Vascular Risk Associated with Asthma

by

Shelby Leigh Henry

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Abstract

Background and Rationale

Asthma is a chronic inflammatory disease of the airways characterized by episodic bronchoconstriction and airway hyper-responsiveness resulting in symptoms such as coughing, wheezing, and shortness of breath. Asthma is one of the most common chronic diseases in the world, effecting an estimated 300 million people. Previous research has demonstrated that individuals with asthma have an increased risk for cardiovascular morbidity and mortality, however the reason for this is unknown. Systemic inflammation, arterial stiffness and vascular dysfunction are independent predictors of cardiovascular risk in health and in disease, all of which have been reported to be abnormal in people with asthma. Approximately 25-35% of people with a physician diagnosis of asthma do not demonstrate asthma with physiological testing. It is unclear if the increased cardiovascular risk reported in people with asthma is observed in those with an asthma diagnosis that cannot be confirmed by physiological testing.

Purpose and Hypothesis

The purpose of this study was to determine if individuals with an asthma diagnosis that cannot be confirmed by physiological testing demonstrate vascular function, arterial stiffness and systemic inflammation similar to individuals with an asthma diagnosis that is confirmed by physiological testing. Additionally, a group of healthy controls were recruited for further comparison. It was hypothesized that individuals with a physician diagnosis of asthma, but no evidence of asthma with physiological testing would have similar vascular function, arterial

stiffness and systemic inflammation compared to those with asthma confirmed by physiological testing.

Methods

Individuals aged 18-55 years with confirmed asthma (n=15), individuals with an asthma diagnosis that cannot be confirmed by physiological testing (n=12), as well as healthy controls (n=13) were recruited for this cohort study. Ultrasound imaging of the brachial artery (FMD) and carotid-radial pulse wave velocity (crPWV) were obtained to assess vascular function and arterial stiffness, respectively. A venous blood sample was acquired for analysis of systemic inflammation (CRP), and a Fitbit was worn for seven days to determine physical activity and reported in average steps per day. All participants completed a full pulmonary function test including spirometry before and after administration of 400µg of salbutamol to assess lung function and presence of reversible airway obstruction. When appropriate, participants completed an exercise challenge as well as a methacholine challenge to objectively confirm an asthma diagnosis.

Results

When comparing the confirmed asthma group to the unconfirmed asthma group, there were no differences found in FMD (confirmed: 7.99 ± 2.70%, unconfirmed: 7.59 ± 2.94%, p=0.72), crPWV (confirmed: 8.33 ± 1.42 m/s, unconfirmed: 8.60 ± 0.97 m/s, p=0.60), or CRP (confirmed: 3.53 ± 2.90 mg/L, unconfirmed: 3.77 ± 1.78 mg/L, p=0.81).

As both asthma groups demonstrated no clinically or statistically significant differences, they were combined and compared to healthy controls. A trend was observed with FMD between

groups, with the asthma group demonstrating lower FMD compared to healthy controls (7.81 ± 2.76%, 9.48 ± 2.55%, p=0.08). There were no differences in crPWV (asthma: 8.47 ± 1.20 m/s, control: 8.00 ± 1.64 m/s, p=0.33), or CRP (asthma: 3.64 ± 2.40 mg/L, control: 4.45 ± 3.37 mg/L, p=0.43) observed between groups. Participants with asthma who used any asthma medication tended to have lower vascular function compared to those using no asthma medication (any: 7.37 ± 2.59% vs. no: 9.75 ± 2.90%, p= 0.08). Additionally, the participants with asthma tended to be less physically active, measured in average steps per day, compared to healthy controls (p=0.05).

Discussion and Significance

The unconfirmed and confirmed asthma groups demonstrated similar FMD, and when combined, tended to have lower FMD compared to healthy controls. The results of this study suggest that objective confirmation of asthma alone is not an important determinant of cardiovascular risk. Rather, other factors associated with a physician diagnosis/clinical history of asthma, such as asthma medication use and physical inactivity, may be more important determinants of cardiovascular risk in people with asthma. It is critical to obtain an objective diagnosis of asthma through physiological testing prior to beginning treatment in order to reduce the unnecessary use of asthma medication. More research is needed to further understand the association of asthma medication use (both medication type and dose) and physical inactivity to the increased cardiovascular risk reported in people with asthma.

Preface

This thesis is an original work by Shelby Leigh Henry. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Vascular Implications of a Naturally Occurring Asthma Exacerbation", ID No. Pro00083372, July 2018.

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List of Symbols and Abbreviations

ACQ: Asthma Control Questionnaire

AQLQ: Asthma Quality of Life Questionnaire

BMI: Body mass index (kg/m2)

COPD: Chronic obstructive pulmonary disease

CPT: Cold pressor test

CRP: C-reactive protein

CV: Coefficient of variance

EIB: Exercise induced bronchoconstriction

ELISA: Enzyme linked immunosorbent assay

eNOS: endothelial nitric oxide synthase

FEV1: Forced expiratory volume in 1-second

FMD: Flow-mediated dilation

FRC: Functional residual capacity

FVC: Forced vital capacity

GINA: Global Initiative for Asthma

HR: Hazard ratio

IC: Inspiratory capacity

ICS: Inhaled corticosteroid

IL-6: Interleukin-6

MI: Myocardial infarction

MSNA: Muscle sympathetic nervous activity

NO: Nitric oxide

OR: Odds ratio

PC20: Provocative concentration needed to decrease lung function by 20%

PWV: Pulse wave velocity

RR: Relative risk

RV: Residual volume

TLC: Total lung capacity

TNF-α: Tumor necrosis factor-α

TV: Tidal volume

V_A: Alveolar volume

VC: Vital capacity

VSMC: Vascular smooth muscle cells

VTI: Velocity time integral

Chapter I: Introduction

Background

Individuals with asthma are at an increased risk for cardiovascular morbidity and mortality[1,2], however the reason for this is unknown. Systemic inflammation, arterial stiffness and vascular dysfunction are independent predictors of cardiovascular risk in health and in disease[3–5], all of which have been reported to be abnormal in people with asthma.[6–8] Approximately 25-35% of people with a physician diagnosis of asthma do not demonstrate asthma with physiological testing[9]. This raises the question as to whether these individuals with unconfirmed asthma have similar cardiovascular risk as compared to those with a confirmed diagnosis of asthma. To better understand the cardiovascular risk observed in asthma, this study compared vascular function, arterial stiffness, and systemic inflammation in individuals with a physician diagnosis of asthma confirmed by physiologically testing to patients who have a physician diagnosis of asthma, but no evidence of asthma on physiological testing. A healthy control group was recruited as an additional comparison.

It is unclear in the current literature if cardiovascular risk reported in asthma is present in individuals that do not demonstrate asthma with physiological testing. Therefore, the results of this study will help interpret the current literature by distinguishing the individuals that may be at an increased risk of cardiovascular disease, and will give insight into the possible cause of this increased risk. This research highlights the importance of obtaining an objective

diagnosis of asthma to ensure a reduction in the unnecessary use of medication, as well as decrease the financial impact on the health care system. This research will inform future rehabilitation studies by better detailing the target population for rehabilitation to reduce cardiovascular risk in people with asthma.

Purpose

The purpose of this study was to examine vascular function, arterial stiffness and systemic inflammation in young adults with a physiologically confirmed asthma diagnosis and to compare these data to young adults with an asthma diagnosis that cannot be physiologically confirmed. In addition, a group of healthy controls were recruited for further comparison.

Hypothesis

It was hypothesized that individuals with an asthma diagnosis that cannot be confirmed by physiological testing would have similar vascular function, arterial stiffness and systemic inflammation to individuals with an asthma diagnosis that is confirmed by physiological testing. Additionally, it was hypothesized that both of these asthma groups would have decreased vascular function, increased arterial stiffness and increased systemic inflammation when compared to age-, sex-, and BMI-matched healthy controls.

Specific Outcomes

The aim of this study was to evaluate vascular function, arterial stiffness and systemic inflammation in young adults with a previous diagnosis of asthma confirmed with physiological assessments compared to those with unconfirmed asthma. Individuals were grouped into 1: those with asthma confirmed by physiological testing (confirmed), 2: those not showing evidence of asthma with physiological testing (unconfirmed).

The primary outcome of this study was vascular function, assessed via flow-mediated dilation (FMD) of the brachial artery determined by ultrasound.

Secondary outcomes of this study included systemic inflammation and arterial stiffness. Systemic inflammation was assessed using serum obtained via antecubital venipuncture, and reported as serum levels of C-reactive protein in mg/L. Arterial stiffness was assessed using tonometry (Complior, Alam Medical, Saint Quentin Fallavier, France) and reported as carotid-radial pulse wave velocity (crPWV) in m/s.

An exploratory sub analysis was completed to investigate whether sex, medication use or physical activity were associated with the primary and secondary outcomes. Physical activity was monitored for 7 days with a Fitbit activity tracking device.

Delimitations

A total of 27 participants with asthma were recruited from the Lung Health Clinic, the University Lung Clinic, and the general population. Exclusion criteria were: age >55 years, current smokers or those with a smoking history >10 pack years, and presence of comorbidities including cardiovascular disease, pulmonary disease other than asthma, and any inflammatory condition or metabolic disease. A total of 13 healthy controls with no current respiratory symptoms, previous diagnosis of asthma, and no known history of cardiovascular disease, or other chronic inflammatory conditions were recruited from the general population. The control group was matched to the asthmatic groups by age, sex, and BMI as best possible.

As within-subject variability of FMD often varies widely between studies (1.8%-50%)[10–12], the primary ultra-sonographer tested three healthy individuals (2 males, 1 female) for three consecutive days to determine within-subject coefficient of variation (CV) prior to the study. All confounding variables were controlled (i.e. fasted state, medications, time of day, phase of menstrual cycle, hours of sleep in previous night) and after adjusting for shear stress, CV was calculated as standard deviation/mean*100. The reported within-subject mean CV was 5.13%, demonstrating competency in this technique.

Limitations

A limitation of this study was the different medications that each participant was receiving for their asthma management. These medications may have varying effects on the outcomes being tested. Along with genetic variation, there were other confounding variables that were not controlled for such as menstrual status. To mitigate this limitation, all confounding variables were documented and controlled for statistically when possible. Serum estradiol levels were analysed to examine estrogen variation and relationship to FMD. No relationship was observed, therefore, estradiol level was not entered as a covariate when evaluating vascular function. To account for potential differences as a result of variable medication use among participants, analysis was completed comparing those with any asthma medication use vs. those who reported no asthma medication use. Individuals were also stratified into groups by medication use (Group 1: no asthma medication, Group 2: SABA as needed only, Group 3: SABA, ICS/combination, Group 4: SABA, ICS/combination, add-on treatment)[9], to determine if type of medication had an effect on FMD results.

A second limitation of this study was the difficulty in obtaining confirmation of asthma with physiological testing after an individual has begun treatment. As the criteria for inclusion requires a prior physician diagnosis of asthma, most participants were currently receiving treatment and were well controlled with medication, making it more difficult to confirm the diagnosis with physiological testing.[9] Therefore, individuals with diagnosed asthma that began treatment prior to physiological testing were asked to withhold their medications for up to 48

hours prior to any tests. If physiological tests following 48 hours of withheld medication did not confirm a diagnosis, the participant was assigned to the unconfirmed asthma group.

Definitions

Asthma is defined as a chronic inflammatory disease of the airways characterized by variable airflow limitation and a history of symptoms such as wheezing, shortness of breath, tightness of chest and coughing, all of which may vary over time.[9] For the purpose of this study, asthma was initially identified by self reported diagnosis or a previous physician diagnosis. Asthma diagnosis was physiologically confirmed by either: 1) a significant improvement (defined as a ≥12% and 200mL increase) in FEV_1 with bronchodilator, 2) a 20% reduction in FEV_1 during a methacholine challenge, 3) a 10% reduction in FEV_1 following an exercise challenge test.[9,13] Patients with unconfirmed asthma were defined as individuals with a physician diagnosis of asthma (with or without current medication use) that cannot be confirmed with physiological testing. Vascular function was defined based on endothelial function (FMD)[14], and arterial stiffness was defined based on peripheral pulse wave velocity (crPWV).[15] Systemic inflammation was defined as the presence of inflammatory marker C-reactive protein.[16] Physical activity levels were defined as average number of steps per day for seven days.[17] Physical activity level was categorized as low activity: <7,500 steps/day, somewhat active: 7,500-9,999 steps/day, and highly active: > 10,000 steps/day.[17]

Chapter II: Literature Review

Introduction

Asthma is a chronic disease of the airways characterized by episodic bronchoconstriction and airway hyper responsiveness leading to symptoms such as wheezing, tightness of chest, and shortness of breath, as well as variable airflow limitation.[9] As one of the most common chronic diseases in the world, an estimated 300 million or more individuals are currently affected by this disease.[18] Asthma is a heterogeneous disease with many different potential causes, triggers, and phenotypes. While there is no cure it is typically quite treatable with the use of medication and avoidance of triggers.

Diagnosis

Asthma diagnosis is guided by a history of symptoms and physical examination revealing wheezing. History of symptoms is generally variable, with symptoms typically worsening at night, and experiencing relief with the use of medication.[19] Along with clinical history of the disease, asthma can be confirmed by different tests/challenges: 1) spirometry , 2) airway responsiveness to inhalation challenge, 3) exercise challenge.[19]

When measuring spirometry, asthma is diagnosed based on significant reversibility. This refers to an increase in FEV_1 of 200mL and 12% after inhalation of a short-acting β-agonist (for this

study salbutamol was used), compared to baseline.[19] For people that show symptoms associated with asthma, but have normal baseline spirometry, an alternative method may be used for diagnosis. Airway responsiveness is commonly measured in two ways: directly with a methacholine challenge or indirectly with an exercise challenge. The methacholine challenge requires a series of inhaled doses of methacholine in increasing quantity. Airway hyper responsiveness is defined as significant airway obstruction (decrease in FEV_1 $\geq$ 20%) within the provocative concentration (PC_{20}) of $\leq$16 mg/ml of inhaled methacholine.[13] Exercise is used to determine exercise induced bronchoconstriction (EIB) in people with symptoms associated with asthma, and is confirmed by a decrease in FEV_1 $\geq$ 10% following an exercise challenge test.[13]

Additionally, since there is such a strong association between asthma and allergy status, the probability of an asthma diagnosis is increased in the presence of allergies or allergic diseases.[19] An allergy test may be useful in helping determine potential risk factors that could be contributing to respiratory symptoms for the individual, as well as provide insight to possible asthma phenotype.

An individual may be diagnosed with asthma and started on medications to treat their symptoms, prior to confirming a diagnosis with the previously mentioned physiological tests. This can make physiological confirmation of a diagnosis more difficult, however, as it is important to obtain this objective assessment to confirm diagnosis and ensure appropriate

treatment. In this case, the individual is asked to refrain from taking their long-acting

(controller) medication for 48 hours and their short-acting (rescue) medication for 8 hours

prior to physiological testing.[9] This should allow for physiological confirmation if asthma is

present. A physician may also increase or decrease the dosage of medication prescribed in

order to monitor changes in airflow limitation and symptoms accordingly.[9] In the event that

neither approach is successful in obtaining confirmation of an asthma diagnosis, it is

recommended to consider an alternative diagnosis or to refer for further investigation.[9]

Phenotypes

Asthma can be divided into subtypes depending on the symptomology as well as the triggers

that initiate an acute asthma response. These recognizable clusters of pathophysiological

characteristics, often referred to as phenotypes, may be helpful in determining risk factors to

avoid. However, at this time there is not a strong relationship seen between the disease

process and treatment response among the different phenotypes.[9]

The most common phenotypes recognized include: allergic (atopic) asthma, non-allergic (non-

atopic) asthma, late-onset asthma, asthma with fixed airflow limitation (refractory), and

asthma with obesity. Atopic asthma is typically characterized with the presence of

eosinophilic airway inflammation within induced sputum samples of these individuals[9] and is

generally triggered by specific allergens depending on their allergic status. Non-Atopic asthma

is usually trigged by environmental factors such as smoke or pollution. The cellular profile of

the sputum of these patients is more variable and they may respond less to inhaled

corticosteroids (ICS).[9] Late-onset asthma is defined by the first presentation of asthma within

adult life. It is more commonly seen in women, tends to be non-allergic, and is more difficult

to control i.e. not responsive or less responsive to standard bronchodilator and corticosteroid

therapy.[9] Asthma with fixed airflow limitation is characterized by long standing asthma

potentially resulting in airway remodelling.[9] Asthma with obesity is characterized by the

presence of prominent respiratory symptoms and a BMI > 30 kg/m^2.[9] As asthma is typically

very heterogeneous, asthma with obesity is further classified by its own phenotypes[20],

however for the purpose of this study, these obesity phenotypes will not be explored.

Asthma Severity and Control

Asthma can be discussed relative to control and severity. The Global Initiative for Asthma

(GINA) refers to control in terms of symptom control and future risk of adverse outcome, with

uncontrolled asthma leading to a higher risk of hospitalization, exacerbation, and death, and

can be assessed using various tools such as the Asthma Control Questionnaire (AQC).[9] There

are many potential reasons for an individual to have uncontrolled asthma, with the most

common reason being non compliance: failure to use, or properly use, medication.[9] A

decreased capacity to obtain, process, and understand basic health information, otherwise

known as low health literacy, also contributes to worse asthma control and increased risk of

exacerbation.[9] Identifying and resolving the barriers an individual may face that would

prevent them from controlling their asthma is an important step in reducing their risk for

future adverse outcomes.

Asthma severity refers to the minimum level of treatment required for the individual's asthma

to remain controlled, as well as the responsiveness to treatment[9]. This is assessed by

evaluating patient history, and categorized as mild, moderate, or severe.[9]

Cardiovascular Disease in Asthma

Asthma is typically referred to as a disease of the airway, with treatment focusing on reducing

the pulmonary symptoms associated with the disease. It has become increasingly more clear

that asthma may have a negative impact that extends past the pulmonary system.

