EFFECT OF DI-BUTYL PHTHALATE EXPOSURE IN THE HUMAN AIRWAY AND SYSTEMIC IMMUNOLOGY: A DOUBLE-BLIND, CROSSOVER STUDY

by

Danay Maestre-Batlle

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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submitted by Danay Maestre-Batlle in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

in Experimental Medicine

Examining Committee:

Dr. Christopher Carlsten
Supervisor

Dr. Scott Tebbutt
Supervisory Committee Member

Dr. Stuart Turvey
Supervisory Committee Member

Dr. Karen Bartlett
University Examiner

Dr. Vincent Duronio
University Examiner

Additional Supervisory Committee Members:

Dr. Neil Alexis
Supervisory Committee Member
Abstract

Phthalates are used as softeners in commercial products. They leak into the environment and become wide-spread contaminants. Epidemiological studies suggest an association between phthalate inhalation and development/worsening of airway diseases, but a firm link has not been established. Asthma is a complex disorder associated with inflammation resulting in airway hyper-responsiveness (AHR). Increasing interest is focused on abnormal immune responses as an underlying mechanism contributing to increasing the risk of asthma.

This study is the first to investigate airway and systemic effects in humans due to inhalation of a known concentration of a single phthalate. Di-butyl phthalate (DBP), exists in high concentrations in indoor air and has shown inflammatory potential. We hypothesize that DBP inhalation, prior to allergen inhalation will: a) enhance airway inflammation and responsiveness to allergen, and b) alter the activation state and functionality of immune cells in the airway and peripheral blood.

In this novel double-blind, order-randomized, crossover study, 16 participants were exposed by inhalation to controlled levels of DBP or clean air (CA) for 3h, followed immediately by an allergen (dust-mite, grass or birch) inhalation. To assess lung function and airway inflammation, spirometry and exhaled nitric oxide was measured before, 3h and 20h post-DBP/CA exposure and allergen inhalation. Blood, bronchoalveolar wash and lavage (BAL) were collected for quantification and measurement of the activation pattern of immune cells and inflammatory mediator release.

DBP inhalation followed by an allergen inhalation, significantly augmented the airflow decline (FEV₁) in response to an inhaled allergen, compared to CA. Moreover, DBP
enhanced the recruitment of BAL macrophages, specifically the M2 phenotype with increased expression of CD206 to the lungs. Meanwhile, the percent of T helper cells increased, while T regulatory and non-classical monocytes decreased, in peripheral blood. Only minor effects were observed for systemic inflammatory mediators. Moreover, significant effect modifications were observed for sex, AHR status and type of allergen inhaled.

The results suggest significant effects of a common commercial chemical, DBP, on clinically relevant airway and systemic endpoints in the context of allergen exposure in sensitized individuals. Future research should aim to validate and connect these findings within relevant policy and public health contexts.
Lay summary

Allergic asthma is a complex disease that involves inflammation in the lungs. Understanding how environmental exposures can modify the immune system is important to minimize the development and exacerbation of symptoms.

Phthalates are plastic softeners present in commercial products. They leak from the plastic into the environment causing continuous exposure of most populations. Several studies suggest an association between phthalate exposure and lung diseases, like allergic asthma, but a firm link has not been established.

This is the first study to investigate how inhalation of dibutyl phthalate (DBP) may alter the human immune system. Lung function measurements, blood and lung samples from 16 volunteers were collected before and after exposure to fresh air or DBP. A worsening of the effects of an inhaled allergen on lung function measures was observed due to DBP exposure. Other pulmonary and systemic endpoints like cells and inflammatory markers were also altered by DBP exposure.
Preface

This dissertation was conceived, conducted, and written by Danay Maestre-Batlle, with direction from Dr. Christopher Carlsten (direct supervisor and PAIR study principal investigator) and Dr. Anette K. Bølling (mentor and study co-investigator).

All studies were conducted after receipt of ethics approval from the University of British Columbia Clinical Research Ethics Board (H14-01119), the Vancouver Coastal Health Authority (V14-01119), and the Norwegian Regional Committees for Medical and Health Research Ethics (2014/1217). This project was registered at clinicaltrials.gov (NCT02688478).

I led the study coordination, recruitment and screening of participants, and the sample processing in the laboratory. I completed the data analysis, interpretation and writing of the published and pending manuscripts in this dissertation. Co-authors designed the study, facilitated international collaborations, and assisted with troubleshooting, sample collection, data interpretation, and manuscript revision. Manuscripts that have been published, or to be submitted for publication are integrated into the body of this dissertation as follows:

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# equal contributors

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To my husband
Chapter 1: Introduction

1.1 Phthalates

1.1.1 Application and classification

The growth of plastic production in the past 65 years has substantially outpaced many other man-made materials, and an estimated 8300 million metric tons of plastic have been produced to date worldwide\(^1\). Phthalates are synthetic diesters of phthalic acid (1,2-benzenedicarboxylic acid)\(^2\), used to enhance the properties of plastics such as flexibility, transparency, durability and longevity\(^3\).

Although phthalates are primarily used as plasticizer agents to soften vinyl products, they are also present in a wide variety of common consumer products\(^4\). Industrial and consumer product applications of phthalates include building materials, household furnishings, clothing, cosmetics, personal care products, pharmaceuticals, nutritional supplements, herbal remedies, medical devices, dentures, children’s toys, glow sticks, modelling clay, food packaging, automobiles, lubricants, waxes, cleaning materials and insecticides amongst others\(^5\). Due to their weak, non-covalent binding, phthalates leak out of their carrier plastics and become ubiquitous environmental contaminants\(^4\).

The general molecular structure of a di-alkyl phthalate is a rigid planar aromatic ring with two flexible, often non-linear, aliphatic side chains\(^2\). Based on the length of the aliphatic side chains and overall molecular weight, phthalates can be classified into two groups: low or high molecular weight phthalates\(^6\). Low molecular weight phthalates (LMW) include dimethyl phthalate (DMP), diethyl phthalate (DEP), and dibutyl phthalate (DBP), whereas high molecular weight phthalates (HMW) generally include diethyl hexyl phthalate (DEHP),
diisononyl phthalate (DINP), diisodecyl phthalate (DIDP), and benzyl butyl phthalate (BBzP)\textsuperscript{7}. LMW phthalates are predominately found in the gas phase, while HMW phthalates are associated with house dust\textsuperscript{7}.

DBP is a LMW phthalate commonly found in high concentrations in indoor environments, both in gas phase and in association with inhalable particulate matter\textsuperscript{7,8}. DBP has two isomers; di-n-butyl phthalate (DnBP, sometimes referred to as DBP), and di-iso-butyl phthalate (DiBP), but DnBP seems to be a commonly applied industrial plasticizer based on its higher prevalence in cosmetics, nail polish, fragrances and as coating of enteric medication compared to DiBP, particularly in the Canadian market\textsuperscript{9,10}.

\textbf{1.1.2 Sources of exposure}

Large population-based studies have demonstrated the extent of the human exposure to phthalates by confirming the presence of phthalate metabolites in nearly all the urine samples analysed\textsuperscript{4,11}.

Dietary consumption is considered the most common source of exposure and frequently occurs due to exposure to food and beverage packaging containing phthalates\textsuperscript{12}. Additionally, inhalation exposure occurs through emissions from vinyl flooring and consumer products such as fragrances, air fresheners or cleaning supplies, whereas dermal exposure can occur through the use of phthalate-containing personal care products such as sunscreens, lotions, and cosmetics\textsuperscript{13}. Moreover, transdermal uptake directly from the air has also been demonstrated to be an indoor exposure route\textsuperscript{14}.

For DBP specifically, ingestion of food generally accounts for approximately 75\% of the total exposure across all consumer groups, with the remainder due to inhalation of indoor air.
and incidental ingestion of dust and personal care products\textsuperscript{4,15}. In some populations, DBP inhalation exposure has been estimated to account for up to 20% of the total intake\textsuperscript{4,7,16}. The phthalate exposure route and the main sources vary with age and sex, but also between different phthalates. For DBP, personal care products are a major source of exposure in female teenagers and adults (15-50%), while contaminated indoor environments are particularly important in infants, toddlers, and children (20–40\%)\textsuperscript{4}.

Phthalates entering the human body are rapidly hydrolyzed to their monoesters through a variety of enzymatic reactions and generally, high molecular weight phthalates undergo further oxidization and glucuronidation processes, turning parent compounds into primary and secondary metabolites that are excreted mainly via urine\textsuperscript{11,13}. The degradation half-time for phthalate metabolism in humans is short and found to be in the order of hours\textsuperscript{17}. A peak from 3-6 hours has been reported and most of an applied dose is excreted in urine within 24 hours\textsuperscript{14,18}.

Both DBP isomers are excreted mainly as glucuronidated monoesters (mono-n-butyl phthalate (MnBP) and mono-iso-butyl phthalate (MiBP)), while minor amounts are excreted as secondary metabolites\textsuperscript{19}. In urine, MBP (the term used to reflect total primary DBP metabolites) represents 64–84\% of the total DBP-derived metabolites, whereas the main secondary metabolite 3-OH-MBP accounts for approximately 7\%\textsuperscript{18,19}. Other secondary metabolites like 2-OH-MBP, 4-OH-MBP and 3-carboxy-mono-propyl phthalate (MCCP) could also be present, however these only make up a small fraction compared to MBP and 3-OH-MBP\textsuperscript{18}. DBP is also excreted via other pathways such as breast milk, sweat and feces, but these are minor compared to the urinary pathway\textsuperscript{20–22}. 


1.1.3 Regulations

Epidemiological studies focusing on exposure to phthalates and respiratory symptoms, pregnancy outcomes, genital development and neurodevelopment in children have suggested that phthalate exposure increases the risk and adverse effects of these outcomes. In recent years, an increasing number of studies have reported associations between allergy and asthma, children’s neurodevelopment and attention deficit hyperactivity disorder to phthalate exposure. The negative impact of phthalates in human health has given rise to restrictions in the use of these chemicals worldwide, particularly in children’s toys and child care products.

In recent years, the European Union (EU) has imposed restrictions on five different phthalates, including DBP, by Commission Directive 2007/19/EC. DBP has been classified by the European Chemicals Agency (ECHA) as a substance of very high concern requiring authorization before its use, by Annex XIV of REACH (Registration, Evaluation, Authorization and Restriction of Chemical substances), and some of its uses are restricted under Annex XVII of REACH. For example, DBP shall not be used as a substance or in mixtures, individually or in combination with other phthalates in toys and childcare articles in a concentration equal to, or greater than 0.1% by weight of the plasticised material, but is still allowed in articles placed on the market before July of 2020. Additionally, the EU has banned DBP from cosmetic and personal care products.

In the United States, the California Toxic Toys Bill (Assembly Bill 1108), bans the manufacture, sale, or distribution in commerce of toys and childcare articles that contain...
DBP in concentrations exceeding 0.1%. Health Canada also regulates the use of phthalates, under the Canada Consumer Product Safety Act, six phthalates (including DBP) have been banned from children's toys and childcare articles, but otherwise (cosmetics, fragrances, etc.) remains unrestricted.

In occupational settings, the National Institute of Occupational Safety and Health (NIOSH) and the Occupational Safety and Health Administration (OSHA), proposed regulations and recommendations that limit the exposure to DBP in the air to a time-weighted average of 5mg/m³ for an 8-hour workday.

1.2 Asthma

1.2.1 Characteristics, prevalence and burden of disease

Asthma is a chronic inflammatory disorder of the airway, characterized by airway hyper-responsiveness, leading to variable airflow obstruction which may occur as a result of both genetic and environmental factors. Airway remodeling, a fundamental pathological characteristic of chronic asthma, has been shown to correlate well with the disease severity.

Approximately 300 million people worldwide have asthma. It is the most common chronic lung disease affecting children globally, and at least 250,000 deaths are attributed to the disease each year. Due to the high number of people affected, especially children, asthma is considered a major public health problem with global treatment costs running at billions of dollars annually. Despite efforts from several research institutions and programs, like the Severe Asthma Research Program by the National Heart, Lung, and Blood Institute (NHLBI) in the United States, or the Global Alliance against Chronic Respiratory Diseases
(GARD)\textsuperscript{39}, with the goal to reduce the global burden of chronic respiratory diseases, the health-care costs from asthma-related complications continues to grow\textsuperscript{37}.

Asthma affects both developed and developing nations\textsuperscript{40} and its prevalence varies in different countries throughout the world\textsuperscript{41}. Previously, the rates were higher in more developed countries, but the gaps are now closing, given the rise in prevalence in low- and middle-income countries and plateauing in high-income countries\textsuperscript{42,43}. Prevalence has been associated with adoption of a modern, urban lifestyle; however, it is unclear what specific factors are causal in nature. Increased prevalence of asthma is also strongly correlated with increases in allergy, a trend observed over the past 40 years\textsuperscript{44}.

