No	Yes	2.6	76%	0.79		a1000	0	PC 20 1.2 mg/ml, reversibility 12%
ES002AA-E	27	M	Yes	Yes	Cat, dust mite, grass, alternaria, cladosporium, aspergillus	Yes	No	5.08	110%	0.78	f250		0	PC20 9.2 mg/ml has shown reversibility in the past
ES004AA-E	45	M	Yes	Yes	Dust mite	Yes	No	3.04	70%	0.71	f400	s600	15	historical reversibility
ES005AA-E	61	F	Yes	Yes	Dust mite, mold	Yes	No	2.12	95%	0.65		f1000 a1000	4	reversibility 14%

ES006AA-0	35	M	Yes	Yes	Dust mite, ragweed, aspergillus, trees, grass, alternaria, cat, horse, dictyoptera	No	No	1.77	59%	0.62		s800	0	reversibility (63% at one visit)
ES007AA-E	63	F	Yes	Yes	Dust mite	Yes	No	1.7	73%	0.63		a750	0	PC20 >2.0 mg/ml
ES008AA-E	58	M	Yes	Yes	Dust mite, mold	Yes	No	1.22	35%	0.62		f1000 a1000		27%reversibility
ES009AA-0	32	F	Yes	Yes	Dust mite, pollens	No	No	3.25	97%	0.8		s800		PC20 2.59 mg/ml
ES010AA-E	22	M	Yes	Yes	Alternaria, cat, horse, feathers, grass	Yes	No	4.52	89%	0.69	f250		0	PC20 0.89 mg/ml
ES011AA-E	28	M	Yes	Yes	Grass, peanuts, shellfish, animals	Yes	No	3.26	68%	0.53		f2000 a2000	0	18 %reversibility
ES012AA-E	55	M	Yes	Yes	Dust mite	Yes	No	1.53	51%	0.51		f1000 a1000	10	13% reversibility
ES013AA-E	74	M	Yes	Yes	Cat, dog, horse	Yes	No	1.77	72%	54		f1000	15	? # too low for PC20, best reversibility is 6%
ES014AA-E	35	F	Yes	Yes	Grass, dust, milk	Yes	No	1.7	66%	72		p2400 s800	5 alt. days	16% reversibility
ES015AA-E	51	F	Yes	Yes	D. far, D. pter, grass, ragweed	Yes	No	2.16	78%	79		p1200 s800	7.5	did not find reversibility or methacholine challenge results ?asthma
ES016AA-E	41	F	Yes	Yes	Dust mite, dictoptera	Yes	No	1.64	69%	81		a1000	0	PC 20 0.26 mg/ml
ES017AA-E	53	F	Yes	Yes	Cat, horse, feathers, dust mite	Yes	No	1.05	41%	62		a1000	10	23% reversibility
ES007NA	36	F	No	No	None	No	No							
ES009NA		F	No	No	None	No	No							
ES010NA		F	No	No	None	No	No							

ES011NA	45	F	No	No	None	No	No	2.95	93%	97				
ES012NA	35	F	No	No	None	No	No	3.15	109%	88				
ES013NA	23	F	No	No	None	No	No	3.4	98%	106				
ES014NA	27	M	No	No	None	No	No	3.97	95%	96				
ES015NA	46	M	No	No	None	No	No	3.48	84%	100				

APPENDIX II: CONSOLIDATED PATIENT RESULTS

Contains:

1. PBMC Proliferation Data
2. Interleukin-5 Data
3. Interleukin-17A Data
4. Interleukin-23 Data
5. Interleukin-10 Data
6. Interferon-γ Data
7. All Cytokines
8. All Cytokines: Skin Prick Test
9. Steroid Dosage Data