Longitudinal studies have shown that people with asthma are at an increased risk of

cardiovascular events compared to the general population.[2,21,22] These studies also concluded

that the cardiovascular risk associated with asthma appears to be stronger in women;

however, the reason for this is currently unknown.

Iribarren et al.[2] conducted a large longitudinal study of 203,595 adults with asthma and

203,595 age-, sex-, and race-matched controls that recorded non-fatal or fatal cardiovascular

disease and all-cause mortality over the course of 12 years. After adjusting for traditional

cardiovascular risk factors, age, sex, race, and allergy, asthma was associated with a 1.4-fold

increased risk of coronary heart disease, a 1.2-fold increased risk of cerebrovascular disease, a 2.14-fold increased risk of heart failure, and a 3.28-fold increased risk of all-cause mortality. They also reported stronger associations for these outcomes among women with asthma compared to men with asthma. Medication use had a negative impact on cardiovascular risk with those reporting any use of asthma medication having increased risk of coronary heart disease (1.52), cerebrovascular disease (1.27), and heart failure (2.24), compared to people with asthma reporting no use of asthma medication. Interestingly, while individuals with asthma who did not take medication were at a reduced risk of cardiovascular disease, they experienced an increased risk in all-cause mortality. It is possible the lower cardiovascular risk associated with no medication use may be a result of individuals with mild asthma requiring little or no medication use as compared to severe asthma. The increased risk of all caused mortality associated with no medication use may be a result of poorly controlled asthma (as they do not use prescribed asthma medication) leading to more asthma related complications and death.

Appleton et. al[21] conducted a cross sectional study on 4060 adults with asthma investigating the prevalence of cardiovascular disease and stroke associated with asthma. This study found that people with asthma had an increased risk of cardiovascular disease and stroke (OR=1.82), which persisted after adjustment for age, lung function, income, and other traditional cardiovascular risk factors. More specifically, asthma was associated with higher prevalence of myocardial infarction (MI) (OR=1.85), stroke (OR=1.84), and angina (OR=1.64).

A recent meta-analysis evaluated the association of asthma and incidence of cardiovascular disease and death in 10 prospective and retrospective cohort studies containing 406,426 participants.[22] Overall, participants with asthma had an increased risk of cardiovascular disease or event (RR=1.33) and all cause mortality (RR=1.36) compared to a reference group. A different meta-analysis looked at the association between asthma and risk of stroke in five cohort studies with a total of 524,637 participants and 6031 reported stroke outcomes.[23] Overall, asthma was associated with an increased risk of stroke (HR=1.32, 1.13-1.76), which was significantly increased in women with asthma (HR=1.42) compared to men with asthma (HR=1.19).

Importantly, the aforementioned studies did not exclusively include individuals with asthma confirmed by physiological testing. Approximately 25-35% of patients diagnosed with asthma in primary care cannot be confirmed as having asthma[9], therefore it is important to investigate the cardiovascular risk of individuals with an asthma diagnosis that cannot be physiologically confirmed in order to better understand the cardiovascular risk that has been reported in these studies.

While it is clear that asthma is associated with increased cardiovascular risk, the reason(s) why are not fully understood. A study done in our lab looked at the vascular health of 16 individuals with physiologically confirmed asthma compared to 16 healthy controls, reporting

significantly higher arterial stiffness in the asthma group even after matching for age, sex, BMI, physical activity and physical fitness levels.[7] This suggests that there are structural changes occurring in the vasculature related to asthma that cannot be explained by previously stated factors. The same study looked at FMD and found no differences between the groups. To the contrary, a study done by Yildiz et al.[8] found significant endothelial dysfunction measured by FMD in individuals with physiologically confirmed asthma compared to healthy controls. Furthermore, there was a significant decrease in endothelial function from mild asthma to moderate asthma, further suggesting increased asthma severity may contribute to increased cardiovascular risk. Yildiz et al. did not match for physical fitness, nor physical activity, suggesting that physical activity levels and physical fitness may be important in attenuating the decline in endothelial function seen in people with asthma.

Asthma medication can have negative physiological affects that could potentially be contributing to the increased cardiovascular risk seen in asthma. A meta-analysis of 13 single dose trials and 20 long-duration trials investigated the cardiovascular safety of β-agonist therapy in patients with COPD and asthma.[24] The authors found that β-agonist use was associated with an increased relative risk (RR) for a cardiovascular event of 2.54 compared to placebo. The increased RR for sinus tachycardia alone was 3.06, with all other major cardiovascular events having a RR of 1.66. A recent study done in our lab on healthy young adults demonstrated that an acute dose of salbutamol significantly increased muscle sympathetic nervous activity (MSNA).[25] This is important as increased MSNA is known to contribute to increased arterial stiffness[26], potentially explaining the structural changes in the

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vasculature seen in asthma. Increased MSNA is also linked to cardiovascular deterioration[27], making it an important factor when investigating cardiovascular risk associated with asthma.

Similarly, a recent study in our lab[28] looked at the vascular effects of acute salbutamol use in 14 participants with a physician diagnosis of asthma and history of salbutamol usage, and 14 healthy (salbutamol-naive) controls at baseline. After administration of 400 μg of salbutamol or placebo, a significant decrease in endothelial function and increase in arterial stiffness was found among the salbutamol/asthma group compared to placebo/asthma and controls. A sub group of 10 controls and 10 people with asthma were tested to investigate if altered neurovascular transduction was responsible for this decline in vascular function using the cold pressor test (CPT)[29] to stimulate an acute increase in sympathetic nerve activity. Interestingly, there were no differences in vascular response to the CPT between groups, suggesting the reason individuals with asthma demonstrated reduced vascular function following salbutamol may be a result of greater sympathetic response to salbutamol rather than altered neurovascular transduction. If asthma medications are contributing to the increased risk of cardiovascular disease seen in people with asthma, it is critically important to ensure individuals with an asthma diagnosis that cannot be confirmed by physiological testing are not being put at an unnecessary risk with the use of these medications.

These studies highlight important vascular changes that occur in people with asthma (i.e. vascular dysfunction and increased arterial stiffness), all of which are independent predictors

of cardiovascular risk. It is possible that these changes are important contributing factors to the increased cardiovascular risk reported in people with asthma. The mechanism of these changes are not well understood, and it remains unclear if these changes are seen in individuals with an asthma diagnosis that cannot be physiologically confirmed.

Inflammation and cardiovascular risk

The systemic inflammatory markers that have been identified as independent predictors for cardiovascular risk include: tumor necrosis factor-α (TNF-α)[30], interleukin-6 (IL-6)[31] and C-reactive protein (CRP)[32]. TNF-α is a complex inflammatory cytokine that has been identified as the initiator of the inflammatory cascade within the arterial wall, as well as having pro-atherosclerotic properties that increase cardiovascular risk.[33] TNF-α is elevated in advanced heart failure, and when elevated in an experimental setting, can result in cardiovascular problems such as left ventricular dystrophy, pulmonary edema, and cardiomyopathy.[30] In health, TNF-α initiates a very important immune response to protect against bacteria, viruses, and parasites; however, when chronically elevated TNF-α can have detrimental effects on the cardiovascular system.[33]

IL-6 is a multifunctional cytokine that regulates multiple physiological responses including the immune and inflammatory response. Specifically, IL-6 mediates the acute-phase response of inflammation, and when this response persists, chronic inflammation results in the stimulation of the immune system.[34] IL-6 production increases as a result of several factors

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including infection and increased production of TNF-α, which then drives the production of CRP.[31] In a large prospective study looking at apparently healthy men, baseline plasma measurements were taken in 202 participants that subsequently developed MI, and 202 age- and smoking status-matched controls which reported no known vascular disease at a 6 month follow up.[31] This study found that increased levels of plasma IL-6 was significantly associated with increased risk of MI. They determined that as elevated levels of IL-6 were seen in a healthy low-risk state prior to MI, the possibility that the cardiac event was a cause of the elevation could be excluded, giving evidence that IL-6 actively contributed to the cardiovascular risk seen in these participants.

CRP is produced in the liver as a result of increased levels of IL-6. CRP has demonstrated a strong association to cardiovascular risk, even after controlling for age, total cholesterol, HDL cholesterol, smoking, body mass index, diabetes, history of hypertension, exercise level, and family history of coronary disease.[3] CRP induces vascular dysfunction by suppressing eNOS expression and activity[35], which in turn increases adhesion of inflammatory cells to the endothelium[36], and activates/attracts inflammatory cells to the site of vascular injury.[37] It has been suggested that increased cell proliferation, vascular inflammation, and vascular fibrosis as a result of such vascular dysfunction may play a direct role in mediating arterial stiffness.[38] Ridker et al. have developed cardiovascular risk stratification groups based on CRP levels as follows: <1mg/L = low risk, 1-3mg/L = moderate risk, and >3 mg/L = high risk.[39] This work gives insight to the clinical relevance of this inflammatory marker in relation to cardiovascular risk. A comparison of multiple inflammatory markers identified CRP as the best predictor of

cardiovascular risk from the perspective of clinical use and chemistry of the marker.[3] CRP is relatively stable, has good interassay precision (<10%), and has World Health Organization standards available. This, along with the availability of assays for this marker, makes CRP a strong choice for evaluating systemic inflammation.

Inflammatory cross-over

While it is clear that both pulmonary and systemic inflammation are increased in people with asthma, the relationship between these is not well understood. There is little research done on the direct mechanisms of the interaction. A murine model study by Kido et al.[40] found that when alveolar macrophages are activated by an inhaled stimulus, there is an increase in expression of IL-6 within the lung, as well as a subsequent increase of IL-6 within the systemic circulation. Another study in a murine model of asthma investigated the contribution of oxidative stress in asthmatic airways in modulation of systemic oxidant-antioxidant balance[41], concluding that when treated with an inhaled irritant, there was a concurrent increase in airway inflammation/oxidative stress with systemic inflammation/oxidative stress. This relationship was further evident when treating the same model with an inhaled antioxidant, resulting in inhibited oxidative stress in the systemic vasculature. These studies provide evidence that inflammation in the airways has the potential to cross over, or stimulate inflammation in the systemic system.

A recent study done in our lab demonstrated that when people with asthma performed an inhaled mannitol challenge, which is an airway challenge shown to cause bronchoconstriction as a result of airway inflammation[42], there was a significant increase in systemic inflammation as measured by serum CRP. In contrast, when bronchoconstriction was stimulated by methacholine, an airway challenge that does not produce airway inflammation, there was no increase in systemic inflammation. These studies provide evidence that the pulmonary inflammation such as what may occur with asthma, and not the associated bronchoconstriction, leads to elevated levels of systemic inflammation.

Systemic inflammation in asthma

Asthma is typically associated with chronic airway inflammation, however there is growing evidence to suggest it may also be associated with an increase in systemic inflammation. A study done that compared serum levels of IL-6 in 17 people with stable asthma and 17 healthy controls found the asthma group had significantly elevated levels of the inflammatory cytokine IL-6.[6] Wood et al. looked at systemic inflammation in relation to asthma phenotypes[43] in 152 people with stable asthma physiologically confirmed with airway hyper-responsiveness to hypertonic saline and 83 healthy controls, finding that the asthma group with neutrophils present in their sputum had significantly elevated levels of systemic inflammation compared to the non-neutrophilic asthma group as well as healthy controls. Individuals with atopic asthma that demonstrate higher levels of sputum neutrophils tend to have characteristics associated with very severe asthma.[44] This suggests there may be an

important relationship between the allergic asthma phenotype, disease severity and cardiovascular risk.

A study done by Zietkowski et al. looked at the exhaled and serum levels of CRP in 62 patients with allergic asthma (20 with steroid-naive mild asthma, 19 with ICS-treated, stable mild-to-moderate asthma, 23 with ICS-treated unstable, severe asthma) and 15 healthy volunteers.[45] They found that there were significantly elevated levels of both exhaled and serum CRP in the asthma groups compared to the healthy volunteers. Importantly, CRP was further elevated in in the patients with unstable asthma as compared to stable asthma, supporting the idea that increased disease severity may contribute to greater cardiovascular risk. Preliminary data from a study done in our lab investigating serum CRP levels in stable asthma, acute asthma exacerbation, and healthy controls, revealed a significantly elevated presence of systemic inflammation during an exacerbation (6.76 ± 5.89) compared to healthy controls (1.18 ± 1.50).

It is evident that people with asthma have increased levels of both pulmonary and systemic inflammation, with inflammation appearing to be more severe in people with more severe or uncontrolled asthma. As systemic inflammation has previously been linked to increased cardiovascular risk in other conditions, this would suggest that the increased inflammation seen in asthma would be contributing to vascular impairment and subsequent increased cardiovascular risk associated with the disease.

It is important to investigate the levels of inflammation in individuals with physiological confirmation of asthma and those with an asthma diagnosis that cannot be confirmed with physiological testing in order to develop a firm understanding of how the inflammatory profiles of these groups may differ. Furthermore, it is important to investigate the relationship between increased levels of inflammation and vascular function in people with asthma in order to better understand the increased cardiovascular risk reported in asthma.

Endothelial dysfunction and systemic inflammation

In health, the endothelium modulates vascular tone by releasing the chemical relaxing factor NO.[46] The endothelium is also responsible for preventing platelet adhesion and cell proliferation, therefore endothelial dysfunction plays a key role in development of atherosclerosis.[47] In resting conditions, NO is released from the endothelium in response to a variety of physiological and pharmacological stimuli[47], an important one being shear stress.[48] Shear stress refers to the frictional force of blood flow on the vessel wall. The usual laminar flow through the vessel changes to a more turbulent flow as blood flow increases, escalating shear stress and stimulating NO to be released from the endothelium. When NO is released, it diffuses into the vascular smooth muscle cells (VSMC) and causes these cells to relax, leading to vasodilation.[49] Importantly, the vasodilation response to shear stress is impaired in atherosclerotic arteries.[48] Risk factors that can lead to endothelial dysfunction tend to parallel coronary risk factors and include: smoking, dyslipidemia, diabetes, hypertension, obesity, cerebral vascular disease, and heart disease.[5] Coronary[50] as well as peripheral[51] endothelial

dysfunction are independent predictors of cardiovascular events. Endothelial dysfunction relates directly to cardiovascular risk because of its involvement in the development and progression or atherosclerosis by promoting platelet adhesion to the arterial wall as a result of reduced bioavailability of NO.[52] Chronic inflammation and oxidative stress resulting from the previously listed risk factors or diseased states are possible mechanisms for this reduced bioavailability of NO.[53]

A study done by Hingorani et al.[54] investigated the effects of an experimental inflammatory stimulus on endothelium-dependent vasodilation in 12 healthy volunteers. This study found that the experimental vaccine generated a mild inflammatory response that resulted in significant dysfunction of the endothelium in response to both physical and pharmacological stimuli. Importantly, CRP has been directly linked to the reduction of endothelial nitric oxide synthase (eNOS) expression, the enzyme in which NO is derived from.[35] As asthma is associated with increased systemic inflammation, chronic systemic inflammation may be the driving factor for increased cardiovascular risk seen in asthma.

Endothelial dysfunction in asthma

A study done by Yildiz et al.[8] looked at the endothelial function assessed by FMD in 49 patients with asthma (24 mild/25 moderate) confirmed by physiological testing and 49 healthy controls. Participants were excluded if they had a known condition associated with endothelial dysfunction such as diabetes or other cardiovascular diseases. They found

significantly lower endothelial function in people with asthma as compared to healthy

controls, with the moderate asthmatics having worse vascular function compared to mild

patients. These findings suggest that more severe asthma is associated with greater

cardiovascular risk. There is currently no literature on the endothelial function in individuals

with an asthma diagnosis that cannot be confirmed by physiological testing. It is important to

determine if these groups have similar or dissimilar endothelial function as compared to

patients with confirmed asthma, as this would give us indications of their future

cardiovascular risk, and would allow us to further understand the contributors to increased

cardiovascular risk in asthma. For the purpose of this study, a clinically significant difference in

FMD was defined as a difference of 1%, as this has been previously shown to impact

cardiovascular risk.[55]

A study done in our lab found that endothelial function was preserved in people with asthma

when matched for age, BMI, physical activity and aerobic fitness, suggesting physical fitness

has a protective effect.[7] This is most likely due to the anti-inflammatory effects of exercise[56]

as well as its positive impact on endothelial function.[57] People with asthma are more likely to

be inactive[58,59], which would inhibit their exposure to these beneficial effects. This could

partially explain the inherent increase in cardiovascular risk in asthma, as well as provide a

potential therapeutic intervention to reduce cardiovascular risk in these individuals.

Physical activity and endothelial function

With a single bout of exercise, NO bioavailability increases in both sedentary and trained adults by 63% and 93% respectively.[57] Interestingly, the same study also showed that in sedentary individuals, the increase in NO returned to baseline approximately 24 hours following exercise; however in the trained individuals, NO bioavailability remained elevated for 48 hours post exercise with both groups showing peak NO bioavailability at 1-hour post exercise. NO bioavailability in short term exercise training increases as a result of the upregulation of endothelial cell nitric oxide synthase (eNOS).[60] The increase in NO seen during acute exercise leads to an increase in artery diameter following chronic exercise[61], which allows for greater amounts of blood to flow without elevated shear stress acting on the endothelium, in turn reducing NO bioavailability. It is important to note that while large artery diameter may be a strong independent predictor of significant coronary artery disease[62], the vascular remodelling as a result of pathophysiological effects is different than that of exercise training.[63]

Not only does the duration of exercise training impact endothelial function, the type of exercise plays a role as well. Green et al.[64] found no significant changes to vascular function with regard to NO moderated vasodilation in response to 4 weeks of hand grip strength training. They hypothesized that perhaps a different result would be present if a different training method was used. These data were supported in a study done by Linke et al.[65] where subjects completed 4 weeks of lower limb exercise training on a cycle ergometer. They found

an improvement in endothelial function in the upper limb indicating that systemic improvements had been achieved. It will be important for future studies investigating the effectiveness of pulmonary rehabilitation on reducing cardiovascular risk in asthma to utilize appropriate exercise protocols (i.e. lower limb aerobic exercise) that will have the potential to positively impact endothelial function.

