Comparison of Immune Reactivity to Respiratory Challenges in Asthmatics with and without Panic Disorder

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This is to certify that the thesis prepared

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THESIS ABSTRACT

Comparison of Immune Reactivity to Respiratory Challenges in Asthmatics with and without Panic Disorder

Alexandre Elhalwi

Objective
Asthma and panic disorder (PD) are highly comorbid conditions. The objective of this study was to examine if PD altered immune reactivity in asthmatics to two acute respiratory stress challenges. We hypothesized that asthmatics with PD would have increased proportions of sputum eosinophils compared to asthmatics without PD in reaction to both challenges.

Methods
Eleven participants (7 PD, 4 non-PD) inhaled methacholine (which produces an asthma attack) on a first day, and on a second, two gases in randomized order: compressed air and a 35% carbon dioxide (CO₂) solution (the latter produces a ‘simulated’ panic attack). Following each challenge, we induced sputum to assess immune cell profiles.

Results
ANCOVA-like GLMs demonstrated that the PD group had a significantly lower proportion of sputum lymphocytes (β=-0.75, 95% CIs = -1.30–0.20) than the non-PD group in response to methacholine. A trend also emerged for the PD group reacting with more eosinophils (β=5.03, 95% CIs = -0.73–10.79). The presence of PD conferred no effect on neutrophils (β=-11.72, 95% CIs = -34.64–11.18) or macrophages (β=0.10, 95% CIs = -22.63–22.82). Analyses did not reveal a significant effect of PD on immune reactivity to CO₂.

Conclusions
PD appears to influence immunological responses in asthmatics by decreasing the proportion of sputum lymphocytes following a methacholine challenge, but does not seem to alter the immunological responses to CO₂ inhalation. Additional studies are indicated to characterize the immunological interrelations between these conditions; these discoveries could allow clinicians to select more targeted treatments for this population.
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First and most importantly, I would like to thank my supervisor, Dr. Simon Bacon, whose guidance and attention over the past four years has been invaluable. Under your supervision, I have grown and developed myself, and your support has allowed me to aim high and to achieve. I have learned tremendously from you, and hope to continue learning from you in the future. Thank you for being an exceptional supervisor!

I would also like to thank the other members of my thesis committee, Dr. Peter Darlington and Dr. Kim Lavoie, with whom I have had many stimulating conversations and who have provided countless insights from their respective fields of expertise for this project.

I would like to recognize the Canadian Institutes of Health Research (CIHR), Concordia University, and the Montreal Behavioural Medicine Centre for supporting me financially through this project with Master’s training scholarships.

I would like to recognize the contributions of my colleagues at the Hôpital du Sacré-Coeur de Montréal: Guillaume, whose familiarity and exceptional capability as lab manager has facilitated my work on this project. Thank you for never making me feel like my questions and requests were bothersome, despite your overwhelming workload. Maxine, with whom I have worked for over two years on this project, thank you for building up this project, for taking the time to mentor me, and for making me feel like my efforts were both valuable and appreciated. It has been a pleasure to work on this project with you!

A special thank you also to Mrs. Jocelyne L’Archevêque and Mrs. Carole Trudeau, who regularly made themselves available to answer countless questions, who readily made themselves available to see our study participants, and without whom testing would have been impossible.

Finally, to my sister and to my mom, your unconditional love and support has carried me through this endeavour and carries me through all others. Thank you!
PREAMBLE

This study, which was a sub-study of a larger study looking to investigate the impact of stress on cardiac and bronchial reactivity in asthmatics, sought to explore the nature of immune reactivity to stress in asthma. This document consists of three parts:

1) Review of the Literature
2) Manuscript prepared for submission to *Psychosomatic Medicine*
3) Appendices

The document has been prepared for submission to *Psychosomatic Medicine* primarily due to the appropriateness of the content for the journal. In addition, our laboratory has submitted articles to this journal and has consistently had them published. Finally, I have also had a good deal of success personally with submitting abstracts to the organization of the journal’s international conference: of the two abstracts I submitted to the American Psychosomatic Society’s Annual International Conference, one was accepted to be presented as a part of an oral symposium, and the second was accepted to be presented at a poster session.

Though *Psychosomatic Medicine* requires a numbered referencing style and page numbers that begin at the start of the manuscript, this document will have numbering start on this page and will have the first reference in the Review of the Literature; a single aggregated list of references will be found at the end of the manuscript, rather than having a different list of references for each section.

All authors participated in the development of the protocol. I generated the main idea for this project, am the main author of the text, developed the database, and assisted with data collection, entry, processing, and analysis. Patient recruitment was conducted by myself and Maxine Boudreau. She, as well as Drs. Kim L. Lavoie and Simon L. Bacon, conceptualized the main study that this present study is a sub-study of, regularly helped me with this project, and were available to take questions and for consultation. Dr. Simon L. Bacon also carried out the statistical analyses used in this article.

With regards to the protocol itself, the methacholine challenge was administered by a laboratory technician employed by the Hôpital du Sacré-Coeur de Montréal who had over 20 years of experience conducting this test, while physiological data and questionnaire data were initially recorded by Maxine Boudreau, and then by me. On the second day of tests, the panic
induction protocol was executed by three individuals: one laboratory technician who handled the metabolic cart and ventilatory maneuvers, Maxine Boudreau administered psychological questionnaires and determined if the participant had experienced a panic attack, and I manipulated the mask, oversaw the data collection by certain pieces of equipment, and administered the gas mixtures. Collecting sputum samples on all three days as well as the cell count analyses were handled by a laboratory technician employed by the hospital who had over 20 years of experience.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADIS-IV</td>
<td>Anxiety Disorders Interview Schedule-IV</td>
</tr>
<tr>
<td>API</td>
<td>Acute Panic Inventory</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in one second</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>HSCM</td>
<td>Hôpital du Sacré-Coeur de Montréal</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC₂₀</td>
<td>Concentration of methacholine required to cause a 20% drop in forced expiratory volume in one second</td>
</tr>
<tr>
<td>PD</td>
<td>Panic disorder</td>
</tr>
<tr>
<td>PRIME-MD</td>
<td>Primary Care Evaluation of Mental Disorders</td>
</tr>
<tr>
<td>Th₁</td>
<td>T helper type 1</td>
</tr>
<tr>
<td>Th₂</td>
<td>T helper type 2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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Asthma

Asthma is a complex respiratory disorder, characterized by reversible and intermittent airway obstruction, inflammation, and hyper-reactivity, that affects over 8% of Canadians over the age of 12 (1). Airway obstruction in asthma is the result of two overlapping processes (2): first, inflammation in the airways causes the formation of mucus, producing a physical obstruction in the airway lumen, and second, if inflammation remains untreated, the smooth muscles that surround the airway become sensitive and contract; this is called “bronchoconstriction.” Aside from also contributing to airway narrowing, bronchoconstriction also produces the common symptoms of asthma: shortness of breath, wheezing, bouts of coughing, and feelings of tightness in the chest (3).