Figure 1.1 Delta: Asthma vs Normal

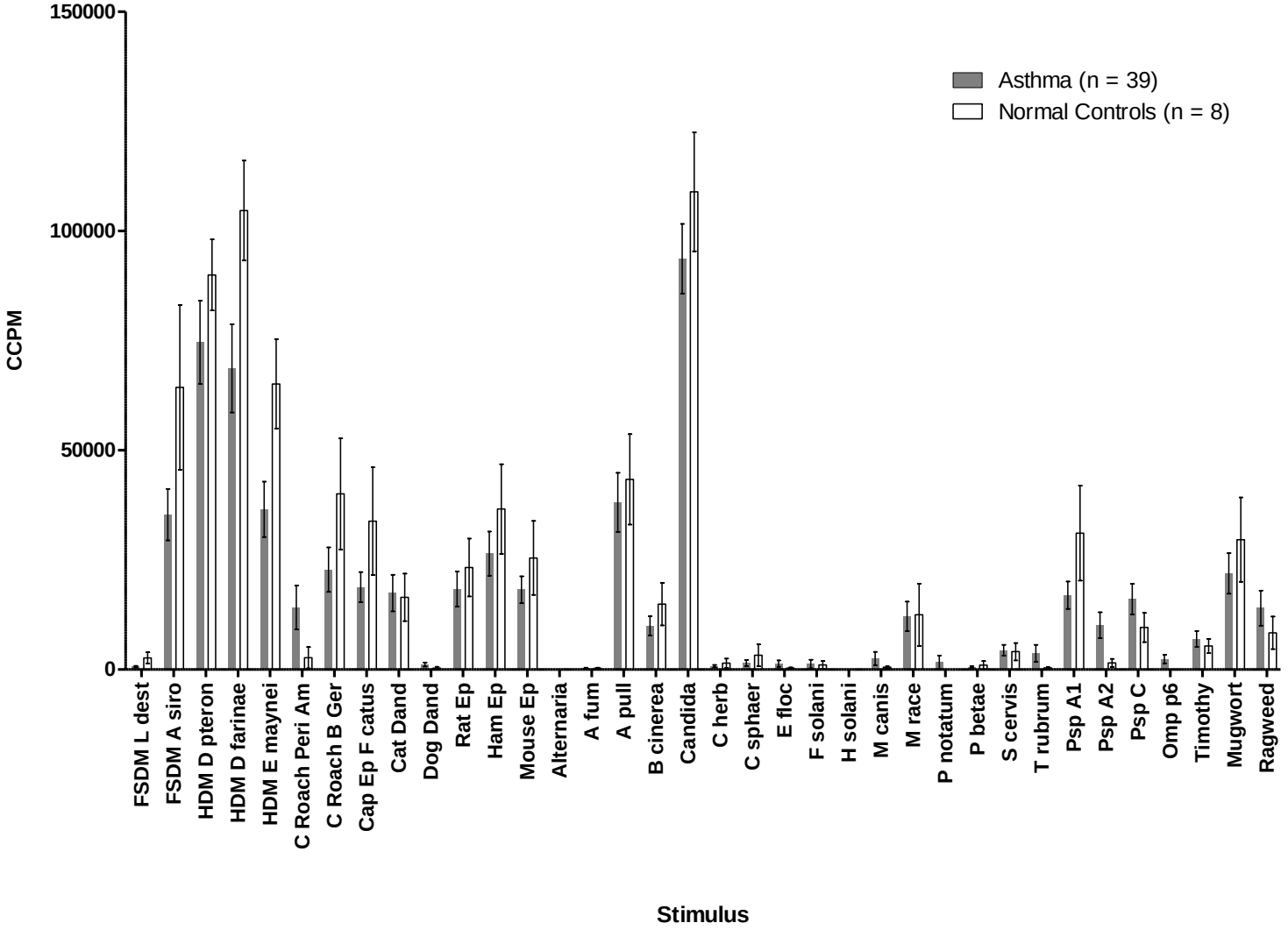


Figure 1.2 Delta Grouped: Asthma vs Normal

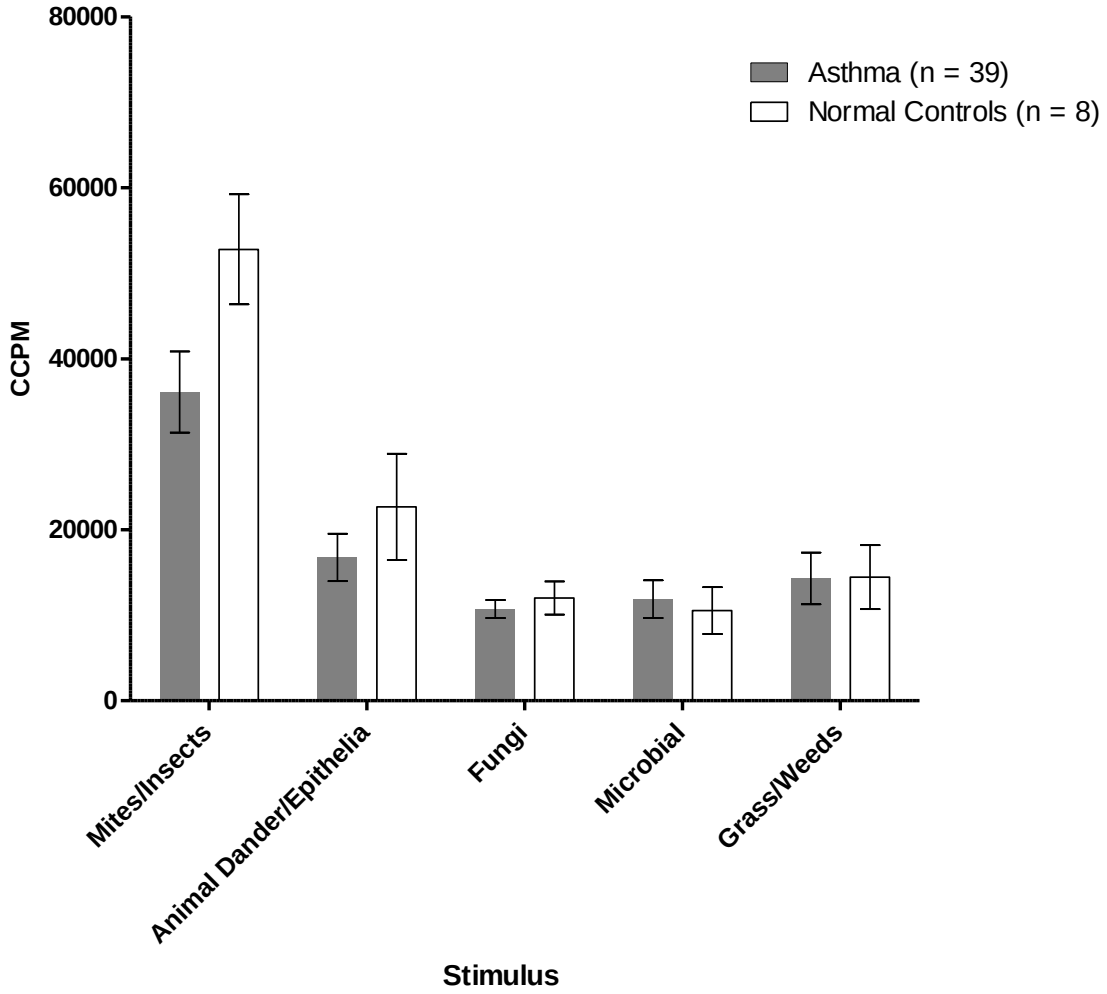


Figure 1.3 Delta: Nonatopic vs Atopic Asthma

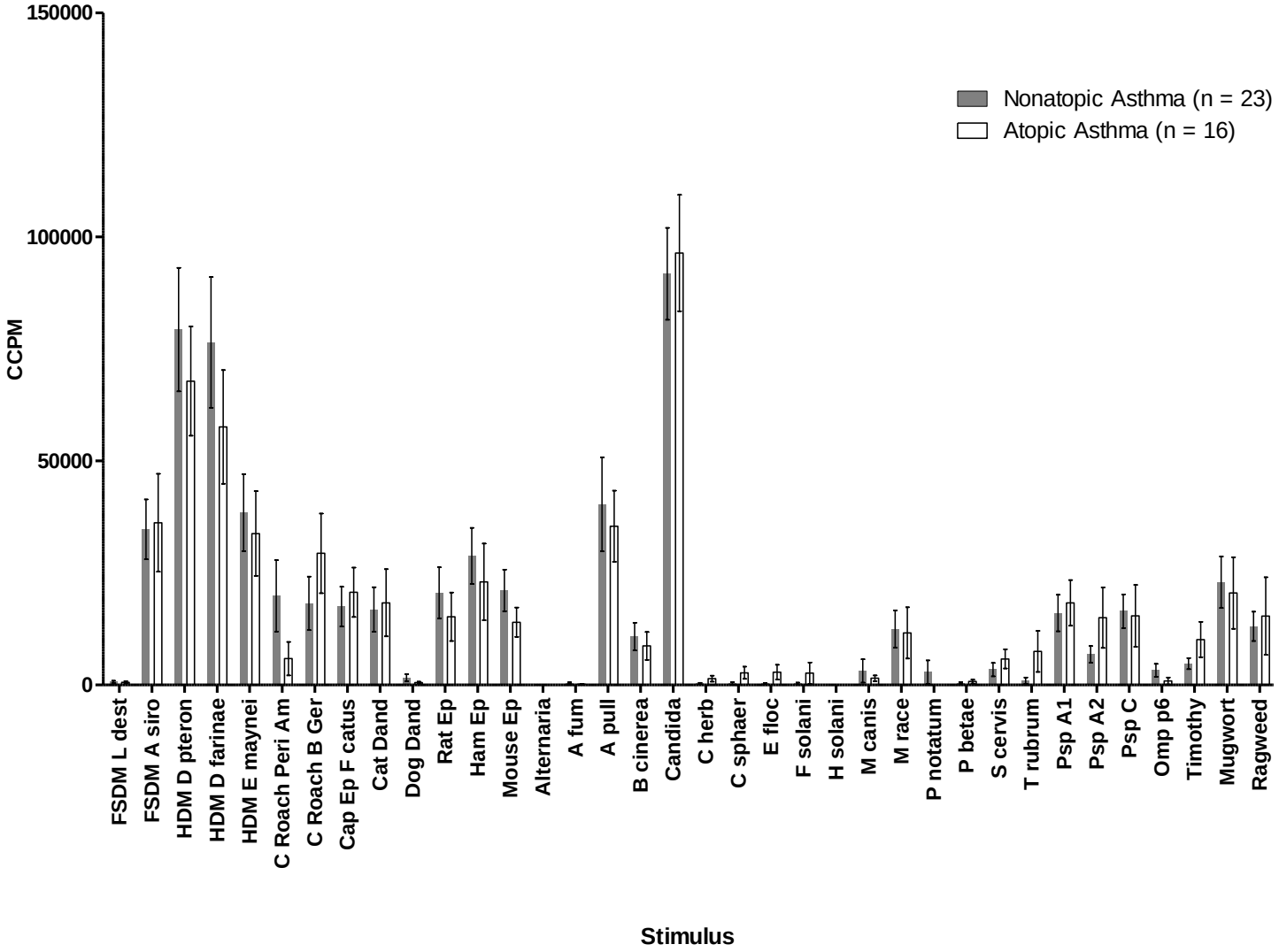


Figure 1.4 Delta Grouped: Nonatopic vs Atopic Asthma

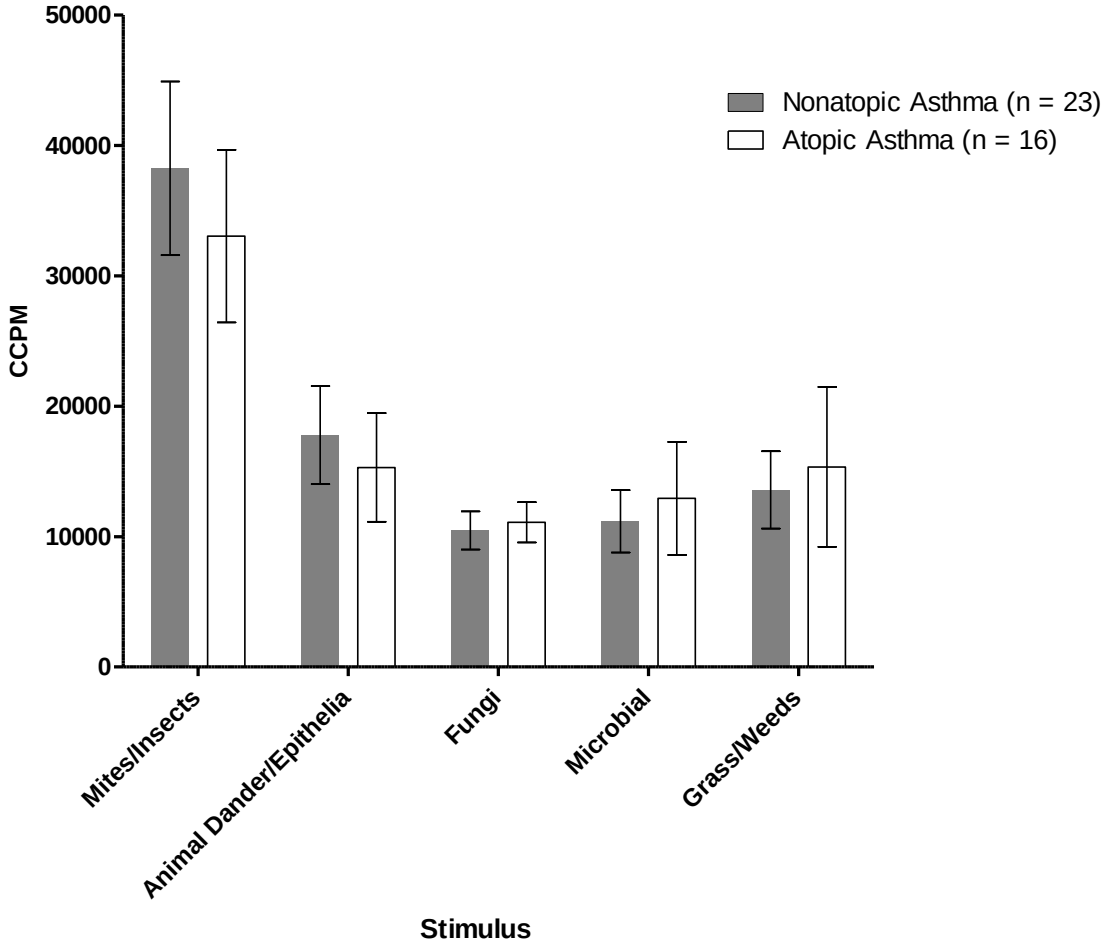


Figure 1.5 Delta: Eosinophilic vs Noneosinophilic

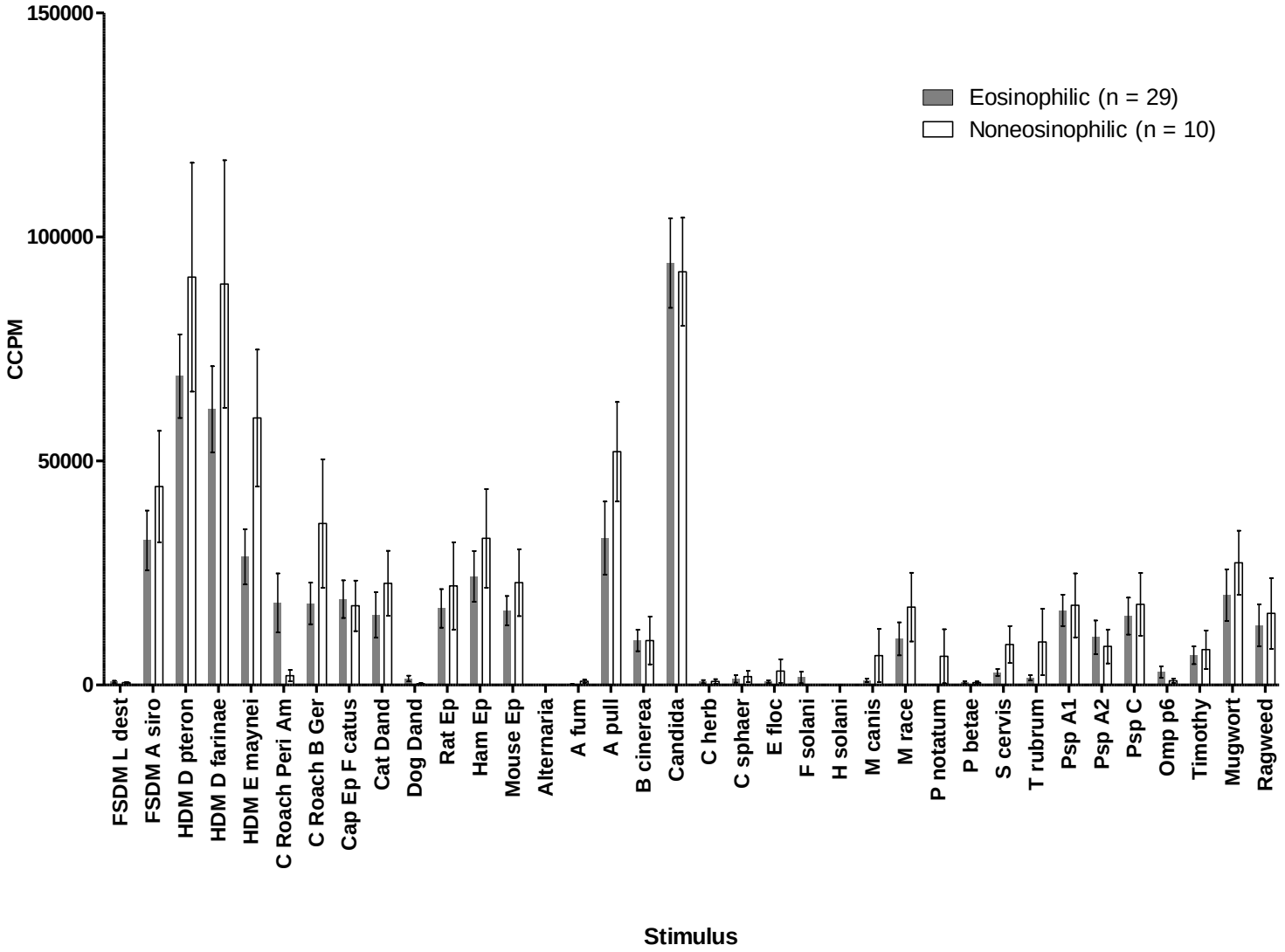


Figure 1.6 Delta Grouped: Eosinophilic vs Noneosinophilic

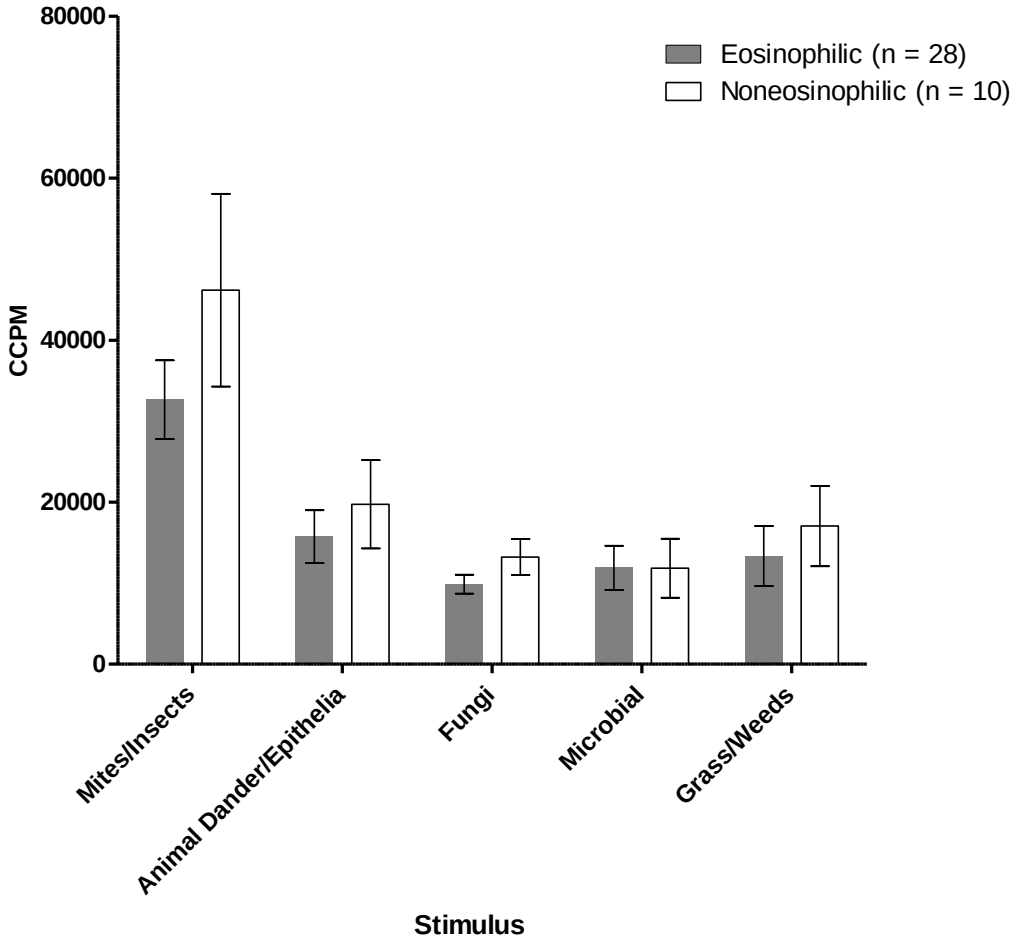


Figure 1.7 Delta Grouped: Nonatopic Asthma vs Normal

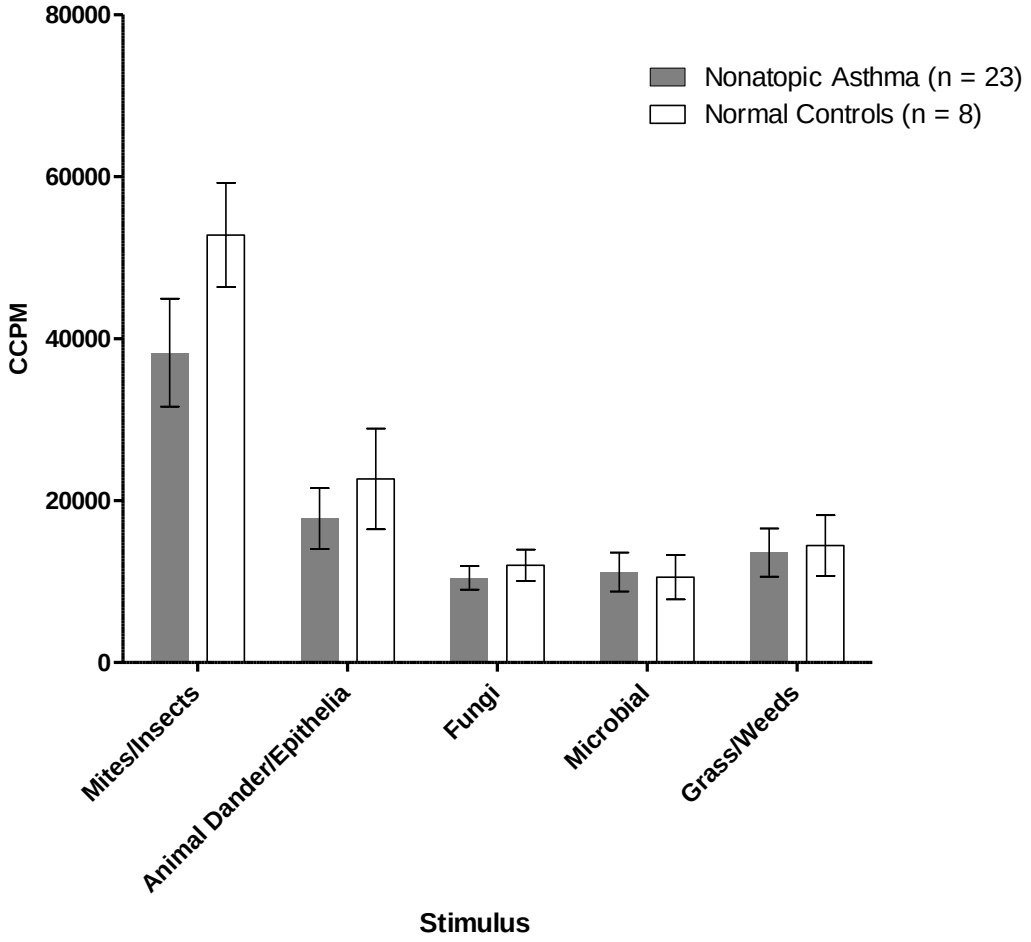


Figure 2.1 IL-5: Asthma vs Normal

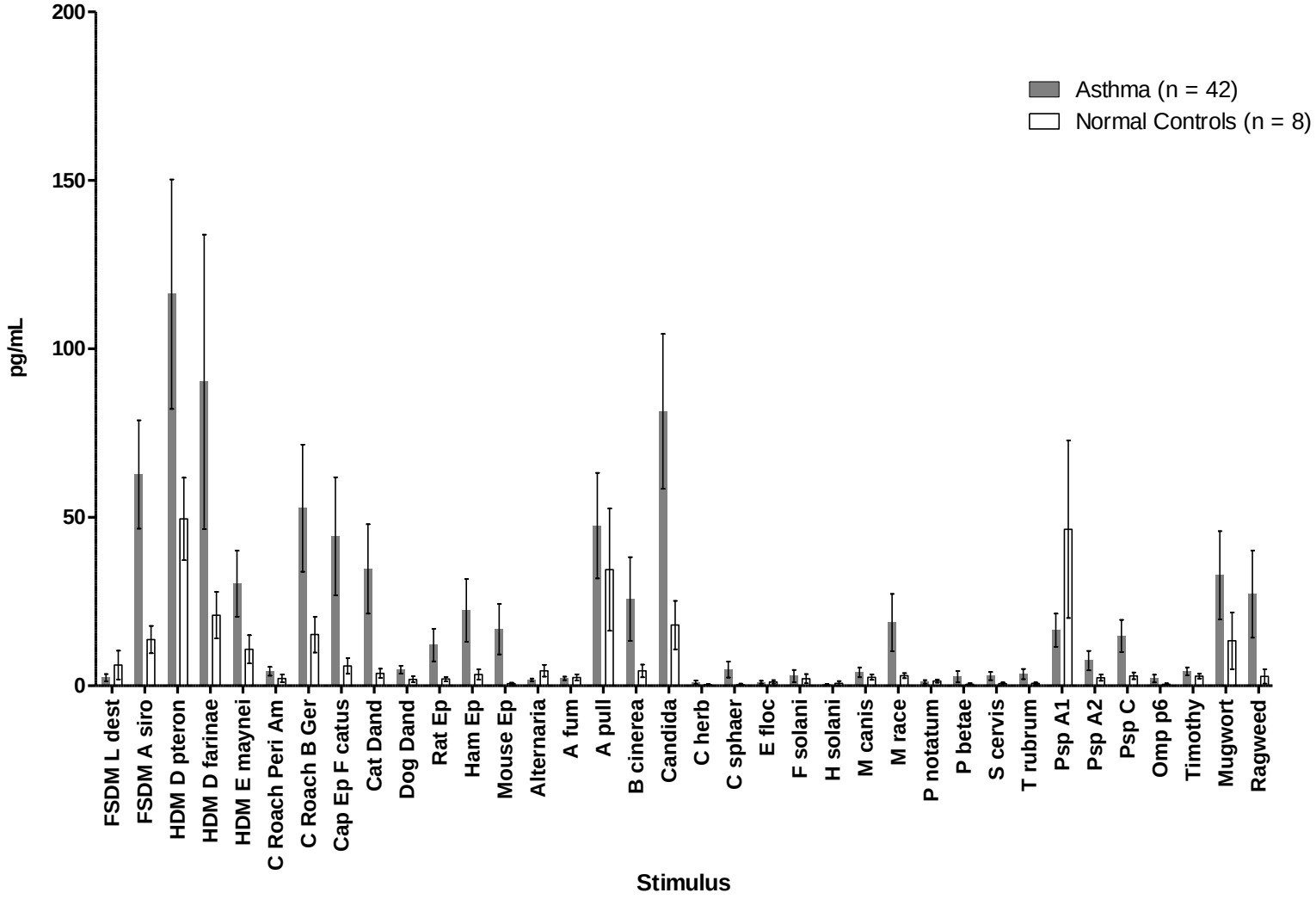


Figure 2.2 IL-5 Grouped: Asthma vs Normal

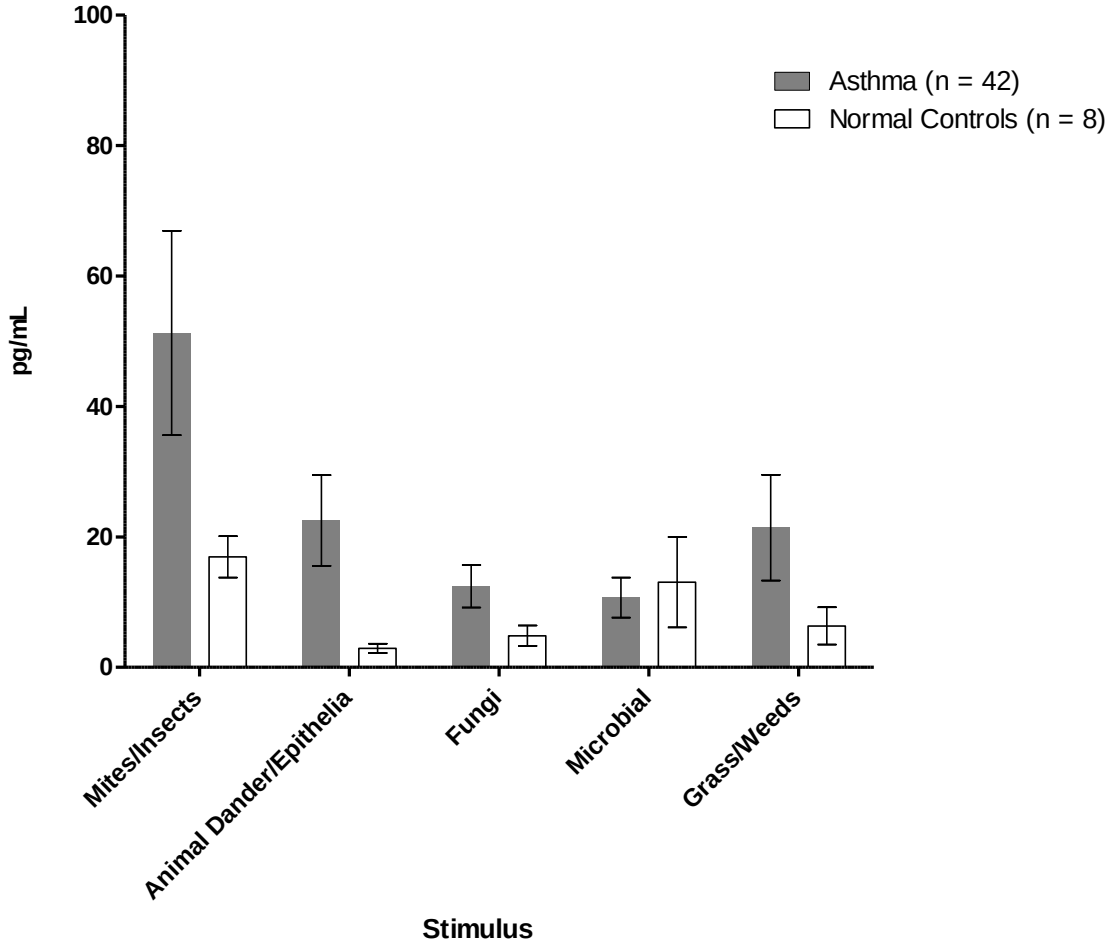


Figure 2.3 IL-5: Atopic vs Nonatopic Asthma

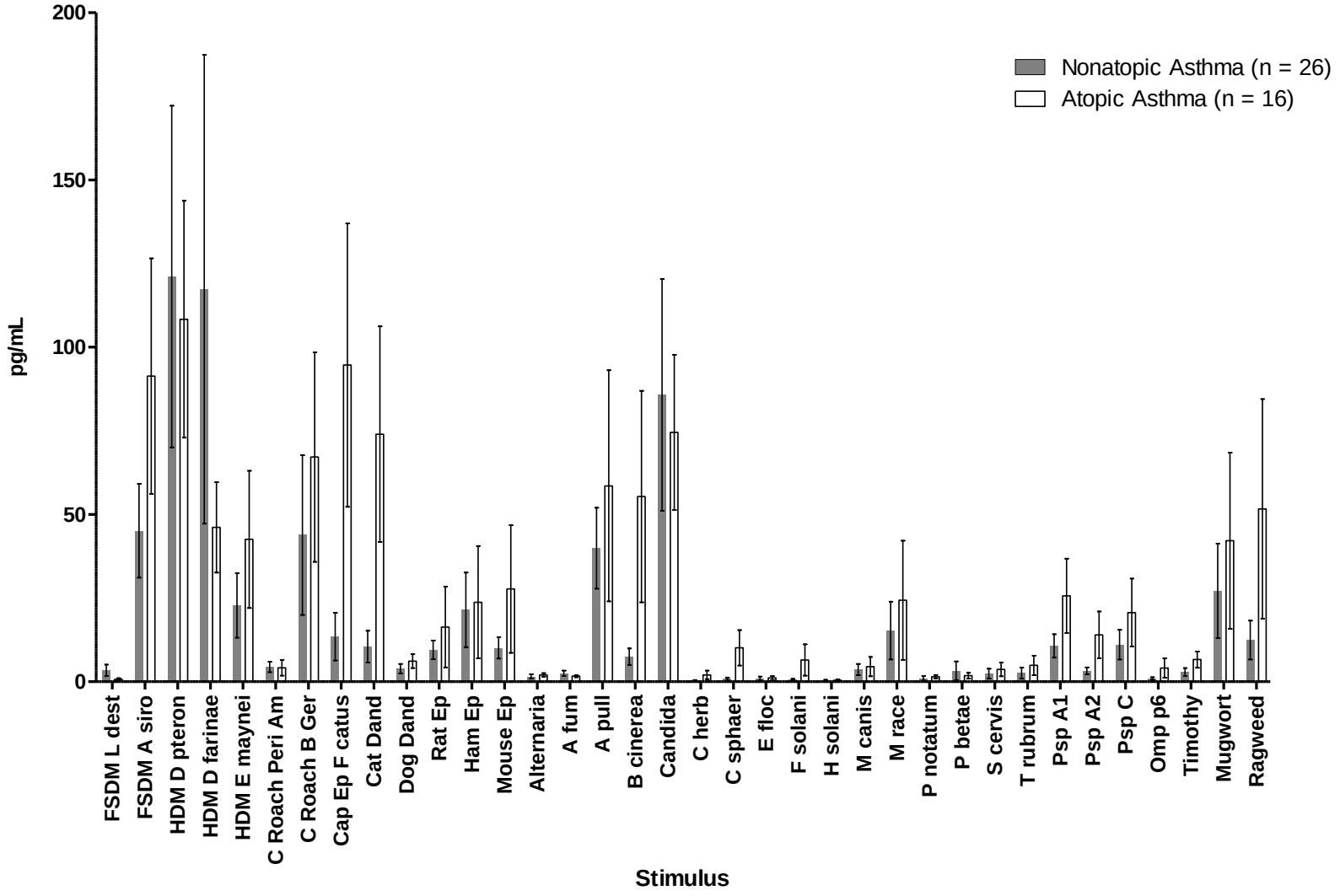


Figure 2.4 IL-5 Grouped: Atopic vs Nonatopic Asthma

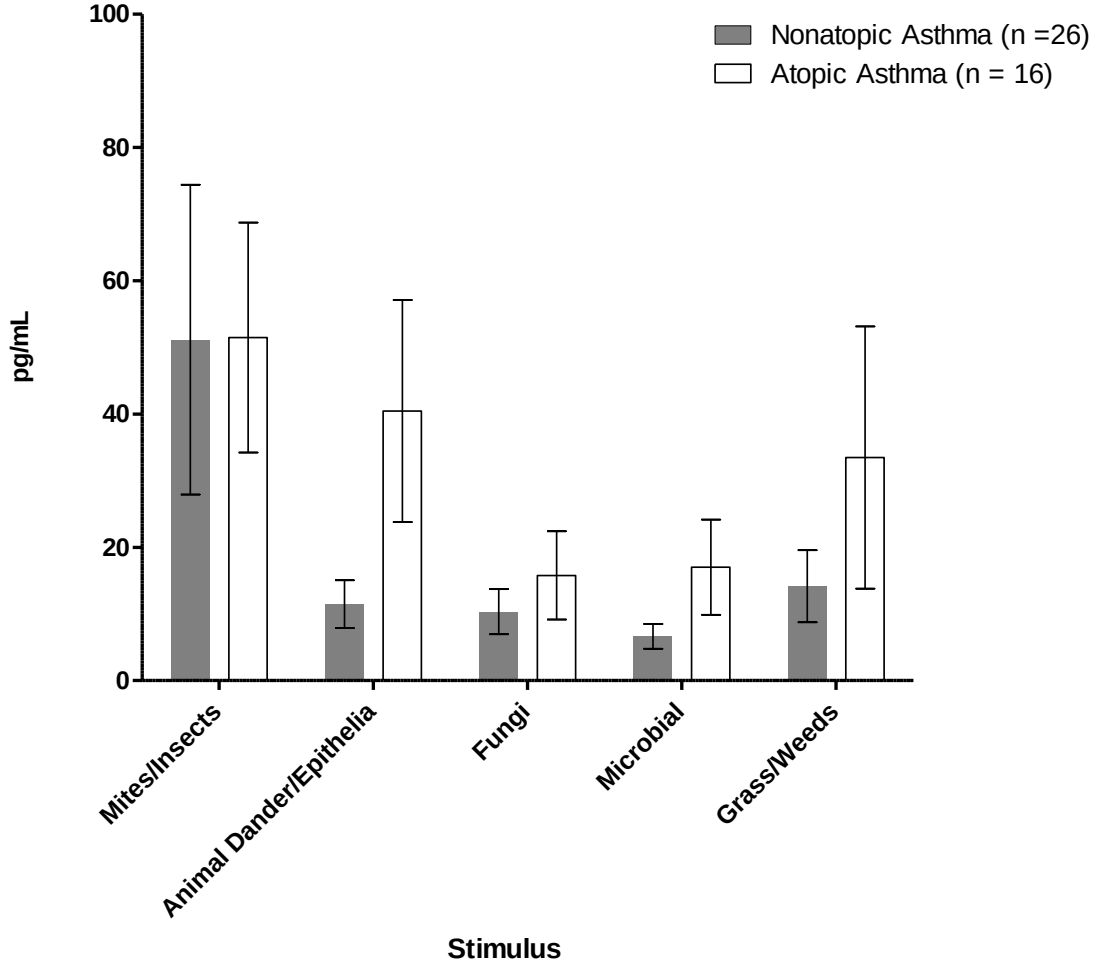


Figure 2.5 IL-5: Eosinophilic vs Noneosinophilic

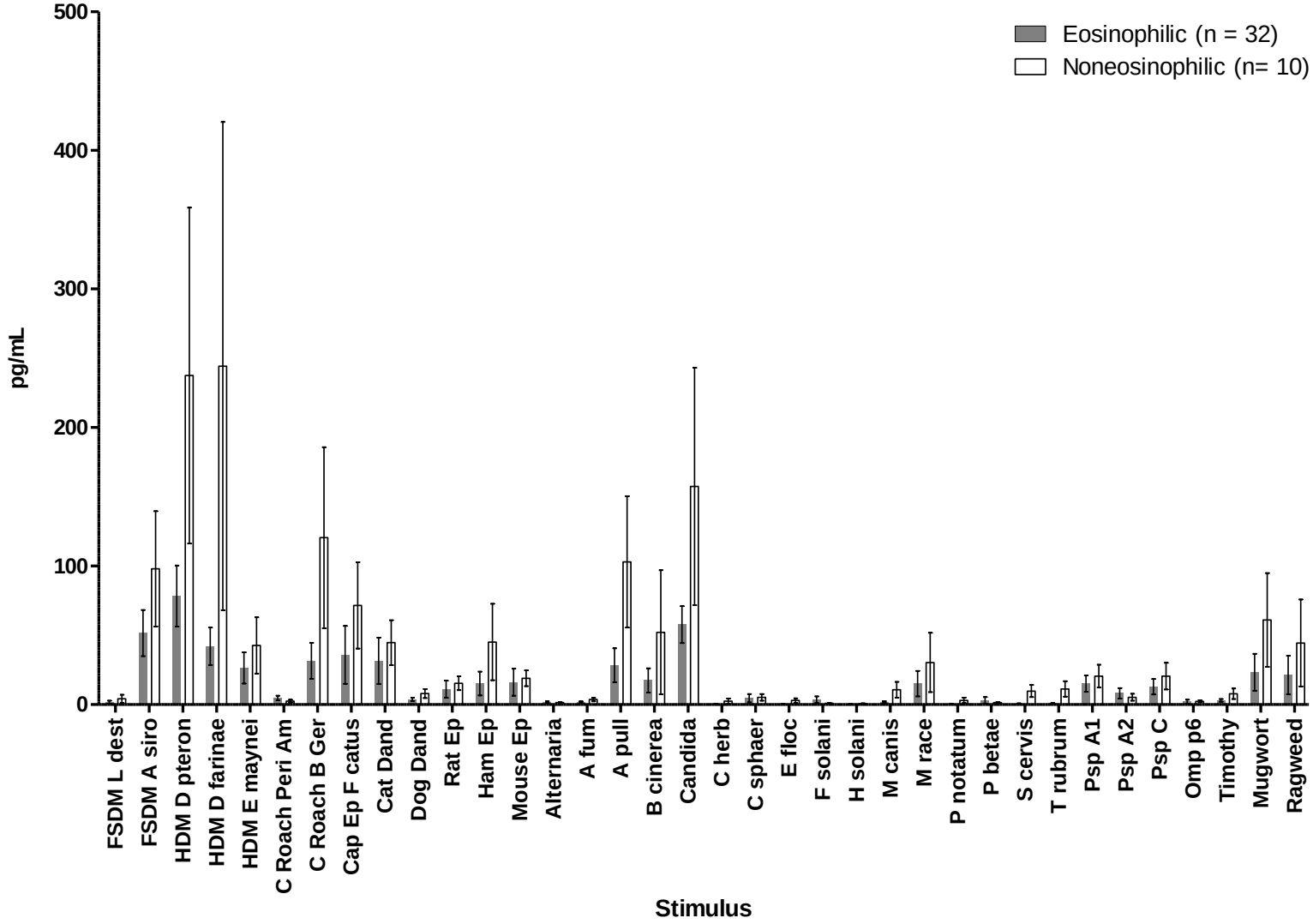


Figure 2.6 IL-5 Grouped: Eosinophilic vs Noneosinophilic

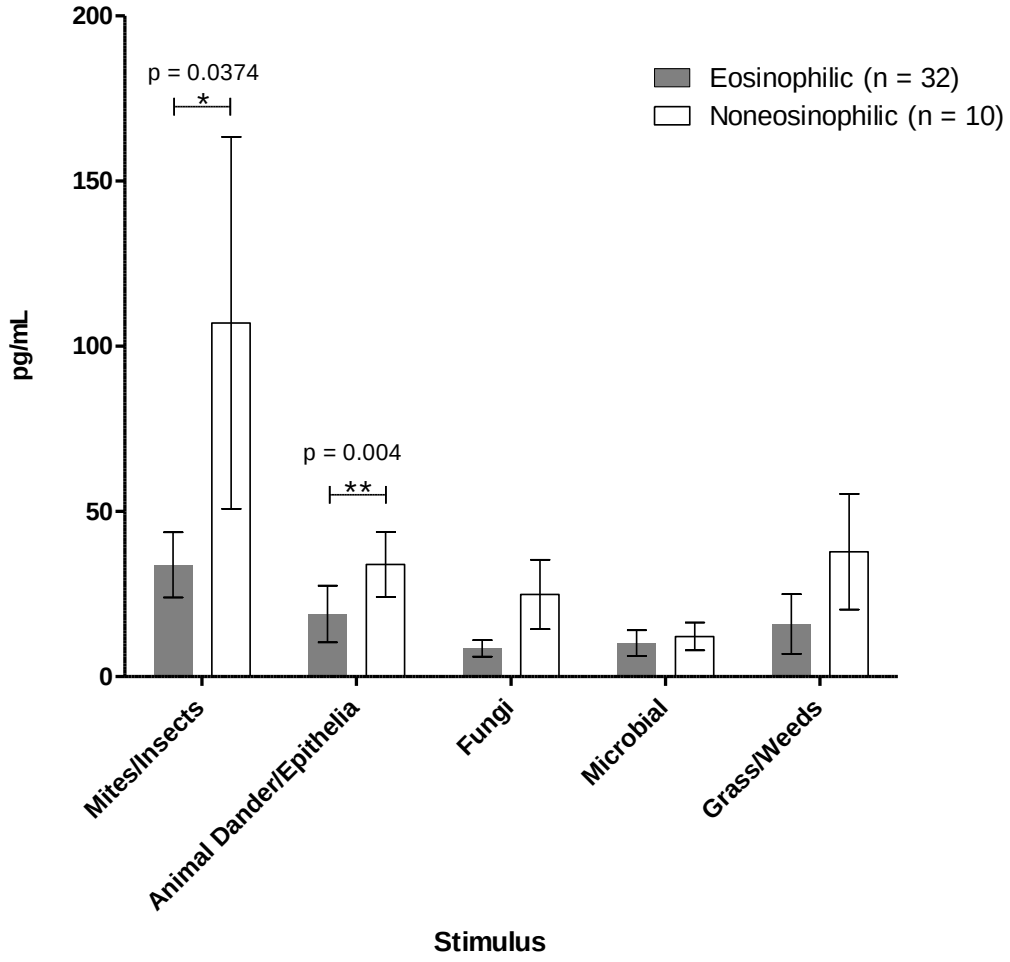


Figure 2.7 IL-5: Neutrophilic vs Nonneutrophilic

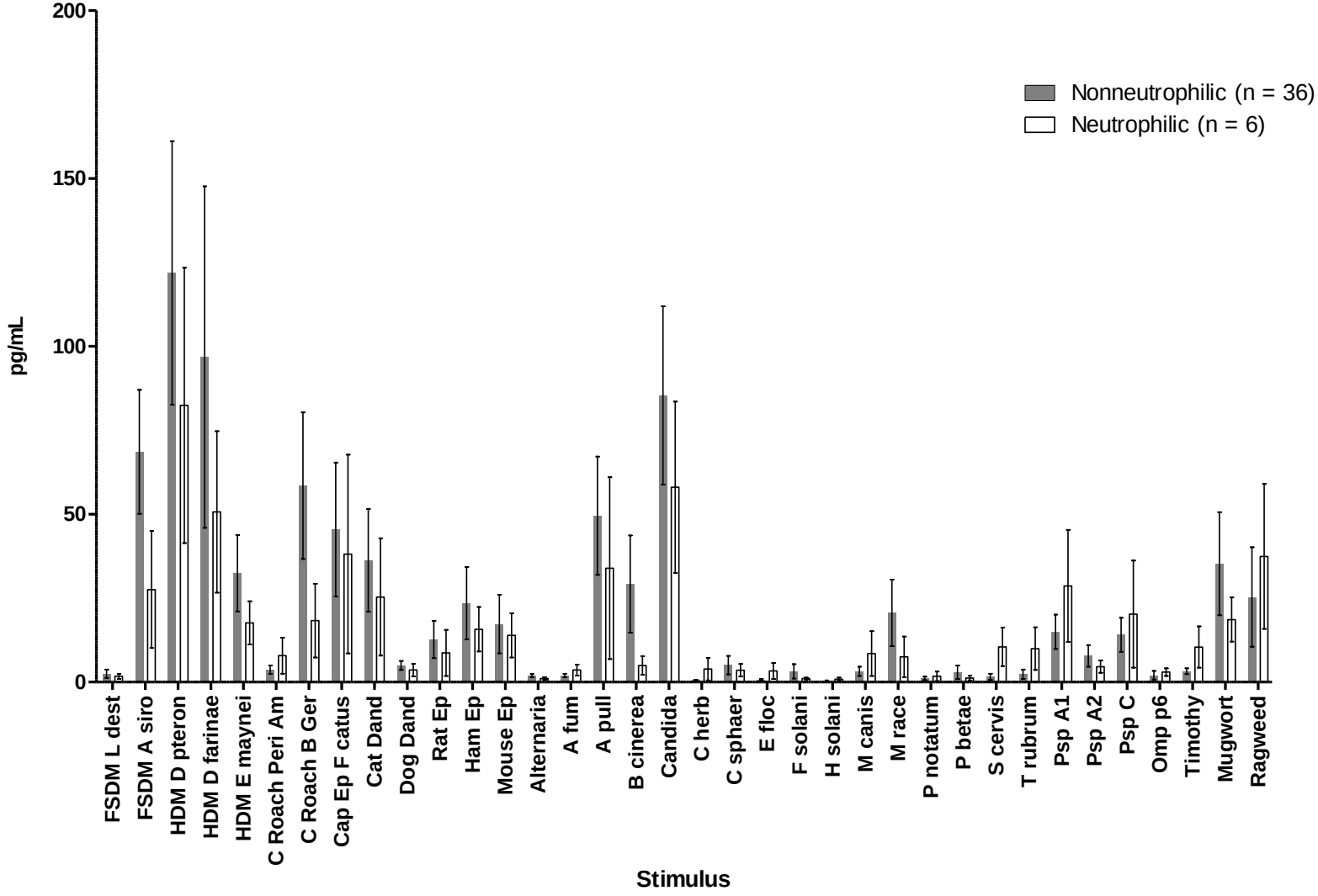


Figure 2.8 IL-5 Grouped: Neutrophilic vs Nonneutrophilic

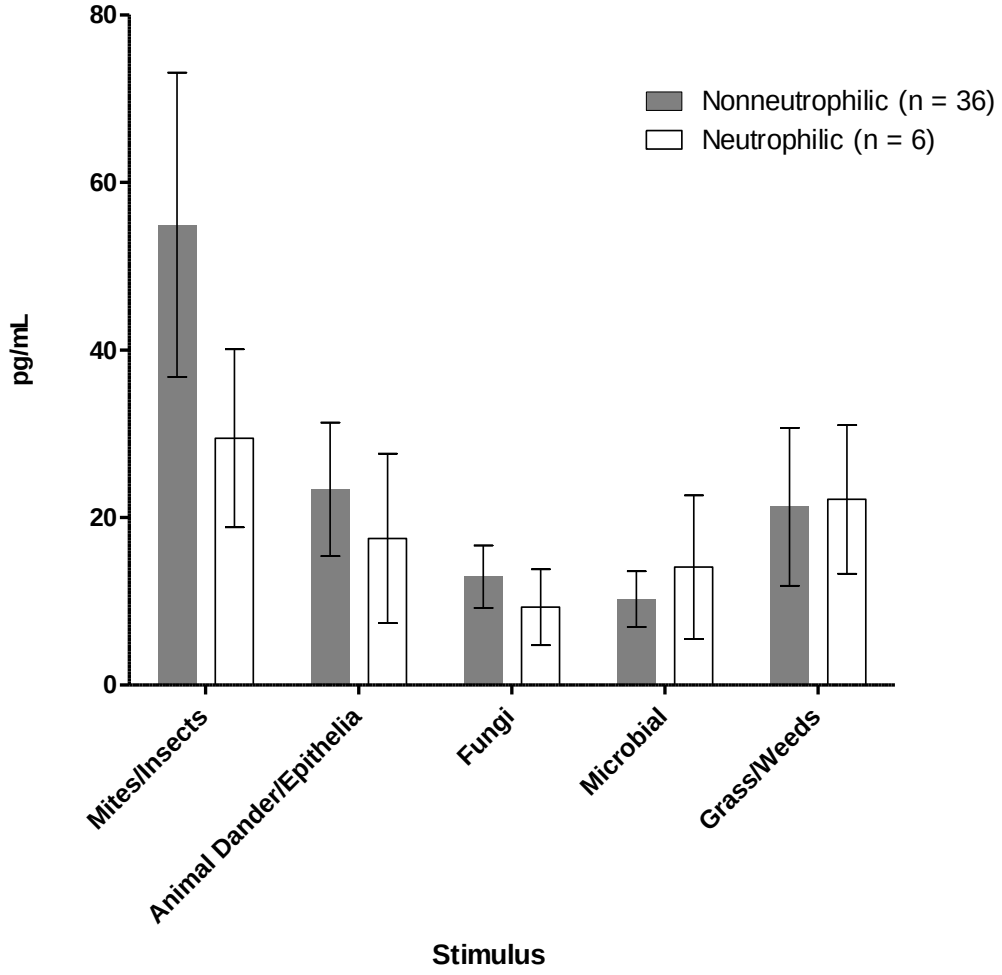


Figure 2.9 IL-5 Grouped: Nonatopic Asthma vs Normal

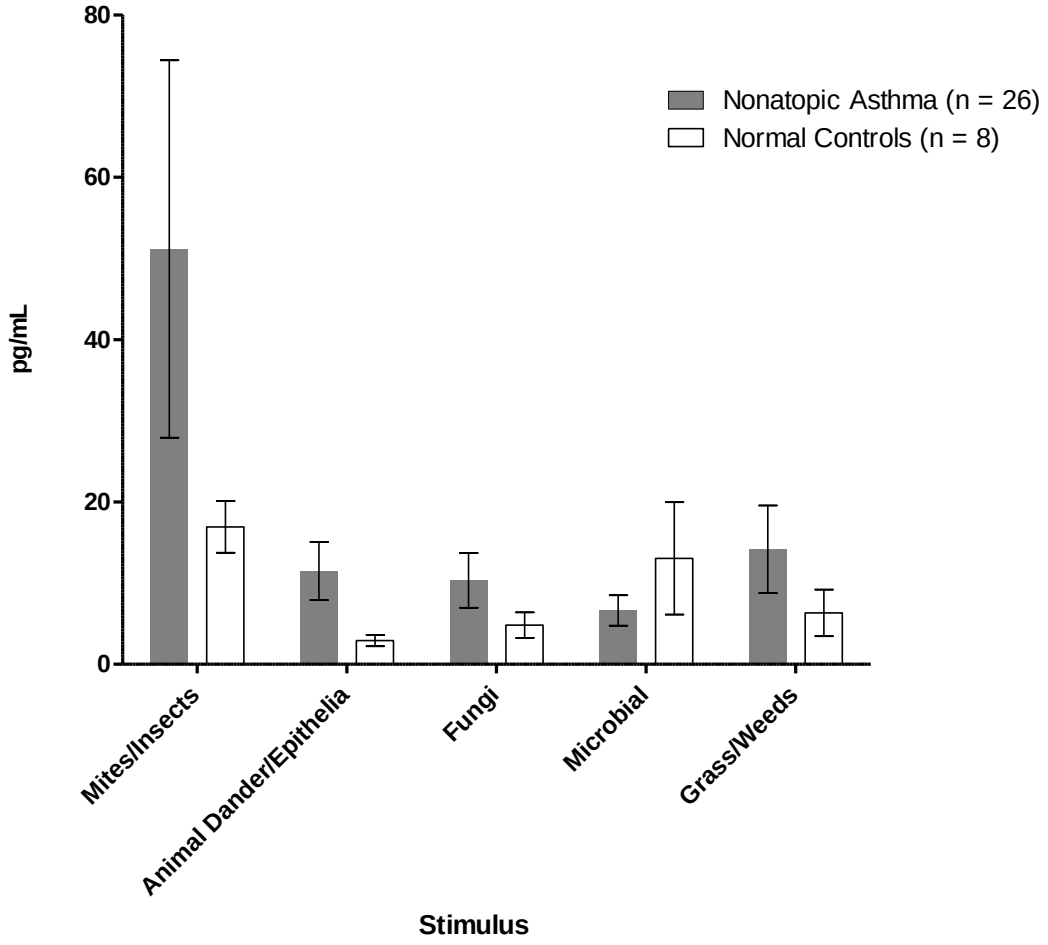


Figure 3.1 IL-17A: Asthma vs Normal

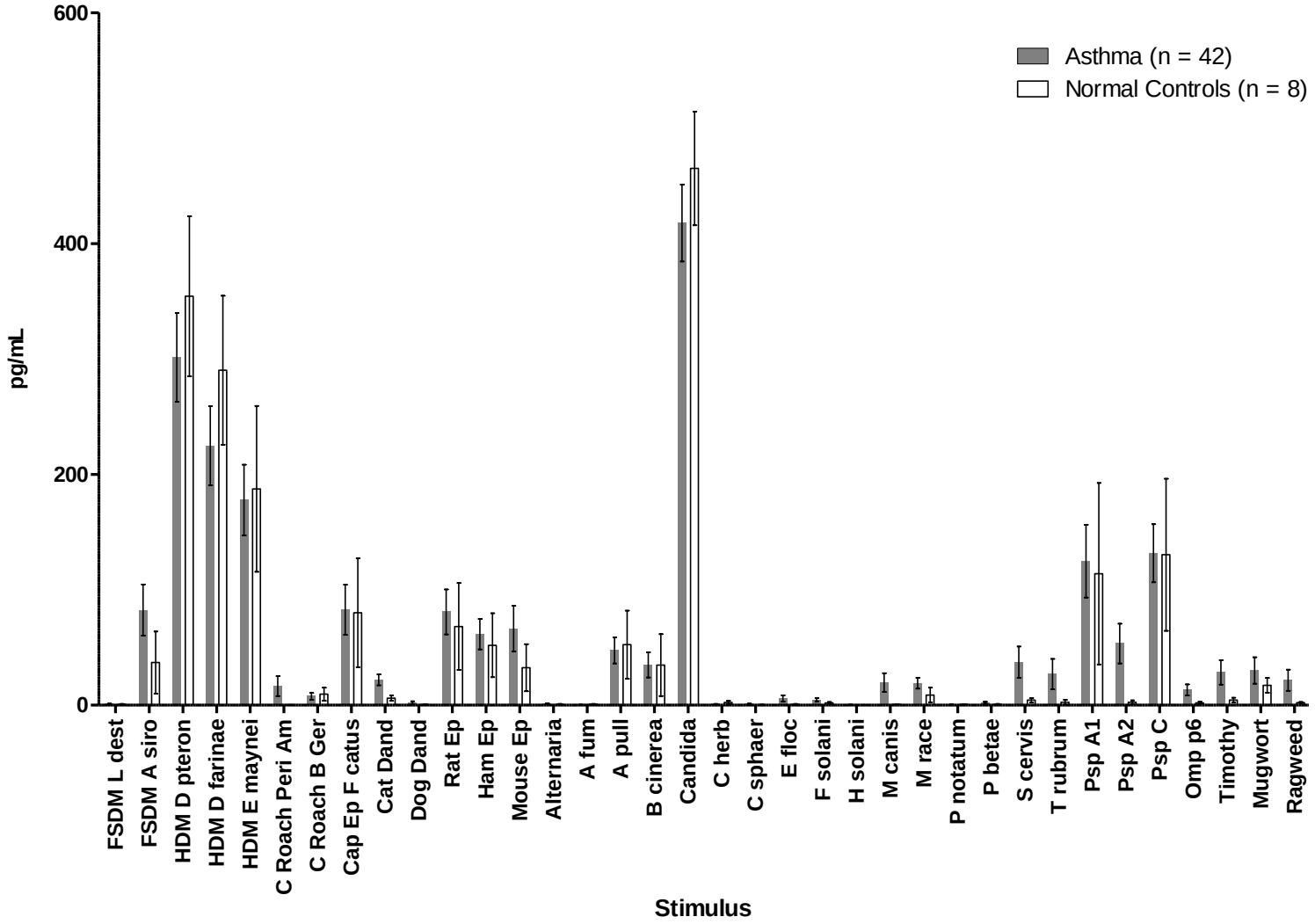


Figure 3.2 IL-17A Grouped: Asthma vs Normal

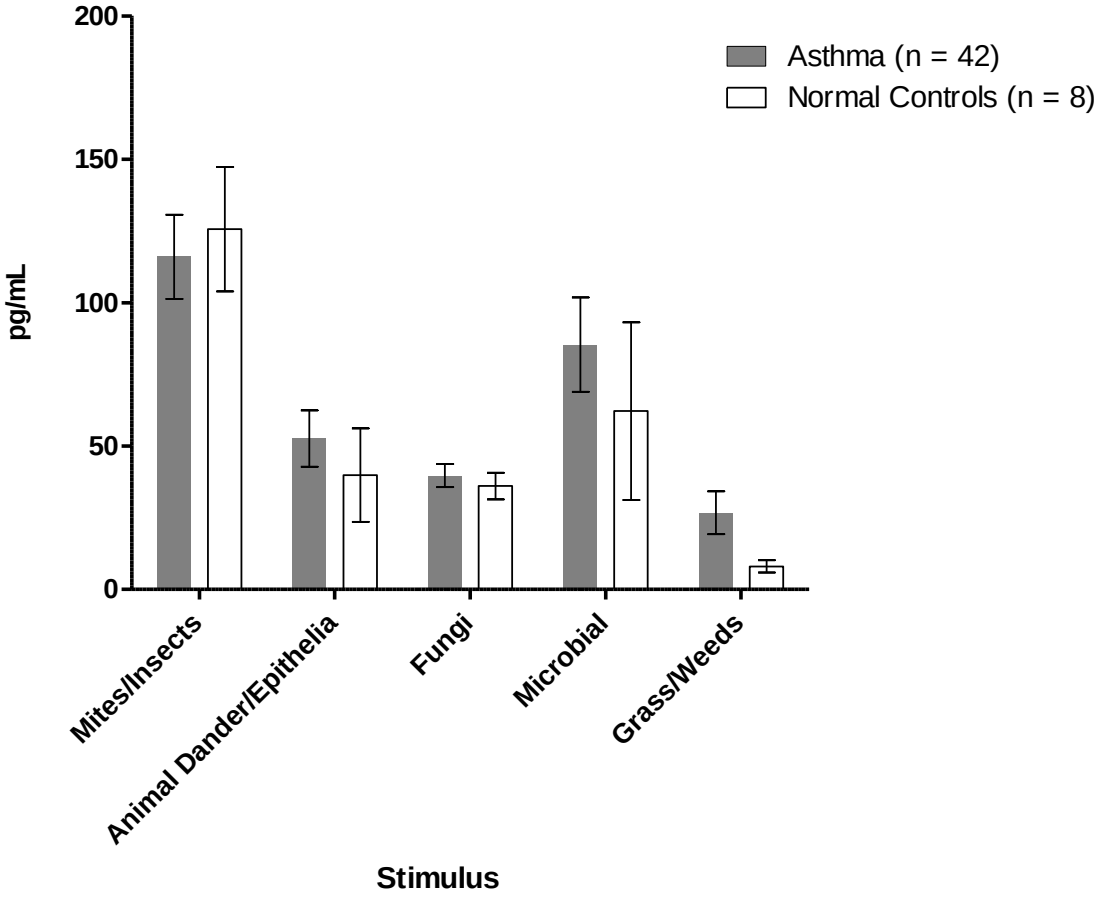


Figure 3.3 IL-17A: Atopic vs Nonatopic Asthma

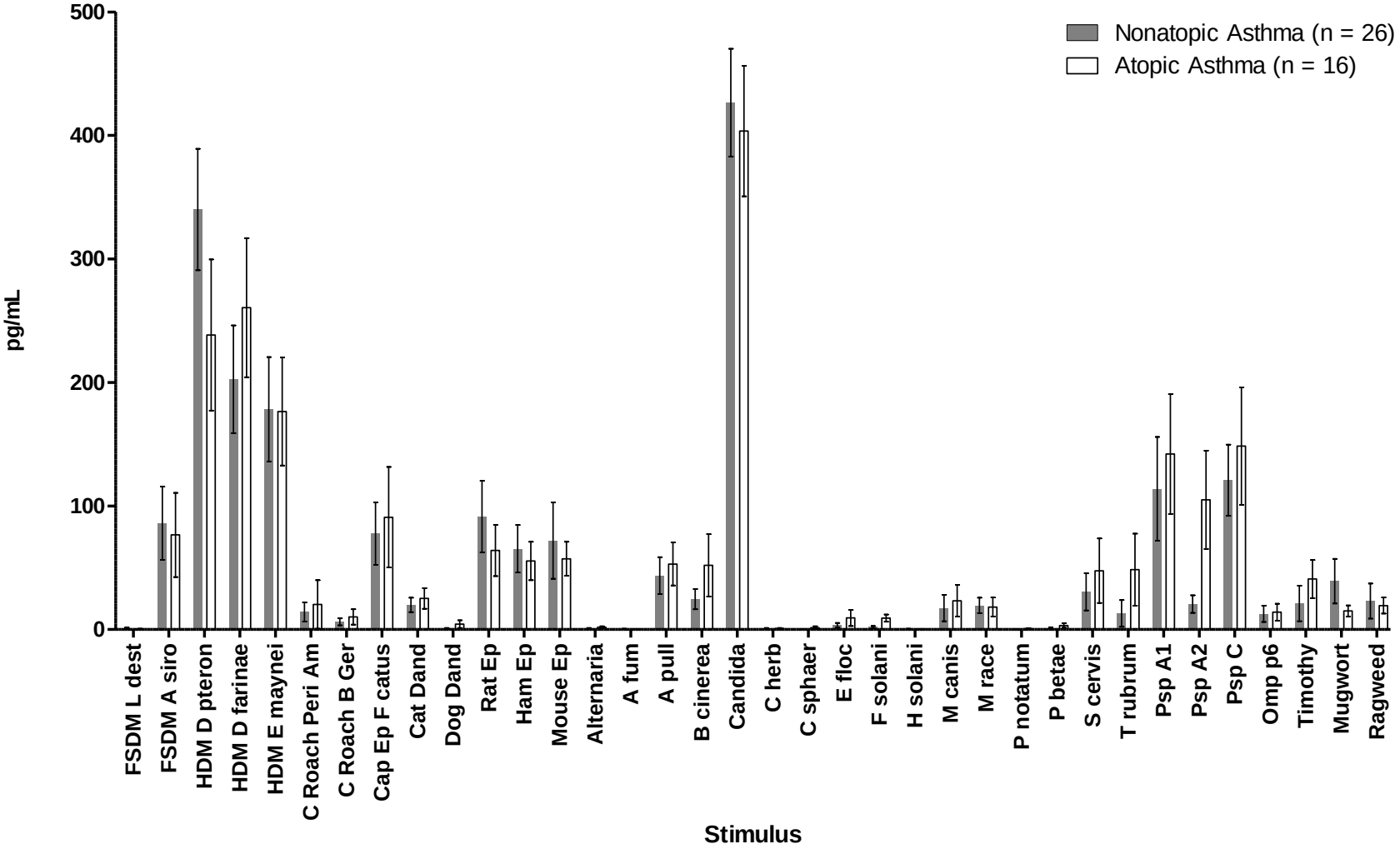


Figure 3.4 IL-17A Grouped: Atopic vs Nonatopic Asthma

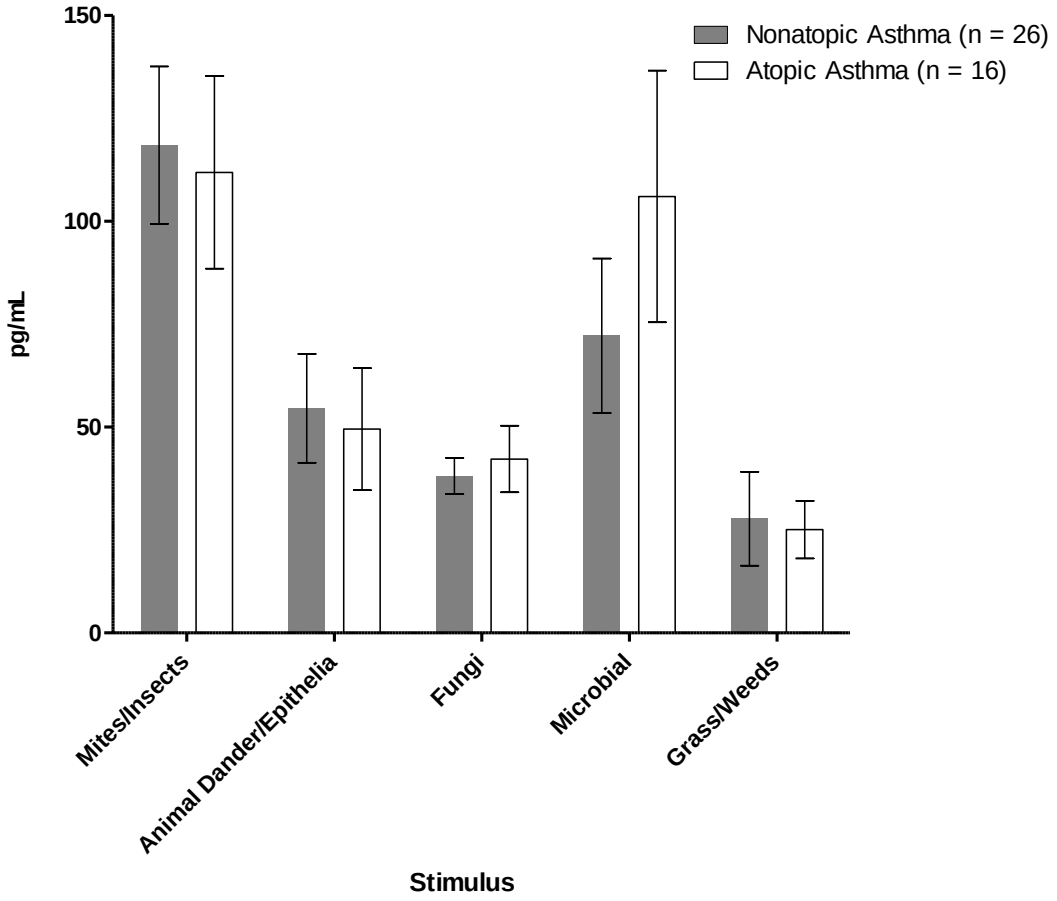


Figure 3.5 IL-17A: Eosinophilic vs Noneosinophilic

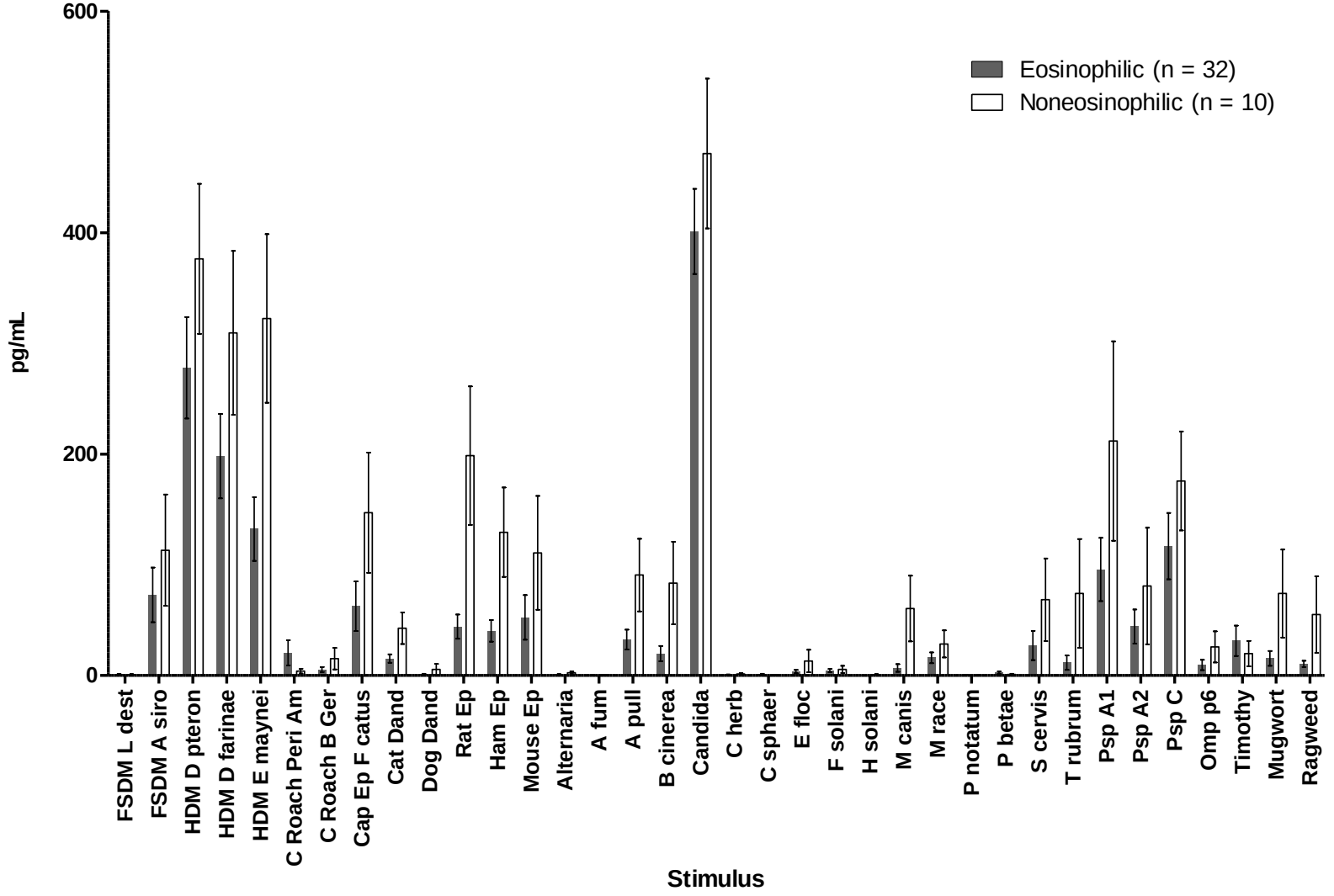


Figure 3.6 IL-17A Grouped: Eosinophilic vs Noneosinophilic

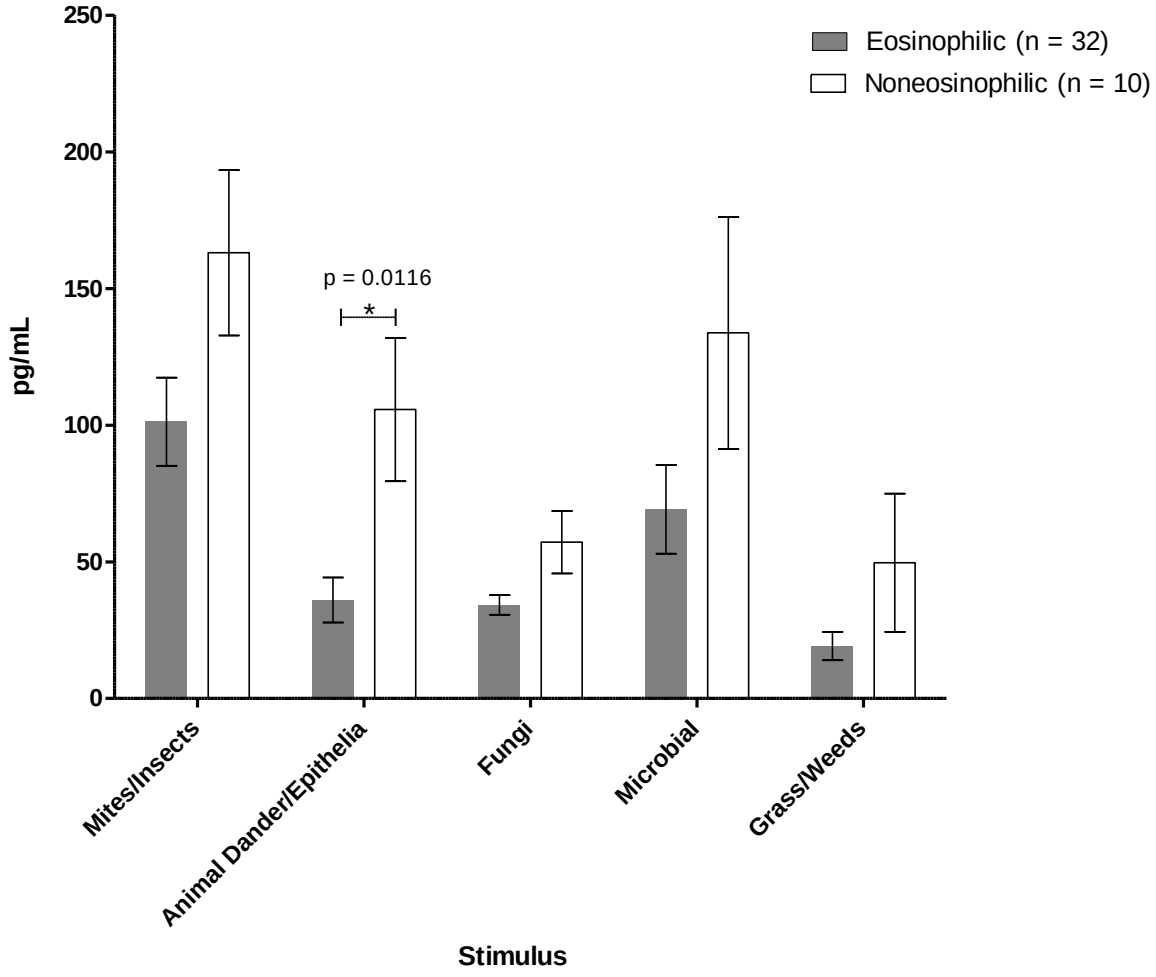


Figure 3.7 IL-17A: Neutrophilic vs Nonneutrophilic

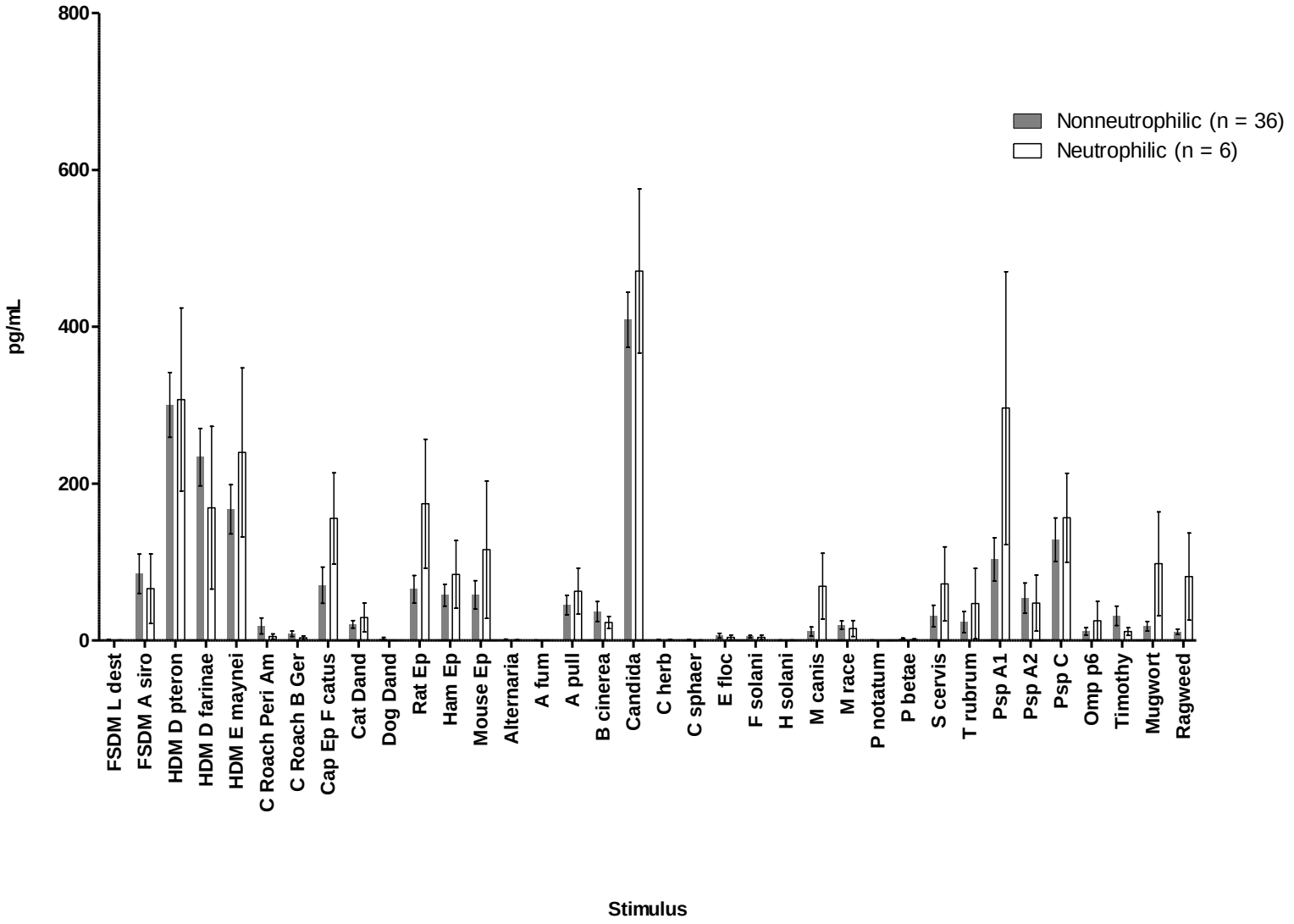


Figure 3.8 IL-17A Grouped: Neutrophilic vs Nonneutrophilic

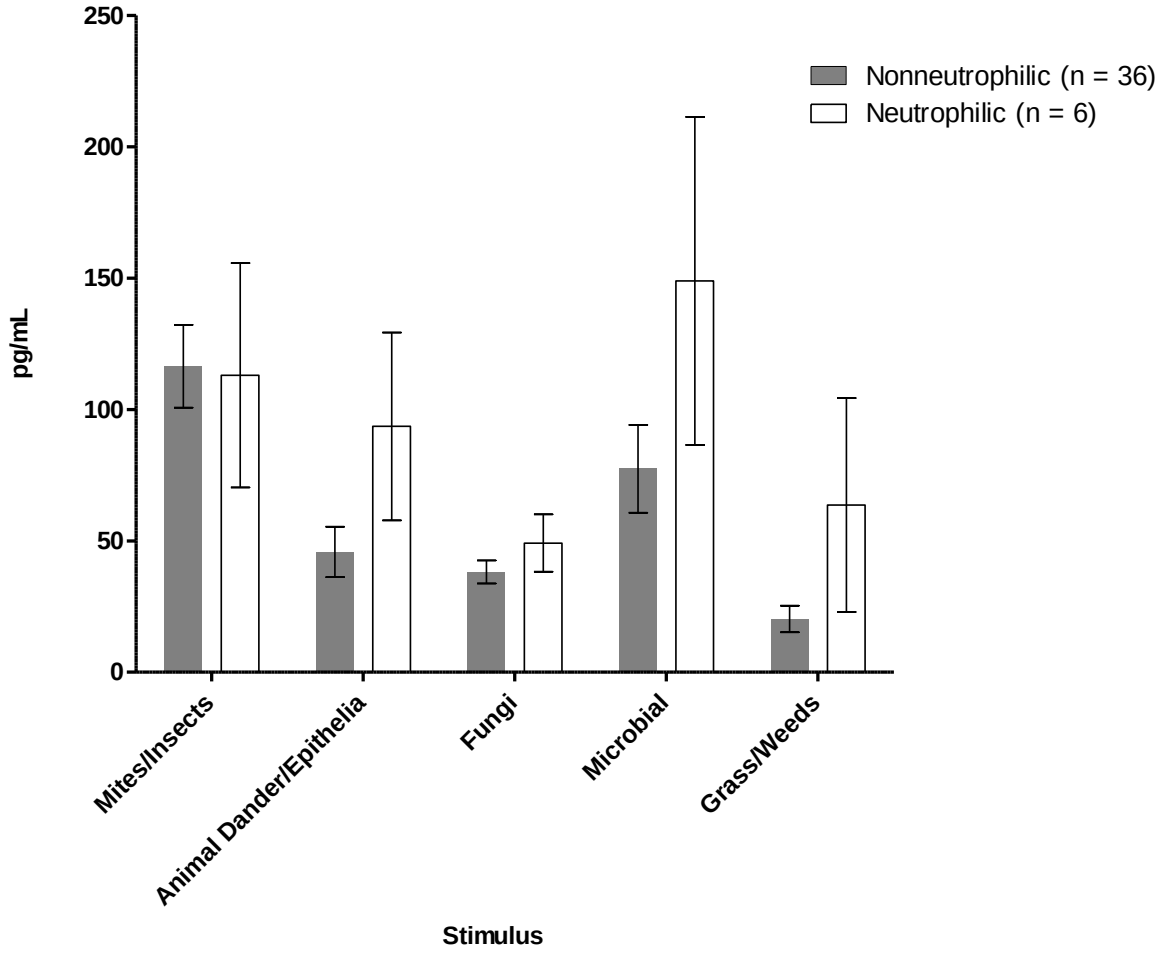


Figure 3.9 IL-17A Grouped: Nonatopic Asthma vs Normal

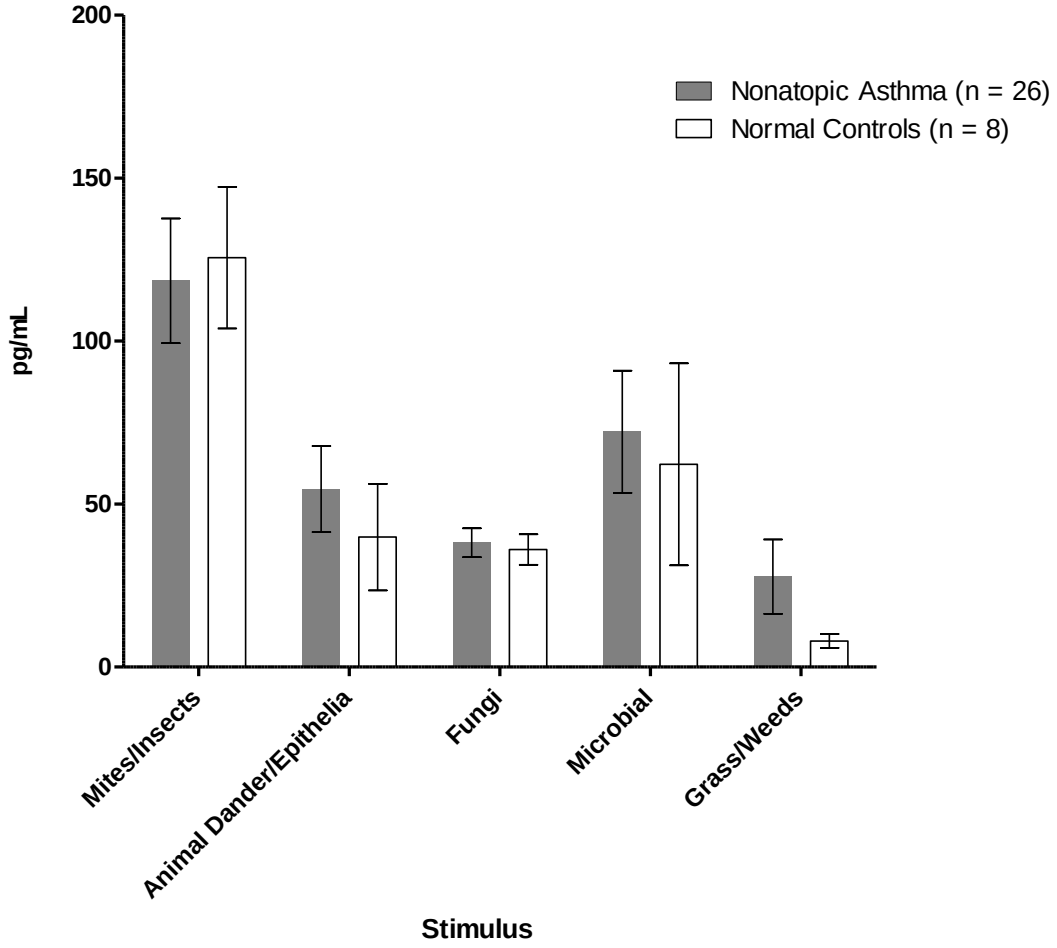


Figure 4.1 IL-23: Asthma vs Normal

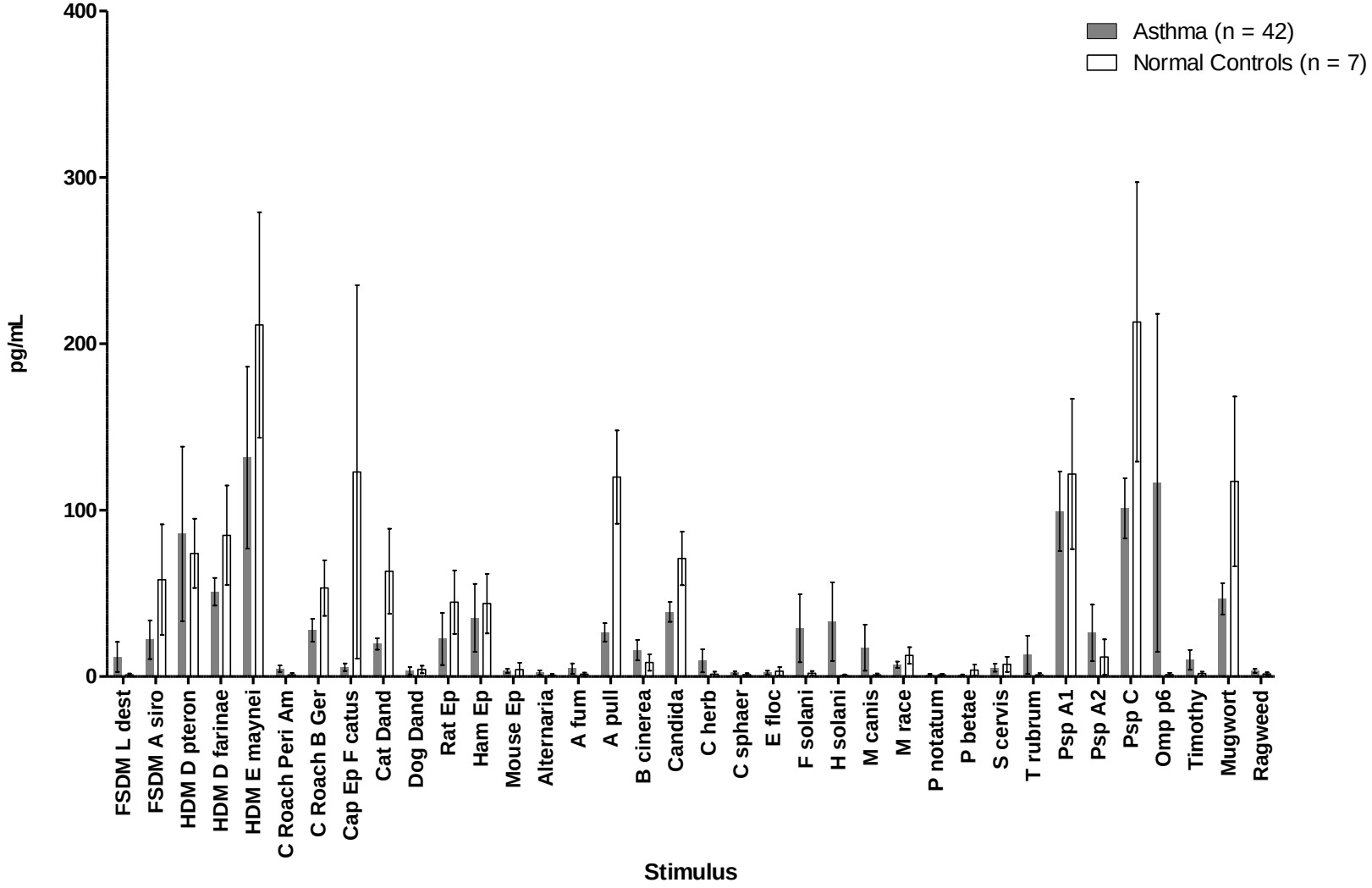


Figure 4.2 IL-23 Grouped: Asthma vs Normal

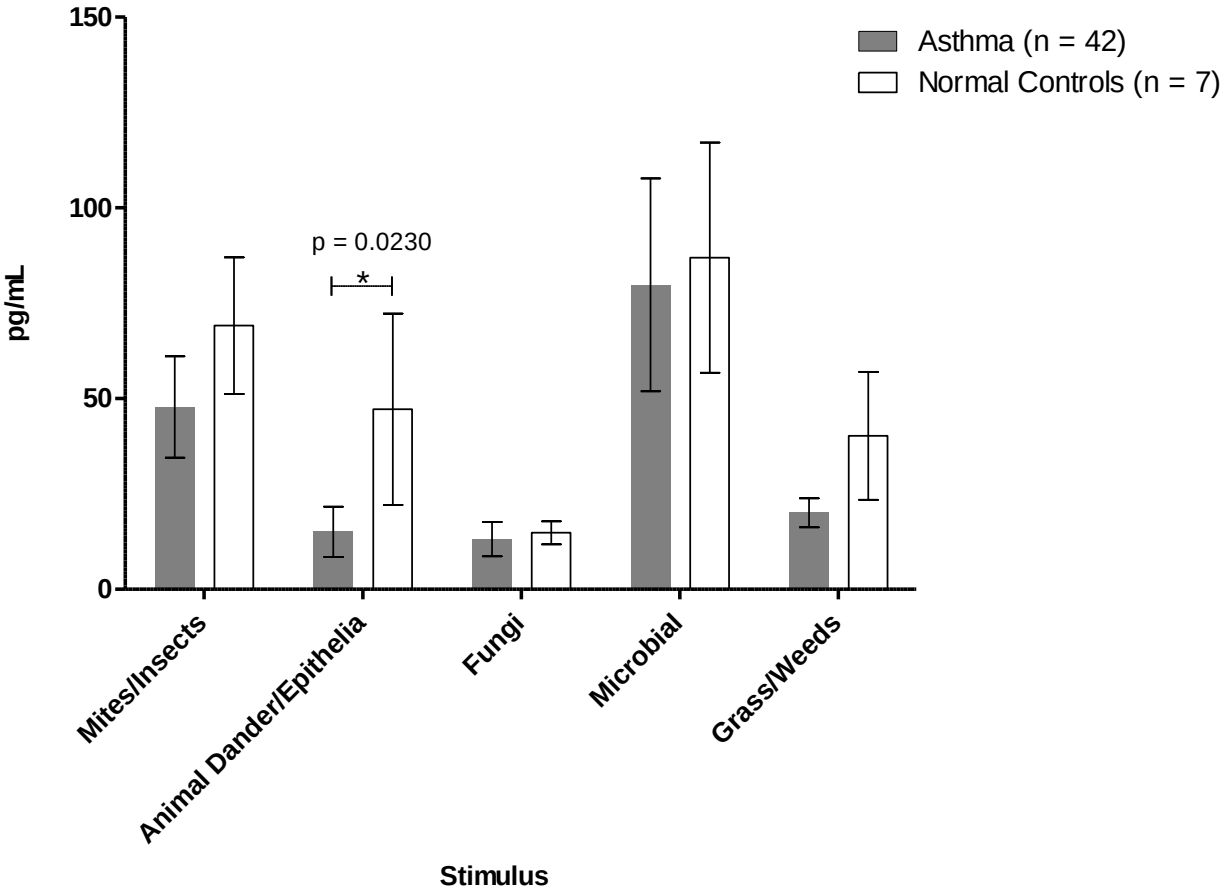


Figure 4.3 IL-23: Atopic vs Nonatopic Asthma

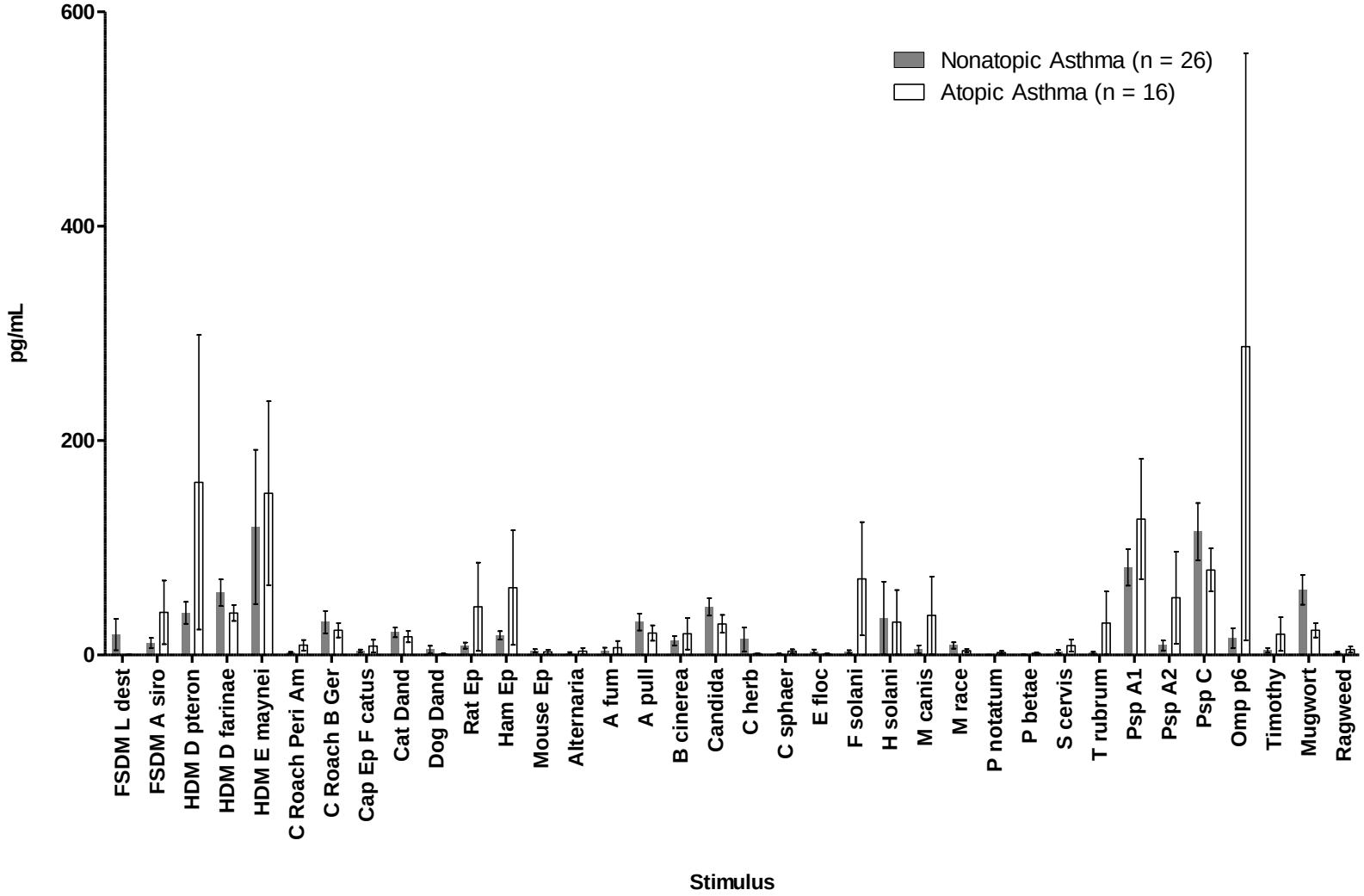


Figure 4.4 IL-23 Grouped: Atopic vs Nonatopic Asthma