In general, increased physical activity is associated with better asthma control and reduced risk of exacerbation.[66,67] Therefore with the demonstrated improvements in endothelial function associated with physical activity[68], it is possible that individuals with asthma who have the highest cardiovascular risk may decrease this risk by increasing physical activity.

Asthma and cardiovascular risk in women

In an apparently healthy younger population, the risk of cardiovascular disease is higher in men than in women.[69] This is most likely due to the sex-hormone differences between men and women.[70] In contrast, young women with asthma have a greater cardiovascular risk compared to young men with asthma.[1,2,71] This suggests there is an important sex difference in how asthma is impacting cardiovascular risk. COPD appears to have similar association of sex, meaning women who smoke are at greater risk of developing COPD as well as have increased cardiovascular risk associated with COPD compared to men who smoke.[72] Women tend to have a faster decline of lung function associated with smoking as well.[73]

It has been reported that polymorphisms in the gene encoding estrogen receptor-α may be associated with the airway hyper-responsiveness seen in asthma, specifically in females.[74] This is potentially because changes in this gene results in altered action of estrogen.[71] Prior to puberty, there is a higher incidence of asthma among boys compared to girls. This difference tends to disappear after puberty when women become more likely to develop asthma, and this yet again changes in older adults, with men being more at risk of developing asthma.[75] This provides evidence that sex hormones may be responsible for severity of cardiovascular risk seen in women with asthma. The role estrogen plays in cardiovascular risk is still much debated as there appears to be both pro- and anti-inflammatory functions.[76] Reduced levels of estrogen have been associated with an increase in circulating levels of IL-6 and TNF-α, and this increase appeared to be attenuated when administered replacement estrogen.[77] To the contrary, a study done in female rats, ovariectomy or treatment with the estrogen receptor antagonist tamoxifen was found to decrease allergic airway inflammation, which returned to levels comparable to intact females when treated with estrogen replacement.[78] Sin et al.[72] have suggested that estrogen may be upregulating the metabolism of toxic metabolites leading to increased oxidative stress within the airways therefore increasing airway inflammation and potentially systemic inflammation as well.

As discussed above, systemic inflammation plays an important mediating role in cardiovascular disease, giving some evidence that hormonal changes may be increasing cardiovascular risk in women with asthma. Overall, it is not fully understood how sex hormones may be impacting asthma, yet it is generally accepted that this is a probable source

of the sex differences seen in asthma. For this reason, and because estrogen levels can impact

endothelial function[79], serum estradiol levels will be analysed and used as a covariate during

the analysis of FMD data if statistically appropriate.

An exploratory analysis done in our lab looked at the vascular and microvascular function of

men with asthma (n=13) and women with asthma (n=11) to determine potential sex related

differences contributing to cardiovascular risk. It revealed significantly lower microvascular

function in women, evaluated by hyperemic blood flow as velocity time integral (VTI),

compared to men.[80] This suggests reduced microvascular function may be a potential factor in

the increased cardiovascular risk seen in women with asthma compared to men with asthma.

A 2017 meta analysis looked at the relationship between asthma and coronary heart disease

within 11 trials totalling 666,355 participants included.[81] Coronary heart disease was

significantly higher in individuals with asthma both for prospective trials (HR 1.34, P = 0.005)

and for retrospective trials (OR 1.29, P = 0.001) when compared to healthy controls.

Interestingly, when put into subgroups according to sex, it was found that females with

asthma remained at significantly higher risk (HR 1.40, P<0.001), but males with asthma were

no longer significantly at risk as compared to their non-asthmatic counterparts (HR 1.19, P =

0.07). The authors continued to subgroup for analysis of risk based on sex as well as late or

early onset of disease. Based on pooled analysis of two trials, they reported that females with

late-onset asthma had the highest risk of CHD (HR 2.06, P<0.001). This suggests the previously

mentioned difficult to treat inflammatory profile of late-onset asthma may be an important

driving factor to increasing cardiovascular risk reported in asthma, and more specifically, women with asthma.

In summary, asthma is associated with an increased risk of cardiovascular disease such as coronary heart disease, cerebrovascular disease, heart failure and myocardial infarction, as well as an increased risk of all-cause mortality.[1,2] The reason(s) for this are unknown, however, evidence suggests that the vascular dysfunction, arterial stiffness and systemic inflammation seen with asthma may have an important role in the development of this increased risk.[8,54,82] Accordingly, the purpose of this study was to investigate the vascular function, arterial stiffness and systemic inflammation of individuals with physiologically confirmed asthma compared to those with an asthma diagnosis that cannot be confirmed by physiological testing. An additional comparison was made to healthy controls. An exploratory analysis explored the effects of sex, physical activity, asthma medication use, and presence of atopy on the previously described outcomes. Together, this provides us with information on the vascular implications of asthma, and helps differentiate the cardiovascular risk of individuals with physiologically confirmed asthma compared to those with an asthma diagnosis that cannot be confirmed through objective testing.

Chapter III: Vascular Risk Associated with Asthma

Introduction

Asthma is a chronic disease of the airways characterized by episodic bronchoconstriction and airway hyper responsiveness leading to symptoms such as wheezing, tightness of chest, and shortness of breath, as well as variable airflow limitation.[9] While typically thought of as a disease that solely affects the pulmonary system, it has become increasingly evident that there are systemic implications associated with asthma. Previous research has reported that individuals with asthma have an increased risk of cardiovascular morbidity and mortality.[2,21,22] Specifically, individuals with asthma have an increased risk of acute cardiovascular events such as myocardial infarction (MI, odds ratio (OR)=1.85), stroke (OR=1.84), and angina (OR=1.64).[21] Additionally, the risk of chronic cardiovascular disease such as coronary heart disease (hazard ratio (HR) 1.40), cerebrovascular disease (HR 1.20), and heart failure (HR 2.14) is also higher in people with asthma[2], however the reason for this is unclear.

Decreased vascular function as well as increased arterial stiffness and systemic inflammation, all of which have been reported in asthma[6–8], are independent predictors of cardiovascular risk in health and in disease.[3–5,15] Increased systemic inflammation has been directly linked to decreasing endothelial function in health[54], however this causal relationship has not yet been investigated in asthma. Past research has shown that people with asthma are generally less active than their healthy counterparts, which would also contribute to an increase in

cardiovascular risk.[59,83] Interestingly, previous research has reported significantly higher arterial stiffness in people with asthma even after matching for age, sex, BMI, physical activity and physical fitness levels.[7] This suggests that there are structural changes occurring in the vasculature related to asthma that cannot be explained by traditional cardiovascular risk factors. The reason for these changes are unclear; however, it has been suggested that asthma medication may be a contributing factor.[24]

Asthma is a heterogeneous disease with many different potential causes, triggers, and phenotypes, making it difficult to determine which individuals diagnosed with asthma may be at risk for cardiovascular morbidity and mortality. Asthma is typically diagnosed by history of symptoms and is physiologically confirmed by different tests/challenges: 1) spirometry, 2) airway responsiveness to inhalation challenge, 3) exercise challenge.[19] However, it is common for a physician to rely on clinical history to diagnose asthma rather than with the addition of objective testing. A cohort study of 90 individuals with physician diagnosed asthma reported that only 58% of participants had undergone previous pulmonary function testing of any kind, and only one participant had undergone an inhalation challenge.[84] Similarly, a retrospective study of 226 people with physician diagnosed asthma found that 45% of patients were prematurely being treated with inhaled corticosteroids prior to obtaining a diagnosis confirmed with objective testing.[85] In addition, approximately 25-35% of patients diagnosed with asthma in primary care cannot be confirmed as having asthma by objective physiological testing[9]. This suggests that a large portion of patients may be receiving asthma treatment/medication inappropriately. Importantly, previous studies[2,22] reporting increased

cardiovascular risk in asthma did not exclusively include individuals with asthma confirmed by physiological testing, therefore, the cardiovascular risk of individuals that do not demonstrate asthma with physiological testing remains unclear.

In order to better understand the increased cardiovascular risk that has been reported in asthma, as well as possible factors which could contribute to this risk, such as asthma medication use, we sought to compare vascular function, arterial stiffness and systemic inflammation in individuals with physician-diagnosed asthma that cannot be physiologically confirmed to those individuals with an asthma diagnosis that can be confirmed by physiological testing. In addition, a group of healthy controls were recruited for further comparison. There are various studies linking respiratory symptoms/diseases to increased cardiovascular risk[71,86–90], making it likely that predictors of cardiovascular risk (i.e. vascular function, arterial stiffness and systemic inflammation) would be similar among individuals with a history of respiratory symptoms, regardless of whether asthma is demonstrated with objective testing. Therefore, it was hypothesized that individuals with an asthma diagnosis that cannot be confirmed by physiological testing would have similar vascular function to individuals with an asthma diagnosis that is confirmed by physiological testing. Additionally, it was hypothesized that both of these asthma groups would have decreased vascular function when compared to age-, sex-, and BMI-matched healthy controls.

Methods

Ethics approval and participant description

This study was approved by the University of Alberta Health Research Ethics Boards (Pro00083372). Healthy controls were recruited from the general population. Individuals with asthma were recruited from the Lung Health Clinic, University Lung Clinic, and the general population. Individuals recruited from the University Lung Clinic or the Lung Health Clinic had their files reviewed for previous objective assessments to confirm an asthma diagnosis. Individuals recruited from the general population were initially identified by self-reported history of asthma diagnosis or asthma medication usage. Participants over the age of 55 and under the age of 18, current smokers or individuals with >10 pack year smoking history, and those with a history of known cardiovascular disease, pulmonary disease other than asthma, or any know chronic inflammatory or metabolic disease were excluded from this study. Fifteen participants with an asthma diagnosis confirmed by physiological testing, 12 participants with an asthma diagnosis that could not be confirmed by physiological testing, and 13 age-, sex-, and BMI-matched healthy controls were recruited for this study (see Table 1 for participant characteristics). All participants provided written, informed consent.

Physiological confirmation of asthma was based on a physician diagnosis/clinical history of asthma and one of: 1) significant reversibility of FEV_1 of $\geq$ 12% and 200mL[19], 2) 20% reduction of FEV_1 following a methacholine challenge[13], or 3) 10% reduction of FEV_1 following an exercise challenge[13]. All participants completed a full pulmonary function test and participants

underwent the appropriate physiological tests, as determined by the research team, in order to confirm an asthma diagnosis. Individuals that had a diagnosis of asthma but were unable to confirm a diagnosis of asthma through objective testing were labelled as unconfirmed asthma (see figure 1 for consort diagram of recruitment and objective assessments).

Study Design

A cross-sectional cohort study design was used to compare vascular function, arterial stiffness and systemic inflammation of individuals with physician-diagnosed asthma that cannot be physiologically confirmed to those with an asthma diagnosis that can be confirmed by physiological testing. Each participant (confirmed asthma, unconfirmed asthma, healthy) underwent the same evaluations, with the exception of asthma questionnaires being administered only to the asthma groups. Participants received instructions prior to testing, requesting they withhold from medication, food, caffeine, alcohol, smoking, and exercise for at least 12 hours prior to the appointment. Height, weight, and waist circumference were measured in all participants. Individuals in either asthma group completed two quality of life questionnaires; the standardized Asthma Quality of Life Questionnaire (AQLQ) in order to assess both the physical and psychosocial effects associated with their asthma diagnosis, and the EQ-5D (5L) in order to evaluate generic health status and health related-quality of life. Additionally, all participants with asthma completed the Asthma Control Questionnaire (ACQ) to assess their symptom control. This was followed by 10 minutes of supine rest in a quiet, dark, temperature controlled room. Applanation tonometry was performed to measure

carotid-radial pulse wave velocity (crPWV), followed by brachial artery flow-mediated dilation

(FMD) to determine endothelial function and lastly a blood sample was obtained to measure

serum C-reactive protein (CRP) and estradiol. The participants then completed a full

pulmonary function test, including spirometry before and after the administration of

salbutamol. A Fitbit was given to track average physical activity over seven days. Individuals

with an asthma diagnosis that could not be confirmed by a significant improvement in FEV_1

were scheduled for a follow-up exercise challenge and methacholine challenge to be

completed on a separate day. Healthy controls that demonstrated >5% increase in FEV_1 after

the administration of salbutamol underwent an exercise challenge to investigate presence of

exercise-induced bronchoconstriction.

Procedures

Arterial Stiffness

After 10 minutes of resting in a dark, quiet, temperature controlled room in the supine

position, two consecutive brachial blood pressure measurements were taken using an

automatic blood pressure monitor (BpTRU Medical Devices, Coquitlam, BC) with the lowest

values being reported. Next, crPWV was recorded using applanation tonometry (Complior,

Alam Medical, Saint Quentin Fallavier, France). A series of 10 consecutive beats were taken

with an average quality of 87% as determined by the Complior analysis software. The distance

between the carotid and radial artery was recorded in mm and input into the program to be

used in the software's algorithm to produce the reported crPWV (see appendix A for detailed calculations).

Endothelial Function

A sphygmomanometer was placed on the right forearm distal to the antecubital fossa. With the arm extended, ultrasound imaging (8L-RS 4.0-13.0MHz probe, Vivid q, GE Healthcare, Mississauga, ON) was used to locate a clear image of the brachial artery proximal of the antecubital fossa, and four 16-second images were stored for baseline values. The cuff was then inflated to 200 mmHg or 50 mmHg above systolic pressure, whichever was higher, and remained inflated for 5 minutes. Prior to release, another 16-second image was stored to analyse diameter and blood velocity during arterial occlusion. Once the cuff was released, a series of 12-13 16-second images were stored for analysis of post-release diameter and blood velocity. Diameter change from baseline was analysed in 8 second averages using Carotid Analyzer (Medical Imaging Applications, LLC, Coralville, IA, USA). Brachial artery blood velocity was averaged in two eight-second time increments for every 16 second sweep, using EchoPAC PC software (version 110.x.x, GE Healthcare, Horten, Norway), and was used to calculate blood flow (see appendix A for detailed calculations).

Time to peak dilation following cuff release was assessed as a supplemental indicator of vascular function, as it has been reported that a delayed time to peak is associated with decreased vascular funciton.[86] In addition, microvascular function was determined by velocity

of reactive hyperemia (VRH) of first beat following cuff release (see appendix A for detailed calculations), as it has also been reported as a significant marker for adverse cardiovascular outcomes.[91]

As within-subject variability of FMD often varies widely between studies (1.8%-50%)[10–12], the primary ultra-sonographer tested three healthy individuals (2 males, 1 female) for three consecutive days to determine intra-rater reliability using coefficient of variation (CV) prior to the study. Participants were asked to standardize all confounding variables (i.e. fasted state, medications, time of day, phase of menstrual cycle, hours of sleep in previous night) and after adjusting for shear stress, CV was calculated as standard deviation/mean*100. The reported intra-rater mean CV was 5.13%, demonstrating competency in this technique.

Venipuncture and Blood Analysis

Venous blood was collected from each participant on the same day as vascular testing via antecubital venipuncture by a certified phlebotomist following specified guidelines.[92] With the participant sitting in a relaxed position, the upper arm was occluded to increase the accessibility of the blood vessels. Once a vein was chosen (based on proximity to the surface, size, position and absence of scar tissue and/or pulsation), a sterile 21- or 23-gauge butterfly needle was inserted at a 30-degree angle or less to initiate blood draw. Blood was collected in anti-coagulant-free polypropylene tubes pre labeled with the participant's identification number and date of collection. After the collection was complete, the tourniquet was

removed followed by the needle and pressure was applied to the venipuncture site and held until the bleeding stopped. The blood was left to clot for a minimum of 30 minutes and a maximum of 90 minutes followed by centrifuging at 1200g for 10 minutes at 4 degrees Celsius. The serum was separated into aliquots of 100 μL immediately after centrifuging and then stored at -80 degrees Celsius until analysis.

Analysis of serum was done at the University of Alberta using enzyme linked immunosorbent assay (ELISA) protocol.[93] High sensitivity human CRP (Catalog # DY1707, R&D Systems, Inc.) and human estradiol (Catalog # KAQ0621, Invitrogen Corporation) ELISA kits were used as per manufacturers' protocols.[94–96]

Initially, estradiol values were obtained in all participants (n=35) to be used as a covariate in the statistical analysis of FMD in order to account for menstrual cycle variation. There was no relationship between estradiol and FMD for all participants (r= -0.17, p=0.33). However, when individual correlations for FMD and estradiol were done for each group: confirmed, unconfirmed, and healthy the respective relationships were found: r=0.18 (p=0.60), and r= -0.55 (p= 0.06), and r=0.26 (p=0.41). Due to the varying relationship between estradiol and FMD between the groups, estradiol was not used during the statistical analysis of FMD. Omission of this covariate did not significantly affect the results reported.

Physical Activity

Physical activity was evaluated by average daily step count using a Fitbit (Fitbit Inc., San Francisco, CA, USA) activity monitor. The Fitbit Flex 2 wristband tracker was to be worn for seven days, only to be removed to shower/bathe and to sleep. After the seven days were completed, the Fitbit was analysed upon return using custom software. The participants were not able to access any information regarding their activity (including step count or heart rate) while they were wearing the device. Total steps in each 24-hour period were averaged and used to determine average physical activity levels. Participants were also categorized by physical activity level; 1) low activity: <7,500 steps/day, 2) somewhat active: 7,500-9,999 steps/day, 3) active: > 10,000 steps/day.[17]

Pulmonary Function Test

The participant was instructed to withhold short-acting medication for at least eight hours, and long acting medication for at least 48 hours prior to testing.[97] Prior to testing, the spirometry system (Vmax, CareFusion, Yorba Linda, CA, USA) was calibrated as per manufacturer's instructions. Participants were coached through the breathing maneuvers before completing the test. Lung volumes[98], plethysmography[98], spirometry[97], and diffusion limitation (DLCO)[99] were all completed and interpreted[100] as per standardized procedures.