While it typically develops in childhood and progresses into adulthood, asthma can develop at any age (4) and its symptoms can vary dramatically between exacerbations or from one person to another (2). Several factors, such as genetics (5) and exposure to occupational allergens (6), have been shown to play a role in the development of asthma, but the number of asthma phenotypes makes the outlining of exact pathogenic mechanisms difficult (3). Asthma is, however, recognized as an immunological disease, and efforts have been made to identify the interactions between leukocytes and cytokines that modulate the disease process in asthma (7).

From an immunological point of view, cytokines are recognized as playing a critical role in the chronic inflammation process of asthma (8). In mild to moderate asthma, a T helper type 2 cell (Th2) cytokine profile dominates over a Th1 profile (9); the Th2 cytokines (such as interleukin(IL)-4 and IL-13) reciprocally inhibit the ability of Th1 cells to produce their own cytokines (including interferon(IFN)-γ and IL-12). Among the Th2 cytokines, eotaxin and IL-5 serve as two important chemoattractants for eosinophils, which promote the infiltration of eosinophils into the lung and contribute to their maturation; lung eosinophilia is one of the hallmarks of allergic asthma (10). Once in the lung, eosinophils become resilient to apoptosis (10), and begin releasing their cytotoxic granules, including proteins such as eosinophilic cationic protein and eosinophil peroxidase (11), some of which contribute to the destruction of the airway epithelium that is typical in asthma (12). In addition, eosinophils release Th2-promoting and Th1-suppressing cytokines and chemokines, such as IL-5 and monocyte...
chemotactic protein-1 (7, 13), thus further propagating inflammation in a positive feedback loop style. Unfortunately, due to the nature of cytokines serving many functions and having many different effects, identifying their involvement in asthma’s pathophysiology may be insufficient, but is nonetheless an important step in understanding the disease’s mechanisms.

Despite the advancements in outlining pathogenic processes in asthma and the development of a multiplicity of asthma medications, asthma control continues to be a problem in Canada. Data from the 2003 Canadian Community Health Survey indicated that more than half of affected Canadians reported having had an asthma attack or having been affected in their daily activities by asthma symptoms in the past 12 months, despite over 65% of these people reporting taking medication (14). More recently, a study noted that 82% of 418 patients who had controlled asthma had times in the previous year where their symptoms became worse. The study also cited that there had been no improvement in asthma control among Canadians since 1999 (15). These figures suggest that factors other than pharmacological treatment may be at play.

*Psychological Factors in Asthma*

Recently, researchers have turned to exploring the realm of health psychology in an effort to explain the continued inability of many asthmatics to keep their asthma under control. Psychiatric disorders are overrepresented in asthmatic populations (16-21). In addition, psychological factors such as stress and anxiety can have a significant influence on respiratory functioning, and have been linked to worse outcomes in asthmatics: anxiety disorders have a negative effect on asthma-related quality of life (22, 23) and asthma control (24). Since the majority of studies assessing the link between psychological factors and asthma have been correlational and limited to examining primarily self-reported outcomes (e.g., asthma control and quality of life), the mechanisms driving the relationship between anxiety and worse asthma remain elusive. In non-asthma patients there are a number of studies which depict a model where an interaction between acute stress and chronic stress mediates immunological pathways (25). Despite the limited literature, the model appears to hold true in the context of asthma, as well (26-28). Physiologically, acute stress is thought to trigger immune reactivity in individuals to protect them during this period of stress. Meanwhile, chronic stress, which has been shown to suppress Th1 immunity, may be driving and amplifying inflammatory reactions in individuals affected by inflammatory conditions such as asthma, where a Th2 immune profile exists (25).
The model would suggest that the occurrence of an acute stress may exacerbate the chronic stress-amplified immune processes present in asthma, thus worsening the condition or producing negative effects. Despite the emergence of this model, a recent review had a very small pool of research to draw from when discussing the immune system’s role in asthma and different types of psychological distress (29). Given that anxiety is pervasive in asthma (some studies report that up to 52% of asthmatics suffer from at least one anxiety disorder (17, 20, 22)), an anxiety disorder which entails both chronic stress and bouts of acute stress would be well-suited in the investigation of this paradigm within the context of asthma.

Recently, the association between asthma outcomes and anxiety sensitivity, a type of trait anxiety defined as fear of physical and psychological anxiety symptoms (30), has been examined. Studies found that patients with more elevated fear of physical symptoms also had worse asthma control and quality of life (31, 32). Interestingly, anxiety sensitivity was, for a cohort of university students, found to be the strongest predictor of the development of panic symptoms and panic attacks (33).

Panic Disorder

Panic disorder (PD) is a recognized anxiety disorder in the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) that is characterized by sudden, unprovoked, and recurrent panic attacks (34), which are episodes of intense fear that are associated with a number of cognitive and physical symptoms. For a list of diagnostic criteria for PD from the DSM-IV, see Appendix A.

PD is of particular interest in asthma: many studies report that PD is quite common in asthma, with one study reporting that 13.9% of their cohort of asthmatic patients had panic disorder (17); in contrast, 1.5% of the regular population is estimated to have PD (35). Like other anxiety disorders, PD also appears to be associated with worse outcomes in asthma. A study that evaluated the last 12 months of a group of asthmatic patients’ medical charts found that the presence of PD resulted in a significantly lower self-reported quality of life, a significantly greater use of short-acting asthma medication compared to the asthma only group, and significantly more visits to primary care physicians (36). PD may influence asthma negatively by virtue of panic attacks having many similar symptoms to asthma attacks (shortness of breath, sudden anxiety, sensations of being smothered, and fear of losing control); this overlap and the
catastrophization of somatic symptoms by PD patients leads them (and medical professionals) to occasionally misinterpret the nature of the crisis, which may delay appropriate treatment; however, it is also possible that panic attacks may trigger asthma attacks through physiological pathways (37). In fact, the unanticipated panic attacks that occur within the context of PD makes it fit well into our current acute and chronic stress model: could panic attacks (i.e., an acute stressor) make asthma worse through amplified immune reactivity? While Feldman et al. found that the presence of PD seemed to confer no effect on asthma severity, asthma severity was evaluated using spirometry, self-report asthma symptoms, and self-reported medication use rather than through an examination of the patients’ immunological profiles. Given that bronchoconstriction is the result of both immunological and parasympathetic nervous system components (38, 39), these findings may not truly reflect the influence of PD on immunological functioning in asthma.