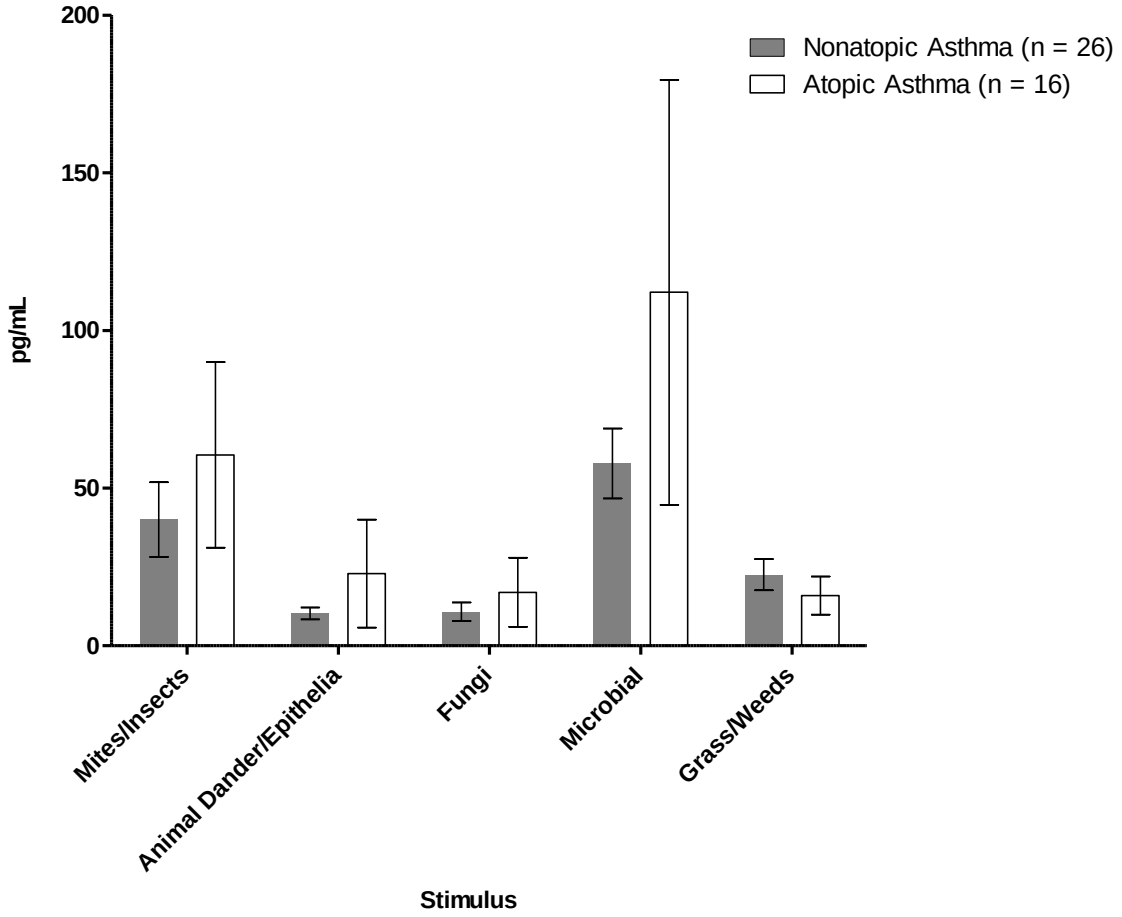


Figure 4.5 IL-23: Eosinophilic vs Noneosinophilic

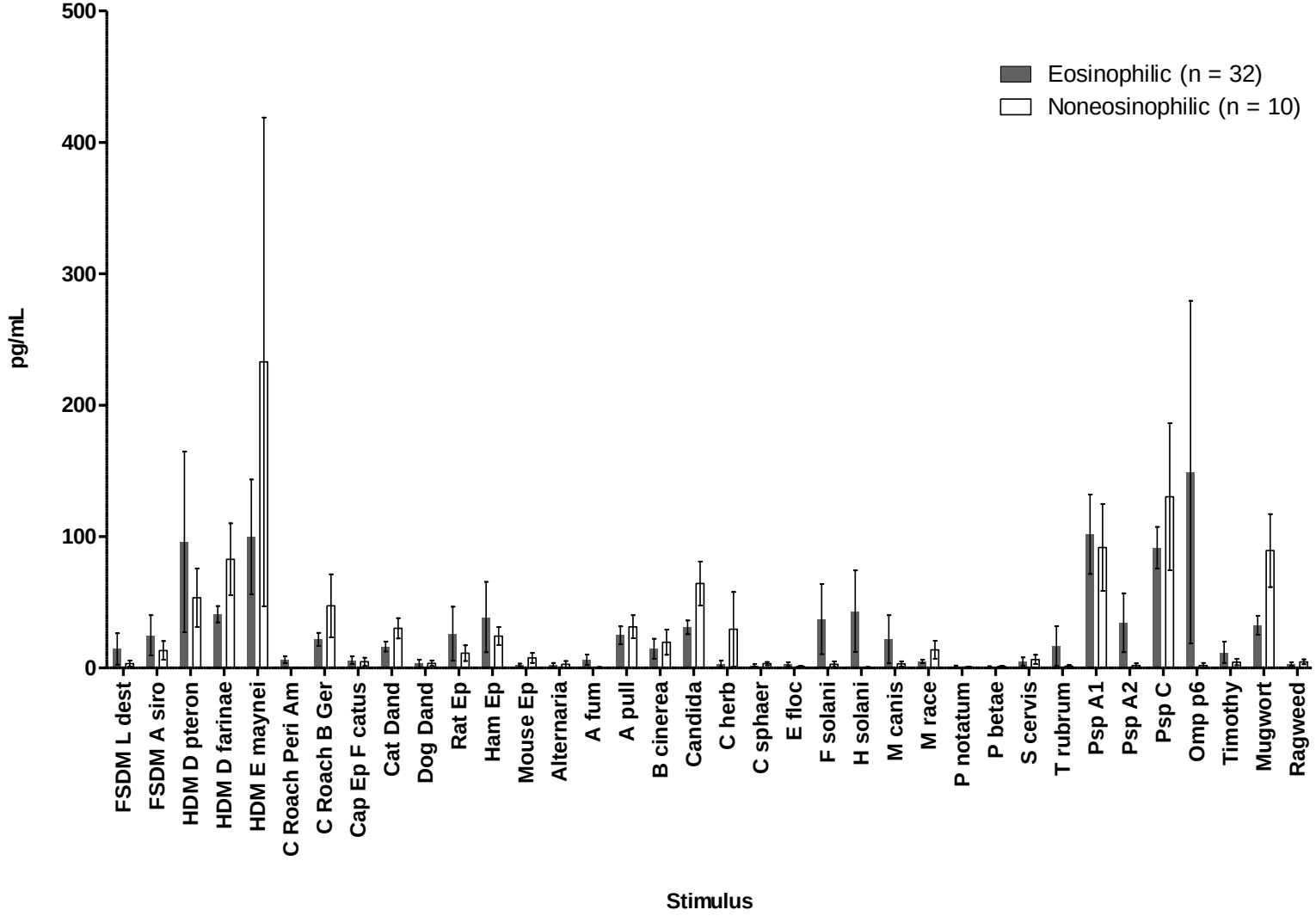


Figure 4.6 IL-23 Grouped: Eosinophilic vs Noneosinophilic

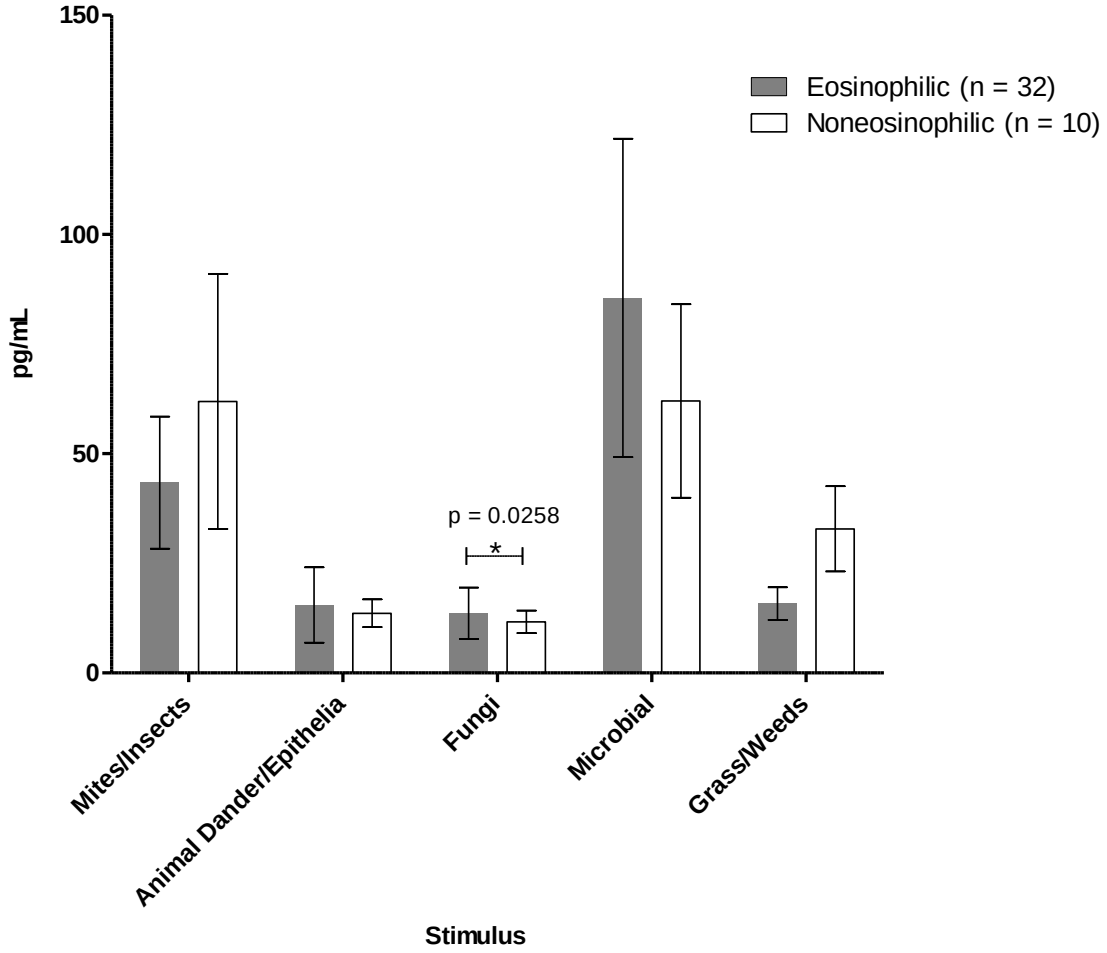


Figure 4.7 IL-23: Neutrophilic vs Nonneutrophilic

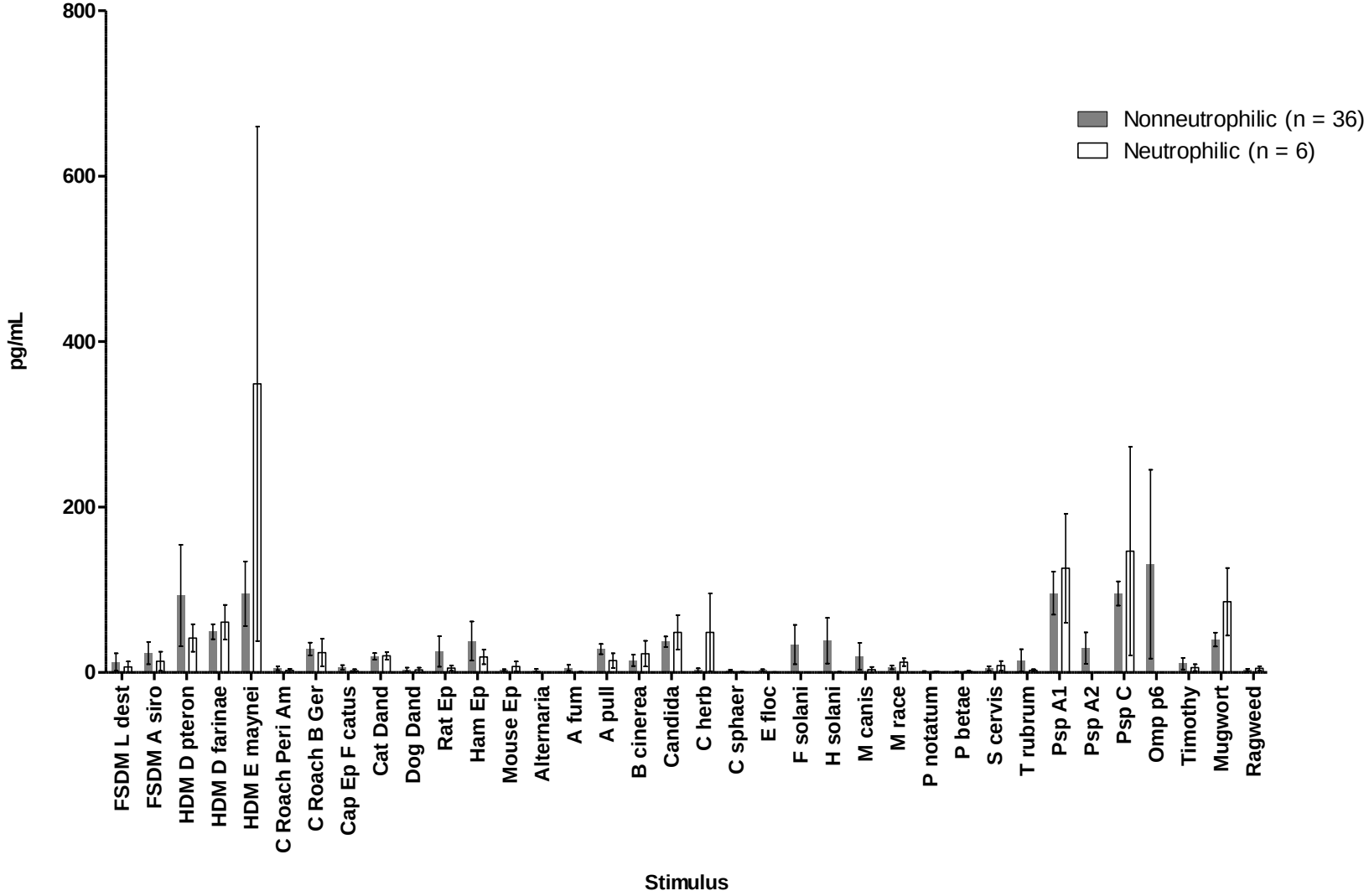


Figure 4.8 IL-23 Grouped: Neutrophilic vs Nonneutrophilic

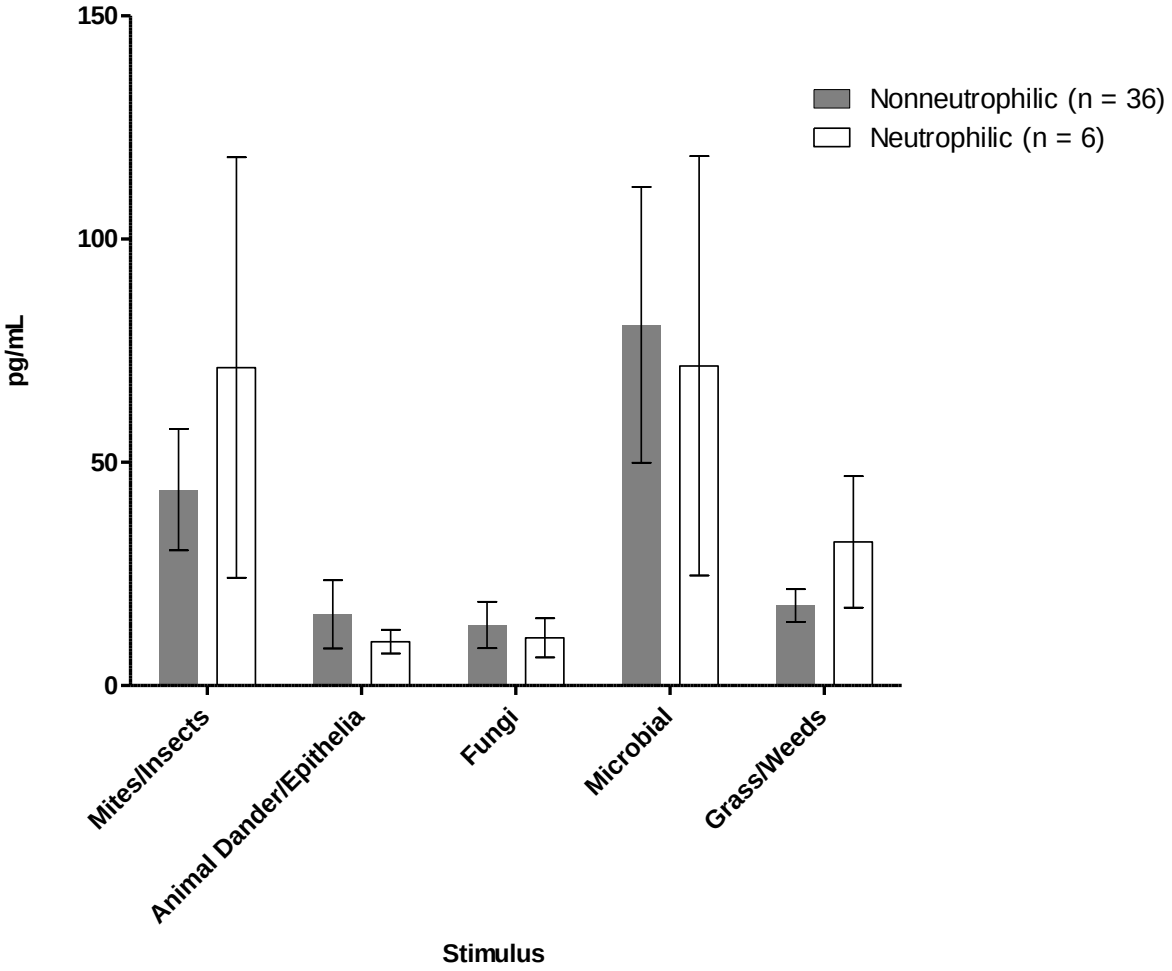


Figure 4.9 IL-23 Grouped: Nonatopic Asthma vs Normal

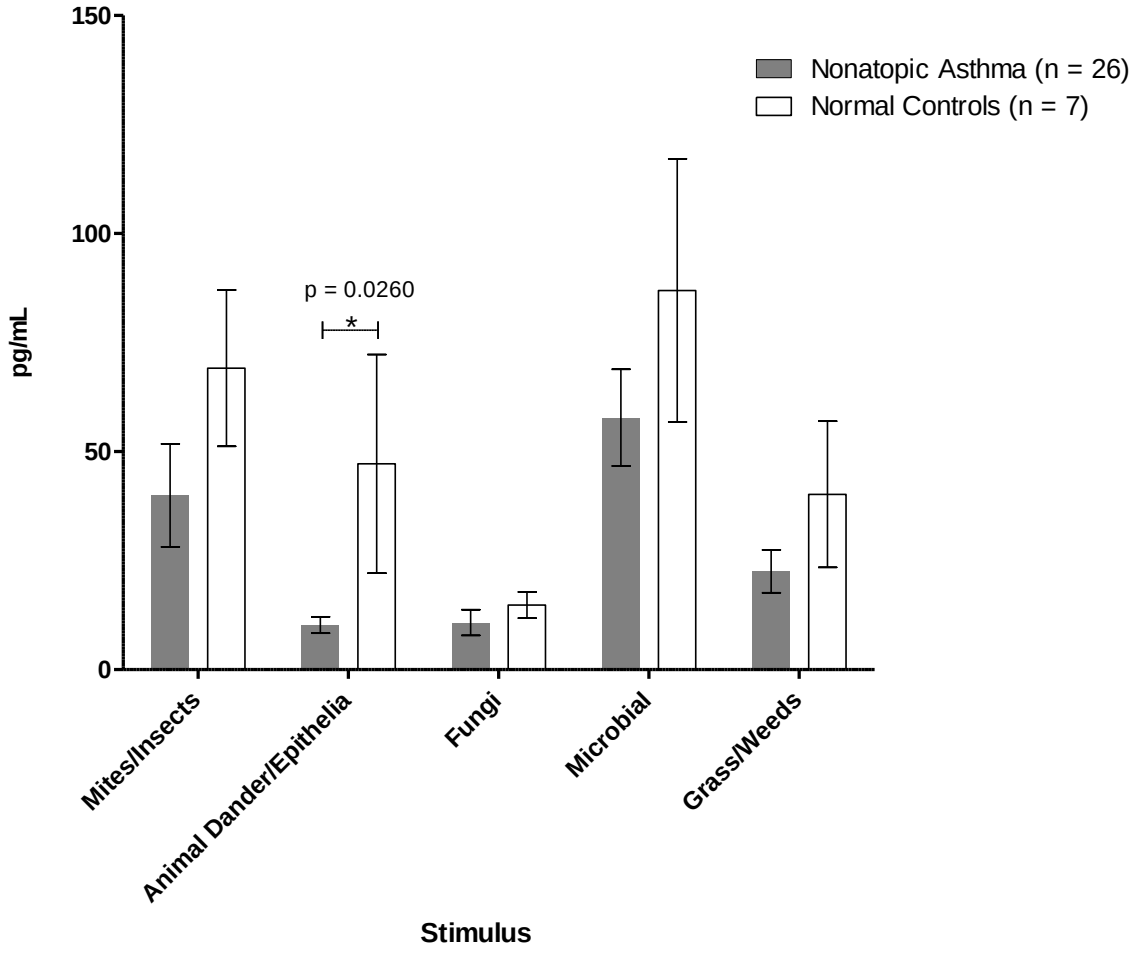


Figure 5.1 IL-10: Asthma vs Normal

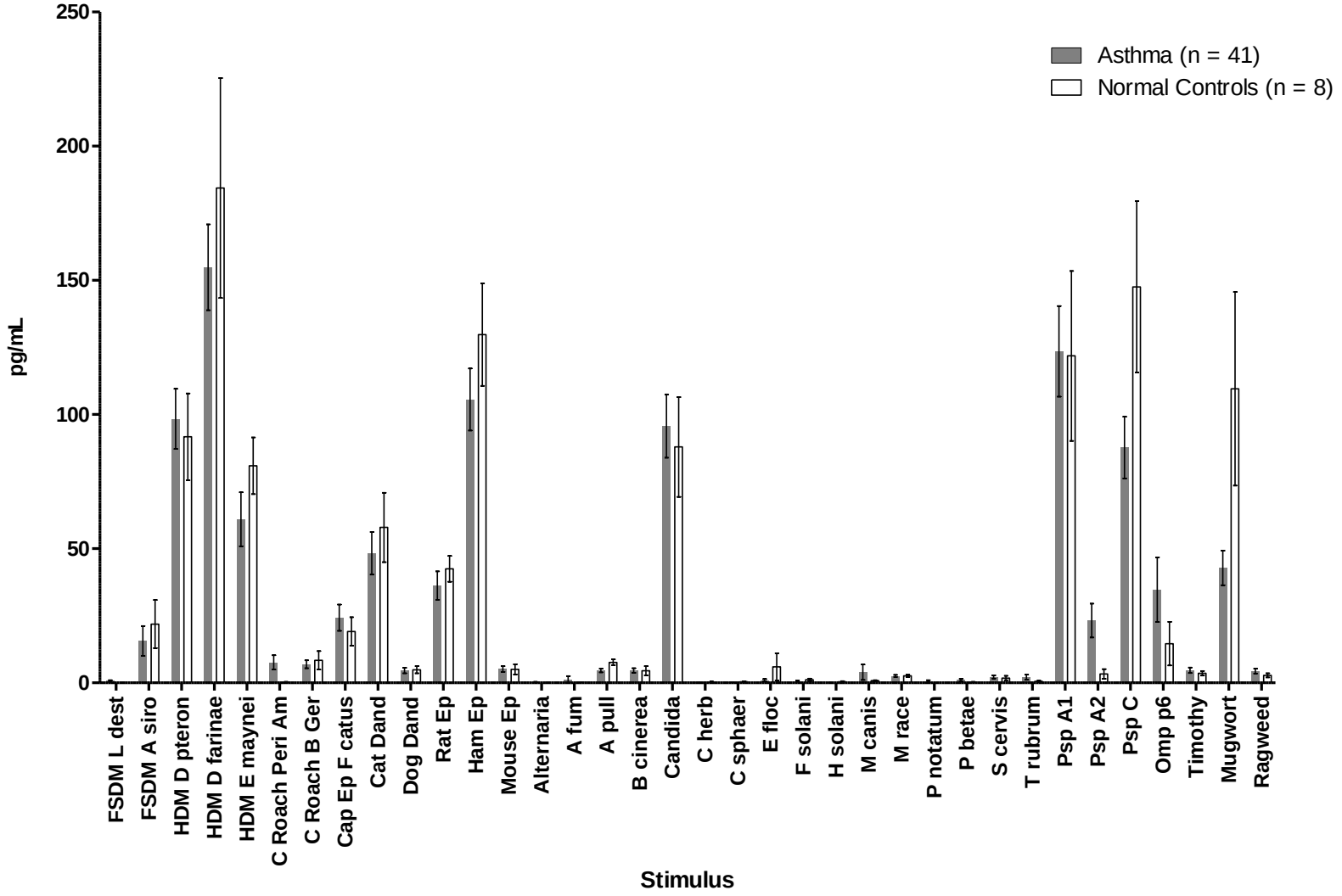


Figure 5.2 IL-10 Grouped: Asthma vs Normal

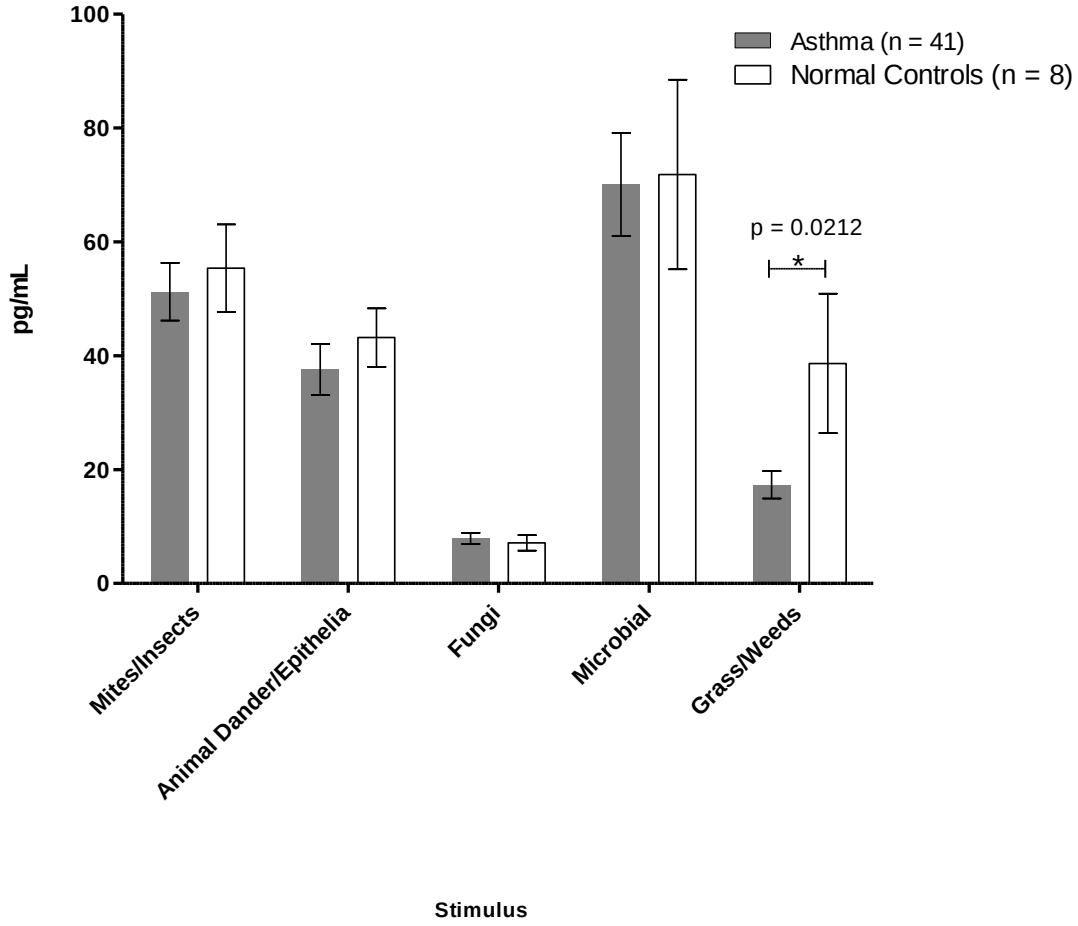


Figure 5.3 IL-10: Atopic vs Nonatopic Asthma

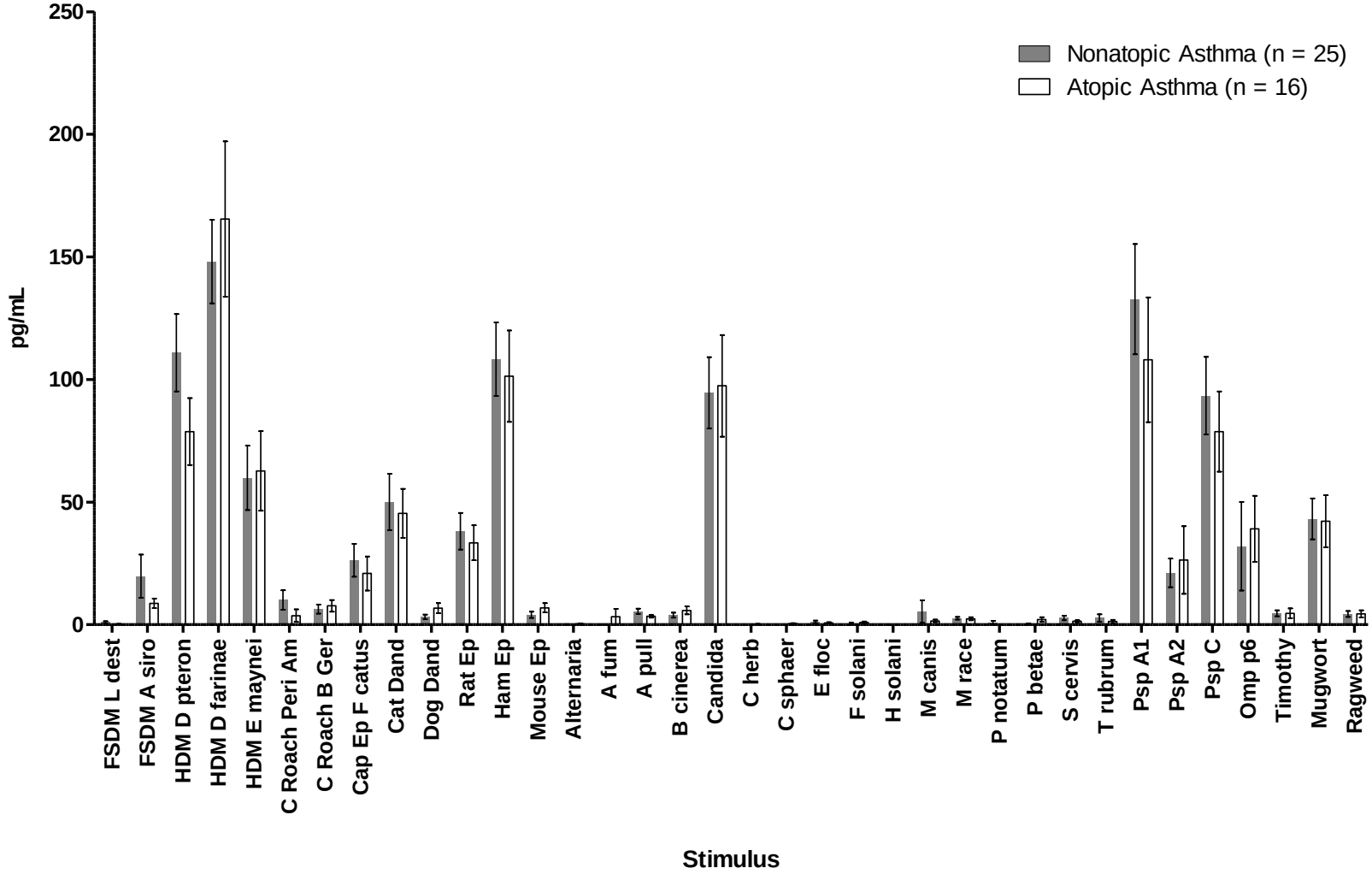


Figure 5.4 IL-10 Grouped: Atopic vs Nonatopic Asthma

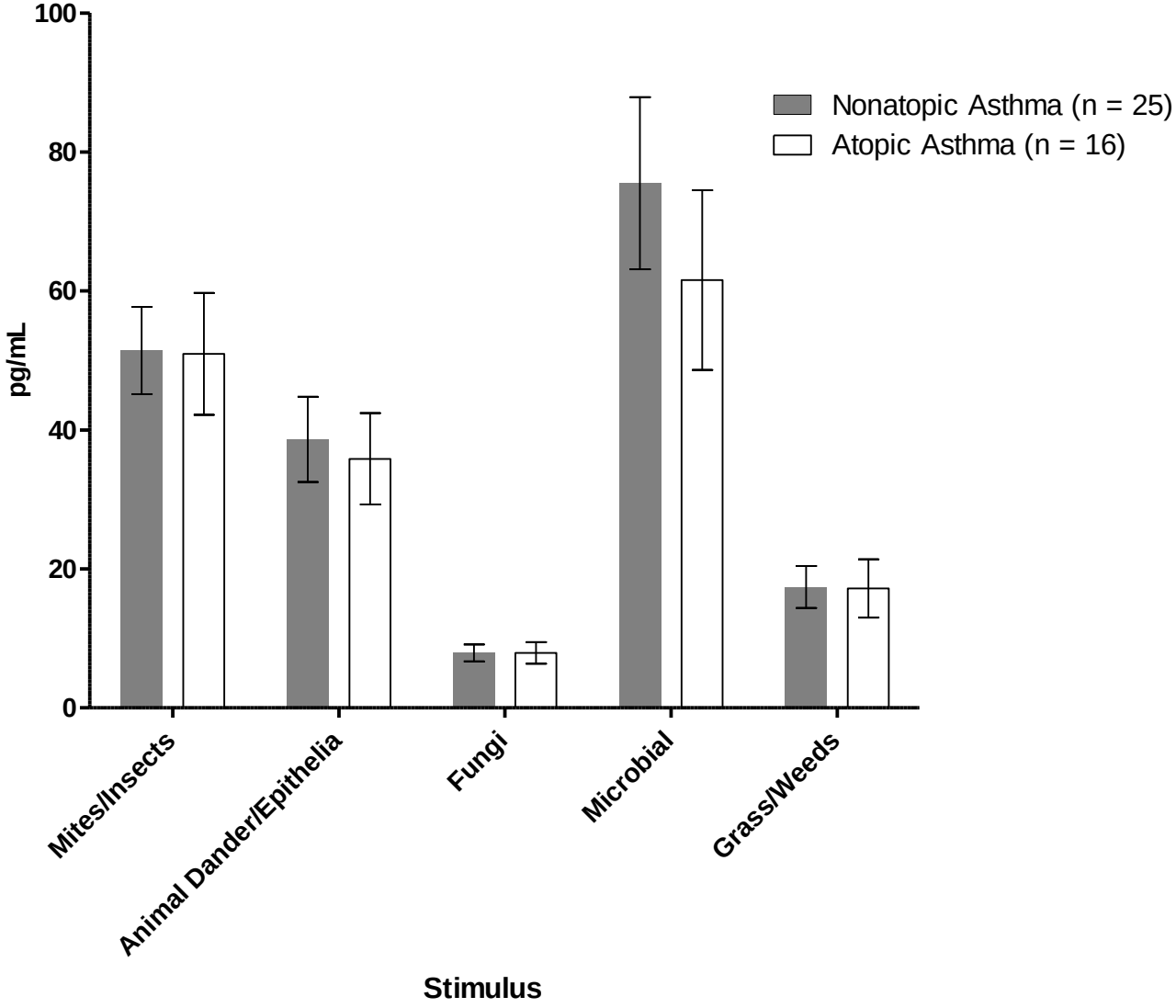


Figure 5.5 IL-10: Eosinophilic vs Noneosinophilic

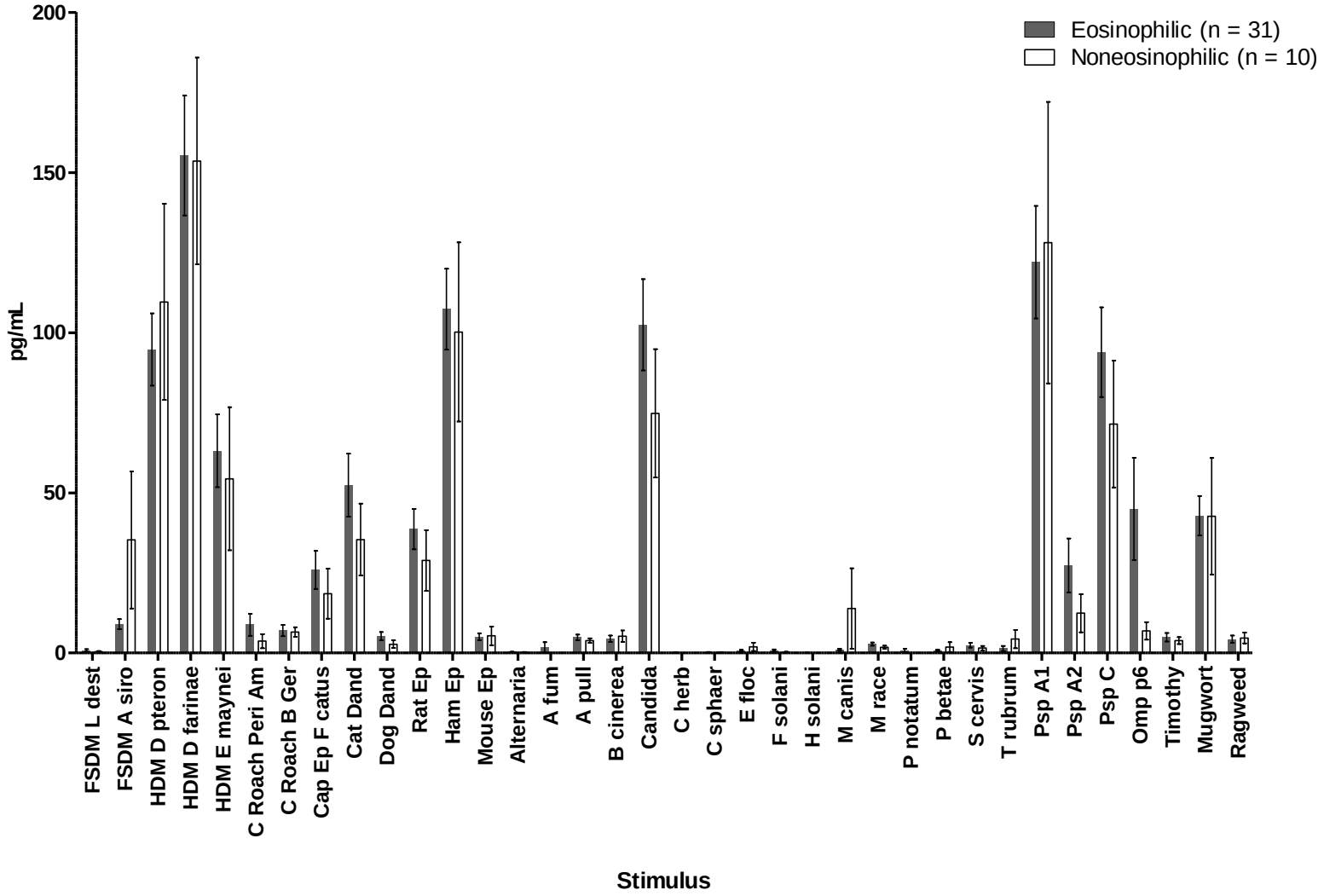


Figure 5.6 IL-10 Grouped: Eosinophilic vs Noneosinophilic

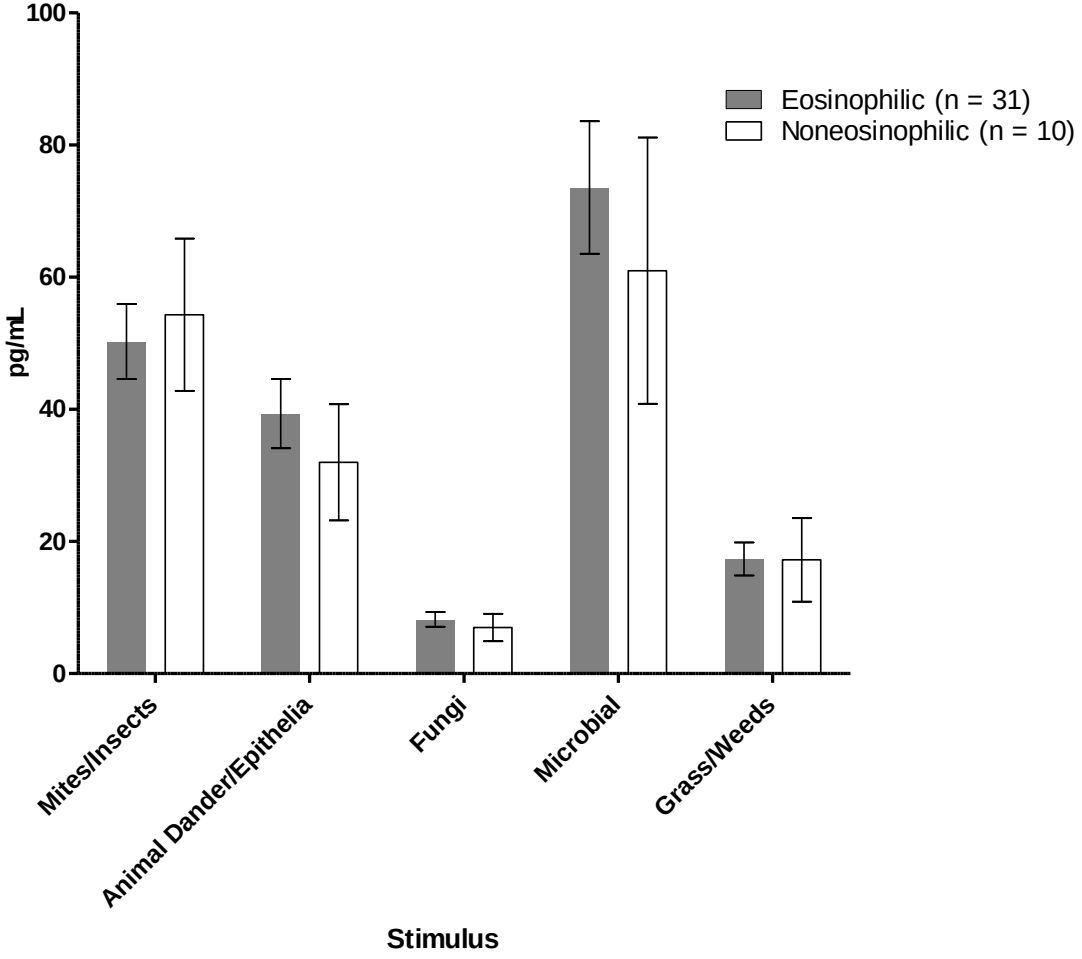


Figure 5.7 IL-10: Neutrophilic vs Nonneutrophilic

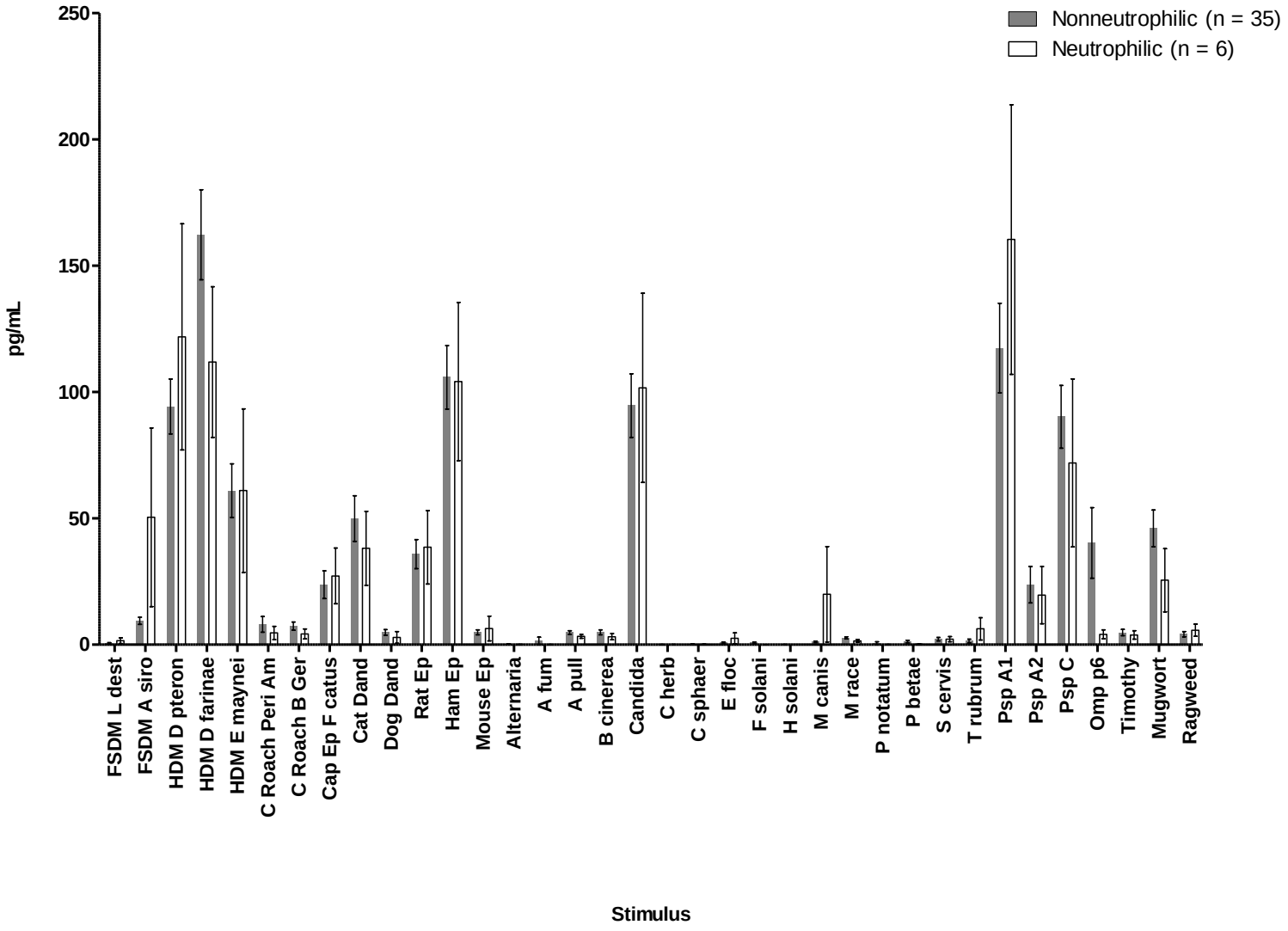


Figure 5.8 IL-10 Grouped: Neutrophilic vs Nonneutrophilic

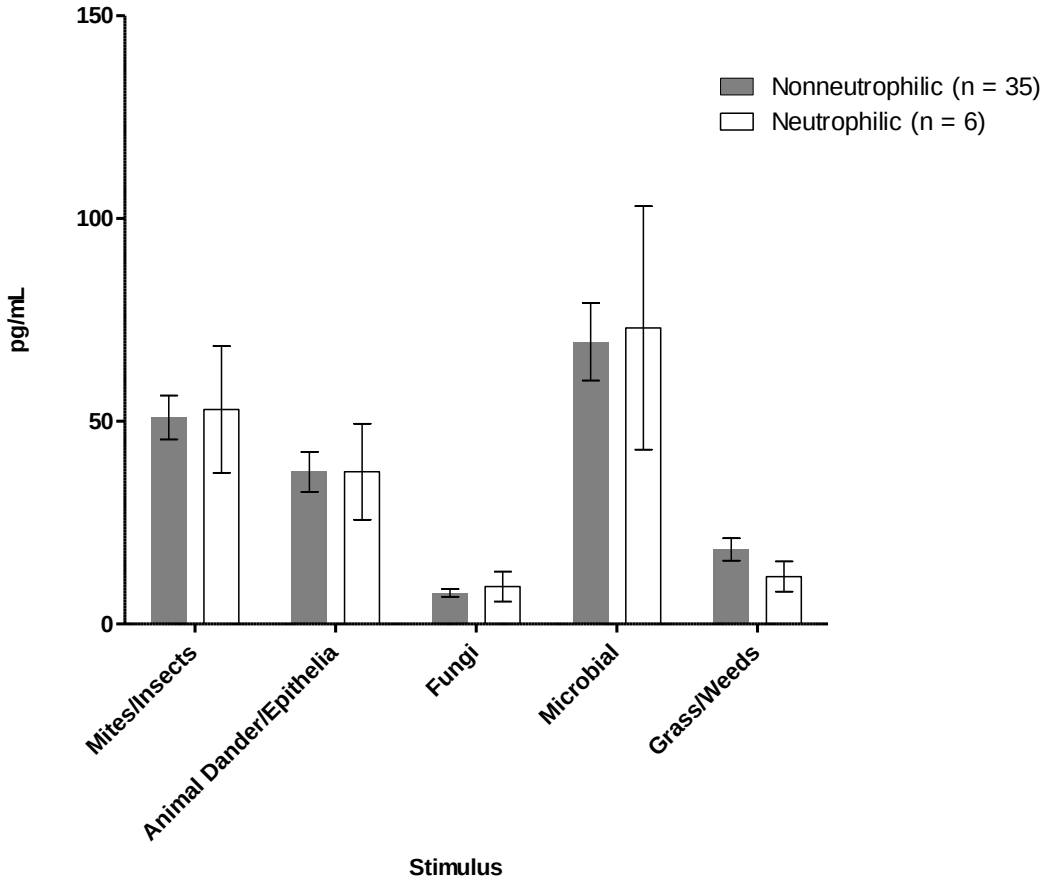


Figure 6.1 IFN-gamma: Asthma vs Normal

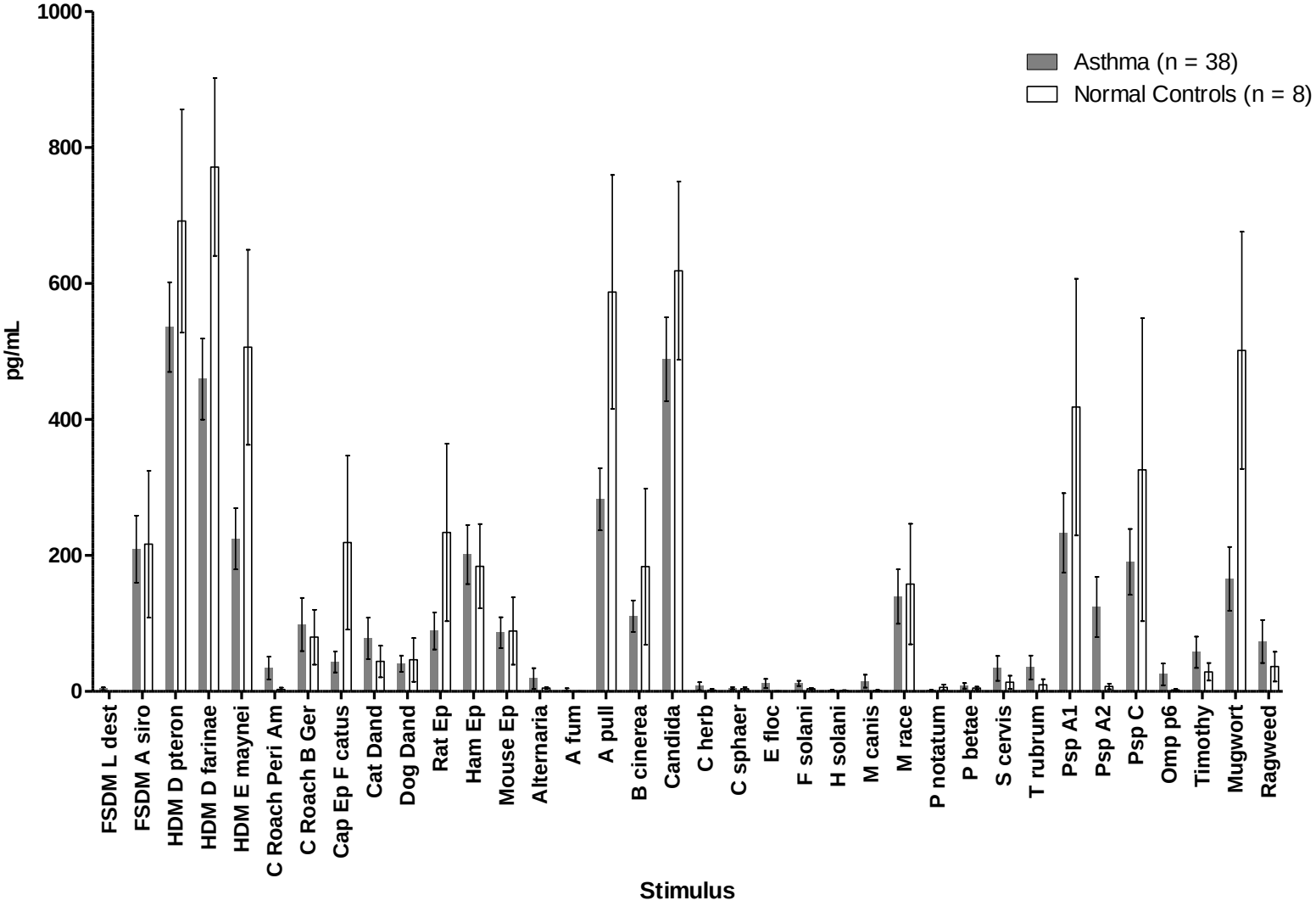


Figure 6.2 IFN-gamma Grouped: Asthma vs Normal

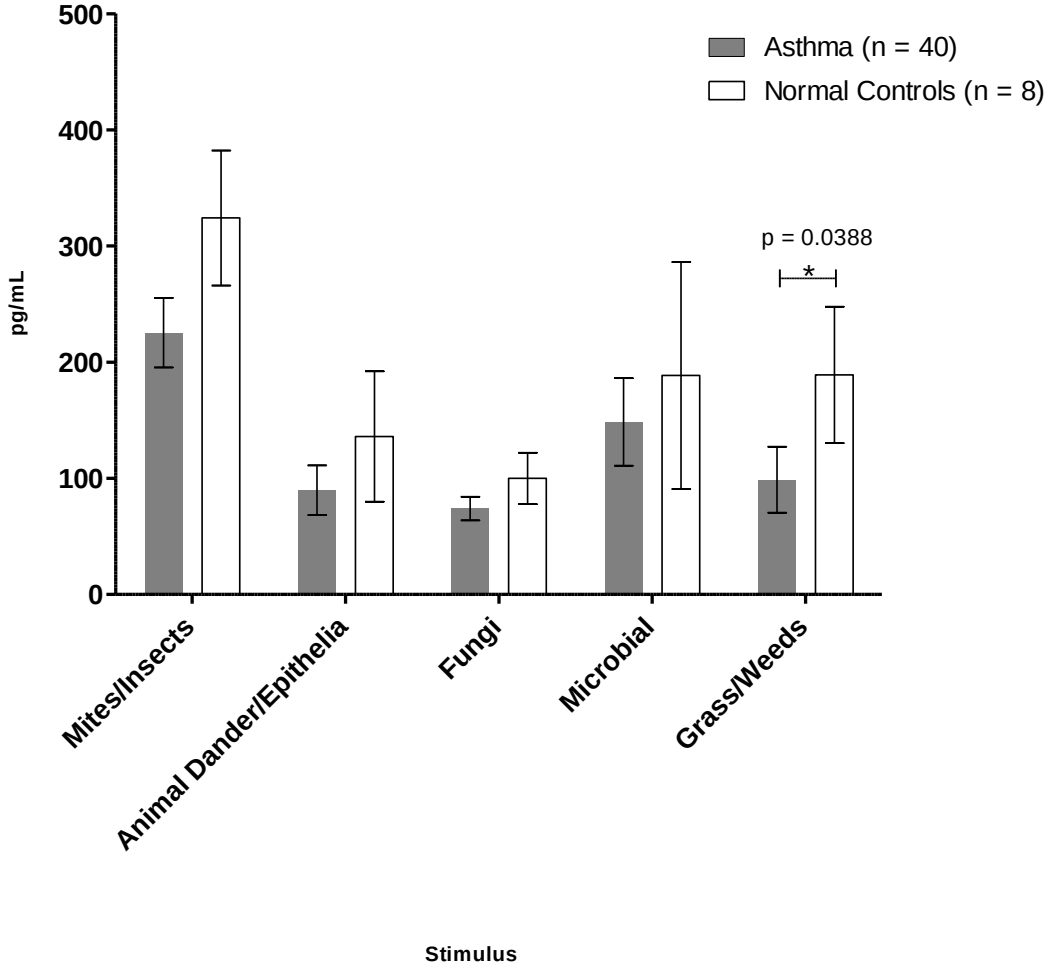


Figure 6.3 IFN-gamma: Atopic vs Nonatopic Asthma

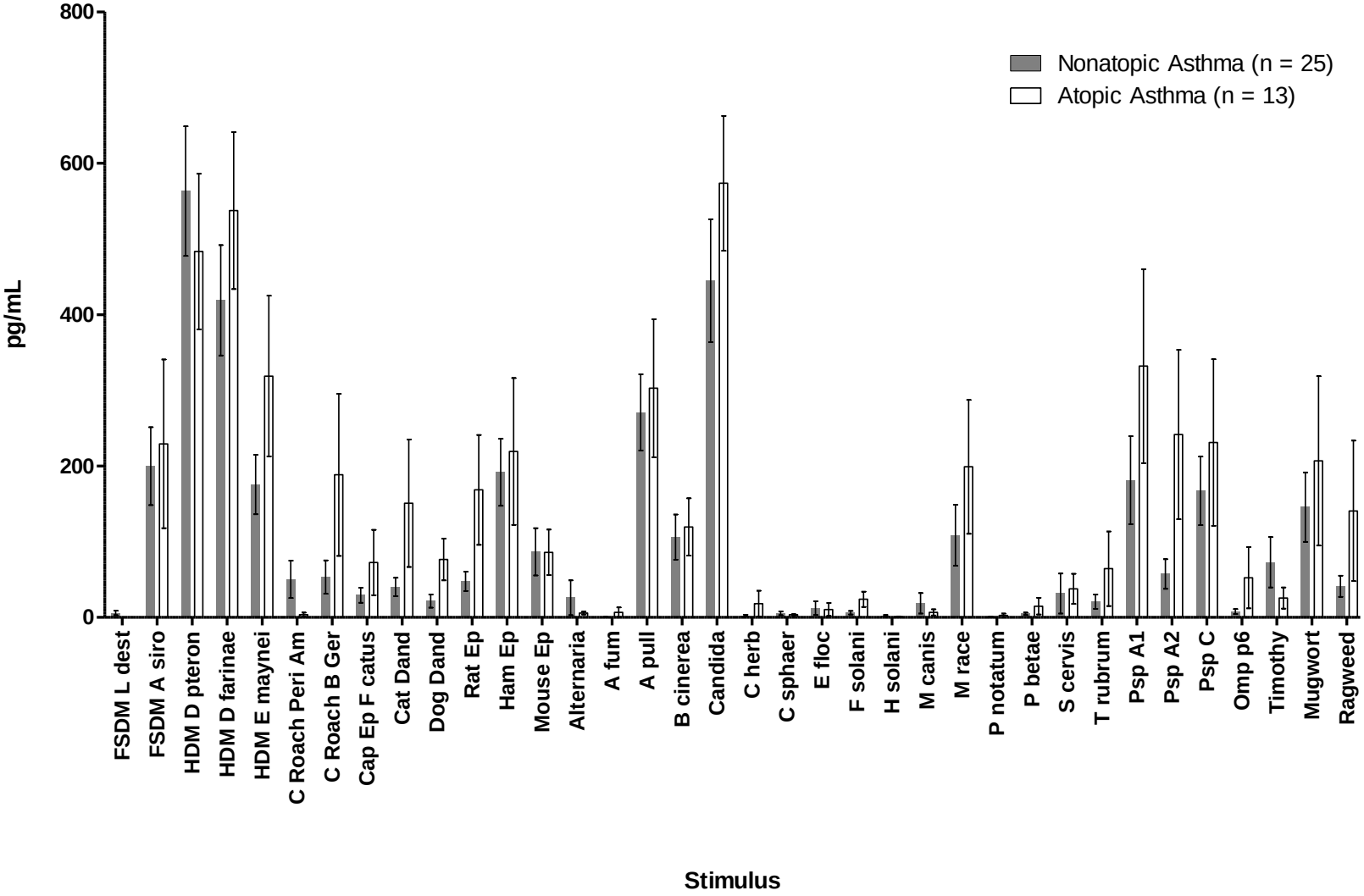


Figure 6.4 IFN-gamma Grouped: Atopic vs Nonatopic Asthma

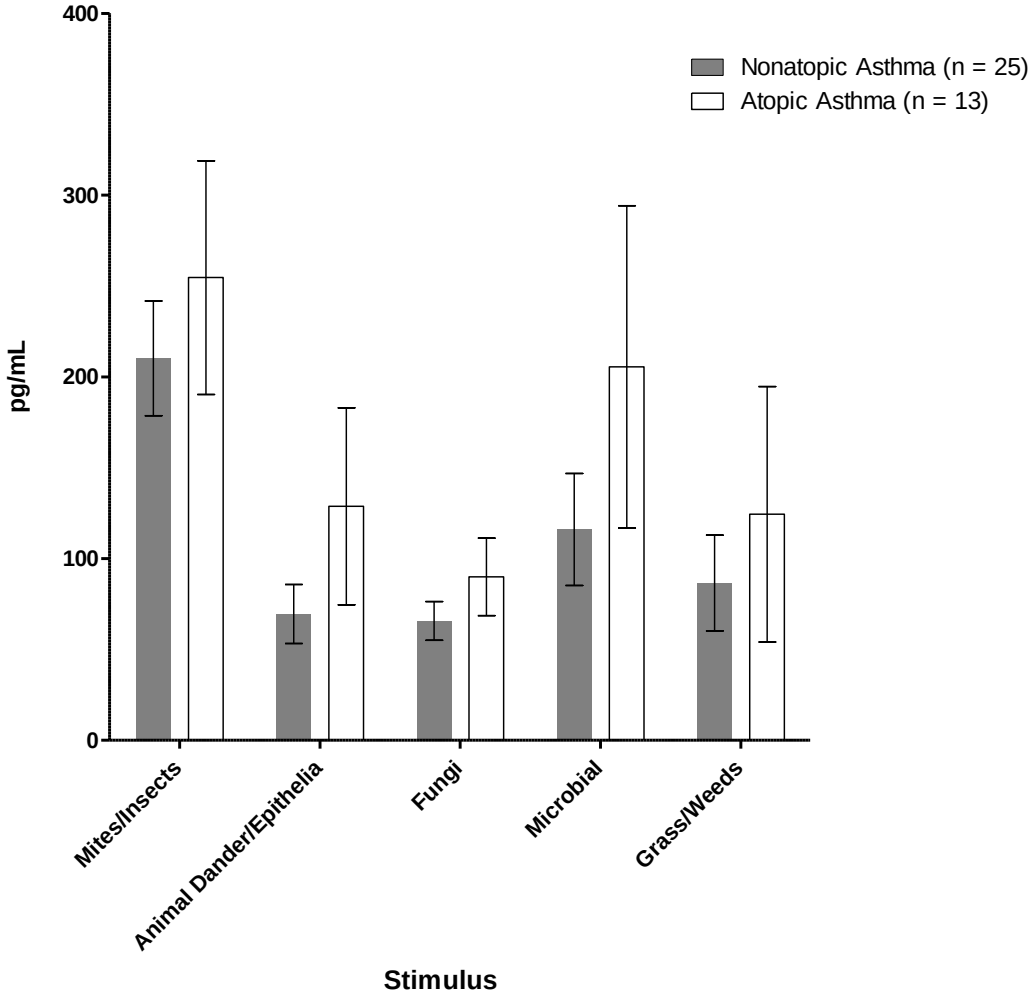


Figure 6.5 IFN-gamma: Eosinophilic vs Noneosinophilic

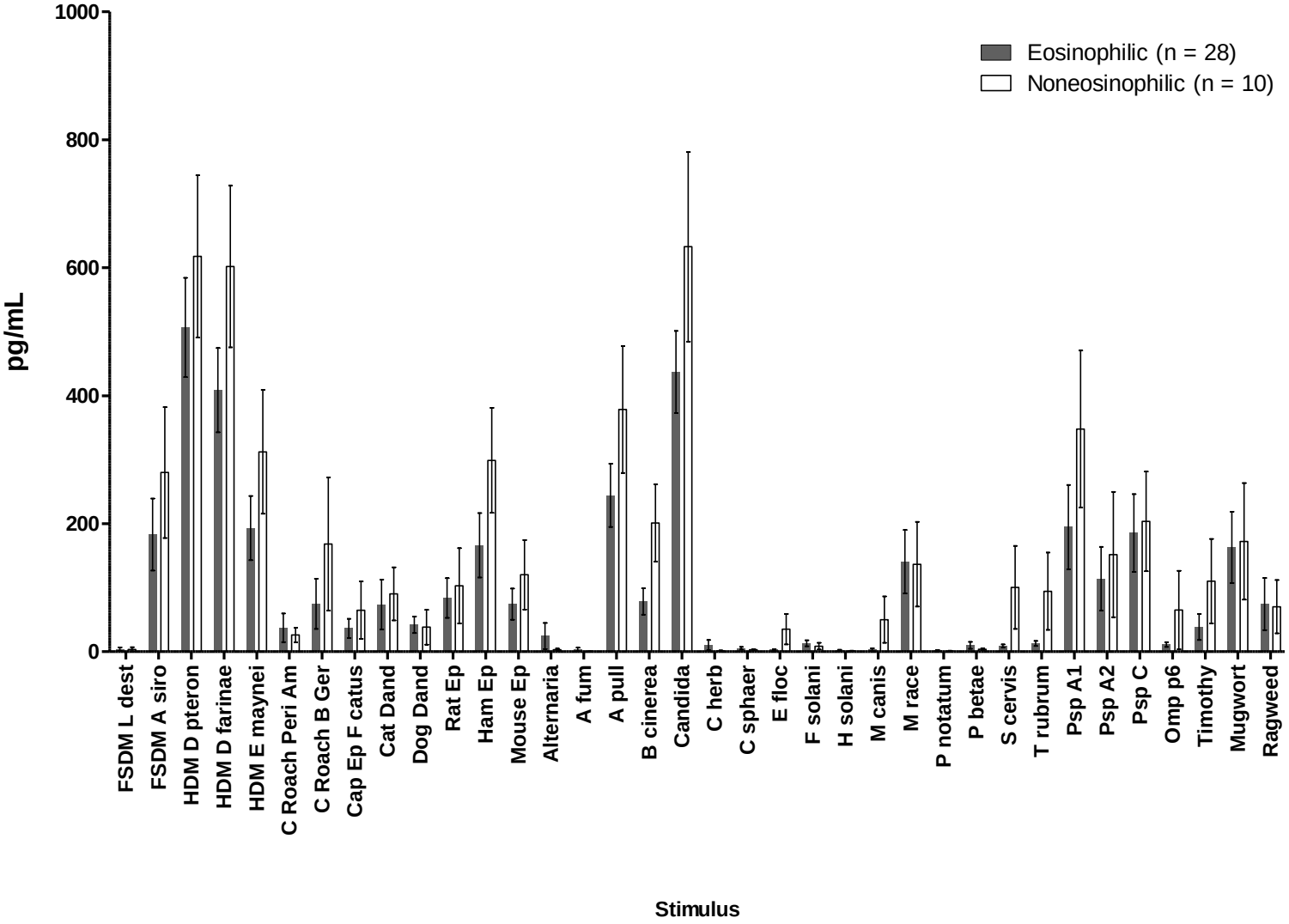


Figure 6.6 IFN-gamma Grouped: Eosinophilic vs Noneosinophilic

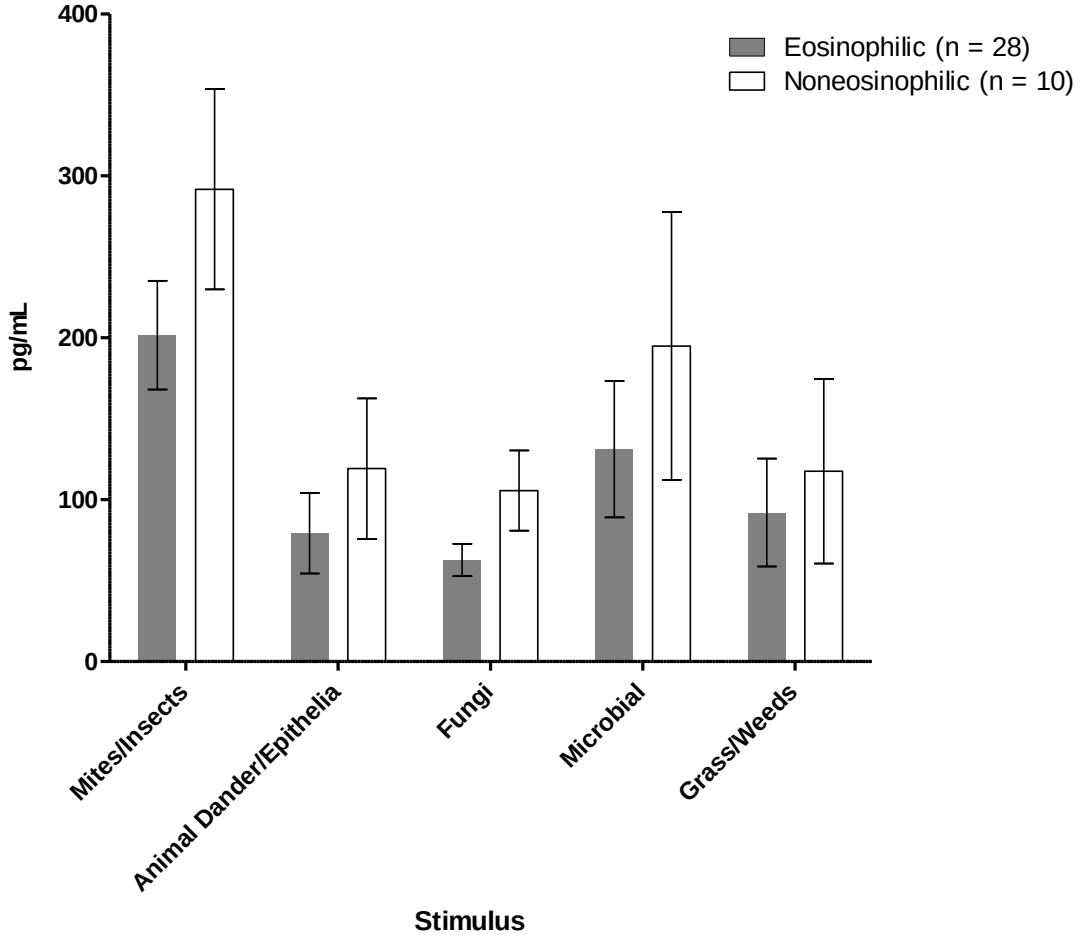


Figure 6.7 IFN-gamma: Neutrophilic vs Nonneutrophilic

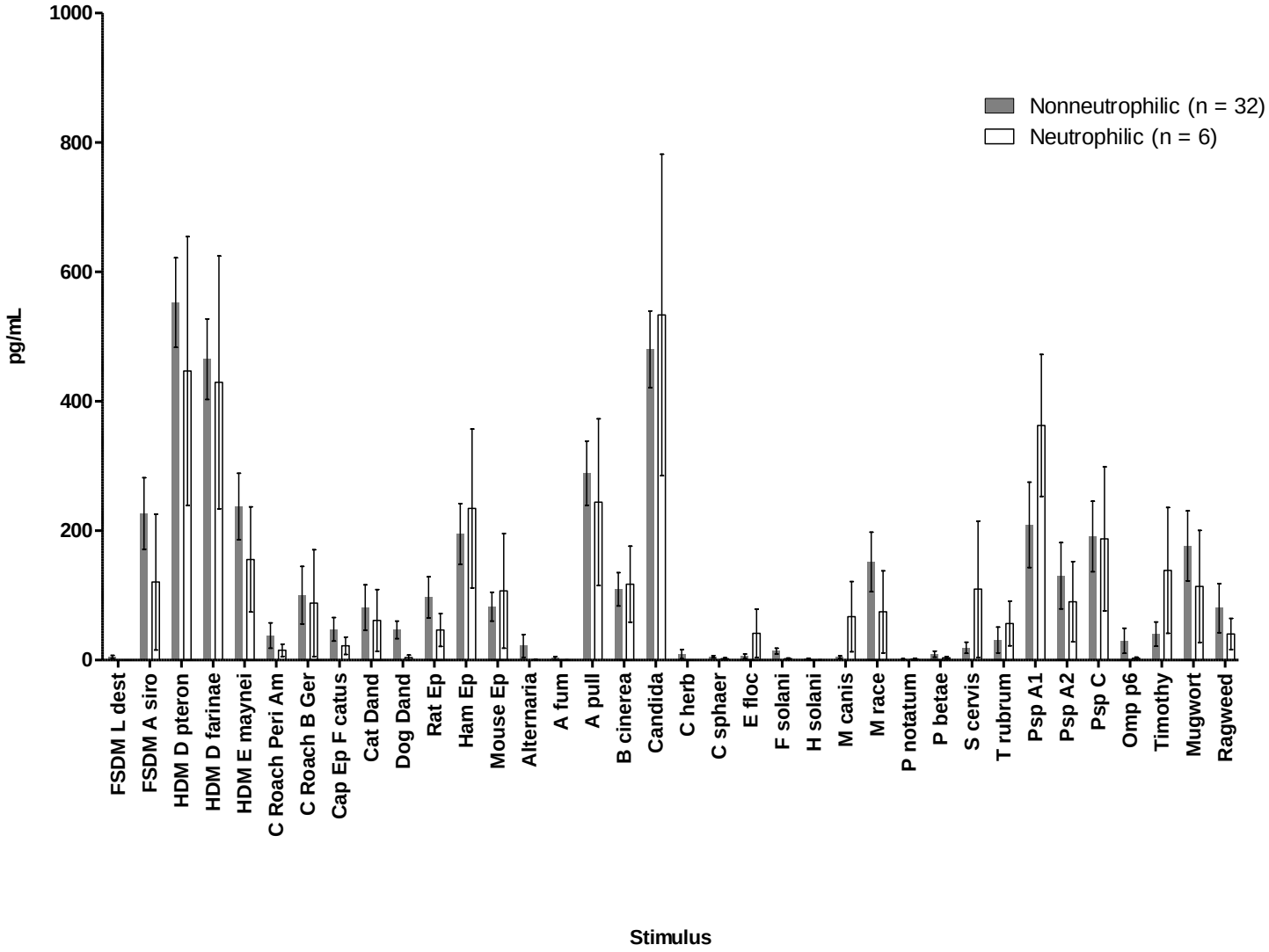


Figure 6.8 IFN-gamma Grouped: Neutrophilic vs Nonneutrophilic

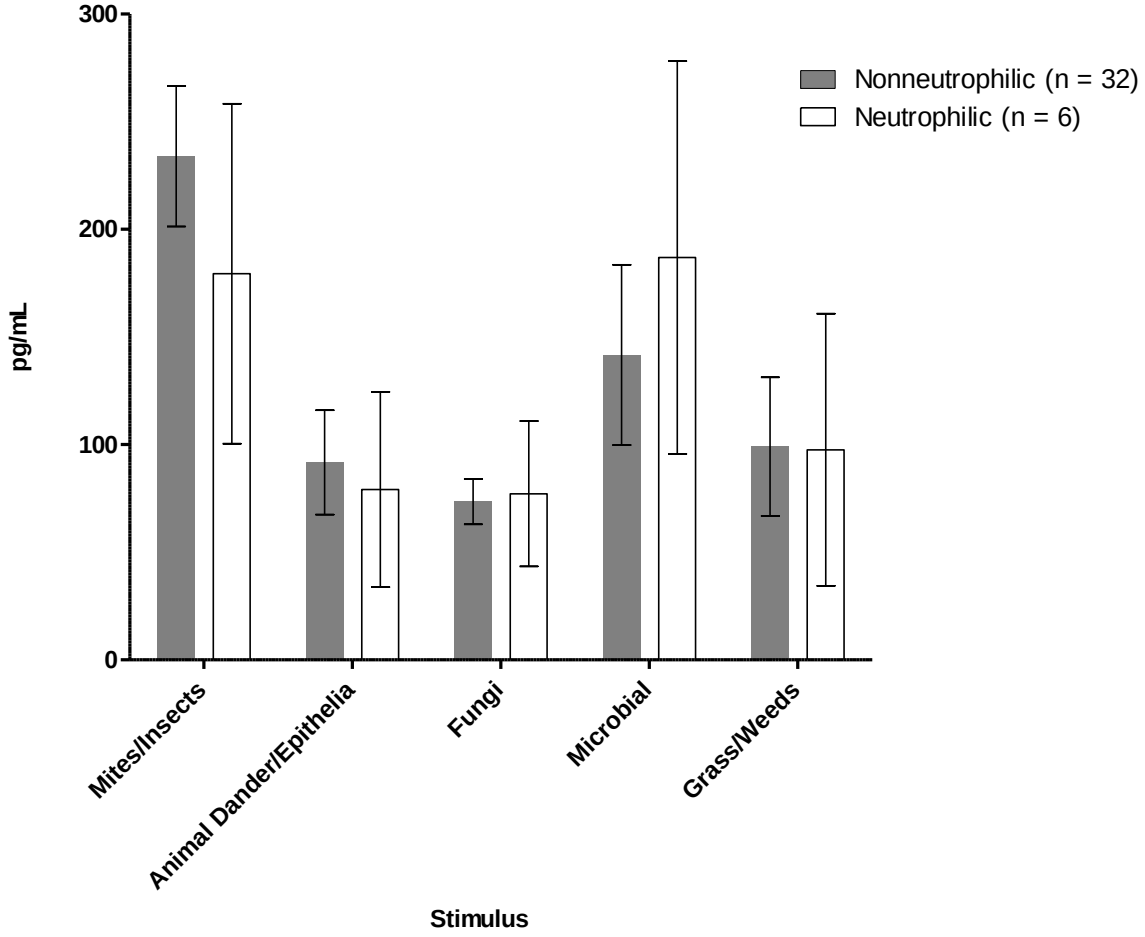


Figure 7.1 All Cytokines: Asthma

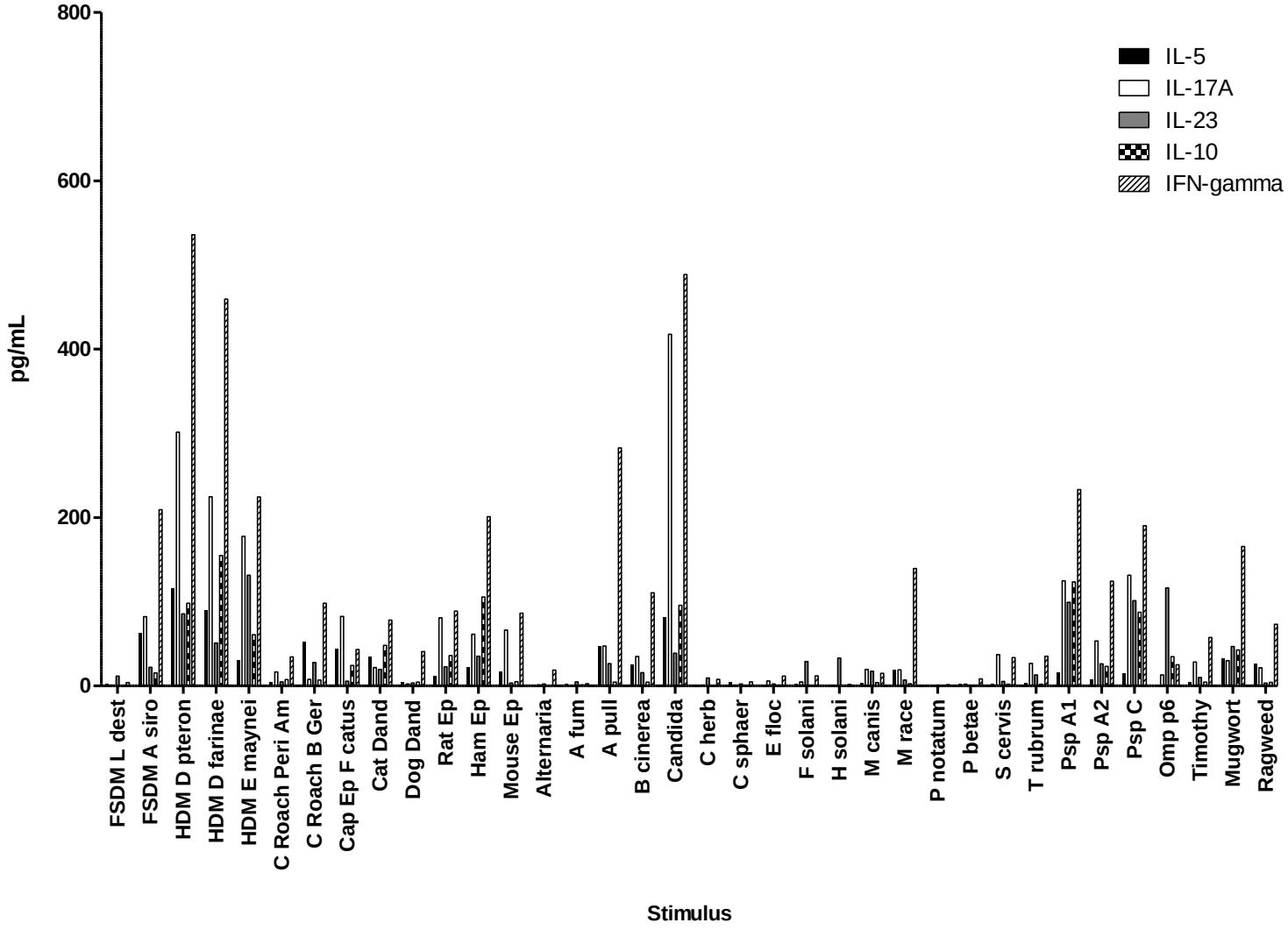


Figure 7.2 All Cytokines: Normal

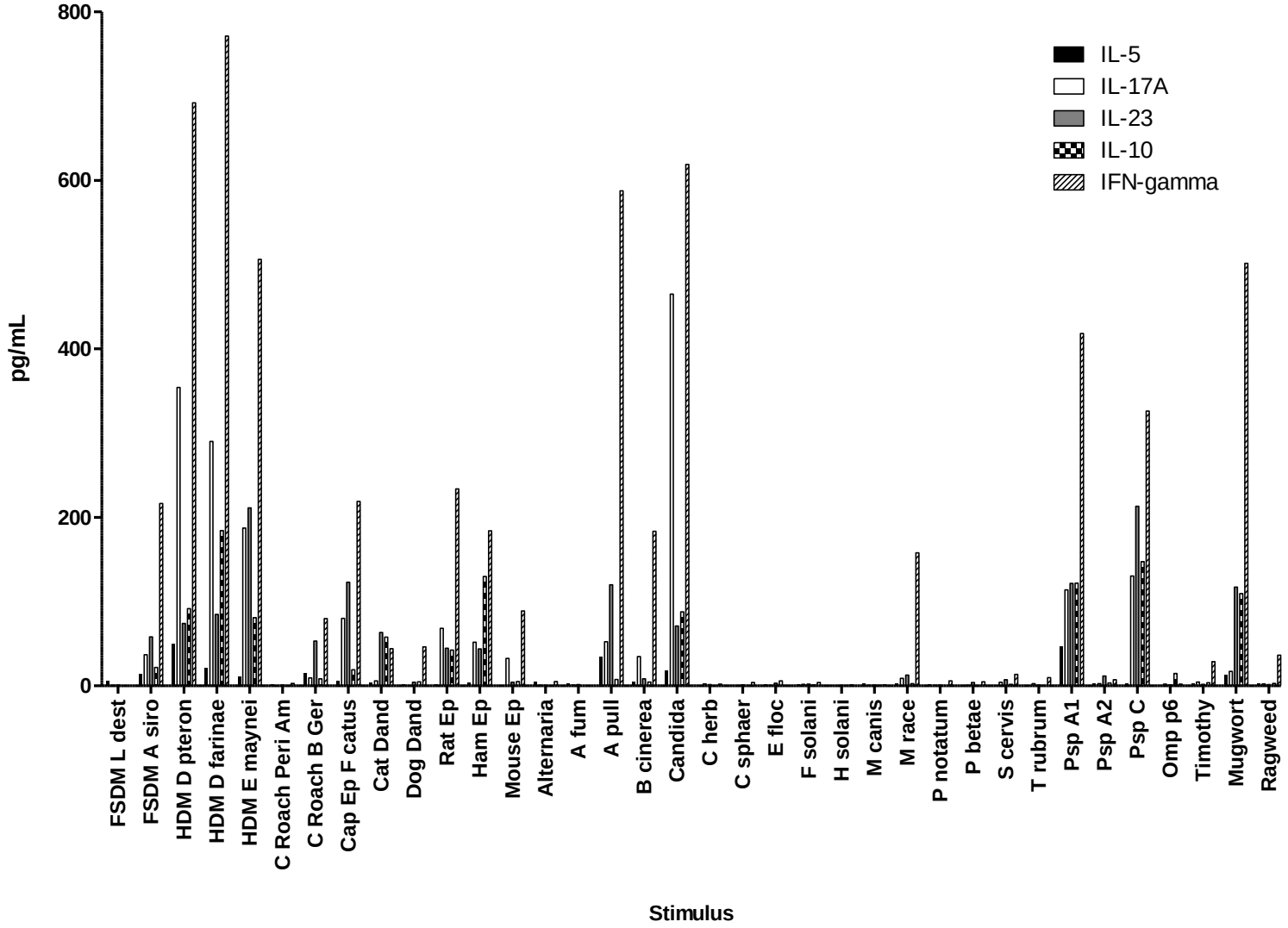


Figure 7.3 All Cytokines: Nonatopic Asthma

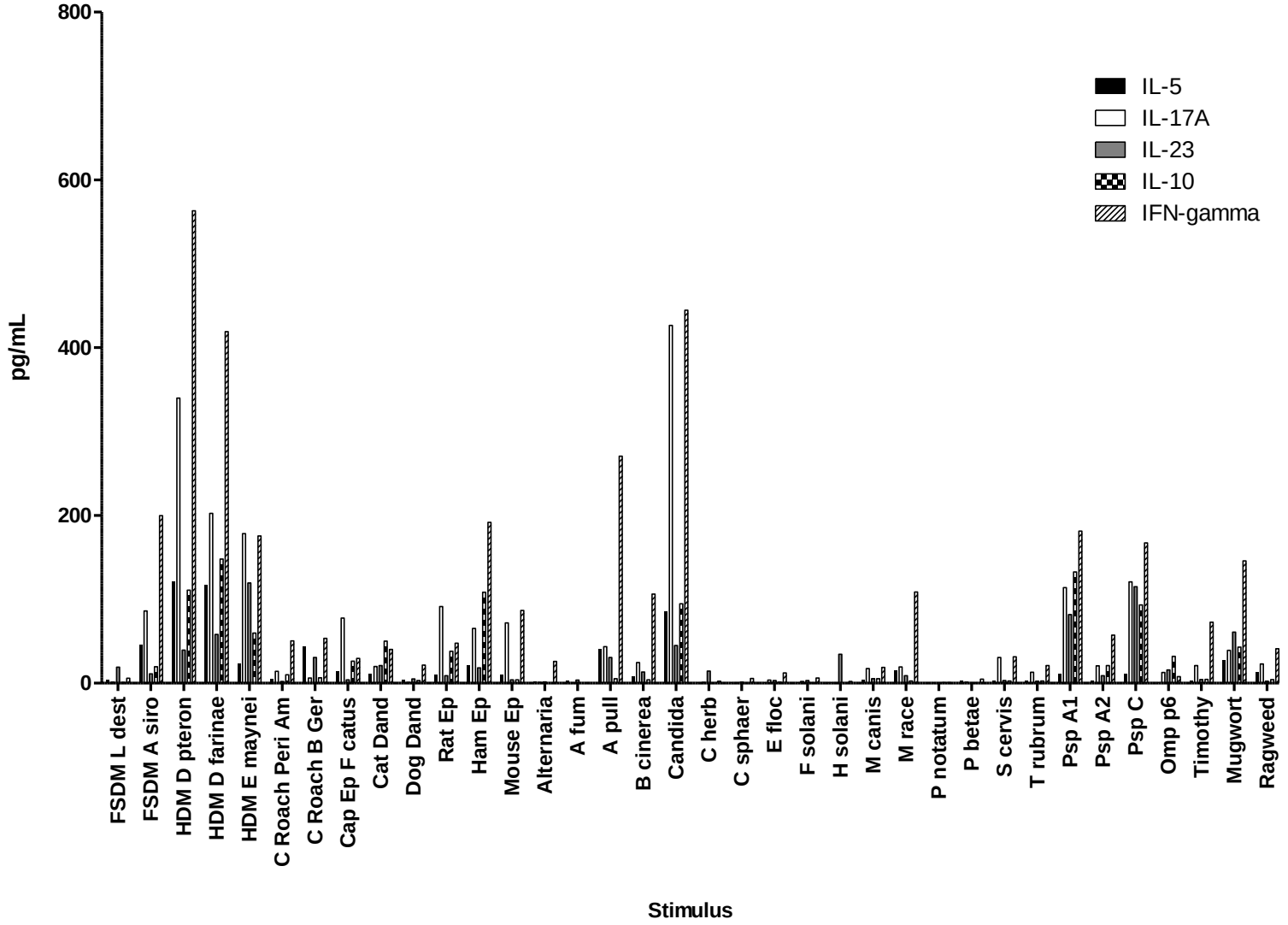


Figure 7.4 All Cytokines: Atopic Asthma

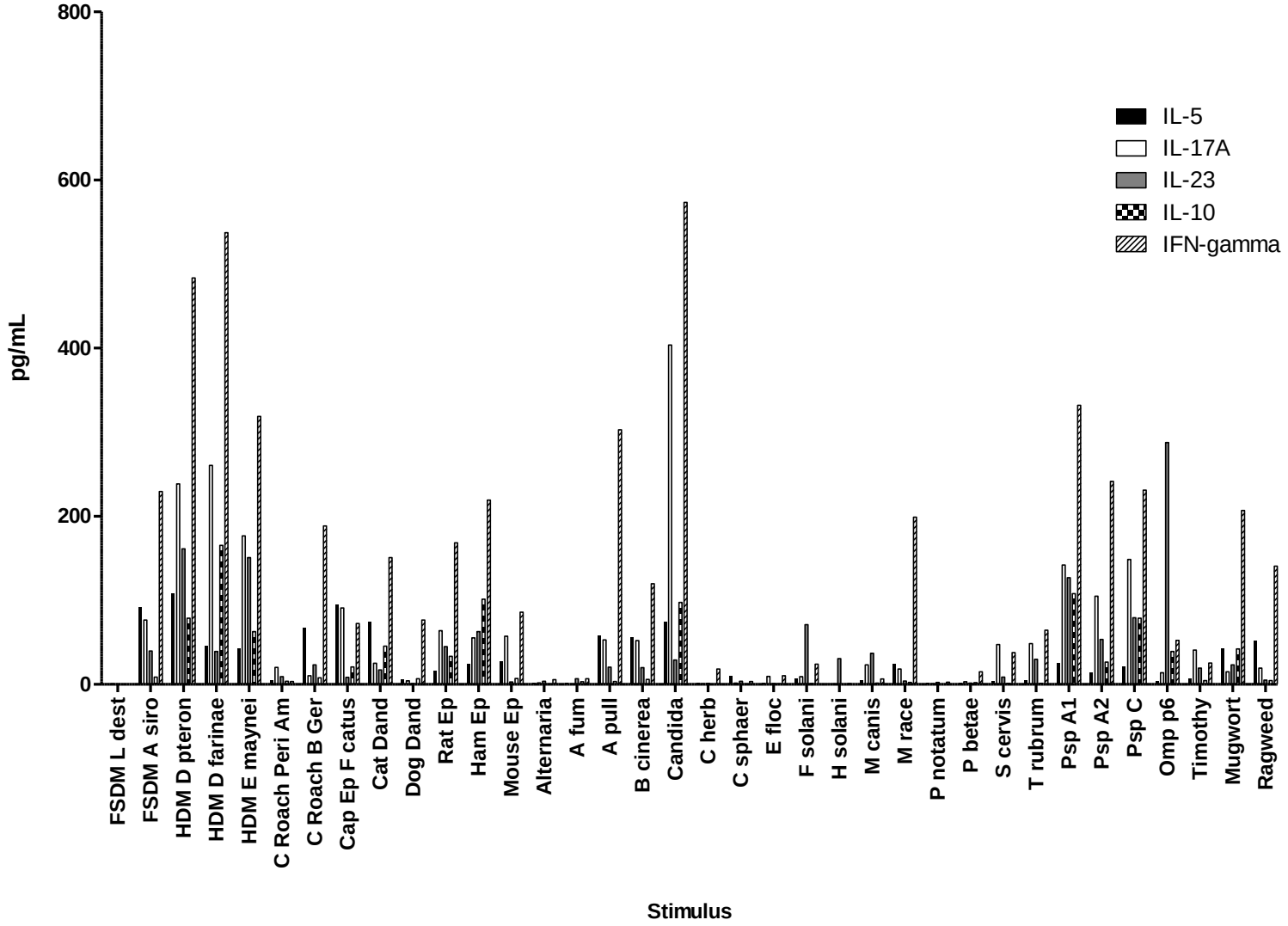


Figure 7.5 All Cytokines: Eosinophilic Asthma

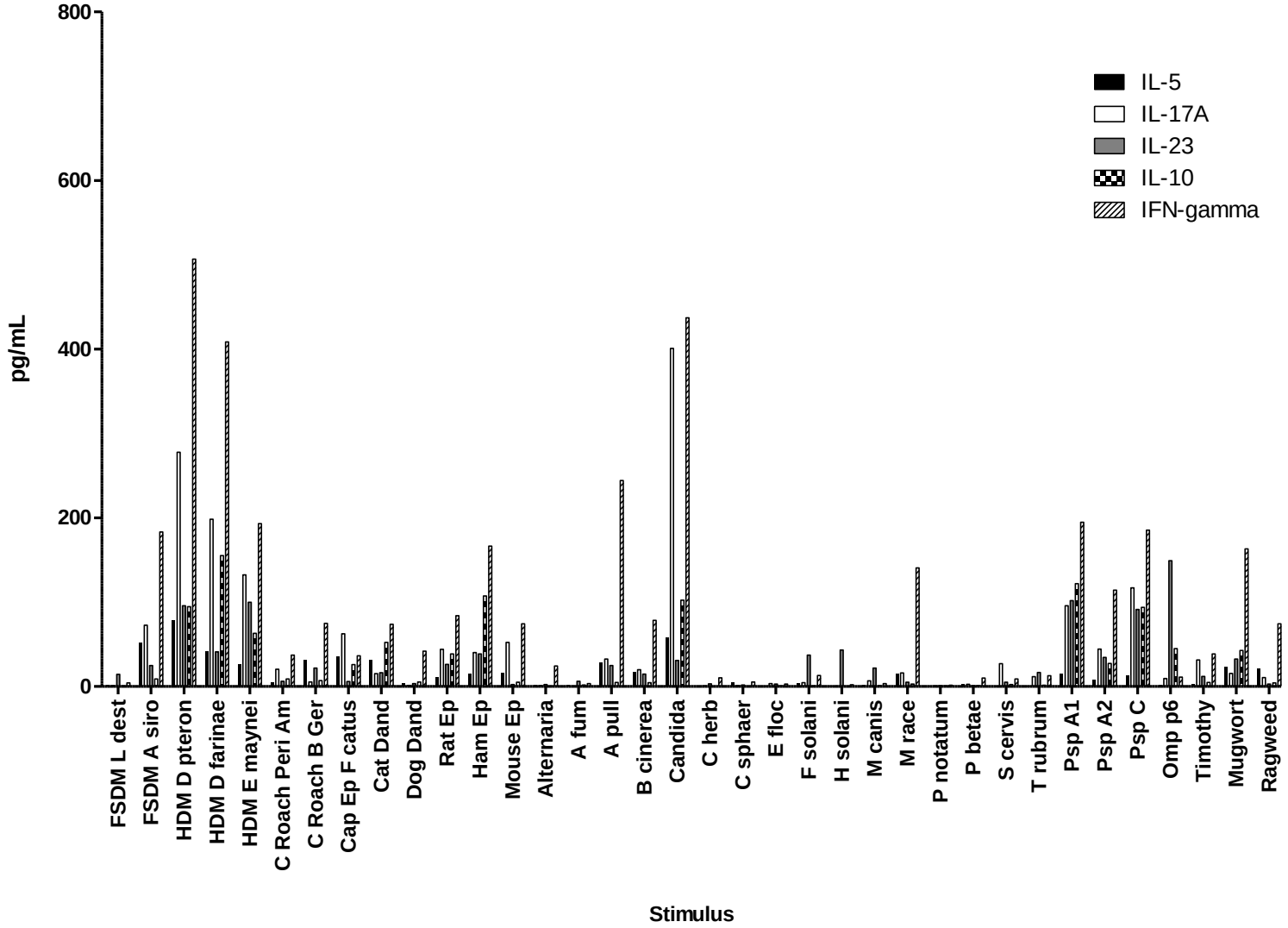


Figure 7.6 All Cytokines: Noneosinophilic Asthma

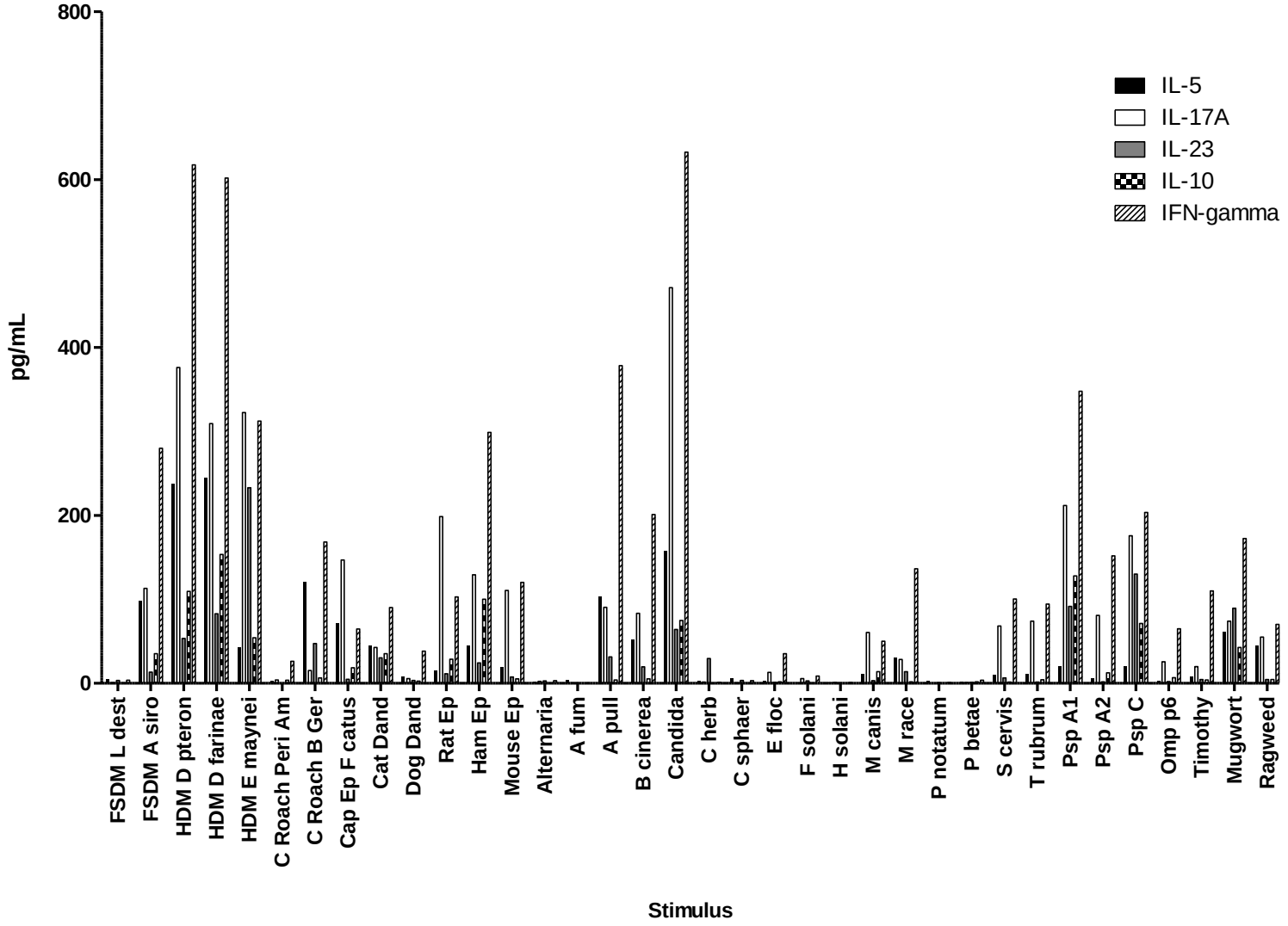


Figure 7.7 All Cytokines: Neutrophilic Asthma