All spirometry values are reported as absolute values (L), and FVC as well as FEV_1 will also be reported as percentage predicted according to calculated reference values (% reference

value). The highest values obtained that met American Thoracic Society quality criteria were used to calculate FEV_1/FVC, even if they were from separate maneuvers, and these values will be reported as % ref as well. [97]

Statistical Analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS) for Windows version 25.0 (SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation (SD) unless indicated otherwise. The probability of a Type I error was set at 0.05. Unadjusted and adjusted FMD values for area under the curve (AUC) shear stress are reported in Table 2. Unadjusted and adjusted crPWV values for diastolic blood pressure are reported in Table 3.

Primary Analysis

A one-way analysis of covariance (ANCOVA) was used to evaluate differences between the three groups: confirmed asthma, unconfirmed asthma, healthy (fixed factor) for FMD (dependant factor) adjusted for AUC sheer stress (covariate). A one-way ANCOVA was used to evaluate differences between the three groups (fixed factor) for crPWV (dependent factor) adjusted for diastolic blood pressure (covariate). A one-way analysis of variance (ANOVA) was used to evaluate the difference between the three groups for: systemic inflammation (CRP), microvascular function (VRH), and time to peak dilation for FMD.

Unpaired Student's t-tests were used to evaluate the same outcomes comparing the unconfirmed asthma group to the confirmed asthma group. Additionally, the same analysis was completed using unpaired Student's t-tests to evaluate the same outcomes comparing all participants with asthma combined (n=27) to healthy controls (n=13).

Exploratory Analysis

An unpaired Student's t-test was also used to determine any differences in FMD based on allergy status (some, none) for all participants combined.

An unpaired Student's t-test was used to determine any differences in FMD based on age of onset (early- or late-) for both asthma groups combined. Unpaired Student's t-tests were used to determine sex differences (male, female) of FMD for all participants combined (n=40) as well as for both asthma groups combined (n=27).

A chi squared test was used to determine between group differences in activity level categories. A one-way ANOVA was used to determine between-group differences in average steps per day. An unpaired Student's t-test was used to compare average steps per day between both asthma groups combined (n=16) and healthy controls (n=13). A one-way ANCOVA was used to determine differences in FMD (co-varied for shear stress) when all participants were combined and stratified by physical activity level categories (low, moderate, high). Pearson's correlation was used to determine relationships for all participants combined (n=29) between FMD and average steps per day. Pearson's correlation was used to determine

a relationship between FMD and average steps per day for both asthma groups combined (n=16).

Both asthma groups were combined and stratified by asthma medication usage (Group 1: no asthma medication, Group 2: SABA as needed only, Group 3: SABA, ICS/combination, Group 4: SABA, ICS/combination, add-on treatment), and a one-way ANCOVA was used to evaluate differences in FMD (co-varied for shear stress) based on medication use. An unpaired Student's t-test was used to determine any differences in FMD based on the use of no asthma medication vs. any asthma medication for both asthma groups combined.

Unpaired Student's t-tests were used to determine difference in ACQ and AQLQ between confirmed and unconfirmed asthma groups. All participants with asthma (n=27) were categorized as well controlled, moderately controlled, and poorly controlled based on ACQ score, and a one-way ANCOVA was used to determine differences in FMD (co-varied for shear stress) between these categories. Unconfirmed asthma and confirmed asthma groups were combined (n=27), and Pearson's correlation was used to determine relationships between FMD and: ACQ and AQLQ.

Pearson's correlation was used to determine relationships between FMD and CRP for all participants combined (n=38), as well as for participants with asthma only (n=26).

Results

Participants

Please refer to Table 1 for participant characteristics. Briefly, all participants had similar weight, height, and age. Pre- and post-bronchodilator lung function (FEV_1 % predicted) was significantly lower in the confirmed asthma group compared to the unconfirmed asthma group (p=0.003, p=0.02, respectively) and trended towards significantly lower compared to healthy controls (p=0.06). Healthy controls were well matched to both asthma groups for BMI and sex. PWV was unavailable for 3/40 participants, blood analysis was unavailable for 2/40 participants, and Fitbits were worn by 29/40 of the participants.

Primary Analysis

When comparing confirmed asthma, to unconfirmed asthma and health controls, there were no between-group differences in FMD (confirmed: 7.97 ± 2.77%, unconfirmed: 7.62 ± 2.77%, healthy: 9.47 ± 2.76, p=0.21, figure 2), crPWV (confirmed: 8.36 ± 1.36 m/s, unconfirmed: 8.51 ± 1.38 m/s, healthy: 8.06 ± 1.37 m/s, p=0.71, figure 3), systemic inflammation (confirmed: 3.53 ± 2.90 mg/L, unconfirmed: 3.77 ± 1.78 mg/L, healthy: 4.45 ± 3.37 mg/L, p=0.72, figure 4), microvascular function (confirmed: 72.19 ± 17.56 cm/s, unconfirmed: 68.67 ± 22.47 cm/s, healthy: 69.28 ± 13.24 cm/s, p=0.86), or time to peak dilation (confirmed: 62.93 ± 19.56 seconds, unconfirmed: 76.00 ± 20.61 seconds, healthy: 68.31 ± 25.43 seconds, p=0.32).

When comparing the confirmed asthma group to the unconfirmed asthma group, there were no differences found in FMD (confirmed: 7.99 ± 2.70%, unconfirmed: 7.59 ± 2.94%, p=0.72), crPWV (confirmed: 8.33 ± 1.42 m/s, unconfirmed: 8.60 ± 0.97 m/s, p=0.60), CRP (confirmed: 3.53 ± 2.90 mg/L, unconfirmed: 3.77 ± 1.78 mg/L, p=0.81), microvascular function (confirmed: 72.19 ± 17.56 cm/s, unconfirmed: 68.67 ± 22.47 cm/s, p=0.65), or time to peak dilation (confirmed: 62.93 ± 19.56 seconds, unconfirmed: 76.00 ± 20.61 seconds, p=0.11).

As both asthma groups demonstrated no clinically or statistically significant differences, they were combined and compared to healthy controls. A trend was observed with FMD between groups, with the asthma group demonstrating lower FMD compared to healthy controls (7.81 ± 2.76%, 9.48 ± 2.55%, p=0.08). There were no differences in crPWV (asthma: 8.47 ± 1.20 m/s, control: 8.00 ± 1.64 m/s, p=0.33), systemic inflammation (asthma: 3.64 ± 2.40 mg/L, control: 4.45 ± 3.37 mg/L, p=0.43), microvascular function (asthma: 70.62 ± 19.57 cm/s, control: 69.28 ± 13.25 cm/s, p=0.82), or time to peak dilation (asthma: 68.74 ± 20.73, control: 68.31 ± 25.43, p=0.95) found between groups.

Exploratory Analysis

Allergy Status

When all participants were included (n=40), and then stratified by allergy status (none vs. some), a trend towards a significant difference in FMD values was observed where individuals

with allergies tended to have lower FMD (allergic: 7.70 ± 2.24% vs. non-allergic: 9.24 ± 3.23%, p=0.08, see figure 5).

Age of Asthma Onset and Sex Difference

With all participants with asthma combined (n=27), age of asthma onset (<18 years old- vs. ≥ 18 years old) did not affect FMD (8.06 ± 3.19% vs. 6.15 ± 2.63%, p=0.22).

With all participants combined (n=40), sex did not influence FMD (male: 7.97 ± 2.94%, female: 8.56 ± 2.72%, p=0.53). Similarly, when only the participants with asthma were examined (n=27), there was no significant difference between males and females (male: 7.16 ± 2.21%, female: 8.13 ± 3.00%, p=0.40).

Physical Activity

Please refer to Table 4 for physical activity data. There was no significant difference in activity level (low, moderate, high) between the three groups (p=0.19). However, there was a trend towards significant difference in steps per day between the three groups (confirmed: 6826.91 ± 3254.01, unconfirmed: 6994.56 ± 3171.36, healthy: 10918.49 ± 6252.29, p=0.11). When both asthma groups were combined (n=16), there was a significant difference observed in average steps per day compared to healthy controls (all asthma: 6931.69 ± 3093.71, control: 10918.49 ± 6252.29, p=0.05, see figure 6). With all participants combined (n=29), stratification

based on physical activity level did not influence FMD (low activity: 8.23 ± 3.02%, moderate

activity: 9.16 ± 3.01%, high activity: 8.55 ± 3.02%, p=0.80). With all participants combined

(n=29), there was no correlation observed between average steps per day and FMD (r=0.06,

p=0.78). However, a significant correlation was observed between average steps per day and

FMD within the participants with asthma (n=16, r=0.60, p=0.02).

Medication Use

Within the participants with asthma (n=27), when patients were grouped by types of asthma

medication use (medication grouping described above) there was no significant difference in

FMD (Group 1: 9.85 ± 2.64%, Group 2: 7.11 ± 2.74%, Group 3: 7.63 ± 2.68%, Group 4: 7.20 ±

2.64%, p=0.31). When participants with asthma (n=27) were grouped into no asthma

medication use vs. any asthma medication use, a trend was observed in FMD between groups,

with the participants with asthma who use any asthma medication demonstrating lower

vascular function (any: 7.37 ± 2.59% vs. no: 9.75 ± 2.90%, p = 0.08, see figure 7).

Quality of life and vascular function

No significant difference in AQLQ was found between confirmed (5.7 ± 1.2) and unconfirmed

asthma (6.5 ± 0.3, p=0.10). There was a significant difference in ACQ score between confirmed

asthma (1.0 ± 0.7) and unconfirmed asthma (0.4 ± 0.5, p=0.02). When all participants with

asthma (n=27) were sub-grouped based on asthma control category (well controlled n=17,

moderately controlled n=5, uncontrolled n=5), no significant differences in FMD were

observed between groups (7.58 ± 2.68, 9.28 ± 2.68, 7.12 ± 2.68, respectively, p=0.39). There

was no relationship between FMD and either ACQ (r= -0.01, p=0.98) or AQLQ (r= -0.02,

p=0.94) for participants with asthma (n=27).

Inflammation and vascular function

There was no relationship between FMD and CRP for all participants (n=40, r=0.15, p=0.39).

Similarly, no correlation was found between FMD and CRP for participants with asthma only

(n=24, r=0.20, p=0.34).

Discussion

The current study aimed to compare vascular function, arterial stiffness and systemic

inflammation in individuals with a physician diagnosis of asthma that was confirmed by

physiological testing, to those with a physician diagnosis of asthma that cannot be confirmed

by physiological testing. Previous research has found that individuals with asthma have an

increased risk of cardiovascular disease or events, however these studies did not differentiate

risk between participants with confirmed and unconfirmed asthma. Importantly, the current

study found there were no differences between confirmed and unconfirmed asthma for FMD,

crPWV, or CRP. As such, confirmed asthma and unconfirmed asthma groups were combined,

and when compared to healthy controls, the participants with asthma demonstrated clinically

significant (i.e. -1.67%) and nearly statistically significant (p=0.08) lower vascular function. The

results of this study suggest that objective confirmation of asthma alone is not an important

determinant of cardiovascular risk. Rather, other factors associated with a diagnosis of asthma, such as asthma medication use or physical inactivity, may be more important determinants of increased cardiovascular risk in people with asthma.

Vascular Function

Endothelial function is an independent predictor of cardiovascular risk.[51] Specifically, the endothelium is responsible for preventing platelet adhesion and cell proliferation.[47] Subsequently, endothelial dysfunction plays a key role in the development of atherosclerosis by promoting platelet adhesion to the arterial wall due to reduced nitric oxide (NO) bioavailability.[52] This directly contributes to increased cardiovascular risk including risk of coronary artery disease, stroke and MI.[5] Previous work has shown that individuals with asthma confirmed by physiological testing have worse vascular function as compared to healthy controls, with those having moderate-to-severe asthma having worse endothelial function as compared to those with mild asthma.[8] The current study found no difference in vascular function between confirmed and unconfirmed participants with asthma, suggesting that the factors related to lung function and inflammation may not be important contributors to cardiovascular risk in asthma.

Previous work has reported that individuals with asthma that use any asthma medication have an increased risk of cardiovascular disease compared to individuals with asthma that use no asthma medication.[2] In the current study, a trend was observed among all participants with

asthma suggesting that those using any asthma medication tended to have lower vascular function compared to those using no asthma medication (p=0.08). It is plausible that the use of asthma medication is contributing to the lower vascular function seen in both asthma groups compared to healthy controls. The negative impact that asthma medication has on vascular function is likely the result of chronic medication use, as all participants withheld long acting medication and ICS for >48 hours and short acting medication for >12 hours prior to testing. Acute cardiovascular effects of asthma medication have been observed, with administration of 400µg of salbutamol temporarily increasing arterial stiffness and decreasing vascular function in participants with asthma.[28] This provides evidence that asthma medication may be a key contributing factor to the reported cardiovascular risk associated with asthma.

Another potential factor contributing to the decreased vascular function in confirmed and unconfirmed asthma is the significantly lower physical activity measured in average steps per day compared to healthy controls (p=0.05). Individuals with asthma tend to be less active compared to their healthy counterparts[58,59] and physical activity is correlated with vascular function. [101] It is well known that decreased physical activity leads to increased cardiovascular risk[102,103], and therefore it is plausible that physical activity/fitness may be a critical factor in the increased cardiovascular risk associated with asthma. Indeed, previous research has shown that when matched for fitness and physical activity, people with asthma have similar vascular function to healthy controls.[7] The current study did not find a correlation between all participants FMD and physical activity(r=0.06, p=0.78), however, a relationship was observed

between FMD and physical activity for the participants with asthma r=0.60, p=0.02, suggesting

physical activity may be an important target to reduce the cardiovascular risk associated with

asthma.

Clinically relevant reference values for FMD are not currently agreed upon within the

literature due to inconsistent imaging and analysis methods, resulting in largely variable FMD

results.[104] The current study used FMD methodology consistent with consensus

guidelines[14,105] in order to reduce variability and increase precision. Because of differences in

methodology, it is difficult to compare vascular function data in the current study to previous

research, and therefore the current study has defined clinical significance as a difference of

1% for FMD, as this has been previously shown to impact cardiovascular risk.[106] The asthma

participants demonstrated 1.67% lower FMD compared to healthy controls. Late-onset

asthma (1.91% lower), presence of atopy (1.54% lower), and asthma medication use (2.38%

lower) was also associated with clinically significantly lower FMD compared to controls.

Arterial Stiffness

Arterial stiffness, which evaluates vascular structure, is also an independent predictor of

cardiovascular risk.[15,107] Vessel diameter as well as increased collagen and decreased elastin

within the vessel contribute to increased vascular stiffness.[38,108,109] It has been suggested that

arterial stiffness precedes hypertension, making it an early predictive marker for adverse

coronary and cardiovascular events.[110] Increased arterial stiffness causes a premature return

of reflected pulse waves back to the heart resulting from increased pulsatile flow in the distal arteries.[110] When this increased stress is chronically present it results in left ventricular hypertrophy, decreased stroke volume, and decreased cardiac output.[111,112] These negative changes may eventually result in heart failure.[113,114]

Previous research has reported that people with asthma have significantly increased arterial stiffness compared to healthy controls.[7] The reason(s) for this are unclear, though it has been suggested that medication use may be an important factor.[24] A previous study done in our lab investigating the effects of salbutamol on arterial stiffness found that after administering a clinically relevant dose of salbutamol, people with asthma demonstrated temporarily increased arterial stiffness.[28] This effect was not found in healthy controls. Importantly, the study by Moore et al. did not exclusively include people with confirmed asthma, but rather used a physician diagnosis and history of salbutamol use as inclusion criteria. This suggests that history of medication use may be an important factor contributing to cardiovascular risk in people with asthma regardless of objective confirmation.

The current study did not find a significant difference in arterial stiffness between the three groups, which is contrary to the previously described research.[7] All three groups demonstrated carotid-radial pulse wave velocity within the healthy range of 8-10 m/s.[115] Similarly, no significant difference in arterial stiffness was found when all participants with asthma were combined and compared to healthy controls. It is important to recognize that

the average age of all participants was <30 years old. At this young age, it is likely that substantial structural changes (i.e. changes to elastin and collagen) are not sufficient to substantially affect arterial stiffness (PWV).

Systemic Inflammation

Systemic inflammation is an independent predictor of cardiovascular risk[3], and is thought to negatively impact endothelial function[54] and arterial stiffness.[108] CRP is an inflammatory cytokine produced in the liver that has demonstrated a strong association to cardiovascular risk.[3] Specifically, CRP suppresses endothelial NO synthase (eNOS) expression and activity[35] resulting in increased adhesion of inflammatory cells to the endothelium[36], and attraction of inflammatory cells to the site of vascular injury.[37] Subsequently, the decrease in bioavailability of NO impairs vascular function, which is demonstrated by decreased FMD.[116] Increased systemic inflammation has been reported in people with asthma when compared to healthy controls.[6,45] Considering the effect of systemic inflammation on the previously mentioned predictors of cardiovascular risk, this is a plausible mechanism behind the increased cardiovascular risk reported in people with asthma.

In the current study, CRP values across all groups were highly variable and followed a contradictory trend compared to previous literature, with the healthy controls tending to have higher systemic inflammation. Importantly, from a clinical perspective all three groups were classified as high risk based on their serum CRP levels.[39] Upon follow-up discussions with

healthy control participants, some individuals revealed health conditions they had in their past (but were not currently experiencing, i.e. childhood cancer) that had previously not been disclosed. It is possible that these past health conditions are currently impacting these individuals' systemic inflammatory response.