There exists scant literature on immunological abnormalities in PD, and results are contradictory. One study in non-asthma individuals by Brambilla found that PD patients had similar levels of tumor necrosis factor(TNF)-α to age- and sex-matched healthy controls (40). Another study reported that PD patients had lower IFN-γ and IL-12 as compared to healthy subjects (41). Given that Th1 cytokines appear to be suppressed by Th2 cytokines, we may be able to infer through these findings that PD may tend to favour a Th2 immune profile, similar to that which exists in asthma. Findings do not seem to be consistent across studies, however: another study found that elevated levels of IFN-γ were detectable in the serum of 75% of PD patients as compared to only 35% of age- and sex-matched healthy controls (42). This last study also had some potentially inconsistent findings: IL-4 was more detectable while IL-10 was less detectable in PD patients. Since both are Th2 cytokines, one might have anticipated that both would be more (or less) detectable together. Of note, the study also reported that eotaxin was significantly more detectable in patients with PD. Unfortunately, we know of no studies in which immunity in PD has been investigated within the context of asthma, but the abundance of Th2 cytokine levels which are seemingly disrupted in PD are enough to suggest that the effects of PD may have an influence on the pathological processes in asthma.
Unanticipated panic attacks are the hallmark of PD; as such, researchers interested in the physiological effects of PD would seek to investigate them. Studying PD-related panic attacks in a research setting is impractical given their unanticipated nature, however. To study them, researchers have sought and determined a number of ways to induce panic. Currently, several challenges and procedures exist which reliably induce a “simulated panic attack,” including lactate infusion (43), caffeine ingestion (44), breath-holding (45), hyperventilation (46), cholecystokinin tetrapeptide injection (47), and carbon dioxide (CO₂) inhalation.

The inhalation of air containing more than 5% CO₂ induces panic and anxiety in both healthy controls and in PD patients, though the effects have been shown to be more pronounced in the latter group (48, 49). CO₂ has been shown to be one of the most panicogenic agents, being a superior stimulus for panic to both caffeine ingestion (50) and hyperventilation of room air (51). In addition, panic induced by CO₂ inhalation is similar to naturally occurring panic (52, 53). Panic disorder patients are thought to be particularly susceptible to the inhalation of carbon dioxide as these individuals are postulated to have hypersensitive CO₂ chemoreceptors, leading them to react to otherwise harmless concentrations of carbon dioxide. This “suffocation false alarm” hypothesis, first described by Klein (54), explains that the “excessive” CO₂ triggers hyperventilation in an effort to eliminate CO₂ from the body.

Two main panic-inducing CO₂ inhalation protocols are currently used in panic research. One involves the continuous breathing of 5% or 7% CO₂ gas mixtures for a period of time until patients are overwhelmed with panic (which usually occurs within minutes (55)). The other involves a single vital capacity inhalation of a 35% CO₂ and 65% oxygen gas mixture, which is held for a few seconds before being exhaled (53). Though both protocols have been shown to reliably induce panic anxiety, it seems that no study has directly examined the efficacy of one method over the other. One study compared the two continuous breathing CO₂ mixtures and found that each had its advantages: the inhalation of the 7% CO₂ solution allowed clinicians to more accurately perceive the occurrence of a panic attack, but the inhalation of the 5% CO₂ solution was better resisted by the patients; since the patients could last longer before being overcome with panic, the researchers could collect more physiological data (51). Another study had healthy participants inhale increasing concentrations of CO₂ gas (0, 9, 17.5, and 35%), and found that participants reported higher anxiety and panic symptoms in a dose-dependent manner.
These studies illustrate that inhaling higher concentrations of CO$_2$ gas will be more likely to cause a panic attack.

Only one study was found to have evaluated immune markers in PD patients before and after a simulated panic attack induced by 35% CO$_2$ inhalation. The study by van Duinen and colleagues reported that, while induced panic attacks caused significantly higher levels of anxiety in the PD patients as anticipated, the CO$_2$ challenge did not cause any significant changes in the serum immune markers that were measured (IL-6, IL-8, IL-10, IL-1 receptor agonist, IFN-$\gamma$, TNF-$\alpha$, soluble IL-6 receptor, and soluble IL-2 receptor) (57). Interestingly, the study reported that there were no immunological differences at baseline between the PD patients and the healthy controls, which contrasts the findings of both Tukel et al. (41) and Hoge et al. (42), who all found that PD patients had different levels of many cytokines than healthy participants. Furthermore, given that the study by van Duinen et al. was neither carried out with asthmatics, nor did it examine key cells and mediators involved in the pathophysiology of asthma, one might wonder how applicable these findings are to an asthmatic population, and within the acute stress/chronic stress model altogether.

The available data addressing the physiological mechanisms of how psychological distress negatively influences asthma is lacking and, in many cases, non-specific; the conclusions that can be drawn from the literature currently are speculative at best. Given the lack of data on the subject and the large potential for more specific treatment strategies, it becomes clear that a closer look at how psychological distress, specifically the presence of PD, can worsen outcomes in asthma is needed, with immune reactivity being a particular mechanism of interest.
Comparison of Immune Reactivity to Respiratory Challenges in Asthmatics with and without Panic Disorder

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MANUSCRIPT ABSTRACT

Objective
Asthma and panic disorder (PD) are highly comorbid conditions. The objective of this study was to examine if PD altered immune reactivity in asthmatics to two acute respiratory stress challenges. We hypothesized that asthmatics with PD would have increased proportions of sputum eosinophils compared to asthmatics without PD in reaction to both challenges.

Methods
Eleven participants (7 PD, 4 non-PD) inhaled methacholine (which produces an asthma attack) on a first day, and on a second, two gases in randomized order: compressed air and a 35% carbon dioxide (CO₂) solution (the latter produces a ‘simulated’ panic attack). Following each challenge, we induced sputum to assess immune cell profiles.

Results
ANCOVA-like GLMs demonstrated that the PD group had a significantly lower proportion of sputum lymphocytes (β=-0.75, 95% CIs = -1.30–0.20) than the non-PD group in response to methacholine. A trend also emerged for the PD group reacting with more eosinophils (β=5.03, 95% CIs = -0.73–10.79). The presence of PD conferred no effect on neutrophils (β=-11.72, 95% CIs = -34.64–11.18) or macrophages (β=0.10, 95% CIs = -22.63–22.82). Analyses did not reveal a significant effect of PD on immune reactivity to CO₂.

Conclusions
PD appears to influence immunological responses in asthmatics by decreasing the proportion of sputum lymphocytes following a methacholine challenge, but does not seem to alter the immunological responses to CO₂ inhalation. Additional studies are indicated to characterize the immunological interrelations between these conditions; these discoveries could allow clinicians to select more targeted treatments for this population.

Keywords (6): Panic disorder, asthma, eosinophil, lymphocyte, stress, panic induction
INTRODUCTION

Asthma is a complex respiratory disease characterized by reversible and intermittent airway narrowing, obstruction, and hyper-reactivity. Despite the advancements in the understanding and treatment of asthma, the disease affects over 8% of Canadians aged 12 and above (1) and many patients struggle to control their asthma symptoms (15). More than half of Canadian asthmatics reported having had an asthma attack or having been affected in their daily activities by asthma symptoms in the past 12 months, despite over 65% of these people reporting taking medication (14).