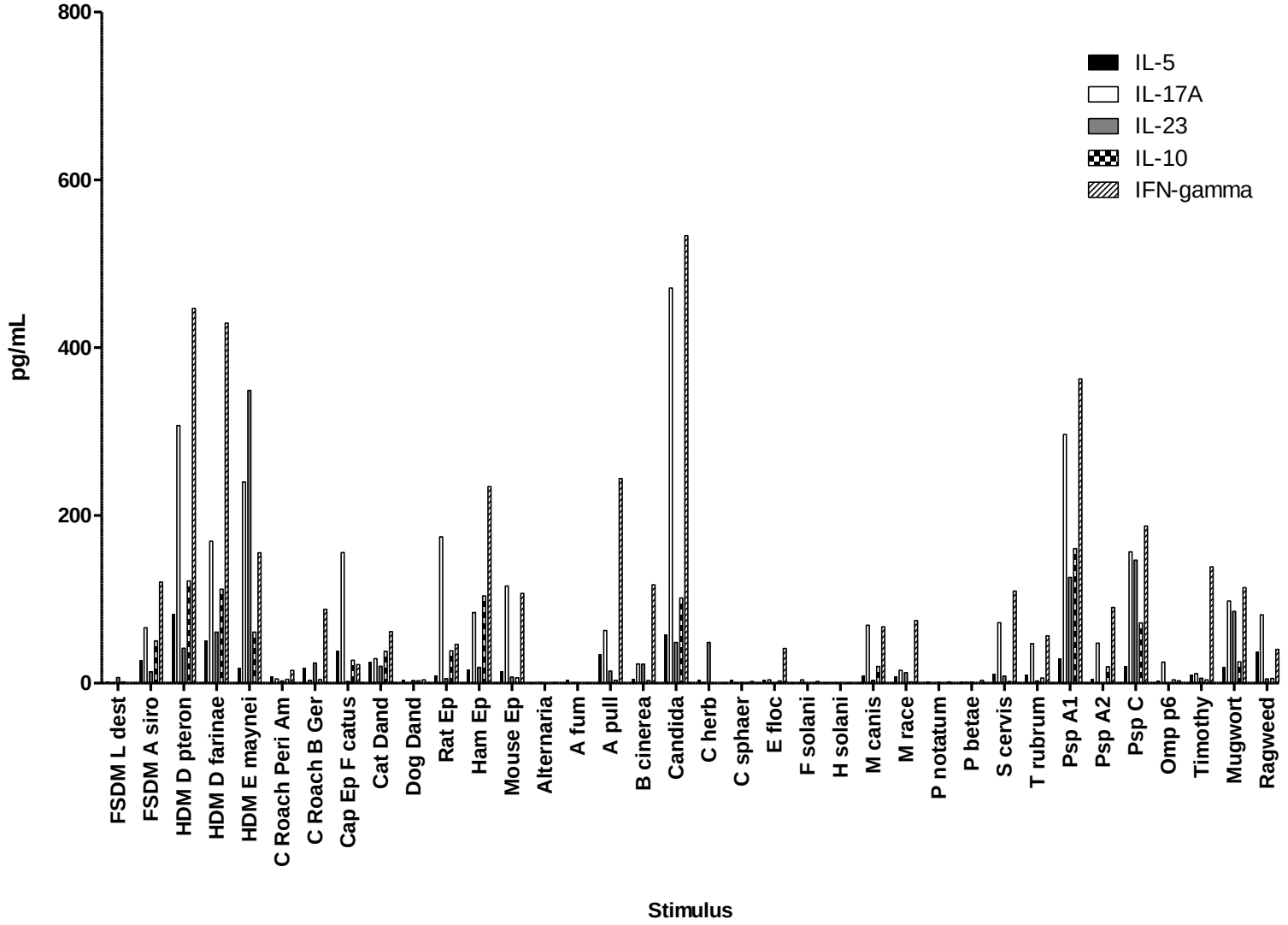


Figure 7.8 All Cytokines: Nonneutrophilic Asthma

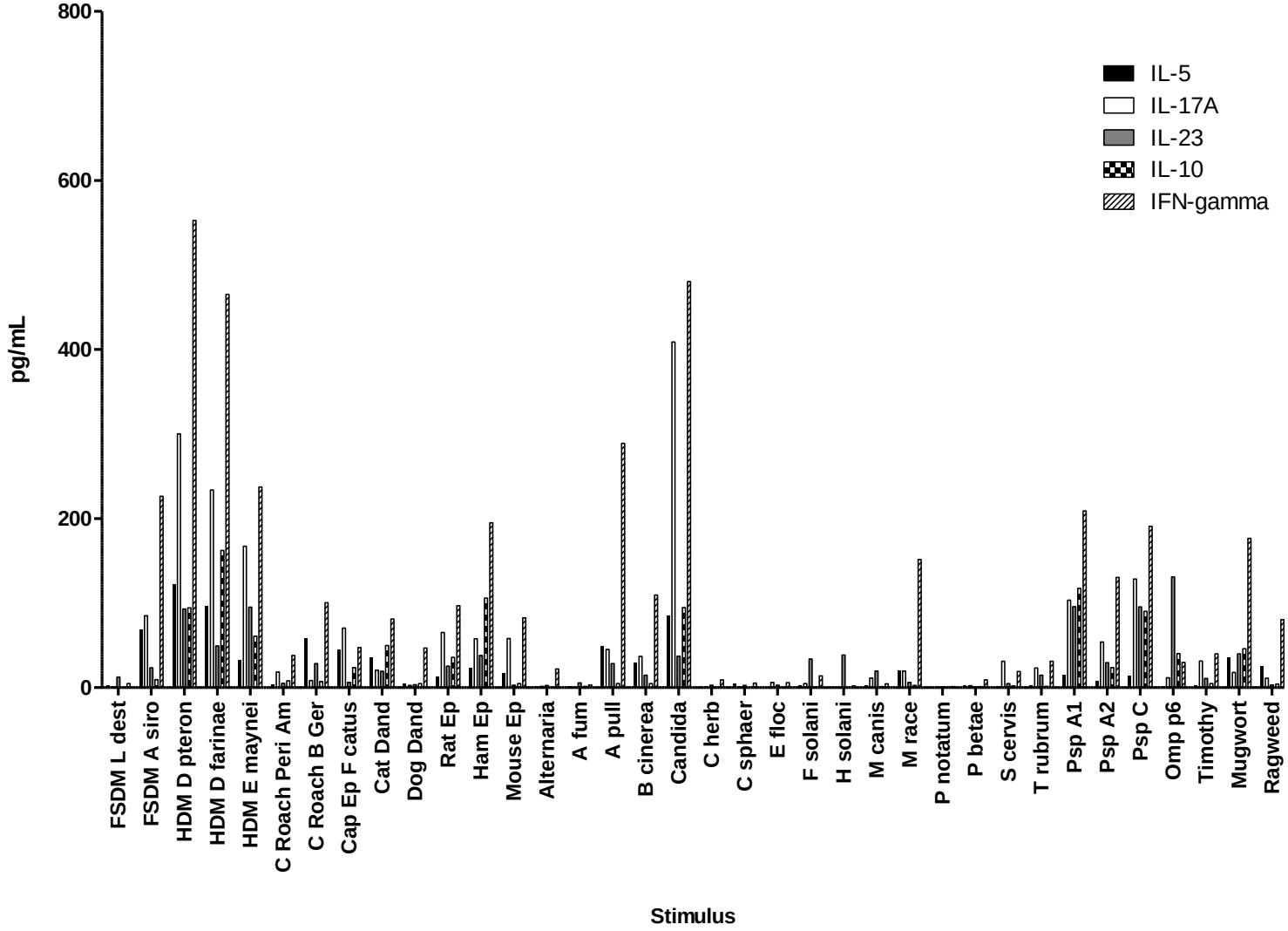


Figure 8.1 Skin Prick Test: Asthma

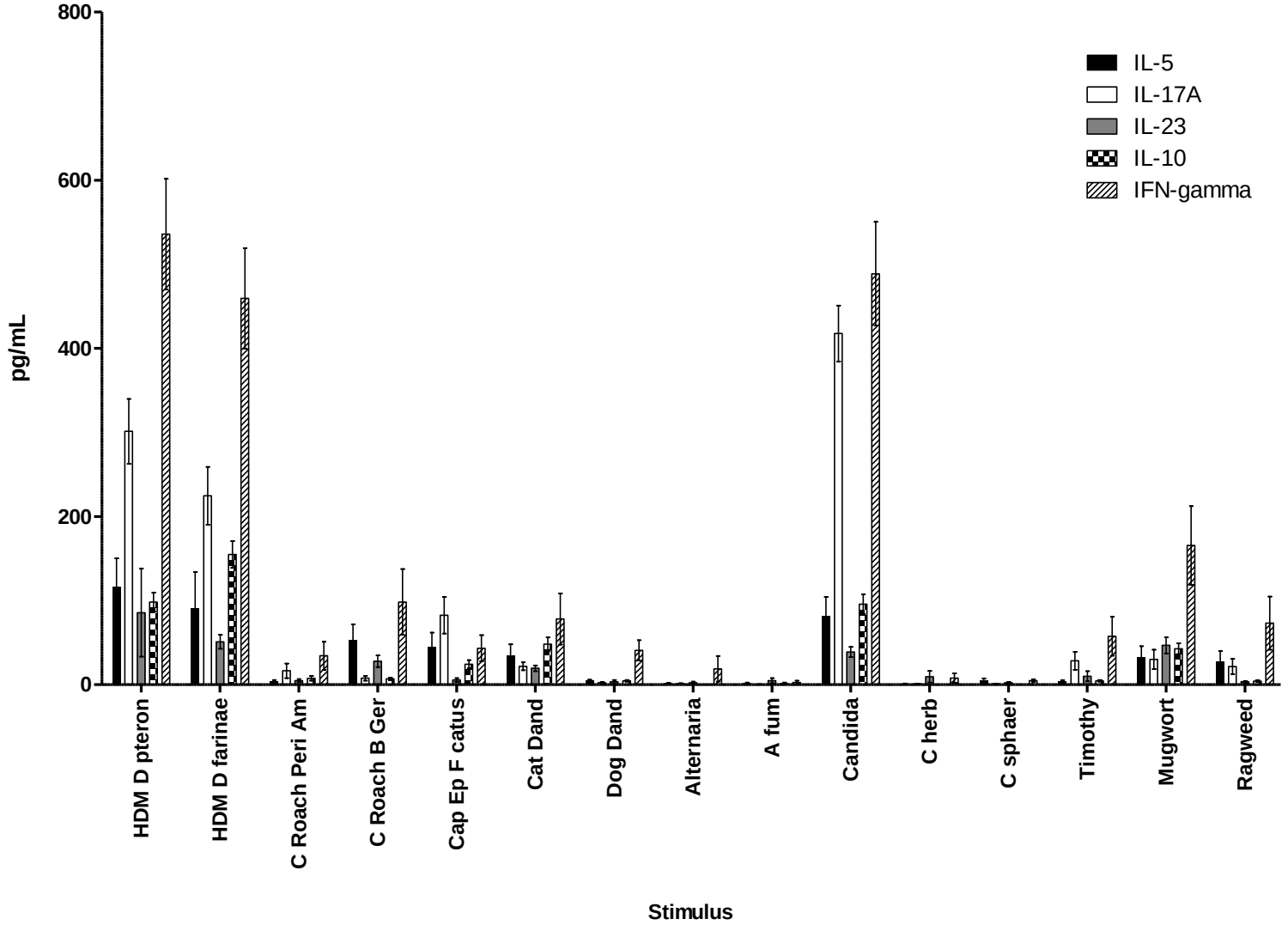


Figure 8.2 Skin Prick Test: Normal

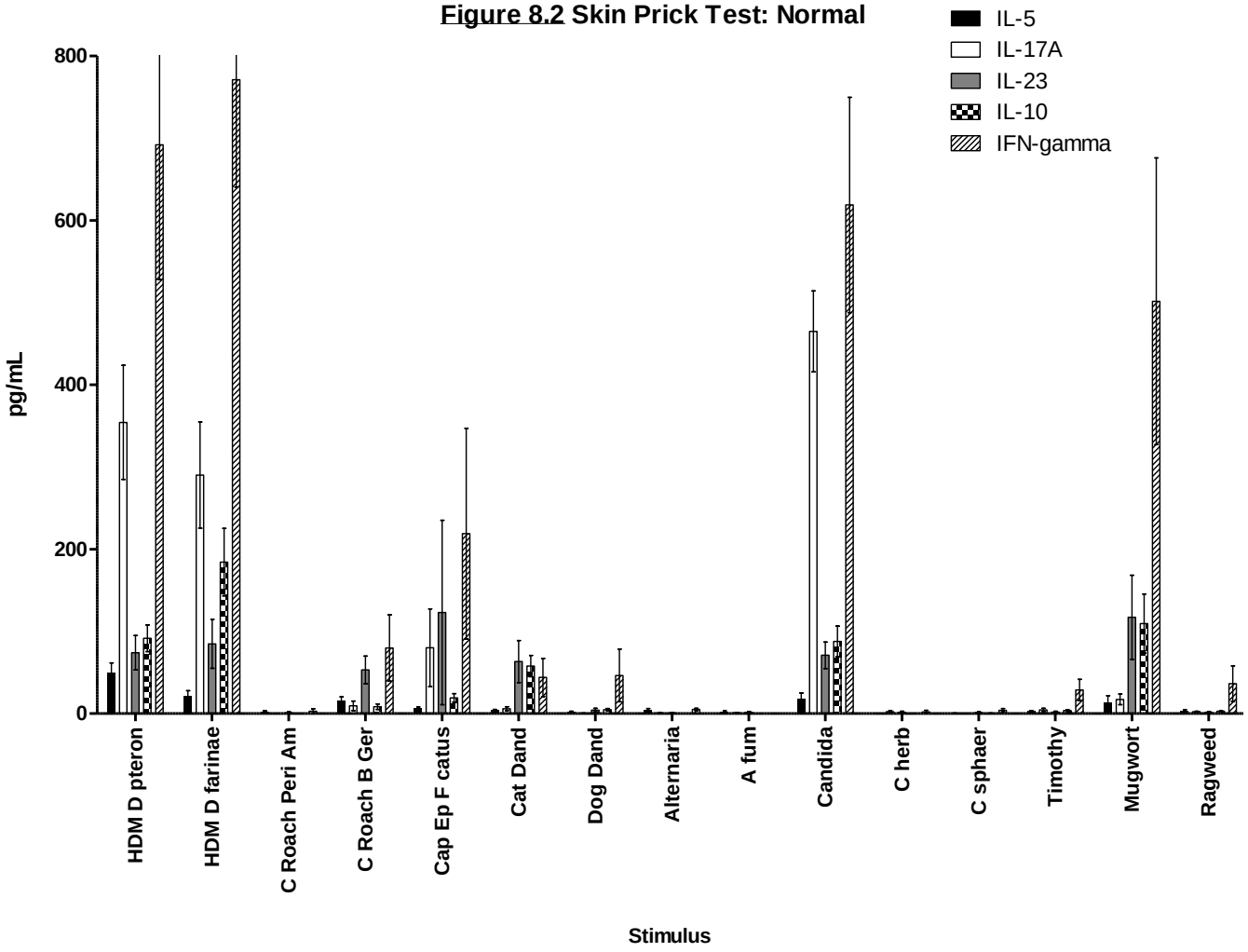


Figure 8.3 Skin Prick Test: Nonatopic Asthma

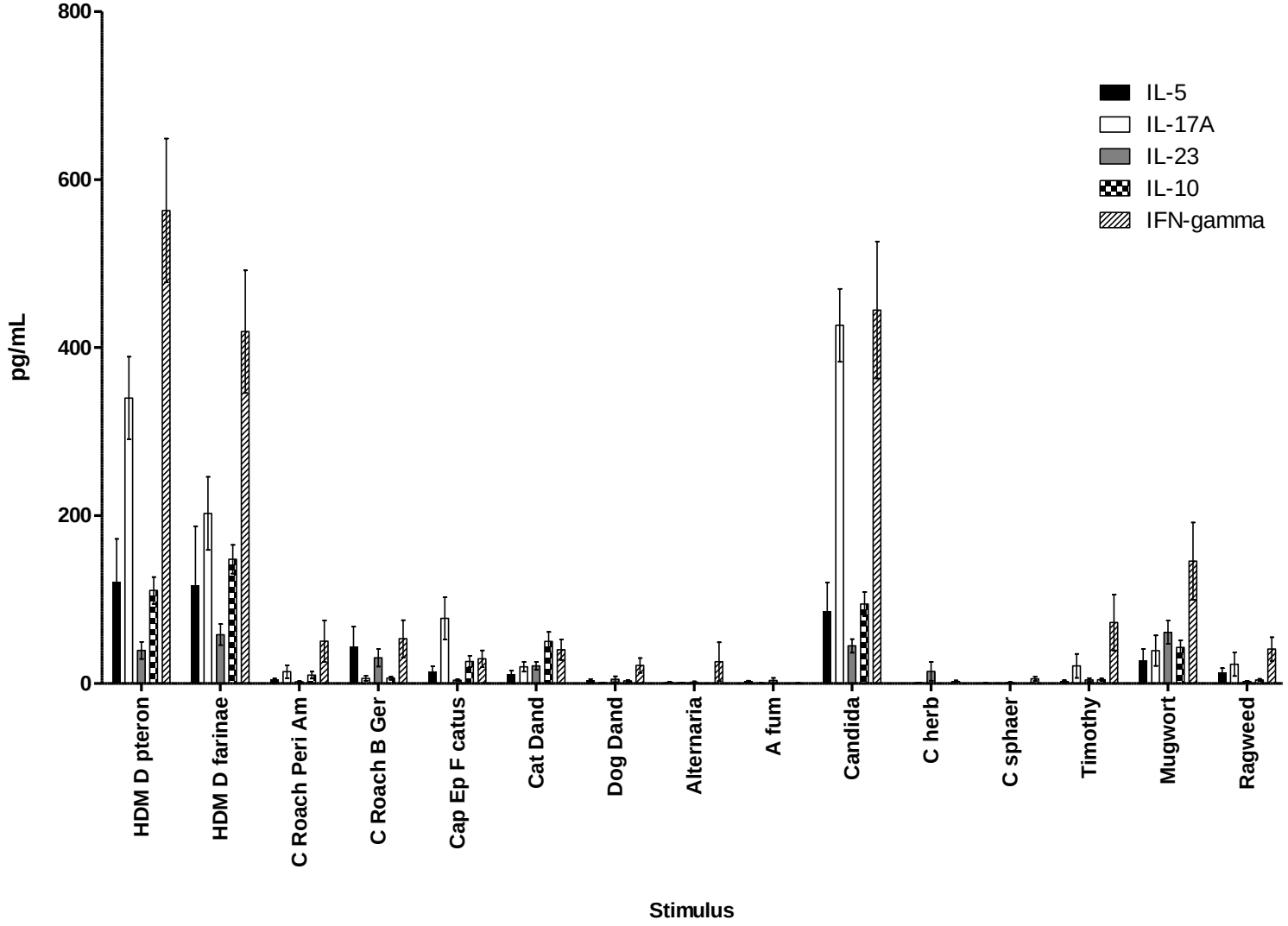


Figure 8.4 Skin Prick Test: Atopic Asthma

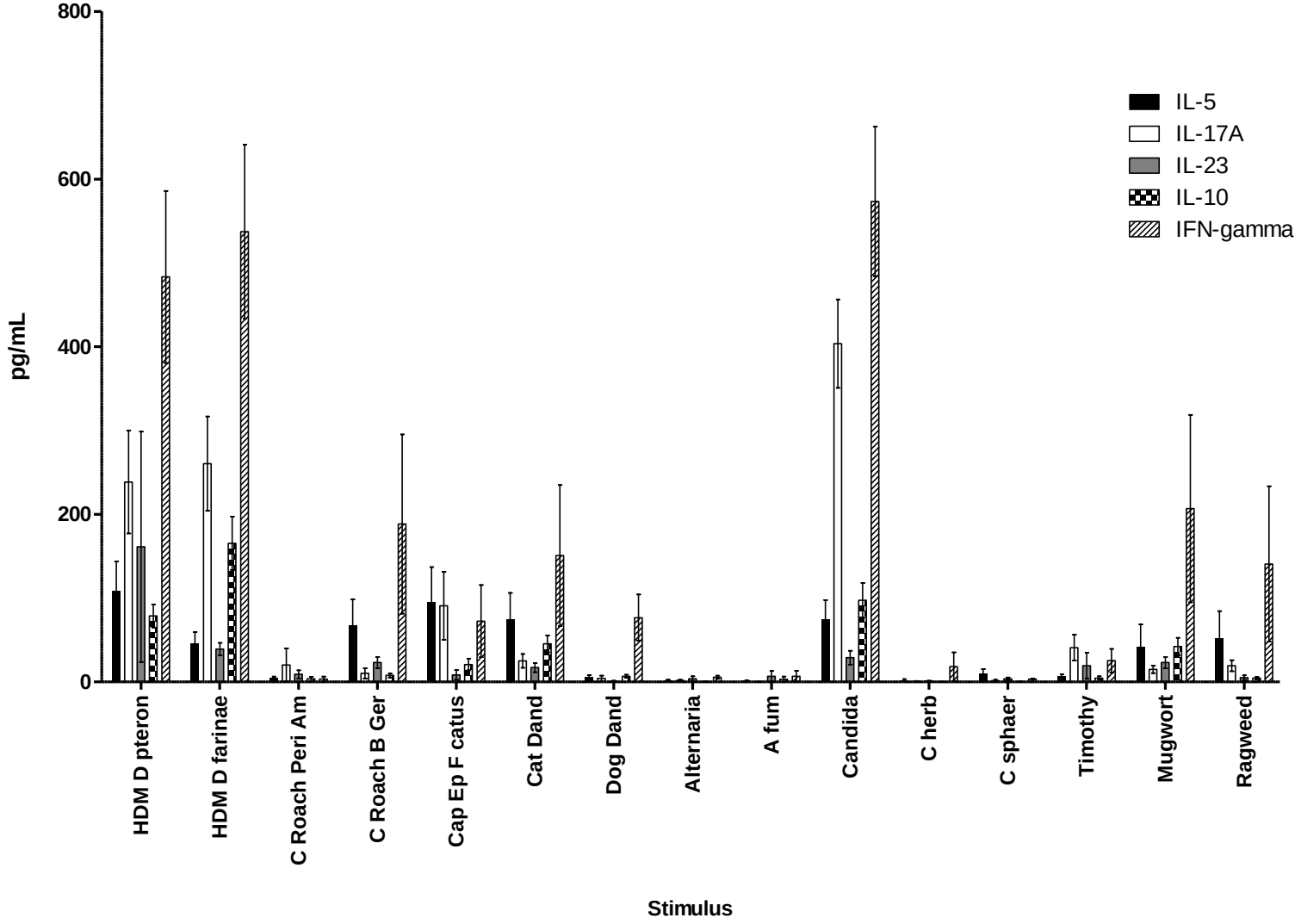


Figure 8.5 Skin Prick Test: Eosinophilic Asthma

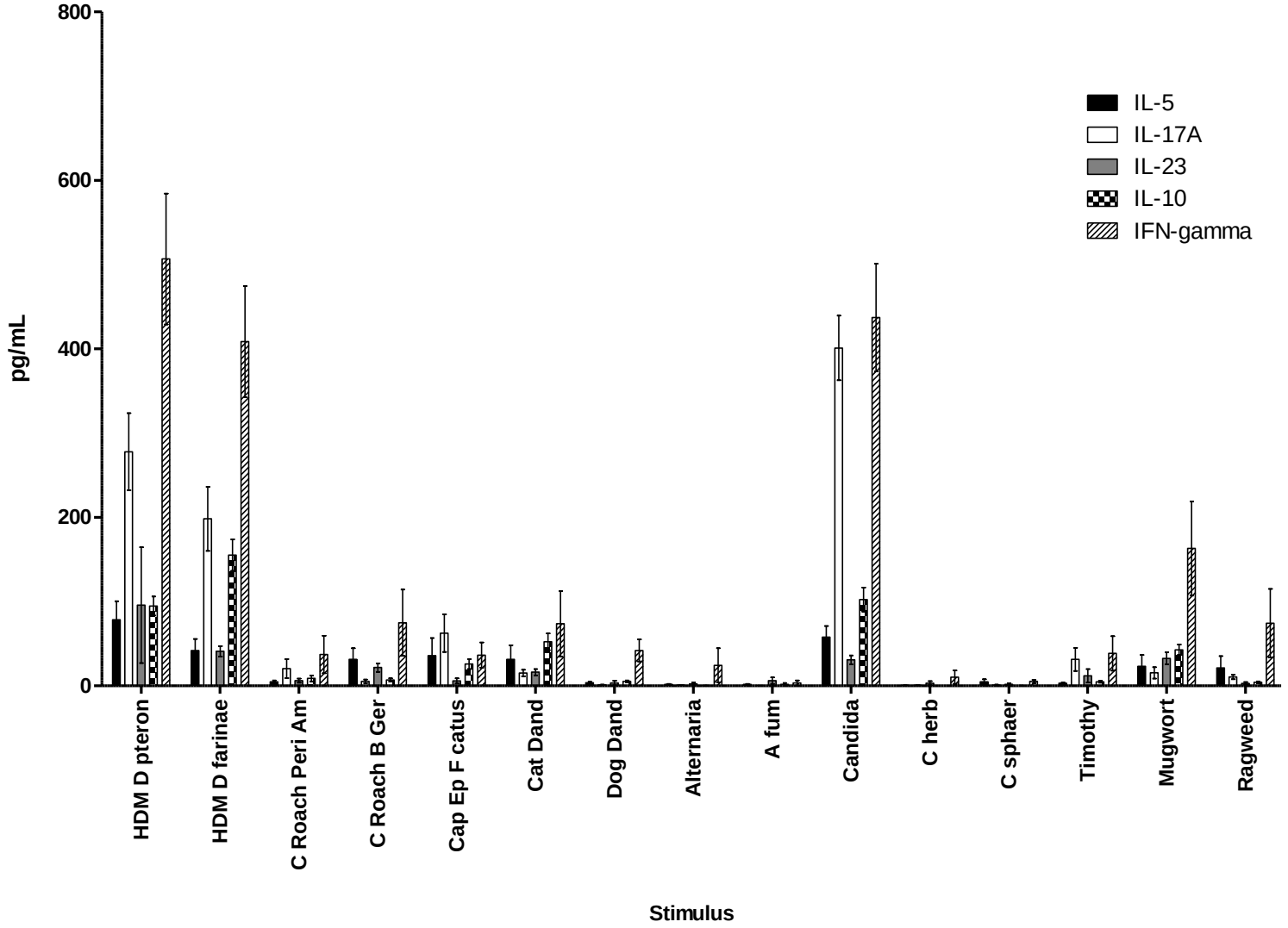


Figure 8.6 Skin Prick Test: Noneosinophilic Asthma

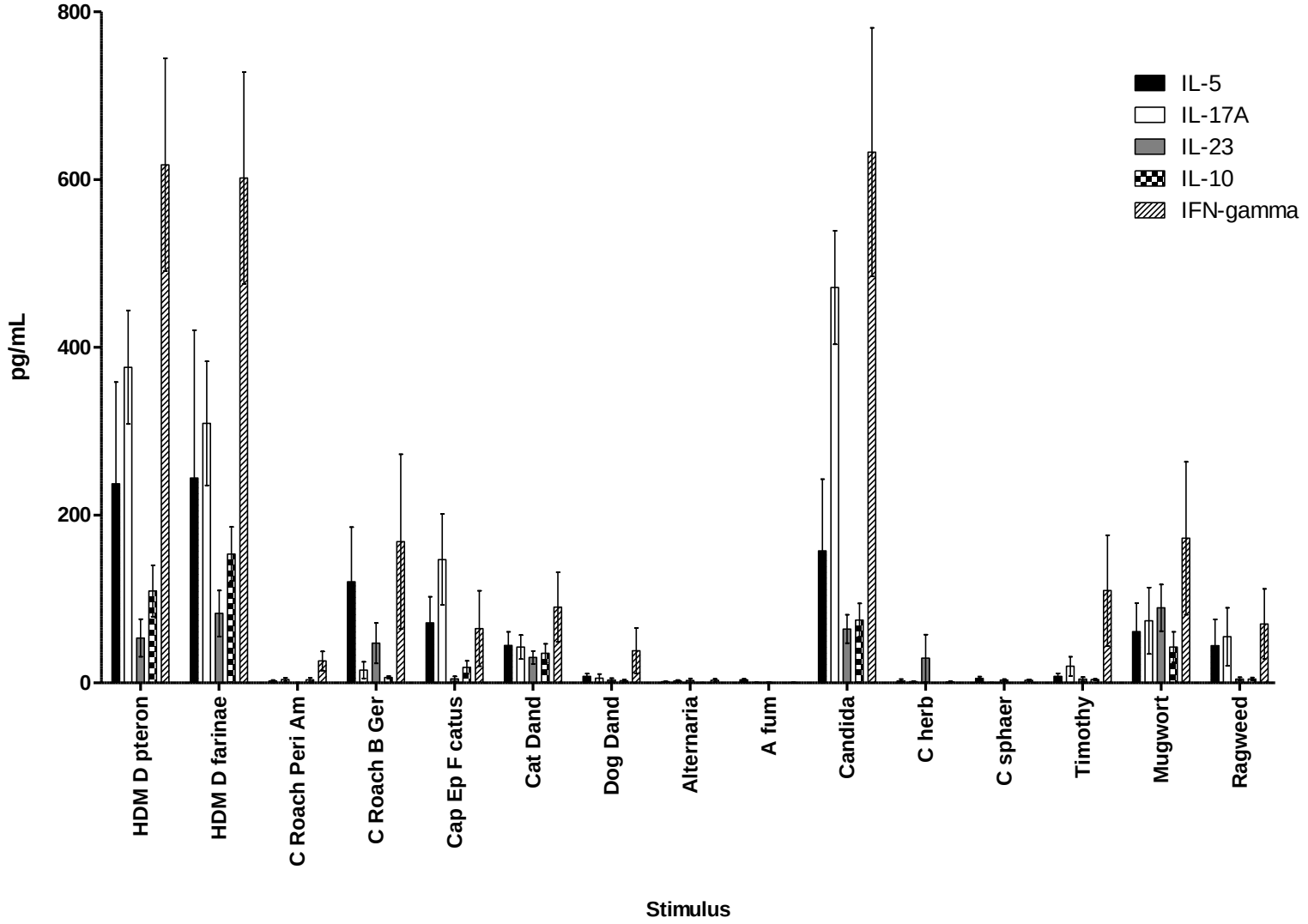


Figure 8.7 Skin Prick Test: Neutrophilic Asthma

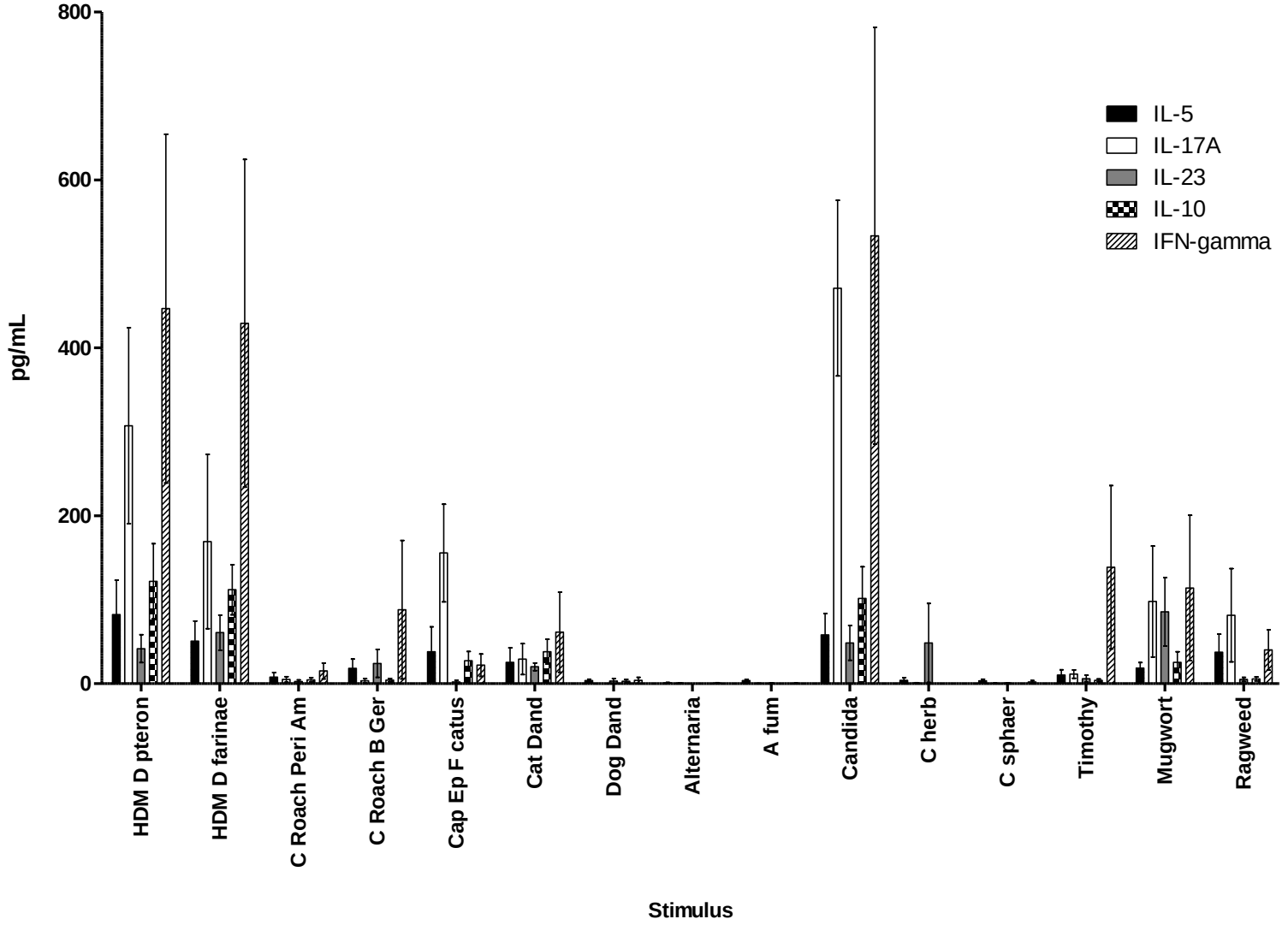


Figure 8.8 Skin Prick Test: Nonneutrophilic Asthma

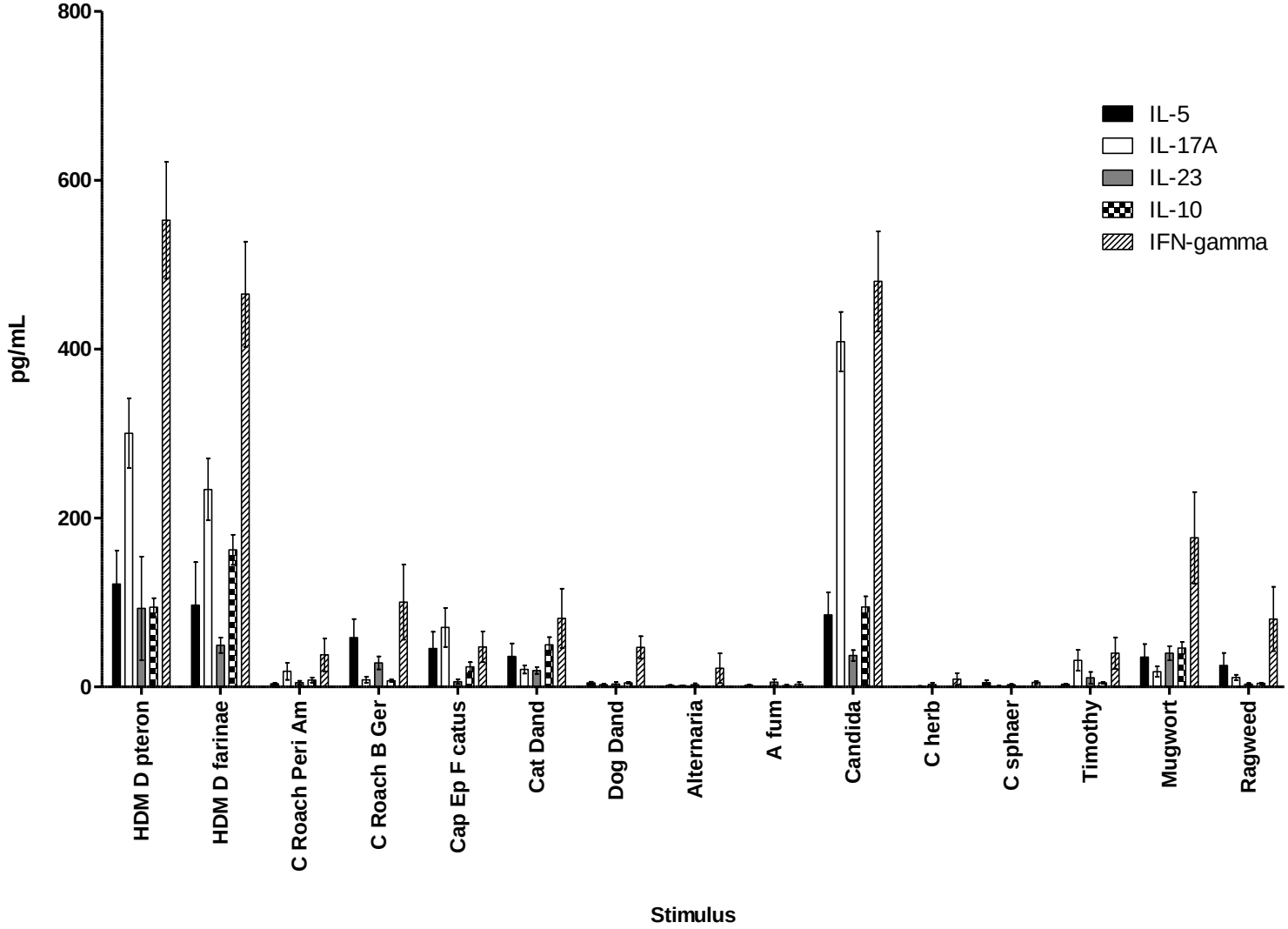


Figure 9.1 Delta Grouped: Steroids

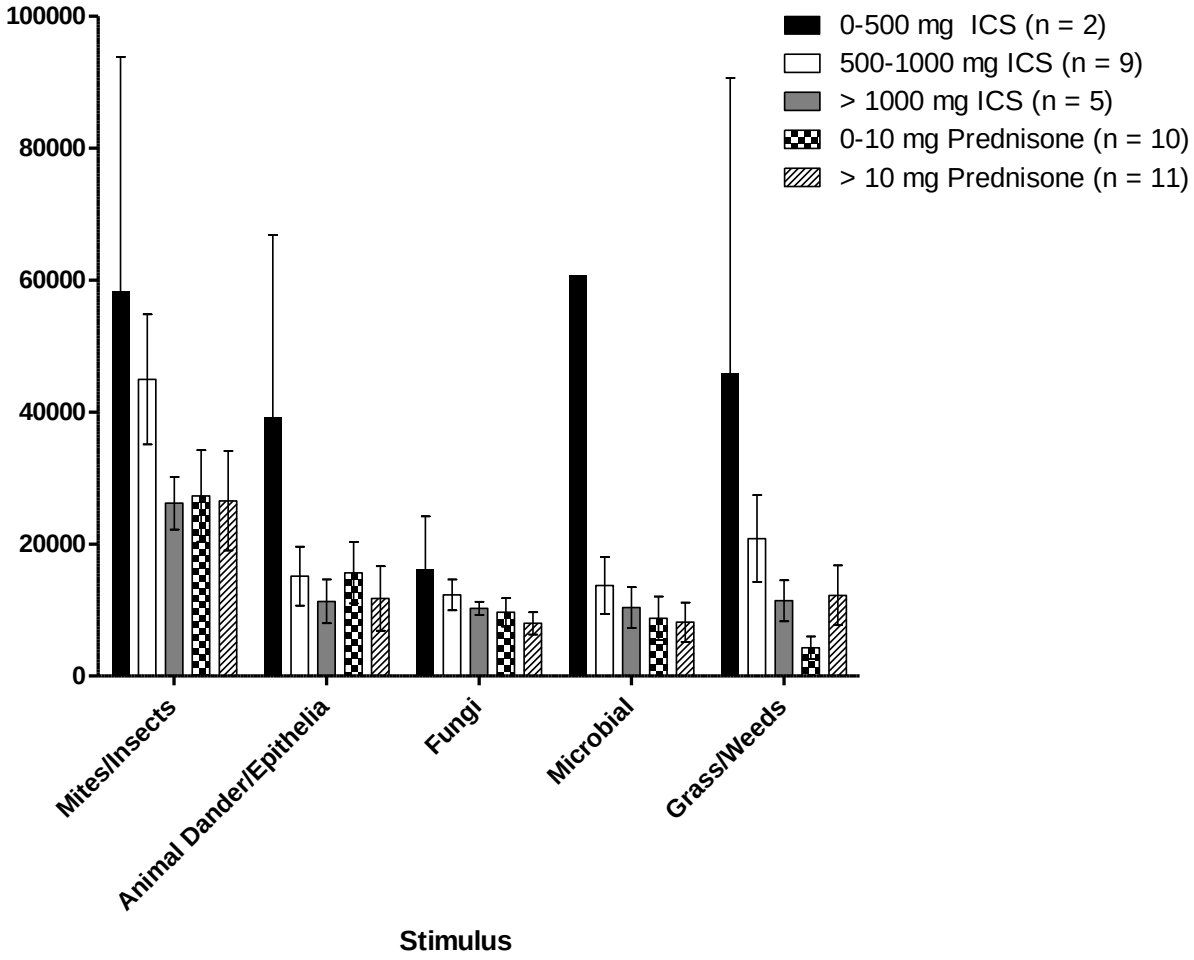


Figure 9.2 IL-5 Grouped: Steroids

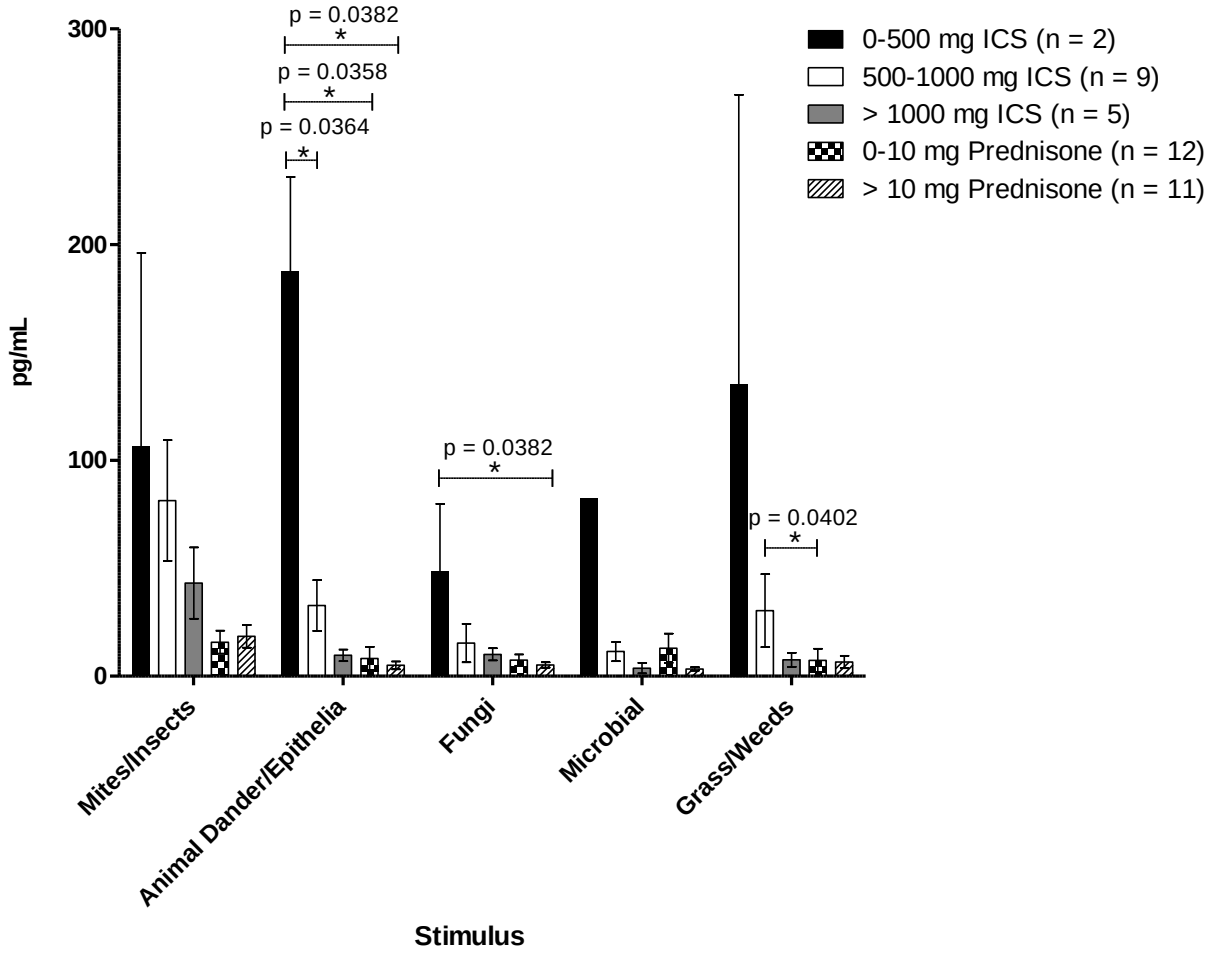


Figure 9.3 IL-17A Grouped: Steroids

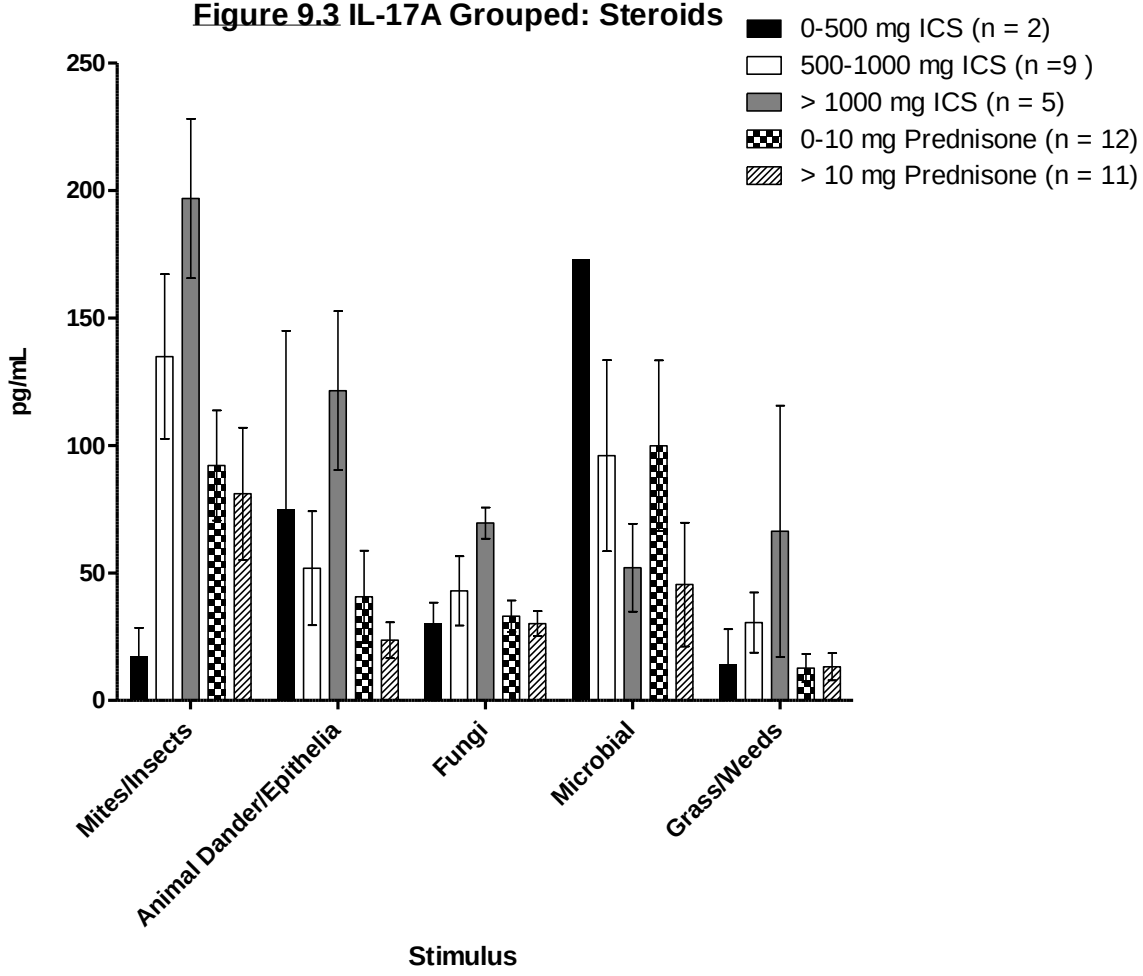


Figure 9.4 IL-23 Grouped: Steroids

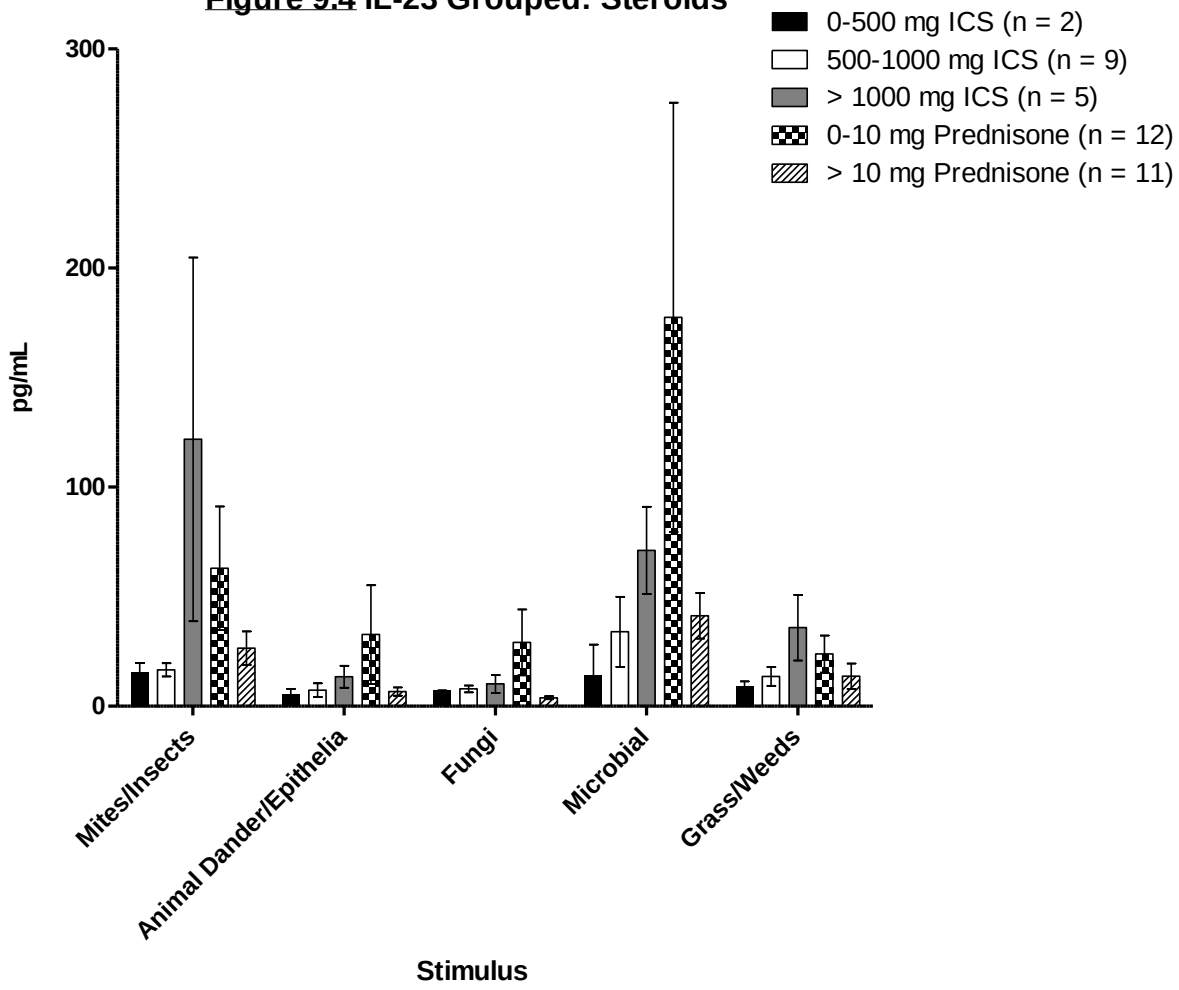


Figure 9.5 IL-10 Grouped: Steroids

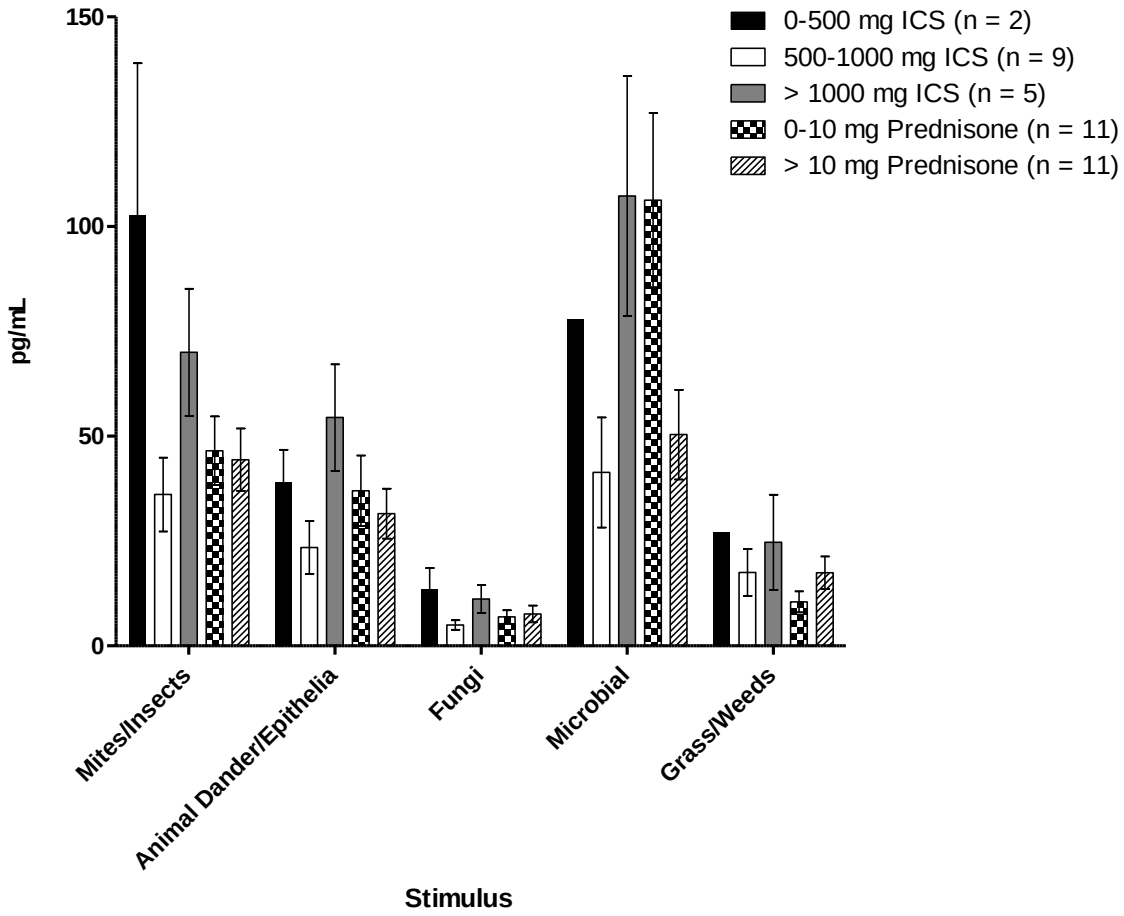


Figure 9.6 IFN-gamma Grouped: Steroids

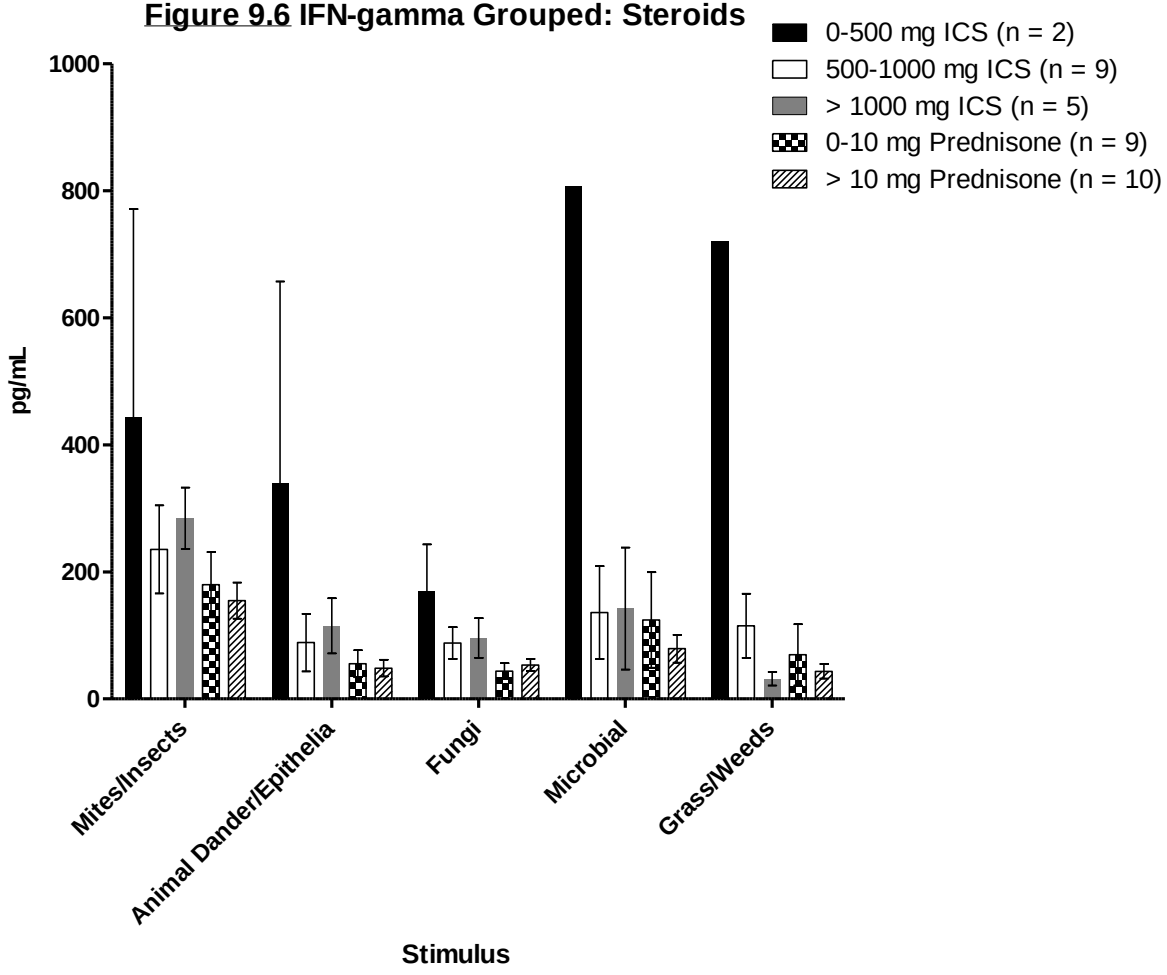
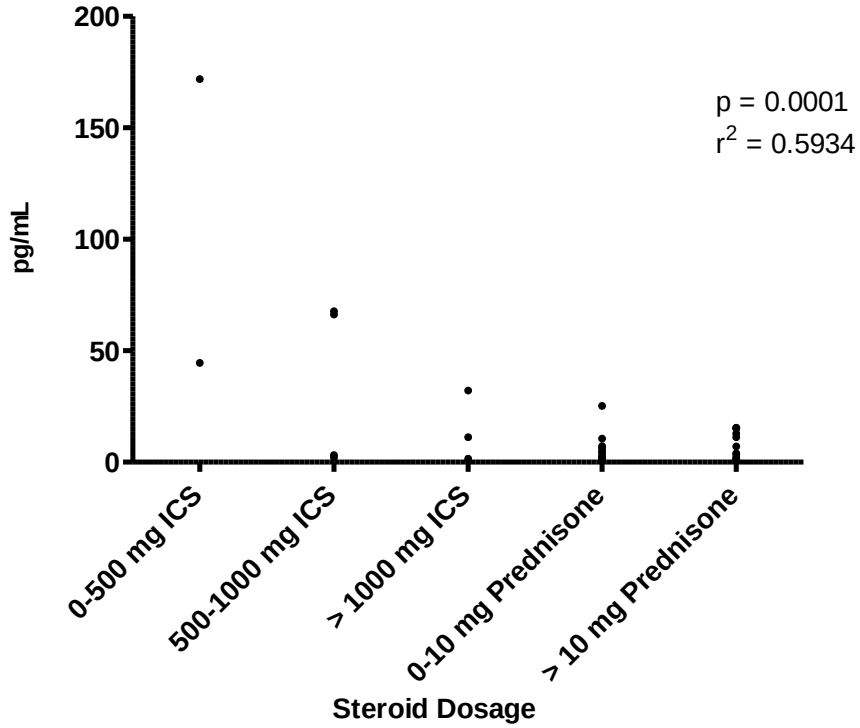


Figure 9.7 IL-5: Steroid Dosage



Average allergen-induced IL-5 production by PBMCs from eosinophilic asthmatic patients compared across 5 steroid treatment groups and analyzed by one-way ANOVA.

APPENDIX III: INDIVIDUAL PATIENT RESULTS

Contains:

1. Nonatopic Asthmatic Patients
2. Atopic Asthmatic Patients
3. Normal Controls

Figure 10.1 Nonatopic Asthmatic Patient 1- Noneosinophilic

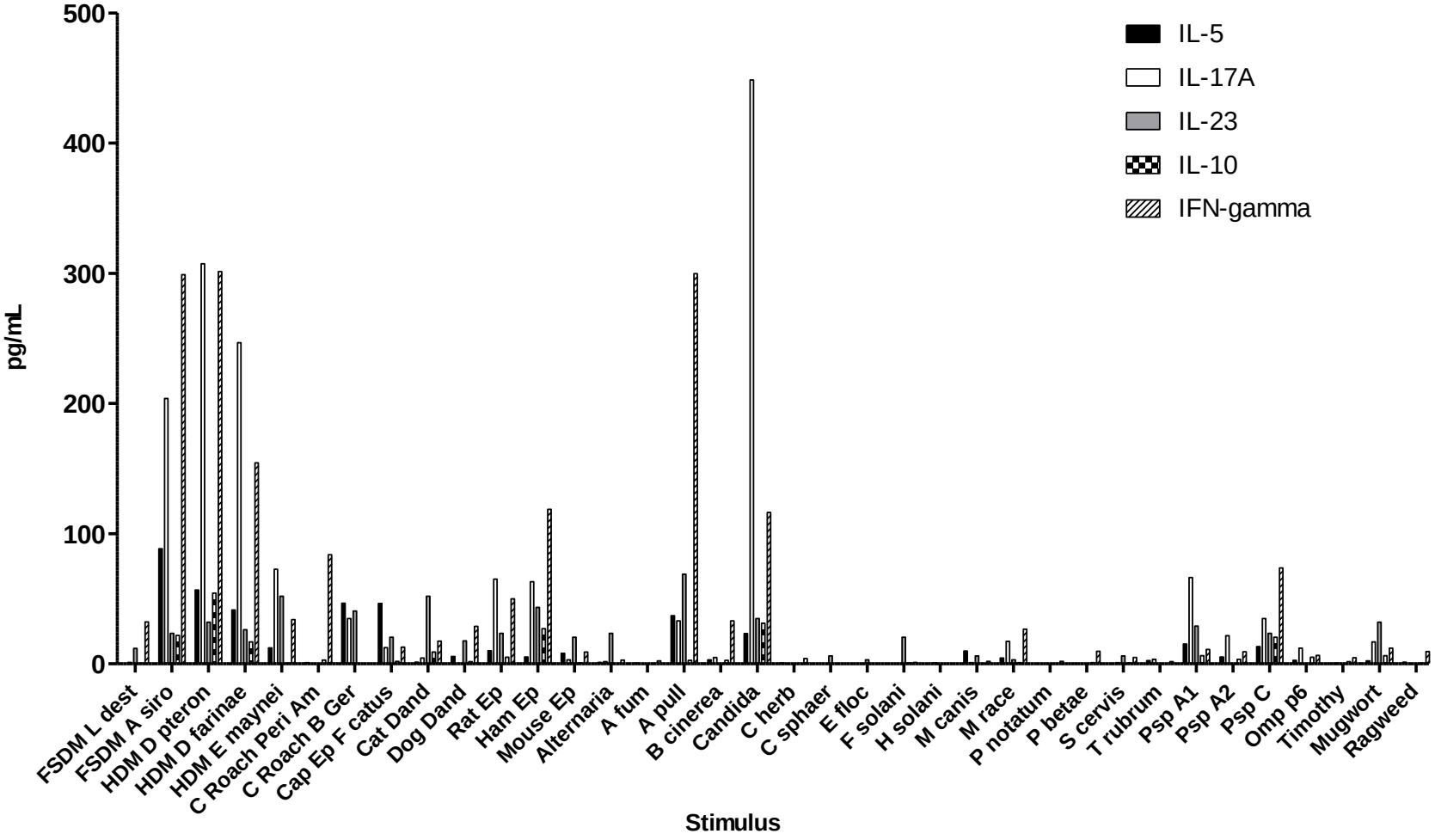


Figure 10.2 Nonatopic Asthmatic Patient 1- Noneosinophilic

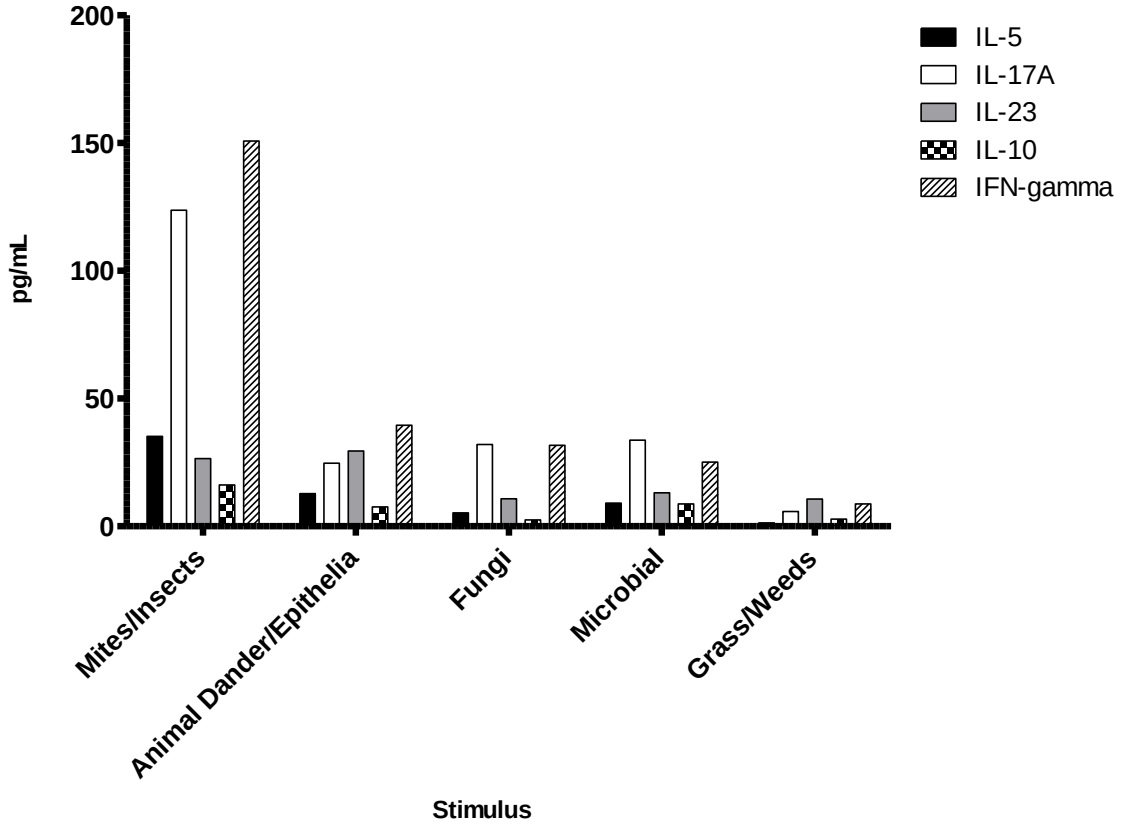


Figure 10.3 Nonatopic Asthmatic Patient 2 - Eosinophilic

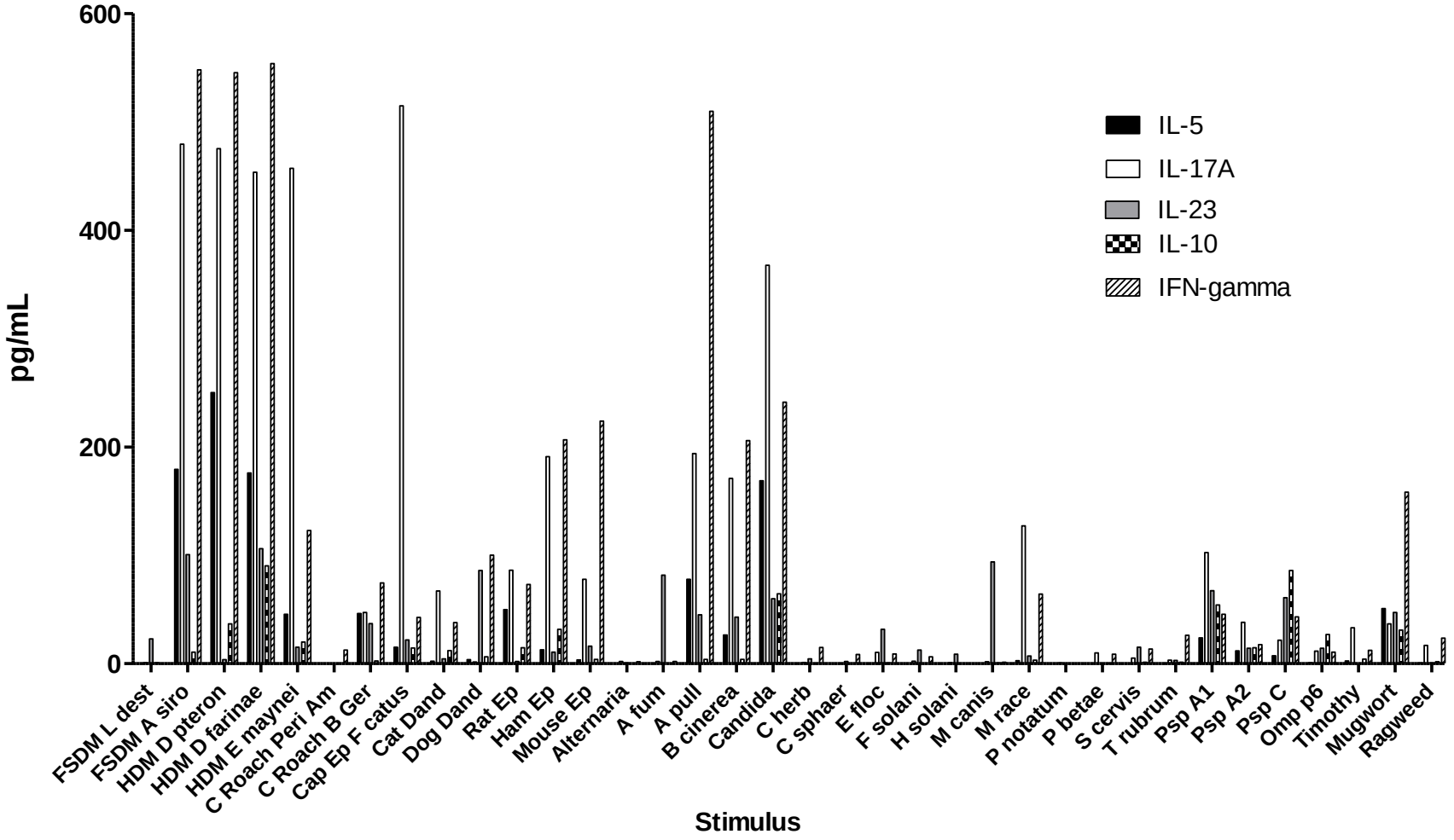
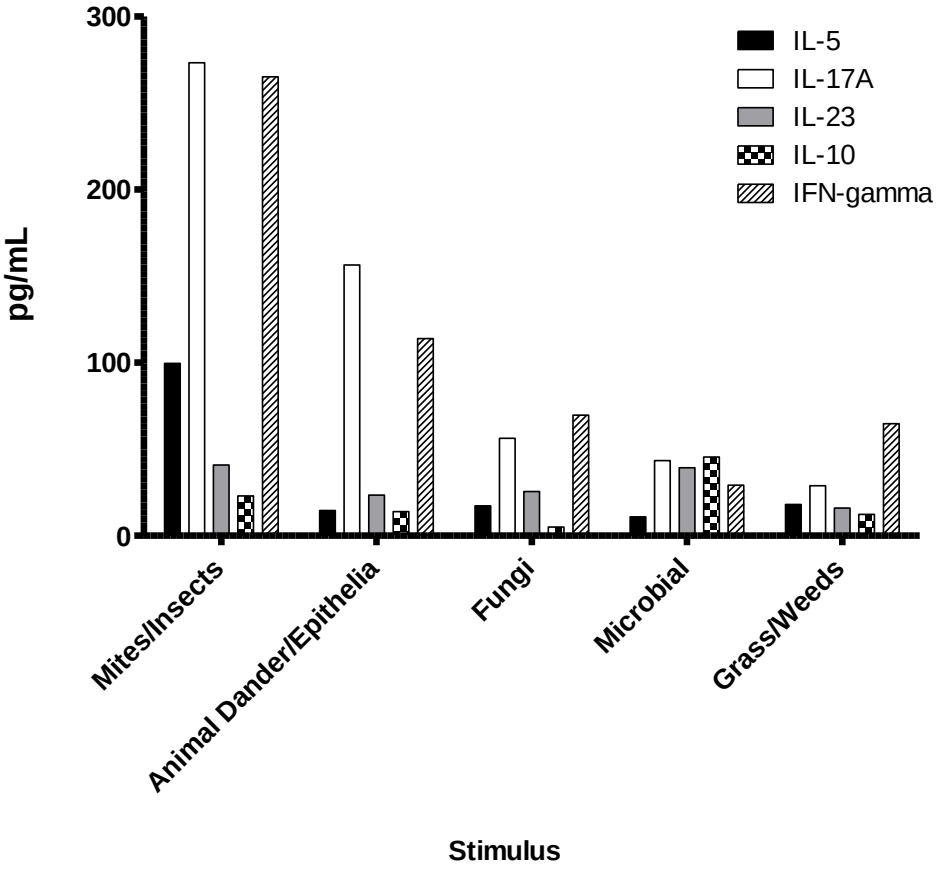


Figure 10.4 Nonatopic Asthmatic Patient 2 - Eosinophilic



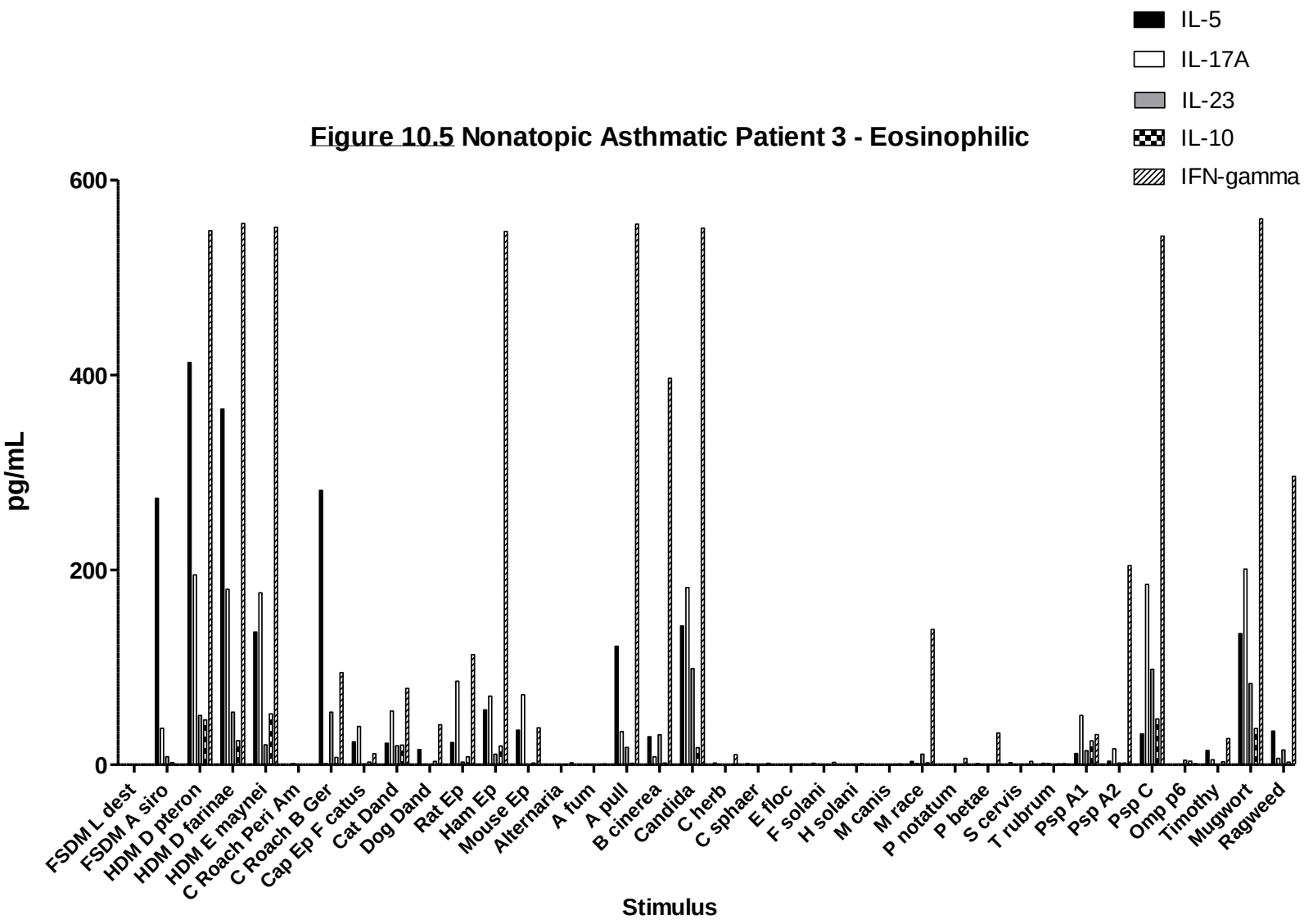

IL-5
IL-17A
IL-23
IL-10
IFN-gamma
Figure 10.5 Nonatopic Asthmatic Patient 3 - Eosinophilic
600
400
200
0
pg/mL
FSDM L dest
FSDM A siro
HDM D pteron
HDM D farinae
HDM E maynei
C Roach Peri Am
C Roach B Ger
Cap Ep F catus
Cat Dand
Dog Dand
Rat Ep
Ham Ep
Mouse Ep
Alternaria
A fum
A pull
B cinerea
Candida
C herb
C sphaer
E floc
F solani
H solani
M canis
M race
P notatum
P betae
S cervis
T rubrum
Psp A1
Psp A2
Psp C
Omp p6
Timothy
Mugwort
Ragweed
Stimulus