Alternatively, this paradoxical inflammation data may be a result of genetics or potentially lifestyle conditions that could not be accurately accounted for. For example, some healthy control participants disclosed following testing that they had a family history of cardiovascular disease, were under stress from school, had decreased sleep the night prior to testing, or were following an unhealthy diet and exercise regime leading up to testing. More rigorous selection for healthy control participants would be an important adjustment to future research to include previous health issues, family history of health issues, exercise and diet information, sleep quality, and current stress levels.

Statistical Power

As no difference in vascular function was found between confirmed asthma and unconfirmed asthma, a power calculation was completed to ensure this negative result was not due to inadequate statistical power. Using the mean ± standard deviation for each group (confirmed: 7.99 ± 2.70%, unconfirmed: 7.59 ± 2.94%) an effect size of 0.14 was determined. In order to detect a significant difference between these two groups a total sample size of 1566

participants would be required to give a power of 0.80 ($\alpha = 0.05$, two tailed). Therefore, it is reasonable to assume that this negative result is not due to a lack of statistical power.

Limitations

Participants were requested to arrive for testing prior to 12PM in an attempt to standardize diurnal variation that has been reported among the outcomes tested. Not all participants were able to do so for a variety of reasons, mainly work scheduling conflicts. Some of the variability observed in FMD, inflammation, and arterial stiffness may be a direct result of the time of testing.

Additionally, there were technical issues with the Fitbit activity tracking devices at the beginning of data collection. As a result, the first 17 participants were asked to wear the device a second time. For these participants, the vascular function and inflammation would have been evaluated between 1-5 months prior to the recorded daily physical activity. Therefore, this decreased accuracy of measurement may introduce variability when evaluating the relationship of vascular outcomes to physical activity, as the physical activity at the time of vascular outcomes may differ from the reported physical activity.

Physiological confirmation of asthma was done using previously mentioned tests. Not all unconfirmed participants with asthma were able or willing to complete the follow up tests

required to objectively confirm an asthma diagnosis. All participants completed a full

pulmonary function test including pre- and post- spirometry. Six of the 12 participants

completed both an exercise and methacholine challenge, while six did not complete a

methacholine challenge. Similarly, three did not complete an exercise challenge, and there

were three participants who did not complete both an exercise and methacholine challenge.

Therefore, it is possible that participants labelled with unconfirmed asthma may have tested

positive to the uncompleted objective tests, placing them in the confirmed asthma group.

As previously reported [117–119], there is the potential of a false negative result with the

objective assessments used. A false negative result to the methacholine challenge may occur

if: 1) the participant was receiving intensive anti-inflammatory treatment prior to the

challenge, supressing airway hyper-responsiveness, 2) the participant was not exhibiting

current symptoms, and 3) the participant experiences occupational asthma which is triggered

by specific chemical sensitivities that are not present during the challenge.[117] Allergens may

induce airway hyper-responsiveness in atopic participants that previously demonstrated

normal airway responsivenss.[118] Therefore, it is possible that if these participants were tested

out of season when allergens are no longer present, that they may demonstrate normal

spirometry and normal airway responsiveness producing a false-negative result (i.e. failing to

detect presence of airway hyper-responsiveness). A previous study found that approximately

10% of participants with a physician diagnosis of asthma and a negative methacholine

challenge were able to obtain a positive methacholine challenge upon tapering of anti-

inflammatory medications.[119] Standardized instructions were given to each participant,

requesting they withhold any long acting asthma medication for 48 hours and short acting

asthma medication for 8 hours prior to testing. This directly aligns with the methacholine

challenge testing guidelines,[117] increasing the sensitivity of the test, and therefore reducing

the likelihood of a false negative as a result of medication supressing airway hyper-

responsiveness.

False positive methacholine challenge results are also of concern when attempting to

objectively confirm an asthma diagnosis. Individuals that have recently had a viral infection,

have allergic rhinitis, and have a smoking history/chronic obstructive pulmonary disease

(COPD) may produce a false-positive methacholine challenge.[13] Specifically, in such cases the

PC_{20} can be <8 mg/ml but the participant does not actually have asthma. About 30% of

patients with allergic rhinitis that do not have asthma will have a PC_{20} in the range of 4 – 16

mg/ml, exhibiting borderline bronchial hyper-responsiveness.[13] Interpretation of results

should consider patient history, as well as pre-test probability of asthma.[13] The current study

included participants with a history of atopy, however, only one confirmed asthma participant

disclosed experiencing symptoms of allergic rhinitis and was being investigated for other

sinus-related conditions. This participant obtained a PC_{20} of 8.60 mg/ml. No participants with

a potential diagnosis of COPD were included in the study as per the exclusion criteria. Though

unlikely, a small number of participants may have been misclassified based on a potential

false-positive or false-negative asthma screening test.

Conclusion

In conclusion, this study examined the vascular function, arterial stiffness and systemic inflammation of individuals with a physician diagnosis/clinical history of asthma that was confirmed by physiological testing, and compared these results to individuals with a physician diagnosis/clinical history of asthma that could not be confirmed by physiological testing. A group of healthy controls were recruited for additional comparison. There were no clinically or statistically significant differences in vascular function, arterial stiffness or systemic inflammation between confirmed and unconfirmed asthma. As no differences were observed, the asthma groups were combined and when compared to healthy controls, the participants with asthma demonstrated clinically significant and nearly statistically significant (p=0.08) lower vascular function. In support of previous research, asthma medication use and physical inactivity were identified as potential contributing factors to the cardiovascular risk associated with asthma. More research is needed to investigate the effects of asthma medications (type, dosage, length of use) and physical activity on the vascular health of individuals with asthma, regardless of whether their asthma can be confirmed with objective testing. Medication use and decreased physical activity may be a strong starting point when determining whom with asthma may be at risk for cardiovascular disease and might best benefit from pulmonary rehabilitation.

Tables

Table 1. Participant characteristics. Values are expressed as mean ± standard deviation.

	Confirmed Asthma	Unconfirmed Asthma	Healthy Control	*p value*
n=(M/F)	15 (6/9)	12 (3/9)	13 (5/8)	0.68
Weight (kg)	77.2 ± 28.6	70.2 ± 19.3	70.4 ± 16.1	0.64
Height (cm)	171.1 ± 10.9	170.8 ± 11.9	167.9 ± 6.7	0.67
BMI (kg/m^2)	25.8 ± 6.1	23.8 ± 4.2	24.9 ± 4.9	0.60
Age (years)	28 ± 6	27 ± 8	24 ± 3	0.23
Waist circumference (cm)	82.8 ± 23.2	77.8 ± 14.1	79.9 ± 12.2	0.81
PRE FEV$_1$ absolute (L)	3.4 ± 0.7	3.9 ± 1.0	3.7 ± 0.6	0.23
POST FEV$_1$ absolute (L)	3.6 ± 0.8	4.1 ± 1.1	3.8 ± 0.8	0.42
PRE FEV$_1$ % predicted	86 ± 14[†]	102 ± 10	96 ± 8	<0.01
POST FEV$_1$ % predicted	93.3 ± 13.9[†]	106.3 ± 9.4	99.4 ± 10.1	0.02
FEV$_1$ % reversibility	9 ± 10	5 ± 3	3 ± 6	0.09
SABA usage (n=)	9	9	-	0.41
ICS usage (n=)	1	3	-	0.18
Combination (ICS + LABA) usage (n=)	6	4	-	0.72
Allergies (n=)	12[*]	8	3	<0.01
ACQ score	1.0 ± 0.7[†]	0.4 ± 0.5	-	0.02
AQLQ score	5.7 ± 1.2	6.5 ± 0.3	-	0.10

Waist circumference measurements in confirmed asthma include n=6 (3/3), *represents statistically significant difference between confirmed asthma and healthy controls, † represents statistically significant difference between confirmed asthma and unconfirmed asthma. BMI: body mass index; PRE: pre-bronchodilator; FEV$_1$: forced expiratory flow in one second; POST: post-bronchodilator; SABA: short acting beta-agonist; ICS: inhaled cortico-steroid; LABA: long acting beta-agonist; ACQ: asthma control questionnaire; AQLQ: asthma quality of life questionnaire.

Table 2. Adjusted and unadjusted flow mediated dilation (FMD) from baseline. Values are expressed as mean ± standard deviation.

	Confirmed Asthma	Unconfirmed Asthma	Healthy Control	*p value*
n=(M/F)	15 (6/9)	12 (3/9)	13 (5/8)	0.68
FMD % Increase	7.99 ± 2.70	7.59 ± 2.94	9.48 ± 2.55	0.20
FMD % Increase[a]	7.97 ± 2.77	7.62 ± 2.77	9.47 ± 2.76	0.21

[a] adjusted for area under the curve shear stress reactive hyperemia (AUC SSHR). FMD: flow mediated dilation.

Table 3. Adjusted and unadjusted carotid-radial pulse wave velocity (crPWV). Values are expressed as mean ± standard deviation.

	Confirmed Asthma	Unconfirmed Asthma	Healthy Control	*p value*
n=(M/F)	12 (6/6)	12 (3/9)	13 (5/8)	0.16
crPWV	8.33 ± 1.42	8.60 ± 0.97	8.00 ± 1.64	0.56
crPWV[a]	8.36 ± 1.36	8.51 ± 1.38	8.06 ± 1.37	0.71

[a] adjusted for diastolic blood pressure (DBP). crPWV: carotid-radial pulse wave velocity.

Table 4. Physical activity. Values are expressed as mean ± standard deviation.

	Confirmed Asthma	Unconfirmed Asthma	Healthy Control	p value
n (M/F)	6 (1/5)	10 (3/7)	13 (5/8)	0.74
average steps/day	6827 ± 3254	6995 ± 3171	10918 ± 6252	0.11
Activity level (low/mod/high)	3/3/0	6/2/2	5/2/6	0.19

Low activity level: <7,500 steps/day; moderate activity level: 7,500-9,999 steps/day; high activity level: >10,000 steps/day.
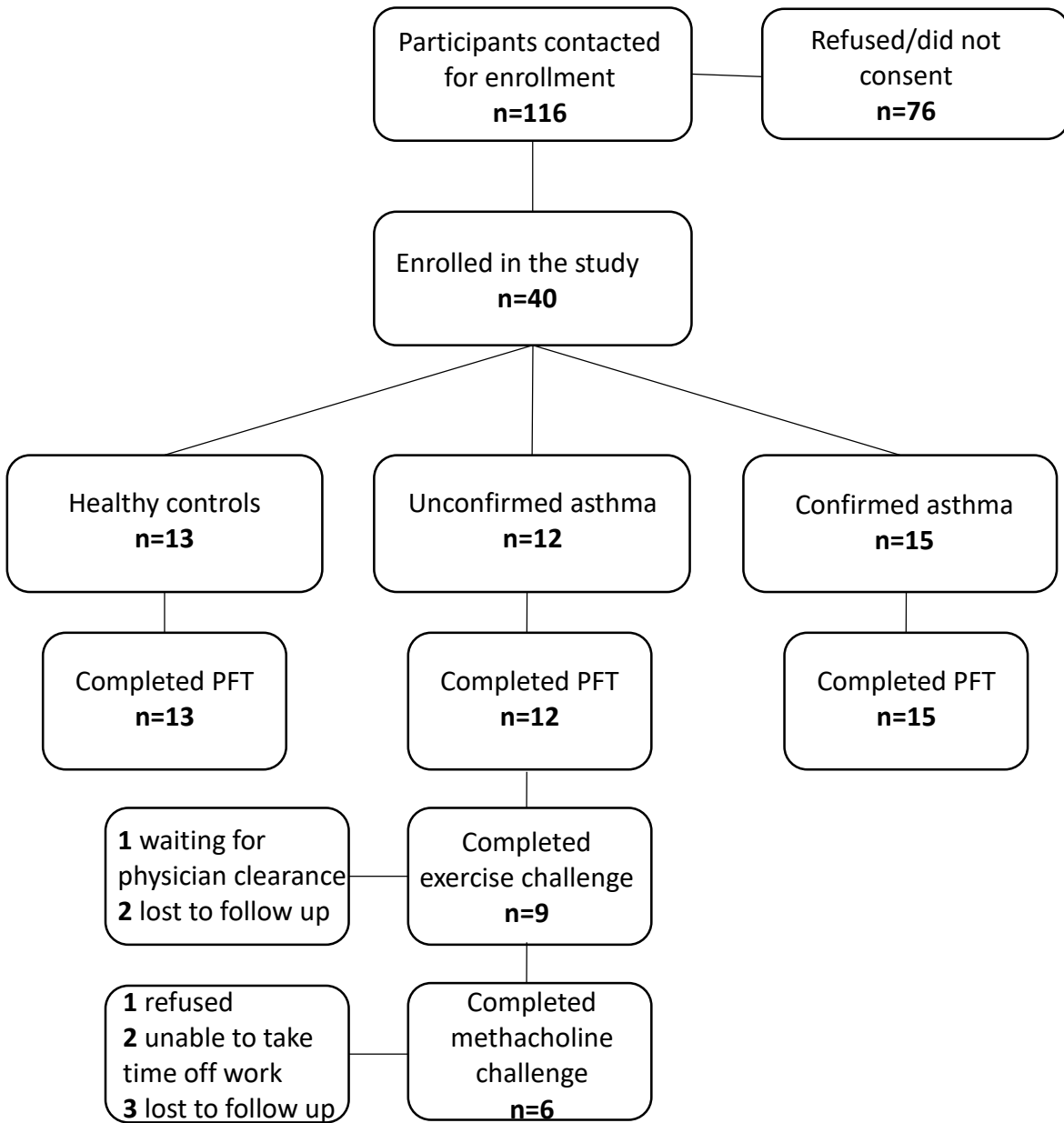
Figures

Figure 1. Consort diagram for recruitment and completion of objective assessments of asthma diagnosis, including explanations for incomplete assessments. PFT: pulmonary function test.
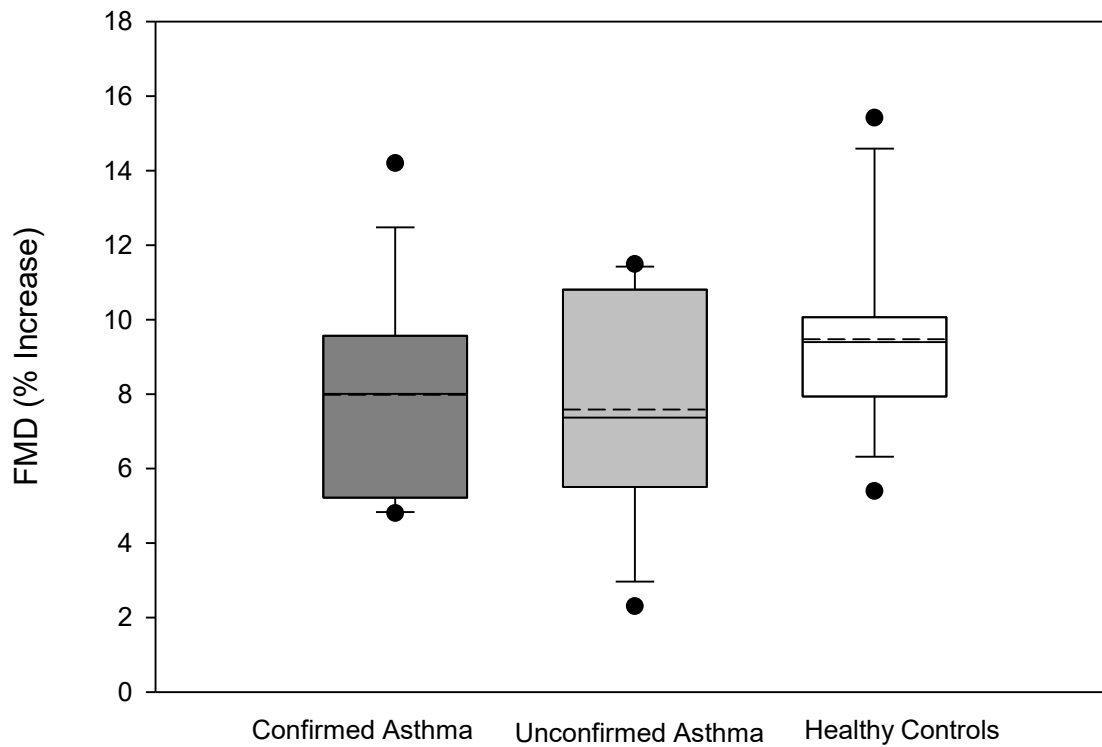
Figure 2. Flow mediated dilation (FMD, % increase from baseline), adjusted for area under the curve shear stress reactive hyperemia in participants with confirmed asthma, unconfirmed asthma and healthy controls. There was no significant difference in FMD across the three groups (p=0.21). Data reported as box and whisker plots. Horizontal line indicates median, horizontal dotted line indicates mean, boundaries of the box indicate inter-quartile range, whiskers indicate 10th and 90th percentile, dots indicate 5th and 95th percentile.

Figure 3. Carotid-radial pulse wave velocity (crPWV, m/s) adjusted for diastolic blood pressure (DBP) in participants with confirmed asthma, unconfirmed asthma and healthy controls. There was no significant difference in crPWV across the three groups (p=0.71). Data reported as box and whisker plots. Horizontal line indicates median, horizontal dotted line indicates mean, boundaries of the box indicate inter-quartile range, whiskers indicate 10th and 90th percentile, dots indicate 5th and 95th percentile.