Airway obstruction in asthma is the result of inflammation (2) which, when untreated, causes the smooth muscles that surround the airway to become sensitive and contract (bronchoconstriction). Asthma is an immunological disease and the chronicity of asthma is believed to be associated with an altered humoral immune system response. Signaling molecules known as cytokines play an important role in orchestrating, perpetuating, and amplifying inflammation in asthma (58, 59). More specifically, the cytokines interleukin(IL)-5 and eotaxin recruit (58), strengthen (7, 10), and activate (60) eosinophils, the white blood cells (leukocytes) implicated in allergic asthma (7, 58) whose excessive presence in the lung are considered one of the hallmarks of allergic asthma (10). Eosinophils release their cytotoxic granules (11), which contribute to the destruction of the airway epithelium that is typical in asthma (12). In addition, eosinophils release T helper type 2 (Th2)-promoting and Th1-suppressing cytokines, such as IL-5 (7, 13), thus further propagating inflammation in a positive feedback loop style.

Stress and anxiety can have a significant negative influence on respiratory functioning and quality of life in individuals with asthma (22, 24, 36), and psychiatric disorders are overrepresented in the asthmatic population (16-21). Psychological factors may be exerting a negative effect through already-existing immunological processes in asthma. The few studies available in the asthma literature depict a model where an interaction between acute stress and chronic stress mediates immunological pathways (25, 26, 28, 61). Physiologically, acute stress activates the immune system in individuals to protect them during this period of stress. Meanwhile, chronic stress, while typically associated to immunosuppression, may be instead
driving and amplifying inflammation in individuals affected by inflammatory conditions such as asthma (25). The model would suggest that the occurrence of an acute stress may aggravate the already existing inflammation-amplifying processes associated with chronic stress; in other words, sudden anxiety may exacerbate the already-existing immune responses present in asthma, thus worsening the condition or producing negative effects. Despite the emergence of this model, very little research discussing the immune system’s role in asthma and psychological distress is available (29).

Panic disorder (PD) is of particular interest in asthma: studies report that PD is up to 12 times more prevalent in asthmatics than in the general population (19, 35), and is associated with worse outcomes in asthma, including a lower quality of life, a greater use of short-acting asthma medication, and more visits to primary care physicians (36). PD is characterized by sudden, unprovoked, and recurrent panic attacks (34), which are episodes of intense fear that are associated with a number of cognitive and physical symptoms. PD may influence asthma negatively by virtue of panic attacks having many similar symptoms to asthma attacks (shortness of breath, sudden anxiety, sensations of being smothered, and fear of losing control); this overlap leads patients and medical professionals to occasionally misinterpret the nature of the crisis, which may delay appropriate treatment. Panic attacks could, however, also be triggering asthma attacks through physiological pathways (37). In fact, the unanticipated panic attacks that occur within the context of PD make it fit well into our current acute and chronic stress model: could panic attacks (i.e.: an acute stressor) make asthma worse through amplified immune reactivity brought on by an anxiety disorder such as PD (i.e., a chronic stress)?

This line of reasoning warrants an examination of the PD-related immunology research. Unfortunately, immunity in PD has not been investigated within the context of asthma, and only scant, contradictory literature is available on immunological abnormalities in PD for non-asthmatics (40-42). It appears nonetheless that both the presence of PD and panic attacks are associated with poorer immune profiles. One study reported that PD patients had lower Th1 cytokines(41). Given the antagonistic relationship between Th1 and Th2 products, these results would suggest that PD patients may tend to have a Th2 immune profile (similar to that which exists in asthma); this would entail the elevated presence of IL-5, which propagates the presence of eosinophils in the asthmatic lung. Furthermore, another study also reported that eotaxin, one of the principal recruiters of eosinophils into the lung in asthma, was significantly more
detectable at rest in patients with PD (42) than in healthy controls. Though these studies report blood serum markers, the findings are enough to suggest that the effects of PD may have an influence on the pathological processes in asthma, and that this possible influence is worthy of preliminary investigation using methods that are more pertinent in the context of asthma.

The objective of this study was to collect pilot data to examine whether PD can alter an asthmatic individual’s immunological reactivity during relevant acute stressors. More specifically, we were interested in knowing 1) Do participants with asthma and PD have a different immunological response to a methacholine challenge (asthma attack simulation) compared to asthmatics without PD, and 2) do participants with asthma and PD have a different immunological reaction to a simulated panic attack compared to asthmatics without PD? Following the acute and chronic stress model cited above, we anticipated that the occurrence of an acute stressor (asthma attack or panic attack) in the presence of a chronic stressor (PD) would exacerbate the immune processes present in a chronic inflammatory disease (asthma). Given the role of eosinophils in propagating asthma, we hypothesized that, compared to asthmatics without PD, those with PD would have increased sputum eosinophil proportions in reaction to both challenges.

METHODS
Study Procedures

This study was a component of a larger study seeking to investigate the impact of stress on cardiac and bronchial reactivity in asthmatics. The data collection took place over three separate visits. There were on average 27 days between visits, and visits were at least five days and at most 77 days apart. For a flowchart of the testing protocol (described in greater detail below), see Figure 1. The extended methodology is available in Appendix B.

Recruitment

Eleven asthmatic patients were recruited from the Hôpital du Sacré-Coeur de Montréal (HSCM). Participants were eligible to participate in the study if they were 18 years of age or older, had an objectively confirmed physician-diagnosis of asthma, if they spoke English or French, if they were current non-smokers, and if they were not suffering from a more severe comorbid condition such as cancer or cardiovascular disease. Participants completed the Primary
Care Evaluation of Mental Disorders to screen for the presence or absence of PD. Prior to the first visit, eligible patients underwent a semi-structured psychiatric interview called the Anxiety Disorders Interview Schedule-IV (ADIS-IV) (62-65), administered by a clinical psychology doctoral student over the phone, to confirm the presence or absence of PD and other comorbid psychiatric disorders. Participants who had a primary PD according to the ADIS-IV were included in the PD group, while those without any history of psychiatric disorders were included in the control (non-PD) group.

**Testing Protocol**

This project was approved by the Human Ethics Committee at the HSCM. Patients coming in for the first visit signed a consent form. Consenting participants completed sociodemographic and medical history questionnaires, then underwent a methacholine inhalation challenge; this test is the diagnostic test for asthma which causes an asthma-like attack by inducing bronchoconstriction, and is used to classify asthma severity (i.e. a patient experiencing a 20% drop in forced expiratory volume in one second [FEV₁] to lower doses of methacholine have more severe asthma). At the end of the test, participants were then given salbutamol (Ventolin) to reverse the airway narrowing, and then underwent an induced sputum test 15 minutes later to collect immunological data (leukocytes). On the second visit, participants underwent a 35% carbon dioxide (CO₂) inhalation challenge, which reliably induces a simulated panic attack. Following this challenge, participants once again underwent a sputum induction, waiting a total of one hour between the first onset of symptoms following an inhalation and the start of the sputum induction. Participants were given the option to participate on a third and final day of testing, where they underwent only an induced sputum test. The data collected on this day served as baseline data. See Figure 1 for a schematic of the protocol.