Figure 10.6 Nonatopic Asthmatic Patient 3 - Eosinophilic

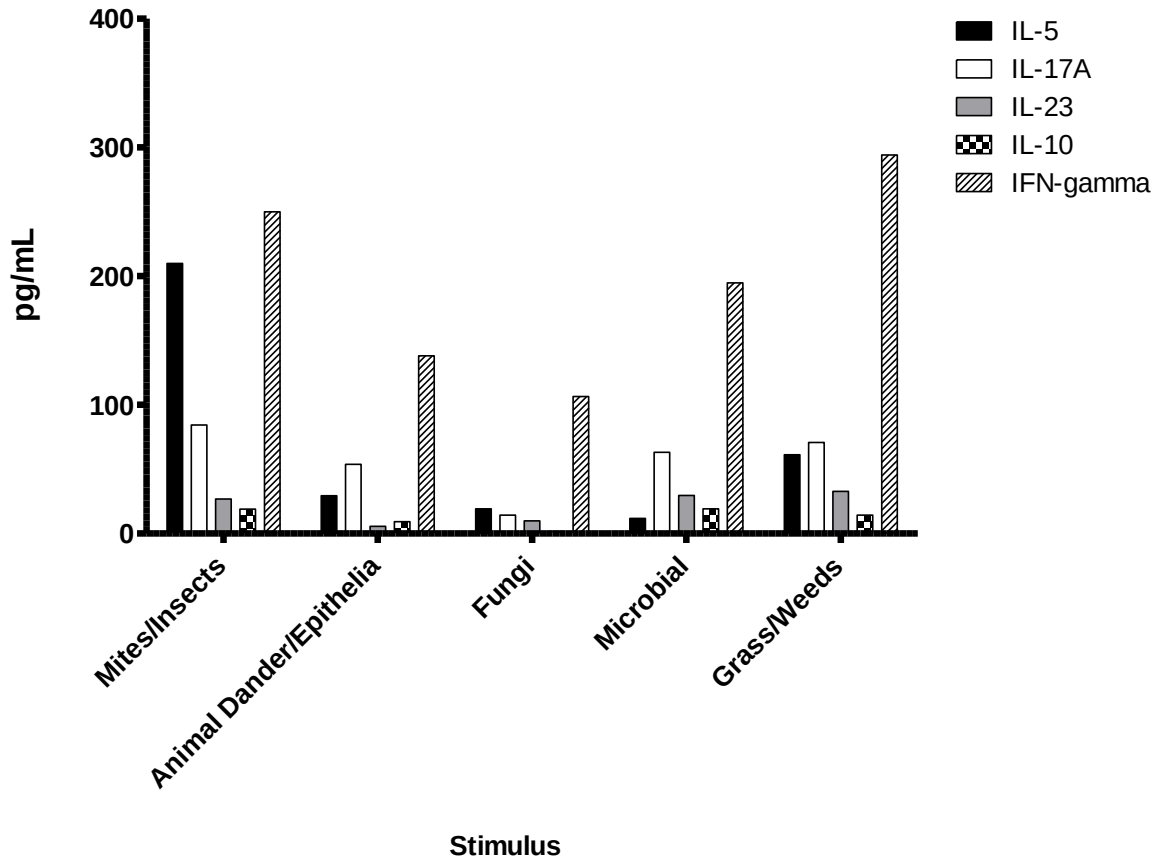


Figure 10.7 Nonatopic Asthmatic Patient 4 - Eosinophilic

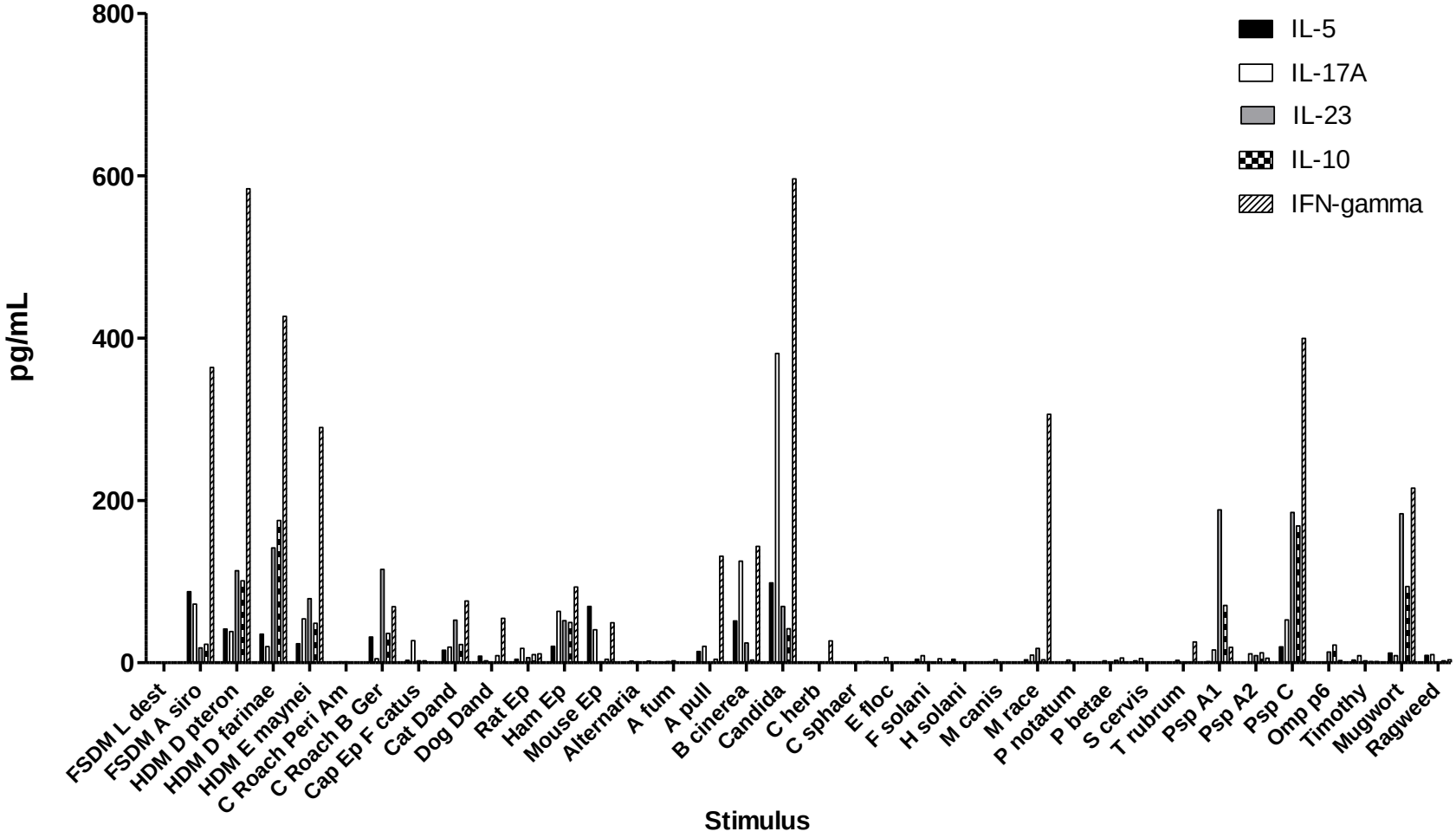


Figure 10.8 Nonatopic Asthmatic Patient 4 - Eosinophilic

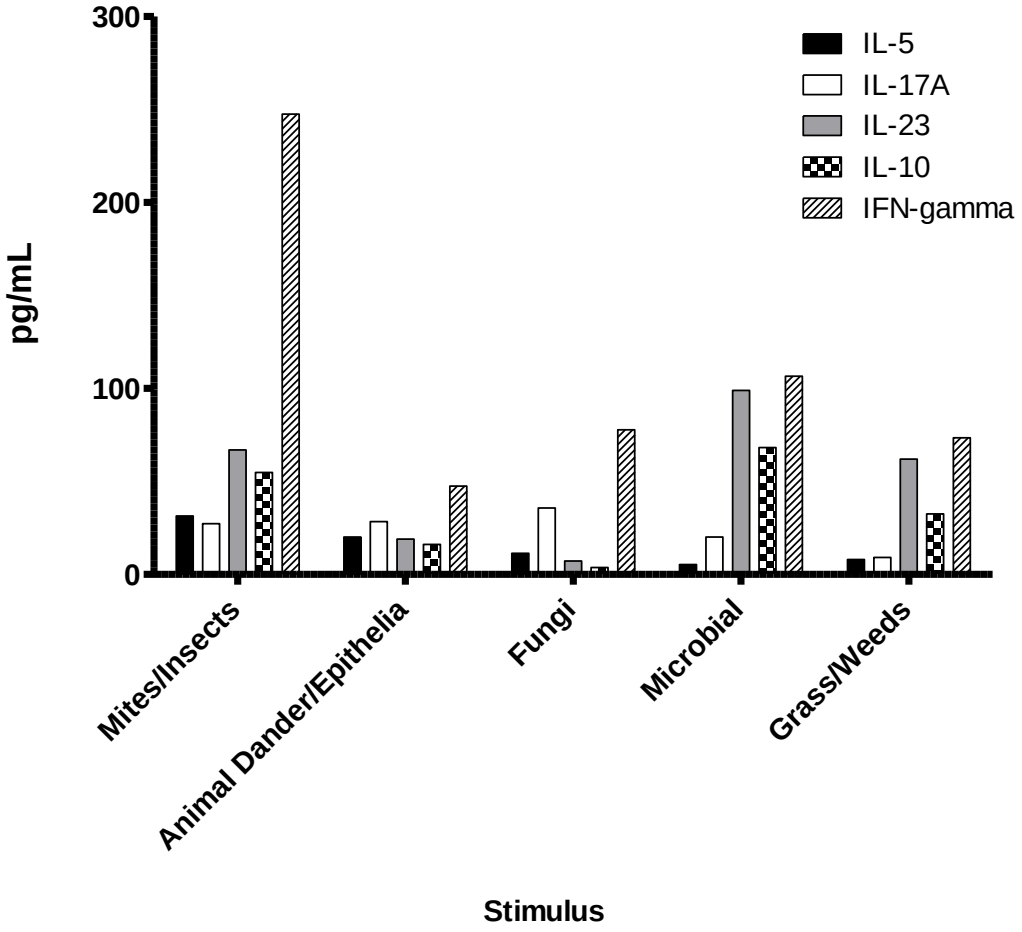


Figure 10.9 Noneosinophilic Asthmatic Patient 5 - Neutrophilic

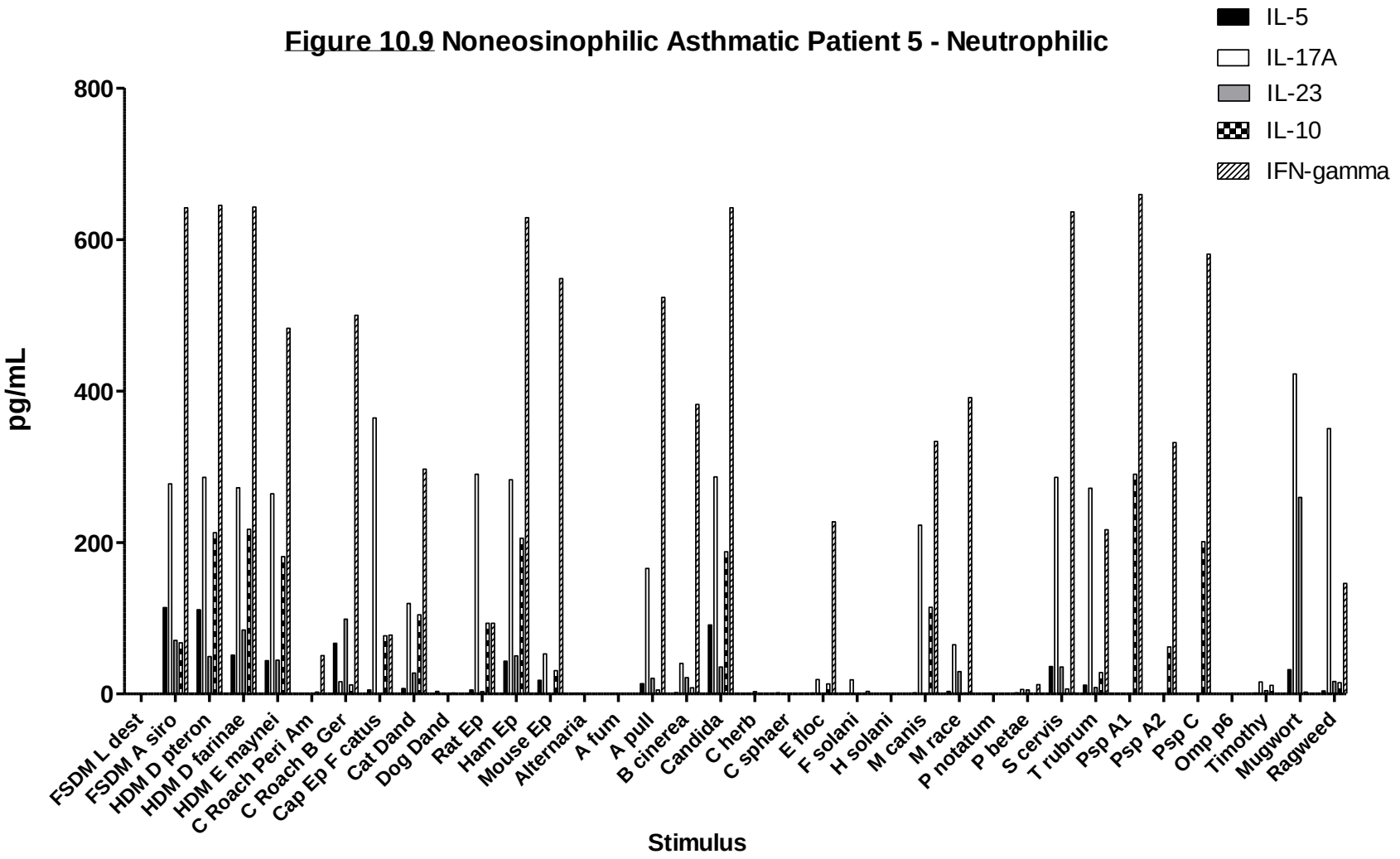


Figure 10.10 Noneosinophilic Asthmatic Patient 5 - Neutrophilic

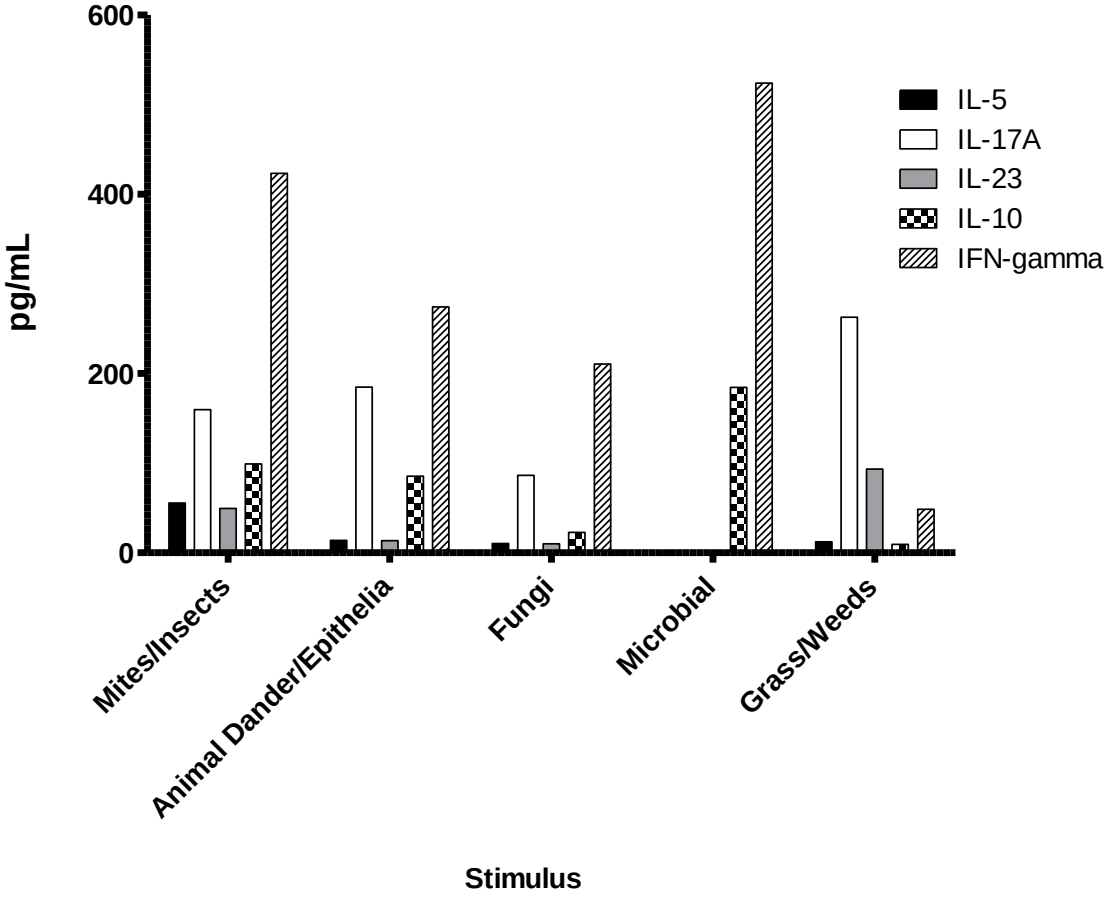


Figure 10.11 Nonatopic Asthmatic Patient 6 - Eosinophilic

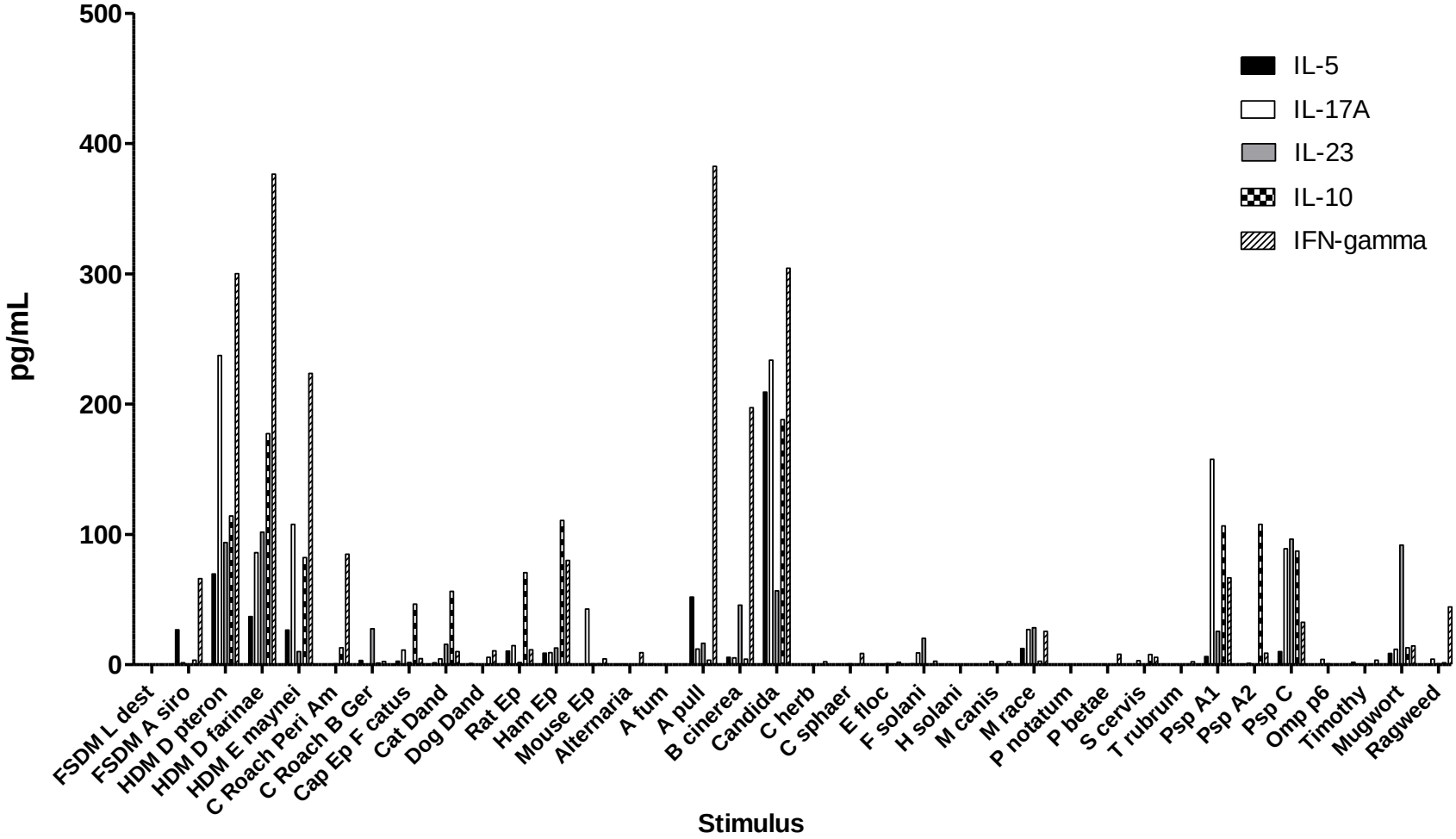


Figure 10.12 Nonatopic Asthmatic Patient 6 - Eosinophilic

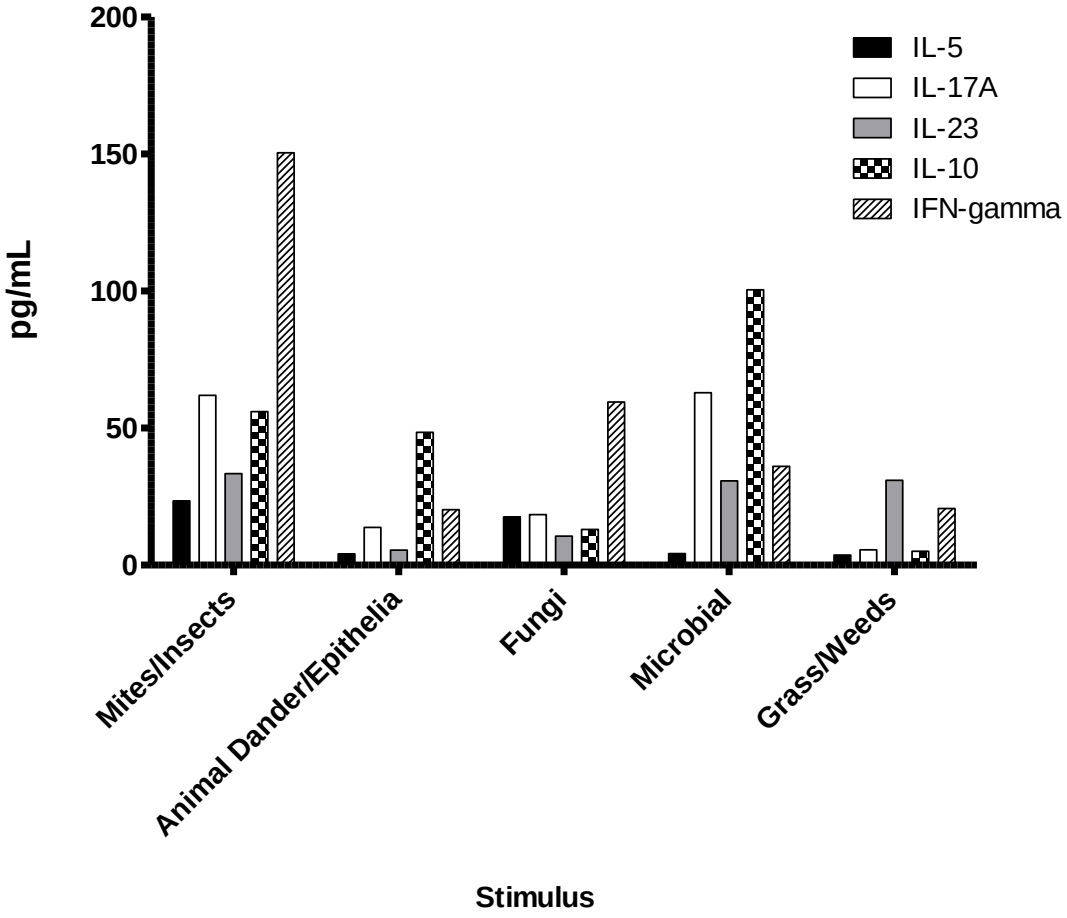


Figure 10.13 Nonatopic Asthmatic Patient 7 - Eosinophilic

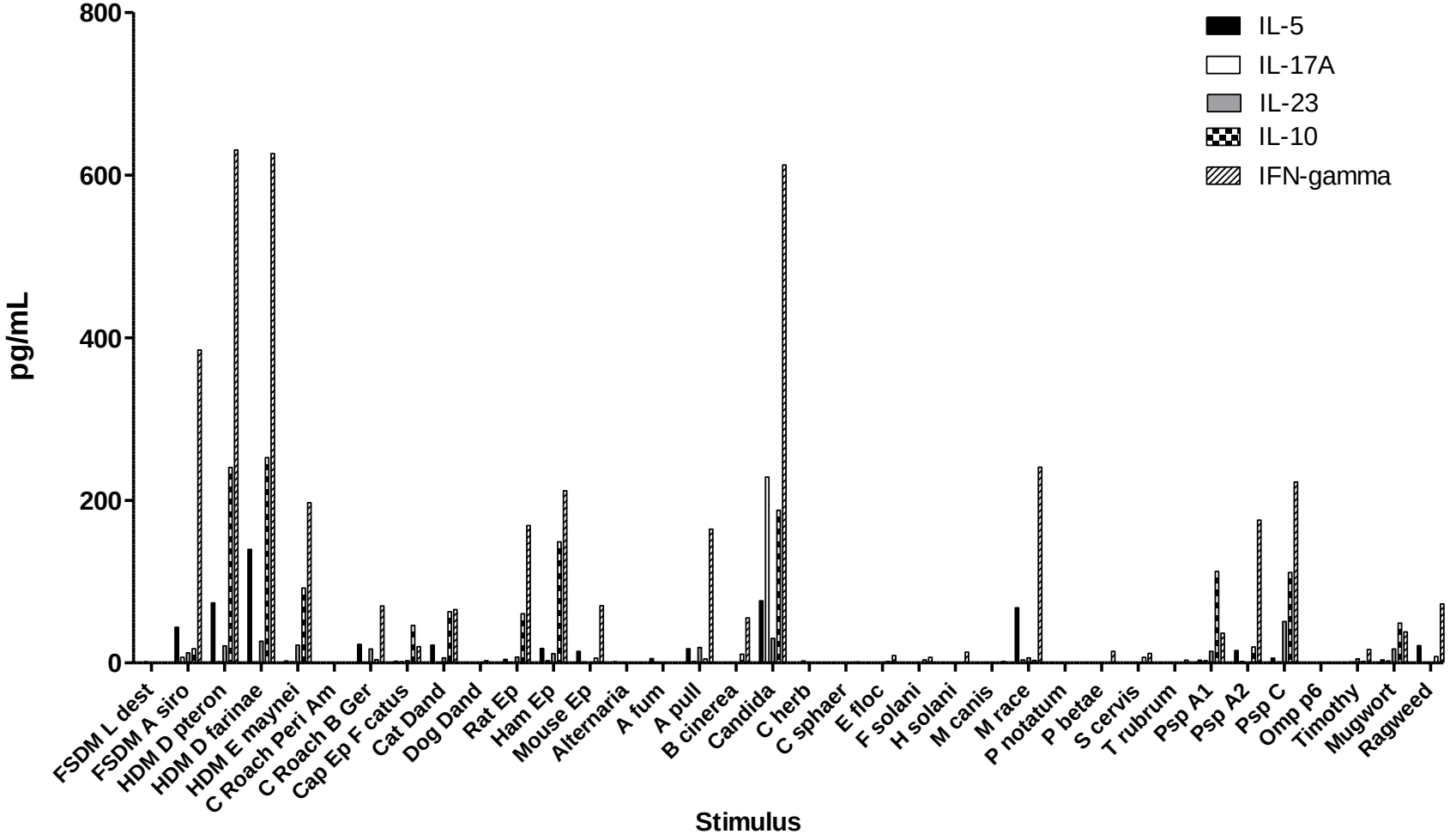


Figure 10.14 Nonatopic Asthmatic Patient 7 - Eosinophilic

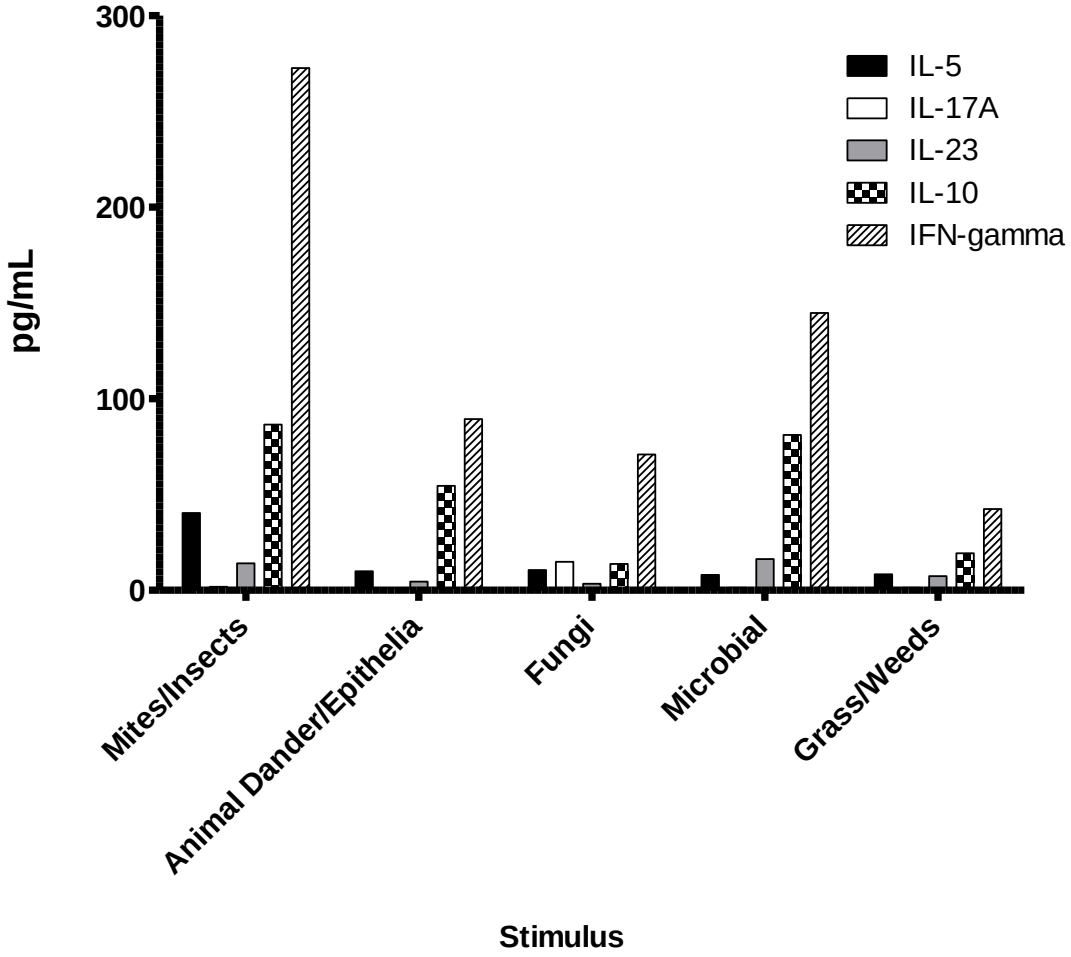


Figure 10.15 Nonatopic Asthmatic Patient 8 - Noneosinophilic

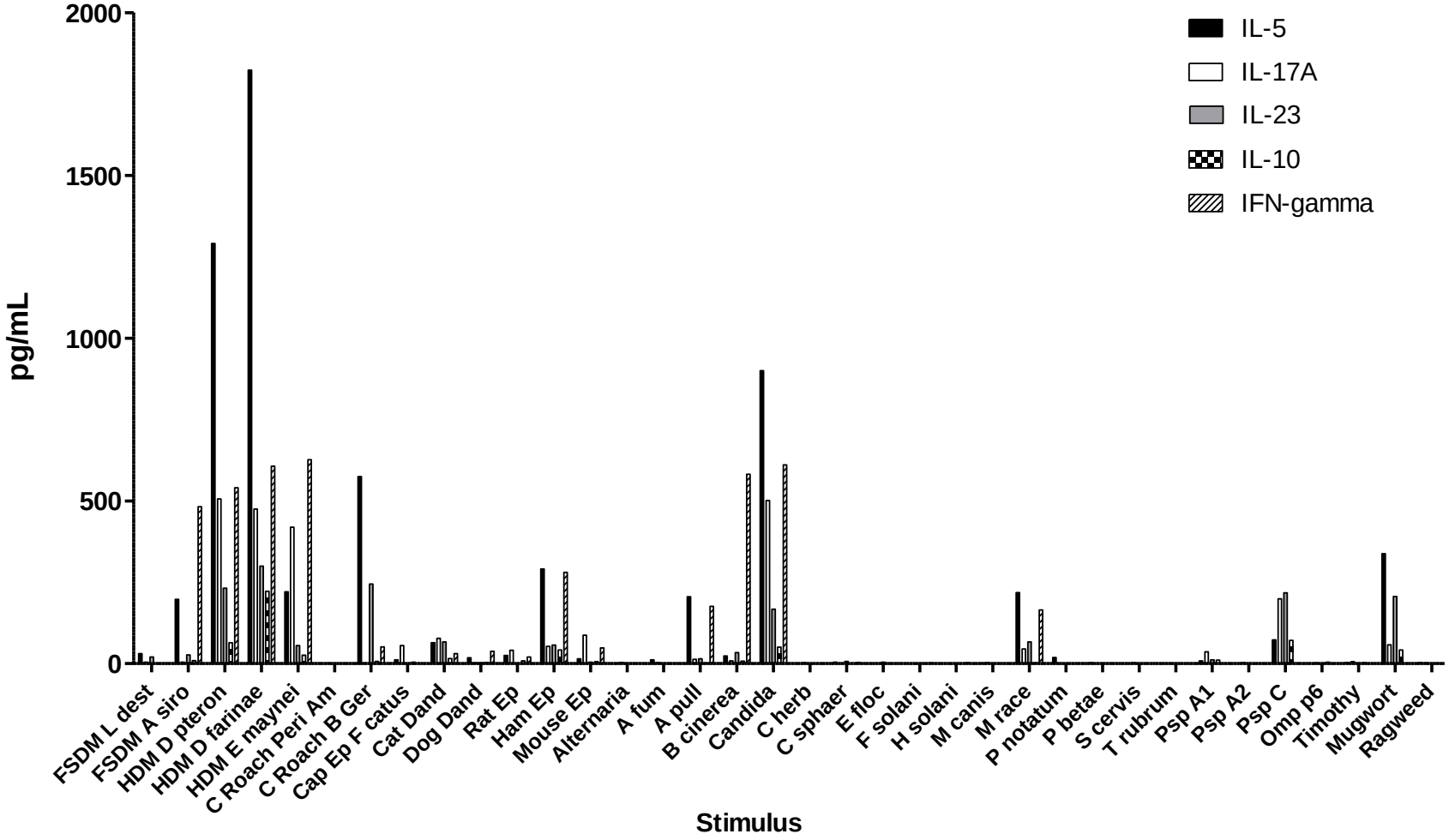


Figure 10.16 Nonatopic Asthmatic Patient 8 - Noneosinophilic

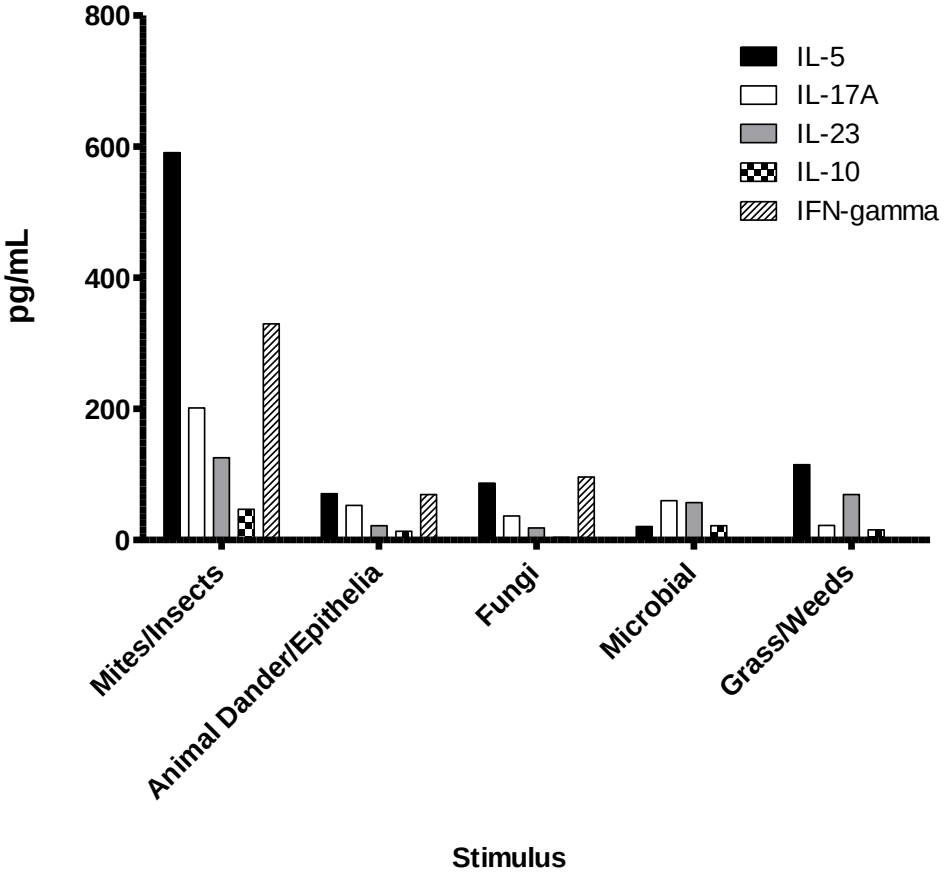


Figure 10.17 Nonatopic Asthmatic Patient 9 - Eosinophilic

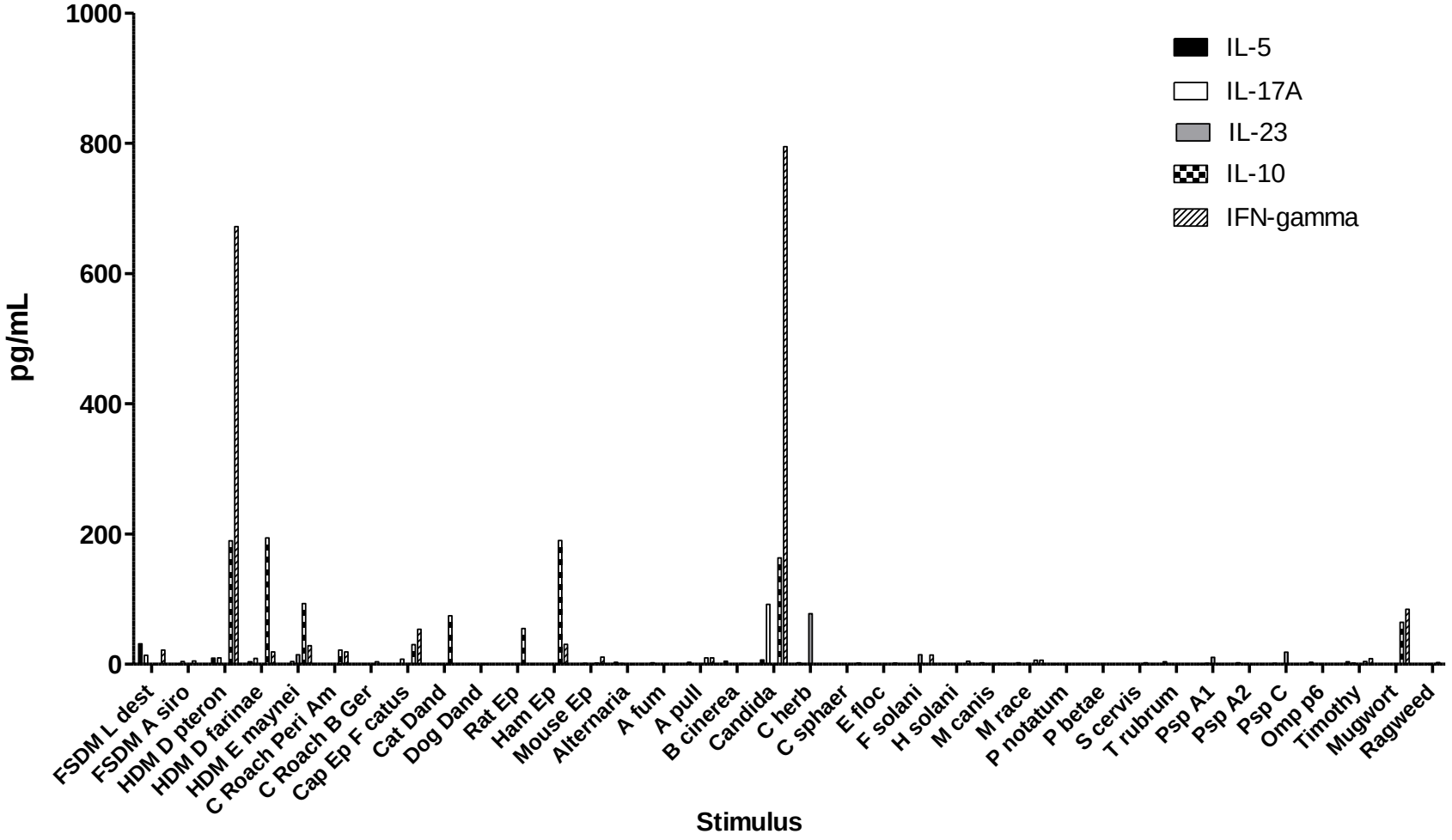


Figure 10.18 Nonatopic Asthmatic Patient 9 - Eosinophilic

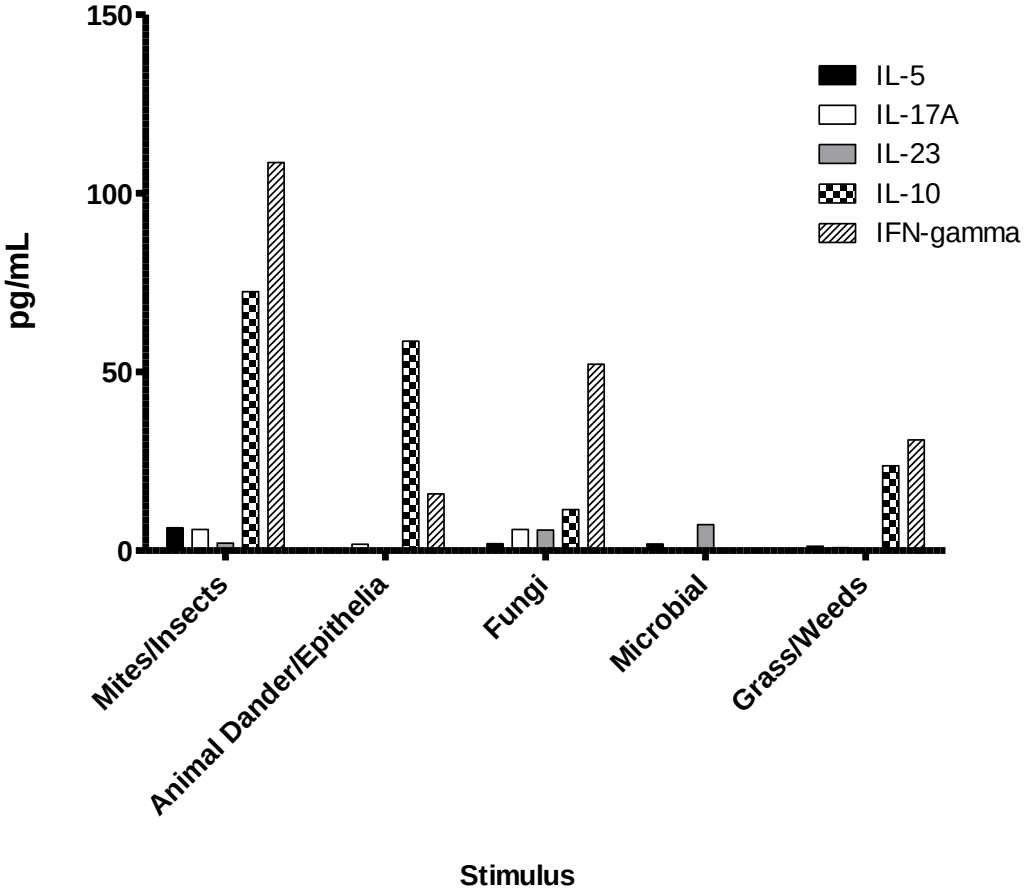


Figure 10.19 Nonatopic Asthmatic Patient 10 - Eosinophilic

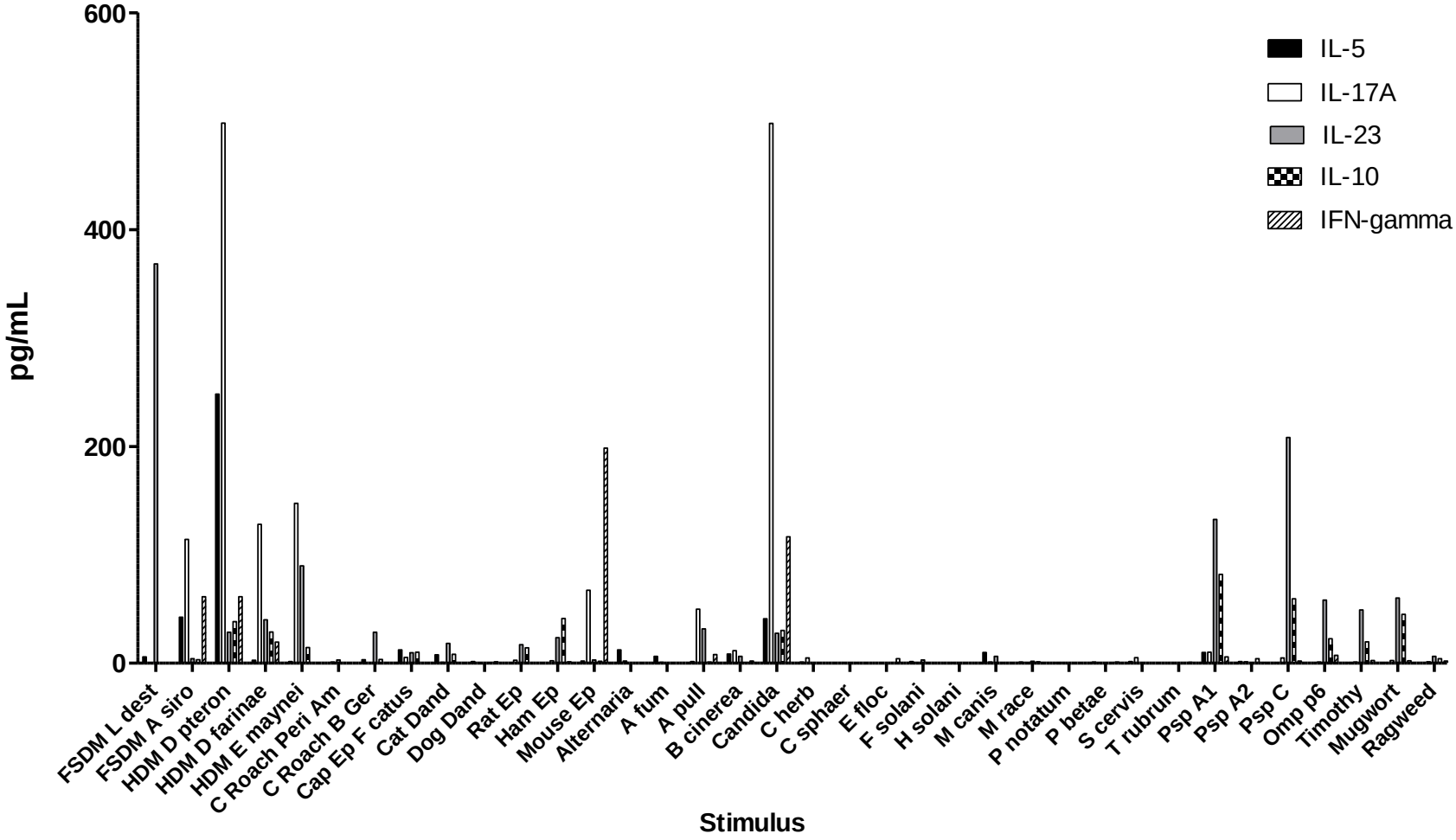


Figure 10.20 **Nonatopic Asthmatic Patient 10 - Eosinophilic**

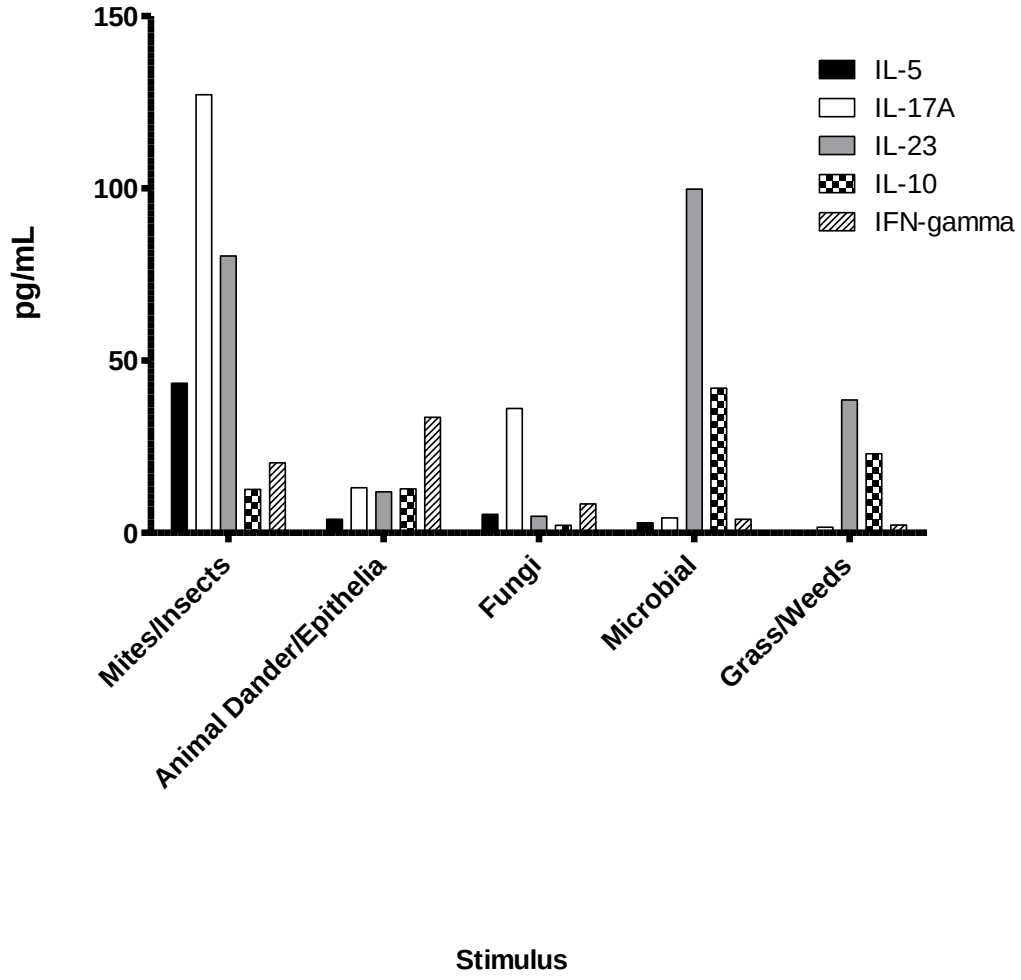


Figure 10.21 Nonatopic Asthmatic Patient 11 - Neutrophilic

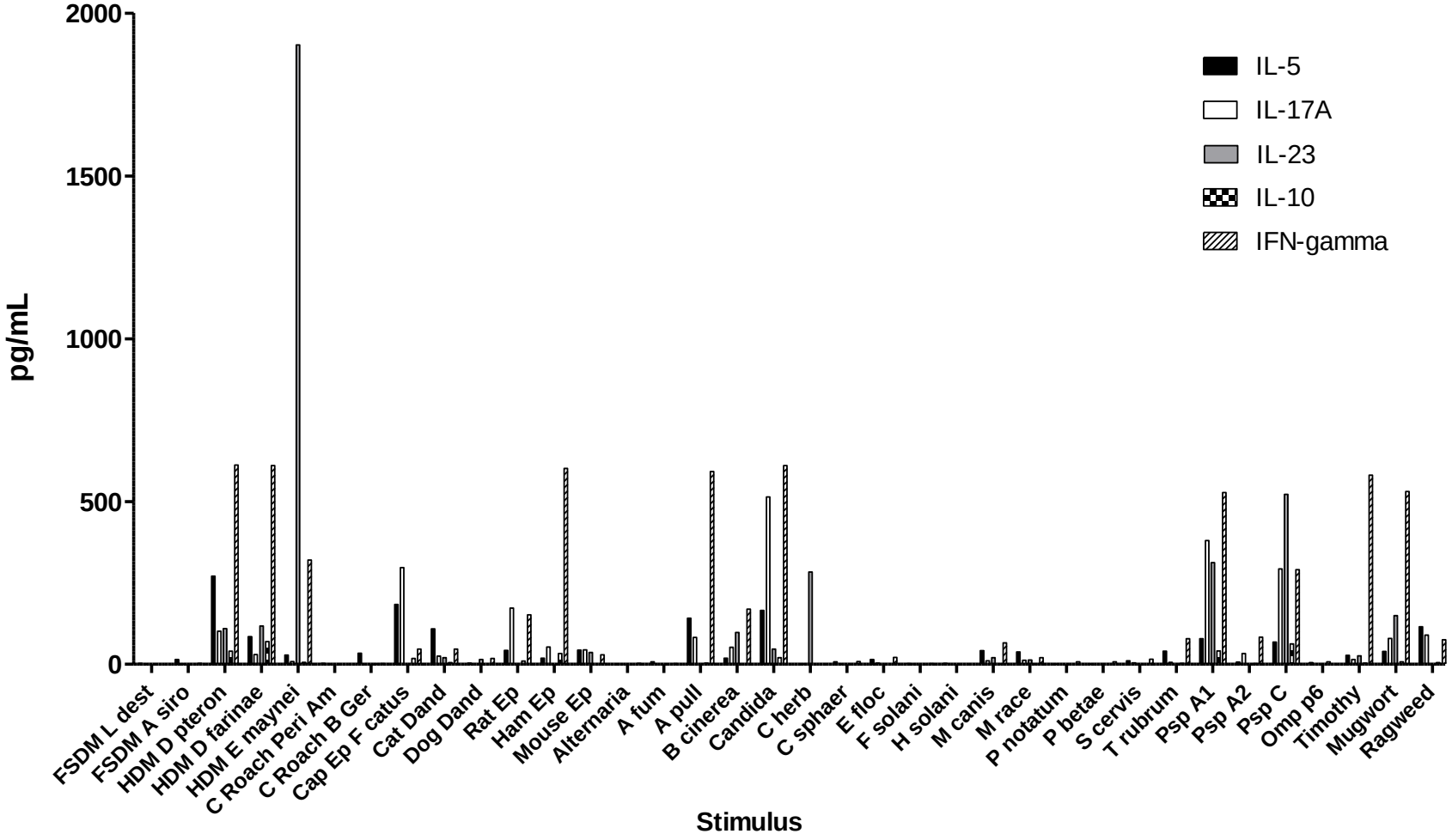


Figure 10.22 Nonatopic Asthmatic Patient 11 - Neutrophilic

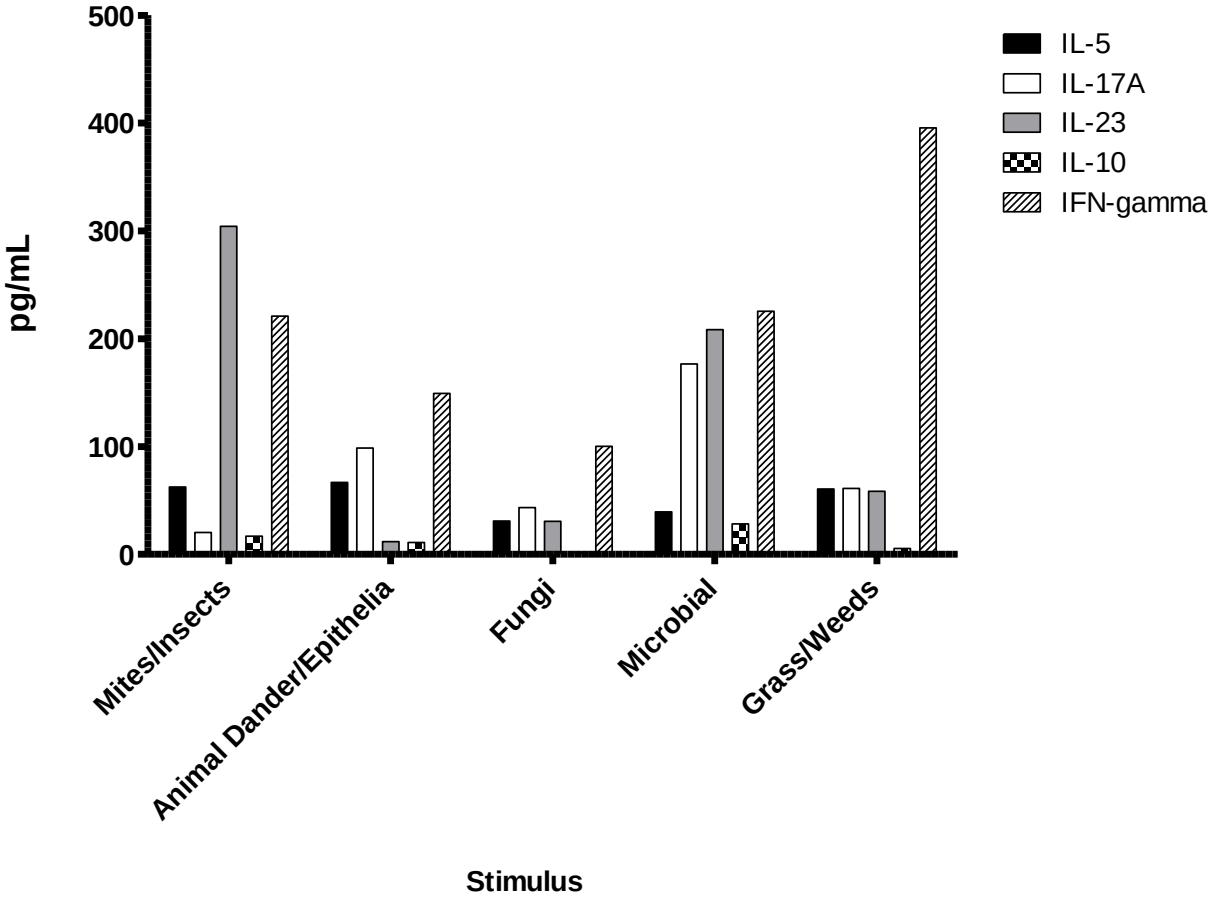


Figure 10.23 Nonatopic Asthmatic Patient 12 - Eosinophilic

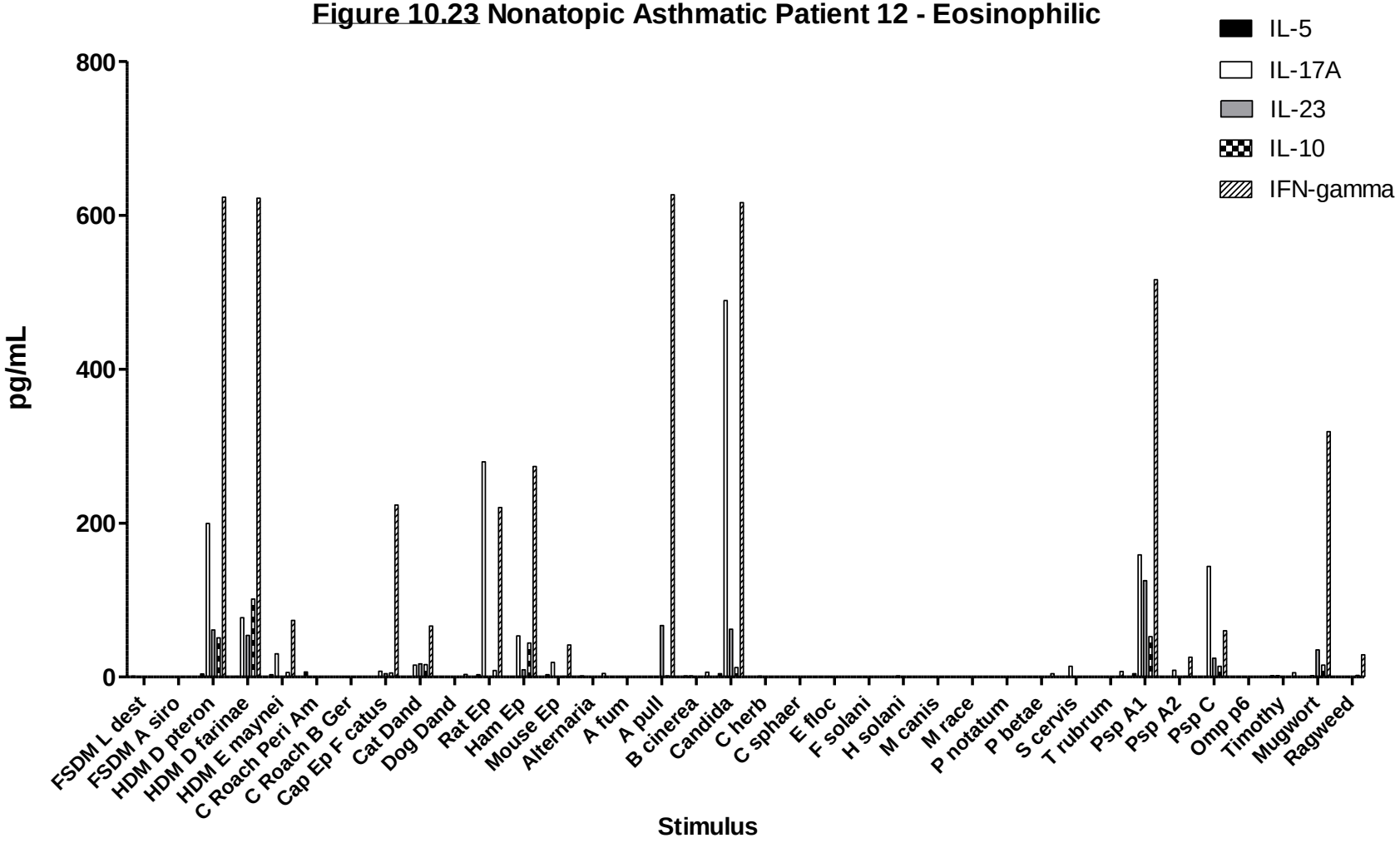


Figure 10.24 Nonatopic Asthmatic Patient 12 - Eosinophilic

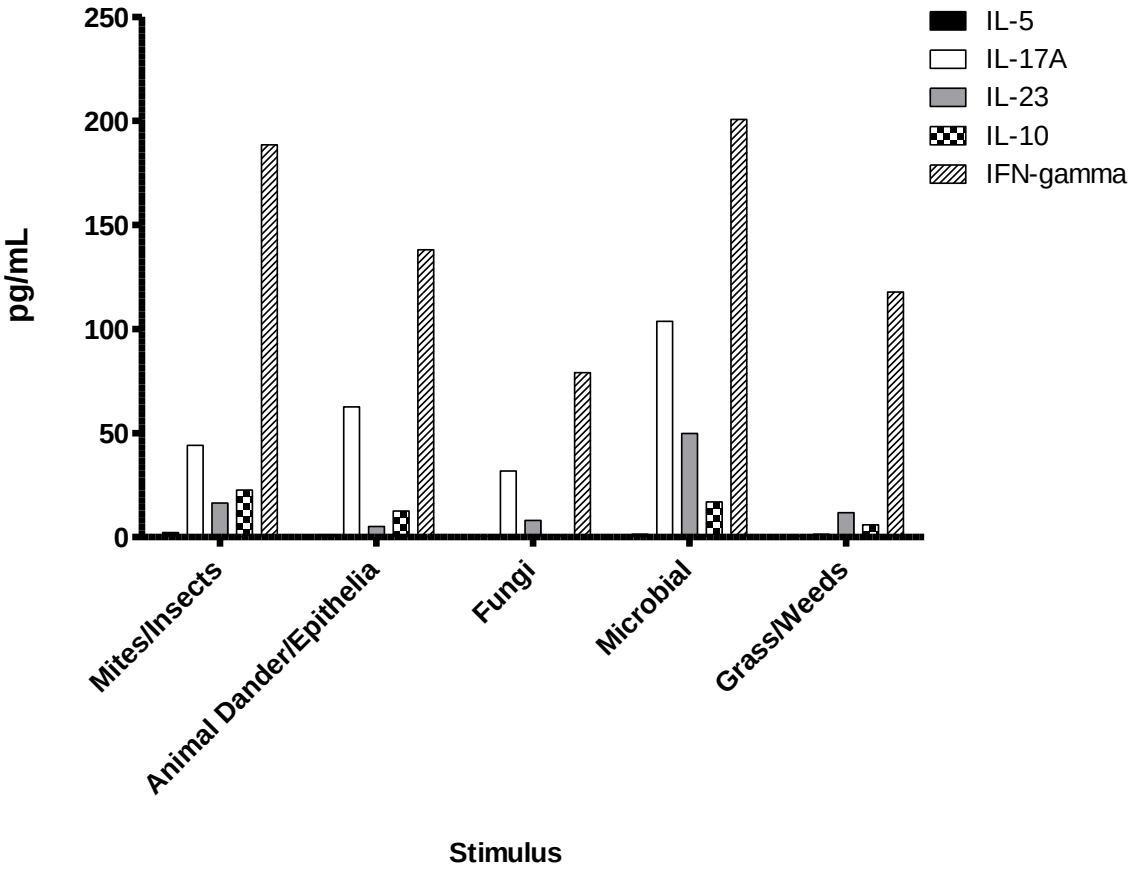


Figure 10.25 Nonatopic Asthmatic Patient 13 - Neutrophilic

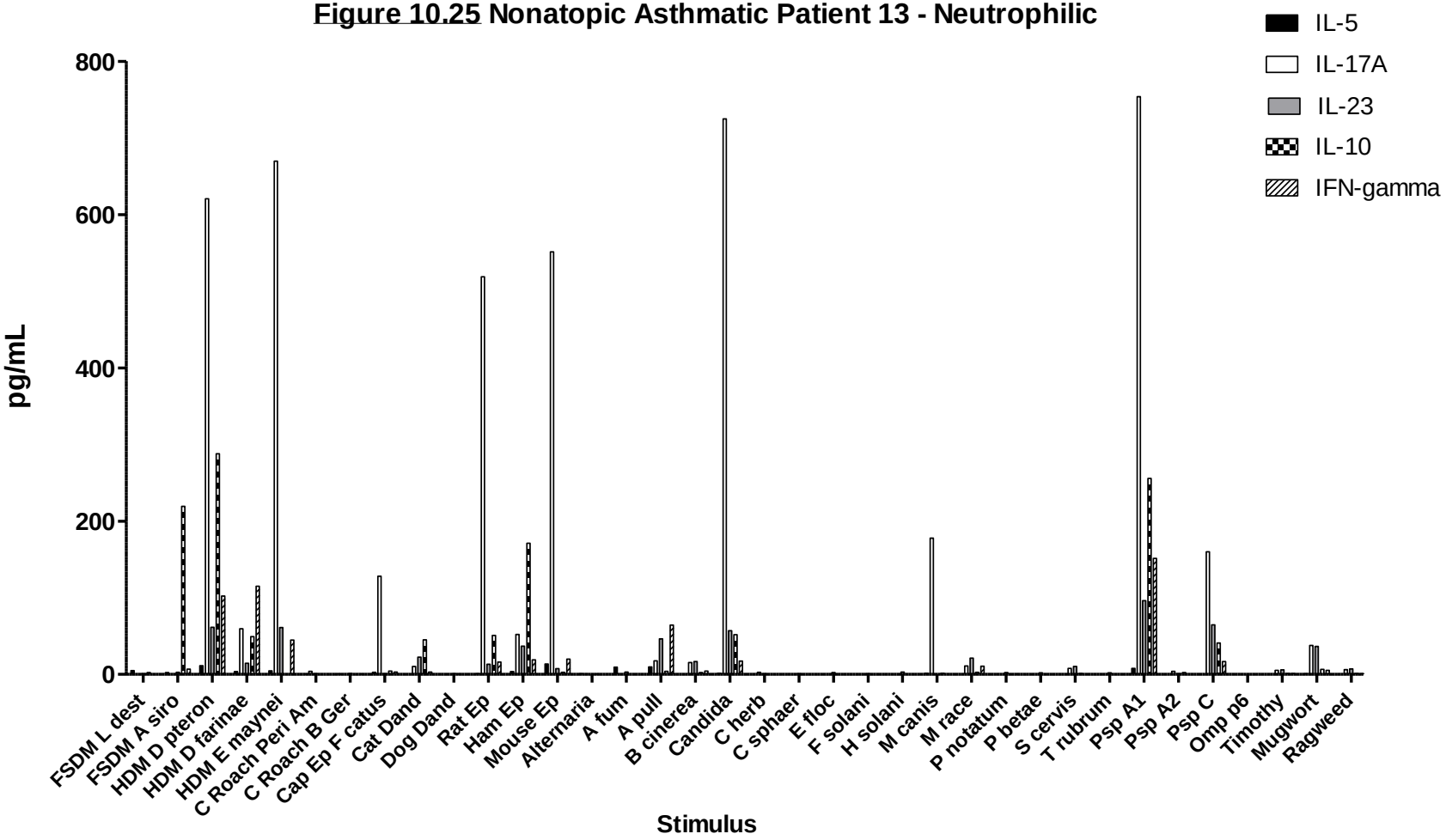


Figure 10.26 Nonatopic Asthmatic Patient 13 - Neutrophilic

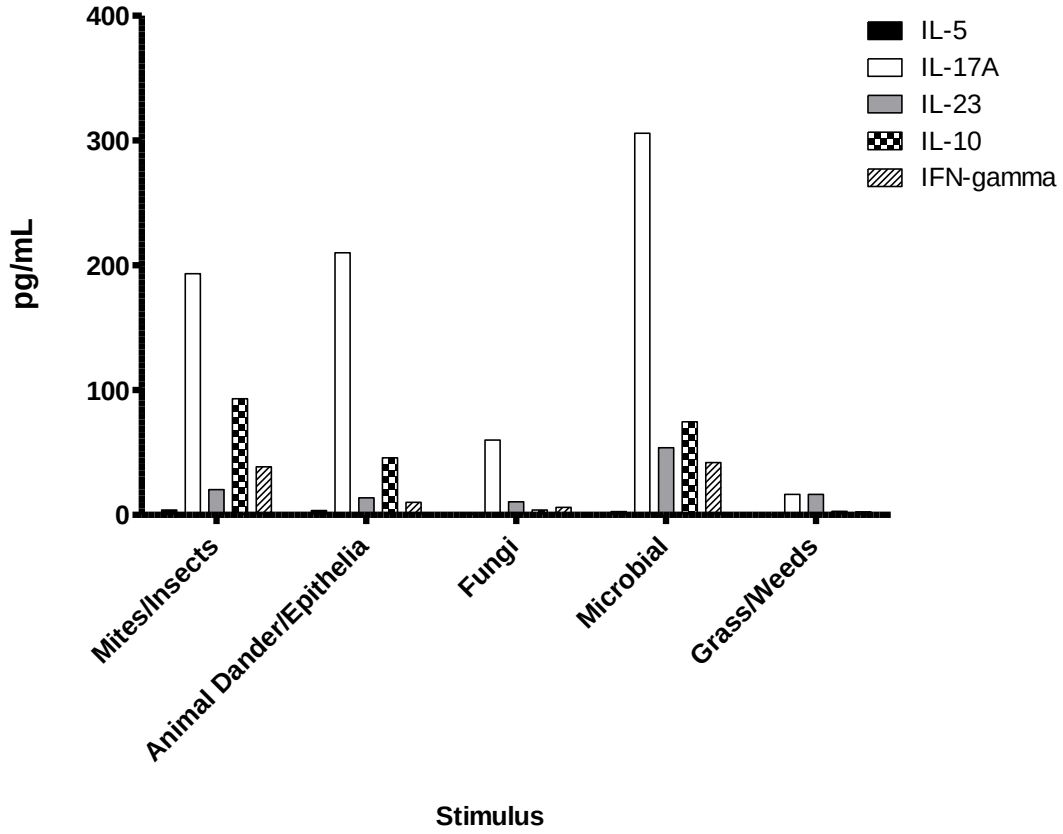


Figure 10.27 Nonatopic Asthmatic Patient 15 - Eosinophilic/Neutrophilic

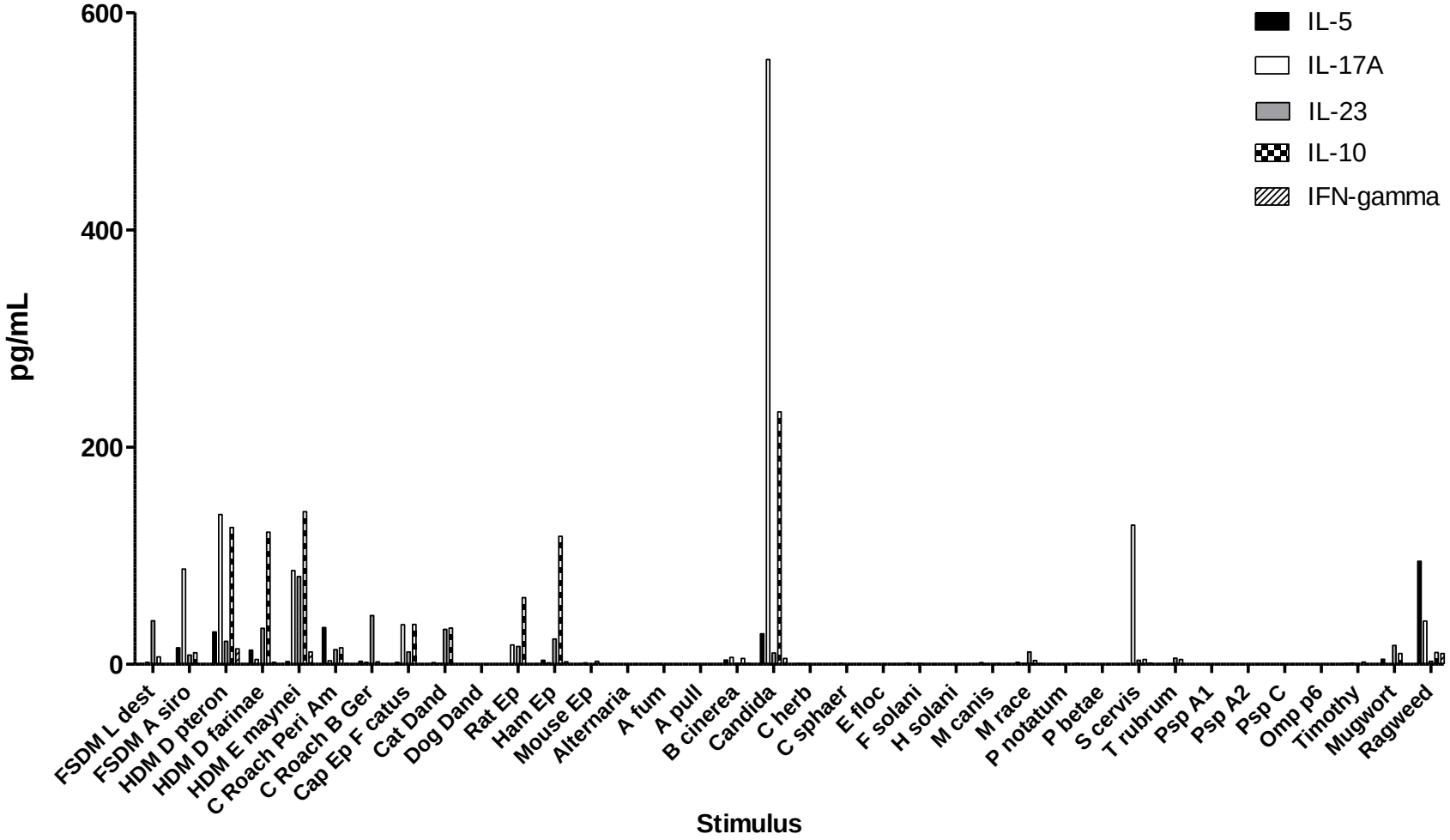


Figure 10.28 Nonatopic Asthmatic Patient 15 - Eosinophilic/Neutrophilic

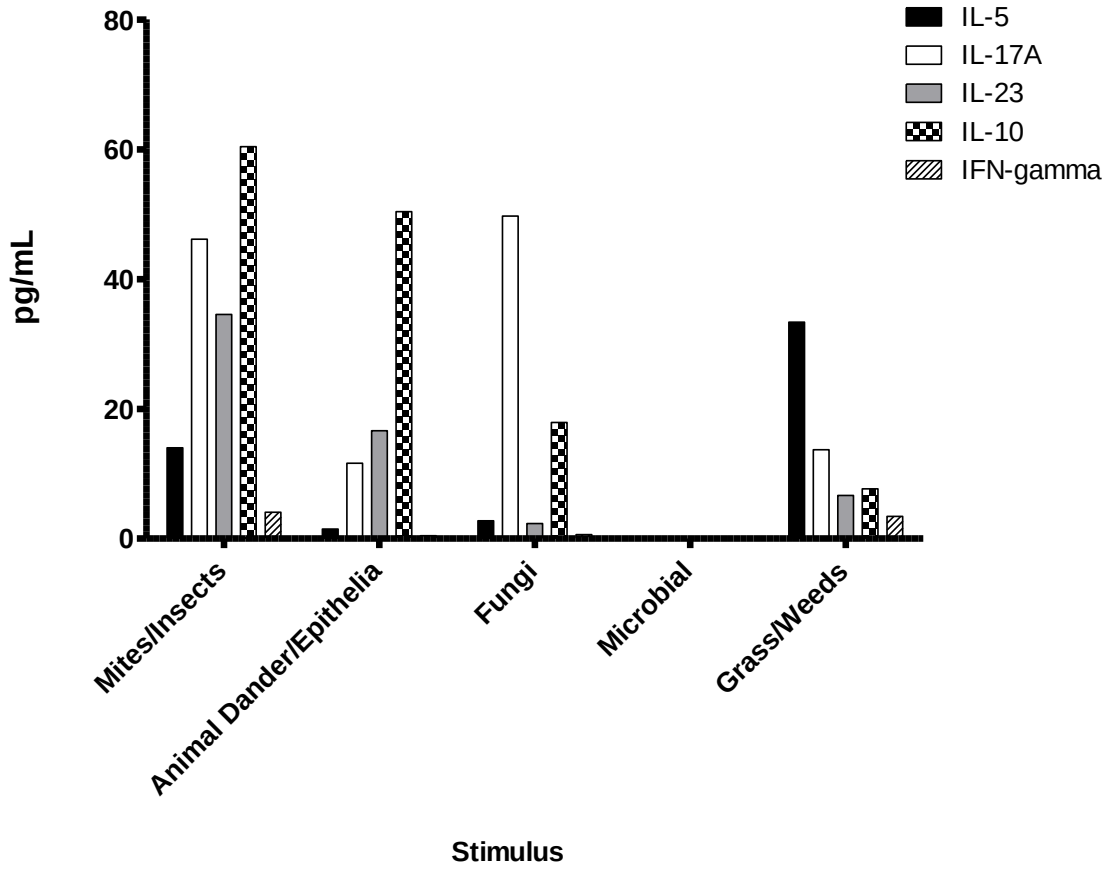


Figure 10.29 Nonatopic Asthmatic Patient 16 - Eosinophilic

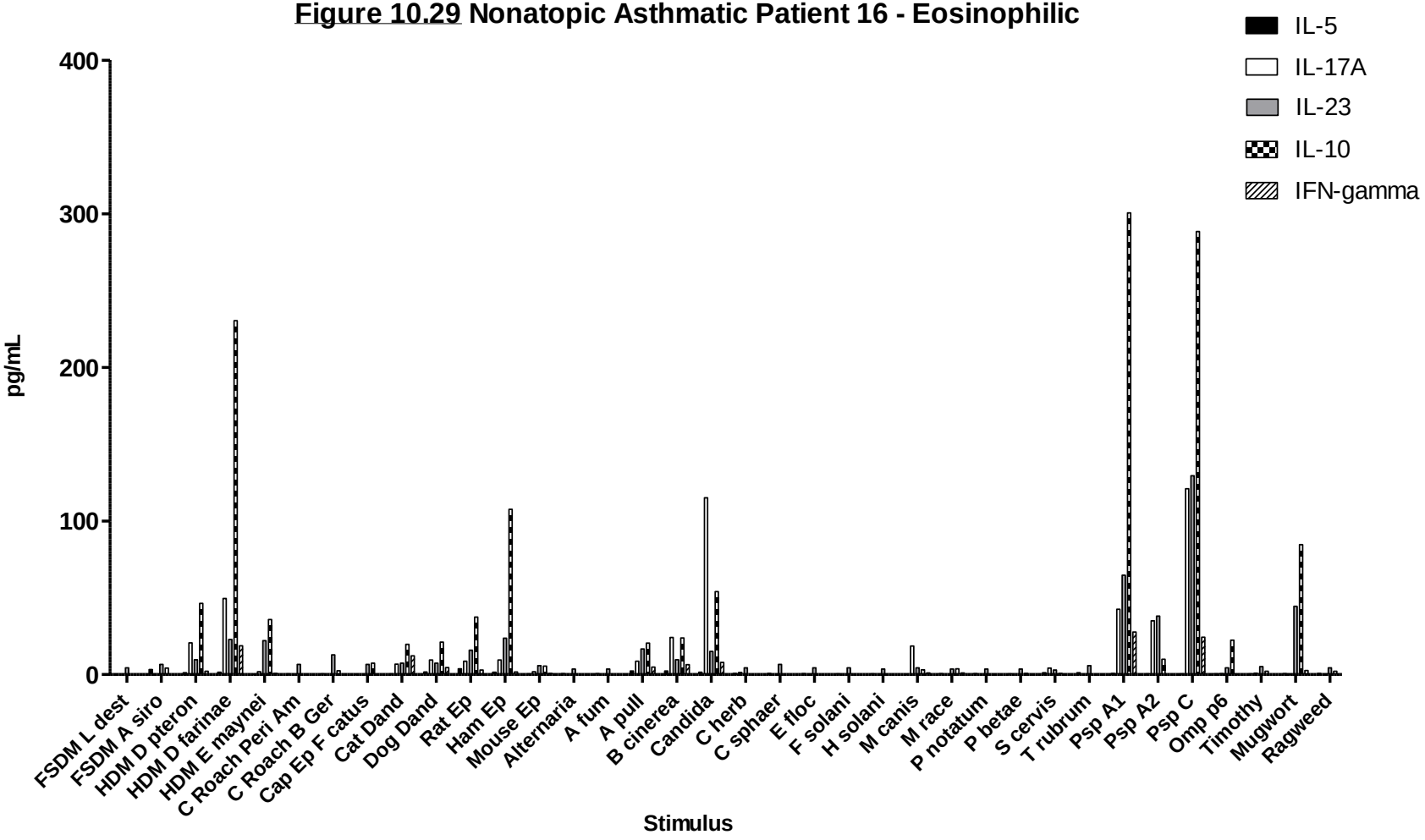


Figure 10.30 Nonatopic Asthmatic Patient 16 - Eosinophilic

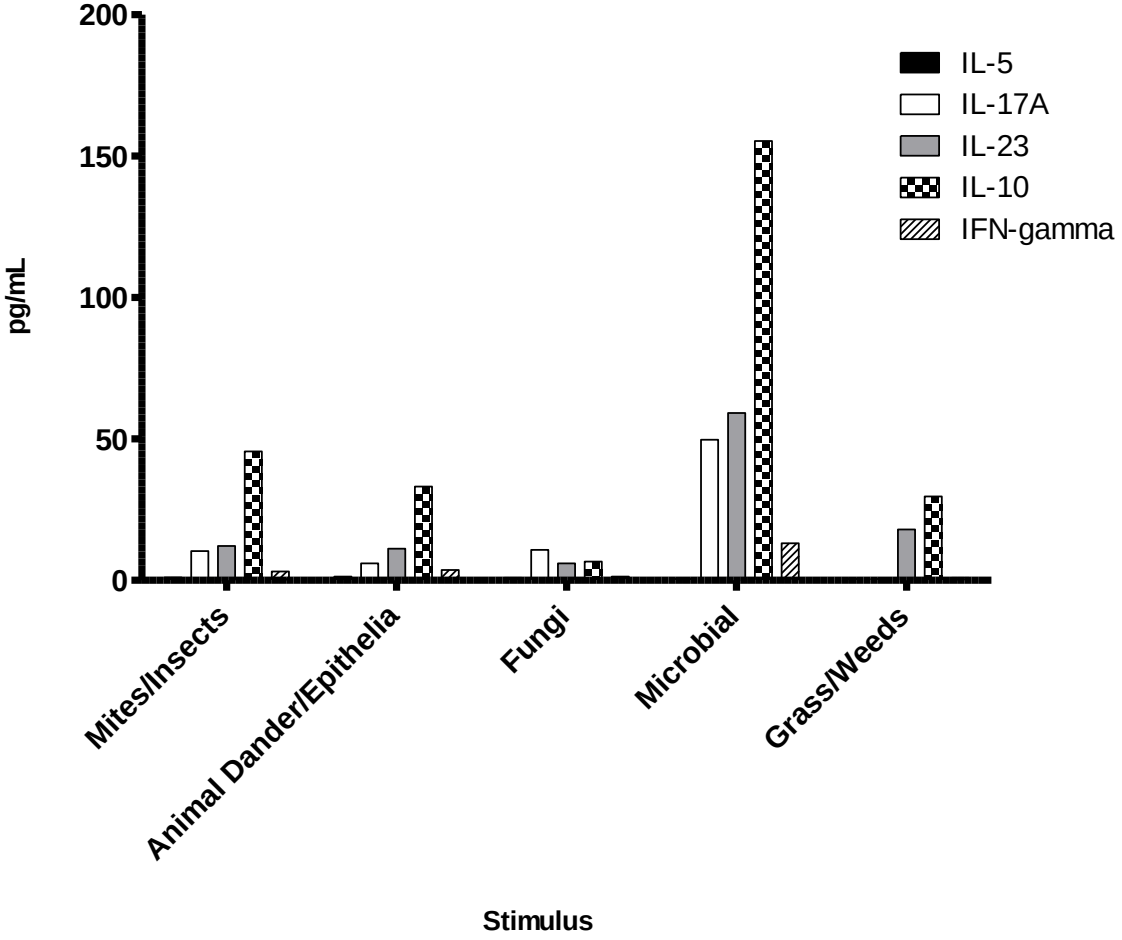


Figure 10.31 Nonatopic Asthmatic Patient 17 - Eosinophilic

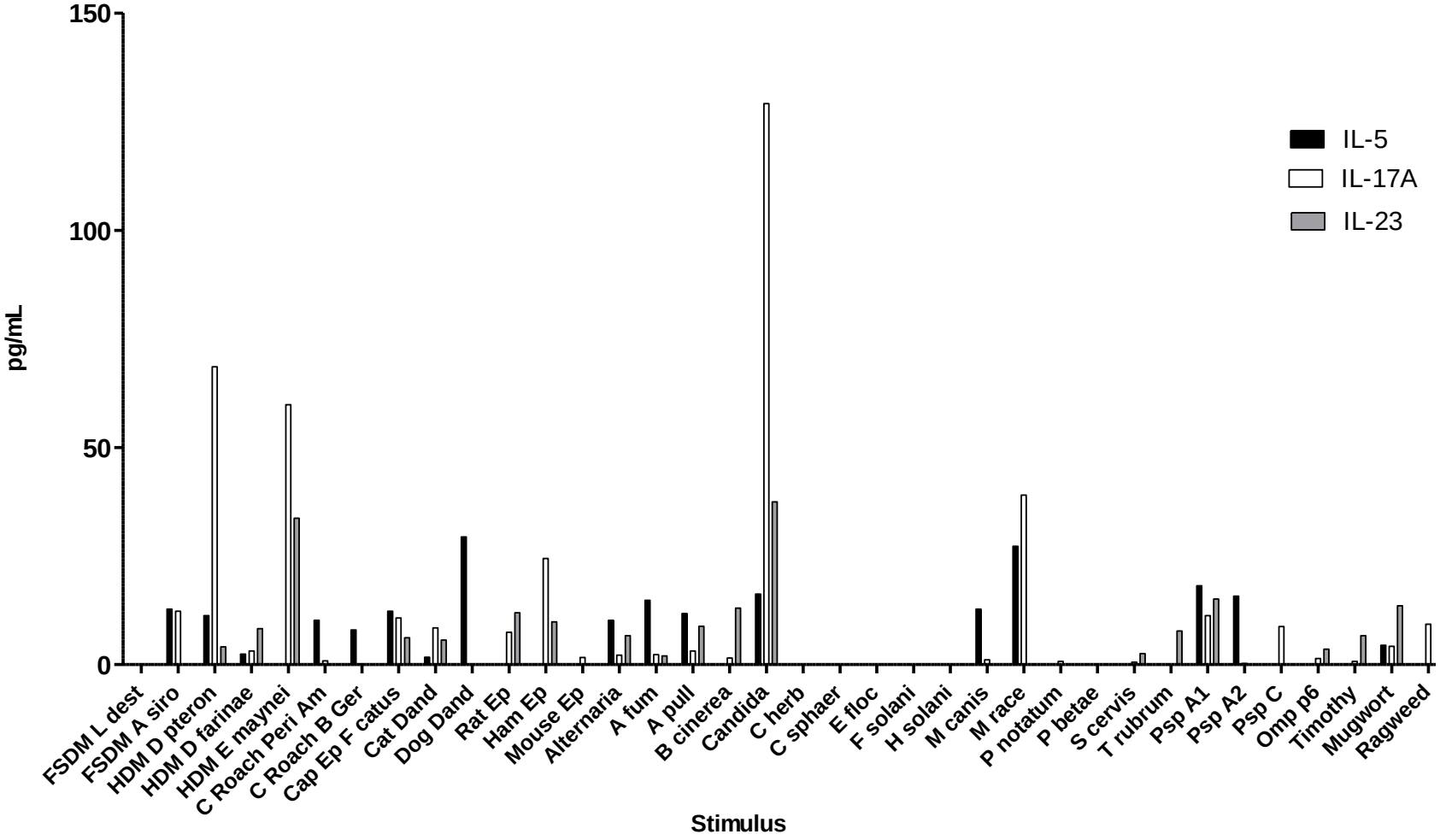


Figure 10.32 Nonatopic Asthmatic Patient 17 - Eosinophilic

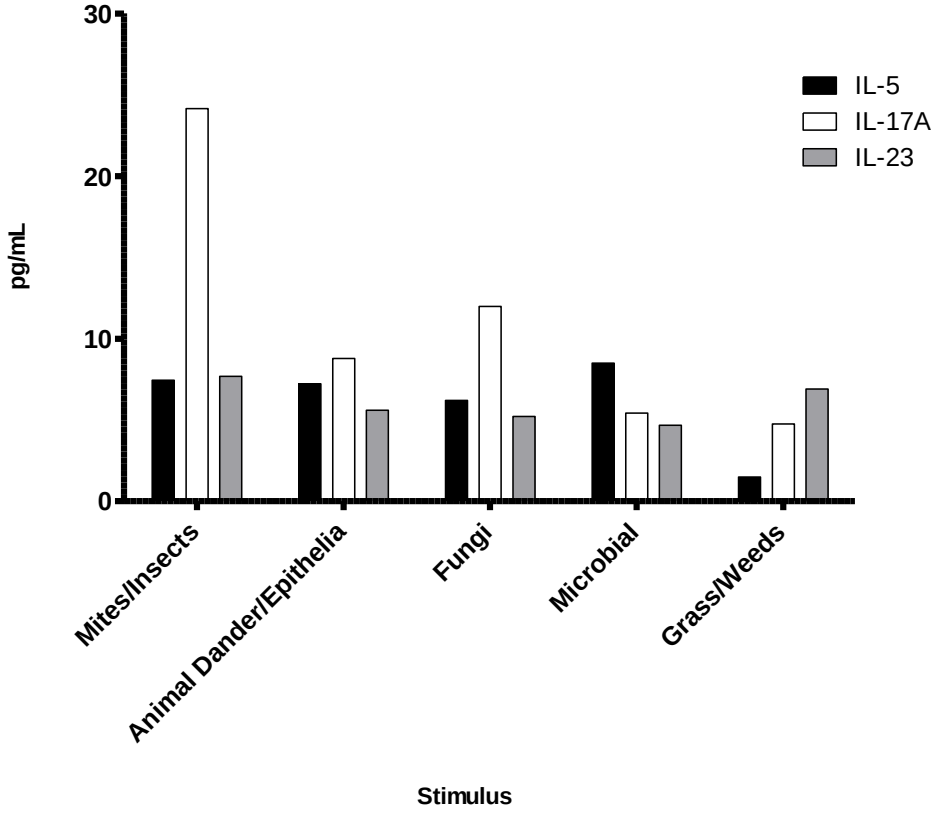


Figure 10.33 Nonatopic Asthmatic Patient 18 - Eosinophilic

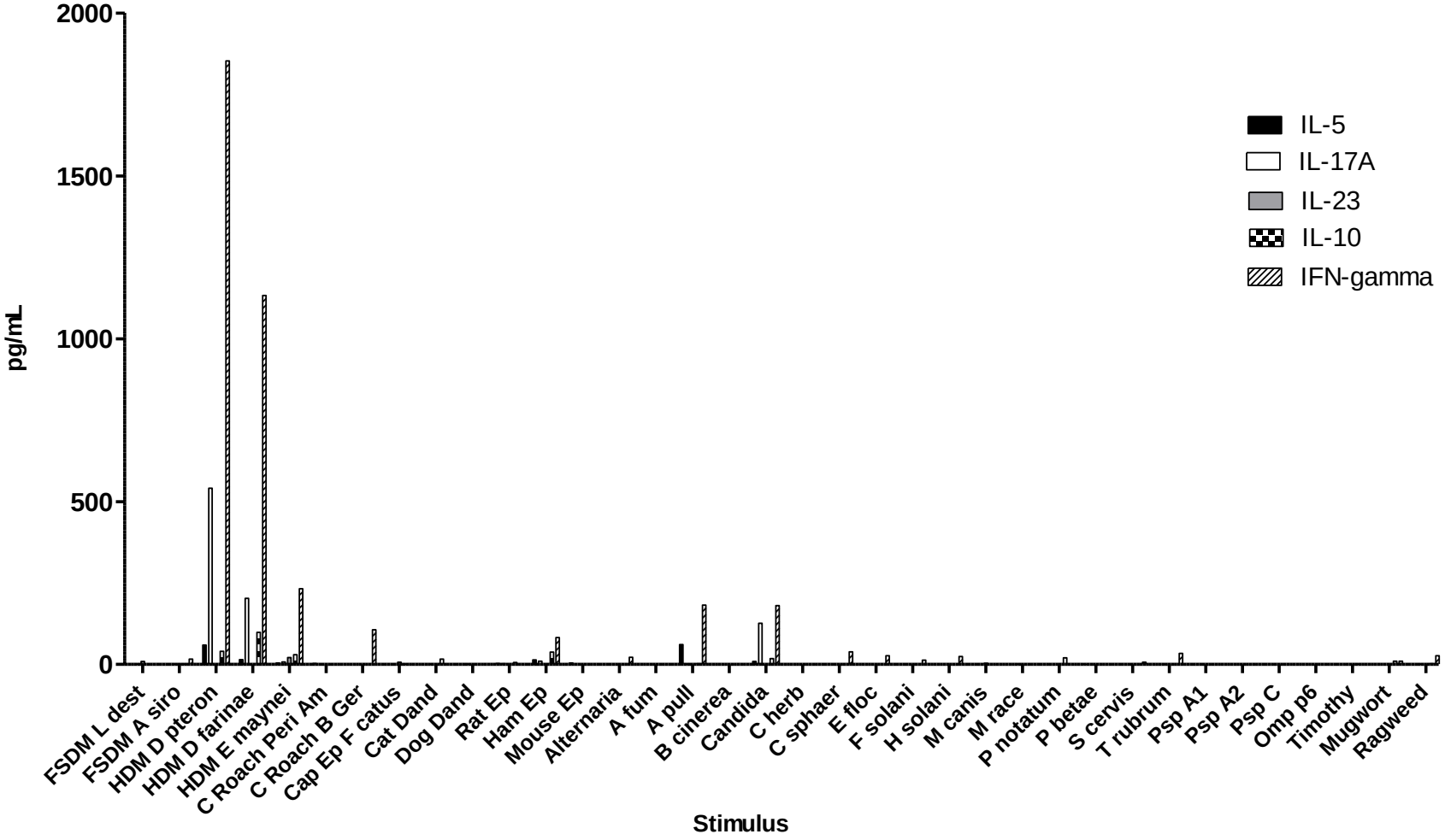