Figure 4. Serum c-reactive protein (CRP, mg/L) in participants with confirmed asthma, unconfirmed asthma and healthy controls. There was no significant difference in CRP across the three groups (p=0.72). Data reported as box and whisker plots. Horizontal line indicates median, horizontal dotted line indicates mean, boundaries of the box indicate inter-quartile range, whiskers indicate 10th and 90th percentile, dots indicate 5th and 95th percentile.
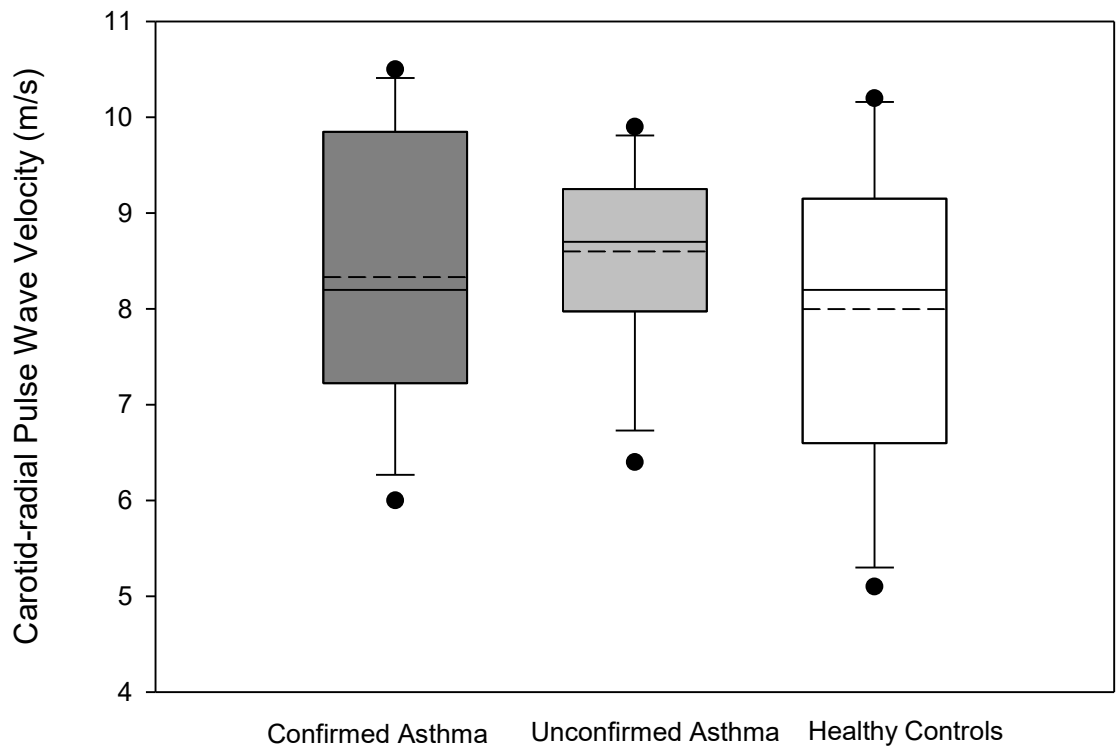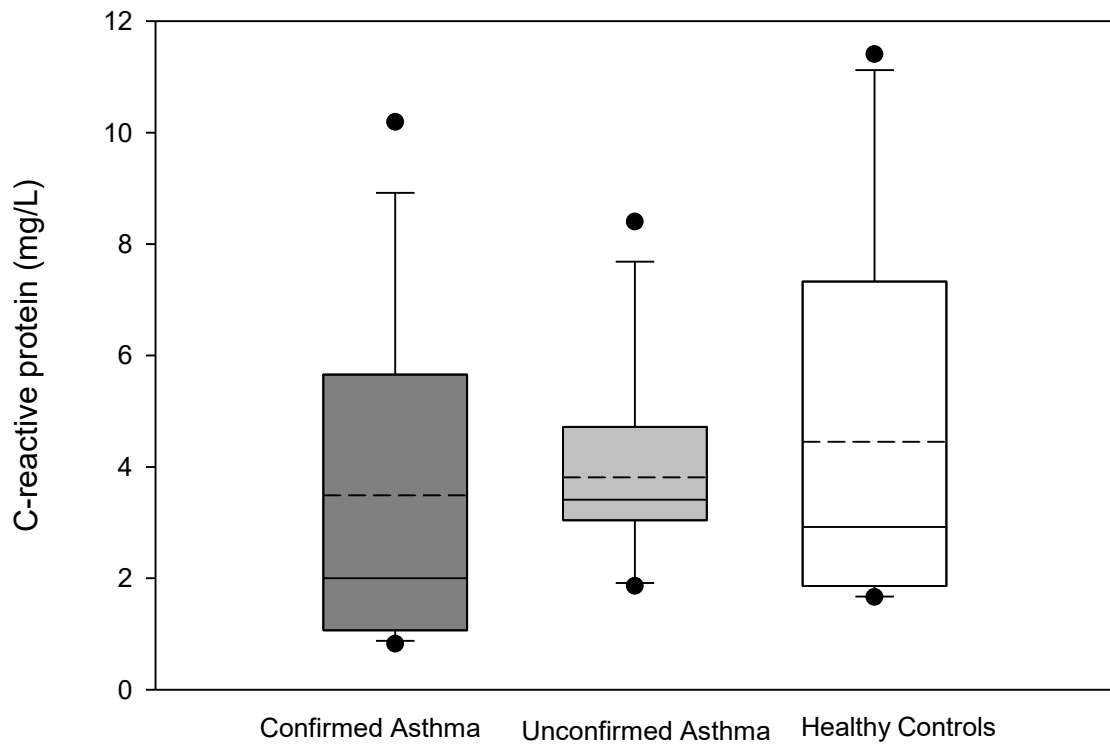
Figure 5. Flow mediated dilation (FMD, % increase from baseline) for all participants (n=40) sub-grouped into presence of atopy vs. no presence of atopy. No significant difference in FMD was found between these sub-groups (p=0.08). Data reported as box and whisker plots. Horizontal line indicates median, horizontal dotted line indicates mean, boundaries of the box indicate inter-quartile range, whiskers indicate 10th and 90th percentile, dots indicate 5th and 95th percentile.

Figure 6. Physical activity (measured in average steps per day) for all participants with asthma (n=16) compared to healthy controls (n=13). Participants with asthma were significantly less active compared to healthy controls (p=0.05). Data reported as box and whisker plots. Horizontal line indicates median, horizontal dotted line indicates mean, boundaries of the box indicate inter-quartile range, whiskers indicate 10th and 90th percentile, dots indicate 5th and 95th percentile.
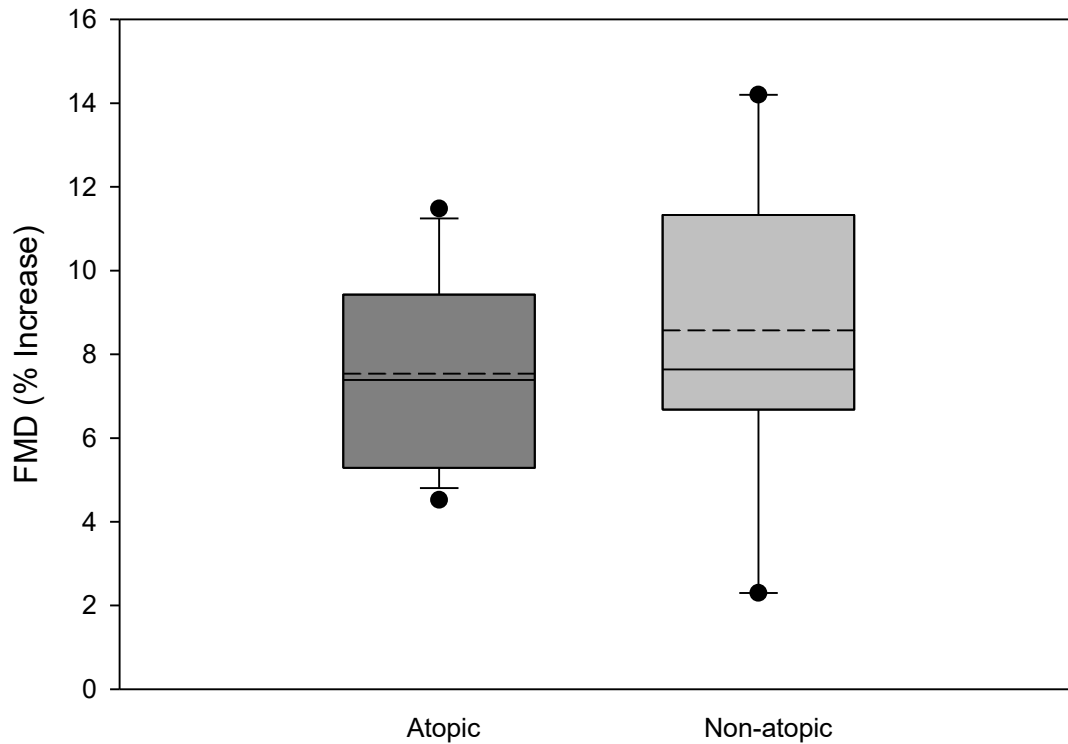
Figure 7. Flow mediated dilation (FMD, % increase from baseline) for all participants with asthma (n=27) sub-grouped into use of any asthma medication vs. no use of asthma medication. No significant difference in FMD was found between these sub-groups (p=0.08). Data reported as box and whisker plots. Horizontal line indicates median, horizontal dotted line indicates mean, boundaries of the box indicate inter-quartile range, whiskers indicate 10th and 90th percentile, dots indicate 5th and 95th percentile.
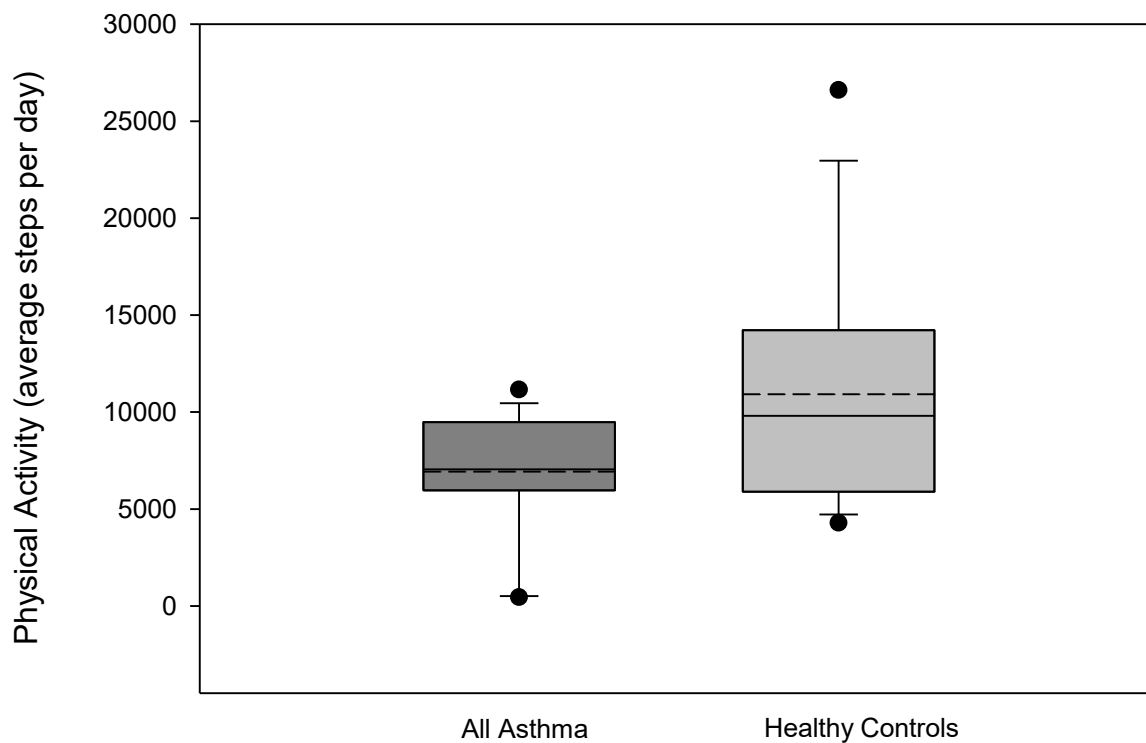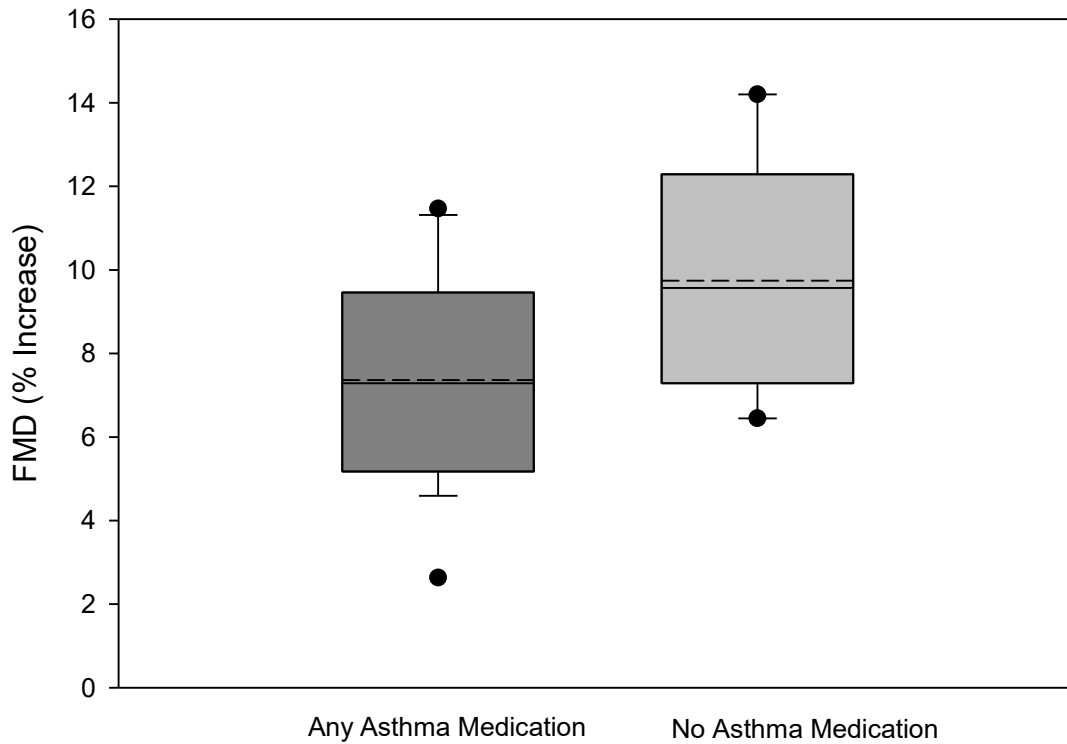
Chapter IV: General Discussion

Key Findings

The purpose of this study was to investigate vascular function, arterial stiffness and systemic inflammation in individuals with a physician diagnosis/clinical history of asthma that was confirmed with physiological testing compared to individuals with a physician diagnosis/clinical history of asthma that could not be confirmed with physiological testing. A group of healthy controls were recruited for further comparison. No significant differences in vascular function, arterial stiffness, or systemic inflammation were found between the unconfirmed and confirmed asthma groups. As no differences were observed, the asthma groups were combined, and when compared to healthy controls the participants with asthma demonstrated a trend towards significantly lower vascular function (p=0.08). This demonstrates that regardless of objective confirmation of asthma, these individuals have clinically and nearly statistically significant reductions in vascular function compared to healthy individuals. An exploratory analysis identified asthma medication use, presence of atopy, and decreased physical activity as potential contributing factors to the increased cardiovascular risk reported in people with asthma.

Types of Cardiovascular Risk Associated with Asthma

Previous work has concluded that there is an increased risk of chronic and acute cardiovascular diseases associated with asthma.[2,21] A prospective study[2] investigated the

association between asthma and coronary heart disease (CHD), cerebrovascular disease, and heart failure. Concurrently, this study assessed the risk specifically for women, atopic individuals, and those using asthma medication to determine if they were at a further elevated risk. The authors reported that all were significantly associated with asthma, with the risk elevated further in women compared to men and in those using any asthma medication compared to none, and all outcomes remained significant after adjustment for prior history of atopy. While the current study did not find any sex related differences in endothelial function, there was a near-significant trend with asthma (confirmed and unconfirmed combined) participants using any asthma medication demonstrating lower endothelial function (p=0.08) compared to the participants using no asthma medication. The current study also observed a trend with all participants (healthy, confirmed asthma and unconfirmed asthma) stratified based on presence of atopy, with the atopic participants demonstrating near-significant lower vascular function (p=0.08) compared to the participant with no history of atopy. Significantly lower physical activity was also demonstrated among the participants with asthma compared to healthy controls (p=0.05), which may be contributing towards the lower vascular function in this group.

There is a gap in the current literature with regards to determining the cause for the increased cardiovascular risk reported in people with asthma. A majority of studies have used occurrence of cardiovascular outcomes (either prospectively or retrospectively) to determine cardiovascular risk associated with asthma. While this provides a necessary foundation of what risks may be connected to a diagnosis of asthma, there is a need for research to

investigate the origins of this risk, and whom it is impacting. The current study aimed to develop further understanding of these questions by evaluating early predictors of cardiovascular risk (endothelial function, arterial stiffness, and systemic inflammation) in people with confirmed and unconfirmed asthma. The results of this study suggest a reduction in vascular function in individuals with an asthma diagnosis, regardless of objective confirmation. It is plausible that endothelial dysfunction is the first sign of the cardiovascular risk associated with asthma, though it remains unclear what is driving this dysfunction, however, airway function does not appear to be one of the primary mechanisms. Medication, decreased physical activity, atopy, and inflammation are likely contributing factors that need to be investigated more thoroughly.