**Assessment**

**Methacholine Challenge**

All spirometric tests (forced vital capacity, FEV₁) were conducted following the American Thoracic Society guidelines (61). Participants inhaled increasing quantities of methacholine (0.0 – 16mg/mL). The test ended when participants experienced a 20% drop in FEV₁ in response to the methacholine inhalation, and participants were included if they had a 20% drop in FEV₁ in
reaction to a dose of methacholine ≤ 16mg/mL (61). We used DSM-IV criteria and the Acute Panic Inventory (API) (66) to determine if participants had a panic attack during the challenge.

**CO\textsubscript{2} Inhalation Challenge**

The use of a single vital capacity inhalation of a 35% CO\textsubscript{2} and 65% oxygen gas mixture, which is held for a few seconds before being exhaled (53), reliably induces a simulated panic attack, allowing researchers to circumvent the difficulty of studying unanticipated PD-related panic attacks. Inhaling CO\textsubscript{2}-rich air reliably induces panic attacks in about 80% of PD patients and 15% of controls (49, 67, 68). Compared to other panic induction challenges, panic induced by CO\textsubscript{2} inhalation is also similar to naturally occurring panic (52, 53) and CO\textsubscript{2} has been shown to be one of the strongest panicogenic agents (50, 51).
For this challenge, participants inhaled either one vital capacity inhalation of regular air (placebo) or one vital capacity of oxygen-balanced CO₂-rich air (delivered in randomized order). After a 30 minute period of seated rest to allow the participants’ respiratory and cardiac measures to return to baseline, participants inhaled a vital capacity of the other gas. Both the participants and the researcher conducting the panic attack assessment following both inhalations were blind as to which gas was being administered, and we used DSM-IV criteria and the API (66) to determine if participants had a panic attack.

Sputum Induction and Processing

Sputum inductions were conducted at the end of each challenge day and followed the hypertonic saline inhalation procedure detailed by Pin et al. (69). Before starting the procedure, participants inhaled 200μg salbutamol to prevent any bronchoconstriction that might result from the sputum induction process. After the samples were collected in a sterile container, the expectorate was analysed for proportions of neutrophils, eosinophils, monocytes/macrophages, lymphocytes, and bronchial epithelial cells present in the sputum. Sputum was processed using standard clinical practices (70), then stained using Wright’s stain, which allowed the visualization of the leukocytes of interest. Relative levels of leukocytes were generated as percentages following cell counts performed on 400 non-squamous cells, with the exception of one methacholine day sample from the PD group and one CO₂ day sample from the non-PD group, which had cell counts of 300 and 344, respectively. Samples had a cell viability greater than 50% and squamous cell contamination less than 20%.

Data Management and Statistical Analyses

We used an ANCOVA-based general linear model to evaluate whether group (PD, no PD) was associated with different methacholine responses for relative levels of sputum neutrophils, eosinophils, macrophages, and lymphocytes. For our second analysis, the same analytical plan was used replacing methacholine responses with simulated panic responses. All analyses included age, sex, inhaled corticosteroid (ICS) medication dose, and the baseline level (visit 3) of the dependent variable as a-priori defined covariates due to their influence on the outcomes. The β value for these statistical tests represents the slope of the regression line; a negative β indicates that the non-PD group had a higher proportion, while a positive β indicates
the opposite. The value of the \( \beta \) represents how much higher the proportion was in one group than the other.

Given the small sample size and exploratory nature of the study, multiple tests were not corrected for. Missing data was handled using Rubin’s multiple imputation method (71) and following Harrell’s guidelines (72), which created twenty parallel datasets and produced one combined statistic per outcome measure.

Given the lack of available data in the literature (see above), power and sample size analyses could not be calculated for the present study. No mean and standard deviation values have been reported previously (required to calculate a sample size (73)) for our outcome measures in the conditions relevant to our study, e.g., in asthmatics with and without PD, at baseline, following a methacholine challenge, and following a CO\(_2\) inhalation challenge. Data was collected for 11 patients; this number was determined on the basis of the time and resources available to conduct testing.

RESULTS

Demographics

Of the 11 asthmatic participants who underwent the testing protocol, seven had PD and four did not have PD. Six participants had complete data including all challenge day and covariate data. Nine patients of 11 agreed to take part in the optional third day of testing to collect baseline immunological sputum data. The mean age of the participants was 47 (±16) years, and the sample contained more female (n=9) than male participants (n=2). As seen in Table 1, there were no significant differences between the two groups (PD or non-PD) for any of the demographic variables.

Three of the seven PD participants had a panic attack in response to the methacholine, compared to none of the four non-PD participants. All seven PD participants had a panic attack in response to the CO\(_2\) inhalation challenge, while only one of the four non-PD participants did.

Objective 1: Methacholine Challenge Immune Reactivity

After adjusting for covariates, the analyses demonstrated that the presence of PD had an effect on the proportions of leukocytes in post-methacholine sputum: lymphocytes occupied on average 0.8% less of the total leukocyte population in the PD group compared to the non-PD group (\( \beta = \)
In addition, a trend emerged for sputum eosinophils, which occupied 5% more of the total leukocyte population in the PD group compared to the non-PD group (β = 5.03, 95% CIs = -0.73 – 10.79, p = .087). PD did not appear to influence the proportions of sputum neutrophils (β = -11.72, 95% CIs = -34.64 – 11.18, p = .31) or macrophages (β = 0.10, 95% CIs = -22.63 – 22.82, p = .99). The covariate-adjusted and imputed post-methacholine findings are reported in Table 2. The unadjusted post-methacholine means are reported in Appendix C.

### TABLE 1. Demographic Characteristics of Patients with and without PD

<table>
<thead>
<tr>
<th>Mean (SD)</th>
<th>PD</th>
<th>Non-PD</th>
<th>Missing Data</th>
<th>F Value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>43 (16)</td>
<td>55 (15)</td>
<td>0</td>
<td>1.34</td>
<td>.28</td>
</tr>
<tr>
<td>Sex (% male [n])</td>
<td>29 [2]</td>
<td>0 [0]</td>
<td>0</td>
<td>1.31</td>
<td>.28</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.6 (8.8)</td>
<td>30.0 (5.5)</td>
<td>0</td>
<td>0.08</td>
<td>.78</td>
</tr>
<tr>
<td>% Predicted FEV₁ (Baseline)</td>
<td>93.1 (17.8)</td>
<td>88.6 (8.6)</td>
<td>0</td>
<td>0.22</td>
<td>.65</td>
</tr>
<tr>
<td>PC20 (mg/mL*)</td>
<td>0.5 (0.06 – 6.33)</td>
<td>0.6 (0.09 – 3.01)</td>
<td>0</td>
<td>0.03</td>
<td>.87</td>
</tr>
<tr>
<td>ICS Dose (µg**)</td>
<td>292 (233)</td>
<td>208 (72)</td>
<td>2</td>
<td>0.35</td>
<td>.58</td>
</tr>
<tr>
<td>% Neutrophils (Baseline)</td>
<td>23.0 (17.4)</td>
<td>27.5 (12.4)</td>
<td>5</td>
<td>0.10</td>
<td>.77</td>
</tr>
<tr>
<td>% Eosinophils (Baseline)</td>
<td>0.7 (0.7)</td>
<td>6.4 (9.0)</td>
<td>5</td>
<td>2.09</td>
<td>.22</td>
</tr>
<tr>
<td>% Macrophages (Baseline)</td>
<td>54.9 (28.0)</td>
<td>62.4 (20.7)</td>
<td>5</td>
<td>0.11</td>
<td>.76</td>
</tr>
<tr>
<td>% Lymphocytes (Baseline)</td>
<td>0.3 (0.2)</td>
<td>0.6 (0.9)</td>
<td>5</td>
<td>0.83</td>
<td>.41</td>
</tr>
</tbody>
</table>

n = number; SD = standard deviation; BMI = body mass index; FEV₁ = forced expiratory volume in one second; PC20 = concentration of methacholine required to cause a 20% drop in FEV₁; ICS = inhaled corticosteroid.