Epidemiological studies have addressed questions regarding the disparity in asthma prevalence between urban (7.1\%) and rural (5.7\%) areas, a disparity that has been also observed in Canada\textsuperscript{45,46}. In recent years, a focus on the link between air pollutants and allergic diseases has gained increased attention, based on the plausibility that irritant pollutants can facilitate the access of allergens to immune-mediating cellular pathways or otherwise synergize allergenic effects\textsuperscript{47}. Exposure to endocrine disruptors, such as phthalates, has been suggested as a contributing factor, through modulation of immune responses and enhanced allergic responses\textsuperscript{48}.

1.2.2 Asthma diagnosis

Asthma is a syndromic disorder, defined by a collection of clinical signs, symptoms, and findings, rather than a single definitive test\textsuperscript{41}. Hallmark characteristics like variable airflow obstruction, airway inflammation and hyper-responsiveness may vary amongst different people and are sometimes inconsistently defined\textsuperscript{41}. This leads to considerable variability in
the diagnosis of asthma and a spectrum of clinical presentations encompassed under the broad umbrella of asthma\textsuperscript{41}. Diagnostic practices that differ among countries and cultures, and depend upon patients access to medical care and an adequate, well-trained health care workforce, can make standardization of asthma diagnosis and surveillance problematic\textsuperscript{49}. Nevertheless, clinical guidelines clearly define the criteria for asthma diagnosis based on patient history, physical examination, symptoms, and pulmonary function testing\textsuperscript{50}. Pulmonary function testing can detect physiological changes for both diagnosis and monitoring of the disease and is extremely important\textsuperscript{51}. Especially because studies have shown that physicians are often inaccurate in diagnosing asthma and predicting reversibility of obstruction based on history and physical examination alone\textsuperscript{51}.

Spirometry is one of the pulmonary function tests commonly employed\textsuperscript{50}. It is often repeated after administering a bronchodilator to confirm reversible/variable airflow obstruction, characterized by a 12\% increase in the FEV\textsubscript{1} value\textsuperscript{50}. In addition, demonstration of airway hyper-responsiveness by a direct bronchoprovocation test is useful in evaluating patients who have asthma symptoms but normal spirometry\textsuperscript{52}. Methacholine, a bronchoconstrictor medication that acts on smooth muscle receptors to constrict the airway, is administered via nebulization during a 2-minute tidal breathing, in doubling concentrations until the FEV\textsubscript{1} falls more than 20\% (methacholine PC\textsubscript{20}) or the highest concentration is reached. A methacholine PC\textsubscript{20} of less than 16mg/ml is characterized as mild airway hyperresponsiveness, less than 4mg/ml is considered moderate hyperresponsiveness, and less than 1mg/ml is severe hyper-reactivity. Values greater than 16mg/ml indicate no significant airway hyperresponsiveness\textsuperscript{41}.
1.2.3 Clinical, cellular and molecular hallmarks of asthma

Clinical diagnosis of asthma can be defined by the history of respiratory symptoms, such as wheeze, shortness of breath, chest tightness and cough, considering variation over time and intensity\textsuperscript{33}. The heterogeneity of asthma is widely recognized, but so far it has been difficult to link molecular mechanisms (endotypes) to clinical phenotypes\textsuperscript{53}. The majority of asthmatic patients are atopic, and allergic reactions to otherwise innocuous environmental agents remains the hallmark of allergic asthma\textsuperscript{41}.

In allergic asthma, many inflammatory cells are recruited from the blood into the lungs under the direction of locally released chemotactic factors\textsuperscript{54}. The inflammatory response involves innate immune cells like eosinophils, neutrophils, macrophages, mast cells, natural killer cells, innate lymphoid cells and dendritic cells, as well as adaptive immune cells like T and B lymphocytes. Finally, structural cells like fibroblasts, epithelial and endothelial cells also play an important role in orchestrating the inflammatory responses\textsuperscript{54}.

The allergic inflammation in asthma is driven by CD4+ T helper 2 (Th2) lymphocytes, which secrete IL-4, IL-5 and IL-13, and is commonly referred to as Type 2 asthma\textsuperscript{55}. Conversely, some asthmatic patients do not have this pattern of inflammation, usually referred to as non-T2 asthma, generally associated with a more severe disease\textsuperscript{55}. The concept of Th1–Th2 lymphocyte imbalance is now decades old, and the evidence for Th2 bias in the development of allergic asthma is compelling\textsuperscript{56}. Unsupervised clustering algorithms have shown that the various asthma endotypes fall into Th2-high or Th2-low clusters, on the basis of the presence or absence of Th2-associated cytokines and eosinophils in blood and lung samples\textsuperscript{57}.
The initial steps in airway responses to an allergen begin with antigen presentation by dendritic cells to naïve T cells\textsuperscript{58}. This presentation stimulates the development of Th2 cells and the production of a variety of cytokines that promote the production of IgE (IL-4), eosinophilopoiesis (IL-5), mast cell development (IL-9), and goblet cell hyperplasia and hyper-responsiveness (IL-13)\textsuperscript{58}. The role of IL-13 in airway hyperresponsiveness appears to be of particular importance, because it allows for mechanistic, diagnostic, and potential therapeutic implications\textsuperscript{58}.

Furthermore, alternatively activated macrophages or “M2” have been increasingly understood to contribute to Th2 immunity through cytokines that amplify type 2 immune responses\textsuperscript{59}. Alveolar M2 macrophages are activated by IL-4, IL-10, immune complexes, glucocorticoids and transforming growth factor-β\textsuperscript{60}. They promote tissue repair, cell proliferation and clearance of apoptotic cells and debris, and express abundant mannose (CD206) and haptoglobin-haemoglobin (CD163) scavenger receptors that mediate detection and capture of airborne pathogens\textsuperscript{61,62}. In the lung, M2 macrophages are associated with asthma, although their exact contribution to the pathogenesis of the disease is currently unknown\textsuperscript{63}. Further research is necessary to better delineate and understand the effects of combined environmental allergens and toxicants on the determination of the asthma phenotype\textsuperscript{41}.

### 1.3 Phthalates and airway effects

Phthalates are considered endocrine disruptors. They are associated with several reproductive disorders as well as tumorigenesis, affecting not only the endocrine or reproductive systems, but also the immune system\textsuperscript{48}. Moreover, several epidemiological
studies have reported an association between phthalate exposure and increased prevalence of wheeze, asthma, and allergies\textsuperscript{13,24,64}. Experimental studies generally support the role of phthalates as potentiators of allergen effects, reporting adjuvant effects\textsuperscript{24,25,65}.

\textbf{1.3.1 Epidemiological studies}

Epidemiological study designs generally favour the investigation of HMW phthalates, like DEHP\textsuperscript{24,66,67}. Many rely on the assessment of urinary metabolites, which reflects the total body burden of phthalate exposure that is dominated by HMW phthalates\textsuperscript{4,68,69}. Moreover, studies that focus on indoor environments measure phthalate concentrations in indoor dust which are dominated by HMW phthalates, as the LMW phthalates are generally present in the gas phase or associated with inhalable particular matter\textsuperscript{7,8}.

Several studies have shown an association between DEHP and airway effects\textsuperscript{24}. In a cross-sectional study assessing potential correlation of phthalate exposure with allergies, exposure to DEHP in house dust was associated with parental-reported rhino-conjunctivitis in children\textsuperscript{66}. After stratification by sex, this trend was found to only occur in boys\textsuperscript{66}.

Interestingly, in the same study, DBP found in dust from multiple surfaces in the indoor environment was significantly associated with allergic rhinoconjunctivitis in girls\textsuperscript{66}.

Some studies have also reported associations with DBP. For example, a study following 14–15-year-old adolescents, found that urinary metabolites from DBP (MBP) and the sum of three DEHP-derived metabolites (MEHP + MEHHP + MEOHP), significantly increased the risk of being diagnosed with asthma. In the same study, urinary levels of oxidative stress marker 8-OHdG, were associated with several urinary phthalate metabolites, particularly in girls\textsuperscript{67}. Amongst children in China, an increased risk of asthmatic and allergic symptoms was
associated with increased levels of urinary MnBP and MiBP metabolites compared to other phthalates, whereas MEHHP and MEOHP (metabolites of DEHP) were associated with an increased risk of allergic rhinitis and eczema\textsuperscript{70}.

Lastly, one epidemiological study assessed the role of indoor exposure using a biomonitoring model reflecting inhalation and dermal exposure as well as intake of dust\textsuperscript{71}. They reported significant associations between non-dietary exposure to DBP and DEHP and allergic sensitization, while urinary metabolite levels were not associated with any of the allergic outcomes. This suggests an important role for the indoor exposure routes, including inhalation and dermal uptake\textsuperscript{71}.

1.3.2 Animal studies

The majority of animal model studies on phthalates have focused on DEHP and its metabolites, highlighting its adjuvant role that leads to enhanced allergic responses\textsuperscript{13}.

The ovalbumin (OVA) model is the most commonly applied model in animal studies. For example, using an OVA sensitization weanling mice model and gastrointestinal exposure to DEHP, demonstrated that DEHP acted as an immuno-adjuvant augmenting the OVA-specific IgE and IgG1 production, and stimulating the expansion of plasma cells\textsuperscript{72}. Another study used an OVA mice model to demonstrate that gastrointestinal exposure to DEHP increased airway reactivity and immune responses\textsuperscript{65}. There was a strong positive association between combined OVA and DEHP exposure and serum total IgE, as well as IL-4, eosinophil recruitment and airway hyper-responsiveness for higher concentrations of DEHP\textsuperscript{65}. Lastly, local (nasal) IL-13 levels were significantly higher in mice treated with DEHP and OVA compared to allergen alone in an OVA-induced rhinitis mice model\textsuperscript{73}.
One study assessed the effect of nasal instillation of DEHP in a cockroach allergen extract (CE)-induced mouse model of asthma. In that study, DEHP promoted a Th2 and Th17 immune response, facilitating the development of a mixed granulocytic airway inflammation instead of the eosinophilic response induced by allergen alone\textsuperscript{74}. This study demonstrated the importance of allergen and phthalate exposure occurring through the same exposure route, and also highlighted that environmentally relevant concentrations of DEHP can induce airway effects alone or in combination with an allergen \textsuperscript{74}.

With regard to animal studies assessing effects of DBP, these are generally focused on skin hypersensitivity models using fluorescein isothiocyanate (FITC)-induced contact hypersensitivity\textsuperscript{75}.

1.3.3 Cell culture studies

Innate immune responses are most commonly assessed in terms of phthalate effects on monocytes, macrophages and dendritic cells, whereas some studies also address adaptive effects through T cell responses\textsuperscript{24}.

In a systemic review, DEHP consistently increased the secretion/production of tumour necrosis factor-\(\alpha\) from monocytes and macrophages, although the \textit{in vitro} studies varied in terms of study design, phthalate concentration and cellular source \textsuperscript{25}. With regard to effects of DBP on innate immune cells, higher doses of DBP had cytotoxic effects on murine peritoneal exudate macrophages (PEMs), whereas moderate doses decreased phagocytosis, cytokine production and antigen-presenting capacity of the PEMs\textsuperscript{76}. Moreover, DBP tended to stimulate the migration of M2 macrophages and not M1, and significantly increased M2 derived IL-1\(\beta\) release\textsuperscript{77}.
With regard to adaptive immune responses, the T cell stimulatory function of plasmacytoid DC (pDCs) pre-treated with DEHP and inflammatory stimuli was impaired, suggesting that phthalates may interfere with immunity against infection\textsuperscript{78}. In the same study, DEHP promoted the deviation of a Th2 response by acting on human pDCs via suppressing IFNα/IFNβ expression, thus modulating the ability to stimulate T cell responses\textsuperscript{78}. Moreover, DEHP has been reported to enhance IL-4 production in activated CD4+ T cells in a concentration dependent manner, providing \textit{in vitro} support for the animal studies suggesting that DEHP can enhance allergic responses\textsuperscript{79}.

\textbf{1.3.4 Human exposure studies}

Despite numerous epidemiological, animal and \textit{in vitro} studies suggesting an association between phthalate exposure and respiratory and allergic diseases, human exposure studies are limited.

A controlled exposure challenge to degraded PVC in 10 workers who had previously experienced respiratory symptoms, suspected to be caused by this kind of exposure in their workplace, found a 50\% increase in the number of respiratory tract symptoms reported compared with 0\% before the challenge. The exposure did not influence lung function parameters like exhaled nitric oxide (NO), nasal NO, or NO in the nasal lavage, and no changes in the proportions of lymphocytes, neutrophils, or eosinophils were observed in the differential cell counts from nasal samples between the conditions\textsuperscript{80}. Another human exposure study investigated the effect of nasal instillation of house dust containing low or high concentrations of DEHP, on the nasal mucosa of 16 healthy and 16 house dust mite (HDM)–allergic subjects. Healthy subjects had almost no response to DEHP, but HDM-
allergic subjects showed varied responses. Low concentrations of DEHP elicited a silent inflammation in the nasal mucosa measured at both the protein and mRNA level whereas high concentrations attenuated this inflammation\(^8\).