Figure 10.34 Nonatopic Asthmatic Patient 18 - Eosinophilic

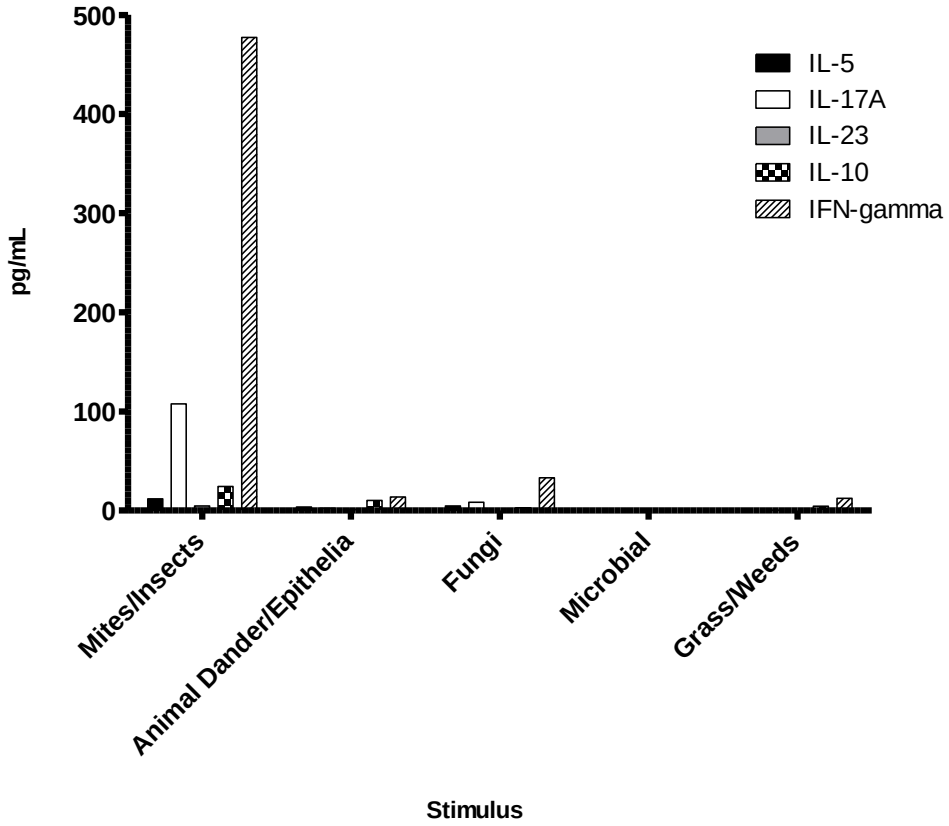


Figure 10.35 Nonatopic Asthmatic Patient 20 - Eosinophilic

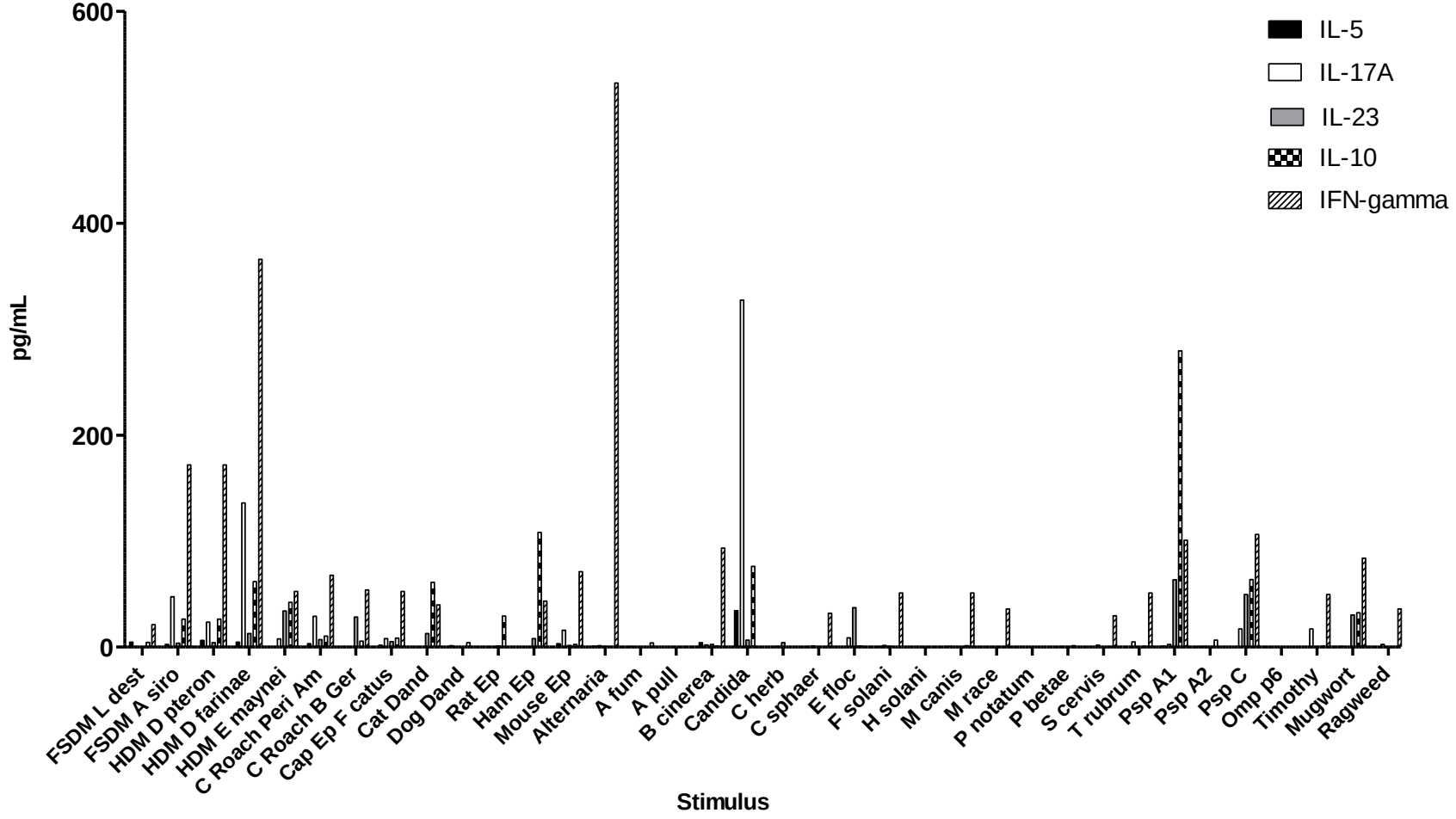


Figure 10.36 Nonatopic Asthmatic Patient 20 - Eosinophilic

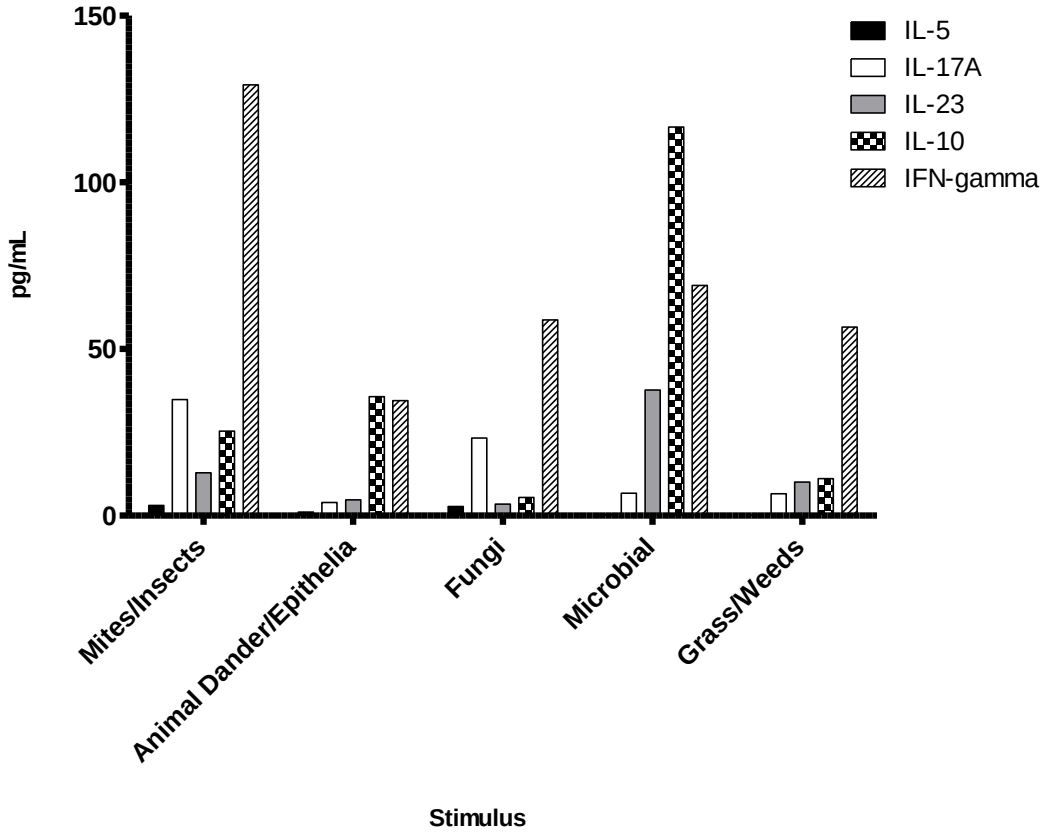


Figure 10.37 Nonatopic Asthmatic Patient 21 - Eosinophilic

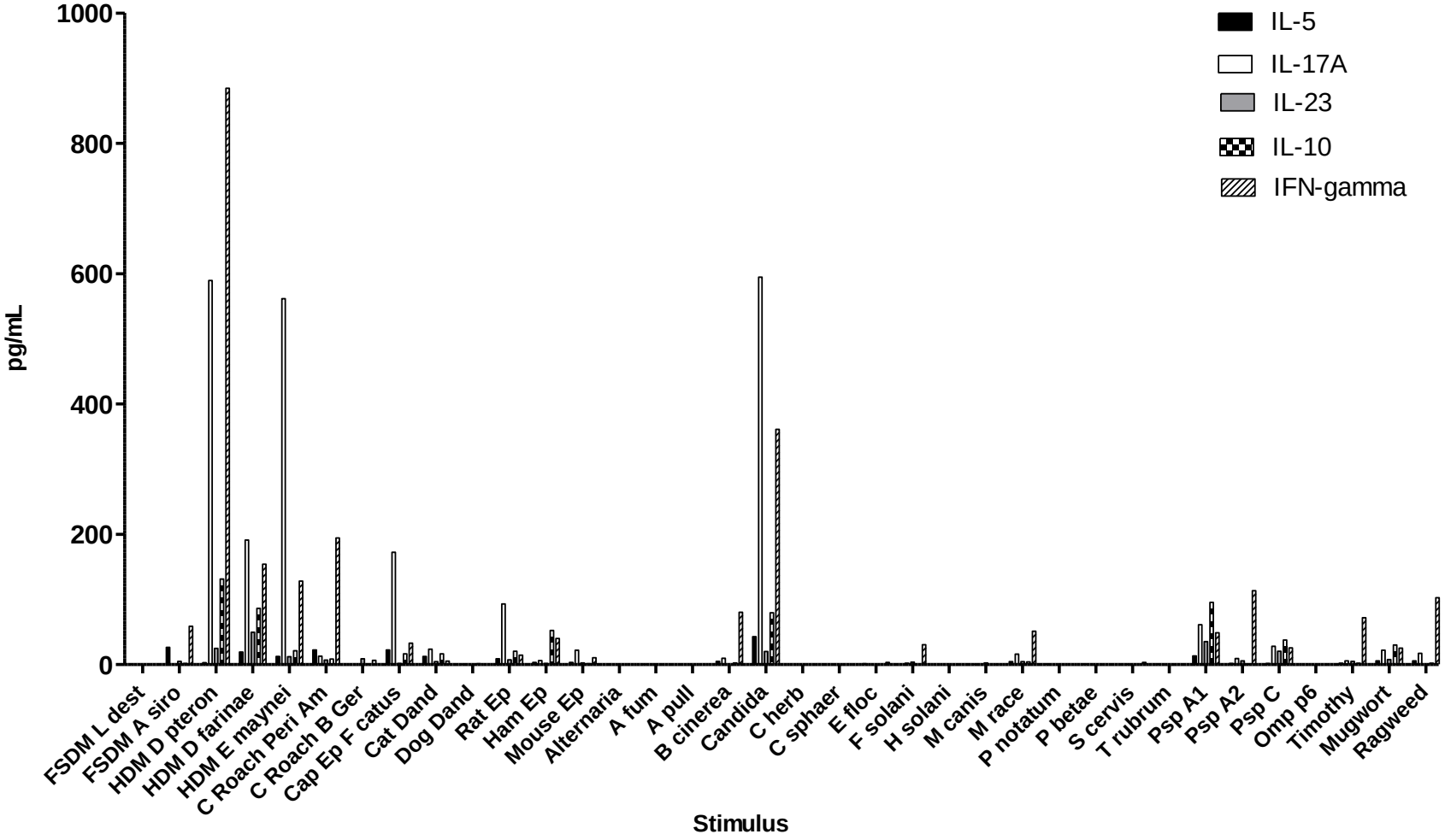


Figure 10.38 Nonatopic Asthmatic Patient 21 - Eosinophilic

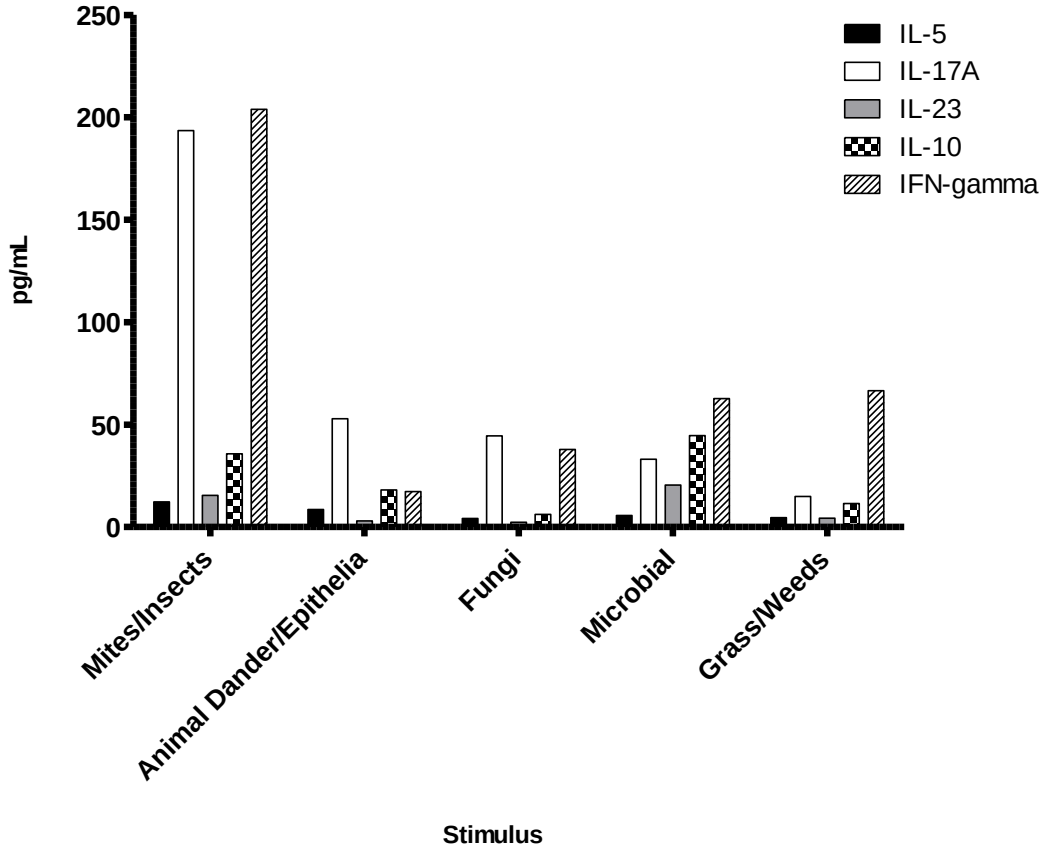


Figure 10.39 Nonatopic Asthmatic Patient 22 - Nonosinophilic

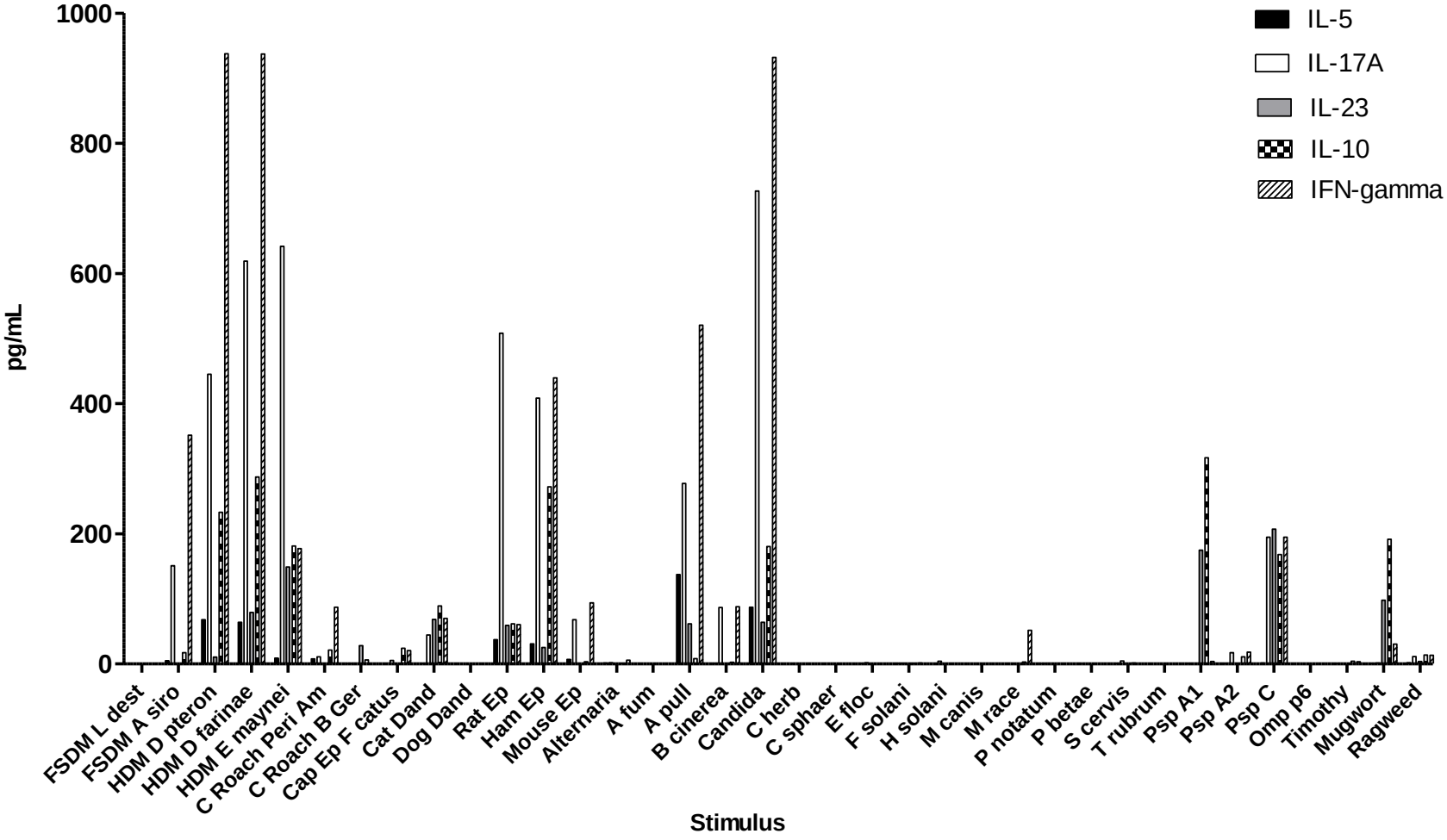


Figure 10.40 Nonatopic Asthmatic Patient 22 - Nonosinophilic

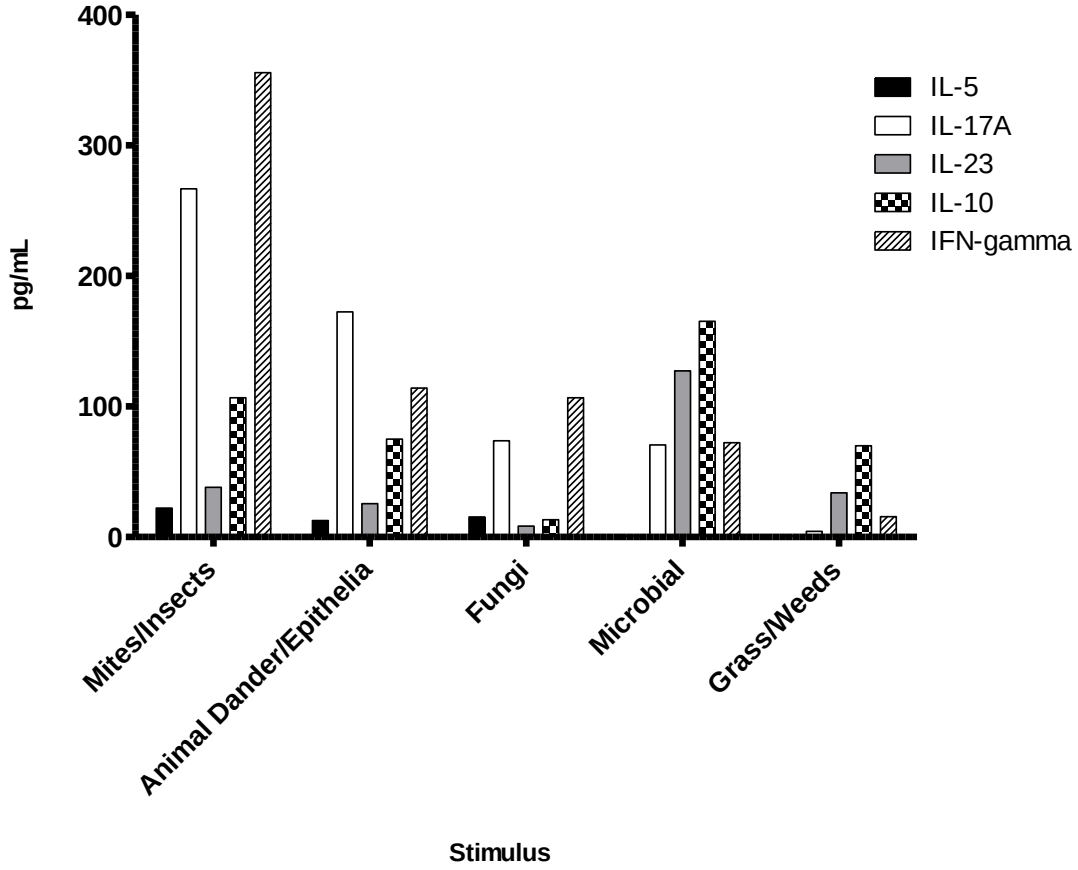


Figure 10.41 Nonatopic Asthmatic Patient 23 - Eosinophilic

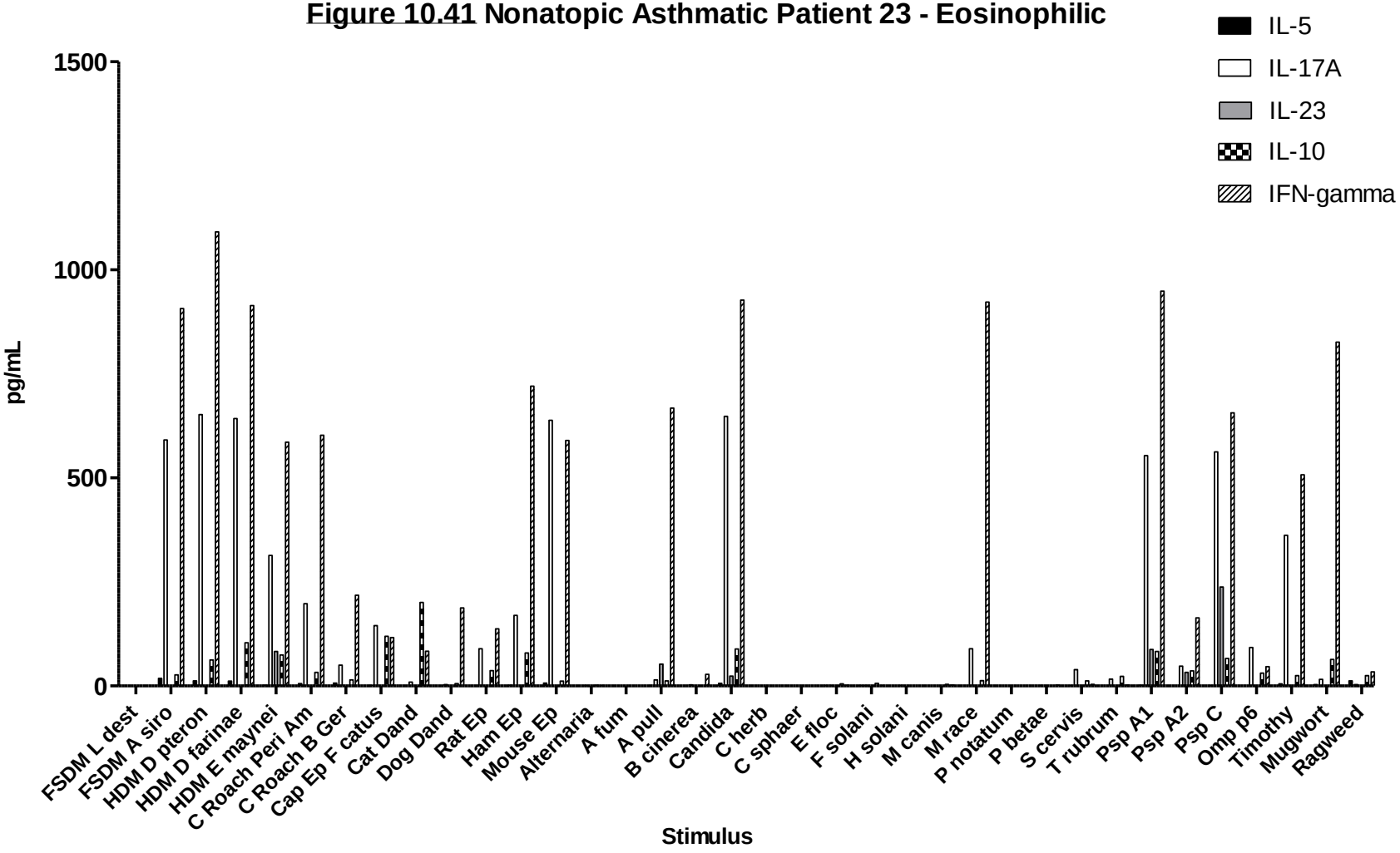


Figure 10.42 Nonatopic Asthmatic Patient 23 - Eosinophilic

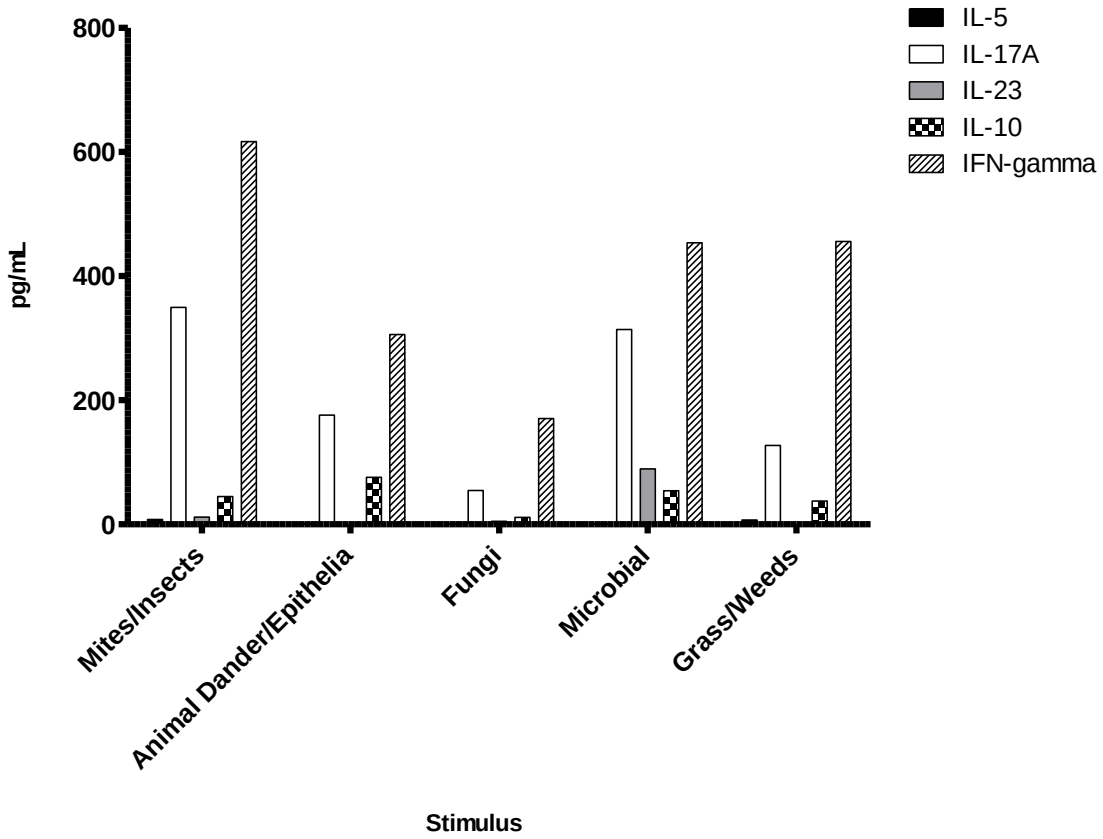


Figure 10.43 Nonatopic Asthmatic Patient 24 - Neutrophilic

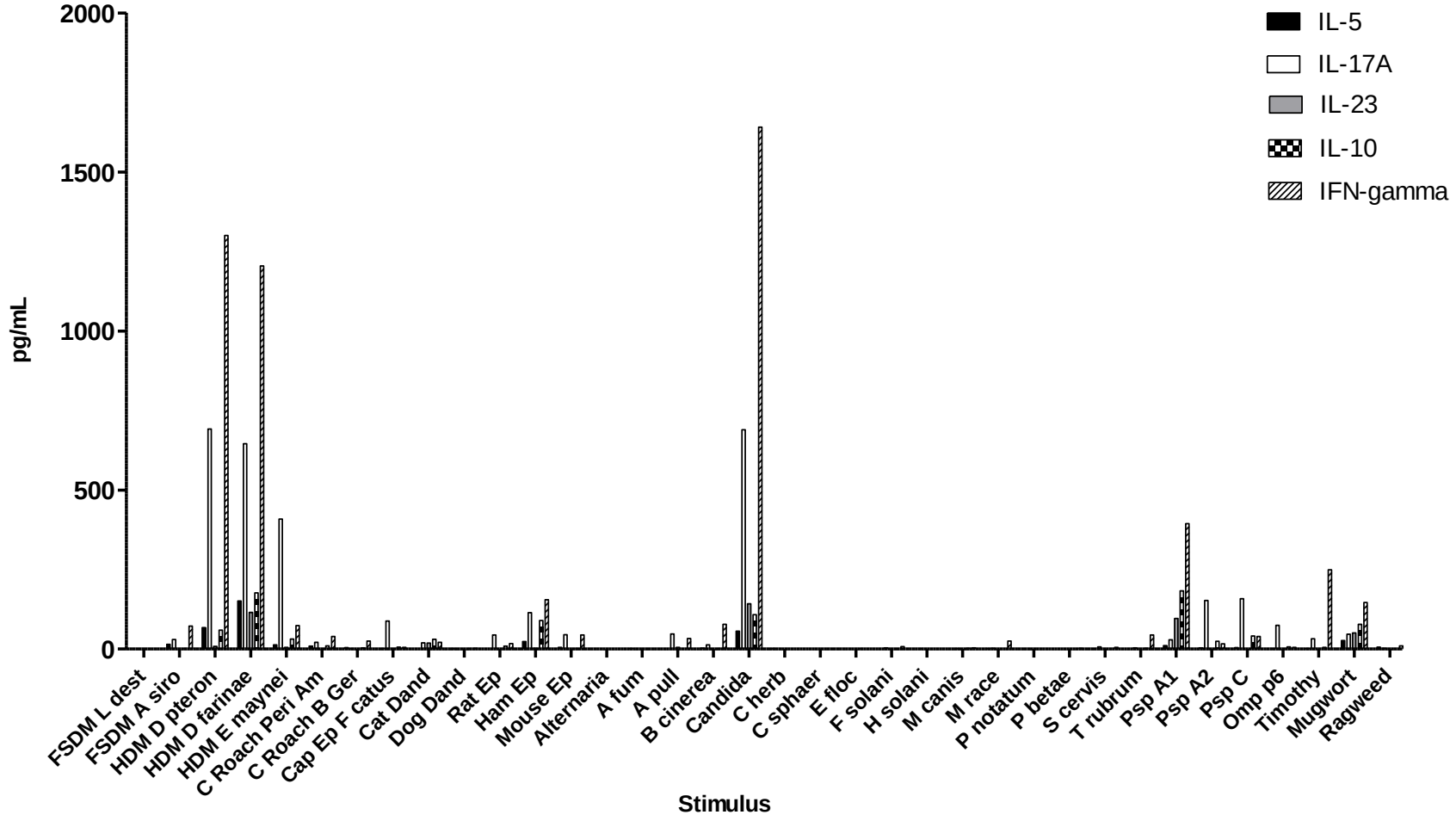


Figure 10.44 Nonatopic Asthmatic Patient 24 - Neutrophilic

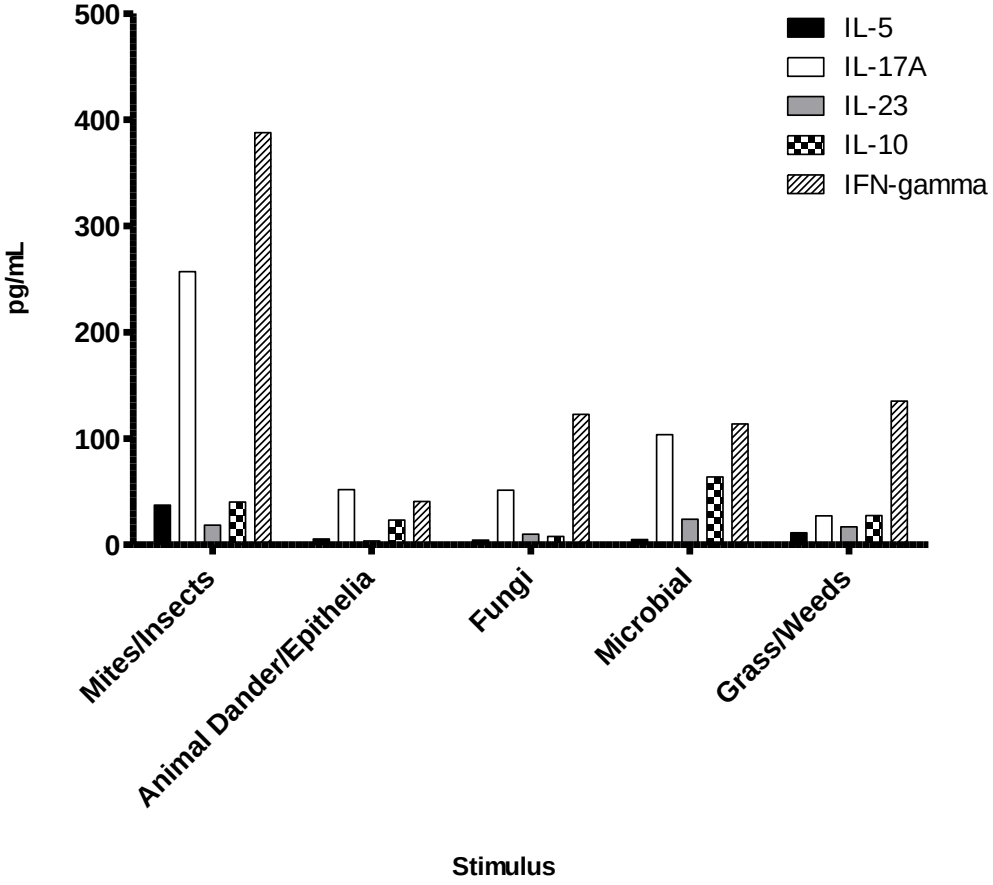


Figure 10.45 Nonatopic Asthmatic Patient 25 - Eosinophilic

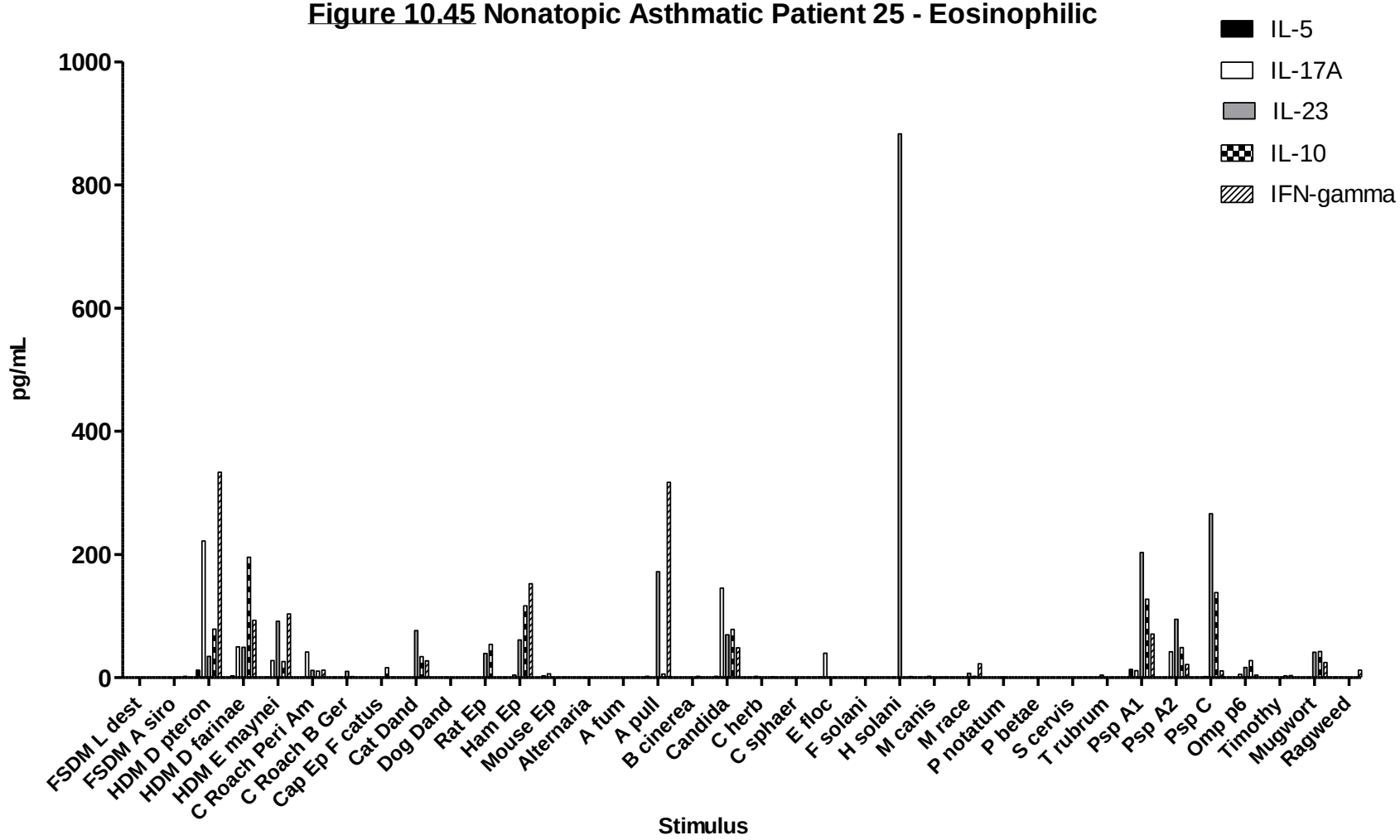


Figure 10.46 Nonatopic Asthmatic Patient 25 - Eosinophilic

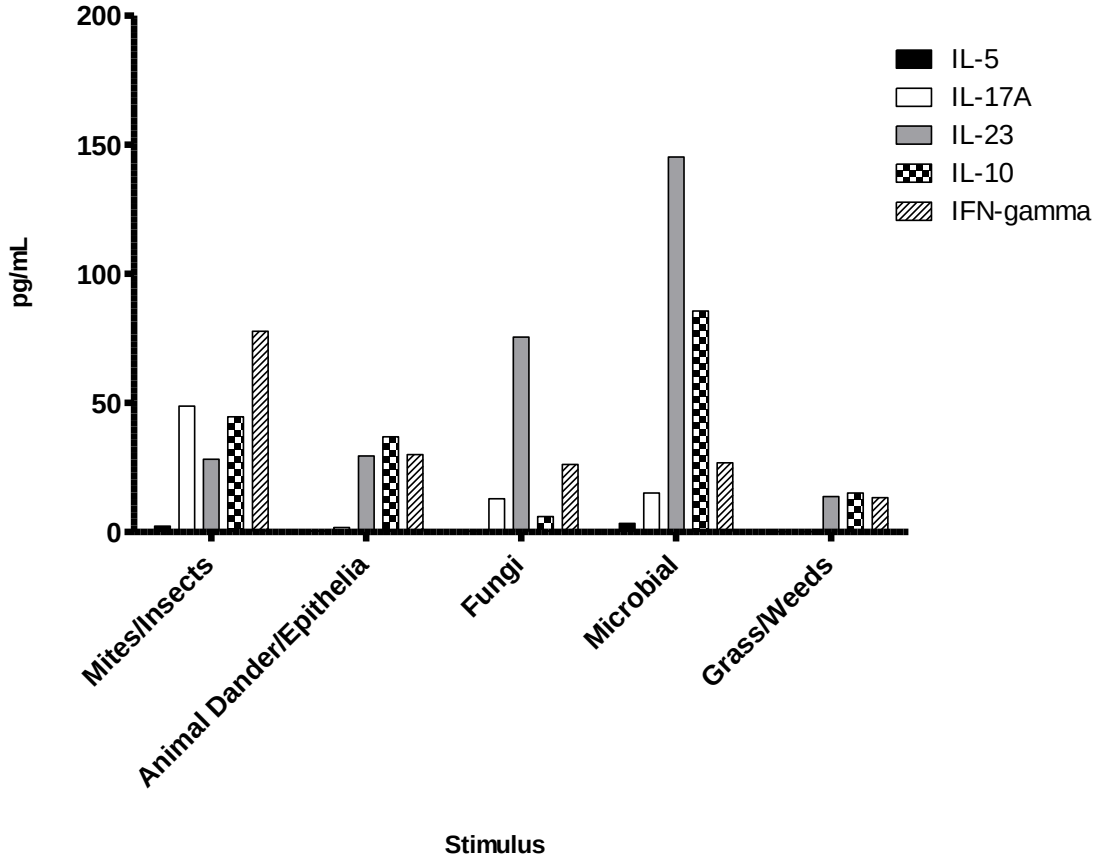


Figure 10.47 Nonatopic Asthmatic Patient 26 - Eosinophilic

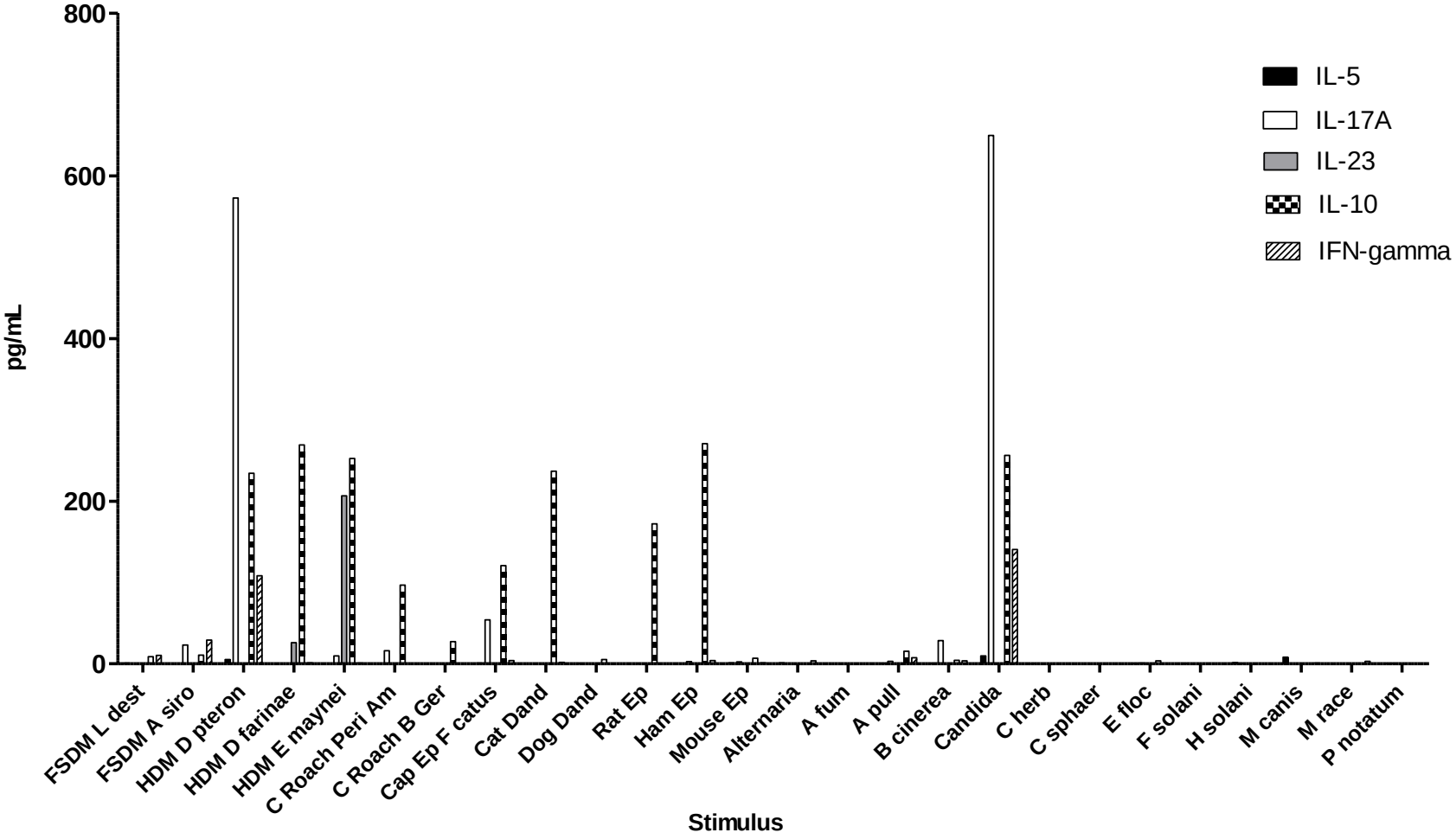


Figure 10.48 Nonatopic Asthmatic Patient 26 - Eosinophilic

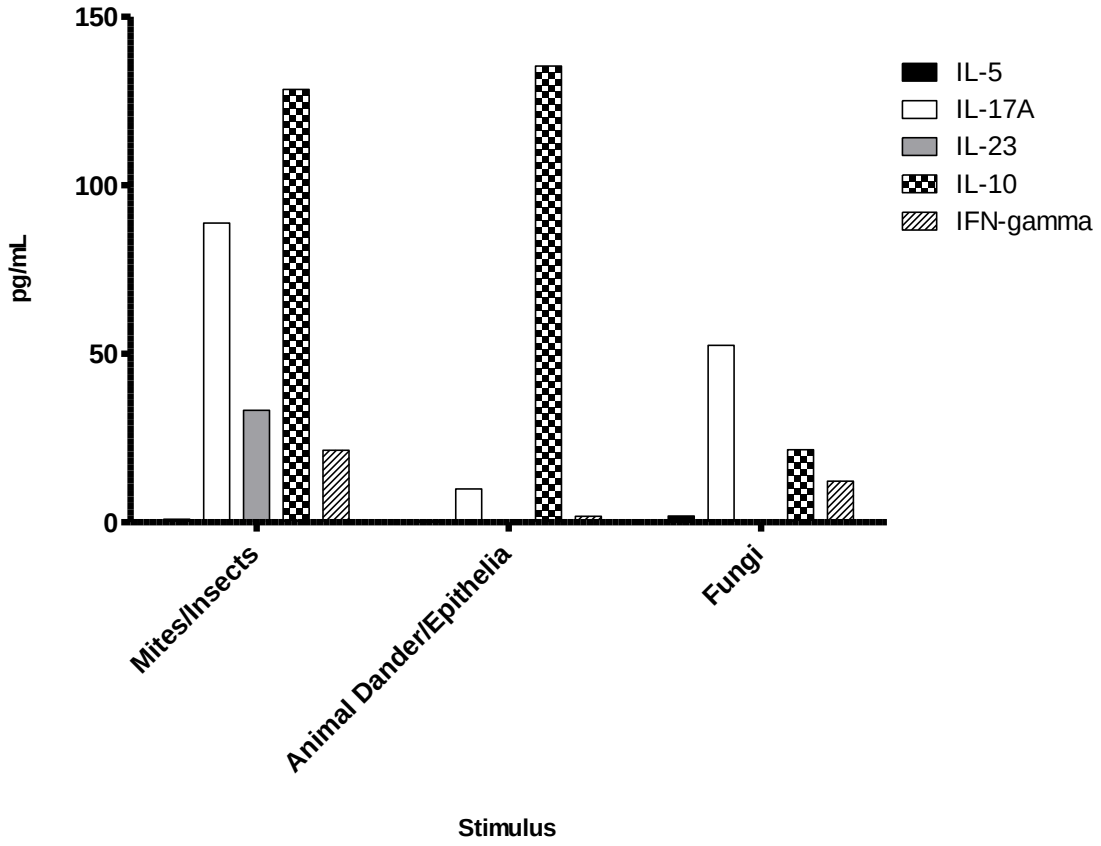


Figure 10.50 Nonatopic Asthmatic Patient 27 - Eosinophilic

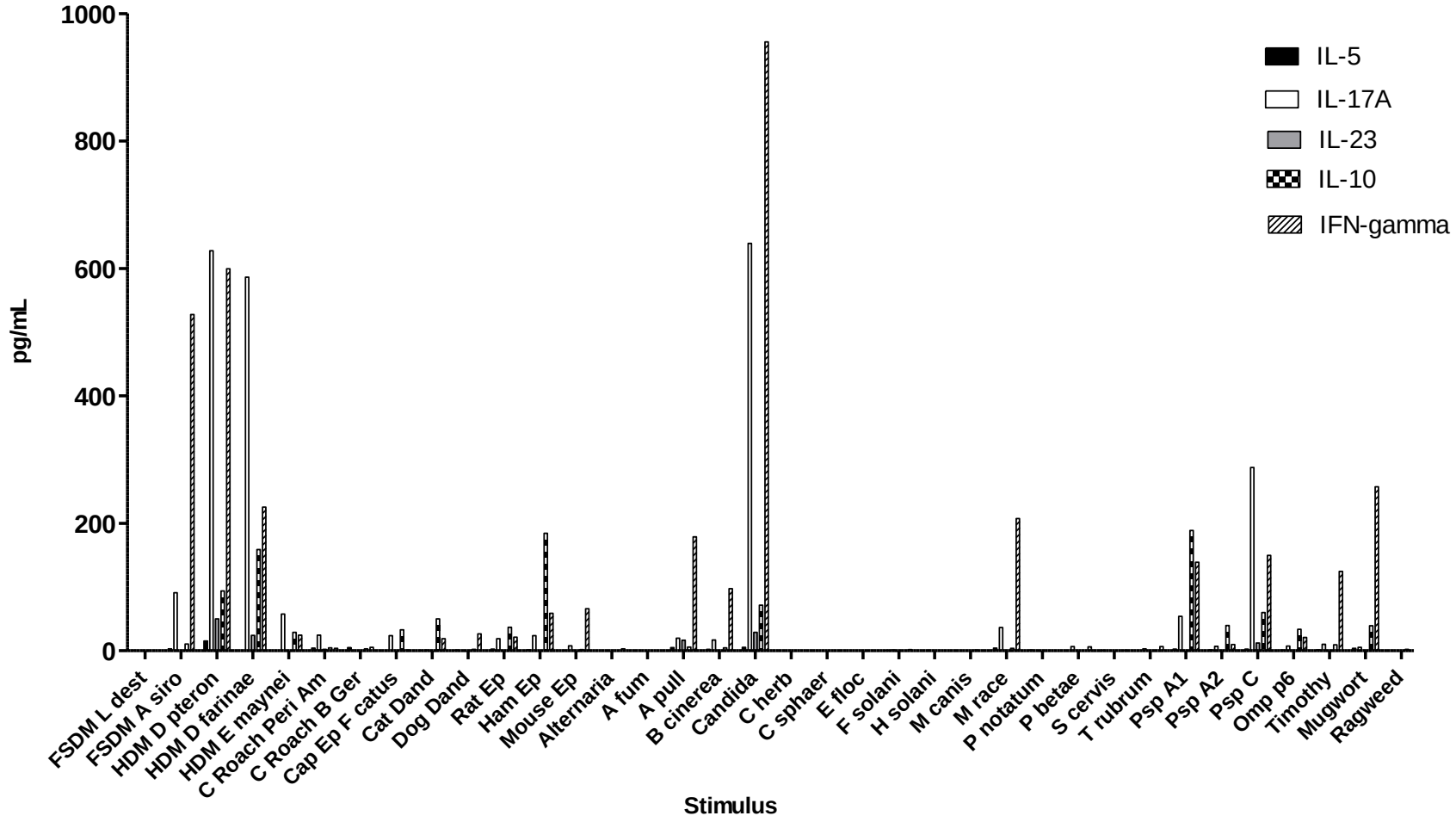


Figure 10.51 Nonatopic Asthmatic Patient 27 - Eosinophilic

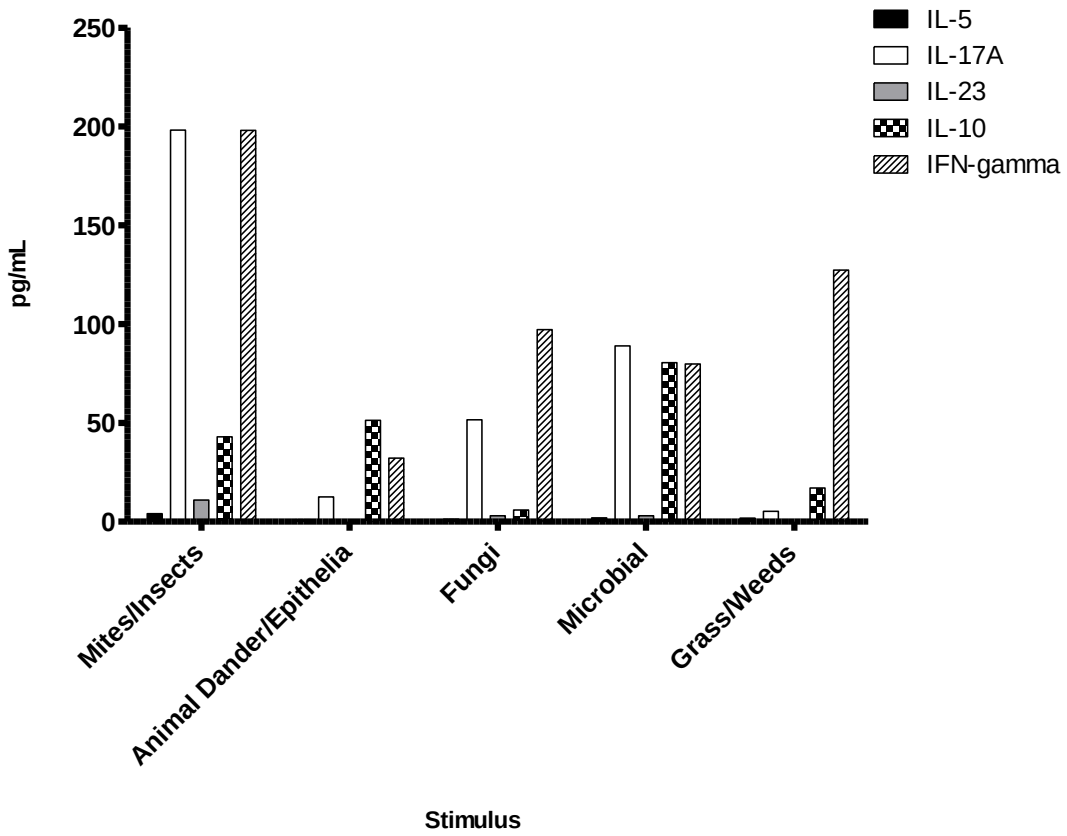


Figure 10.52 Nonatopic Asthmatic Patient 28 - Eosinophilic

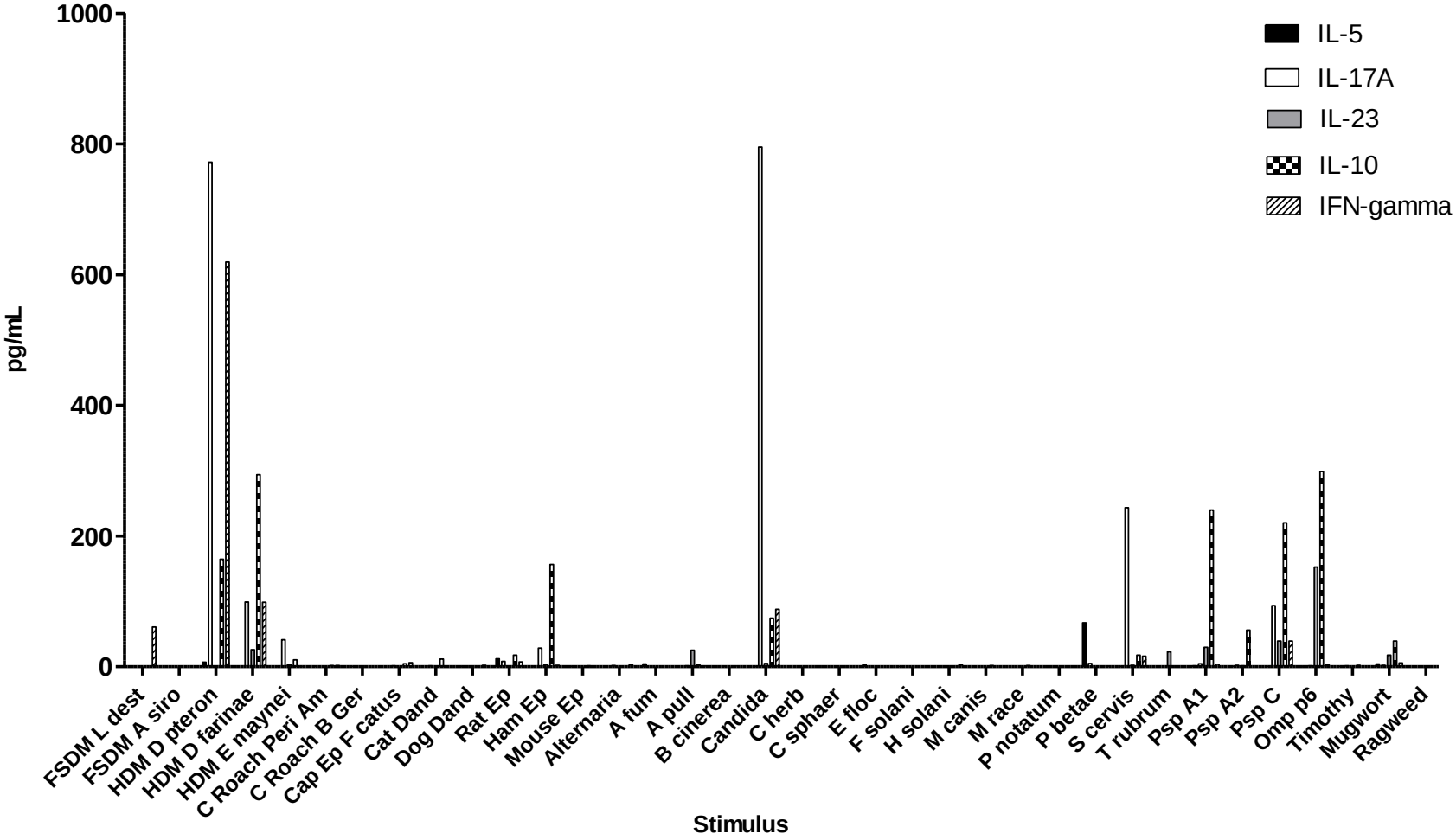


Figure 10.53 Nonatopic Asthmatic Patient 28 - Eosinophilic

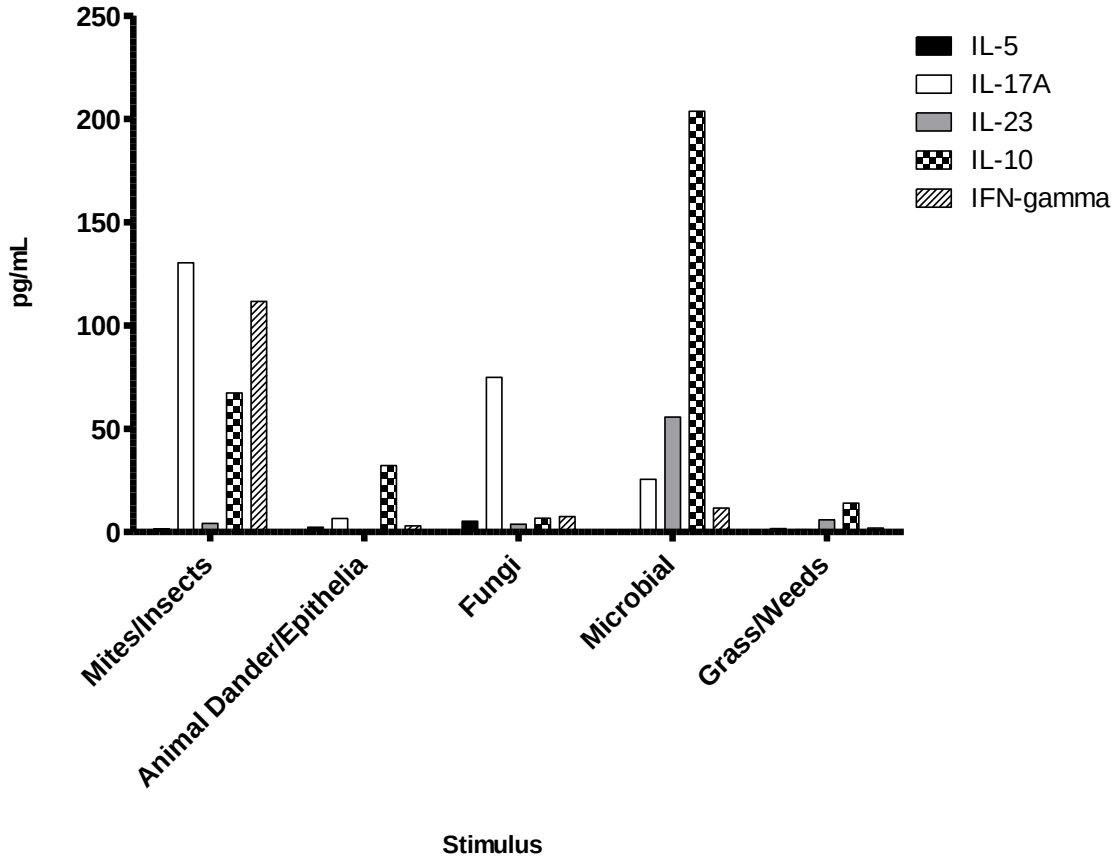


Figure 11.1 Atopic Asthmatic Patient 1 - Neutrophilic
Allergies: Ragweed, mold, dust mite, peanut, birch tree

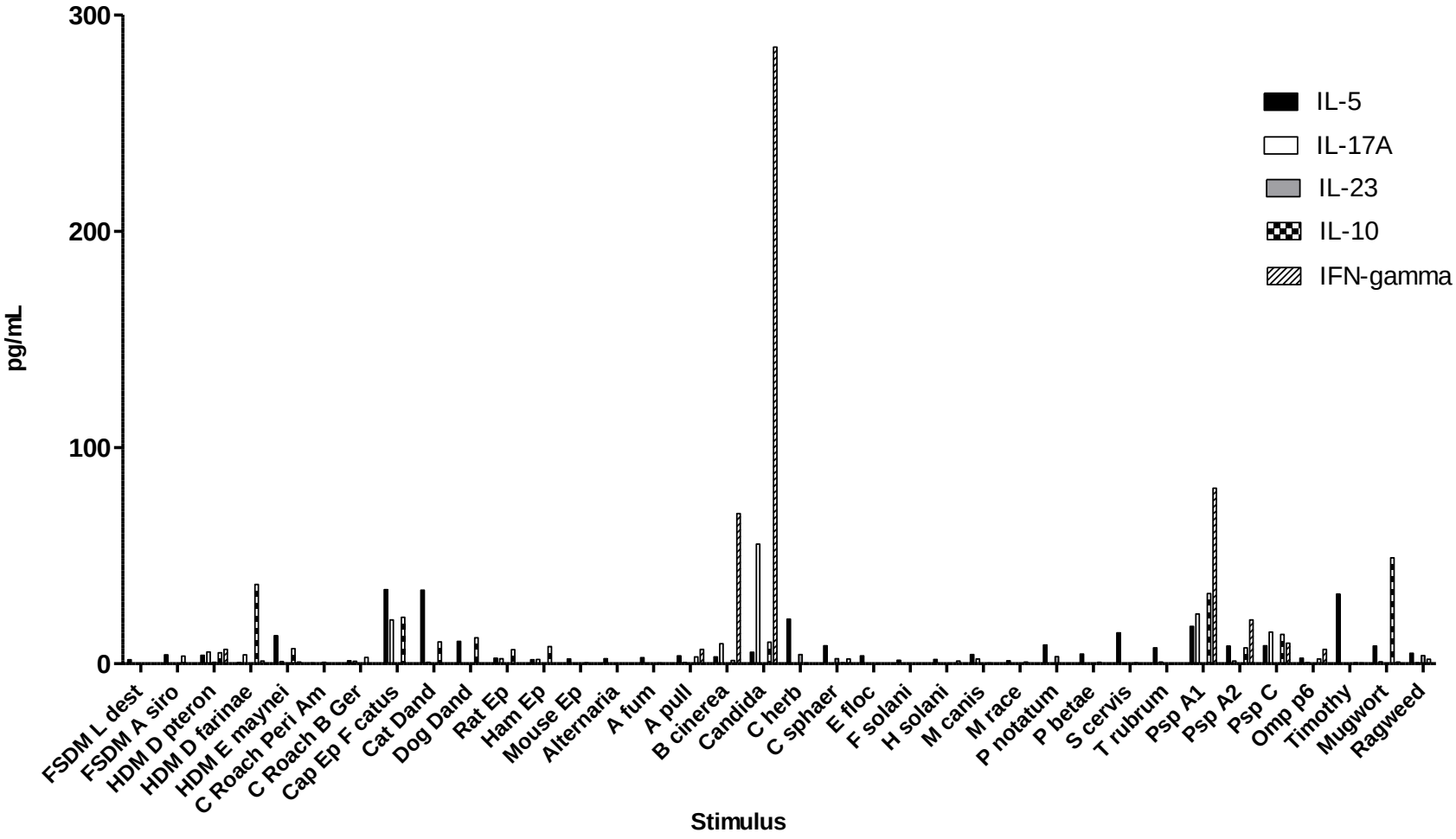


Figure 11.2 Atopic Asthmatic Patient 1 - Neutrophilic

Allergies: Ragweed, mold, dust mite, peanut, birch tree

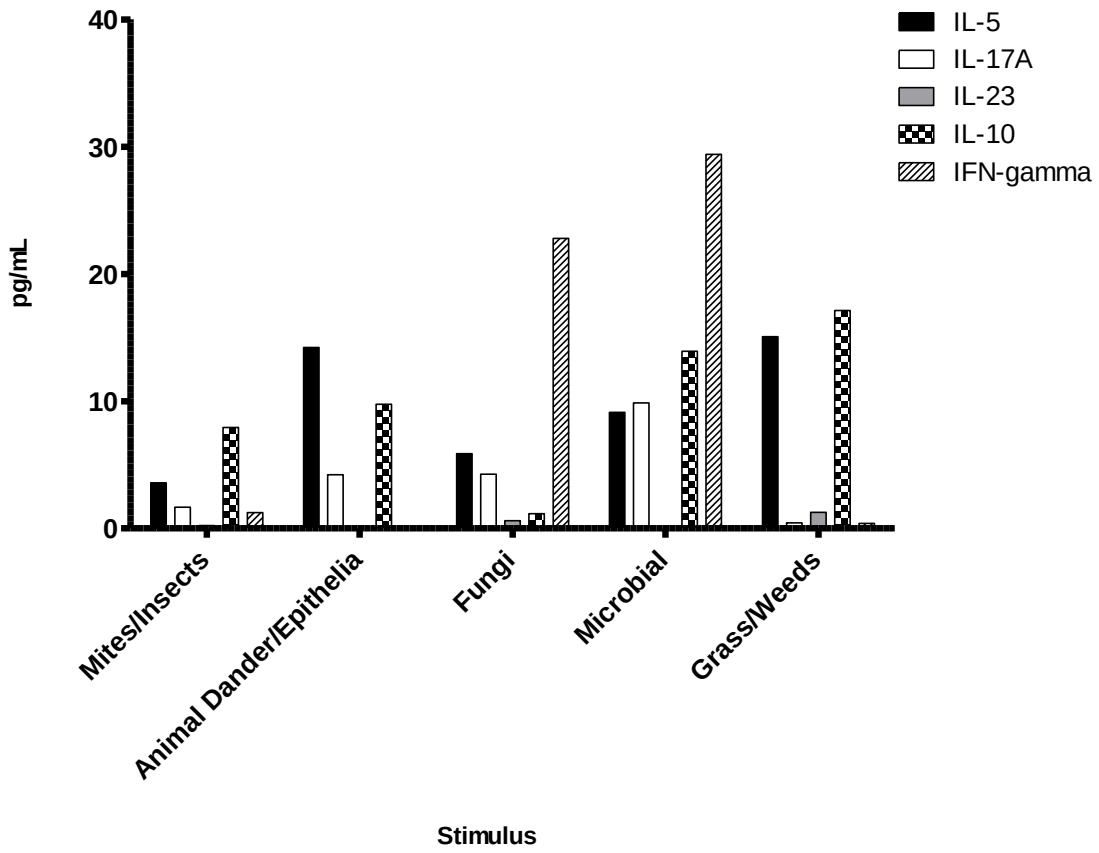


Figure 11.3 Atopic Asthmatic Patient 2- Eosinophilic
Allergies: cat, dust mite, grass, alternaria

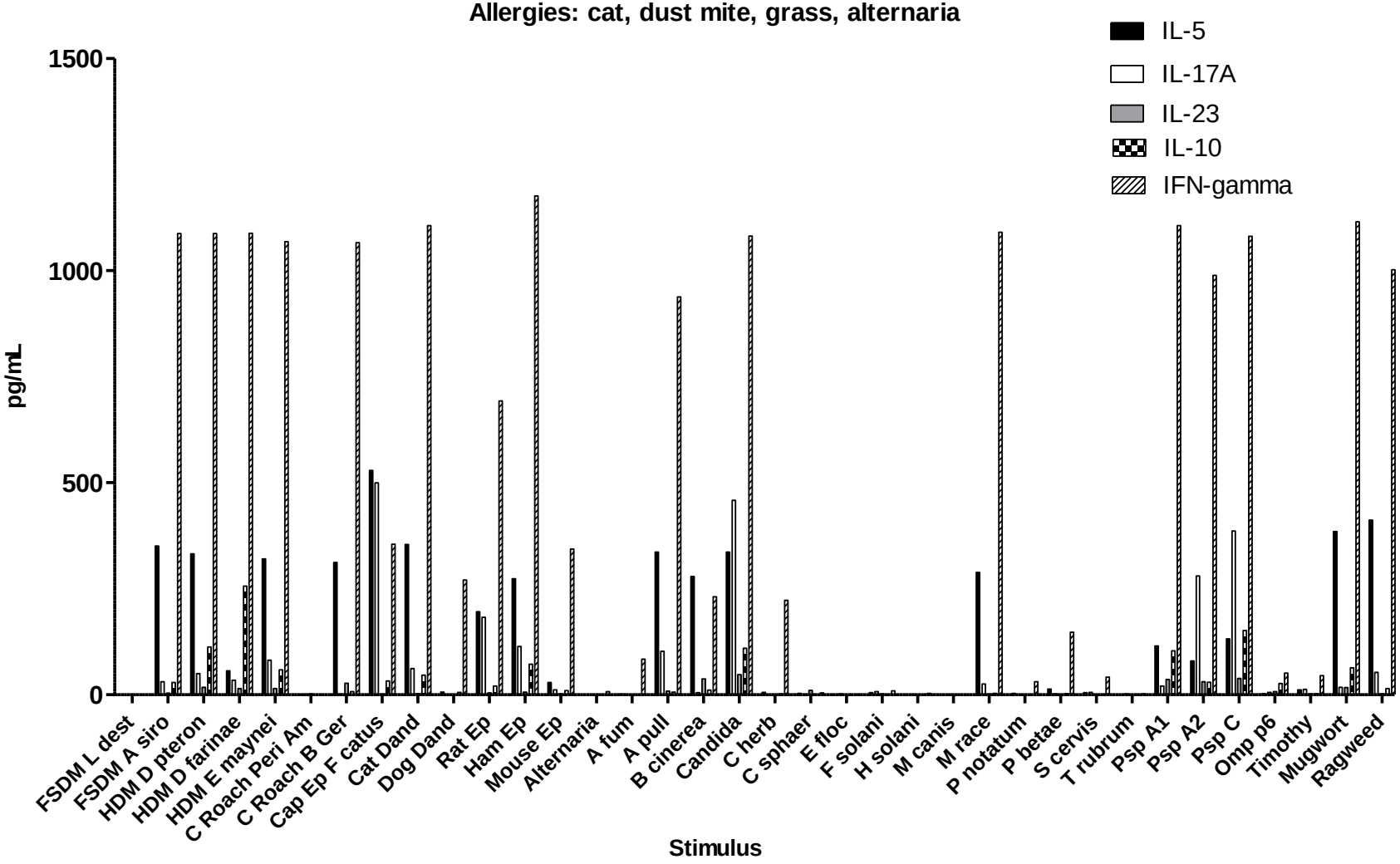


Figure 11.4 Atopic Asthmatic Patient 2 - Eosinophilic
Allergies: cat, dust mite, grass, alternaria

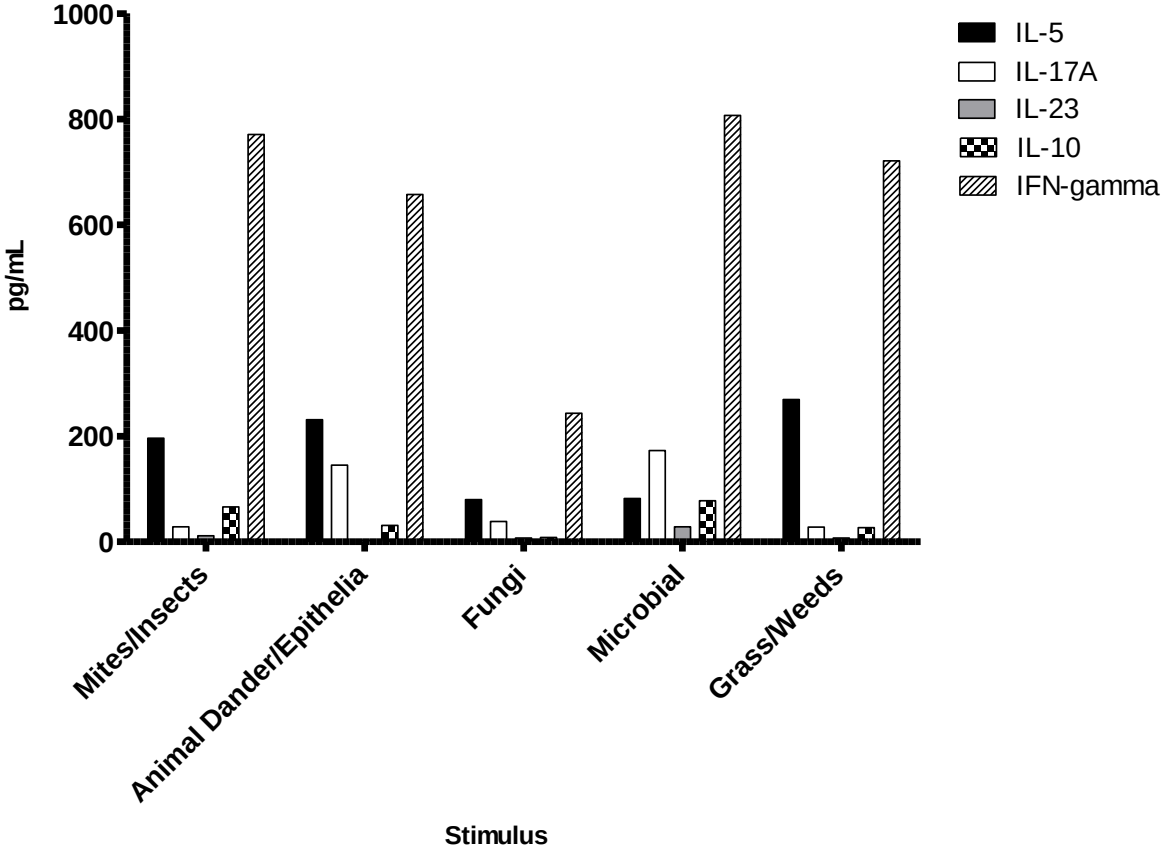


Figure 11.5 Atopic Asthmatic Patient 4 - Eosinophilic
Allergies: dust mite

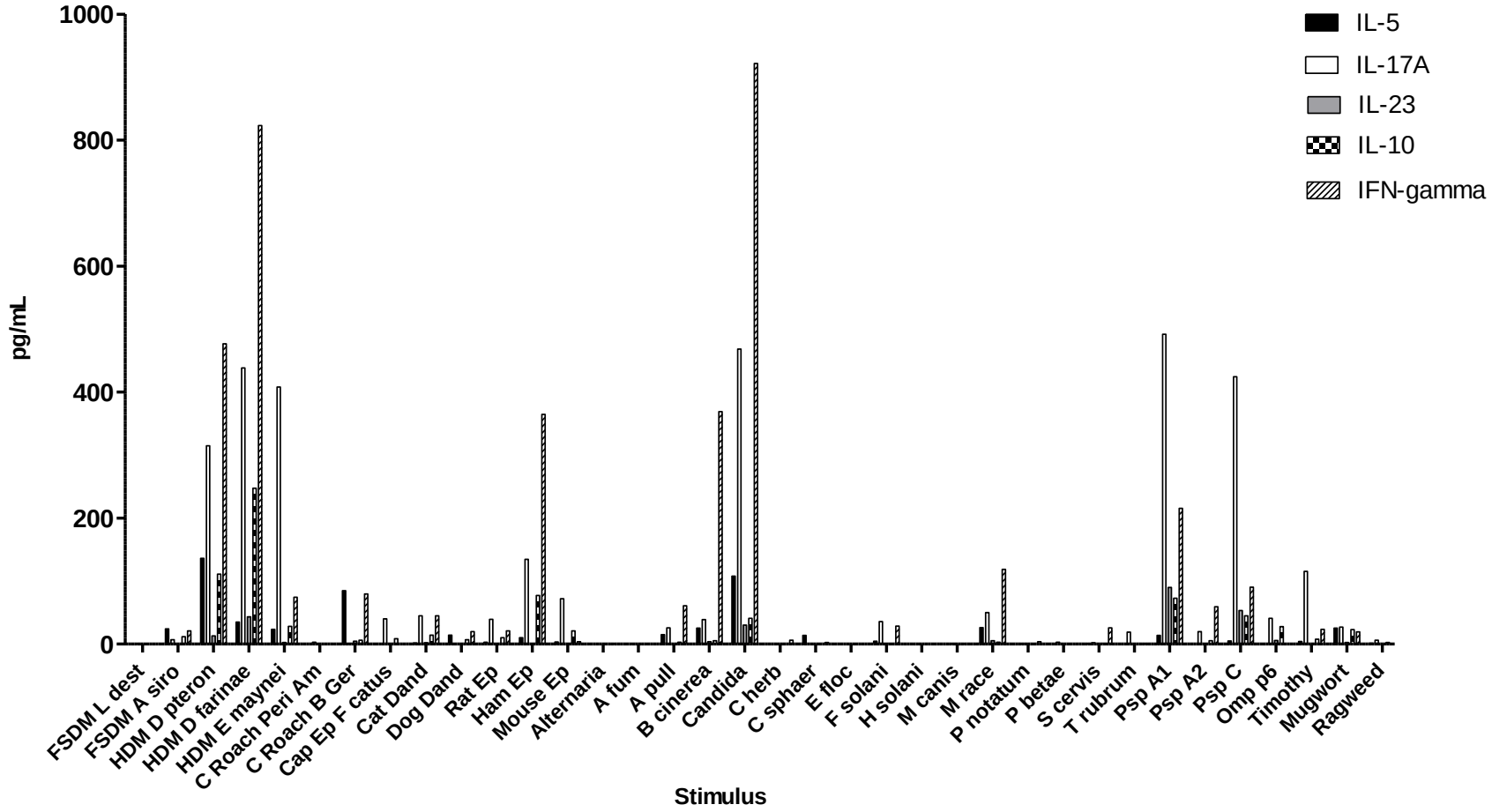


Figure 11.6 Atopic Asthmatic Patient 4 - Eosinophilic

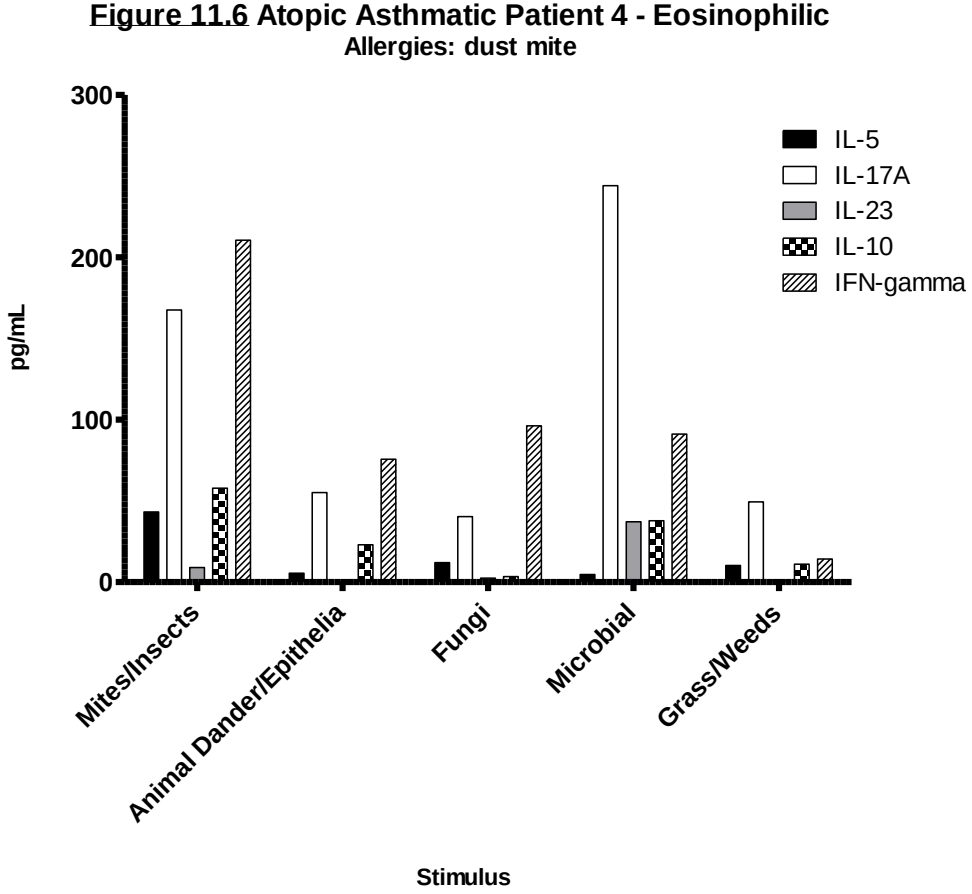


Figure 11.7 Atopic Asthmatic Patient 5 - Eosinophilic
Allergies: dust mite, mold

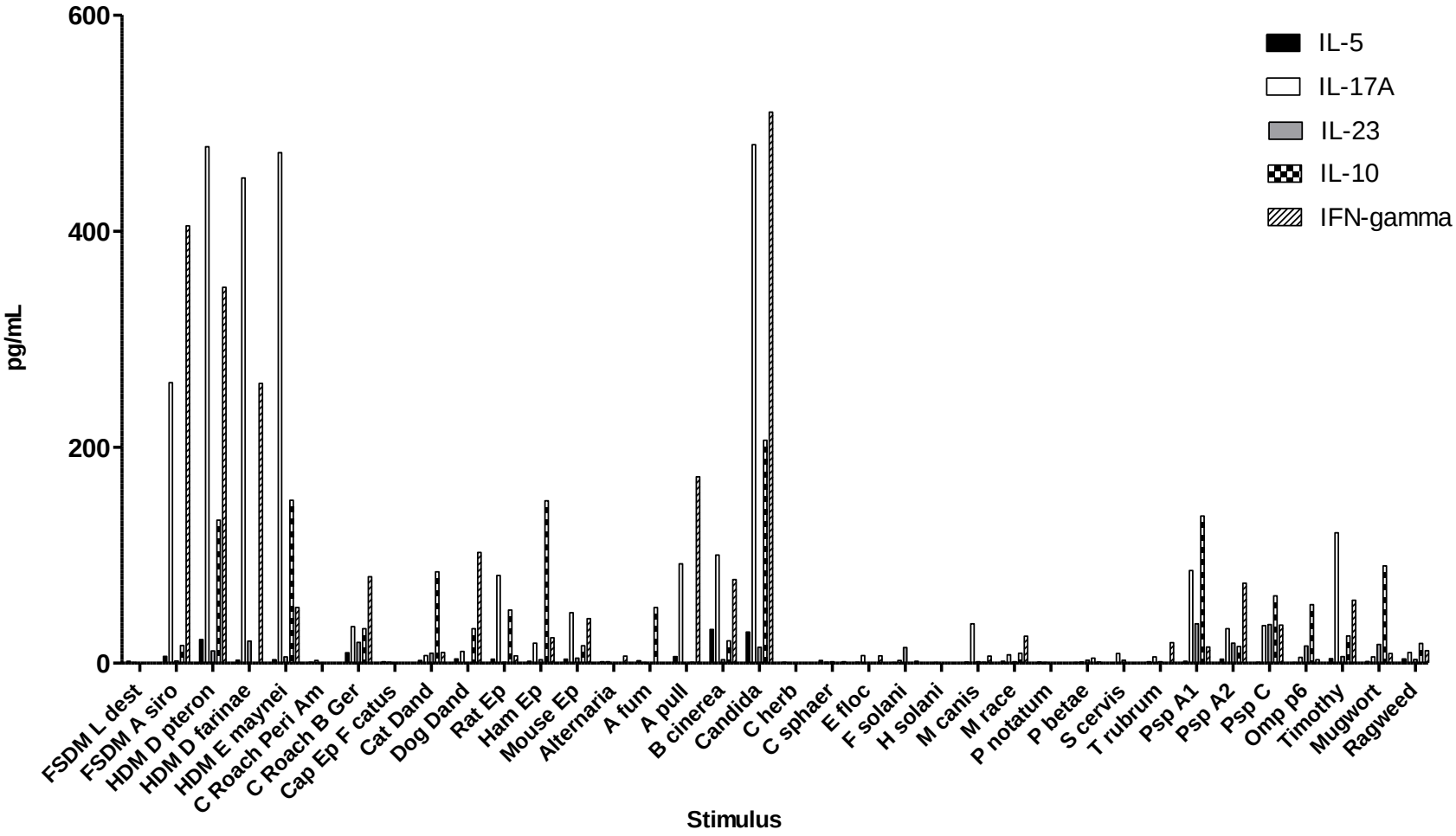
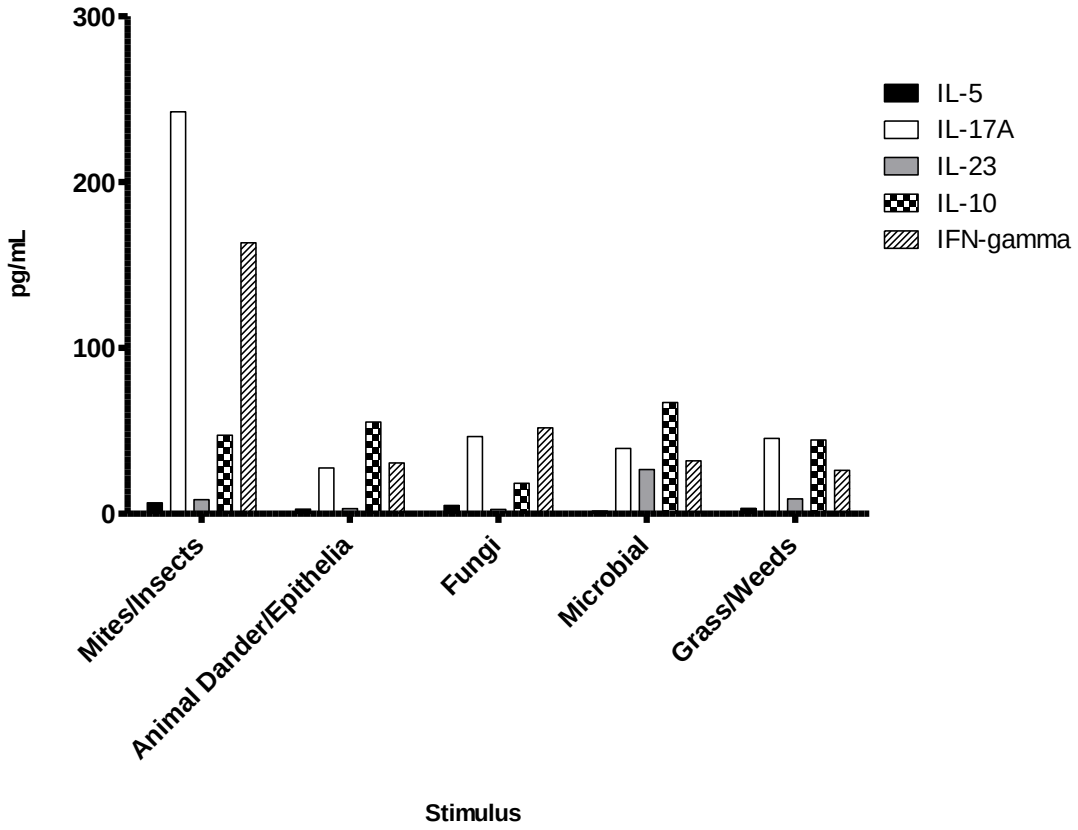