* Reported as Geometric mean (95% Confidence Intervals)

** Fluticasone equivalent

### TABLE 2. Influence of Panic Disorder on Sputum Composition in Asthmatics following the Methacholine Challenge

<table>
<thead>
<tr>
<th>Marker (Mean)</th>
<th>PD</th>
<th>Non-PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Neutrophils</td>
<td>45.0</td>
<td>56.8</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>8.6</td>
<td>3.9</td>
</tr>
<tr>
<td>% Macrophages</td>
<td>33.8</td>
<td>34.2</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>0.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Covariates included age, sex, ICS dose, and baseline proportion of the leukocyte being evaluated.
Objective 2: CO₂ Challenge Reactivity

After adjusting for covariates, the analyses demonstrated that the presence of PD did not impact the proportions of sputum neutrophils ($\beta = 31.25$, 95% CIs = -9.91 – 72.41, $p = .14$), eosinophils ($\beta = -9.78$, 95% CIs = -49.50 – 29.95, $p = .63$), macrophages ($\beta = -5.96$, 95% CIs = -32.55 – 20.63, $p = .66$), or lymphocytes ($\beta = -0.22$, 95% CIs = -2.06 – 1.63, $p = .82$) in the participants’ sputum following the CO₂ inhalation challenge. The findings are reported in Table 3. The covariate-adjusted and imputed post-CO₂ findings are reported in Table 3. The unadjusted post-CO₂ means are reported in Appendix C.

<table>
<thead>
<tr>
<th>Marker (Mean)</th>
<th>PD</th>
<th>Non-PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Neutrophils</td>
<td>47.4</td>
<td>18.1</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>7.4</td>
<td>22.7</td>
</tr>
<tr>
<td>% Macrophages</td>
<td>38.1</td>
<td>45.9</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>0.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Covariates included age, sex, ICS dose, and baseline proportion of the leukocyte being evaluated.

DISCUSSION

To our knowledge, this was the first study to assess the role of inflammation in acute-stress responses in patients with asthma and PD, two highly comorbid conditions. PD appears to influence immune reactivity in asthmatics following the methacholine challenge. Unexpectedly, lymphocytes were significantly less present in PD group sputum following the challenge. Lymphocytes are a type of leukocyte, some of whose subsets (such as T cells and B cells) are implicated in the pathogenesis and propagation of asthma (7). Given the nature of some subsets of lymphocytes being pro-inflammatory and others anti-inflammatory (such as the Th1 and Th2 cells described above), the nature of this decrease as beneficial or detrimental in the asthma process depends heavily on which subset of lymphocytes were found in the sample. Unfortunately, the Wright’s stain used to identify cells does not give us additional information about subsets of lymphocytes present in sputum (70).
Though PD did not significantly alter the proportion of sputum eosinophils, a trend did emerge, and the PD group appeared to have higher eosinophils in response to the challenge than the non-PD group, which is consistent with our hypotheses. Recent trends in the diagnosis of asthma attempt to qualify asthma according to their inflammatory phenotype. Some asthma phenotypes include eosinophilic asthma, where ≥ 3% of the sputum cells are eosinophils, and neutrophilic asthma (neutrophils ≥ 76%) (74, 75). Our finding appears to indicate that PD participants tend to have an eosinophilic response to the methacholine challenge. A closer examination of our methacholine reactivity data revealed that six of the PD group participants were eosinophilic asthmatics (six samples available of seven), while none of the non-PD group participants were eosinophilic asthmatics (two samples available of four). This may corroborate the findings in the PD-related immunology research, which suggested that a Th2 cytokine profile may be present in PD patients (41, 42). This raises an interesting question about the nature of causality; unfortunately, it is impossible to determine with this data if eosinophilic reactivity in asthma predisposes an individual to the effects of PD, or if PD alters the pattern of immunological reactivity in asthma.

The data above also indicate that the presence of PD does not confer a statistically significant effect on immunological reactivity to the CO₂ inhalation challenge. This would indicate that both the asthmatics with and without PD are reacting immunologically in the same way. A simple examination of the results illustrates, however, that despite the small sample size, there is a great deal of difference in the sputum leukocyte profiles, which may be clinically meaningful. It appears that the cytokine profiles produced following the CO₂-induced panic attack are the reverse of those produced following the methacholine-triggered asthma attack, with the exception of lymphocyte proportions. One interpretation of these data might suggest that panic might have a protective effect in asthma; surprisingly, this notion has been documented in bronchial reactivity: Lehrer and Carr reported in 1996 that asthmatics with PD had significantly lower levels of airway impedance (as measured by forced oscillation) than asthmatics without PD (76).

The fact that both acute stress challenges produced different outcomes raises the question of the nature of both challenges; it has been demonstrated that different stressor types have different physiological effects in asthma (77). Further analysis of the relative sympathetic and parasympathetic patterns in responses to both simulated asthma attacks and panic attacks may
help explain the difference in reactivity in a similar manner to the differences seen between active and passive stressors (77). Of course, the wide variability of the data, which may also indicate that panic may be a more potent immunological trigger for some rather than for others, and the lack of statistical significance, means that great caution should be used in making any interpretations about these data.

Only one of four non-PD asthmatics experienced a panic attack compared to all seven asthmatics with PD in response to the 35% CO₂ inhalation challenge. These differences in panic attack reactivity to the CO₂ challenge seems to be supported in an article by Fleet and colleagues, who reported that, in patients with coronary artery disease, those with PD reacted significantly more to the challenge than those who did not have PD (78). The fact that less than half of the non-PD group experienced a panic attack may have affected the results: a difference might not have emerged because the participants in both groups did not all react with a panic attack. This same circumstance may have also affected our post-methacholine values, where three PD participants of seven (but none of the non-PD participants) reacted to the methacholine with a panic attack. We conducted secondary analyses (ANOVA-like GLMs) using unadjusted and non-imputed data to determine how having a panic attack during the challenges affected reactivity in the presence and absence of PD. For the post-methacholine data, the lymphocyte results were maintained: those individuals who had PD and experienced a panic attack (3 of 6) had a lower proportion of lymphocytes than the non-PD group, where none reacted with a panic attack. Furthermore, the PD participants who had experienced a panic attack also had a lower proportion of lymphocytes than the PD participants who did not experience a panic attack (3 of 6). For the CO₂ results, the same statistical approach revealed that the non-PD group participant who experienced a panic attack had a higher proportion of eosinophils than both the non-PD group who had no panic attack, as well as the PD group (all of which had a panic attack). While this finding might suggest that the panic attack might have a more profound impact than the presence of PD, it is important to recall that this comparison was made with only a single participant who appeared to have a very pronounced eosinophilic reactivity, even compared to the other participants in the study. The means and additional analyses can be found in Appendix D. These preliminary data support the idea that there may be an interaction between acute and chronic stress in inflammatory responses; however, studies with larger samples are needed to confirm this.
This study has some limitations, the most important of which is the already-mentioned small sample size. As with all underpowered studies, the true effects may not have been revealed by the analyses, and findings should be considered with caution. In addition, the sample of 11 included only two men. While the inclusion of sex as a covariate may have accounted for sex differences (such as hormonal levels), the sample would arguably have benefited from additional male participants.