Finally, an exposure verification study comparing transdermal and inhalation exposure to DEP and DBP concluded that dermal uptake directly from air is a meaningful exposure pathway for DEP and DBP\(^{14}\). The study measured phthalate metabolites in urine over time, but no other sample type was collected or analyzed\(^{14}\).

1.4 PAIR study

1.4.1 Study overview

To date, no studies have performed a controlled human phthalate inhalation exposure to assess implications on lung function and the airway’s cellular and inflammatory mediator milieu. To provide human data that could strengthen the associations reported in epidemiological and experimental studies, a double-blinded, randomized, controlled, crossover study enrolling 16 allergen-sensitized participants was approved by the research ethics board at the University of British Columbia (H14-01119), the Norwegian Regional Committees for Medical and Health Research Ethics (2014/1217) and registered at ClinicalTrials.gov (NCT02688478).

The Phthalate-Allergen Immune Response (PAIR) study, is the first to investigate airway and systemic effects in humans due to inhalation of a known concentration of a single phthalate. DBP was chosen as the model phthalate since it is one of the phthalates found in highest concentrations in indoor air\(^7\) and it appears to have a higher inflammatory potential compared to other phthalates \textit{in vitro}\(^8\).
1.4.2 PAIR study aim and thesis hypotheses

The overall aim of the PAIR study is to understand how DBP may alter the immune response to an allergen in the human airway and at a systemic level.

The research project presented here tested the following hypotheses, in order to address a substantial portion of the PAIR study’s specific aims. Each hypothesis below is described in a corresponding chapter of this doctoral dissertation.

- DBP in the presence of an inflammatory stimuli in vitro, will downregulate the surface marker expression and increase the activation state of innate immune cells from peripheral blood, and decrease the production of pro-inflammatory cytokines. (Chapter 2).

- Inhalation of DBP prior to allergen will
  
  - enhance airway inflammation and responsiveness, and promote immune cell recruitment and activation in the lower airway (Chapter 3)
  
  - enhance allergen-induced systemic inflammation and promote immune cell recruitment and activation in peripheral blood (Chapter 4).
Chapter 2: Dibutyl phthalate modulates phenotype of granulocytes in human blood in response to inflammatory stimuli.

2.1 Synopsis

Phthalates are plasticizers used in many common commercial products. They are ubiquitous environmental contaminants and epidemiological studies suggest that phthalate exposure is associated with development or worsening of airway diseases. DBP is a type of phthalate, found in high concentrations in indoor air. *In vitro* studies on innate immune cells like macrophages have shown a reduction in phagocytic and antigen-presenting capacity and decreased production of stimuli-induced cytokines after DBP exposure. We aimed to assess how DBP may alter the *in vitro* cellular and humoral innate immune response to inflammatory stimuli in blood innate immune cells.

Human whole blood was stimulated with inflammatory stimuli (lipopolysaccharide (LPS), resiquimod (R848) or phorbol 12-myristate 13-acetate (PMA)) in the presence or absence of DBP. The expression of surface markers CD16, CD24, CD69 and CD14 on granulocytes and monocytes was quantified by flow cytometry analysis. The release of TNFα, IFNγ, IL8 and IL10 cytokines was measured by ELISA. The presence of DBP reduced the inflammatory stimuli-induced expression of CD24 on neutrophils and eosinophils and CD69 on activated eosinophils, whereas expression of CD16 on neutrophils was increased. DBP also had a dampening effect on the release of pro-inflammatory mediators TNFα and IFNγ in response to the inflammatory stimuli.

These responses reflect an immunosuppressive effect of DBP that may occur through impairment of immune cell function.
2.2 Background and objective

Phthalates are diesters of benzenedicarboxylic acid (phthalic acid) that are produced in large quantities worldwide. Broadly used in industry as plastic softeners, phthalates can be found in a variety of common commercial products including clothing, cosmetics, lubricants, waxes, cleaning materials, medical devices and building materials such as polyvinyl chloride (PVC) flooring. Due to weak binding between phthalates and their carrier plastic, phthalates are continuously released into the environment, and are considered ubiquitous environmental pollutants. The general population is continuously exposed to phthalates as confirmed by the presence of phthalate metabolites in nearly all analysed urine samples in large epidemiological studies. In addition, a positive correlation between the amount of indoor air phthalates and the presence of their primary and secondary (biotransformed) metabolites in urine samples has been observed.

The main routes of phthalate exposure are ingestion, inhalation and dermal uptake. Ingestion, typically by dietary exposure, is considered the most common and it occurs when food or beverages are exposed to packaging containing phthalates. Inhalation exposure occurs through inhalation of contaminated indoor air due to emissions from building materials and consumer products such as fragrances, dryer sheets, air fresheners and sunscreens. Dermal exposure consists of both direct trans-dermal uptake from the environment, and absorption after application of lotion and other personal care products containing phthalates.

The contribution of these various sources and exposure routes to the total phthalate exposure depends on age and sex. For DBP, food consumption contributes to 40–90% of the total exposure across all consumer groups. In infants, toddlers and children indoor air contributes...
between 20–40% of the total DBP intake, whereas dust contributes to 10%. In teenagers, indoor air contributes less to the total DBP intake (14–22%). Moreover, the use of personal care products amongst teenagers and female adults causes exposure via skin and inhalation constituting between 15–50% of the total DBP intake^4.

In addition to their known endocrine disruptive function^84, phthalate exposure has been associated with other adverse health effects including altered genital development^88, altered neural development^89, diabetes^90, oxidative stress^91, obesity^92, as well as adverse respiratory outcomes that include increased risk of bronchial obstruction, susceptibility to respiratory infections^93,94, wheezing and asthma^64. Justifiably, increasing interest is being focused on deepening the understanding of these associations and the mechanisms involved^7,13,64.

However, real life exposure effects and the identification of molecular targets that could explain mechanism of action and underlying molecular interactions remain undetermined^6,24.

Several potential molecular mechanisms involved in the asthma promoting effect of phthalates have been suggested. For instance, DNA hypermethylation and a subsequent global dysregulation in DNA methylation in the offspring of mice. The epigenetic changes led to altered expression of genes that played a crucial role in immune regulation, including hypermethylation of GATA-3 repressor zinc finger protein 1 (Zfpm1). This is a highly relevant candidate for mediating the enhanced susceptibility for Th2-driven allergic asthma^95.

Peroxisome proliferation activated receptors (PPARs), have also been identified as phthalate targets and suggested as a possible mechanistic link through partial antagonist effects^7.

Phthalates have been presented as modulators of the immune system, influencing synthesis of cytokines, immunoglobulins, and cell mediators, as well as immune cell activation and survival^48. Interestingly, several studies also report an attenuating rather than stimulatory
effect of phthalates in vitro\textsuperscript{24}. For example, DBP reduced tumor necrosis factor $\alpha$ (TNF$\alpha$) secretion or production in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells\textsuperscript{96}, in the mouse macrophage cell line RAW 264.7\textsuperscript{97} and in murine peritoneal exudate macrophages\textsuperscript{76}. Modulating host defense mechanisms, by interfering with the elimination of bacteria by alveolar macrophages or dampening the expression of pro-inflammatory mediators, may have a significant impact on the development of respiratory infections, particularly in susceptible populations exposed to phthalates\textsuperscript{7}.

The whole blood assay (WBA) has been used to investigate cellular responsiveness to a variety of stimuli because it mimics the natural environment\textsuperscript{98}. Natural cellular crosstalk is preserved and circulating stimulatory and inhibitory mediators remain present\textsuperscript{99}. Through the recognition of pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs), specific signaling pathways that influence the orientation of the immune response can be triggered\textsuperscript{100}.

LPS is a major component of the cell wall of gram-negative bacteria and a commonly used TLR ligand that binds to TLR4\textsuperscript{101}. After LPS interaction with signal adaptor myeloid differentiation primary response 88 (MyD88), the transcription factor nuclear factor kappa beta (NF-κB) can be activated to produce pro-inflammatory cytokines; whereas receptor interaction with the signal adaptor TRIF (TIR-domain-containing adapter-inducing interferon-β) activates the interferon pathway\textsuperscript{102}.

On the other hand, Resiquimod (R848) is an imidazoquinolinone compound that binds to TLR7/TLR8 triggering viral responses through MyD88-dependent and activation of interferon regulatory factor 5 (IRF5) and IRF7 pathways\textsuperscript{103,104}. 
Lastly, phorbol 12-myristate 13-acetate (PMA) is a surrogate of diaclylglycerol and known activator of protein kinase C (PKC) pathways. In the context of a WBA, the use of LPS, R848, and PMA permit the assessment of a wide range of inflammatory responses through partially overlapping immune pathways. It is important to highlight that although the WBA system provides a robust and more physiologically relevant environment compared to other in vitro designs, very few studies report cellular surface marker expression. The majority focus on the assessment of serum biomarkers and cytokine profiles.

DBP is a low molecular weight phthalate and ideal candidate for analysis due to its high inflammatory potential compared to other phthalates and relatively high abundance in indoor air. To inform the choice of endpoints in an anticipated, funded and approved in vivo study, we investigated the effects of DBP on the in vitro cellular and humoral innate immune response to inflammatory stimuli (IS) in human whole blood. We hypothesized that DBP in the presence of an IS would downregulate the surface marker expression and increase the activation state of innate immune cells and decrease the production of pro-inflammatory cytokines.

2.3 Methods

2.3.1 Ethics statement and sample collection

The study was approved by the research ethics board at the University of British Columbia (H14-01119) and the Norwegian Regional Committees for Medical and Health Research Ethics (2014/ 1217). The study conforms to the standards of the Declaration of Helsinki and no deviations were made from the approved protocol.
Written informed consent was signed by healthy volunteers between the ages of 19 to 49 years, from whom whole peripheral blood was collected into sodium heparin tubes (Vacutainer, BD, New Jersey, USA). Blood samples were kept at room temperature and processed immediately.

2.3.2 Whole blood assay

Human peripheral whole blood was added to a 24-well plate (VWR, PA, USA) and diluted 1:1 vol/vol with RPMI-1640 Medium (Millipore- Sigma, St. Louis, USA) plus penicillin-streptomycin (Thermo Fisher Scientific, MA, USA) totaling 1 ml per well. A 5mM stock of DBP (Millipore-Sigma, Ontario, Canada) was prepared in dimethyl sulfoxide (DMSO, Millipore-Sigma, St. Louis, USA) and 2 μl added to the wells for a target final concentration of 10μM of DBP. Control wells were exposed to the same concentration of DMSO.

After a 20-hour incubation at 37 °C with 5% CO2, the IS were added, and the samples were incubated for 4 more hours. The IS used were lipopolysaccharide (LPS, from E. coli 026:B6, Millipore-Sigma, St.Louis, USA), Resiquimod (R848, Enzo Life Sciences, NY, USA,) and phorbol 12-myristate 13-acetate (PMA, Ann Arbor, USA). LPS stock solution (800 μg/ml) was diluted in RPMI-1640 media and added at a final concentration of 1 μg/ml, while stocks of R848 (3.14 mg/ml) and PMA (0.5 mg/ml) prepared in DMSO, were further diluted in RPMI media and added at a final concentration of 314 ng/ml and 25 ng/ml respectively. During the cellular exposure, the DMSO concentration remained below 0.2% of the total volume for all conditions.

After a total of 24 h incubation, cells were harvested by first pipetting the blood up and down to mix the cell suspension and centrifuging at 450×g for 5 min. The supernatant was then
removed by pipette and stored at −80 °C for subsequent analysis by ELISA, while the cellular fraction was processed immediately for flow cytometry analysis. **Figure 2.1** shows the applied model system.

**Figure 2.1** Experimental design of whole blood assay. *In vitro* stimulation of human whole blood with dibutyl phthalate (DBP) and inflammatory stimuli. LPS: lipopolysaccharide, R848: resiquimod, PMA: phorbol 12-myristate 13-acetate.

2.3.3 Flow cytometric analysis

The cellular fraction was stained with an antibody cocktail designed for the identification of human innate immune cells’ common surface and activation markers (Table 2.1).

**Table 2.1** Fluorochrome-conjugated antibodies and viability dye used to identify innate immune cells in human whole blood.