Clinical Significance of Outcomes

As mentioned previously, endothelial function[120], arterial stiffness[15], and systemic inflammation[31] are all independent and early predictors of cardiovascular risk, and all have been reported to be negatively altered in people with asthma.[7,8,45] For this reason, these were the outcomes chosen for the current study. Endothelial dysfunction resulting from a decreased bioavailability of nitric oxide (NO) has been previously reported in people with asthma.[8] Reduced bioavailability of NO may also have negative implications with respect to arterial structure. NO directly acts to relax vascular smooth muscle cells, which in turn dilates the artery. Therefore, it is plausible that a lack of NO bioavailability contributes to increasing arterial stiffness. It is suggested that endothelial dysfunction and arterial stiffness due to

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increased systemic inflammation is one of the mechanisms driving the cardiovascular risk

associated with asthma. This is possible, as asthma is a chronic inflammatory condition of the

airways, and it is becoming more apparent that this has systemic inflammatory consequences.

However, in the current study the systemic inflammation for all groups was elevated,

suggesting there are more important mechanisms driving cardiovascular risk in asthma.

Another speculated cause of arterial stiffness in people with asthma is the chronic use of

asthma medications, specifically short-acting β-agonists.[7,25]

Previous research has determined that a 1% increase in endothelial function was clinically

significant, as it translated to a 9% decrease in risk. [106] The current study found that presence

of atopy, late-onset asthma, and use of any asthma medication resulted in 1.5% (p=0.08),

1.9% (p=0.22), and 2.4% (p=0.08) lower endothelial function, respectively. This would

theoretically translate to an increased risk of cardiovascular disease of 14% in those with

atopy, 17% with early onset asthma, and 21% who use asthma medications.

 A meta-analysis[121] informed the clinically relevant changes to central (carotid-femoral) pulse

wave velocity as an increase of 1 m/s , which translated to 15% increase in cardiovascular risk.

Importantly, the current study evaluated peripheral (carotid-radial) PWV, therefore the

clinically significant difference of this outcome may not directly translate to central (carotid-

femoral) pulse wave velocity. The current study found no statistically or clinically significant

differences in carotid-radial PWV between the three groups. Similarly, no difference in arterial

stiffness was found between all participants with asthma combined when compared to

healthy controls. It is suggested that the reason all groups demonstrated similar arterial

stiffness is the young age of the participants. Though the participants with asthma tended to

demonstrate lower vascular function, the decreased bioavailability of NO may not have

impacted the arterial stiffness as it is in the early stages of dysfunction. It is speculated that if

these participants were tested again in the future, there would likely be increases in crPWV

found with the asthma group. Additionally, the increased arterial stiffness previously reported

in asthma[7] is potentially a result of chronic us of asthma medication[25], therefore it is possible

the participants in the current study had a shorter duration of medication use.

C-reactive protein, a marker of systemic inflammation, is used clinically to stratify participants

into cardiovascular risk groups based on serum concentration as low risk: <1mg/L; moderate

risk: 1-3 mg/L, and high risk: >3 mg/L.[39] Although it has been previously reported that people

with asthma have increased systemic inflammation compared to healthy controls[45,82], the

current study did not observe the same findings. Rather, the healthy control group had the

highest level of inflammation, with all three groups categorized as high risk based on this

accepted stratification. As explained in the previous chapter, the healthy control group

disclosed potential reasons for increased inflammation (stress, lack of sleep, poor diet, past

medical conditions, socioeconomic status) which may have increased their systemic

inflammation.[122,123] Alternatively, it is possible that an error occurred throughout the analysis

process, though this is unlikely as the assay procedure requires control samples in order to

produce a standard curve, for which the R^2 was > 0.99. More rigorous inclusion criteria to

include family history, sleep index, stress, and other environmental factors would be beneficial to reduce the possibility of confounding results.

As all groups demonstrated systemic inflammation that is considered high risk, it is difficult to differentiate the impact of this high inflammation on the vascular function of people with asthma. It is possible that the high levels of systemic inflammation reported are negatively impacting the vascular function within the participants with asthma, however, it is unclear why this effect would not be seen with the healthy controls. It could be speculated that the healthy controls demonstrated an acute increase in systemic inflammation prior to testing (lack of sleep, stress, etc.), whereas the participants with asthma experienced more chronic disease related elevations in systemic inflammation translating to a stronger impact on vascular function.

Heterogeneity of Asthma and Difficulty with Confirmation

Asthma is a largely heterogeneous disease with many different phenotypes and inflammatory profiles.[9] Various phenotypes may be associated with greater cardiovascular risk, however the current study did not recruit based on phenotype and therefore is not able to examine this question.

Along with the heterogeneity in asthma is the heterogeneity of triggers that cause asthma-related symptoms. Participants in the current study who were classified as unconfirmed asthma most commonly reported the following triggers for their symptoms: cats/animals, house dust mites, indoor mold, exercise, outdoor environment, weather, pollution, and cold/flu. The vascular testing was completed in a controlled and ventilated indoor environment, between October and March, and was not completed if the participant indicated they felt unwell. With the exception of exercise, none of the triggers listed would have been present for this testing. Previous literature reports that false negatives to hyper-responsive tests may occur if the asthma trigger is not present.[117,118] As a result, it is possible that some participants may have been misclassified in the unconfirmed asthma group.

It is difficult to differentiate if the unconfirmed participants had grown out of their asthma, had asthma but were currently not hyper-responsive (because of treatment or lack of triggers), or were misdiagnosed and have a separate health condition. Confirmation of asthma would ideally be consistently assessed over an extended period of time in order to confidently determine presence, or lack of, disease.[124] Previous research indicates a low likelihood of confirmation of diagnosis during long term follow up in this population[117,124] , therefore the current study results would likely not change drastically if repeat assessments were completed. Importantly, some individuals in a previous study[124] that were misdiagnosed with asthma, were diagnosed with serious health conditions upon further investigation of their respiratory symptoms that required immediate alternative treatment. As such, all participants in the current study that were classified as unconfirmed were encouraged to discuss the

results of their objective assessments with their physicians to allow the opportunity to assess for other potential causes to their respiratory symptoms when appropriate and adjust treatment as necessary.

Importance of Exploratory Analysis and Findings

An exploratory analysis was completed for the current study which revealed three notable findings: 1) Presence of atopy (regardless of history of asthma) resulted in clinically significant lower vascular function, which was trending towards statistical significance, 2) Use of any asthma medications resulted in clinically significant lower vascular function, which was trending towards statistical significance, 3) with all participants with asthma combined, the asthma group demonstrated significantly lower physical activity compared to healthy controls and there was a positive relationship found between FMD and physical activity for the participants with asthma.

Allergies can lead to increases in systemic inflammation[125], and consequently may have negative effects on vascular function. Previous research found that individuals with allergic disorders were at a significantly increased risk for high intima-media thickness and atherosclerosis development and progression.[126] It has also been reported that adults with asthma, allergies, and both asthma and allergies demonstrate a significantly increased risk for coronary heart disease, cerebrovascular disease, and heart failure.[2] This gives evidence that those with allergies, even without the presence of asthma, are at an increased risk for

cardiovascular disease. Additionally, it also provides evidence that those with asthma, without

the presence of allergies, remain at an elevated cardiovascular risk, and highlights that there

are additional mechanisms contributing to cardiovascular risk in asthma outside of atopic

inflammation.

Asthma medication use has been suggested as a plausible mechanism behind cardiovascular

risk in asthma.[2,7] A large proportion of people diagnosed with asthma cannot be confirmed as

having asthma with objective physiological testing[9], with many of these individuals being

prematurely treated with asthma medication[85] This may be increasing cardiovascular risk in

these individuals unnecessarily. The current study found that individuals using any asthma

medications displayed clinically significant lower vascular function compared to those using

no asthma medication, regardless of objective confirmation of asthma. This supports previous

research suggesting that asthma medication contributes to the cardiovascular risk reported in

asthma.[2,25] It is critically important to objectively confirm a diagnosis of asthma prior to

beginning treatment with medication. If an individual requires treatment with asthma

medication it is important to ensure that the individual is utilizing the lowest dose needed to

control symptoms, as this may help mediate this risk. Additionally, an important change has

been made to asthma management guidelines recommending to avoid symptom-driven

treatment with short-acting β2-agonists (SABA) alone. Rather, it is recommended that

individuals with asthma receive a daily low dose of inhaled corticosteroids to reduce airway

inflammation and avoid reliance on SABA medication.[127]

It is well understood that decreased physical activity can lead to increased cardiovascular risk.[102,103] Previous research has demonstrated that people with asthma are significantly less active that their healthy counterparts[58,59], which was demonstrated within the current study. Exercise avoidance due to barriers (actual or perceived) that are associated with an asthma diagnosis, such as increased dyspnea during exercise,[128] may lead to reduced physical activity in people with asthma. In the current study, some participants showed a strong broncho-constrictive response to exercise demonstrating a drastic reduction in lung function (>30%) following only a few minutes of intense exercise. In these individuals it would be important to provide them with education on how to best manage their asthma while still remaining physically active; pulmonary rehabilitation would be an excellent method of executing this task. However, the majority of participants with asthma (both confirmed and unconfirmed) were able to complete a maximal exercise test without a drastic reduction in lung function, with some demonstrating no reduction in lung function. It is likely that their avoidance of exercise is due to perceived barriers that may be modifiable. If an individual is diagnosed with asthma without objectively confirming the diagnosis, or without investigating the triggers of their symptoms, they may incorrectly assume they cannot be as physically active or exercise as much as a healthy individual. Inactivity is detrimental to their cardiovascular health, and could potentially explain why those with confirmed and unconfirmed asthma demonstrated similar vascular function. Previous research has found that individuals with asthma demonstrate similar vascular function compared to healthy controls when they are matched for physical activity or physical fitness.[7] The positive relationship found in the current study

between physical activity and FMD within the participants with asthma supports this finding. It is possible that people with asthma may reduce their cardiovascular risk by increasing their daily physical activity.

Current Study within the Context of Rehabilitation Sciences and Practice

The International Classification of Functioning, Disability and Health (ICF) describes a person's functioning as "a dynamic interaction between her or his health conditions, environmental factors, and personal factors."[129] Impairments of body structure and function associated with asthma (i.e. airway hyper-reactivity and bronchoconstriction)[9] along with environmental barriers (i.e. fear, anxiety, or lack of supports)[130] contribute to reduced participation and activity limitation in people with asthma.[58,59] The current study found that although participants with confirmed asthma had significantly lower lung function and significantly worse symptom control compared to participants with unconfirmed asthma, both groups demonstrated lower daily physical activity compared to healthy controls. This suggests that the activity limitation demonstrated among participants with asthma is a result of environmental barriers rather than structural or functional impairments only. This emphasizes the importance of obtaining objective confirmation of an asthma diagnosis in order to reduce the unnecessary barriers to physical activity associated with a physician diagnosis of asthma.

It is important to provide individuals that are diagnosed with asthma education on how to overcome apparent barriers to physical activity. Fear and anxiety around exercise can be

reduced with a rehabilitation program that provides supervised exercise at relatively high intensity by giving participants confidence that they can exercise safely, regardless of their diagnosis.[130]

It is speculated that asthma medication use and physical inactivity may be important determining factors of cardiovascular risk associated with asthma, both of which may be modified with the utilization of pulmonary rehabilitation. The relationship between asthma medication and reduced vascular function highlights the potential risk associated with beginning treatment with asthma medication prior to objectively confirming a diagnosis of asthma. Importantly, some participants did not experience any symptoms immediately prior to or during the current study, while some participants experienced symptoms which they believed were exacerbated when asked to withhold medication prior to testing. This suggests that some participants may not have a confirmation of asthma, but clearly have a respiratory issue that requires treatment through medication. Medication may not always be a modifiable factor (depending on clinical assessment), however, the type of medication used may be important. Daily inhaled corticosteroids tend to be preferred to short-acting beta-agonist medication, as they target the airway inflammation associated with asthma rather than just bronchoconstriction.[127] However, previous research has shown that inhaled corticosteroids are also associated with an increased risk of cardiovascular disease[2], so the role different medications have in the cardiovascular risk associated with asthma remains unclear. While people with asthma are typically encouraged to utilize their short acting medication prior to exercise to reduce feelings of breathlessness[19], this suggestion is not supported by evidence.

Previous research has reported that administration of salbutamol prior to an exercise test did not effect operating lung volumes, nor exertional dyspnea.[128]

Physical inactivity can be targeted within pulmonary rehabilitation with the aim of reducing cardiovascular risk and improving asthma related and general quality of life. By providing education, through pulmonary rehabilitation, to individuals with an asthma diagnosis about how to better control their symptoms during physical activity, it may reduce barriers (i.e. fear and anxiety) and increase their average daily physical activity. This is especially important for individuals that experience debilitating reductions in lung function during exercise, as they likely avoid exercise on a consistent basis. Additionally, for those participants that do not experience an increase in symptoms with exercise, pulmonary rehabilitation would help increase their confidence in their ability to exercise, leading to increased daily physical activity. Though pulmonary rehabilitation for people with asthma has not been shown to improve lung function[131], it is plausible that it would have beneficial effects on the cardiovascular system by improving vascular function and subsequently reducing cardiovascular risk. In addition, rehabilitation in patients with asthma has demonstrated the ability to improve quality of life, reduce reliance on short acting medication, and reduce feelings of fear associated with exercise, acting as an important facilitator to participation and increasing activity in people with asthma.[130,132–134]

Future Directions

While the current study suggests that individuals with confirmed and unconfirmed asthma have similar vascular function, arterial stiffness, and systemic inflammation, the reason for this warrants further investigation. Cardiovascular risk in people with confirmed and unconfirmed asthma may potentially be mediated by asthma medication use, presence of atopy, or physical inactivity. Currently, a majority of research evaluating cardiovascular effects of asthma medication utilize prospective or retrospective reporting of cardiovascular events and administrative data for medication use. Randomized controlled trials and longitudinal studies investigating the impact of different types and dosages of asthma medication on predictors of cardiovascular risk in people with asthma as well as healthy controls are needed. These studies should include participants with asthma that are matched to healthy controls for physical fitness or physical activity to further differentiate the origins of demonstrated cardiovascular risk. Additionally, research on the effectiveness of pulmonary rehabilitation for people with asthma is needed. There is previous research looking at the effects of pulmonary rehabilitation in people with asthma[131,133], however, these studies did not evaluate predictors of cardiovascular risk. This research should address the appropriate type of rehabilitation (aerobic vs. anaerobic. vs. resistance training), as well as the demographics that may benefit most from attending. Importantly, these studies should include measures to assess health-related quality of life as well as fear and anxiety towards exercise prior to beginning a rehabilitation program and directly following completion in order to fully understand the biopsychosocial impacts of rehabilitation on people with asthma.

Summary

Vascular function, arterial stiffness and systemic inflammation were evaluated in participants with a physician diagnosis/clinical history of asthma that was confirmed by physiological testing, and compared to age-, sex- and BMI-matched participants with a physician diagnosis/clinical history of asthma that was unable to be confirmed by physiological testing. Confirmed and unconfirmed asthma groups did not demonstrate any differences in vascular function, arterial stiffness, or systemic inflammation, therefore they were combined and compared to healthy controls. Participants with asthma demonstrated clinically significant and nearly statistically significant lower vascular function compared to healthy controls. Exploratory analysis demonstrated significantly lower physical activity assessed by average steps per day in participants with asthma, and suggested that asthma medication use and presence of atopy may result in lower vascular function. Asthma medication and physical inactivity may be contributing to the increased cardiovascular risk associated with asthma. Physical inactivity is a modifiable factor that can be targeted with pulmonary rehabilitation to reduce cardiovascular risk associated with a sedentary lifestyle. More researched is needed to further understand the interaction of asthma medication use and vascular dysfunction, and the mechanisms as well as types of asthma medications contributing to cardiovascular risk associated with asthma.

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Appendix A: Detailed Methods

Sample Size Calculation

There is limited literature regarding NO-dependant vasodilation in people with asthma. A previous study that examined the vascular function of people with mild and moderate asthma compared to healthy controls[8] was used to determine an estimated effect size of 0.86. To detect a significant difference (α = 0.05 and β = 0.2) between groups, 18 participants were required for each group for a total of n=54. In order to perform a sub analysis on sex differences, a total of 23 men with asthma and 23 women with asthma were required.

Measurements

Arterial stiffness

Arterial stiffness is an independent predictor of cardiovascular risk,[15,107] which increases with age and to a higher degree in women than in men.[135] A longitudinal study by Kaess et al. assessed 1759 participants 60 ± 9 years of age and demonstrated that higher arterial stiffness was predictive of incident hypertension, whereas higher initial blood pressure was not predictive of an increase in arterial stiffness. [136] This provides evidence that arterial stiffness is not the result of hypertension, rather that hypertension is a consequence of stiffening arteries which can be used as an early predictor for future risk.[110] Arterial compliance is the elasticity of the artery allowing for the pulsatile blood flow of the left ventricle to be translated into a less pulsatile flow in the distal arteries leading to a non-pulsatile flow in the capillaries.[137]

Reduced compliance, or increased arterial stiffness, causes a premature return of reflected

pulse waves back to the heart due to the strong pulsatile pressure in distal arteries. This

increases the stress put on the left ventricle and when arterial stiffness is chronically

increased, it results in left ventricular hypertrophy, decreased stroke volume, and decreased

cardiac output[111,112], putting an individual at risk for cardiovascular events. Oxidative stress[138]

and inflammation[38,108] leading to an increase in collagen and decrease in elastin within the

ECM as well as stiffening of VSMC[109] are thought to be responsible for arterial stiffness. NO

acts on the VSMC by relaxing them which in turn dilates the artery, so endothelial dysfunction

leading to unavailability of NO may also contribute to arterial stiffness.