There was a great deal of variability in the time between visits across patients, which is not ideal. This was usually the result of scheduling conflict, but some participants felt that the temperature and humidity also affected their level of airway obstruction. In all cases where testing was conducted, however, all patients were stable and started with at least 80% of their percent predicted FEV$_1$ as per the American Thoracic Society guidelines (61). In addition, patients were not tested on days where they felt sick because this would likely have biased the immunological results.

Missing data was also a limitation: on the methacholine challenge day, three participants did not produce a sputum sample, while only one participant did not produce a sample on the CO$_2$ inhalation day. For the baseline day assessment, five samples could not be collected: three participants could not expectorate and two others had declined to participate. Looking only at the numbers, one might speculate that the CO$_2$ challenge may have contributed somewhat to the participants’ ability to expectorate or produce a sample; this line of thinking would corroborate the thought that CO$_2$ inhalation could indeed present a physiological challenge for asthmatics. However, with approximately 27% missing data overall, 20 imputations were conducted to ensure that the imputation efficiency would be preserved (71).

Finally, the true effects of PD may have been revealed had we evaluated certain immune cell markers rather than the leukocytes themselves. While the asthma literature regularly evaluates both sputum leukocyte and cytokines, the PD literature consists only of the evaluation of serum cytokines (40-42, 57). As a result, the evaluation of a number of cytokines and chemokines relevant in the pathophysiology of asthma may have been indicated. These markers would include IL-5 and eotaxin, which are important for shuttling eosinophils into the lungs (10), as well as IL-4, IL-12, IL-13, and IFN-γ, which might reflect the dominance of the Th2 profile over Th1. Though additional, more rigorous and comprehensive studies are necessary to confirm
these findings, the results presented here may nonetheless shed an important light on the possible mechanisms linking PD to worse outcomes in asthma.

**CONCLUSION**

Since the majority of the relatively few studies conducted on the topic have been correlational and have been limited to examining non-physiological outcomes, the mechanisms driving the relationship between psychological factors and worse asthma remain elusive. This study is the first to explore PD’s association to asthma from an immunological standpoint, and presents novel, preliminary data in the investigation of PD’s effect as a moderator of immunological reactivity in response to stress in asthma. The presence of PD appears to have an influence on immunological reactivity in asthmatics such that the proportion of sputum lymphocytes are decreased, and while it seems that PD does not alter the immunological reactivity to CO₂ inhalation, additional, more rigorous and comprehensive studies may be indicated. Future studies in this domain should seek to investigate the immunological changes in asthmatics conferred by the presence of PD by looking more specifically at cytokine reactivity in an acute stress context. Accurately elucidating the mechanisms and interrelations between these two conditions may allow us to identify specific immunological markers common to both conditions, which may lead to the development of new therapeutics and treatment strategies, but may also give clinicians the ability to select and prescribe more specific, targeted treatments.
REFERENCES

APPENDIX A

Diagnostic Criteria for Panic Disorder from the DSM-IV
List of Panic Attack Symptoms from the DSM-IV
Diagnostic Criteria for Panic Disorder from the DSM-IV (34)

1) Recurrent unexpected panic attacks (see below)
2) Persistent concern about having additional attacks, including worry about the implications of attack or its consequences
3) Significant change in behavior as a result of the attacks

Panic Attack:
A discrete period of intense fear or discomfort in which four or more of the following symptoms develop abruptly and reach a peak within 10 minutes:

(1) Palpitations or accelerated heart rate
(2) Sweating
(3) Trembling or shaking
(4) Shortness of breath (dyspnea)
(5) Choking
(6) Chest pain or discomfort
(7) Nausea or abdominal discomfort
(8) Feeling dizzy, unsteady, or faint
(9) Numbness or tingling sensations (paresthesias)
(10) Chills or hot flashes
(11) Derealization (feelings of unreality) or depersonalization (being detached from oneself)
(12) Fear of losing control or going crazy
(13) Fear of dying
APPENDIX B

Extended Methodology
Recruitment

Eleven asthmatic patients were recruited from the asthma clinics at the Hôpital du Sacré-Cœur de Montréal. Participants were considered eligible to participate in the study if they were 18 years of age or older, had an objectively confirmed physician-diagnosis of asthma, if they spoke either English or French, if they were non-smokers, and if they were not suffering from a more severe comorbid condition such as cancer or cardiovascular disease. Participants completed the Primary Care Evaluation of Mental Disorders (PRIME-MD) to screen for the presence or absence of PD. Prior to the first visit, eligible patients underwent a semi-structured psychiatric interview called the Anxiety Disorders Interview Schedule-IV (ADIS-IV) (62), administered by a clinical psychology doctoral student over the phone, to reconfirm the presence or absence of PD and other comorbid anxiety disorders. The ADIS-IV, which rates anxiety disorder-related symptoms on a 0 – 8 Likert scale (63), has good psychometric properties (63, 64), with good inter-rater agreement for PD in terms of diagnostic reliability (κ=0.72) (65).

General Protocol Parameters

This study was a component of a larger study seeking to investigate the impact of stress on cardiac and bronchial reactivity in asthmatics. This project was approved by the Ethics Committee at the HSCM. The data collection takes place over three separate visits.

All spirometric tests (forced vital capacity [FVC], forced expiratory volume in one second [FEV₁]) were conducted following the American Thoracic Society guidelines (61). Percent predicted FEV₁ values were obtained by comparing the recorded FEV₁ values to predicted FEV₁ values for patients under the age of 70 (79).

Day 1: Methacholine Challenge

Patients coming in for the first day signed a consent form. Consenting participants then completed sociodemographic and medical history questionnaires, and underwent a methacholine inhalation challenge, where they inhaled increasing quantities of methacholine, a histamine-like substance that causes bronchoconstriction. Each participant inhaled every increasing dose of nebulised methacholine for two minutes, starting with a diluent (0.0mg/mL methacholine), and followed by 0.03mg/mL, 0.06mg/mL, 0.125mg/mL, 0.25mg/mL, 0.5mg/mL, 1.0mg/mL, 2.0mg/mL, 4.0mg/mL, 8.0mg/mL, and finally, 16.0mg/mL (this is the maximum dose that any
participant received). Participants were included in the study if they had mild to moderate asthma, defined by having had a 20% drop in FEV₁ in reaction to a dose of methacholine ≤ 16mg/mL (61).