<table>
<thead>
<tr>
<th>Conjugated antibody [Clone]</th>
<th>Quantity/sample</th>
<th>Supplier</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16 FITC [3G8]</td>
<td>3µl</td>
<td>BD Biosciences</td>
<td>555406</td>
</tr>
<tr>
<td>CD24 PE [ML5]</td>
<td>3µl</td>
<td>BD Biosciences</td>
<td>555428</td>
</tr>
<tr>
<td>CD69 PE-Cy7 [FN50]</td>
<td>3µl</td>
<td>BD Biosciences</td>
<td>557745</td>
</tr>
<tr>
<td>CD14 APC [M5E2]</td>
<td>3µl</td>
<td>BD Biosciences</td>
<td>555399</td>
</tr>
<tr>
<td>CD45 APC-Cy7 [2D1]</td>
<td>3µl</td>
<td>BD Biosciences</td>
<td>557833</td>
</tr>
<tr>
<td>Fixable Viability Dye eFluor® 450</td>
<td>2µl</td>
<td>eBioscience</td>
<td>65-0863-14</td>
</tr>
</tbody>
</table>
Samples were stained for 20 min in the dark at room temperature. Red blood cells were lysed with BD FACSTM lysing solution (BD Biosciences, San Jose, CA, USA) for 15 min and washed with phosphate buffered saline (PBS, Millipore-Sigma, St. Louis, USA) according to the supplier’s protocol. Flow cytometry was performed using FACS Canto II (BD Biosciences, New Jersey, USA) with a 3-laser and 6-colour configuration.

Flow cytometric analysis was performed using FlowJo software version 10.1r5 (Tree Star, San Carlos, CA, USA) and FCS Express version 6.04.0034 (DeNovo Software, Los Angeles, CA, USA). Spectral spillover signals into adjacent detectors were corrected by applying an auto-compensation matrix created using single-stain compensation beads (BD Biosciences, New Jersey, USA) for all the channels, except the viability channel. Since compensation beads are not designed to bind live/dead dye, a combination of live/dead cells were used to compensate the viability signal. Settings were updated and saved before each acquisition by running BD Cytometer Setup and Tracking (CS&T) beads. The cell gating hierarchy used is shown in Figure 2.2A.

To determine a positive/negative cut-off for the gating strategy, fluorescence minus one (FMO) controls were used, as shown in Figure 2.2B. Following exclusion of debris, doublets and dead cells, surface marker CD14 and granularity (light scatter) characteristics separated monocyte, granulocyte and lymphocyte populations within the CD45+ cells (Figure 2.2C). Three different monocyte subsets were identified by their CD14/CD16 expression level. Neutrophils and eosinophils were identified by their expression of CD24 and (or lack of) CD16 surface marker, within the granulocyte population, respectively. Activation marker CD69 was observed in eosinophils in response to various experimental conditions.
The flow cytometry data were evaluated in terms of (i) the percentage of cells present in each population identified in the gating strategy (Figure 2.2), and (ii) the expression of surface markers (listed in Table 2.1) as mean fluorescence intensity (MFI) for each of these populations.

**Figure 2.2 Flow cytometry analysis.** A: Cell gating hierarchy. B: Fluorescence minus one (FMO) controls (CD45 not shown) displaying the unstained sample (grey), multi-stained sample (black) and FMO (colored). C: Density plots showing the gating strategy followed for the identification of populations of interest in whole blood (here, using an example of PMA plus DBP). Values inside the gates represent the percentages relative to the parent gate.
2.3.4 Cytokine production quantification by ELISA

Pro- and anti-inflammatory mediators TNFα, IL8, IL10 and interferon gamma (IFNγ), were measured with ELISAs (TNFα, IFNγ, and IL10: BD Biosciences, CA, USA; IL8: R and D Systems, MN, USA) following supplier’s instructions.

2.3.5 Statistical analysis and data processing

All statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, Inc., CA, USA). Most of the data was analyzed using either repeated measures one-way ANOVA (to test the effect of the inflammatory stimuli alone) or two-way ANOVA (to test the modulatory effect of DBP in the presence or absence of the inflammatory stimuli), with Sidak’s correction for multiple comparisons as indicated in the figure legends.

For the surface markers where DBP had a significant modifying effect on the response, and for all the measured cytokines, the percent change was calculated for the individual data (Table 2.2). The purpose was to highlight the size of the DBP-induced effect. These data were analyzed using a one-sample t-test using zero as the fixed value for comparison. A Bonferroni test for correction for multiple testing was used within each surface marker or cytokine.

2.4 Results

2.4.1 Effect of IS and DBP on the percentage of innate immune cells

The percentages of neutrophils, eosinophils, monocytes and their subsets, were not affected by exposure to DBP or IS, alone or in combination. Likewise, the cellular viability was not affected by any of these in vitro exposures (Figure 2.3).
Figure 2.3 Effect of inflammatory stimuli and DBP on the percentage and viability of cells. Data from whole blood assay for 5 donors after exposure to 10µM DBP or DMSO control for 20h followed by 4h co-
stimulation with DBP and inflammatory stimuli. In (A) effect of the inflammatory stimuli alone, displayed as the mean with SEM (one-way-ANOVA with Sidak’s post-test), and (B) effect of inflammatory stimuli in the presence of DBP, displayed as independent values (two-way-ANOVA with Sidak’s post-test). LPS: lipopolysaccharide, R848: resiquimod, PMA: phorbol 12-myristate 13-acetate.

2.4.2 Effect of IS and DBP on cell surface marker expression

The MFI's of the analysed surface markers used in the panel (CD14, CD16, CD24 and CD69) were moderately affected by the different exposures. With regard to effects of the IS alone, the expression of CD16 on intermediate monocytes was reduced by LPS (43%, p=0.005), while the expression of CD24 on neutrophils increased with the addition of LPS (39%, p=0.05) and PMA (29%, p=0.04) when compared to the unstimulated control (Figure 2.4A). A similar non-significant trend was observed for the expression of CD24 on eosinophils and CD69 on activated eosinophils. Exposure to DBP had a dampening effect on the expression of CD24 on neutrophils (p=0.004), CD24 on eosinophils (p=0.002) and CD69 on activated eosinophils (p=0.001), while CD16 expression on neutrophils increased when compared to the respective control (p=0.025) (IS stimulated sample without DBP; Figure 2.4B). The magnitude of these changes was as follows: CD24 expression on neutrophils and eosinophils was reduced by 5–11% (significantly for neutrophils/PMA), CD69 expression on activated eosinophils decreased by 9% (LPS), 18% (R848, significantly) and 5% (PMA); CD16 expression on neutrophils increased by 2–8% (Table 2.2).
Figure 2.4 Effect of inflammatory stimuli and DBP on surface marker expression. Data from whole blood assay for 5 donors after exposure to 10µM DBP or DMSO control for 20h followed by 4h co-stimulation with DBP and inflammatory stimuli. In (A) effect of the inflammatory stimuli alone, displayed as the mean with SEM (one-way-ANOVA with Sidak’s post-test), and (B) effect of inflammatory stimuli in the presence of DBP, displayed as independent values (two-way-ANOVA with Sidak’s post-test). In panel A: * indicates significant differences (p<0.05) relative to the unstimulated control. In panel B: # indicates significant effects of the DBP.
exposure factor and * indicates significance between pre-selected pairs (no-DBP vs 10µM DBP). LPS: lipopolysaccharide, R848: resiquimod, PMA: phorbol 12-myristate 13-acetate.

Table 2.2 Percent change due to DBP exposure. This table shows the percent change in the MFI of surface markers expressed on innate immune cells of interest, and in the cytokine release as the mean ± SEM of 5 independent experiments. As shown in Figure 2.4B and 2.5B, two-way ANOVA (considering the modifying effect of DBP upon the primary effect of each stimulus) was significant for each of the surface markers shown in this table, and for TNFα and IFNγ release, but not for IL8 and IL10. Positive values represent an increase while negative values represent a decrease in the MFI or cytokine release due to an effect of DBP relative to control (no inflammatory stimulus). The percent changes significantly different from zero when analysed with a one-sample t-test combined with Bonferroni correction for multiple correction (see statistics section for explanation) are indicated (*) and reflect a significant effect of DBP.

<table>
<thead>
<tr>
<th>% Change (Mean ± SEM)</th>
<th>CD24 on Neutrophils</th>
<th>CD24 on Eosinophils</th>
<th>CD69 on Activated Eosinophils</th>
<th>CD16 on Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>-5 ± 1</td>
<td>-7 ± 1</td>
<td>-9 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>R848</td>
<td>-7 ± 1</td>
<td>-10 ± 2</td>
<td>-18 ± 2*</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>PMA</td>
<td>-10 ± 1*</td>
<td>-11 ± 2</td>
<td>-5 ± 1</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% Change (Mean ± SEM)</th>
<th>TNFα</th>
<th>IFNγ</th>
<th>IL8</th>
<th>IL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>16 ± 20</td>
<td>-1 ± 3</td>
<td>1 ± 4</td>
<td>-36 ± 6</td>
</tr>
<tr>
<td>R848</td>
<td>-30 ± 7</td>
<td>-35 ± 5</td>
<td>-9 ± 4</td>
<td>-10 ± 3</td>
</tr>
<tr>
<td>PMA</td>
<td>-50 ± 10</td>
<td>-9 ± 2</td>
<td>-9 ± 6</td>
<td>-38 ± 9</td>
</tr>
</tbody>
</table>

2.4.3 Effect of IS and DBP on cytokine release

The 4 h exposure to the IS alone significantly increased the release of TNFα (409-fold) and IL10 (27-fold) for R848 when compared to the unstimulated control (Figure 2.5A). IL-8 and IFNγ levels were also considerably increased after IS exposure, but did not reach statistical significance, presumably due to large inter-individual variations. DBP reduced the overall IS-induced release of TNFα and IFNγ significantly (Figure 2.5B). In the presence of DBP, TNFα release increased by 16% after LPS stimulation, and was reduced by 30% and 50% after R848 and PMA stimulation respectively (p=0.034). DBP also reduced the IFNγ release by 1, 35 and 9% (p=0.047) after LPS, R848 and PMA stimulation respectively (Table 2.2).
2.5 Discussion

Several recent epidemiological studies have revealed a correlation between the levels of phthalates present in indoor air (including DBP) and the level of phthalate metabolites present in human urine\(^4\). While epidemiological studies suggest a link between phthalates and respiratory diseases\(^9\), the molecular mechanisms and pathways related to phthalate exposure in humans are still insufficiently understood.

We investigated the effects of DBP on the \textit{in vitro} cellular and humoral innate immune response to inflammatory stimuli using human whole blood. The obtained data also add complementary data to the limited body of knowledge regarding DBP-induced effects in human primary immune cells.
The observed increase of the cell surface marker CD24 in neutrophils following treatment with LPS (39%) and PMA (29%) is consistent with findings from another research group, where LPS increased CD24 expression on neutrophils in a whole blood assay. In that study, LPS did not affect purified neutrophils, suggesting that cellular crosstalk is necessary for altered CD24 expression. CD24 is a danger-associated molecular pattern (DAMP) costimulatory receptor with the ability to discriminate DAMPs from PAMP molecules.

Our results were consistent with the current understanding of the LPS-induced TLR4-dependent inflammatory response and the PMA-induced PKC-mediated activation of NF-κB response, typical of inflammation. To our knowledge, there are currently no other reports describing CD24 expression in the context of a whole blood stimulation with inflammatory stimuli.

We also observed a reduction (43%) in the expression of CD16 in the surface of intermediate monocytes, but not on the non-classical monocytes, following LPS stimulation. A number of studies have reported loss of CD16 expression after stimulation of whole blood with LPS, which could impair the ability to identify non-classical and intermediate monocyte subsets, while some report increased CD16 expression after LPS stimulation. Our results revealed monocyte subset specific responses to LPS, as we detected a reduction in the CD16 expression after LPS stimulation but not a complete loss, allowing us to distinguish between the three monocyte subsets under every condition tested.

In addition to modulating cell surface marker expression, IS induced a robust cytokine response in our WBA (Figure 2.5A). We observed a significant increase of TNFα and IL10 release (409-fold, and 27-fold respectively), after stimulation with R848, a potent TLR7/8 ligand. IS-induced release of IFNγ and IL8 was also evident, especially in response to LPS.
stimulation, but did not reach statistical significance probably due to large individual variations or possibly the relatively short IS incubation time of 4 h. It is known that R848 induces the expression of TNFα, interferons, and other pro-inflammatory cytokines through a (MyD88)-dependent pathway and through the activation of IRF5 and IRF7\textsuperscript{103,104}. Other studies have also observed a specific R848-induced increase in TNFα using the WBA\textsuperscript{110}, while increased IL10 release does not appear to be reported previously in the same context. Overall, the IS affected the cytokine release to a much larger extent than the surface marker expression, suggesting that the former may be a better choice of endpoint for the WBA approach.