PWV is expressed as m/s and is calculated as:

$$PWV = L/\Delta t$$

with L being the distance between the two points and Δt being the time it takes for the pulse

to get from site 1 to site 2.[139] The gold standard for non-invasive measurement of arterial

stiffness is carotid-femoral pulse wave velocity (cfPWV).[15] For every 1 m/s increase in cfPWV,

there is an increase in total cardiovascular events, cardiovascular mortality and all-cause

mortality by 15%, 14% and 15%, respectively. For every 1 standard deviation increase of

cfPWV there is an increase of relative risk in the same domains by 47%, 47%, and 42%,

respectively.[121] As the femoral artery can be difficult to palpate with the participants comfort

in mind, carotid-radial PWV (crPWV) is an alternative measurement that is easily accessible

and was recorded for all tests.

After 10 minutes of resting in a dark, quiet room in the supine position, crPWV was recorded using applanation tonometry (Complior, Alam Medical, Saint Quentin Fallavier, France). A series of 10 consecutive beats were taken with a minimum quality of 85%. The distance between the carotid and radial artery was recorded in mm. PWV was measured prior to FMD and blood collection.

Various factors can affect arterial stiffness leading to variability in measurements.[15] For this reason, all PWV assessments were done initially and between the hours of 7:00am and 12:00PM when possible. Participants were asked to arrive in a fasted state (~8 hours), avoiding alcohol, caffeine, smoking, medications as possible, and strenuous physical activity for 12 hours prior to testing. Menstrual status as well as any contraceptives used were recorded as endogenous hormones also affect arterial stiffness.[79] All confounding variables were documented.

Endothelial function

Endothelial dysfunction is an independent predictor for cardiovascular risk in both healthy[120,140] and diseased populations.[141] Flow mediated dilation (FMD) in response to reactive hyperemia is a safe, non-invasive way to accurately assess NO-dependent endothelial function by brachial artery ultrasound.[142] Occluding the limb distal to the brachial artery stimulates the production and release of NO which activates a vasodilation response that can be measured and quantified as an index of vascular function/dysfunction.[143] The dilation of

the brachial artery occurs in response to an increase in shear stress due to reactive hyperemia (SSRH) caused by the occlusion, and is commonly reported as the percent change in diameter. FMD is calculated using the following formula:

$$\%FMD = [(max\ diameter - baseline\ diameter)\ /\ baseline\ diameter]x100$$

A sphygmomanometer was placed on the right forearm distal to the antecubital fossa. With their arm extended, ultrasound imaging (8L-RS 4.0-13.0MHz probe, Vivid q, GE Healthcare, Mississauga, ON) was used to find a clear image of the brachial artery proximal of the antecubital fossa for baseline values. Four baseline images were then stored. The cuff was then inflated to 200 mmHg or 50 mmHg above systolic pressure, whichever was higher, and remained inflated for 5 minutes. Prior to release, an image was stored to analyse blood velocity and diameter during arterial occlusion. Once the cuff was released, a series of 12-13 images were stored in 16 second frames for analysis of post release state. Diameter change from baseline was analysed in 8 second averages using Carotid Analyzer (Medical Imaging Applications, LLC, Coralville, IA, USA). With regards to clinical relevance, a 1 % increase in FMD is associated with a 9% decrease of cardiovascular risk.[106]

Brachial artery blood velocity was averaged in two eight-second time increments for every 16 second sweep, using EchoPAC PC software (version 110.x.x, GE Healthcare, Horten, Norway). Flow was calculated as:

$$Flow = blood\ velocity * \pi r^2 * 60$$

Where r is the radius of the artery, and 60 is the constant used to convert seconds to minutes (ml/min).

A study done by Philpott et al. reported that SSRH as well as velocity of reactive hyperemia (VRH) provided a stronger correlation with the presence of risk factors than did FMD.[144] This is potentially because SSRH and VRH represent NO-dependent microvascular function[145], which can detect cardiovascular risk with greater sensitivity.[91,146] SSRH can be calculated as follows:[14]

$$SSRH = 8\ x\ flow\ /\ diameter$$

and is calculated for baseline as well as each post release 16 second frame in two eight-second averages. SSRH is an important regulator for magnitude of FMD and therefore should be used to normalize this response using the following formula:[14,147]

$$FMD\%\ normalized = peak\ diameter\ /\ AUC\ SSRH$$

where AUC refers to the area under the curve, i.e. the sum of all SSRH post release values up to and including the peak diameter, as these all contribute to the FMD response.[147] For the current study, SSRH was used as a covariate during the statistical analysis of FMD.

Like SSRH, VRH is important in the prediction of cardiovascular risk[144] and can also be used to normalize for shear stress in FMD analysis.[91] VRH is simply the velocity time integral (VTI)

normalized to a heart rate of 60 beats per second. VTI will be found by tracing the first

waveform following cuff release and used to calculate VRH as follows:

$$VRH = VTI \ x \ heart \ rate \ / \ 60$$

It has been suggested that this assessment tool may be more sensitive than using FMD

therefore may be able to detect earlier consequences of cardiovascular risk factors.[144] Healthy

microvasculature will dilate in response to ischemia, therefore based on the principles of flow,

a lower VRH will be indicative of microvascular dysfunction.

Various factors may impact vascular function and were considered for each assessment. In

terms of medication, it is recommended to withhold from medication for ≥ 4 half lives of the

drug before taking FMD measurements.[143] This may not always be possible depending on the

type of medication, so all medications were documented and the potential effects were

considered during analysis. Aspirin and nonsteroidal anti-inflammatory drugs (NSAIDS) should

be avoided for 1 and 3 days prior to testing.[14] Smoking negatively impacts endothelial

function[148] and should be avoided for ≥ 12 hours prior to testing.[14] Caffeine has also been

shown to attenuate FMD response so should also be avoided ≥ 12 hours before vascular

function assessments.[14] Menstrual state impacts vascular function due to the variation in

hormones produced over the menstrual cycle. Particularly progesterone and estrogen

increases may increase endothelial function.[149] As this study tested premenopausal women, it

is best to be performed at the same time in their menstrual cycle, and if possible it is best to

test day 1-7 of the menstrual cycle as this is when these hormones are at their lowest.[143,150]

As this was not always possible due to the nature of the study recruitment, cycle day as well as any contraceptives used were noted. Serum estradiol levels were analysed to be used as a covariate during statistical analysis. However, as no linear relationship between estradiol and FMD was found, estradiol was not used in this manner. Exercise is known to increase FMD therefore it is recommended to abstain from exercise for ≥ 12 hours prior to testing. FMD should be done under fasting conditions if possible, or after consumption of a standardized low fat meal[14] as ingesting a high fat meal may attenuate the FMD response.[151] When performing repeated measurements, it is optimal to test at the same time of day as FMD experiences diurnal variation.[152] For these reasons, when possible, all assessments were completed between 7:00 AM and 12:00 PM in a fasting state. All confounding variables were documented.

Blood collection and analysis

Blood was collected from each participant on the same day as vascular testing via antecubital venipuncture by a certified phlebotomist following specified guidelines.[92] With the participant sitting in a relaxed position, the upper arm was occluded to increase the accessibility of the blood vessels. Once a vein was chosen (based on proximity to the surface, size, position and absence of scar tissue and/or pulsation), a sterile 21- or 23-gauge needle was inserted at a 30-degree angle or less to initiate blood draw. Blood was collected in anti-coagulant-free polypropylene tubes pre labeled with the participant's identification number and date. After the collection was complete, the tourniquet was removed followed by the needle and pressure was applied to the venipuncture site and held until the bleeding stops. The blood

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was left to clot for a minimum of 30 minutes and a maximum of 90 minutes followed by centrifuging at 1200 g for 10 minutes at 4 degrees Celsius. The serum was separated into aliquots of 100 μL immediately after centrifuging and then stored at -80 degrees Celsius until analysis.

To control for natural fluctuations within participants all samples were taken in a fasted state, between 7:00 AM and 12:00 PM when possible. The participant was also asked to avoid physical activity[153], caffeine, and medications when possible for at least 12 hours prior to blood collection. Women have demonstrated variability in CRP levels at different menstrual states[154] therefore cycle day as well as any contraceptives used were documented, and serum estradiol levels were analysed within the same sample.

Analysis of blood was done at the University of Alberta using enzyme linked immunosorbent assay (ELISA) protocol.[93] High sensitivity human CRP (Catalog # DY1707, R&D Systems, Inc.) and human estradiol (Catalog # KAQ0621, Invitrogen Corporation) ELISA kits were used as per manufacturers' protocols.[94–96] Plate preparation for CRP analysis consisted of diluting the capture antibody and coating the 96-well microplate with 100 μL per well, sealing the plate, and allowing it to sit overnight prior to the assay procedure. After approximately 24 hours, the plate was washed three times with the Wash Buffer solution. Next, 300 μL of Reagent Dilution was added to each well and left to incubate for one hour. During this time, samples (already brought to room temperature) were prepared by two 100-fold dilution (10 μL of sample + 990

µL diluent, repeated for a total dilution of 10000-fold), and after the plate had incubated and been washed three times, 100 µL of sample or standards (already diluted) was added in duplicate to each well. The plate was then sealed and left to incubate for two hours. After incubation, the plate was washed three times and 100 µL of Detection Antibody (previously diluted) was added to each well. The plate was then sealed and left to incubated for another two hours. After incubation and washing, 100 µL of the working dilution of Streptavidin-HRPA was added to each well. The plate was covered and left to incubate for 20 minutes out of direct light (wrapped in foil, placed in drawer). After incubation and washing, 100 µL of Substrate Solution was added to each well and the plate was covered and left to incubate out of direct light. Lastly, 50 µL of Stop Solution was added to each well. The plate was read at an optical density of 450 nm and 570 nm, using the delta optical density (450 nm − 570 nm) values for calculations. Using a curve-fitting software (SigmaPlot version 13, Systat Software, San Jose, CA), a four parameter algorithm standard curve was generated with an R^2 of 0.9999 using the formula:

$$x = c\left(\frac{a - d}{y - d} - 1\right)^{\frac{1}{b}}$$

Where c is the EC50, a is the minimum value, d is the maximum value, y is the mean of the two duplicate samples, and b is the hillslope. The value x was then multiplied by 10000 (the dilution), and converted from pg/ml to mg/L to obtain the actual amount of CRP present in the sample.

No plate preparation was necessary for analysis of estradiol (E2), as the kit came with a pre-coated 96-well plate. All reagents and samples were brought to room temperature prior to carrying out the assay. Samples did not require dilution for this procedure. To begin, 50 µL of each standard, control, or sample was added in duplicate to the wells. Next, 50 µL of estradiol-HRP conjugate was added into all wells, followed by 50 µL of anti-estradiol into each well. The plate was sealed and left to incubate for two hours at room temperature on a horizontal shaker set at 600 RPM. After incubation, the wells were thoroughly washed with Wash Buffer four times, followed by the addition of 200 µL of Chromogen solution to each well. The plate was sealed and left to incubate for 30 minutes at room temperature in the dark (stored in a drawer with thick paper on top). Next, 50 µL of Stop Solution was added to each well and the plate was read immediately afterwards at an optical density of 450 nm. Using a curve-fitting software (SigmaPlot version 13, Systat Software, San Jose, CA), a four parameter algorithm standard curve was generated with an R^2 of 0.9902 using the formula:

$$\underline{x = c \left(\frac{a - d}{y - d} - 1 \right)^{\frac{1}{b}}}$$

Where c is the EC50, a is the minimum value, d is the maximum value, y is the mean of the two duplicate samples, and b is the hillslope. Percent bound was calculated for each sample and standard using the formula:

$$\frac{B}{Bo} * 100 = \frac{OD(standard\ or\ sample)}{OD(zero\ standard)} * 100$$

Where B/Bo x 100 represent percent bound, and OD represents optical density. These values were then interpolated to determine the concentration of estradiol (pg/ml) in each sample from the reference curve.

Physical activity

Physical activity was evaluated by step count using a Fitbit activity monitor. Participants were instructed to wear the monitor on their wrist for seven days only removing it to shower/bathe and to sleep. Total steps in each 24-hour period were averaged and used to determine average physical activity levels. Low active was defined as <7,500 steps/day. Somewhat active was defined as 7,500-9,999 steps/day. Highly active was defined as > 10,000 steps/day.[17]

Pulmonary Function Test

The participant was instructed to withhold short-acting medication for at least 8 hours, and long acting medication for at least 48 hours prior to testing, as well as to avoid smoking for 12 hours prior to the test and throughout.[97] Prior to testing, the spirometry system (Vmax, CareFusion, Yorba Linda, CA, USA) was calibrated as per manufacturer's instructions. Participants were coached through each breathing maneuver before completing the test. After connecting the participant to the system with a mouthpiece, a nose clip was used to prevent air leakage.

Spirometry

After a few breaths of normal tidal breathing, the participant was instructed to take a big breath in filling the lungs to total lung capacity (TLC). Once TLC was achieved they were instructed to forcefully exhale until residual volume (RV) was reached. The one-second forced expiratory volume (FEV_1) represents the volume of air expired within the first second. Forced vital capacity (FVC) represents the total volume of air exhaled following a full inspiration.

A minimum of 3 acceptable maneuvers were taken for both pre and post bronchodilator. An acceptable maneuver requires: 1) no hesitation at the start of expiration, 2) no coughing during first second of expiration and no presence of second breath taken, 3) reaches full expiration before terminating maneuver, 4) no presence of Valsalva maneuver or any hesitation that causes a cessation of airflow, 5) no leak within the closed system, and 6) no presence of obstruction in mouth piece i.e. by the tongue.[97]

Between-maneuver criteria require the two largest values of FVC to be within 150 mL of each other as well as the two largest values of FEV_1 to be within 150 mL of each other. If a total of 8 tests were performed or the subject could not continue with testing, a minimum of 3 satisfactory tests were saved.[97]

Three acceptable baseline spirometry tests were taken (PRE measurements) followed by administration of four doses of 100µg of a short-acting β-agonist, 30 seconds apart. Each dose was inhaled in one breath to TLC from a spacing device, with a 10 second breath hold before exhaling. Three additional spirometry tests were taken ≥ 10 minutes up to 15 minutes after medication (POST measurements).[97] Significant reversibility was defined as the increase in FEV_1 and/or FVC of ≥ 12% and 200 mL.[100]

All spirometry values were reported as absolute values (L), and FVC as well as FEV_1 were also reported as percentage predicted according to calculated reference values (% ref). The highest values available were used to calculate FEV_1/FVC, even if they were from separate maneuvers, and these values were reported as % ref as well. [97]

Lung Volumes and Plethysmography

Tidal volume (TV or V_T) is the volume of gas inhaled or exhaled with regular breathing. Functional residual capacity (FRC) represents the volume of gas remaining in the lung at the end of expiration during tidal breathing. Expiratory reserve volume (ERV) is the volume of gas that can be maximally exhaled following expiration during tidal breathing. Inspiratory capacity (IC) is the volume of gas that can be inhaled following expiration during tidal breathing. Inspiratory reserve volume (IRV) is the volume of gas that can be inhaled forcefully following inspiration during tidal breathing. Reserve volume (RV) is the volume of gas that remains in the lung after maximal expiration. Total lung capacity (TLC) is the volume of gas in the lungs

following maximal inspiration (including RV). Vital Capacity (VC) is the volume of gas that is 1) maximally inhaled following maximal exhalation (IVC), or 2) maximally exhaled following maximal inspiration (EVC).[98]

Participants completed both IVC and EVC maneuvers, and the one that produced the highest VC value was repeated. After a few baseline breaths of tidal breathing, the participant was instructed to either: 1) at the end of expiration during tidal breathing, exhale fully to RV, then inhale maximally to TLC, followed by a maximal expiration to RV, or 2) at the end of expiration during tidal breathing, inhale maximally to TLC, then exhale maximally to RV, followed by a maximal inhale to TLC.[98]

Next, plethysmography was performed using a constant-volume chamber in order to measure changes in pressure during the manoeuver. Participants were seated in the chamber with the door sealed. They were instructed to place their hands on their cheeks to ensure there was no air inappropriately displaced. After at least four resting tidal breaths when the participant was near FRC, the shutter was closed and the participant was instructed to pant gently for approximately 3 seconds. A minimum of three satisfactory manoeuvres were recorded (within 5%) with the mean value reported. [98]

Single-breath determination of diffusing capacity of the lung for carbon monoxide (DLCO)

Single-breath DLCO refers to the capacity of the lung to exchange gas across the alveoli and capillaries based on the structure and function of this interface.[99] After a minimum of four tidal breaths, the participant was instructed to exhale to RV (in an unforced manner). Once RV was reached, the test gas was released and the participant was instructed to maximally inhale to TLC. The participant performed a 10-second breath hold at TLC followed by a maximal, but not forced, expiration to RV that did not exceed four seconds. At least four minutes was allowed between repeated tests. Unadjusted DLCO, %ref, and DLCO adjusted for alveolar volume(DLCO/V_A), %ref were reported. [99]

Questionnaires

Individuals with asthma (confirmed and unconfirmed) completed the Asthma Control Questionnaire (ACQ) to assess asthma control. The ACQ is a validated, simple 7 item questionnaire that asks questions regarding symptoms and rescue inhaler use in the last week.[155] The minimally important difference for the ACQ is 0.5 with scores ranging from 0 (totally controlled) to 6(severely uncontrolled).[156] Next the participant was asked to fill out the standardized Asthma Quality of Life Questionnaire, which is a validated, disease specific health-related quality of life assessment that encompasses both physical and emotional impacts of asthma.[157] This questionnaire contains 32 items across 4 domains, with a minimally important difference of 0.5 with scores ranging from 1-7 with higher scores representing better quality of life.[158] Lastly, the EQ-5D (5L) was completed by the participant to assess their generic health status and all related aspects in their quality of life.[159] The EQ-5D (5L) is a

validated and reliable questionnaire consisting 5 domains, along with a visual scale to

measure the participants own judgement on current health status.[160] Together, these

questionnaires will give us a representations of the individuals experience with asthma, along

with the physical and psychosocial impacts of the disease.