The test ended when participants experienced a 20% drop in FEV₁ in response to the methacholine inhalation. Participants were then given salbutamol (Ventolin) to reverse the airway narrowing, and then underwent an induced sputum test, which served to collect immunological data (leukocytes).

**Day 2: Induced Panic Attack**

On the second day, participants underwent the 35% CO₂ respiratory challenge, where they inhaled both one vital capacity inhalation of regular air (placebo) and one vital capacity of oxygen-balanced CO₂-rich air, delivered in randomized order. We used DSM-IV criteria and the Acute Panic Inventory (API) (66) to determine if participants had a panic attack. (For a list of panic attack symptoms, see Appendix A.) The API is a checklist with 17 items, each ranked on a 4-point scale from 0 (no symptom) to 3 (severe), that asks questions pertaining to panic attack symptoms, such as “Do you feel nauseous?” and “Do you feel faint?” Both the participants and the researcher conducting the panic attack assessment following both inhalations were blind as to which gas was being administered, and a 30 minute period followed the inhalation of each gas in order for the participants’ respiratory measures to return to baseline. Following this challenge, participants once again underwent a sputum induction, waiting a total of one hour between the first onset of symptoms following an inhalation and the start of the sputum induction.

**Day 3: Baseline**

On the third and final day, participants underwent only an induced sputum test. The data collected on this day served as baseline data.

**Sputum Induction**

Sputum inductions were conducted as per standard clinical procedures (69). Before starting the procedure, participants inhaled salbutamol (200μg) to prevent any bronchoconstriction that might result from the sputum induction process. Participants then inhaled increasing concentrations of vaporized hypertonic saline solution (3%, 4%, 5%) for
seven minutes each. Between each inhalation period, participants were instructed to blow their nose and rinse their mouth with water, then to expectorate into a sterile container.

**Inflammatory Markers**

The expectorate collected at the end of each testing day was analysed for immune cells. We were mainly interested in relative levels of leukocytes present in the sputum, including neutrophils, eosinophils, macrophages, and lymphocytes. The expectorate was processed by first separating the sputum samples from the saliva and placing these samples in a test tube. The sputum samples were then treated using dithiothreitol and placed on a test tube rocker for 15 minutes. Following this period, the sample was resuspended with phosphate buffered saline (PBS) and then filtered through a nylon mesh. To determine the total cell count and cell viability, the cells were mixed with an equal amount of trypan blue and were counted under the microscope. To begin the process of identifying the cells and calculating cell proportions, the cell concentration was adjusted using PBS to obtain a concentration of $1.0 \times 10^6$ cells per mL. To identify the cells, 60μL of the suspension was centrifuged at 450rpm for six minutes, and then stained using Wright’s stain (which allowed the visualization of eosinophils, neutrophils, lymphocytes, monocytes/macrophages, and bronchial epithelial cells).

**Remuneration**

For their participation in the project, participants had their hospital parking stubs and bus tickets refunded. In addition, participants were remunerated for the total amount of $120.00, consisting of $50.00 for the first day, $50.00 for the second day, and $20.00 for the third day. Participants who dropped out of the study or were deemed ineligible to participate were paid for the testing days in which they presented themselves at the laboratory.
APPENDIX C

Unadjusted Post-Challenge Means
### TABLE C1. Influence of Panic Disorder on Sputum Composition in Asthmatics following the Methacholine Challenge using non-imputed data with no adjustment for covariates

<table>
<thead>
<tr>
<th>Marker (Mean, SD)</th>
<th>PD</th>
<th>Non-PD</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>33.0 (26.2)</td>
<td>68.1 (3.7)</td>
<td>.12</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>9.9 (5.8)</td>
<td>1.4 (0.9)</td>
<td><strong>.099</strong></td>
</tr>
<tr>
<td>% Macrophages</td>
<td>43.7 (26.3)</td>
<td>26.3 (3.5)</td>
<td>.41</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>0.2 (0.1)</td>
<td>0.9 (0.5)</td>
<td><strong>.013</strong></td>
</tr>
</tbody>
</table>

SD = Standard deviation.

### TABLE C2. Influence of Panic Disorder on Sputum Composition in Asthmatics following the CO\(_2\) Inhalation Challenge using non-imputed data with no adjustment for covariates

<table>
<thead>
<tr>
<th>Marker (Mean, SD)</th>
<th>PD</th>
<th>Non-PD</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>40.6 (28.2)</td>
<td>32.9 (27.2)</td>
<td>.70</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>5.4 (7.6)</td>
<td>26.5 (45.1)</td>
<td>.23</td>
</tr>
<tr>
<td>% Macrophages</td>
<td>42.1 (18.0)</td>
<td>35.3 (16.0)</td>
<td>.59</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>0.8 (1.1)</td>
<td>0.2 (0.3)</td>
<td>.40</td>
</tr>
</tbody>
</table>

SD = Standard deviation.
APPENDIX D

Secondary Analyses on the Influence of Panic Attacks on Immunological Reactivity in the Presence and Absence of PD
### TABLE D1. Influence of Panic Attacks on Sputum Composition in Asthmatics following the Methacholine Challenge using non-imputed data with no adjustment for covariates

<table>
<thead>
<tr>
<th></th>
<th>Non-PD (No PA)</th>
<th>PD (No PA)</th>
<th>PD (PA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>68.1 (3.7)</td>
<td>45.5 (34.3)</td>
<td>20.5 (8.1)</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>1.4 (0.9)</td>
<td>6.9 (1.5)</td>
<td>12.8 (7.5)</td>
</tr>
<tr>
<td>% Macrophages</td>
<td>26.3 (3.5)</td>
<td>31.0 (33.0)</td>
<td>56.4 (12.3)</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>0.9 (0.5)</td>
<td>0.3 (0.0)</td>
<td>0.1 (0.1)</td>
</tr>
</tbody>
</table>

SD = Standard deviation; PA = Panic attack.

### TABLE D2. Influence of Panic Attacks on Sputum Composition in Asthmatics following the CO₂ Inhalation Challenge using non-imputed data with no adjustment for covariates

<table>
<thead>
<tr>
<th></th>
<th>Non-PD (No PA)</th>
<th>Non-PD (PA)</th>
<th>PD (PA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>2</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>48.3 (7.0)</td>
<td>2.0 (-)</td>
<td>40.6 (28.2)</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>0.5 (0.6)</td>
<td>78.5 (-)</td>
<td>5.4 (7.6)</td>
</tr>
<tr>
<td>% Macrophages</td>
<td>44.5 (2.4)</td>
<td>17.0 (-)</td>
<td>42.1 (18.0)</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>0.3 (0.4)</td>
<td>0.0 (-)</td>
<td>0.8 (1.1)</td>
</tr>
</tbody>
</table>

SD = Standard deviation; PA = Panic attack.