Our results demonstrated that DBP had an overall dampening effect over the IS-induced expression of surface markers on granulocytes, and over the IS-induced cytokine release. The decreased expression of CD24 on eosinophils and neutrophils in the presence of DBP (Figure 2.4B, Table 2.2), although modest in terms of percent change, was consistent amongst all the IS tested (LPS, R848 and PMA). Decreased CD24 expression on neutrophils has been observed during sepsis and although it is unclear which factors play a role in the regulation of CD24 on neutrophils during inflammatory processes, this downregulation has been associated with decreased activity of CD24 regulated pathways and delayed apoptosis\textsuperscript{106}. If DAMP costimulatory receptor’s expression and function is compromised in the presence of DBP, this impaired ability to recognize an external insult may lead to a reduced or defective innate immune response.

The reduction in IS-induced expression of CD69 on activated eosinophils in the presence of DBP further support immunosuppressive effects of DBP. Eosinophils under resting conditions have intracellular pools of CD69, a marker of cytokine-induced eosinophil
activation\textsuperscript{111}, that are mobilized to the surface in response to various stimuli\textsuperscript{112}. Consistent with these studies, we also observed increased expression of CD69 after LPS, R848 and PMA stimulation. The implications of a dampened expression of this molecule are considerable. The structure, chromosomal localization, expression and function of CD69 suggest that it is likely a pleiotropic immune regulator\textsuperscript{113}. Downregulation of its expression could affect not only the function of eosinophils, but a variety of immune cells and consequently the inflammatory mediators they produce\textsuperscript{113}.

In response to DBP, we also observed increased expression of CD16 on neutrophils when compared to the IS alone. Shedding of CD16 is considered a hallmark of apoptosis\textsuperscript{114}. Neutrophils are constitutively apoptotic cells, unless they are functionally defective, as observed in sepsis patients, in which case apoptosis is delayed or shedding does not occur\textsuperscript{106}. To our knowledge, effects of DBP or other phthalates on IS-induced expression of CD16, CD69 or CD24 have not been reported in the literature.

The overall dampened release of TNF\protect\textalpha{} and IFN\gamma{} (Figure 2.5B) we observed in response to IS in the presence of DBP further strengthens the potential immunosuppressive effect of DBP in terms of impaired immune cell functionality. Consistent with our results, DBP reduced TNF\protect\textalpha{} and IFN\gamma{} secretion from LPS and phytohemagglutinin-P stimulated mononuclear cells, whereas IL10 was not affected\textsuperscript{25}.

A proposed mechanism of action involved modulation of prostaglandin (PG) metabolism and signaling based on the resemblance between the structure of phthalates and PGs\textsuperscript{115}. On the other hand, PGs have been reported to induce a cytokine secretion pattern similar to that observed in phthalate-exposed monocytes/ macrophages\textsuperscript{25}. Phthalate exposure also
suppressed secretion of IFNγ from plasmacytoid dendritic cells and enhanced IL13 production by CD4+ T cells, suggesting that they may interfere with immunity against infection and promote the deviation towards a T helper 2 response\textsuperscript{78}. Another possible mechanism explaining the reduced production of pro-inflammatory cytokines is the interaction between phthalates, including DBP, and peroxisome proliferator activated receptors (PPARs)\textsuperscript{116}. Mice and in vitro studies using cell lines have shown that DBP activates, albeit weakly, all three PPAR subtypes\textsuperscript{117}.

Overall, our study suggests immunosuppressive effects on the cellular response to IS challenge. The downregulation of CD24 and increased expression of CD16 on neutrophils as well as the reduced expression of CD69 on activated eosinophils strongly suggests an impaired functionality of the innate immune cells. This compromised ability to detect and respond to the IS challenge in the presence of DBP, led to a defective immune response observed through the overall dampened release of inflammatory mediators like TNFα and IFNγ.

This is the first study to report altered expression of surface markers in human granulocytes in response to DBP in vitro. In terms of magnitude of the response, the surface marker expression was less affected by all treatments than the cytokine release in vitro. These observations served as an input during the planning of our human controlled exposure study and could also inform other researchers considering one of these two measured outcomes. Since the use of flow cytometry is relatively costly and time consuming, only cytokine release was chosen for analysis of effects of IS in vitro in the anticipated in vivo human DBP exposure study. However, both surface marker expression and cytokine release are to be analyzed in freshly collected samples to examine in vivo effects of controlled DBP exposure.
The WBA is emerging as a useful assay to study immune cell function *in vitro* in epidemiological and clinical studies. For example, the presence of gram-positive bacteria in house dust, was associated with a reduction in Th1 cytokine (IFN-γ) production capacity in infant blood, suggesting that the microbial indoor air exposure influenced the infants’ allergy development through changes of immune responses\textsuperscript{118}. Moreover, LPS-induced IL-10 release was found to reflect individual susceptibility to adverse respiratory symptoms in response to high occupational endotoxin exposure\textsuperscript{119}. Although the WBA requires a minimal amount of blood and relatively simple sample processing in comparison to assays that isolate PBMCs and other cell types\textsuperscript{98}, it has not been used extensively for the study of immunomodulatory effects of environmental factors. However, WBA provides higher reproducibility in terms of cytokine responses than PBMCs, as demonstrated by its smaller intra-individual variations\textsuperscript{120}. The natural physiological environment in the blood is also well represented in WBA, which retains growth factors, cytokines and all cell types\textsuperscript{120}. Moreover, the current data demonstrate the WBA is a valuable tool also for the assessment of interactions between environmental pollutants and immune cells *in vitro*.

In summary, we observed significant modulatory effects of DBP upon the IS-induced responses *in vitro*. Consistent with observations by other groups, DBP dampened the innate immune responses to IS *in vitro*. Inflammatory stimuli exposure *in vitro* has limited effect on surface marker expression, but strong effect on cytokine release. Thus, cytokine production and release appear to be a more suitable endpoint in WBA analyses. Additionally, this emphasizes the value of the WBA as a model to study environmental pollutant effects *in vitro*. 

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\textsuperscript{118} Density of LPS

\textsuperscript{98} LPS concentration

\textsuperscript{119} In vitro

\textsuperscript{120} WBA analyses
Chapter 3: Dibutyl phthalate (DBP) augments allergen-induced lung function decline and alters lower airway innate immunology in crossover human study

3.1 Synopsis

Phthalates are a group of chemicals used as plastic softeners in many common commercial products. They leak out of their carrier plastic and become ubiquitous environmental contaminants. Epidemiological studies suggest that phthalate exposure is associated with development or worsening of airway diseases like asthma, but causation is not currently established and further longitudinal, molecular, and intervention studies are needed. Dibutyl phthalate (DBP) is a type of phthalate found in high concentrations in indoor air.

We performed a controlled, randomized, double-blind crossover study of 16 allergen-sensitized participants each exposed for 3 hours to control air and DBP, with a 4-week washout between exposures. We hypothesized that DBP inhalation prior to allergen inhalation would enhance airway inflammation and alter macrophage phenotype in the lower airway. Lung function measures FEV₁, FeNO, and response to an inhaled allergen and methacholine challenge were assessed. BW and BAL were obtained 24h post-exposure. The cellular fraction of each was used for immunophenotyping by flow cytometry and \textit{ex vivo} functional assays, while the supernatant was examined for immune mediators.

DBP exposure augmented the allergen-induced FEV₁ decline (p=0.032), while FeNO and methacholine PC₂₀ were not affected. DBP increased the recruitment of total macrophages to the lower airway measured in BAL (p=0.07) and enhanced the M2 skewing by specifically increasing the M2 phenotype (p=0.04) and their expression of CD206 (p=0.07), whereas the
number of Th1 cells decreased (p=0.07). This conventional (manual gating) analysis was supported by unsupervised computational analysis.

DBP exposure had modest effects on inflammatory mediator levels in the airways. In the BW fraction, DBP exposure increased MIP-1α (p=0.07) and fractalkine (p=0.07), while it decreased Flt-3L (p=0.02) and SCF (p=0.07) compared to control air. The functionality of alveolar macrophages, measured by the response to inflammatory stimuli ex vivo, was generally suppressed by DBP exposure.

In summary, we demonstrated that DBP inhalation augments an allergen-induced lung function decline in atopic individuals. Moreover, DBP has immuno-modulatory effects by altering the airway cellular and inflammatory milieu induced by an allergen inhalation in vivo. This is the first human exposure study of a specific phthalate, providing biological evidence that expands the epidemiological and experimental literature linking phthalates to airway diseases.

3.2 Background and objective

Phthalates are a group of chemicals used as plastic softeners in many common commercial products like cosmetics, toys, building materials and medical devices. Due to the weak, non-covalent binding to their carrier plastics, phthalates leak into the environment and are considered ubiquitous environmental contaminants. The extent of the exposure has been demonstrated by the presence of phthalate metabolites in the majority of the human urine samples analysed in several epidemiological studies.
Dietary consumption, inhalation and dermal absorption are the main exposure routes for phthalates in humans, and of these ingestion is the dominating route\textsuperscript{11,15}. For dibutyl phthalate (DBP), one of the phthalates found in high concentrations in indoor air\textsuperscript{7,85}, inhalation may contribute to more than 20\% of the daily internal dose\textsuperscript{4,15}.

More than 30 epidemiological studies have linked phthalate exposure to allergic diseases in the last decade, using exposure indicators like house dust levels of phthalate, presence of urinary metabolites and prenatal phthalate exposure\textsuperscript{121–124}. These epidemiological studies generally favour high molecular weight phthalates like diethylhexyl phthalate (DEHP) and are not designed to study inhalation exposure. They do however support a link between overall phthalate exposure and asthma and allergic diseases\textsuperscript{121–124}. The role of inhalation and dermal uptake is challenging to address in epidemiological studies, but through a modelling approach, Bekö et al (2015) identified an important role for low molecular weight phthalates through these exposure routes in allergic sensitization\textsuperscript{71}.

Exposure to phthalates has been proposed to contribute to the dramatically increasing prevalence of allergic respiratory diseases\textsuperscript{6,34}. A possible physiological mechanism for this association is that phthalates act as adjuvants, enhancing the effects of an allergen\textsuperscript{24,64}. Accordingly, animal studies reported that ingestion of DEHP induced adjuvant effects (\textit{eg}, augmented specific IgE and histopathological effects), increased airway inflammation and hyper-responsiveness (AHR) in an ovalbumin mouse model\textsuperscript{65,72}. Although very few animal studies apply phthalates directly to the airway, two recent studies report that intranasal exposure to environmentally relevant doses of DEHP augmented inflammatory allergen responses in mice\textsuperscript{73,74}. 
Approximately 300 million people worldwide have asthma and at least 250,000 deaths are attributed to the disease each year, making it the most common chronic lung disease\textsuperscript{41}. An important molecular mechanism of asthma is type 2 inflammation, driven by T helper 2 (Th2)-derived cytokines, eosinophils and B cells, which occurs in many but not all patients\textsuperscript{125,126}. Furthermore, alternatively activated macrophages or “M2” have been increasingly understood to contribute to Th2 immunity through cytokines that amplify type 2 immune responses\textsuperscript{59}. Alveolar M2 macrophages express abundant mannose receptor (CD206) which mediates detection and capture of air-borne pathogens but also limits the phagosome-lysosome interaction during resolution of inflammation\textsuperscript{62}.

Since there is a paucity of direct evidence of effects of phthalates in humans, studies of inhalation exposure to phthalates through controlled chamber studies have been recommended\textsuperscript{64}. Inhalation exposure is highest to low molecular weight phthalates, and of these, DBP has been shown the highest inflammatory potential\textsuperscript{82}. The Phthalate-Allergen Immune Response (PAIR) study, reported here, is the first to investigate airway effects in humans due to inhalation of a known concentration of a single phthalate, DBP.

Using a randomized controlled crossover design to enhance power, we tested the hypothesis that DBP inhalation prior to allergen inhalation, enhances airway inflammation and responsiveness, promoting immune cell recruitment and activation in the lower airway. The PAIR study, designed to extend the current literature, aims to demonstrate that the inhalation exposure route, neglected in the design of epidemiological studies, may cause airway effects. Since effects of phthalates alone are seldom reported in the experimental literature, the allergen inhalation challenge was included in both exposure conditions.
3.3 Methods

3.3.1 Ethics statement

The study was approved by the research ethics board at the University of British Columbia (H14-01119), the Norwegian Regional Committees for Medical and Health Research Ethics (2014/1217) and registered at ClinicalTrials.gov (NCT02688478). No deviations were made from the approved protocol and it conforms to the standards of the Declaration of Helsinki.

3.3.2 Participant recruitment and screening

Study participants were recruited through posters and online notices. Written informed consent was signed and inclusion/exclusion criteria followed during the screening process. Allergen-sensitized, healthy or mildly asthmatic participants between the ages of 19 to 49 years enrolled in the study. At screening, baseline AHR status was assessed through a methacholine challenge (Table 3.1).