Figure 11.8 Atopic Asthmatic Patient 5 - Eosinophilic
Allergies: dust mite, mold



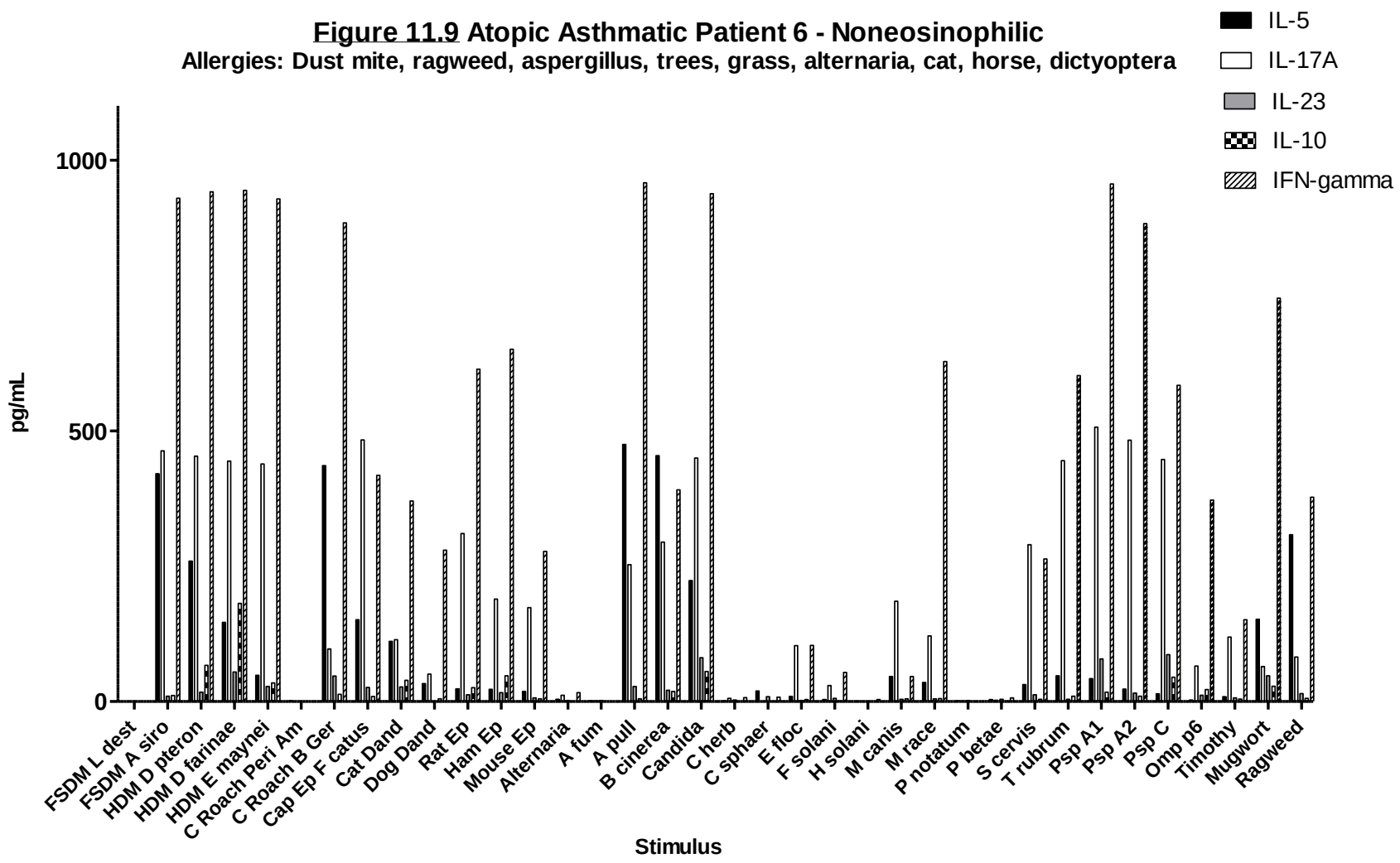


Figure 11.9 Atopic Asthmatic Patient 6 - Noneosinophilic
Allergies: Dust mite, ragweed, aspergillus, trees, grass, alternaria, cat, horse, dictyoptera
IL-5
IL-17A
IL-23
IL-10
IFN-gamma
pg/mL
1000
500
0
FSDM L dest
FSDM A siro
HDM D pteron
HDM D farinae
HDM E maynei
C Roach Peri Am
C Roach B Ger
Cap Ep F catus
Cat Dand
Dog Dand
Rat Ep
Ham Ep
Mouse Ep
Alternaria
A fum
A pull
B cinerea
Candida
C herb
C sphaer
E floc
F solani
H solani
M canis
M race
P notatum
P betae
S cervis
T rubrum
Psp A1
Psp A2
Psp C
Omp p6
Timothy
Mugwort
Ragweed
Stimulus

Figure 11.10 Atopic Asthmatic Patient 6 - Noneosinophilic

Allergies: Dust mite, ragweed, aspergillus, trees, grass, alternaria, cat, horse, dictyoptera

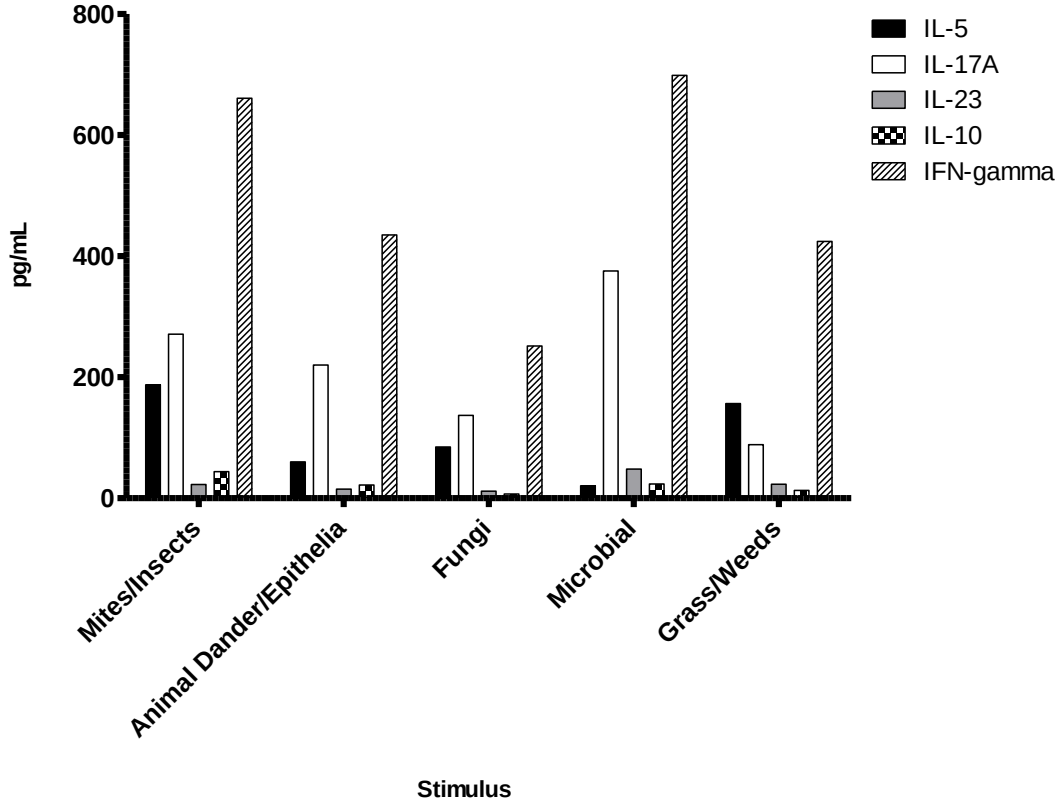


Figure 11.11 Atopic Asthmatic Patient 7 - Eosinophilic
Allergies: dust mite

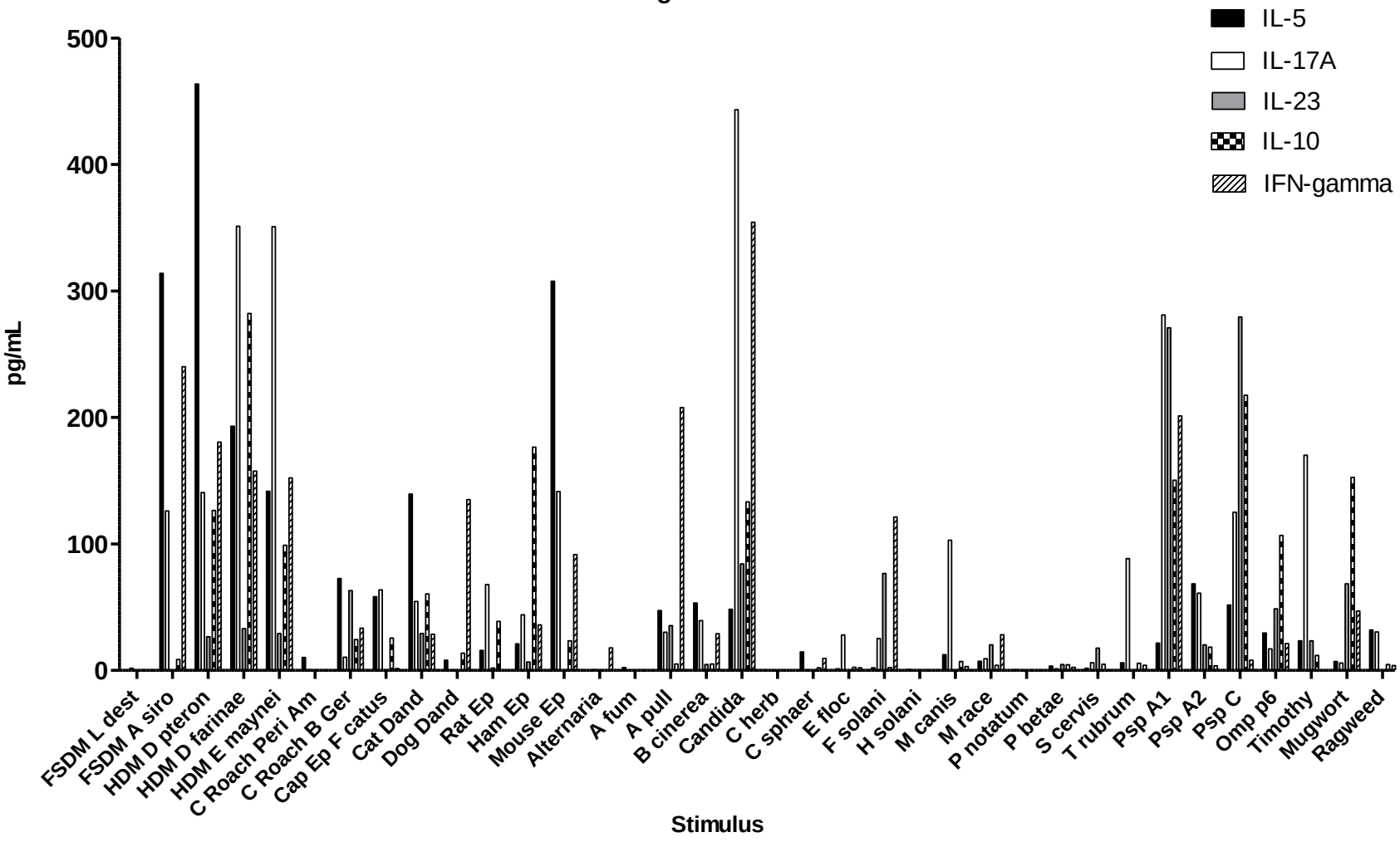


Figure 11.12 Atopic Asthmatic Patient 7 - Eosinophilic

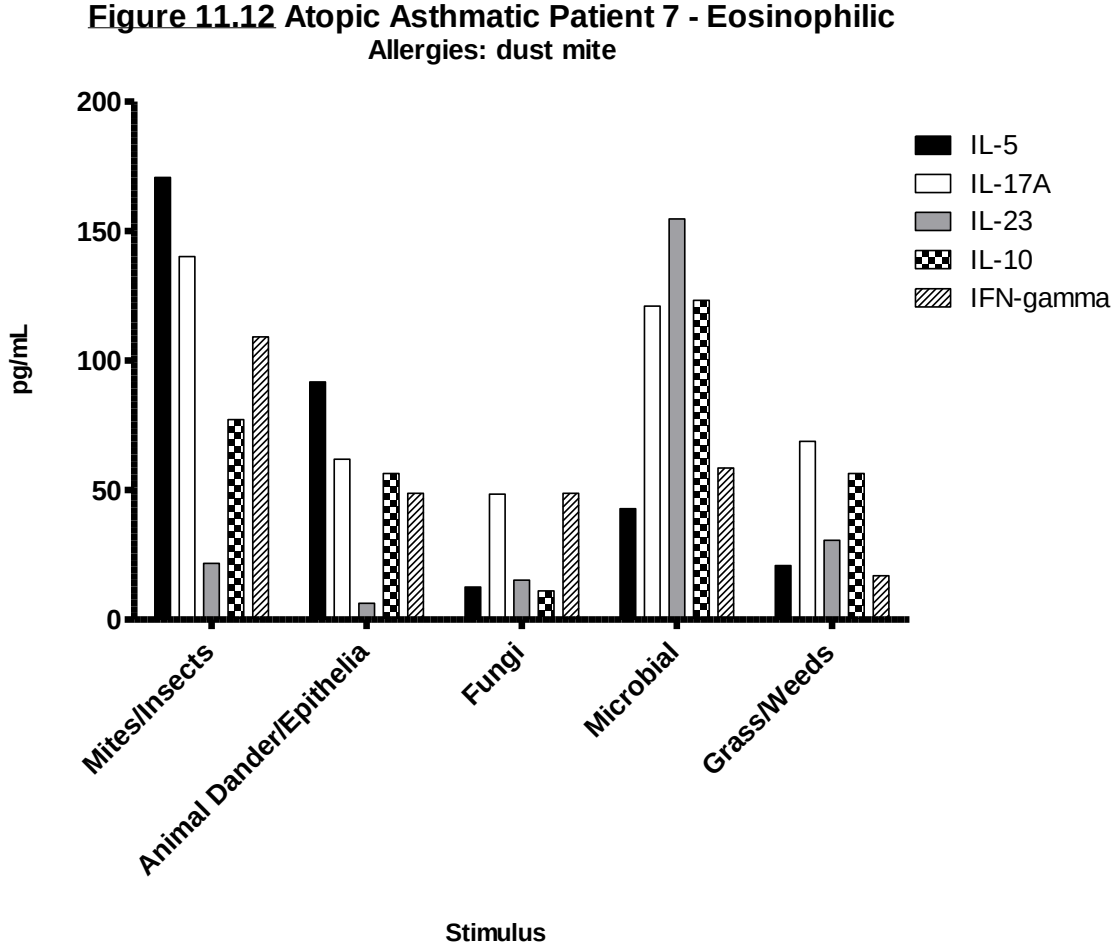


Figure 11.13 Atopic Asthmatic Patient 8 - Eosinophilic
Allergies: dust mite, mold

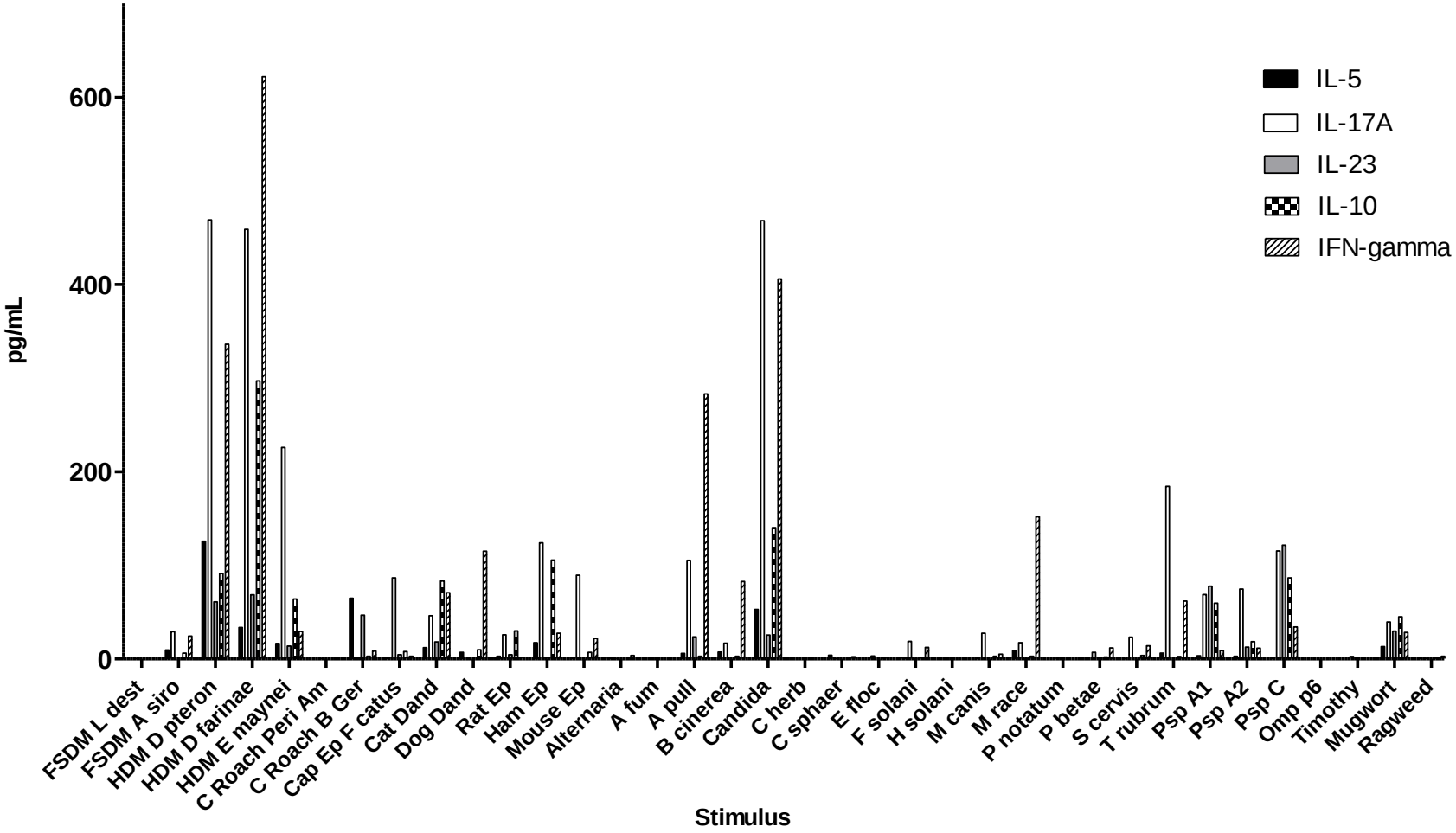


Figure 11.14 Atopic Asthmatic Patient 8 - Eosinophilic
Allergies: dust mite, mold

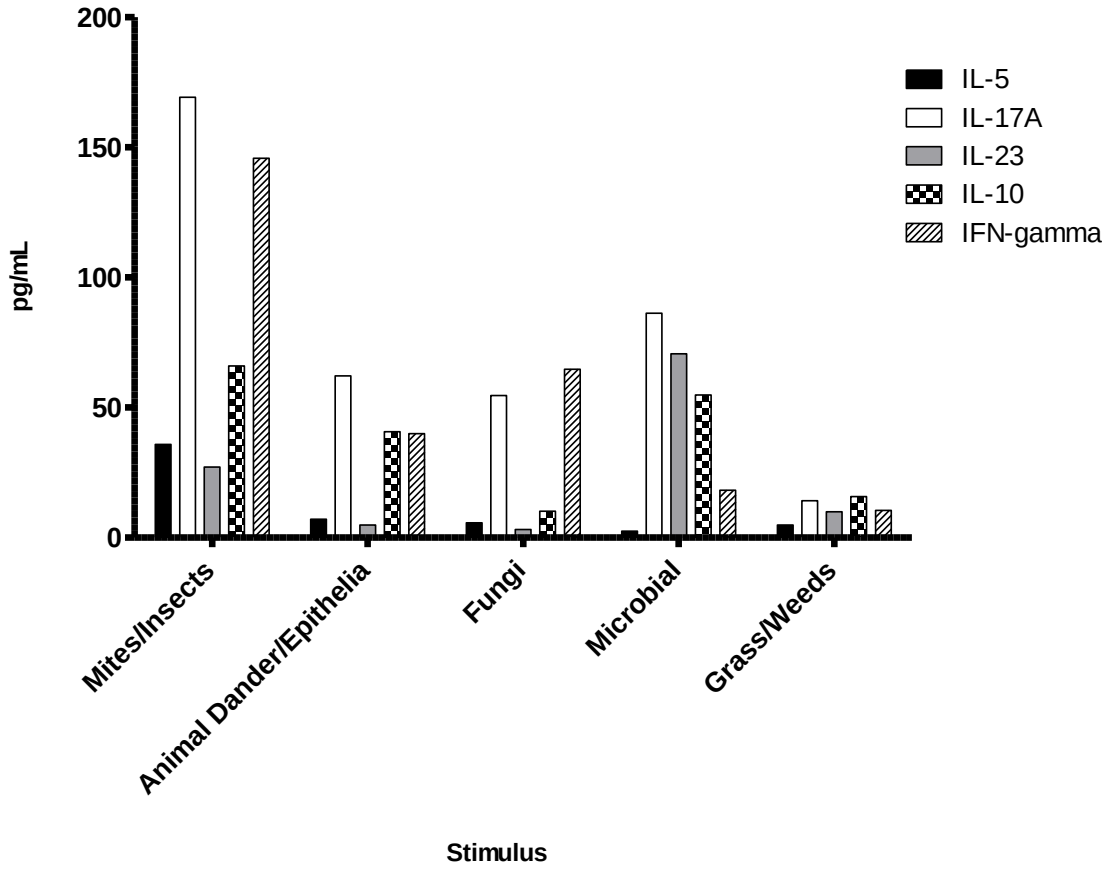


Figure 11.15 Atopic Asthmatic Patient 9 - Noneosinophilic
Allergens: dust mite, pollens

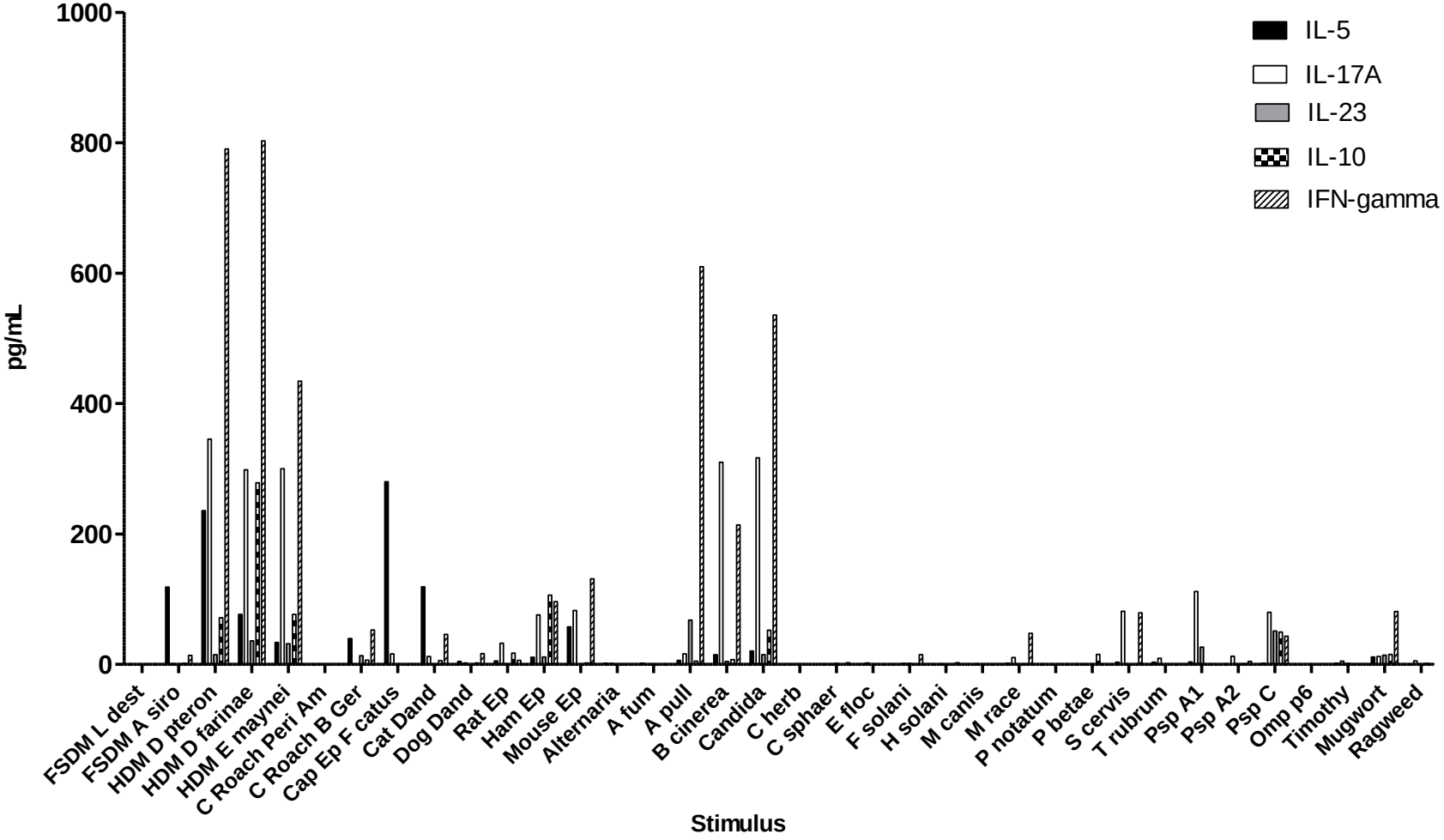


Figure 11.16 **Atopic Asthmatic Patient 9 - Noneosinophilic**
Allergens: dust mite, pollens

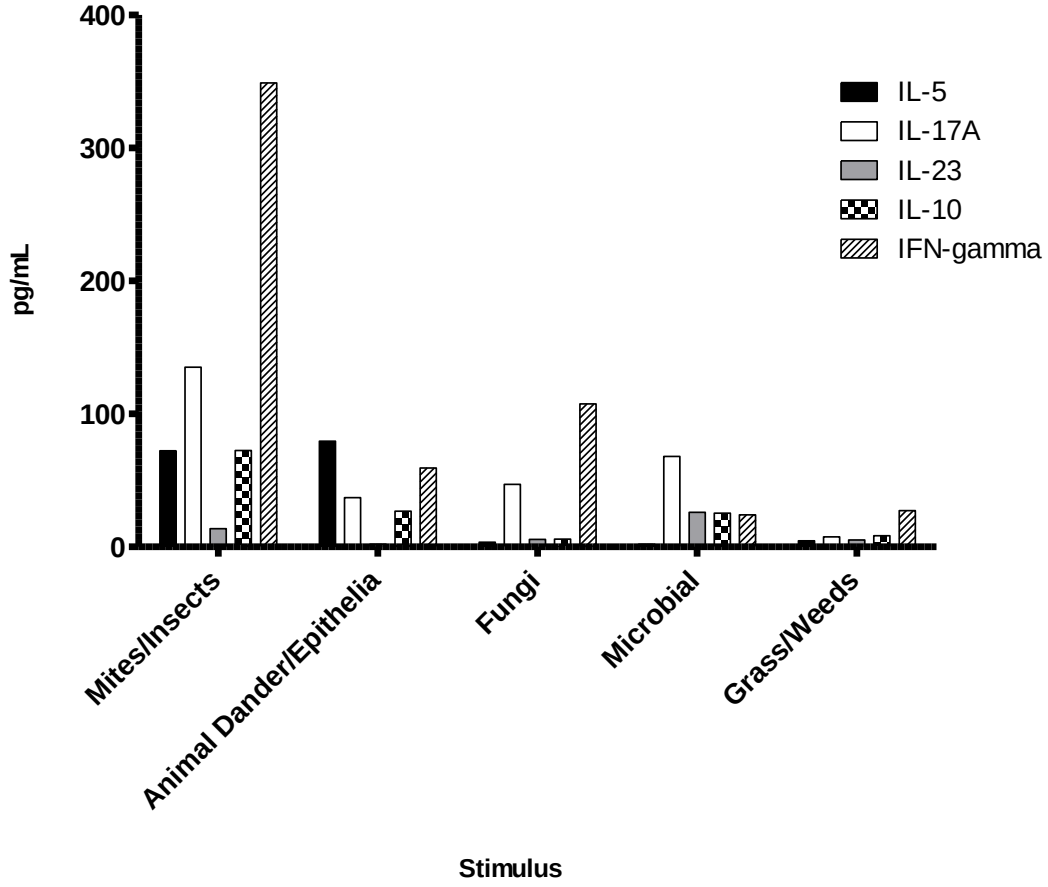


Figure 11.17 Atopic Asthmatic Patient 10 - Eosinophilic
Allergies: Alternaria, cat, horse, feathers, grass

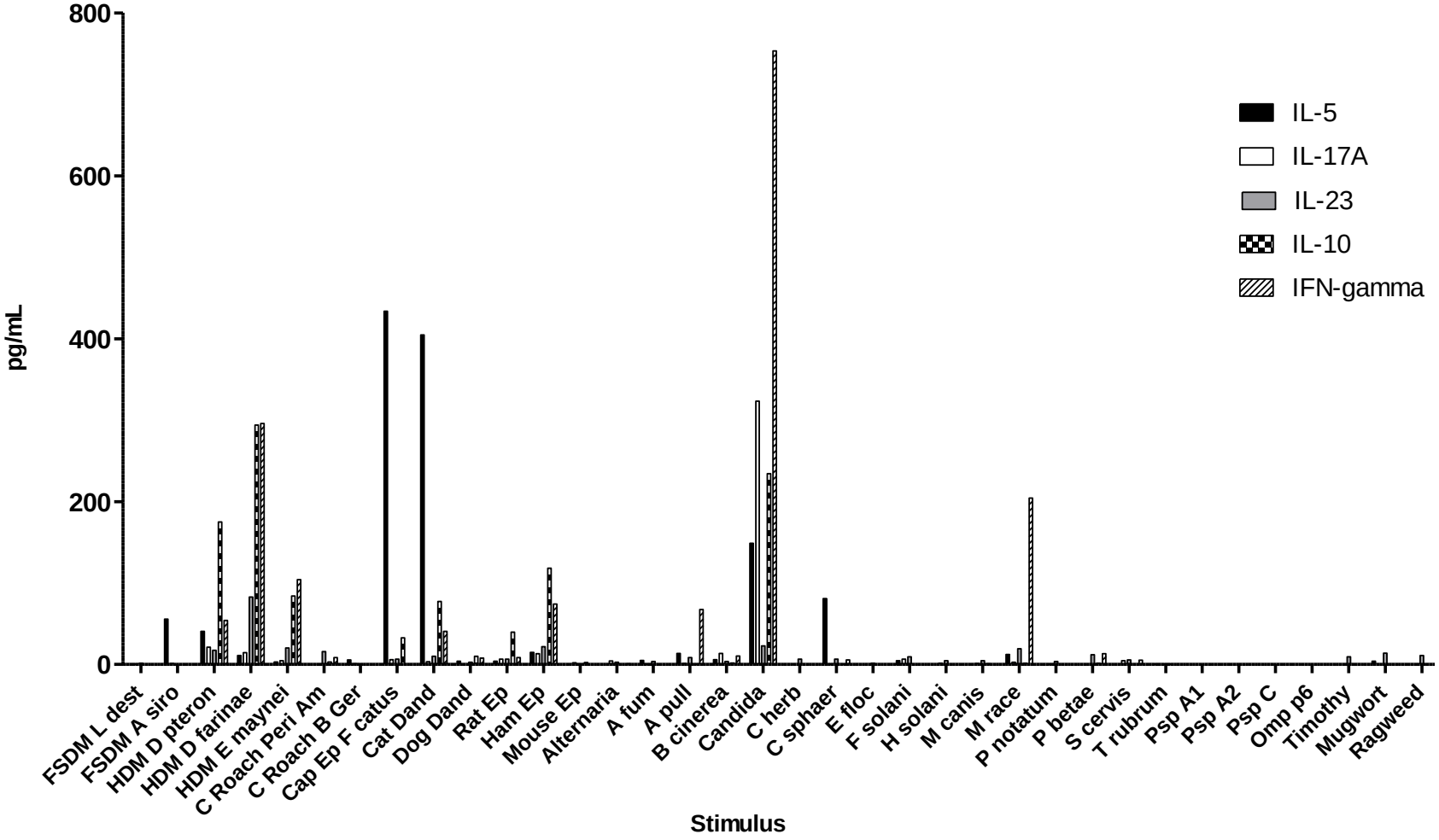


Figure 11.18 Atopic Asthmatic Patient 10 - Eosinophilic
Allergies: Alternaria, cat, horse, feathers, grass

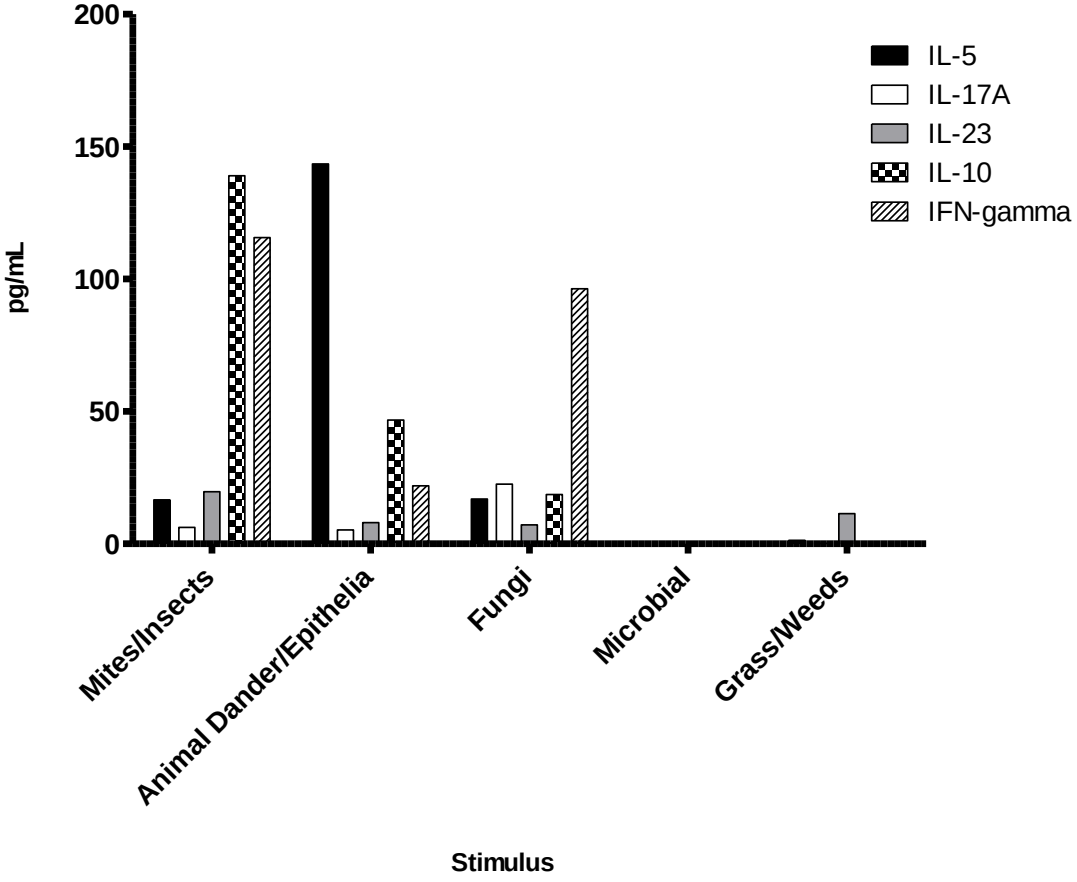


Figure 11.19 Atopic Asthmatic Patient 11 - Eosinophilic
Allergies: grass, peanuts, shellfish, animals

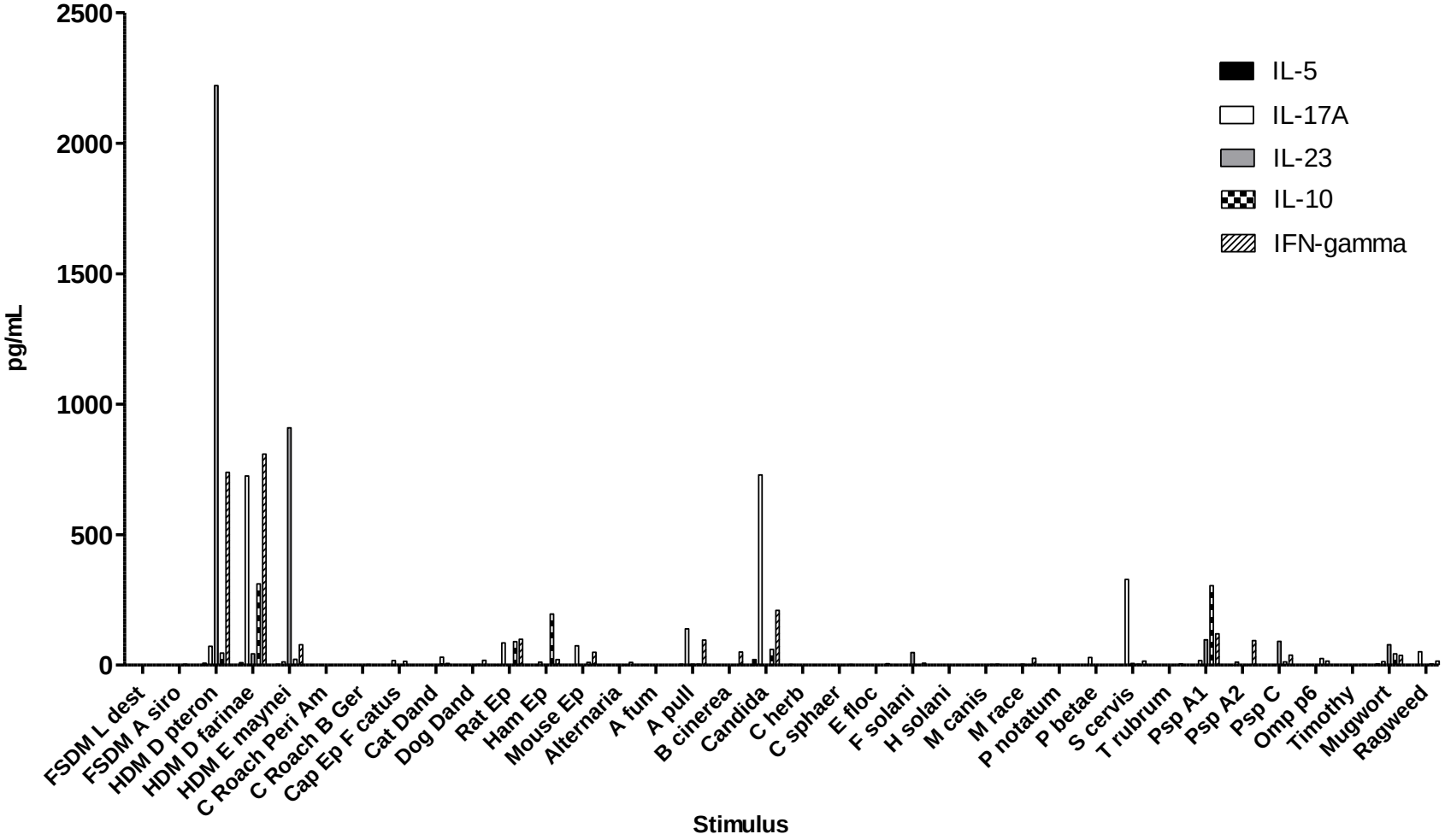


Figure 11.20 Atopic Asthmatic Patient 11 - Eosinophilic
Allergies: grass, peanuts, shellfish, animals

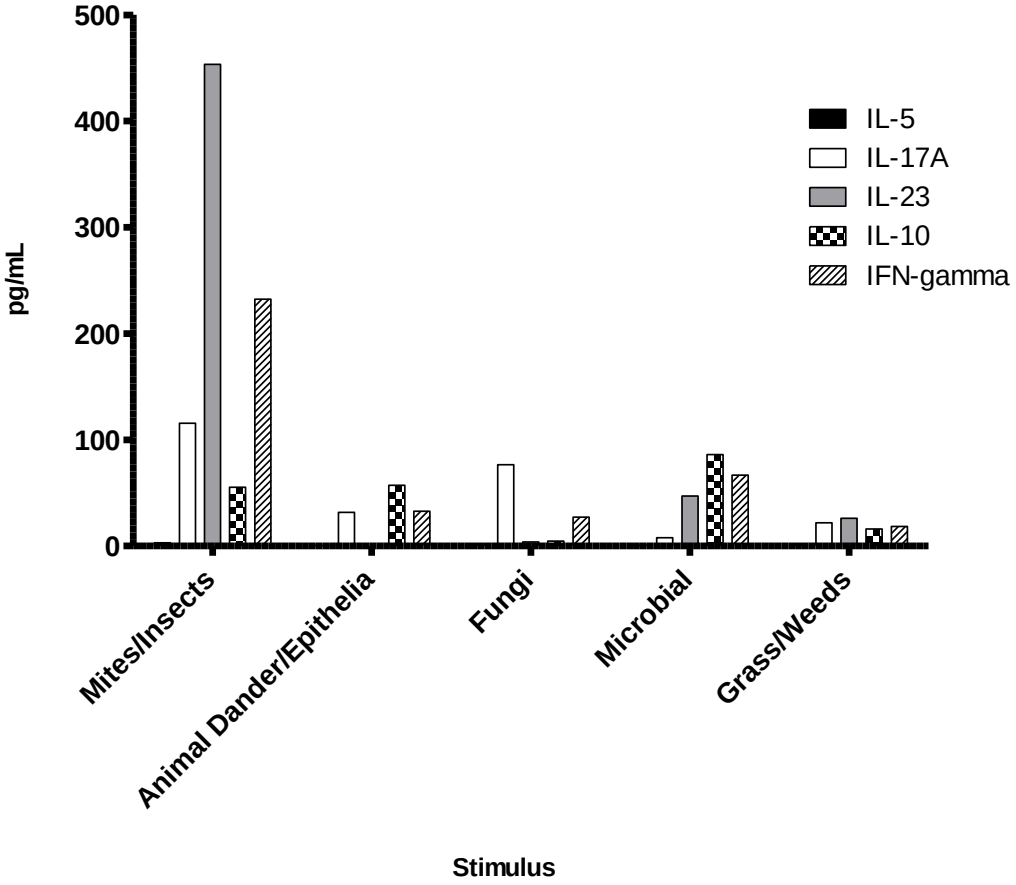


Figure 11.21 Atopic Asthmatic Patient 12 - Eosinophilic

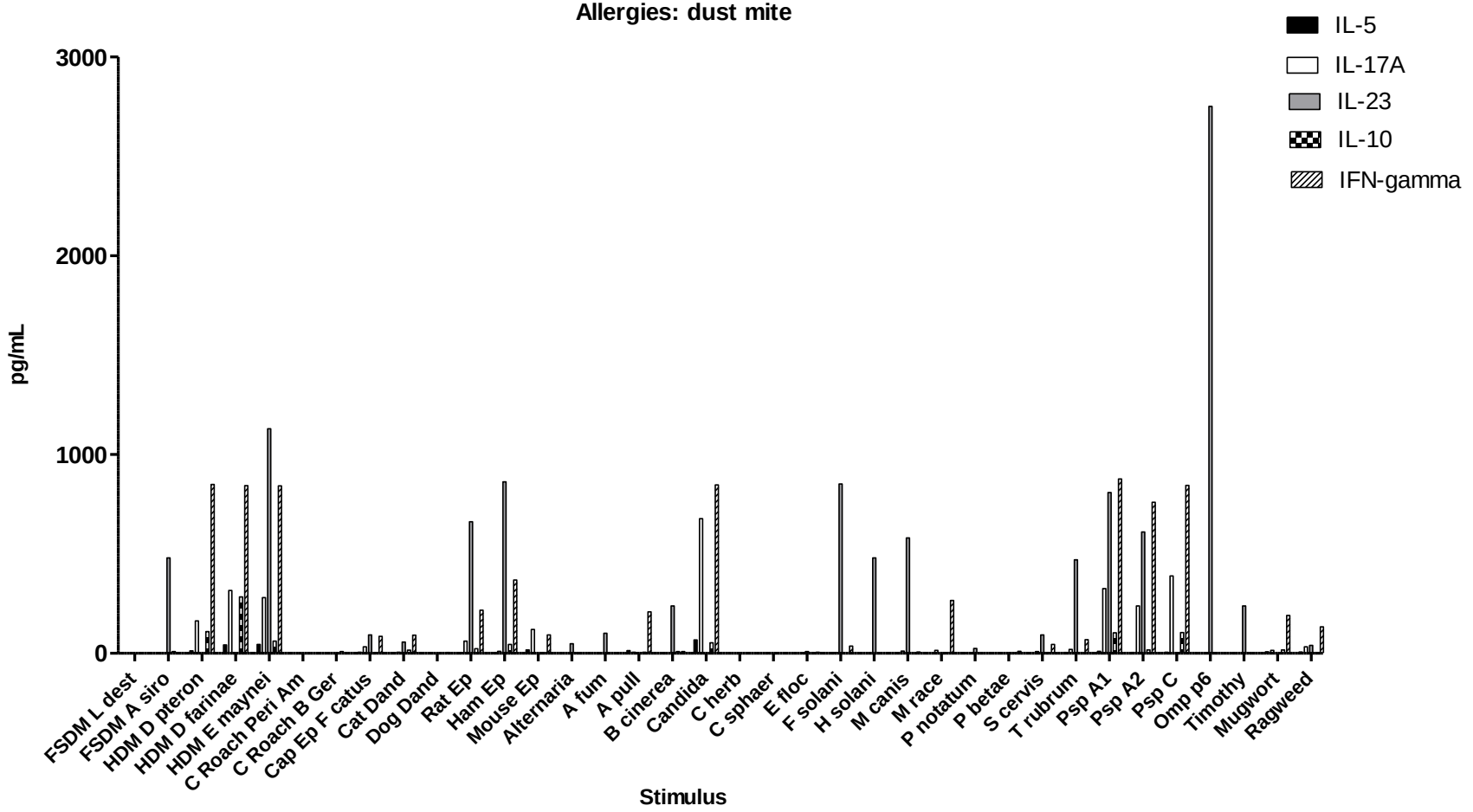


Figure 11.22 Atopic Asthmatic Patient 12 - Eosinophilic
Allergies: dust mite

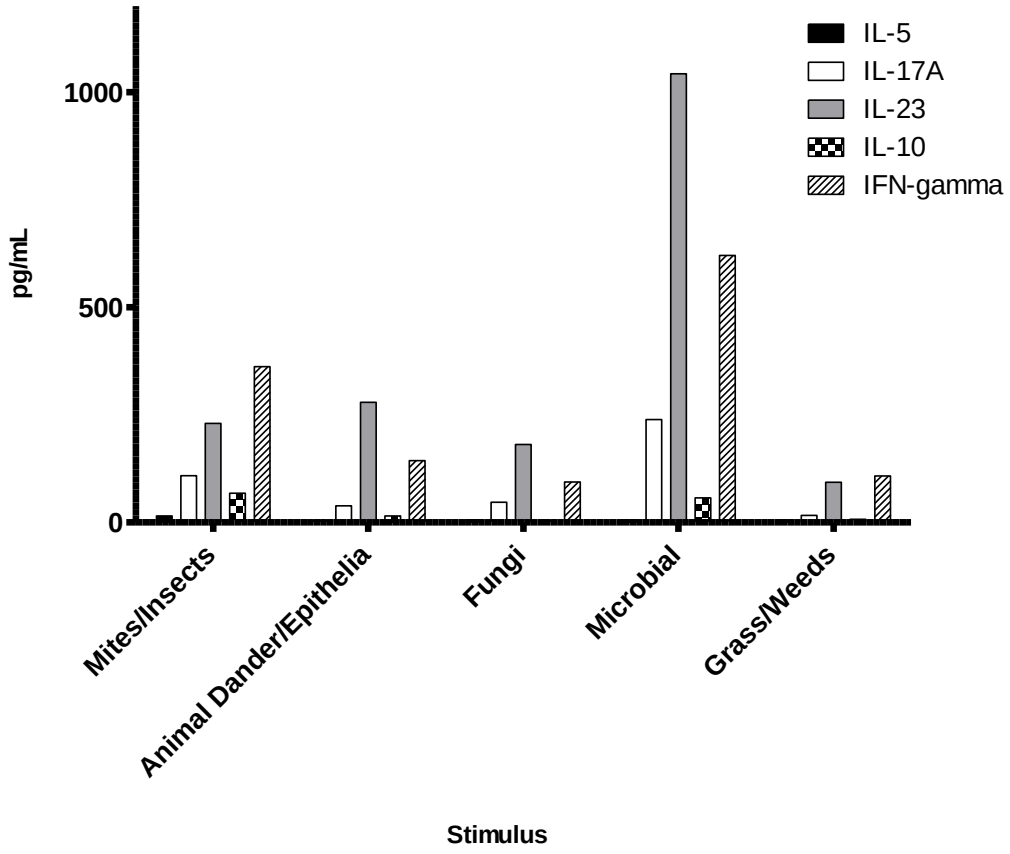


Figure 11.23 Atopic Asthmatic Patient 13 - Eosinophilic
Allergies: cat, dog, horse

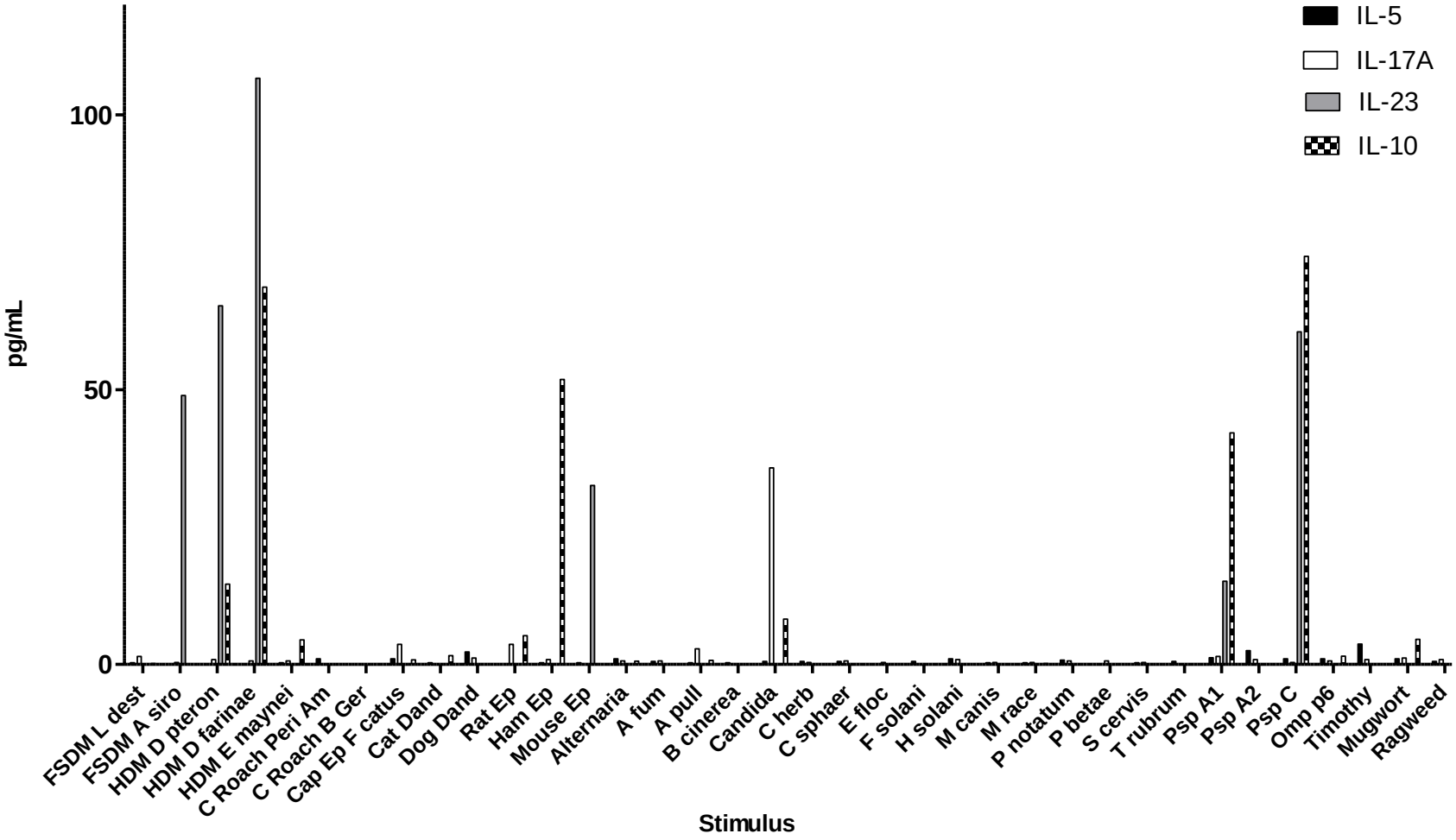


Figure 11.24 Atopic Asthmatic Patient 13 - Eosinophilic
Allergies: cat, dog, horse

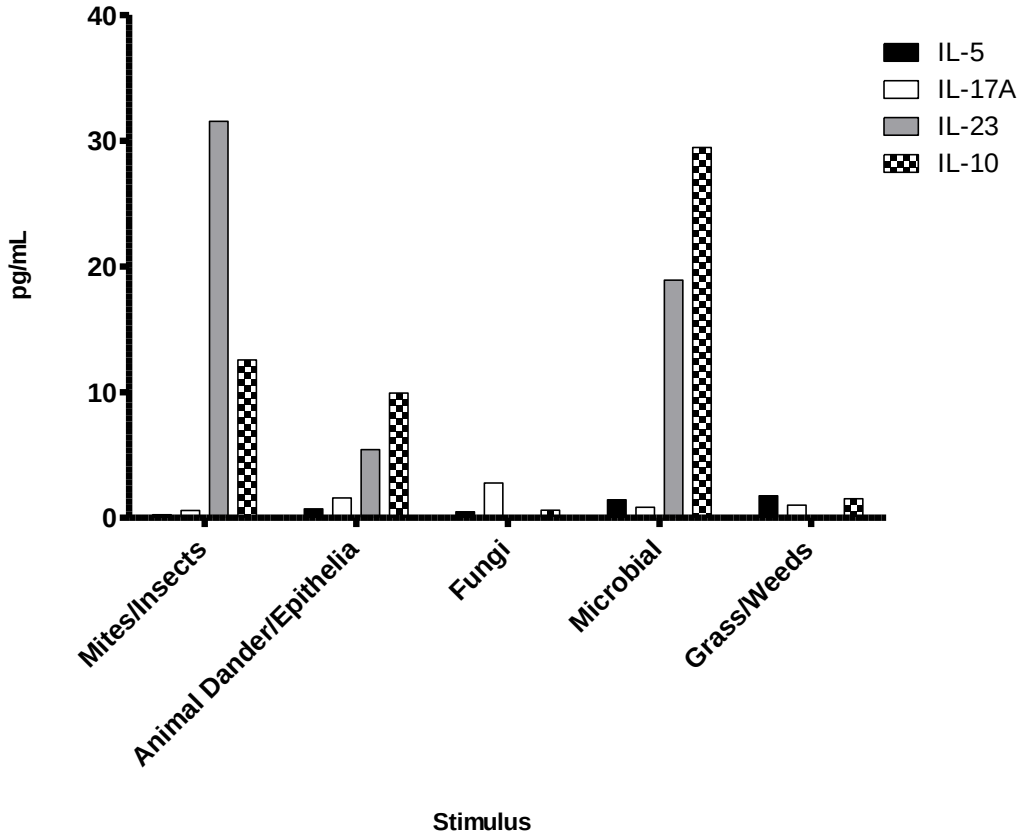


Figure 11.25 Atopic Asthmatic Patient 14 - Eosinophilic
Allergies: grass, dust, milk

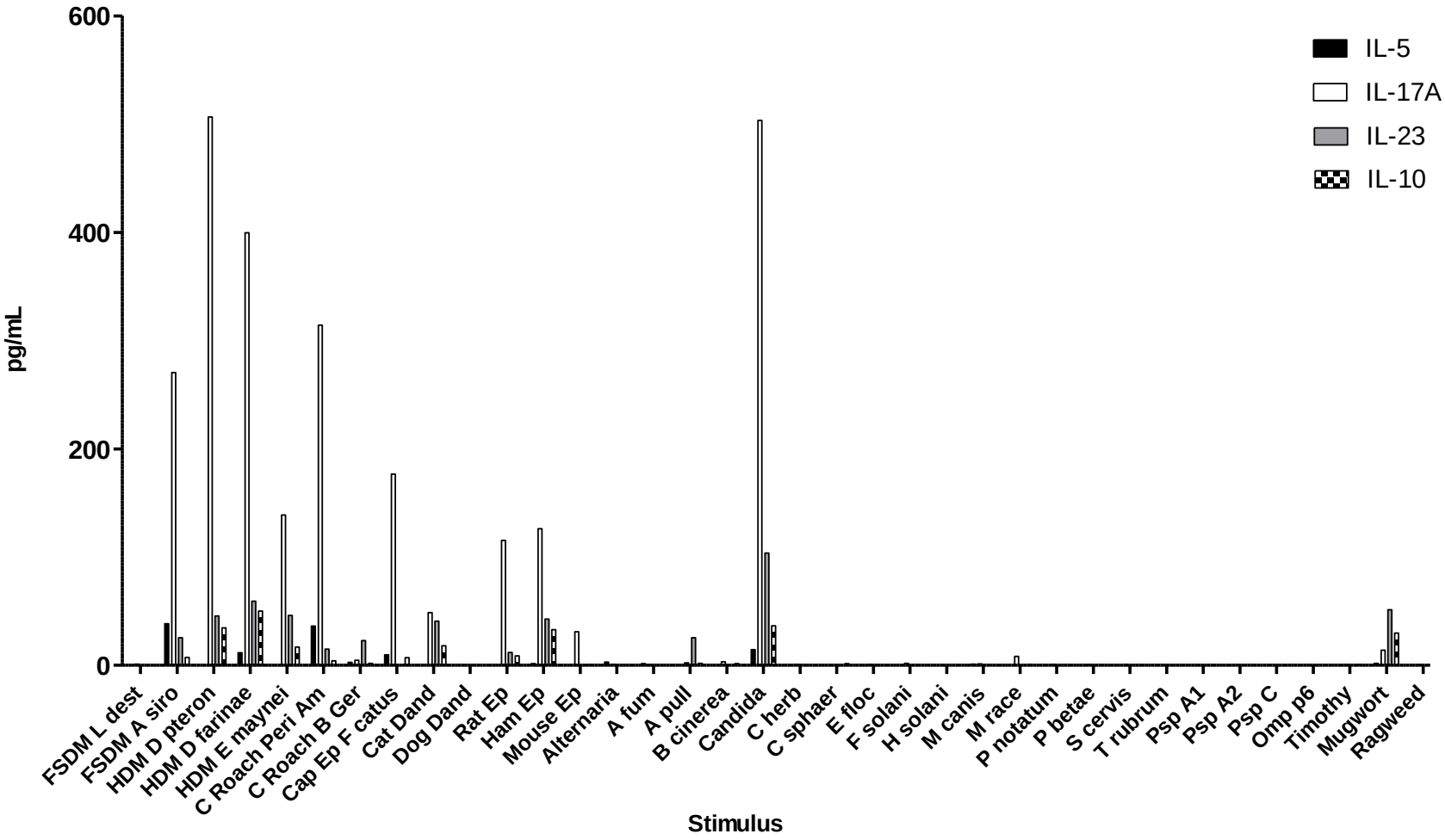


Figure 11.26 Atopic Asthmatic Patient 14 - Eosinophilic
Allergies: grass, dust, milk

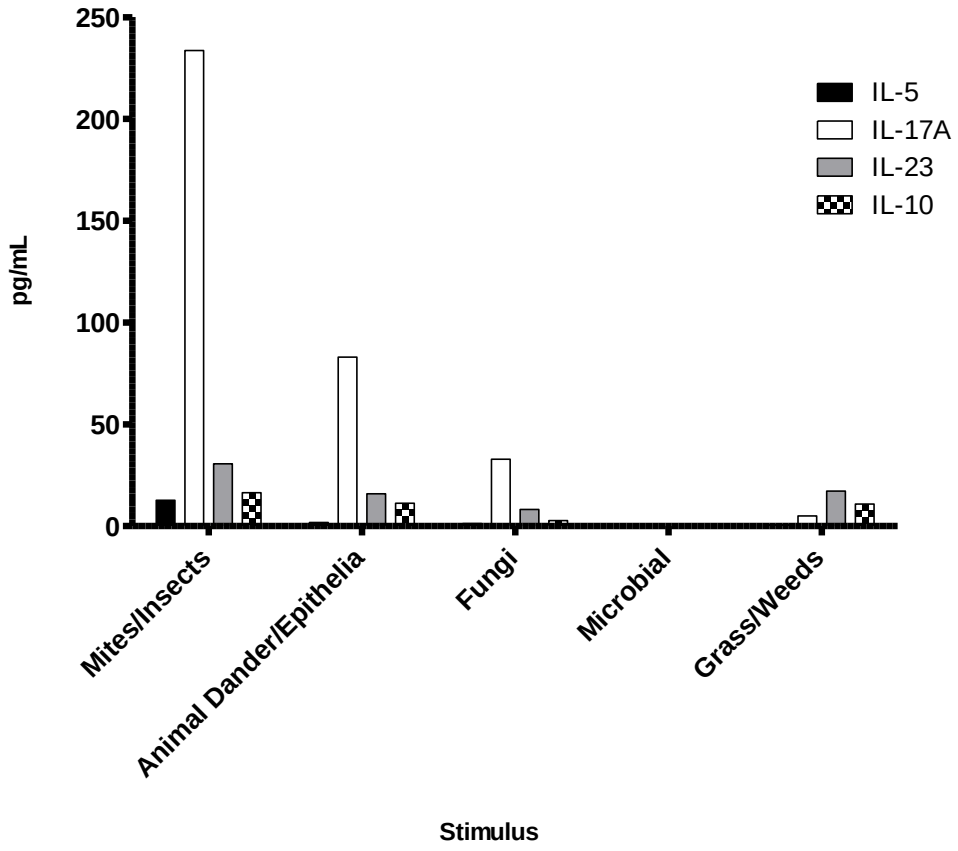


Figure 11.27 Atopic Asthmatic Patient 15 - Eosinophilic
Allergies: d. far, d. pter, grass, ragweed

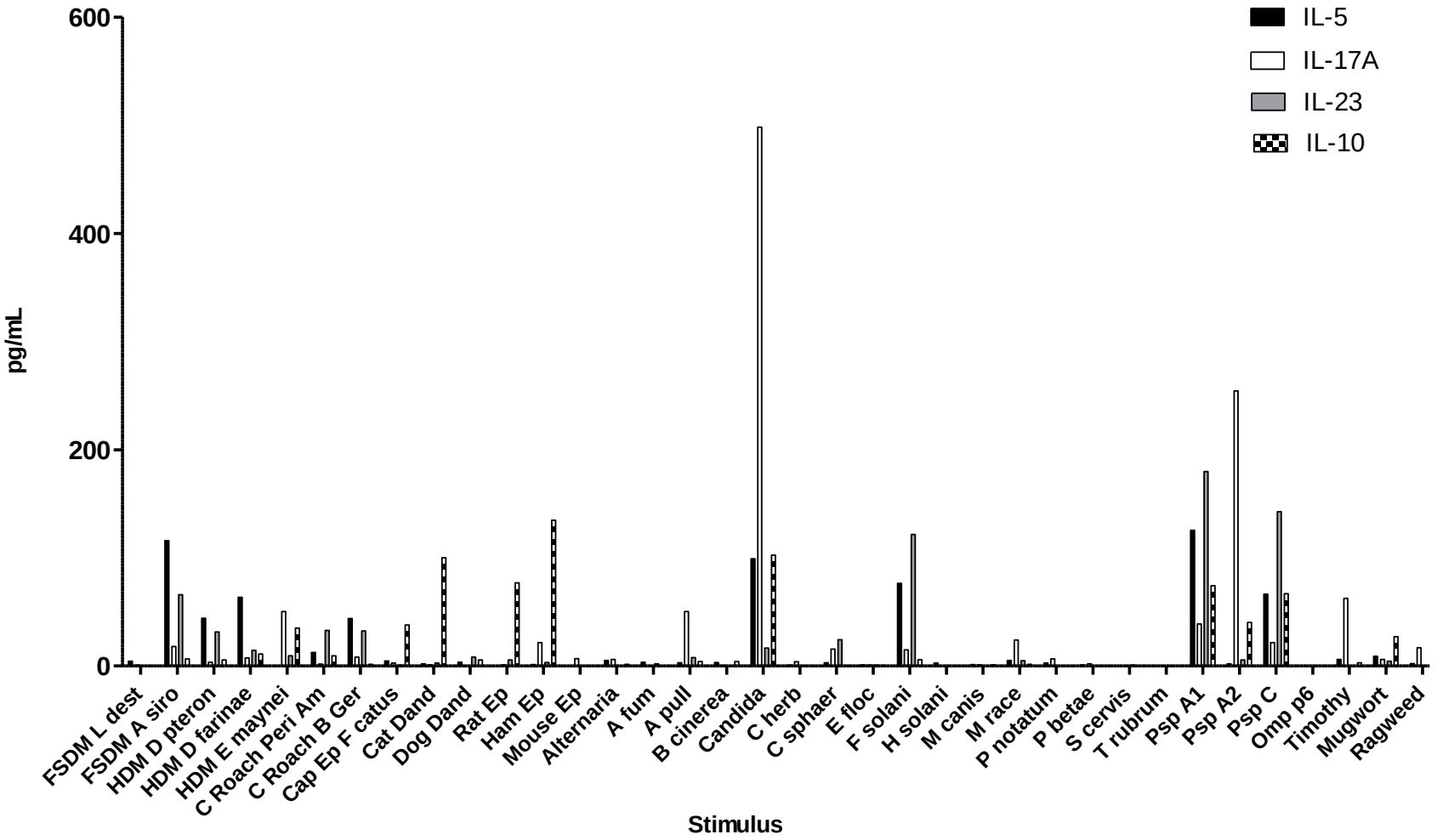


Figure 11.28 Atopic Asthmatic Patient 15 - Eosinophilic
Allergies: d. far, d. pter, grass, ragweed

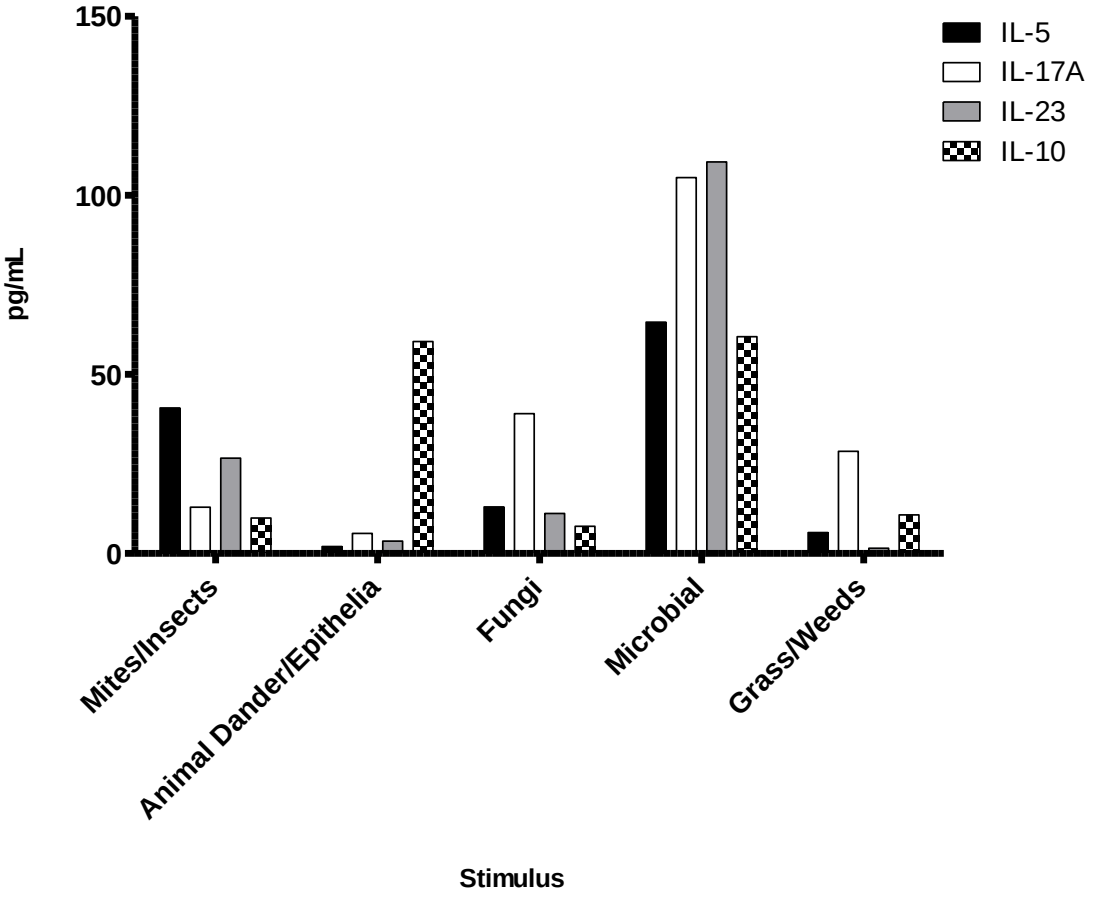


Figure 11.29 Atopic Asthmatic Patient 16 - Eosinophilic
Allergies: dust mite, dictoptera

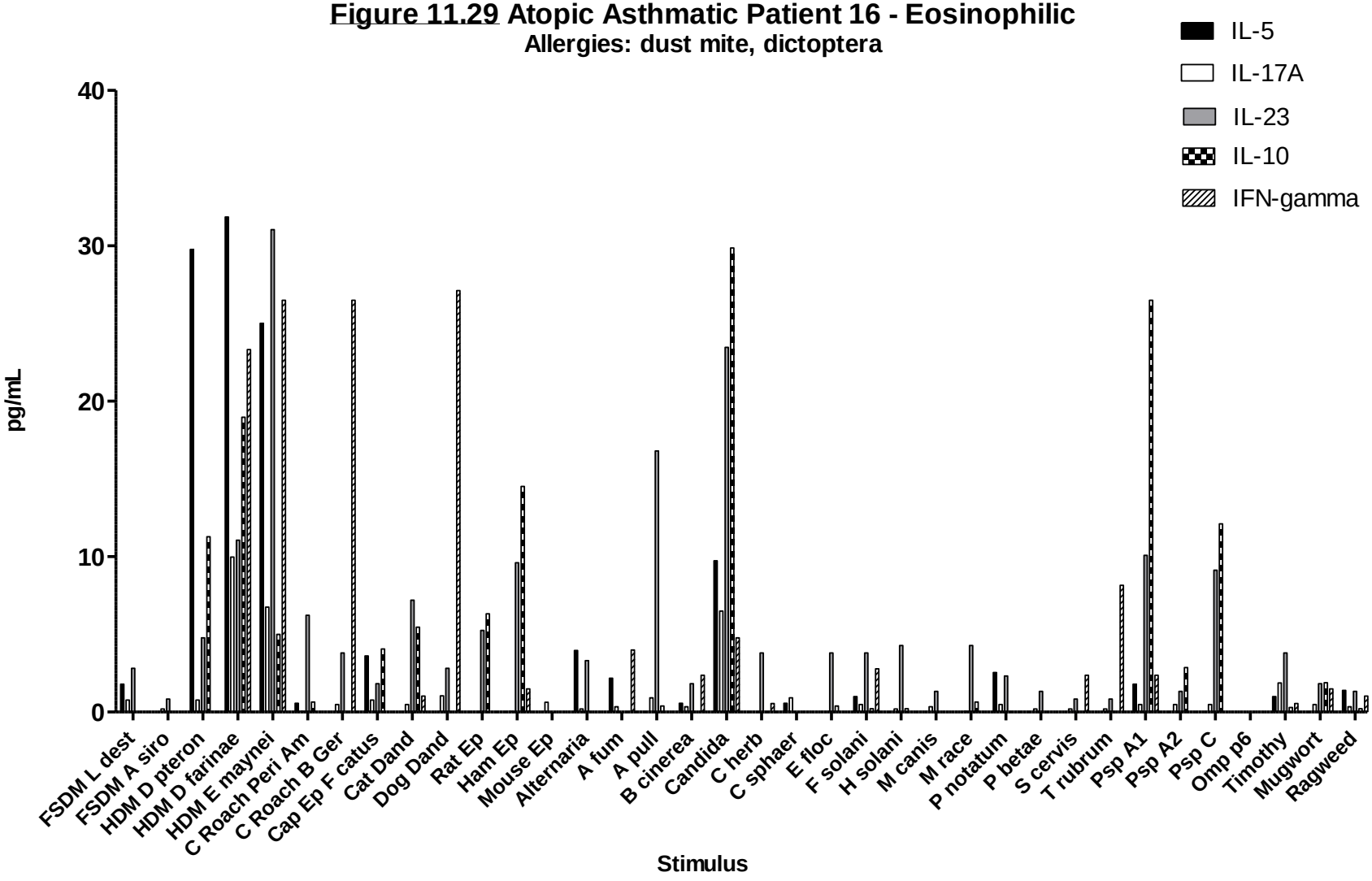


Figure 11.30 Atopic Asthmatic Patient 16 - Eosinophilic
Allergies: dust mite, dictoptera

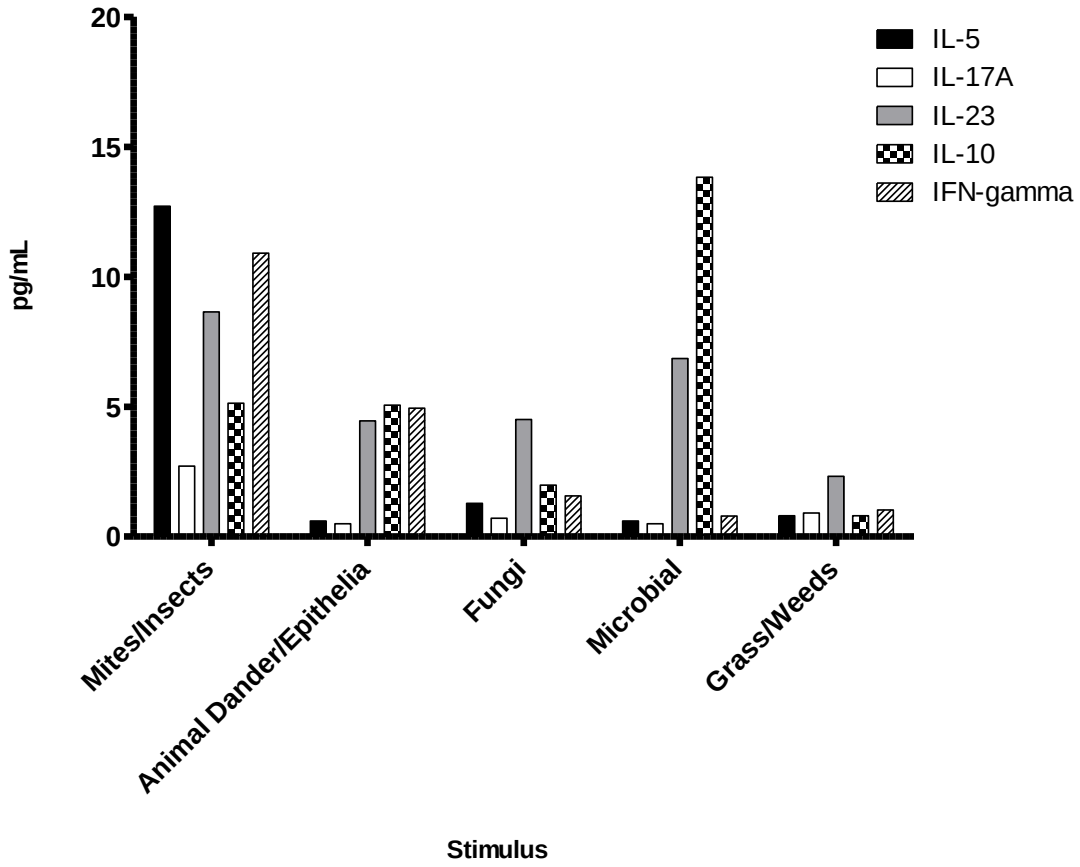


Figure 11.31 Atopic Asthmatic Patient 17 - Eosinophilic
Allergies: cat, horse, feathers, dust mite