Table 3.1 Participants characteristics in PAIR study

<table>
<thead>
<tr>
<th>Participant</th>
<th>Methacholine PECO₂ (mg/mL)</th>
<th>AHR status</th>
<th>Allergen (Concentration)</th>
<th>Sex</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;128</td>
<td>No</td>
<td>Grass (1/256)</td>
<td>M</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>&gt;128</td>
<td>No</td>
<td>Birch (1/128)</td>
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<td>29</td>
</tr>
<tr>
<td>3</td>
<td>6.9</td>
<td>Yes</td>
<td>HDM (1/4)</td>
<td>F</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Yes</td>
<td>Grass (1/256)</td>
<td>M</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>&gt;128</td>
<td>No</td>
<td>Grass (1/512)</td>
<td>F</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>&gt;128</td>
<td>No</td>
<td>HDM (1/32)</td>
<td>M</td>
<td>31</td>
</tr>
<tr>
<td>7</td>
<td>2.9</td>
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<tr>
<td>8</td>
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<td>F</td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td>64</td>
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<td>Birch (1/128)</td>
<td>F</td>
<td>34</td>
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<tr>
<td>10</td>
<td>&gt;128</td>
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<td>M</td>
<td>26</td>
</tr>
<tr>
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<td>1.7</td>
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<td>33</td>
</tr>
<tr>
<td>12</td>
<td>121.1</td>
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<td>HDM (1/32)</td>
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<td>21</td>
</tr>
<tr>
<td>13</td>
<td>47.9</td>
<td>No</td>
<td>Birch (1/16)</td>
<td>F</td>
<td>21</td>
</tr>
<tr>
<td>14</td>
<td>0.3</td>
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<td>15</td>
<td>16</td>
<td>Yes</td>
<td>Birch (1/32)</td>
<td>M</td>
<td>27</td>
</tr>
<tr>
<td>16</td>
<td>14.5</td>
<td>Yes</td>
<td>HDM (1/32)</td>
<td>M</td>
<td>36</td>
</tr>
<tr>
<td>n = 11</td>
<td>No = 8</td>
<td>Grass = 5</td>
<td>HDM = 6</td>
<td>M = 6</td>
<td>Mean ± SD = 31 ± 8</td>
</tr>
<tr>
<td></td>
<td>Yes = 8</td>
<td>Birch = 5</td>
<td></td>
<td>F = 10</td>
<td></td>
</tr>
</tbody>
</table>
Baseline response to methacholine during screening visit was used to classify participants based on airway hyper-responsiveness (AHR) status as either hyper-responsive (PC20 ≤ 16mg/mL) or normally responsive (PC20 > 16mg/mL). Specific allergen and concentration inhaled are also shown. (b) indicates participants consenting and undergoing a bronchoscopy procedure. HDM: House dust mite.

3.3.3 Study design

Sixteen participants enrolled in a double-blind, randomized, crossover study that was counter-balanced to order between two exposure conditions (DBP or control air, Figure 3.1). Each exposure period was separated by at least 4 weeks (washout period) to avoid carryover effects of inhaled allergen¹²⁷.

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**Figure 3.1 PAIR study design and endpoints.** Consentig participants were screened and randomly assigned to two groups for a double-blind crossover exposure to DBP or control. FEV₁: Forced expiratory volume in 1 second. FeNO: Fractional exhaled Nitric Oxide. MFI: Median fluorescence intensity.
3.3.4 Exposure method and considerations

The three-hour inhaled and dermal exposure to approximately 150 (mean 147.5, standard deviation 63.4) µg/m³ of DBP or control air (15.7 ± 8.5) occurred at the Air Pollution Exposure Laboratory (APEL). The time-weighted average concentration for our controlled exposure was approximately 2% of the 8h time-weighted exposure limit (5000 µg/m³) recommended by the National Institute for Occupational Safety and Health.

The chosen exposure level was approximately 2 to 3 magnitudes greater than the background level reported in indoor air (average is 0.1 - 2.9 µg/m³) and 10 times the maximum reported (15 µg/m³) indoor level, to ensure a gradient by which to observe a potential effect⁷. A well-characterized exposure method¹²⁸ used previously in a human exposure study¹⁴ was followed, adapted to the APEL room specifications, using latex paint doped with 10% DBP (Sigma Aldrich #18281) by weight. The room’s air exchange rate, temperature, volatile organic compounds and humidity were measured and controlled throughout the duration of exposure.

3.3.5 Lung function measures and analysis

Clinical measurement of lung function was assessed by spirometry tests, conducted according to the American Thoracic Society (ATS) and the European Respiratory Society (ERS) standards¹²⁹ and considerations¹³⁰. Forced expiratory volume in 1 second (FEV₁) and fractional exhaled nitric oxide (FeNO) measurements occurred at different timepoints throughout the study (Figure 3.1). Response to the bronchoconstrictor methacholine to determine the provocative concentration that induces a 20% fall in FEV₁, or PC₂₀, and the dose-response slope (DRS) was calculated.
An inhaled allergen challenge, using a specific concentration determined during screening, was conducted immediately after each exposure to assess the early allergen response (EAR). The area under the curve (AUC), measured as FEV$_1$ % fall from baseline to intervals spanning 0h to 3h post-allergen challenge, was calculated.

### 3.3.6 Sample collection and analysis

Bronchoalveolar wash (BW) and lavage (BAL) were obtained 24h post-exposure as previously described$^{131}$. In both samples, BW and BAL, the cellular fraction was assessed by immunophenotyping and the supernatant evaluated for the levels of immune mediators (Human Cytokine/Chemokine 65-Plex Panel, Eve Technologies, AB).

In addition, the BAL cellular fraction was used for *ex vivo* functional assays (Figure 3.1). The BAL fraction contained a higher number of cells compared to the BW fraction. In contrast, the BW fraction was collected in a smaller volume than BAL, and therefore considerably less diluted.

Cells were stained with antibodies against surface and intracellular markers using 5 different panels (Table 3.2), following supplier’s instructions (BD Biosciences, New Jersey, USA) and using a BD FACSCanto II instrument (BD Biosciences, San Jose, CA). Conventional flow cytometric analysis (manual gating) was performed using FCS Express version 6.04.0034 (De Novo Software, Los Angeles, CA, USA) while computational analysis (CITRUS) was performed using Cytobank (http://www.cytobank.org/).
Table 3.2 Standardized antibody panels for flow cytometry immunophenotyping of lung samples.

<table>
<thead>
<tr>
<th>Panels</th>
<th>Macrophages</th>
<th>Dendritic Cells</th>
<th>Granulocytes</th>
<th>TBNK</th>
<th>T helper/T regulatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorochrome</td>
<td>Cat. No</td>
<td>Marker</td>
<td>Cat. No</td>
<td>Marker</td>
<td>Cat. No</td>
</tr>
<tr>
<td>V450</td>
<td>**</td>
<td></td>
<td></td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>BV510</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.7 Ex-vivo stimulation of lung macrophages

Human BAL-derived primary macrophages were seeded in a 24-well plate (VWR, PA, USA). Each well had 1×10⁵ cells diluted in 0.5ml of in-house macrophage media, containing RPMI-1640 with L-Glutamine (VWR, PA, USA), FBS (Millipore-Sigma, St. Louis, USA) and penicillin-streptomycin (Thermo Fisher Scientific, MA, USA). After a 2h incubation at 37 °C, non-adherent cells were washed out with PBS (Thermo Fisher Scientific, MA, USA) leaving adherent primary macrophages attached to the wells.

Freshly made inflammatory stimulant media was added to the respective wells and after a 24-hour incubation, the conditioned media was collected and examined for immune mediators (Custom Human Cytokine 10-Plex Panel, Eve Technologies, AB). The three inflammatory stimulant media used and the final concentration per well were LPS (10ng/ml, from E. coli 026:B6, Millipore-Sigma, St. Louis, USA), Resiquimod (R848, 314 ng/ml, Enzo Life Sciences, NY, USA,) and phorbol 12-myristate 13-acetate/ Ionomycin (PMA/Ionomycin, 25/1000 ng/ml, Ann Arbor, USA/ Abcam, San Francisco, USA). Unstimulated and dimethyl...
sulfoxide (DMSO)-spiked media (DMSO was used to dissolve R848 and PMA/Ionomycin) were used as controls.

### 3.3.8 Statistical analysis

A linear mixed effects (LME) model was used to evaluate the overall effect of the DBP exposure. Specifically, the model included exposure condition (DBP or control air) and outcome (FEV$_1$, FeNO, etc.) as fixed effects, and participant number as a random effect. Moreover, AHR status (yes or no), sex (male or female) and the type of allergen inhaled (grass, house dust mite or birch) were included in 3 additional models wherein condition-by-group interaction were considered as an additional fixed effect. To assess the DBP effect on each group separately, the reference group in the LME model was changed from non-AHR to AHR, male to female, for example.

The analysis was done in RStudio (R version 3.4.1). The p-values are specified when at least marginally significant (p<0.1), for comparisons between control air and DBP exposure conditions. Significant interaction effects of effect modifiers (AHR status, sex and type of allergen inhaled) are specified. For the FEV$_1$ analysis, delta values were calculated for 3h and 20h timepoints relative to the baseline (-4h), for each individual and each exposure condition.

This paper reports primary outcomes originally submitted to clinicaltrials.gov. In this novel study, we purposefully included a wide range of endpoints, and we propose that our results be put in context of the broader literature when used to suggest policy changes. That said, the allergen challenge result for airflow drop, expressed in terms of AUC, is robust both statistically and in terms of effect size and it reflects a primary outcome of concern in this setting. Still, since it was not the primary objective of our study that a particular study
outcome be itself put forth in isolation for definitive clinical or policy implementation (which would typically require a standard statistical threshold), a multiple comparison analysis was not performed. Instead associated interpretation is at the discretion of the reader. Our approach is consistent with expert statistical guidance.\textsuperscript{132–134}

3.4 Results

3.4.1 Enrolment and participant characteristics

We enrolled 16 participants (10 females and 6 males), aged 19-49 (Table 3.1). Of these, half (8) exhibited baseline AHR (methacholine PC\textsubscript{20} ≤16 mg/mL at screening). Atopy to grass (5), birch (5) and HDM (6) was also balanced. Methacholine PC\textsubscript{20} and FeNO were obtained from all 16 study participants, while the early allergen response and FEV\textsubscript{1} were available for 15 participants. The optional bronchoscopy procedure was performed on 11 participants.

3.4.2 DBP exposure augmented allergen-induced lung function decline

DBP exposure increased the allergen challenge AUC by 21.4\% compared to control air (p=0.03, Figure 3.2a, Table 3.3). Effect modification by AHR-status, sex and type of allergen was not significant, although the effect of DBP upon AUC was significant in the non-AHR group only (p=0.07, Figure 3.2b) and the HDM and birch groups (HDM p=0.04, birch p=0.09, grass p=0.9, Figure 3.2c).
Figure 3.2 Effect of DBP over lung function measures. Linear mixed effects model analysis showing a, d, f) the overall DBP effect over a) FEV$_1$ (at timepoints before and after the allergen challenge, comparing AUCs §).
d) methacholine PC\textsubscript{20}, f) FeNO and FEV\textsubscript{1}, (b, c, e, g) role of airway hyper-responsiveness and type of allergen inhaled as effect modifiers over b-c) allergen inhalation challenge, e) methacholine challenge and g) FEV\textsubscript{1}. The AUCs reflects the area between each curve and the dotted line. Effect of DBP vs. control air: (*) p<0.1 and (**) p<0.05, or interaction effect: (#) p<0.05 of AHR-status. Allergen inhalation challenge and FEV\textsubscript{1}, n=15. Methacholine challenge and FeNO, n=16.

Overall, exposure to DBP did not alter the other pulmonary function endpoints; methacholine PC\textsubscript{20}, FeNO or FEV\textsubscript{1} significantly (Figure 3.2d, f). However, the effect modification of AHR status was significant for methacholine PC\textsubscript{20}, and DBP exposure reduced methacholine PC\textsubscript{20} (p=0.02) and FEV\textsubscript{1} (20 hours post-exposure, p=0.05) in non-AHR participants only (Figure 3.2e-g, Table 3.3). Sex or type of allergen inhaled did not modify the effect of DBP upon the methacholine challenge, FeNO or FEV\textsubscript{1}, although significant effects of DBP were observed in some groups (Table 3.3).

Table 3.3 Lung function measures. The table summarizes the effect of DBP overall (LME model), and the role of AHR, sex and type of allergen inhaled (LME with interaction term) over lung function measures. Each DBP effect represents the mean increase or decrease relative to control air (control intercept).

<table>
<thead>
<tr>
<th></th>
<th>Control Intercept</th>
<th>DBP Effect (% change from control)</th>
<th>SE</th>
<th>DBP Effect (% change from control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Non-AHR</td>
</tr>
<tr>
<td>AUC</td>
<td>2320.2</td>
<td>496.6 (21.4)*</td>
<td>208.5</td>
<td>571.9 (28.5)</td>
</tr>
<tr>
<td>Δ FEV\textsubscript{1} 20h</td>
<td>-7.8</td>
<td>-3.4 (-43.6)</td>
<td>2.4</td>
<td>-6.6 (-108.2)*</td>
</tr>
<tr>
<td>LogPC\textsubscript{20}</td>
<td>0.7</td>
<td>-0.46 (-66.0)*</td>
<td>0.3</td>
<td>1.1 (-66.2)**</td>
</tr>
</tbody>
</table>

Effect of DBP vs. control air: (bold) p<0.1, (bold*) p<0.05. Interaction effect: (#) p<0.05. AUC: area under the curve. FEV\textsubscript{1}: forced expiratory volume in 1 second. LogPC\textsubscript{20}: Log methacholine PC\textsubscript{20}. Allergen challenge and FEV\textsubscript{1}, n=15. Methacholine challenge and FeNO, n=16.
3.4.3 DBP exposure enhanced allergen-induced M2 skewing in the lower airway

The cellular environment in the lower airway was altered by DBP exposure (Table 3.4, n=11). Overall, the number of total BAL macrophages increased by 4.6% (p=0.07). In this population, macrophages expressing CD206 and CD163, generally termed “M2 macrophages” increased by 46.9% (p=0.04) with their expression of CD206 increased by 62.8% (p=0.07). On the other hand, the number of CD169+ macrophages decreased by 10% (p=0.02). Similarly, the number of Th1 cells in BAL decreased by 14% (p=0.07) while the presence of CD4 in the surface of all T helper cells increased by 18% (p=0.07) with DBP exposure.

Unsupervised computational analysis using the clustering algorithm CITRUS supported the findings from conventional “manual gating” analysis (Figure 3.3), since DBP exposure increased the expression of CD206 on several cell clusters, within a false discovery rate of 5% (p<0.05). In contrast, expression of surface marker CD169 was increased by DBP in one cluster of phenotypically similar cells.

The effect modification of AHR-status, sex and type of allergen was limited, although the effect of DBP on the BAL cellular fraction was significant for several endpoints for male and AHR participants (Table 3.4). Since only 2 out of 11 participants that underwent bronchoscopy were birch-sensitized, results from the interaction model for allergen should be interpreted with caution.

The overall effect of DBP on the cellular fraction of BW samples showed some overlap with the results from the BAL samples, since DBP exposure increased the expression of CD4 on the surface of T helper cells by 23.5% (p=0.08) compared to control air. However, the
macrophage populations were not significantly affected by DBP exposure in BW samples, while the percentage of Th17 cells decreased by 31.9% (p=0.07). Note, that the BW data should be interpreted with caution due to the smaller group size (n=8), and therefore the interaction model was not applied for this dataset.

Table 3.4 Cellular endpoints in BAL. LME model, and the role of AHR, sex and type of allergen inhaled (LME with interaction term) over cellular endpoints. Each DBP effect represents the mean increase or decrease relative to control air (intercept). n=11.

Figure 3.3 CITRUS analysis of BAL macrophages. Based on the unsupervised clustering algorithm, the identified clusters are colored by MFI of surface markers CD206, CD163, CD169, CD40 and CD80 respectively. Bubbles indicate clusters where DBP exposure induced changes (p<0.05) in marker expression.

3.4.4 DBP exposure had modest effects on inflammatory mediators in the airway

Relatively few of the analysed inflammatory markers were affected by the DBP exposure overall (Table 3.5). In the BW fraction, macrophage inflammatory protein-1 alpha (MIP-1α) and fractalkine levels increased by 60 (p=0.07) and 25.4% respectively (p=0.07) with DBP exposure compared to control air, whereas FMS-like tyrosine kinase 3 ligand (Flt-3L) and stem cell factor (SCF) decreased by 78 (p=0.02) and 55.2% (p=0.07). In the BAL fraction, DBP exposure decreased the levels of SCF by 34.5% (p=0.02), monocyte chemoattractant I-309 by 29% (p=0.05) and platelet-derived growth factor AA (PDGF-AA) by 23.5% (p=0.02).

The effect modification of AHR status was significant for a range of inflammatory mediators (Table 3.5). Generally, AHR participants increased the cytokine release in response to DBP whereas non-AHR showed a reduction in BW. Sex also had a modifying effect over the response to DBP in BW, in which male participants (similarly to cellular effects) showed significant responses. In BAL, DBP exposure generally caused a reduction of cytokine release, especially in males and AHR participants.
Table 3.5 Inflammatory mediators in the lung. LME model, and the role of AHR, sex and type of allergen inhaled (LME with interaction term) over inflammatory mediators. Each DBP effect represents the mean increase or decrease relative to control air (intercept). n=11.

<table>
<thead>
<tr>
<th></th>
<th>Control Intercept</th>
<th>DBP Effect (% change from control)</th>
<th>SE</th>
<th>DBP Effect (% change from control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-AHR (n=5)</td>
<td></td>
<td>AHR (n=6)</td>
</tr>
<tr>
<td><strong>Bronchoalveolar wash</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractalkine</td>
<td>10.2</td>
<td>2.6</td>
<td>1.3</td>
<td>(25.4)</td>
</tr>
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<td>0.5</td>
<td>0.2</td>
<td>(60.0)</td>
</tr>
<tr>
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<td>30.3</td>
<td>9.3</td>
<td>10.6</td>
<td>(30.7)</td>
</tr>
<tr>
<td>CTACK</td>
<td>0.009</td>
<td>0.01</td>
<td>0.02</td>
<td>(355.6)</td>
</tr>
<tr>
<td>MCP-4</td>
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<td>0.1</td>
<td>(-43.2)</td>
</tr>
<tr>
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<td>0.03</td>
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</tr>
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<td>0.1</td>
<td>(-14.2)</td>
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<td>1.3</td>
<td>(-17.0)</td>
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<td>-0.5</td>
<td>0.2</td>
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<td>(-55.2)</td>
</tr>
<tr>
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<td>3.4</td>
<td>(-39.6)</td>
</tr>
<tr>
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<td>0.04</td>
<td>(60.0)</td>
</tr>
<tr>
<td>EGF</td>
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<td>0.6</td>
<td>0.4</td>
<td>(75.9)</td>
</tr>
<tr>
<td>IL-2</td>
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<td>0.04</td>
<td>0.1</td>
<td>(27.9)</td>
</tr>
<tr>
<td>IL-20</td>
<td>23.2</td>
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<td>3.1</td>
<td>(22.9)</td>
</tr>
<tr>
<td>TRAIL</td>
<td>3.4</td>
<td>1.9</td>
<td>1.3</td>
<td>(53.4)</td>
</tr>
<tr>
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<td>0.09</td>
<td>0.3</td>
<td>(10.5)</td>
</tr>
<tr>
<td>IL-8</td>
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<td>-1.1</td>
<td>5.8</td>
<td>(-3.1)</td>
</tr>
</tbody>
</table>

Effect of DBP vs. control air: (bold) p<0.1, (bold*) p<0.05. Interaction effect: (#) p<0.05. Values are in pg/ml.


3.4.5 DBP exposure had modest immuno-suppressive effects over the functionality of alveolar macrophages

Exposure to DBP reduced the R848-induced release of TNFα in alveolar macrophages by 22% (p=0.02). Although the effect of DBP was significant for several mediators/stimuli in non-AHR, AHR, female and HDM-atopic subjects (Table 3.6), there was no or limited effect modification of AHR status, sex or type of allergen inhaled, and no clear pattern between groups.

Table 3.6 DBP effect on inflammatory mediator release from lung macrophages stimulated ex vivo. LME model, and the role of AHR, sex and type of allergen inhaled (LME with interaction term) over inflammatory mediator release from stimulated primary lung macrophages from PAIR study participants. Each DBP effect represents the mean increase or decrease relative to control air (intercept). n=10.
Effect of DBP vs. control air: (bold) p<0.1, (bold*) p<0.05. Interaction effect: (#) p<0.05. Values are in pg/ml and the delta calculated from unstimulated controls. TNFα: Tumour necrosis factor alpha. TGFβ: Transforming growth factor beta. IFNγ: Interferon gamma.

3.5 Discussion

In this novel controlled inhalation study, featuring inhalation of a known concentration of a single phthalate, exposure to gas-phase DBP significantly augmented airflow decline in response to an inhaled allergen challenge. Moreover, DBP inhalation enhanced the recruitment of macrophages, specifically the M2 phenotype with increased expression of CD206 to the lungs. Only minor effects were observed for inflammatory mediators and the *ex vivo* functionality of primary BAL macrophages.

The allergen challenge, also known as allergen bronchoprovocation test, is a long-standing exacerbation model of allergic asthma that can induce several clinical and pathophysiologic features of asthma in sensitized individuals. The PAIR study is the first study utilizing this research tool to provide evidence of a direct negative impact on lung function due to phthalate inhalation, focused on the acute response.

The increased AUC reflects that DBP enhanced the effect of allergen alone, which can be interpreted as an adjuvant effect of DBP upon lung function decline. Animal studies have investigated the effect of DEHP on AHR and reported that phthalate ingestion induced adjuvant effects on AHR in an OVA model, measured as an increased response to a methacholine challenge. In the PAIR study, we also looked at AHR but did not see an overall effect of DBP. Interestingly, there was a significant effect of DBP in the non-AHR participants only, when analysed in the interaction model. Also, exposure to DBP
significantly decreased methacholine PC\textsubscript{20} in non-AHR participants only, as well as the FEV\textsubscript{1} 20h post-exposure, providing further support for an effect of DBP on lung function measures.

Both the methacholine PC\textsubscript{20} and FEV\textsubscript{1} effects of DBP were considered adjuvant since they enhanced the already present inhaled allergen effect. In contrast, two human inhalation studies in workers exposed to PVC emissions reported no effects on pulmonary function, measured by changes in FEV\textsubscript{1} before and after their workshift\textsuperscript{80,135}. In both studies, there was no allergen inhalation component, which might be a reason for the lack of effect. While we focused on the baseline AHR classification as an objective and physiologically relevant way of interpreting the results, we believe that the absence of a consistent effect in the response from AHR participants, as opposed to the more uniform pattern from non-AHR, could be due to an admixture of disease phenotypes within our study.

M2 macrophages have a distinct cell surface and effector phenotype, playing an important role in allergic asthma through their bidirectional interactions with other immune and structural cells, and inflammatory mediators\textsuperscript{59}. The number of M2 macrophages in the lung and their expression of mannose receptor (CD206) has been correlated with peak expiratory flow variation, a marker of asthma severity and airway inflammation\textsuperscript{136}. Thus, the increased numbers of total and M2 macrophages and the M2 skewing in the airways observed after DBP inhalation could be linked to the parallel observation of reduced lung function measures. However, using a correlation analysis we could not link the various increased macrophage percentages to the decreased lung function measures logPC\textsubscript{20} and AUC (data not shown). Thus, the increased M2 skewing did not appear to be related to the decrease in lung function in our subjects.
In line with the observed increase in macrophages after DBP inhalation, inhalation of the DEHP metabolite MEHP also led to an increase in the number of alveolar macrophages in mice, reaching a maximum infiltration at 16 hours post-exposure\(^{137}\). The majority of the animal studies reported adjuvant effects particularly involving eosinophils\(^{65,72}\) using ingestion as the exposure route, unlike our inhalation exposure route. The one study applying DEHP through nasal instillation\(^{74}\) reported a skewed cellular infiltration from eosinophilic to neutrophilic, in the presence of cockroach allergen extract\(^{74}\). The authors hypothesize that the type of cellular infiltration depends upon the allergen model used, for example, eosinophil-dominated infiltration is driven by OVA, whereas cockroach allergen extract induced a mixed granulocytic inflammation in the airways\(^{74}\). DEHP alone also had an effect, causing neutrophilic infiltration\(^{74}\).

DBP exposure decreased the percentage of CD169+ macrophages in the lung. The recently described M169 macrophages appear to regulate the immune system, specifically through development of immuno-tolerance to pathogens and self antigens, and not so much mediating phagocytosis\(^{138,139}\). In addition, M169 macrophages enables the induction of CD8+ T cell responses to viruses and blocking the CD169 macrophage-T cell interaction impairs the response to viral infections\(^{140}\). Thus in susceptible populations, this could lead to respiratory infections, previously correlated to acute asthma symptoms\(^{141}\).