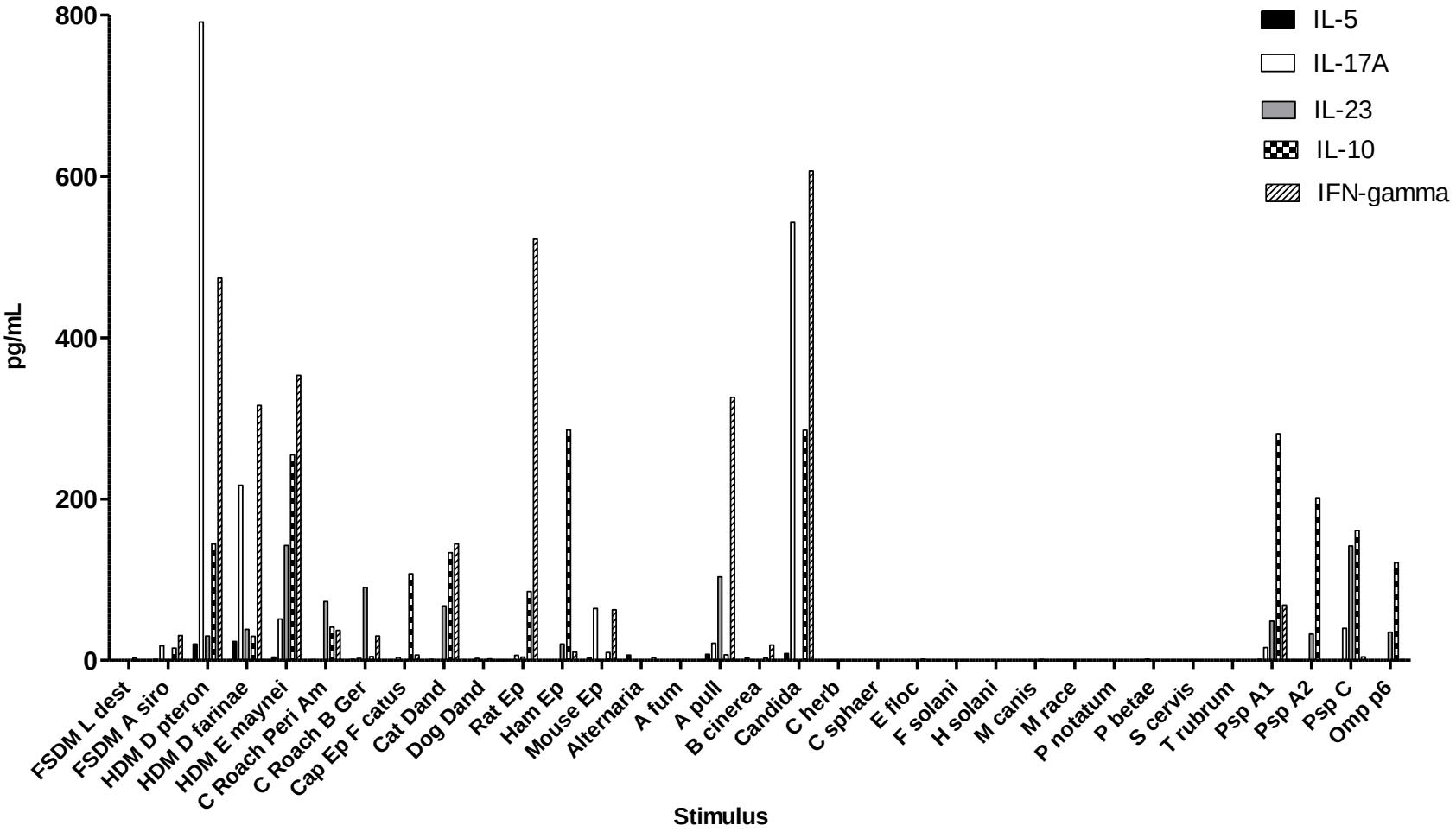


Figure 11.32 Atopic Asthmatic Patient 17 - Eosinophilic

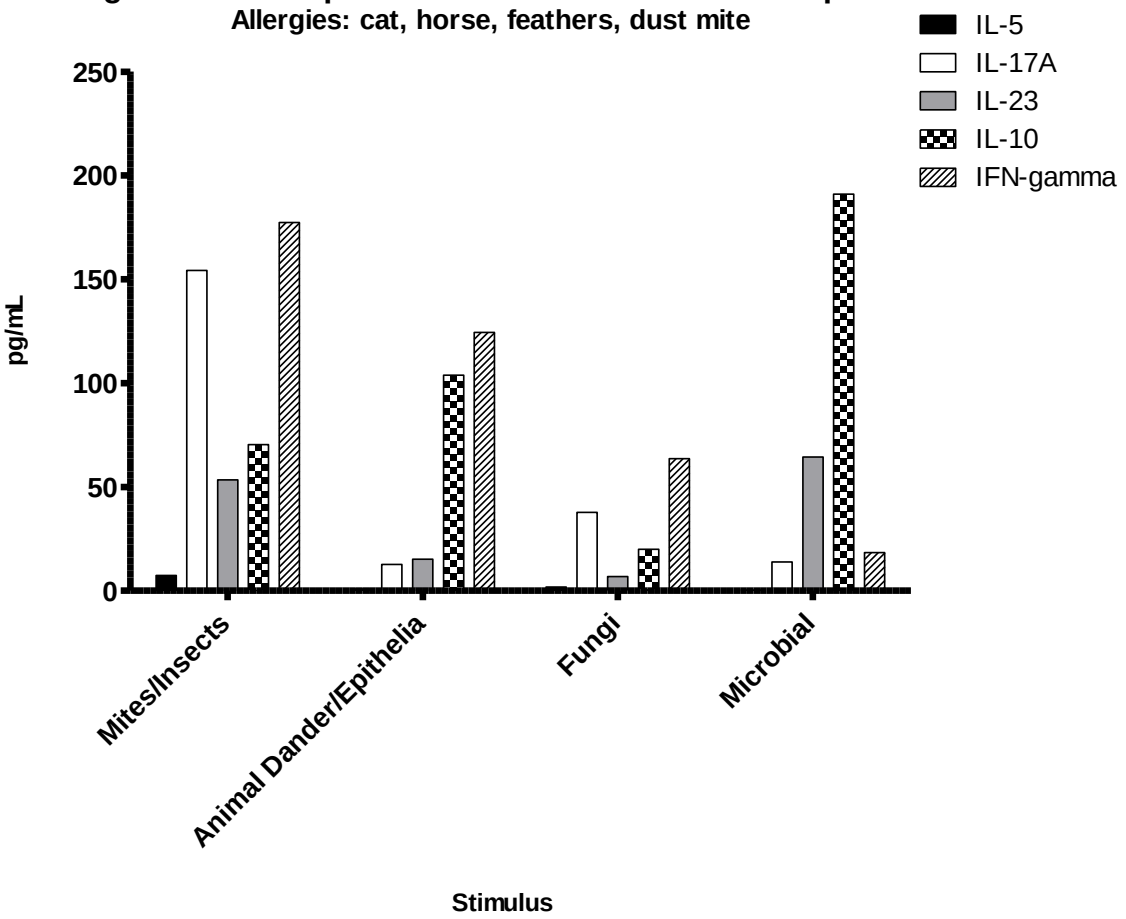


Figure 12.1 Normal Control 7

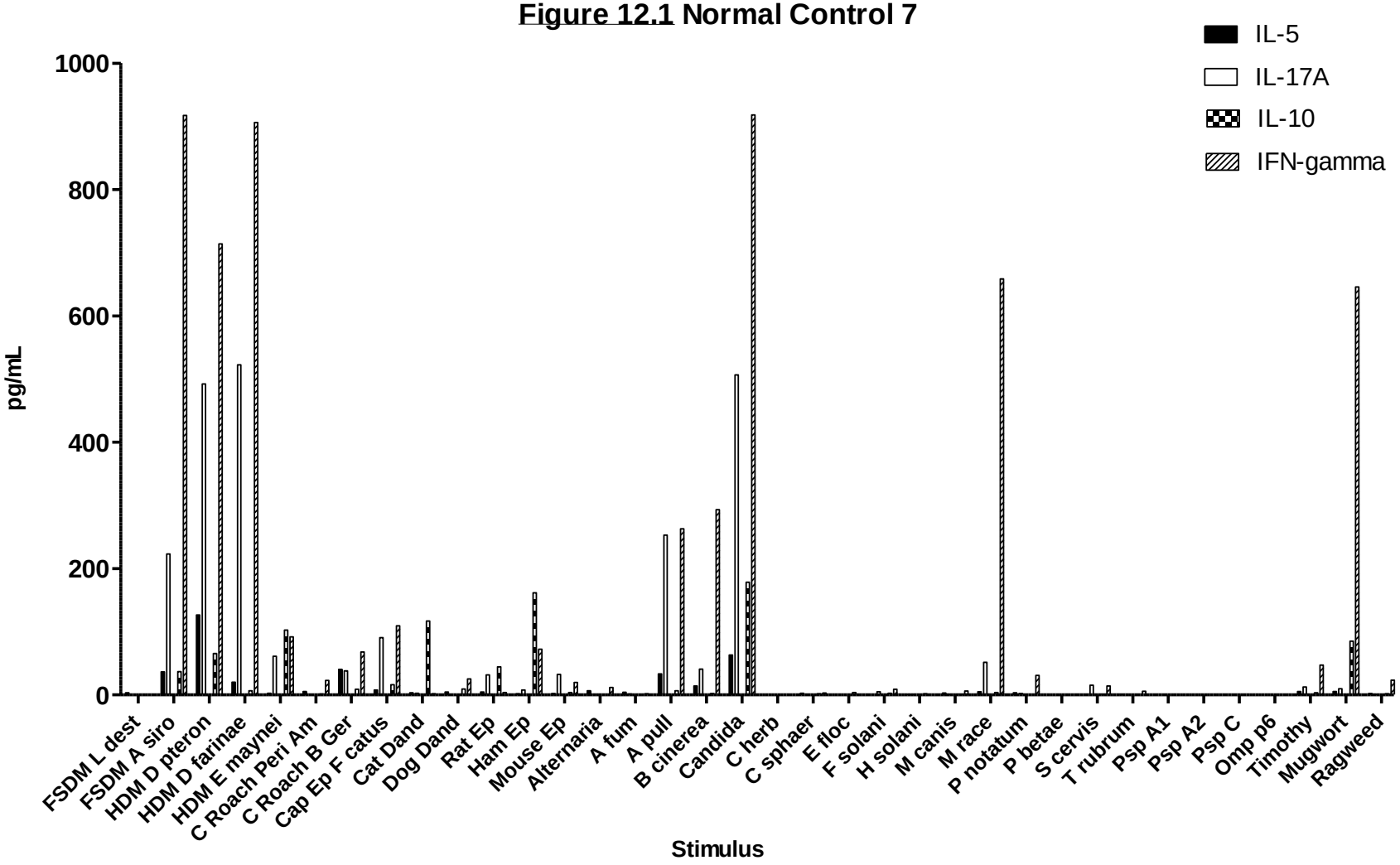


Figure 12.2 Normal Control 7

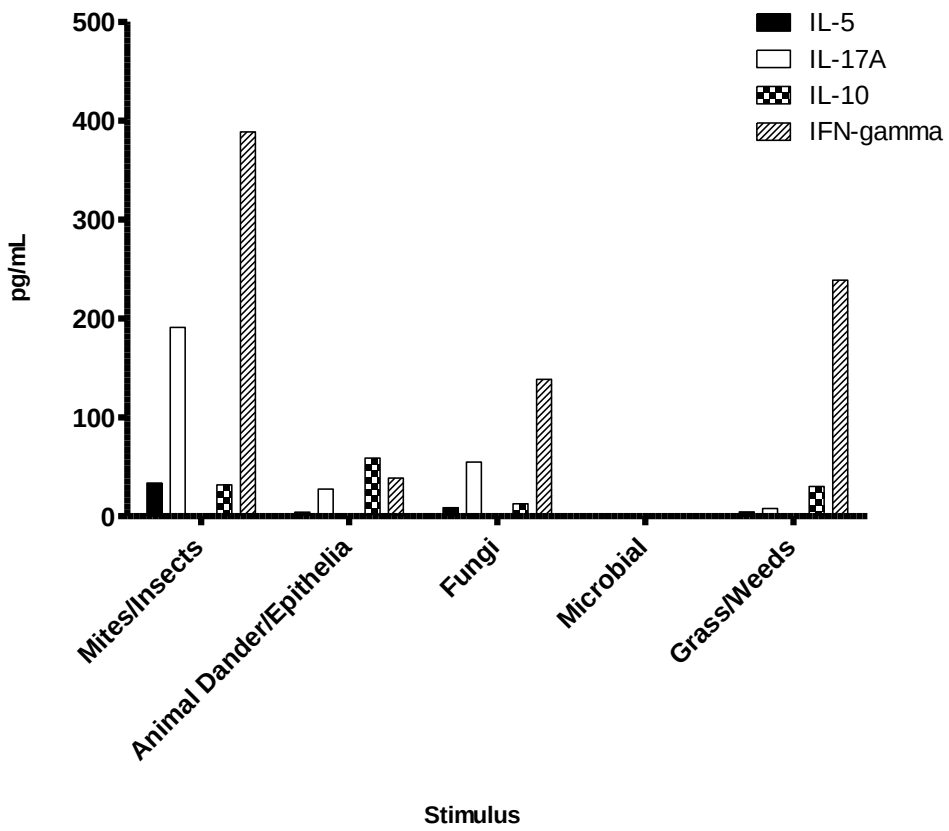
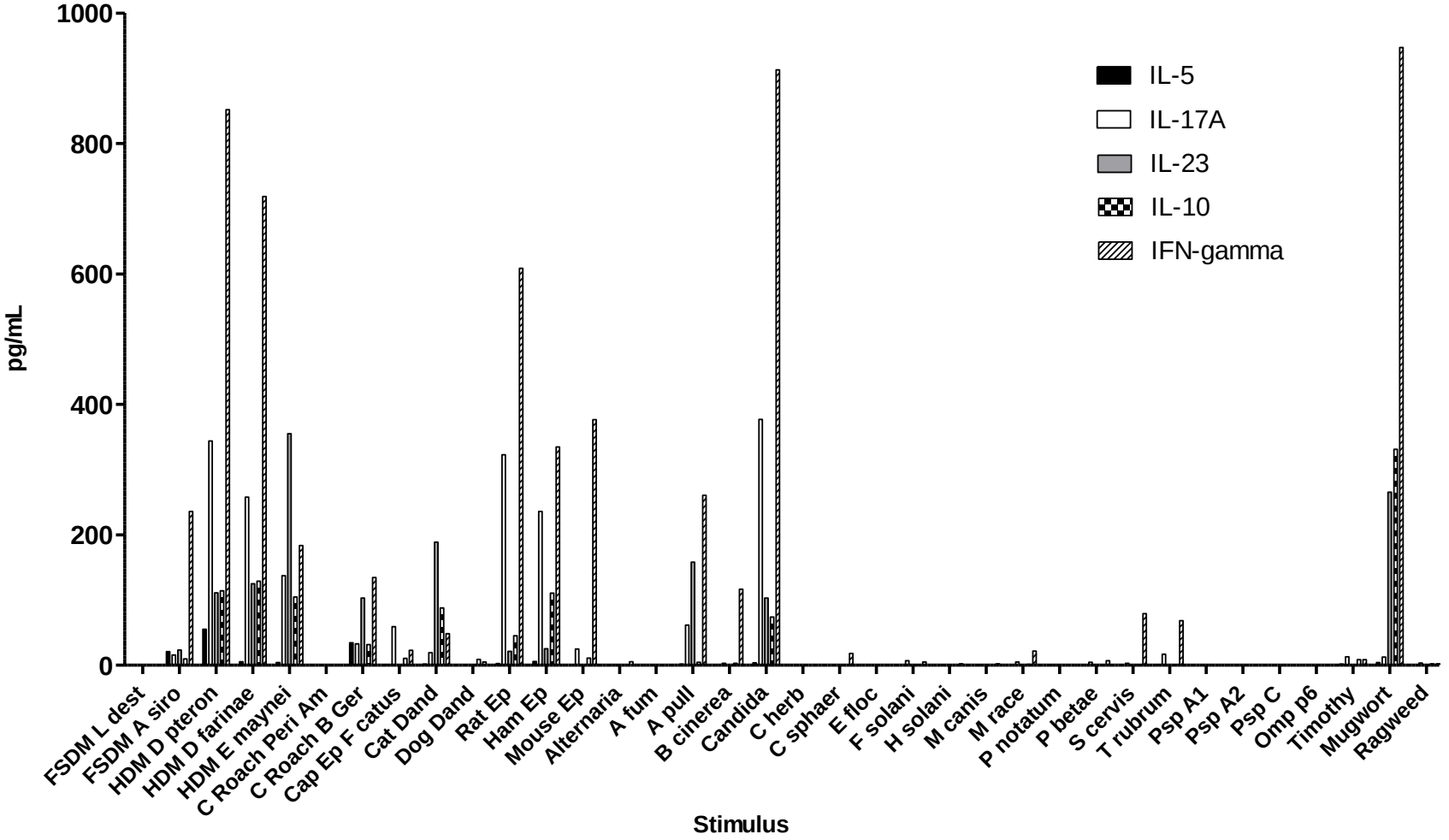


Figure 12.3 Normal Control 9



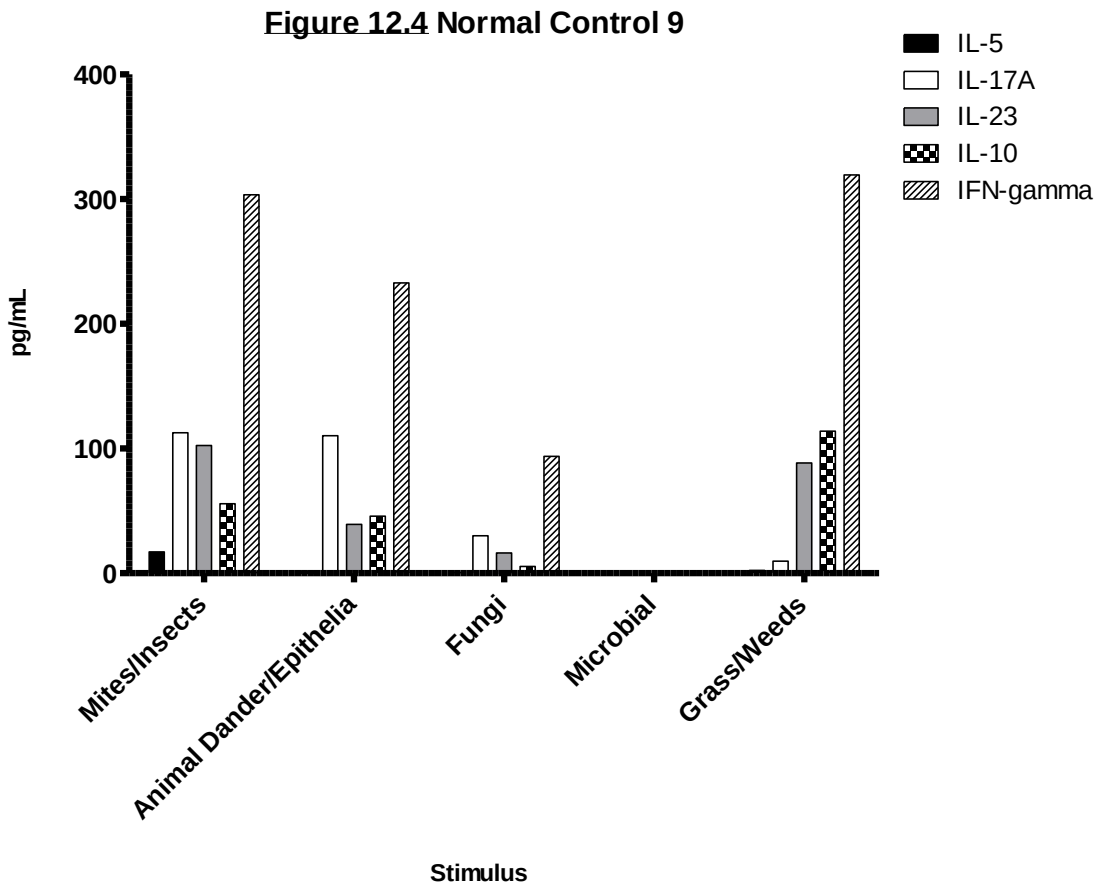


Figure 12.4 Normal Control 9
IL-5
IL-17A
IL-23
IL-10
IFN-gamma
400
300
200
100
0
pg/mL
Mites/Insects
Animal Dander/Epithelia
Fungi
Microbial
Grass/Weeds
Stimulus

Figure 12.5 Normal Control 10

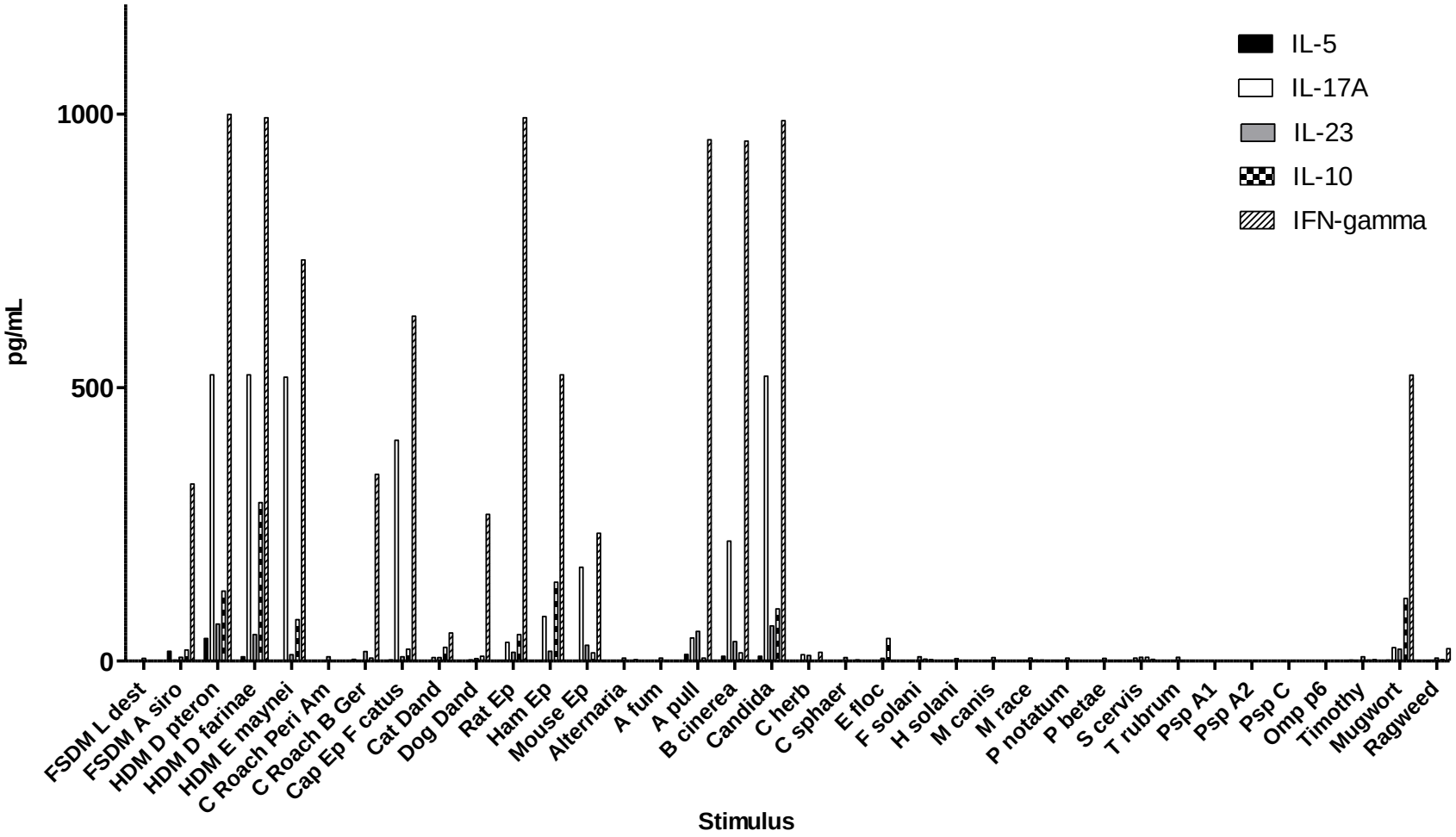


Figure 12.6 Normal Control 10

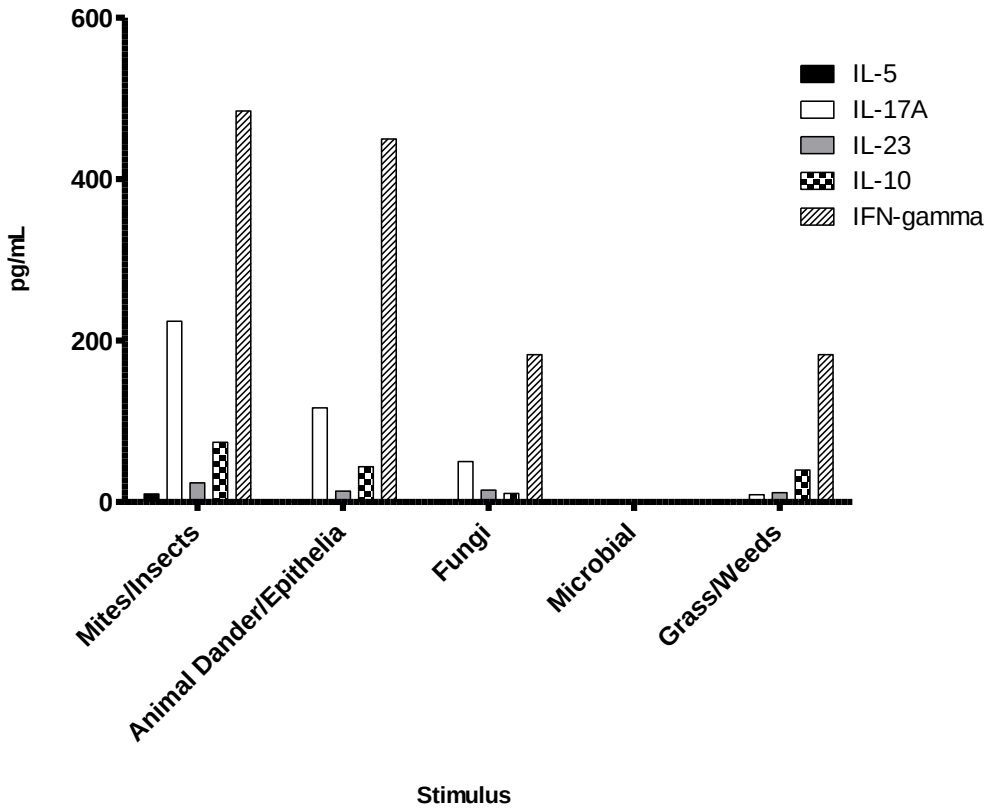


Figure 12.7 Normal Control 11

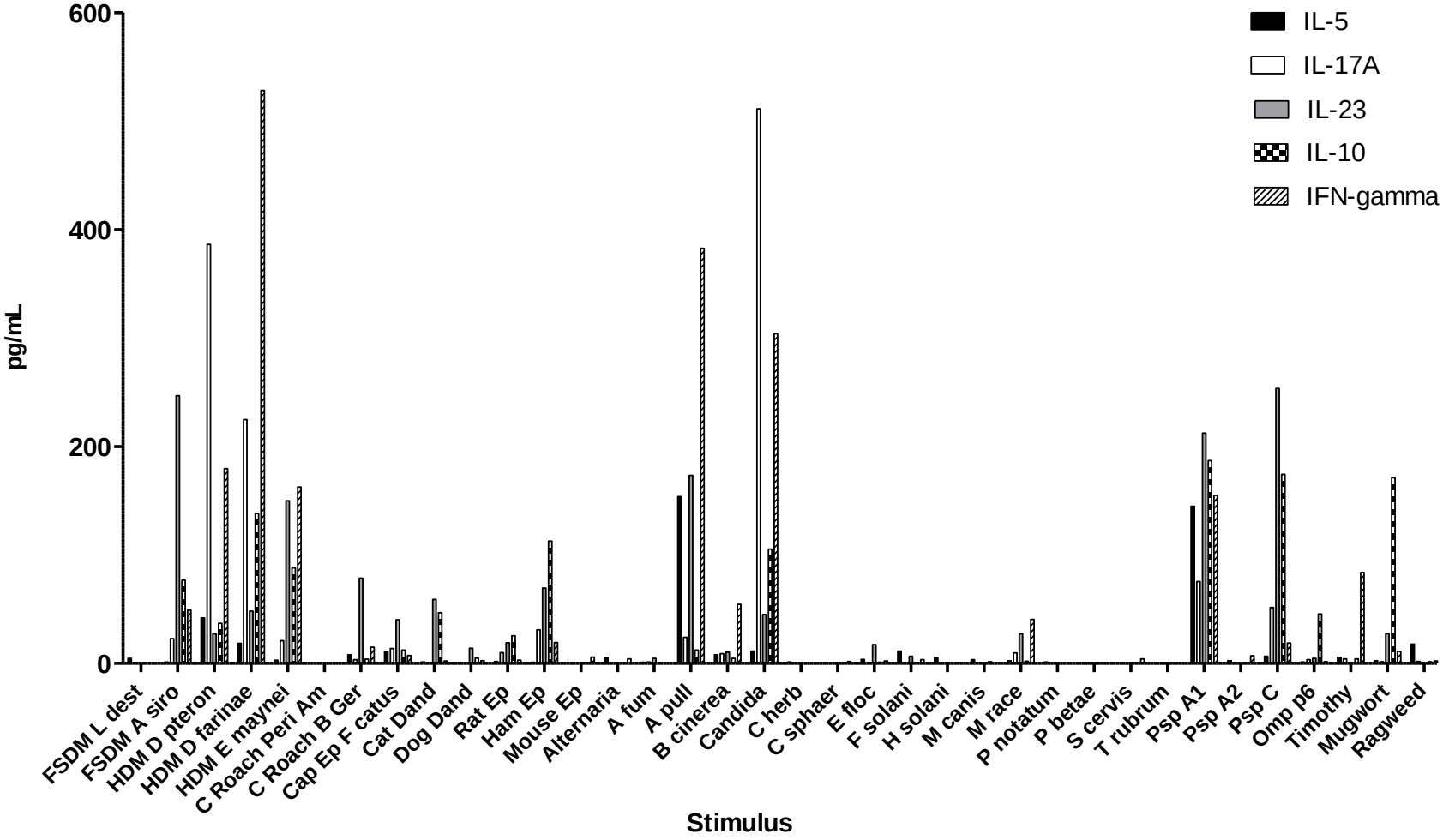


Figure 12.8 Normal Control 11

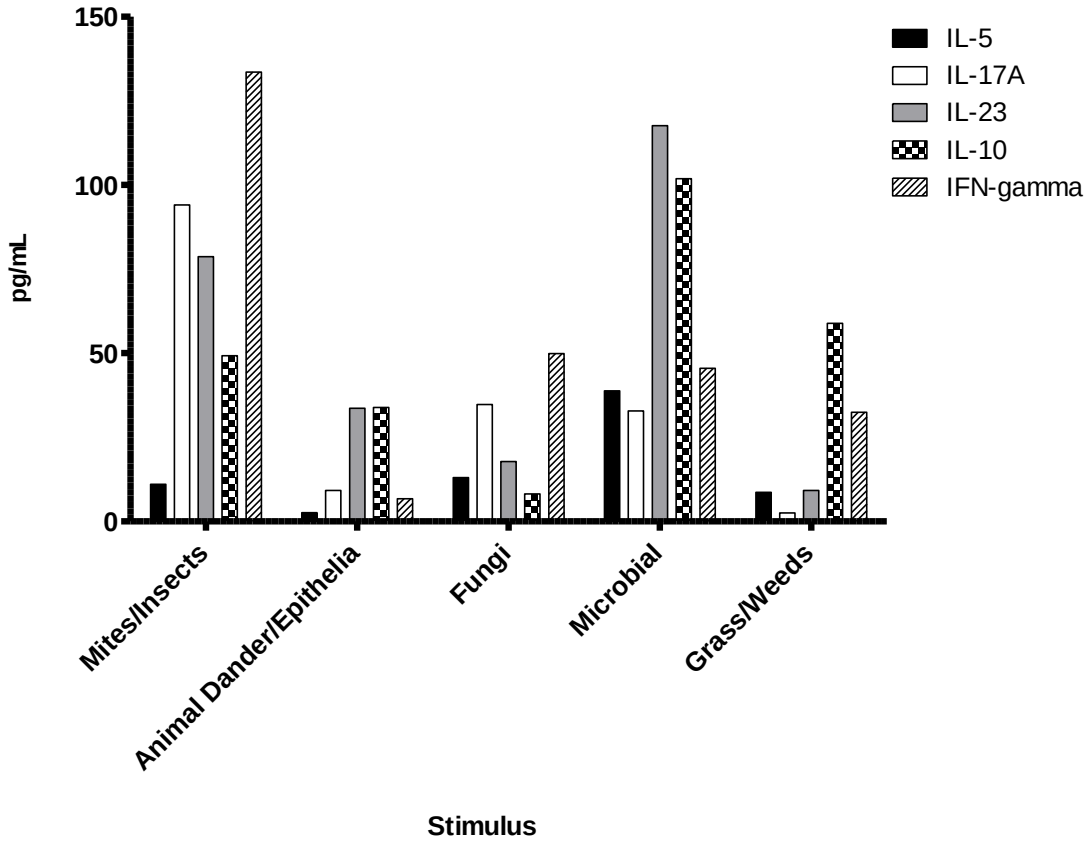
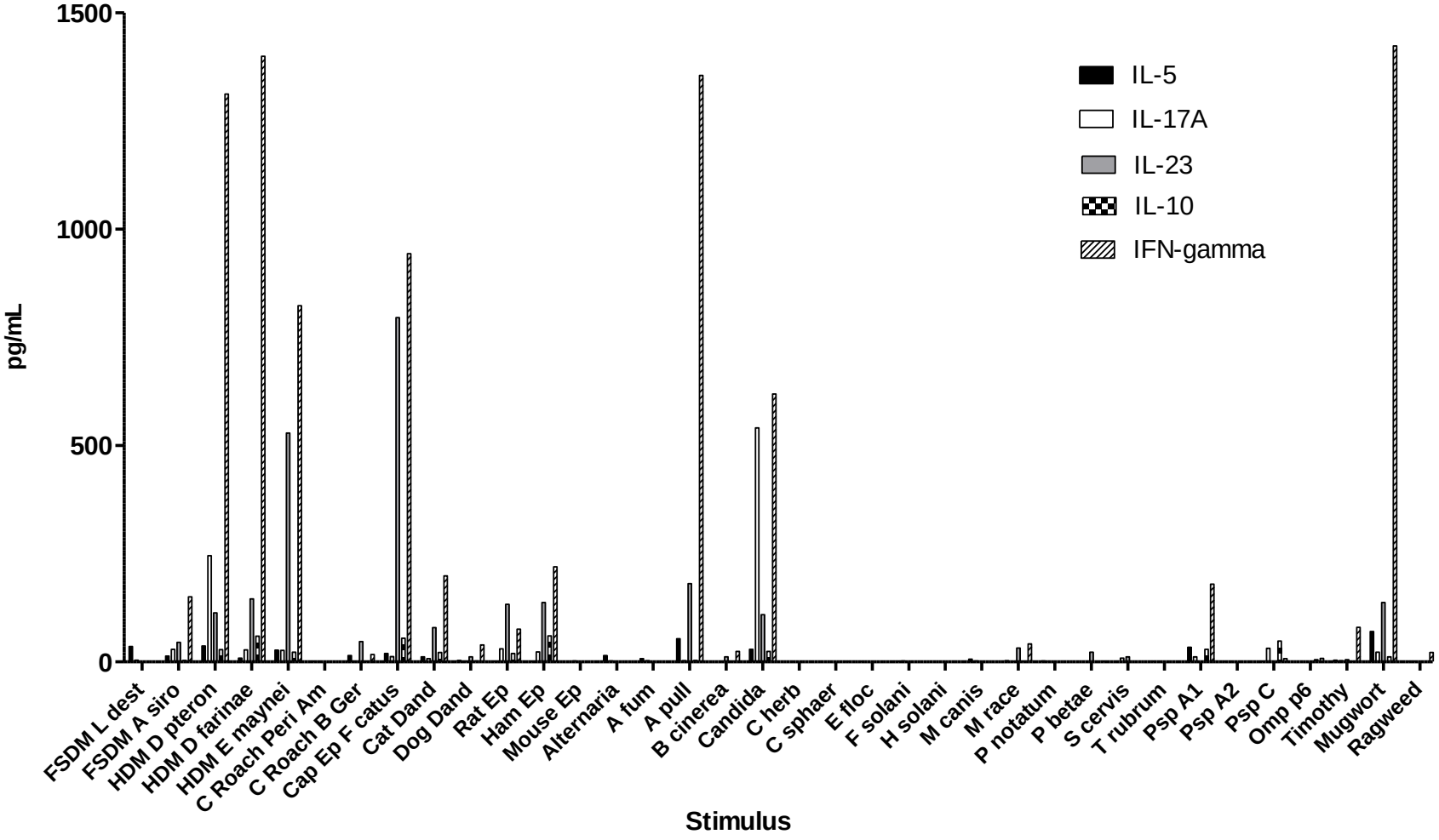


Figure 12.9 Normal Control 12



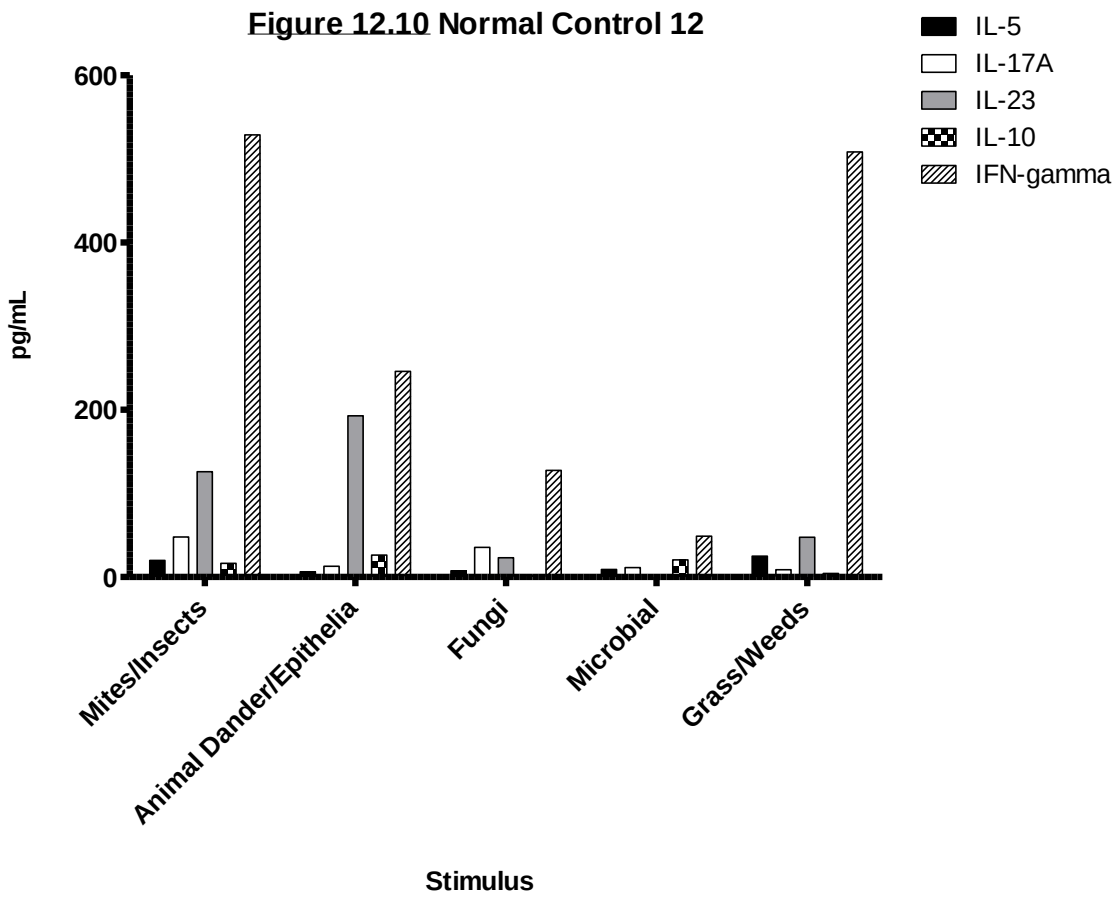


Figure 12.10 Normal Control 12
IL-5
IL-17A
IL-23
IL-10
IFN-gamma
600
400
200
0
pg/mL
Mites/Insects
Animal Dander/Epithelia
Fungi
Microbial
Grass/Weeds
Stimulus

Figure 12.11 Normal Control 13

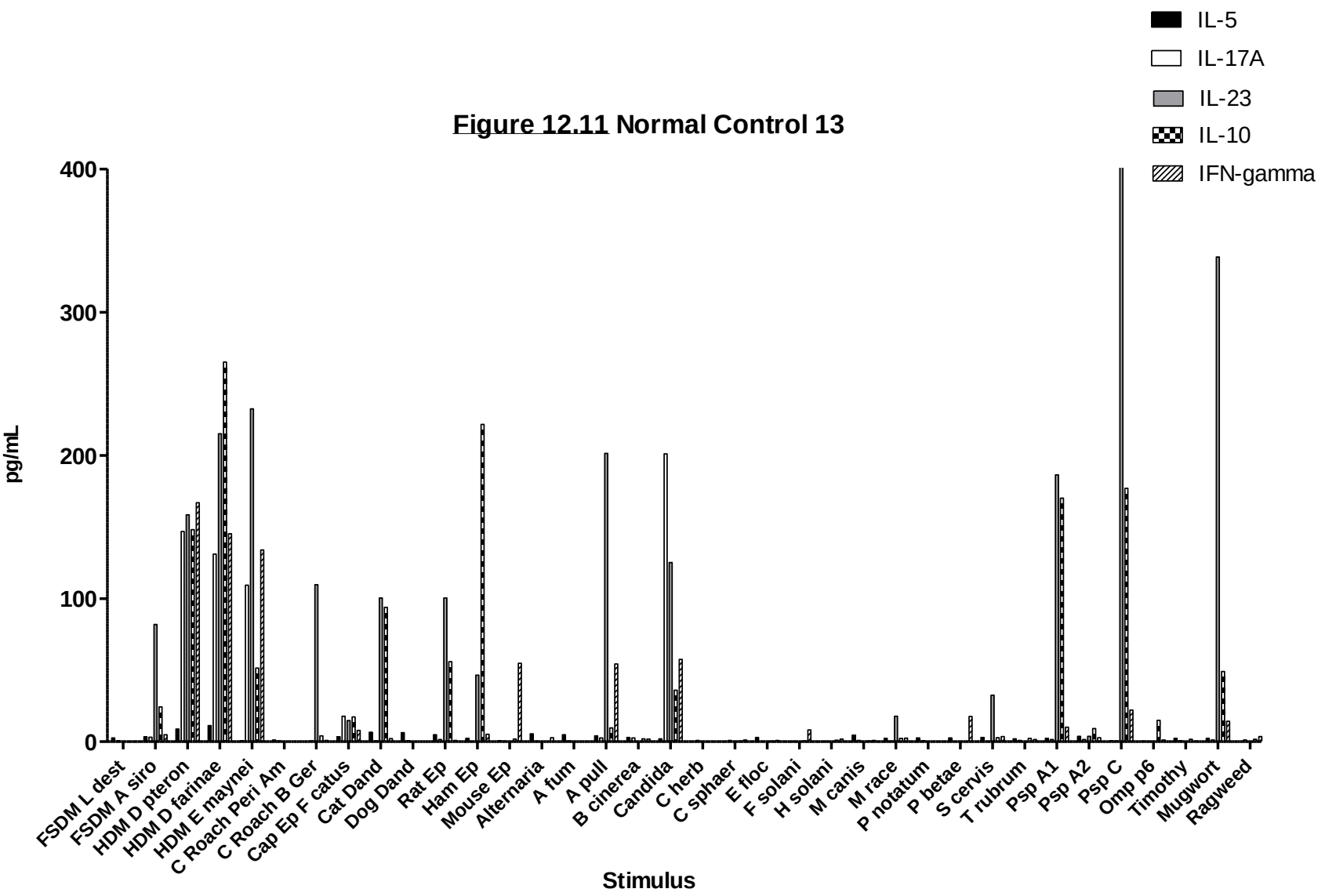


Figure 12.12 Normal Control 13

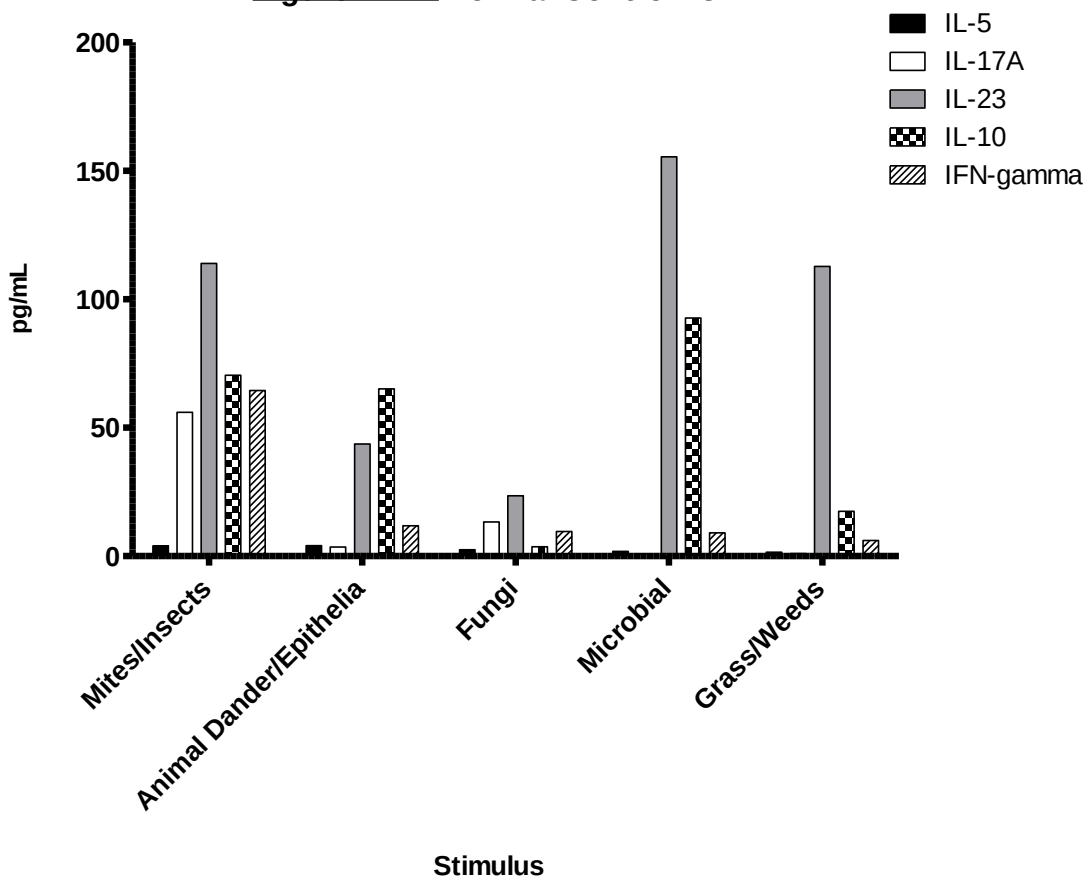


Figure 12.13 Normal Control 14

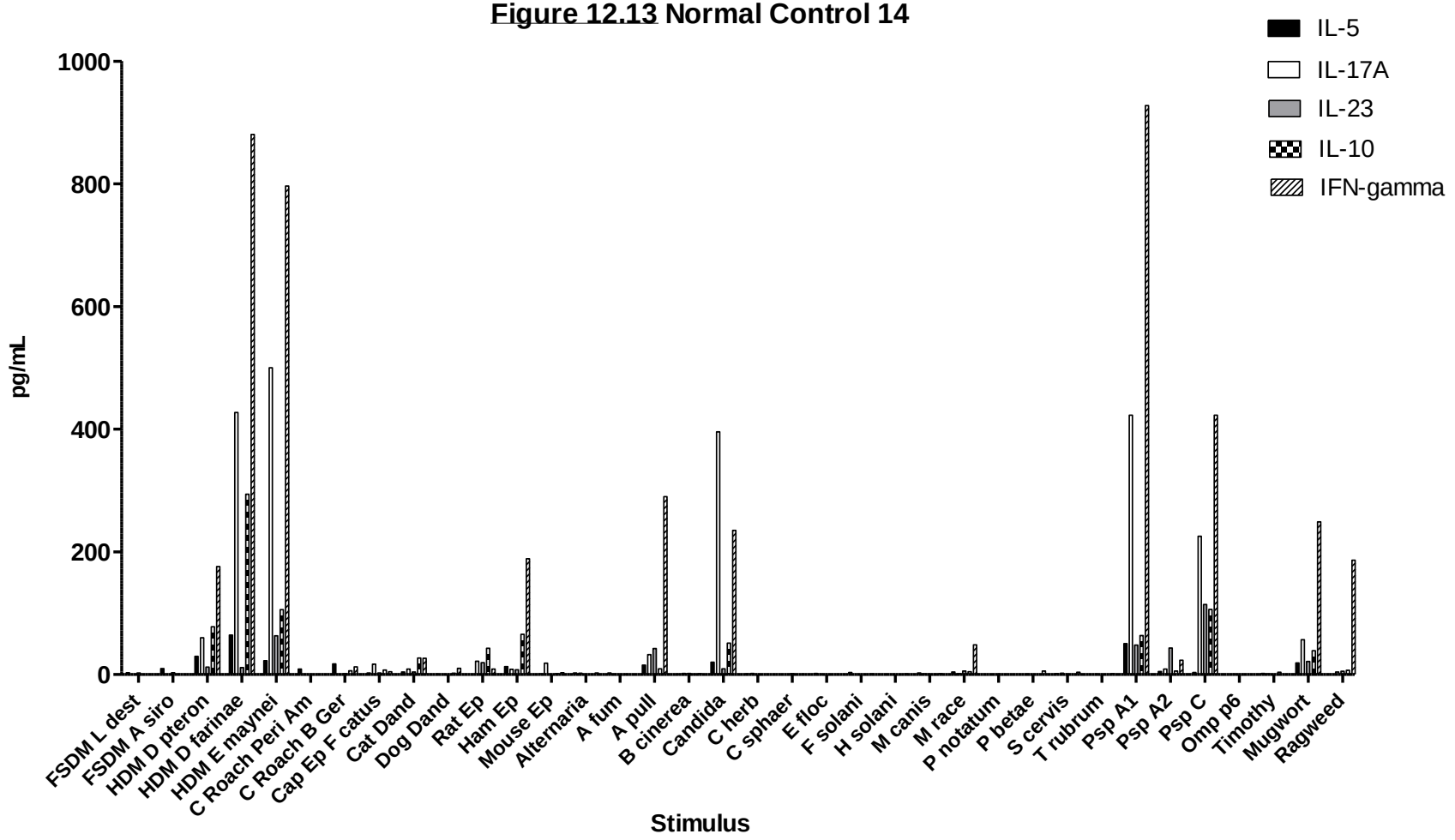
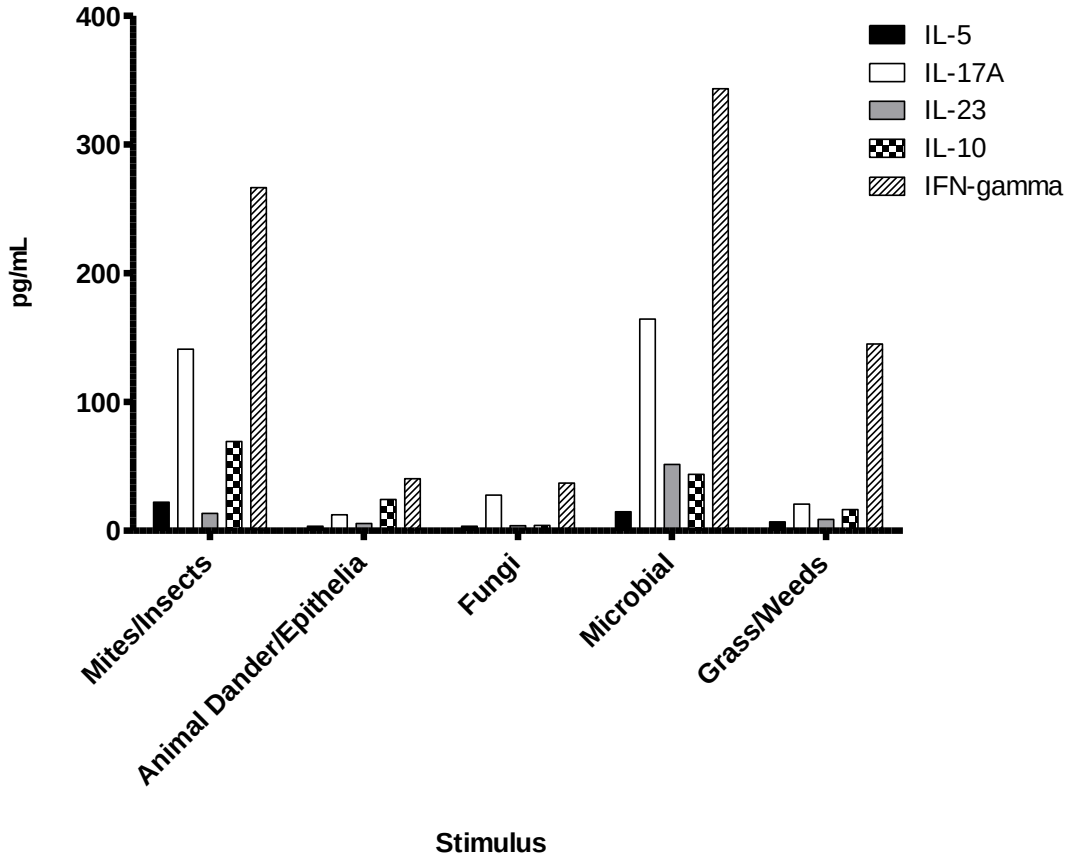


Figure 12.14 Normal Control 14



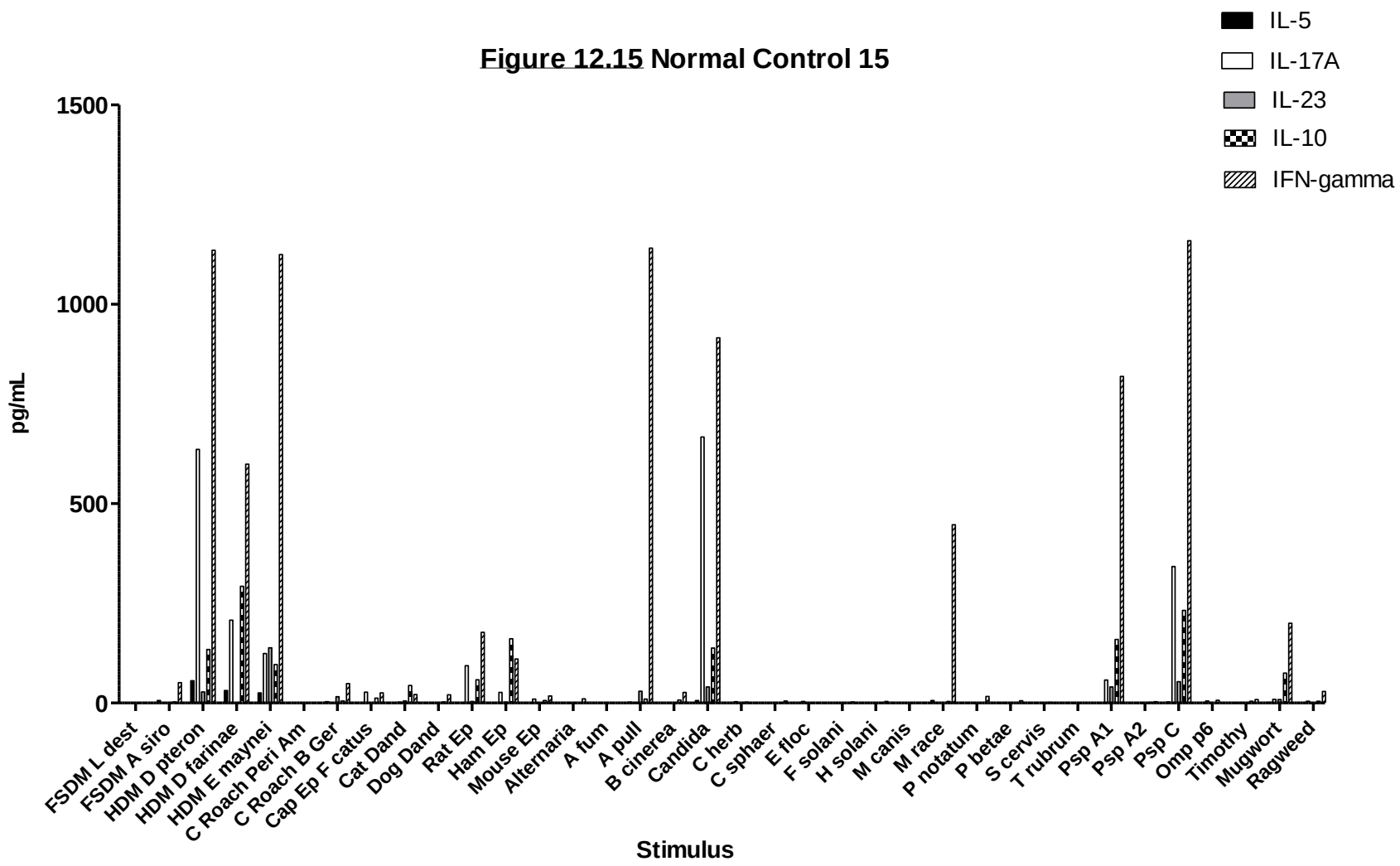
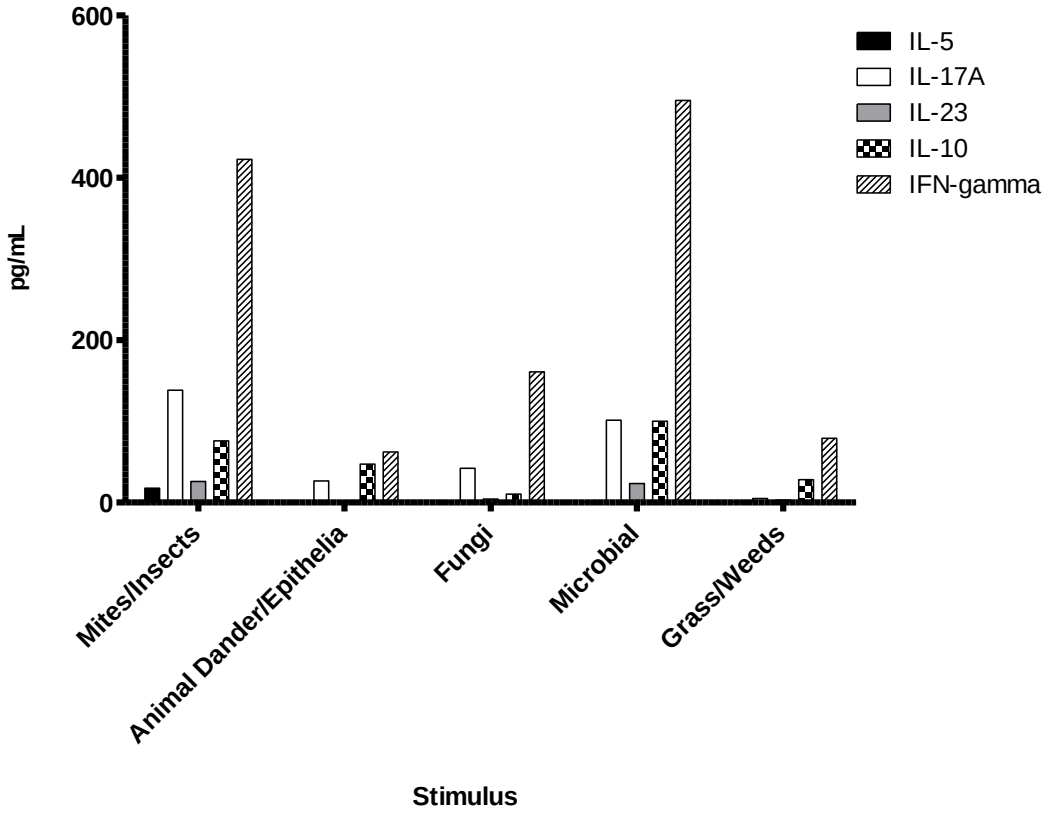


Figure 12.15 Normal Control 15
IL-5
IL-17A
IL-23
IL-10
IFN-gamma
1500
1000
500
0
pg/mL
FSDM L dest
FSDM A siro
HDM D pteron
HDM D farinae
HDM E maynei
C Roach Peri Am
C Roach B Ger
Cap Ep F catus
Cat Dand
Dog Dand
Rat Ep
Ham Ep
Mouse Ep
Alternaria
A fum
A pull
B cinerea
Candida
C herb
C sphaer
E floc
F solani
H solani
M canis
M race
P notatum
P betae
S cervis
T rubrum
Psp A1
Psp A2
Psp C
Omp p6
Timothy
Mugwort
Ragweed
Stimulus

Figure 12.16 Normal Control 15



REFERENCES

1. Akdis, M., Verhagen, J., Taylor, A., Karamloo, F., Karagiannidis, C., Crameri, R., Thunberg, S., Deniz, G., Valenta, R., Fiebig, H., et al. (2004). Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J. Exp. Med. 199, 1567–1575.
2. Akutsu, I., Kojima, T., Kariyone, A., Fukuda, T., Makino, S., Takatsu, K. (1995). Antibody against interleukin-5 prevents antigen-induced eosinophil infiltration and bronchial hyperreactivity in the guinea pig airways. Immunol Lett. 45: 109-116.
3. Barnes, P.J. (2007). New molecular targets for the treatment of neutrophilic diseases. J Allergy Clin Immunol. 119: 1055-1062.
4. Barnes, P.J. (2008b). Role of GATA-3 in allergic diseases. Curr Mol Med. 8: 330-334.
5. Bateman, E. D., Hurd, S. S., Barnes, P. J., Bousquet, J., Drazen, J. M., FitzGeralde, M., et al. (2008). Global strategy for asthma management and prevention: GINA executive summary. Eur Respir J. 31: 143-178.
6. Barbato, A., Turato, G., Baraldo, S., Bazzan, E., Calabrese, F., Panizzolo, C., Zanin, M.E., Zuin, R., Maestrelli, P., Fabbri, L.M., Saetta, M. (2006). Epithelial damage and angiogenesis in the airways of children with asthma. Am J Respir Crit Care Med. 174: 975-981.
7. Barczyk, A., Pierzchala, W., Sozanska, E. (2003). Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respir Med. 97: 726-733.
8. Boniface, K., Blom, B., Liu, Y., De Waal Malefyt, R. (2008) From interleukin-23 to T-helper 17 cells: human T-helper T cell differentiation revisited. Immunol Reviews. 226: 132-146.
9. Buc, M., Dzurilla, M., Vrlik, M., Bucova, M. (2009). Immunopathogenesis of bronchial asthma. Arch Immunol Ther Exp. 57: 331-344.
10. Burney, P.G.J., Newson, R.B., Burrows, M.S., Wheeler, D.M. (2008). The effects of allergens in outdoor air on both atopic and nonatopic subjects with airway disease. Allergy. 63: 542-546.
11. Bradding, P., Okayama, Y., Howarth, P.H., Church, M.K., Holgate, S.T. (1995). Heterogeneity of human mast cells based on cytokine content. J Immunol. 155: 297-307.
12. Carroll, N., Carello, S., Cooke, C., James, A. (1996). Airway structure and inflammatory cells in fatal attacks of asthma. Eur Respir J. 9: 709-715.
13. Comhair, S.A., Xu, W., Ghosh, S., Thunnissen, F.B.J.M., Almasan, A., Calhoun, W.J., Janocha, A.J., Zheng, L., Hazen, S.L., Erzurum, S.C. (2005). Superoxide dismutase inactivation in pathophysiology of asthmatic airway remodeling and reactivity. Am J Pathol. 166: 663-674.
14. Corrigan, C. J., & Kay, A. B. (1992). T cells and eosinophils in the pathogenesis of asthma. Immunol Today. 13: 501-507.
15. Cox, G. (1995). Glucocorticoid Treatment Inhibits Apoptosis in Human Neutrophils. J Immunol. 154: 4719-4725.
16. Coyle, A.J., Tsuyuki S., Bertrand C., Huang S., Aguet, M., Alkan S.S., Anderson, G.P. (1996). Mice lacking the IFN-γ receptor have impaired ability to resolve a lung

eosinophilic inflammatory response associated with a prolonged capacity of T cells to exhibit a Th2 cytokine profile. J Immunol. 156: 2680-2685.
17. Dahlen, B., Shute, J., Howarth, P. (1999). Immunohistochemical localization of the matrix metalloproteinases MMP-3 and MMP-9 within the airways in asthma. Thorax. 54: 590-596.
18. Dent, L.A., Strath, M., Mellor, A.L., Sanderson, C.J. (1990). Eosinophilia in transgenic mice expressing interleukin 5. J Exp Med. 172: 1425-1431.
19. Fahy, J.V., Kim, K.W., Liu, J., Boushey, H.A. (1995). Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. J Allergy Clin Immunol. 95: 843-852.
20. Finotto, S., Neurath, M.F., Glickman, J.N., Qin, S., Lehr, H.A., Green, F.H., Ackerman, K., Haley, K., Galle, P.R., Szabo, S.J., Drazen, J.M., De Sanctis, G.T., Glimcher, L.H. (2002). Development of spontaneous airway changes consistent with human asthma in ice lacking T-bet. Science. 295: 336-338.
21. Flood-Page, P.T., Menzies-Gow, A.N., Kay, A.B., Robinson, D.S. (2003a). Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. Am J Respir Crit Care Med. 167: 199-204.
22. Grunig, G., Corry, D.B., Leach, M.W., Seymour, B.W., Kurup, V.P., and Rennick, D.M. (1997). Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. J. Exp. Med. 185, 1089–1099.
23. Haldar, P., Brightling, C., Hargadon, B., Gupta, S., Monteiro, W., Sousa, A., Marshall, R.P., Bradding, P., Green, R.H., Wardlaw, A.J., Pavord, I.D. (2009). Mepolizumab and Exacerbations of Refractory Eosinophilic Asthma. N Engl J Med. 360: 973-984.
24. Hamid, Q., Azzawi, M., Ying, S., Moqbel, R., Wardlaw, A.J., Corrigan, C.J., Bradley, B., Durham, S.R., Collins, J.V., Jeffrey, P.K., Quint, D.J., Kay, A.B. (1991). Expression of mRNA for Interleukin-5 in Mucosal Bronchial Biopsies from Asthma. J Clin Invest. 87: 1541-1546.
25. Hammond, H. and Lambrecht, B.N. (2008). Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. Nat Rev Immunol. 8:193-304.
26. Hargreave, F.E. and Nair, P. (2009). The definition and diagnosis of asthma. Clin Exp Allergy. 39: 1652-1658.
27. Hartl, D., Koller, B., Mehlhorn, A.T., Reinhardt, D., Nicolai, T., Schendel, D.J., Griese, M., Krauss-Etschmann, S. (2007). Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. J Allergy Clin Immunol. 119: 1258-1266.
28. Haselden, B.M., Kay, A.B., Larché, M. (1999) Immunoglobulin E-independent Major Histocompatibility Complex-restricted T Cell Peptide Epitope-induced Late Asthmatic Reactions. J.Exp. Med. 189: 1885-1894.
29. Hashimoto, T., Akiyama, K., Kobayashi, N., Mori, A. (2005). Comparison of IL-17 production by helper T cells among atopic and nonatopic asthmatics and control subjects. Int Arch Allergy Immunol. 137(suppl 1): 51-54.
30. Holgate, S.T. (2008). Pathogenesis of Asthma. Clin and Exp Allergy. 38: 872-897.

31. Hoshino, H., Laan, M., Sjostrand, M., Lotvall, J., Skoogh, B.E., Linden, A. (2000). Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways in vivo. J Allergy Clin Immunol. 105: 143-149.
32. Humbert, M., Corrigan, C.J., Kimmitt, P., Till, S.J., Kay, A.B., Durham, S.R. (1997). Relationship between IL-4 and IL-5 mRNA Expression and Disease Severity in Atopic Asthma. Am J Respir Crit Care Med. 156: 704-708.
33. Humbert, M., Durham, S.R., Ying, S., Kimmitt, P., Barkans, J., Assoufi, B., Pfister, R., Menz, G., Robinson, D.S., Kay, A.B., Corrigan, C.J. (1996). IL-4 and IL-5 mRNA and protein in bronchial biopsies from atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathological disease. Am J Respir Crit Care Med. 154: 1497-1504.
34. Humbert, M., Menz, G., Ying, S., Corrigan, C.J., Robinson, D.S. Durham, S.R., Kay, B.A. (1999). The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. Immunol Today. 20: 528-533.
35. Humbles, A. A., Lloyd, C. M., McMillan, S. J., Friend, D. S., Xanthou, G., McKenna, E. E., Ghiran, S., Gerard, N.P., Yu, C., Orkin, S.H., Gerard, C. (2004) A critical role for eosinophils in airway remodeling. Science. 305: 1776-1779.
36. Ito, T., Wang, Y.H., Duramad, O., Hori, T., Delespesse, G.J., Watanabe, N., Qin, F.X., Yao, Z., Cao, W., Liu, Y. (2005). TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. J Exp Med. 202: 1213-1223.
37. Iwamoto, I., Nakajima, H., Endo, H., Yoshida, S. (1993). Interferon γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4+ T cells. J Exp Med. 177: 573-576.
38. Jatakanon, A., Uasuf, C., Maziak, W., Lim, S., Chung, K.F., Barnes, P.J. (1999). Neutrophilic Inflammation in Severe Persistent Asthma. Am J Respir Crit Care Med. 160: 1532-1539.
39. Janeway, C.A. *et al.* Immunobiology. 6th Edition. Garland Science (2005).
40. Jayaram, L., Pizzichini, M.M., Cook, R.J., Boulet, L., Lemiere, C., Pizzichini, E., Cartier, A., Hussack, P., Goldsmith, C.H., Laviolette, M., Parameswaran, K., Hargreave, F.E. (2006). Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations. Eur Resp J. 27: 483-494.
41. Jeannin, P., Lecoanet, S., Delneste, Y., Gauchat, J.F., Bonnefoy, J.Y. (1998). IgE versus IgG4 can be differently regulated by IL-10. J Immunol. 160: 3555-3561.
42. Kaur, D., Saunders, R., Berger, P., Siddiqui, S., Woodman, L., Wardlaw, A., Bradding, P., Brghtling, C.E. (2006). Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. Am J Respir Crit Care Med. 174: 1179-1188.
43. Kearley, J., Barker, J.E., Robinson, D.S., Lloyd, C.M. (2005). Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+ CD25+ regulatory T cells is interleukin 10 dependent. J Exp Med. 202: 1539-1547.
44. Lama, M., Chatterjee, M., Nayak, C.R., Chaudhuri, T.K. (2011). Increased interleukin-4 and decreased interferon-γ levels in serum of children with asthma. Cytokine. doi:10.1016/j.cyto.2011.05.011.

45. Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., Cua, D.J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. 201: 233-240.
46. Leckie, M.J., ten Brinke, A., Khan, J., Diamant, Z., O'Connor, B.J., Walls, C.M., Mathur, A.K., Cowley, H.C., Chung, K.F., Djukanovic, R., Hansel, T.T., Holgate, S.T., Sterk, P., Barnes, P.J. (2000). Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsivenes, and the late asthmatic response. The Lancet. 356: 2144-2148.
47. Lee, J.J., McGarry M.P., Farmer, S.C., Denzler, K.L., Larson, K.A., Carrigan, P.E., Brenneise, I.E., Horton, M.A., Haczku, A., Gelfand, E.W., Leikauf, G.D., Lee, N.A. (1997). Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. J Exp Med. 185: 2143-2156.
48. Lewkowich, I.P., Herman, N.S., Schleifer, K.W., Dance, M.P., Chen, B.L., Dienger, K.M., Sproles, A.A., Shah, J.S., Kohl, J., Belkaid, Y., Wills-Karp, M. (2005). $CD4^+CD25^+$ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. J Exp Med. 202: 1549-1561.
49. Lewkowich, I.P., Lajoie, S., Clark, J.R., Herman, N.S., Sproles, A.A., Wills-Karp, M. (2008) Allergen Uptake, Activation, and IL-23 Production by Pulmonary Myeloid DCs Drives Airway Hyperresponsiveness in Asthma-Susceptible Mice. PLos. 3: e3879.
50. Li, Y., Sun, M., Cheng, H., Li, S., Liu, L., Qiao, H., Hua, S., Lu, J. (2011). Silencing IL-23 expression by small hairpin RNA protects against asthma in mice. Exp Mol Med. 43: 197-204.
51. Ling, E.M., Smith, T., Nguyen, X.D., Pridgeon, C., Dallman, M., Arbery, J., Carr, V.A., Robinson, D.S. (2004). Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet. 363: 608-615.
52. Liu, Y.J. (2006). Thymic stromal lymphopoietin: master switch for allergic inflammation. J Exp Med. 203: 269-273.
53. Lloyd, C.M., Hawrylowicz, C.M. (2009). Regulatory T cells in asthma. Immunity. 31: 438-449.
54. McKinley, L., Alcorn, J.F., Peterson, A., Dupont, R.B., Kapadia, S., Kapadia, S., Logar, A., Henry, A., Irvin, C.G., Piganelli, J.D., Ray, A., Kolls, J.K. (2008). TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. J Immunol. 181: 4089-4097.
55. Molet, S., Hamid, Q., Davoine, D., Nutku, E., Taha, R., Pagé N., Olivenstein, R., Elias, J., Chakir, J. (2001) IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol. 108: 430-438.
56. Mori, A. *et al*. Presented at World Allergy Organization. 2005.
57. Mori, A., Ikeda, Y., Taniguchi, M., Aoyama, C., Maeda, Y., Hasegawa, M., Kobayashi, N., Akiyama, K. (2001) IL-5 Production by Peripheral Blood Th Cells of Adult Asthma Patients in Response to *Candida albicans* Allergen. Int Arch Allergy Immunol. 125 (suppl 1): 48-50.

58. Mori, A., Kaminuma, O. Suko, M., Mikami, T., Nishizaki, Y., Ohmura, T., Hoshino, A., Asakura, Y., Miyazawa, K., Ando, T., Okumura, Y., Yamamoto, K., Ojudaira, H. (1997). Cellular and molecular mechanisms of IL-5 synthesis in atopic disease: A study with allergen-specific human helper T cells. J Allergy Clin Immunol. 100: S56-64.
59. Murphy, D.M. and O'Byrne, P.M. (2010). Recent Advances in the Pathophysiology of Asthma. Chest. 137: 1417-1426.
60. Nair, P., Pizzichini, M.M.M., Kjarsgaard, M., Inman, M.D., Efthimiadis, A., Pizzichini, E., Hargreave, F.E., O'Byrne, P.M. (2009). Mepolizumab for Prednisone-Dependent Asthma with Sputum Eosinophilia. N Engl J Med. 360: 985-993.
61. Nakajima, H., Hirose, K. (2010) Role of IL-23 and Th17 Cells in Airway Inflammation in Asthma. Immune Network. 10: 1-4.
62. Nakajima, H. and Takatsu, K. (2007). Role of Cytokines in Allergic Airway Inflammation. Int Arch Allergy Immunology. 142: 265-273.
63. Nembrini, C., Marsland, B.J., Kopf, M.K. (2009). IL-17-producing T cells in lung immunity and inflammation. J Allergy Clin Immunol. 123: 986-994.
64. Ogawa, Y., Clhoun, W.J. (2006). The role of leukotrienes in airway inflammation. J Allergy Clin Immunol. 118: 789-798.
65. Ordonez, C.L., Shaughnessy, T.E., Matthay, M.A., Fahy, J.V. (2000). Increased Neutrophil Numbers and IL-8 Levels in Airway Secretions in Acute Severe Asthma. Am J Respir Crit Care Med. 161: 1185-1190.
66. Peng, J., Yang, X.O., Chang, S.H., Yang, J., Dong, C. (2010) IL-23 signaling enhances Th2 polarization and regulates allergic airway inflammation. Cell Research. 20: 62-71.
67. Plante, S., Semlali, A., Joubert, P., Bissonnette, E., Laviolette, M., Hamid, Q., Chakir, J. (2006). Mast cells regulate procollagen I (alpha 1) production by bronchial fibroblasts derived from subjects with asthma through IL-4/IL-4 delta 2 ratio. J Allergy Clin Immunol. 117: 1321-1327.
68. Robinson D.S., Hammond, Q., Ying S., Tsicopoulos A., Barkans, J., Bentley, A.M., Corrigan, C., Durham, S.R., Kay, A.B. (1992). Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med. 326: 298-304.
69. Robinson, D.S. (2010). The role of the T cell in asthma. J Allergy Clin Immunol. 126: 1081-1091.
70. Robinson, D.S., North, J., Zeibecoglou, K. (1999). Eosinophil development and bone marrow and tissue eosinophils in atopic asthma. Int Arch Allergy Immunol. 118: 98-100.
71. Scadding, J.G. (1963). Meaning of Diagnostic Terms in Broncho-Pulmonary Disease. BMJ. 1425-1430.
72. Shaw, D.E., Berry, M.A., Hardadon, B., McKenna, S., Shelley, M.J., Green, R.H., Brightling, C.E., Wardlaw, A.J., Pavord, I.D. (2007). Chest. 132: 1871-1875.
73. Takjar, P., Corrigan, C.J., Smurthwaite, L., O'Connor, B.J., Durham, S.R., Lee, T.H., Gould, H.J. (2007). Class switch recombination to IgE in the bronchial mucosa of atopic and nonatopic patients with asthma. J Allergy Clin Immunol. 119: 213-218.

74. Tosca, M., Silvestri, M., Morandi, F., Prigione, I., Pistorio, A., Ciprandi, G., Rossi, G.A. (2011). Impairment of lung function might be related to IL-10 and IFN-γ defective production in allergic children. Immunol Let. doi:10.1016/j.imlet.2011.05.004.
75. Van Oosterhout, A.J., Ladenius, A.R., Savelkoul, H.F., Van Ark, I., Delsman, K.C., Nijkamp, F.P. (1993). Effect of anti-IL-5 and IL-5 on airway hyperreactivity and eosinophils in guinea pigs. Am Rev Respir Dis. 147: 548-552.
76. Wakashin, H., Hirose, K., Maezawa, Y., Kagami, S., Suto, A., Watanabe, N., Saito, Y., Hatano, M., Tokuhisa, T., Iwakura, Y., Puccetti, P., Iwamoto, I. (2008) IL-23 and Th17 Cells Enhance Th2-Cell-mediated Eosinophilic Airway Inflammation in Mice. Am J Respir Crit Care Med. 78: 1023-1032.
77. Wang, Y.H., Ito, T., Wang, Y.H., Homey, B., Watanabe, N., Martin, R., Barnes, C.J., McIntyre, B.W., Gilliet, M., Kumar, R., Yao, Z., Liu, Y.J. (2006). Maintenance and polarization of human TH2 central memory T cells by the thymic stromal lymphopoietin-activated dendritic cells. Immunity. 24: 827-838.
78. Wang, Y.H. and Liu Y.J. (2008). The IL-17 cytokine family and their role in allergic inflammation. Curr Opin Immunol. 20:697-702.
79. Wark, P.A., Johnston, S.L., Bucchieri, F., Powell, R., Puddicombe, S., Laza-Stanca, V., Holgate, S.T., Davies, D.E. (2005). Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. J Exp Med. 201: 937-947.
80. Wong, C.K., Ho, C.Y., Ko, F.W.S., Chan, C.H.S., Ho, A.S.S., Hui, D.S.C., Lam, C.W.K. (2001). Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-γ, IL-4, IL-10 and IL-13) in patients with allergic asthma. Clin Exp Immunol. 125: 177-183.
81. Wong, C.K., Lun, S.W., Ko, F.W., Wong, P.T., Hu, S.Q., Chan, I.H., Jui, D.S., Lam, C.W. (2009). Activation of peripheral Th17 lymphocytes in patients with asthma. Immunol Invest. 38: 652-664.
82. Zhao, Y., Yang, J., Gao, Y., Guo, W. (2010) Th17 Immunity in Patients with Allergic Asthma. Int Arch Allergy Immunol. 151: 297-307.
83. Zhao, Y., Yang, J., Gao, Y. (2011). Altered Expression of Helper T cell (Th)1, Th2, and Th17 Cytokines in CD8(+) and γδ T Cells in Patients with Allergic Asthma. J Asthma. 48: 429-436.