In the literature, the response to an inhaled allergen challenge, in terms of percent of macrophages in BAL is conflicting. Some studies report a decrease or unchanged percentage 24h after an inhaled allergen challenge\(^{142-144}\), while others reported an increase or unchanged number of macrophages\(^{142,145}\). One study reported increased numbers of freshly recruited, immature macrophages, identified by their expression of CD14 and CD86\(^{145}\). In our study,
we did not include a bronchoscopy without an allergen challenge, therefore we can not determine whether the allergen challenge caused increased percentages of alveolar macrophages in our participants. Consequently, our study design does not allow us to determine whether the DBP-induced effect on macrophage recruitment is adjuvant, immuno-suppressive or independent from the allergen-induced effects. Although our panel does not allow for the identification of freshly recruited macrophages, our finding of increased M2 skewing does not point towards recruitment of immature macrophages. Therefore, the identified macrophage population does not appear to be due to the allergen inhalation and pointing towards the effect of DBP not being adjuvant.

The molecular mechanisms of phthalates in promoting allergic airway effects are still unknown but it has been suggested that DBP may interact with peroxisome proliferator-activated receptor (PPARγ) receptor and, through partial antagonist effects, interrupt anti-inflammatory functions and so promote airway inflammation. Recently, DBP was reported to modify monocyte-to-macrophage differentiation in vitro through interaction with PPARγ. PPARγ is highly expressed in M2 macrophages and known to interact with CD206 receptor through NF-κB activation independent pathways. Although PPARγ expression has not yet been assessed in the PAIR study, PPARγ activation is a possible explanatory hypothesis for the observed effects of DBP. More specifically, DBP exposure may lead to an impaired PPARγ anti-inflammatory response, promoting upregulation of CD206 in M2 macrophages as a host response to restore tissue homeostasis and inflammatory balance in a discrete way. Co-expression of CD206 and CD163, scavenger receptors in M2 macrophages, is indicative of a high apoptotic cell uptake and resolution of inflammation.
Inflammatory mediators in the lung were generally not affected by DBP exposure. Modest alterations, including increased levels of MIP-1α and fractalkine, implicated in migration of macrophages and leukocytes to the lung\textsuperscript{149,150}, and decreased Flt-3L and SCF levels, implicated in hematopoiesis\textsuperscript{151}, were observed. We believe that the increase in MIP-1α could be associated to the increase in macrophages in the lung. Allergen inhalation has been linked to increased Th2 cytokines like IL-4 and IL-13\textsuperscript{152}, but in the PAIR study none of these were affected by DBP. Moreover, the mediators affected by DBP, to our knowledge, have never been reported to be altered by an allergen inhalation challenge in the literature. Thus, the effects of DBP on inflammatory mediators do not appear to be adjuvant. This is in contrast with the adjuvant effects in inflammatory mediators reported in mice after nasal instillation with DEHP\textsuperscript{73,74}.

Limited effects on the functionality of alveolar macrophages were detected after DBP exposure, generally following a pattern of immuno-suppression. The overall reduction of R848-induced TNFα release, and other pro-inflammatory mediators (IL-1α, IL-6, IL-8 and IFNγ from different groups) in response to inflammatory stimuli, demonstrated a dampening effect of DBP. In line with this, DBP had immuno-suppressive effects \textit{in vitro} dampening the release of inflammatory mediators like TNFα and IFNγ in stimulated whole blood\textsuperscript{153}. Similarly, DBP has been reported to impair phagocytosis and reduce release of LPS-induced pro-inflammatory cytokines in macrophages \textit{in vitro}\textsuperscript{76}.

In the PAIR study, lung function effects of DBP seem to be adjuvant whereas cellular and inflammatory mediators’ effects do not appear to be adjuvant. The cellular effects of DBP may be independent of the allergen exposure. This is supported by the large body of \textit{in vitro} studies in macrophages and other cell types reporting effect of phthalates independent of an
allergen challenge, Additionally, effects of DEHP alone, have been reported, supporting that effects of phthalate alone can occur, and that the airway may be a particularly important pathway.

Although the PAIR study is unprecedented and is the first study to conduct a controlled exposure to a single phthalate and measure the biological impact on the human airway immunology, we acknowledge some limitations in the study design and implementation. For example, the 3-hour exposure duration is unlikely to be precisely recapitulated in typical life. Moreover, to assure that the participants were exposed to a higher concentration than in their home environment, the applied exposure concentration is somewhat higher than the reported environmental exposure. Finally, the conditions tested always included an allergen inhalation component, therefore our study only allows for assessment of allergen-induced DBP effect. Although this was justified by a range of animal studies only reporting effects of phthalates in the presence of an allergen, a more recent study suggests that airways may be prone to effects of phthalates alone.

PAIR is the first controlled human exposure study to assess airway effects due to inhalation exposure to a known concentration of a single phthalate. We demonstrate that inhalation of DBP significantly augmented the allergen-induced lung function decline, suggesting adjuvant effects in the airways. Moreover, DBP inhalation enhanced macrophage recruitment and M2 skewing of the macrophage population, although the relative importance of the allergen inhalation for these cellular effects need further clarification. These findings expand the existing literature and bridge the knowledge from other experimental and epidemiological studies.
3.6 Supplemental material

Allergen challenge

Sensitization to grass, house dust mite or birch was determined by skin prick test (wheal size of at least 3 mm considered positive) during the first screening visit, along with the methacholine PC\textsubscript{20}. The specific allergen inhalation dose was estimated by inputting these values (minimal concentration to provoke a 3 mm wheal size and methacholine PC\textsubscript{20} percent drop and concentration) in a formula, as previously described\textsuperscript{155}. In a second screening visit, concentrated allergen extract (Birch Cat# LH1169ED, Grass Cat# LH0831TS, and D. pteronyssinus Cat# LH6692UP, Omega Laboratories, Montreal QC, Hollister Stier) was diluted with 0.9\% normal saline and the allergen challenge conducted, starting with a dilution 4-times lower than the estimated allergen PC\textsubscript{20} dose, until a 20\% drop in FEV\textsubscript{1} was achieved. During the exposure visits, a 2-min inhaled allergen challenge was performed immediately after exposure, to assess the early allergen response. The area under the curve (AUC), measured as FEV\textsubscript{1} % fall from baseline from 0h and up to 3h post-allergen challenge, was calculated using GraphPad (version 6.01). Visits that included an allergen challenge were strategically scheduled outside of the allergy season for each participant.

Methacholine challenge

Baseline airway responsiveness was evaluated by a methacholine test during the first screening visit using the 2-min tidal breathing technique\textsuperscript{130}. The provocative concentration of methacholine eliciting a 20\% fall (PC\textsubscript{20}) in forced expiratory volume in 1 second (FEV\textsubscript{1}) was used to define participants as either hyper-responsive (PC\textsubscript{20} ≤ 16 mg/mL) or normally responsive (PC\textsubscript{20} > 16 mg/mL). The dose-response slope (DRS) was calculated following a
previously described method that uses the percent fall at the highest methacholine concentration divided by the cumulative dose\textsuperscript{156}.

\textit{Cellular endpoints in BAL}

Male, birch-atopic, AHR participants experienced the overall DBP effects to a higher level compared to other groups (Table 3.4). In AHR participants, total and M2 macrophages increased by 7.8\% and 60\% (p=0.03, p=0.06), as did the M2/M1 ratio (144.4\%, p=0.05). Expression of CD4 on T helper cells and CD8 on T cytotoxic cells also increased by 26\% and 97\% (p=0.06, p=0.05). A decrease in plasmacytoid DC (40\%, p=0.03), which provide intrinsic protection against inflammatory responses\textsuperscript{157} and the increased expression of CD4 and CD8 on effector T cells respectively, supports the hypothesis that DBP has immunomodulatory effects in the lungs, specifically in susceptible populations. Males, but not females also experienced an increased expression of CD4 and CD8 on effector T cells (26.6\% and 162.7\%, p=0.05). Also, in males, the presence of M2 and their expression of CD206 increased with DBP exposure (60.8\% and 92.3\%, p=0.08). Meanwhile, in non-AHR participants, DBP exposure decreased the number of B and T helper cells by 48.8\% and 12.9\% (p=0.08). B and T helper cells are hallmarks of the immunological response to inhaled allergens and inability to respond to environmental insults can lead to opportunistic viral infections and respiratory disease in this group.

\textit{Inflammatory mediators in BW}

AHR participants experienced an increase in IL-6 (p=0.09), previously linked to CD206 expression in M2 macrophages\textsuperscript{59}, and IL-2 (p=0.03), linked to stimulation of naïve T cells into effector T cells and further differentiation into Th1 or Th2 cells\textsuperscript{158}. Other immune-
regulator and chemotactic factors increased in AHR participants after DBP exposure, supporting cellular observations, for example IL-20 (p=0.02), TRAIL (p=0.03), CTACK (p=0.04) and TGFα (p=0.04, Table 3.5). Members of the IL-20 subfamily facilitate the communication between leukocytes and epithelial cells, enhancing innate defence mechanisms and tissue repair processes\textsuperscript{159}. TRAIL has been implicated in immuno-regulatory and immuno-effector functions, playing an important role in response to viral infections and immune surveillance\textsuperscript{160}. Lastly, under inflammatory conditions, CTACK regulates the specific migration behavior of skin-homing lymphocytes\textsuperscript{161} while TGFα stimulates keratinocyte migration\textsuperscript{162}.

In non-AHR participants, DBP exposure also had an impact on the permeabilization and immune cell migration to the lower airway reducing the release of VEGF-A (p=0.04), which plays an important role in wound healing and tissue repair\textsuperscript{162}. Furthermore, the DBP-induced dampened release of BCA-1 (p=0.04), a chemokine that selectively attracts B cells and induces chemotaxis of activated T cells\textsuperscript{163}, is consistent with the decrease observed in B cells and to a lesser extent T cell recruitment in non-AHR participants (Table 3.4). Lastly, monocyte chemo-attractant protein (MCP)-4, a chemokine with potent chemotactic activities for eosinophils, monocytes, T lymphocytes, and basophils and signaling associated with the allergic response\textsuperscript{164} was reduced by DBP (p=0.04) in non-AHR participants. Although not as evident as AHR status, sex and type of allergen also influenced the immune mediator’s release pattern following DBP exposure compared to control air (Table 3.5).
Chapter 4: Dibutyl phthalate (DBP) inhalation prior to allergen challenge increased the CD4+ T helper cells and decreased T regulatory cells in blood of human volunteers

4.1 Synopsis

Epidemiological and experimental studies in the last decades have increasingly associated the ubiquitous presence of phthalates with allergic diseases. Dibutyl phthalate (DBP), found in high concentration in indoor air, has shown inflammatory potential and immuno-modulatory effects through animal and in vitro models.

The phthalate-allergen immune response (PAIR) study, using DBP as a model phthalate, is the first controlled human exposure study to provide experimental evidence of systemic immuno-modulatory effects of DBP in humans. This double-blind, randomized, crossover of two exposure conditions (DBP or control air) enrolled 16 allergen-sensitized participants. Whole blood collected before and after exposure was used to analyse the cellular fraction, while inflammatory mediators were analysed in serum.

DBP inhalation, prior to allergen inhalation, increased the CD4+ T helper cells and decreased T regulatory cells in blood at 3h and 20h, and decreased non-classical monocytes at 20h. Modest effects were observed for inflammatory mediators, with increased levels of chemoattractant SDF-1α+β and decreased levels of IFNα2, PDGF-AA and TSLP at 20h. With regard to effect modification, the type of allergen inhaled generally modified the effect of DBP upon cellular endpoints, while sex and airway hyper-responsiveness status influenced the DBP effect on inflammatory mediator levels. Interestingly, the observed systemic effects of DBP could not be classified as adjuvant, since they did not reflect enhancement of the allergen-induced responses.
4.2 Background and objective

Phthalates are synthetic diesters of phthalic acid, present in a variety of consumer products\(^4\) to enhance properties such as flexibility, transparency, durability and longevity\(^3\). Industrial applications of phthalates include building materials, household furnishings, clothing, cosmetics, personal care products, pharmaceuticals, medical devices, children’s toys, food packaging and cleaning materials amongst others\(^5\). Due to their weak, non-covalent binding, phthalates leak out of their carrier plastics and become ubiquitous environmental contaminants\(^4\).

The presence of polyvinyl chloride (PVC) materials in homes and the phthalate levels in house dust have been associated with allergic diseases like asthma and rhinitis in epidemiological studies\(^64,71,121,165,166\). Experimental animal studies support that phthalates may enhance allergen-induced effects, i.e. exert adjuvant effects on basic mechanisms of allergic sensitization\(^24\). In the airways, adjuvant effects have been reported both in terms of increased recruitment of inflammatory cells and airway hyper-responsiveness\(^65\). Although systemic inflammation has been reported due to phthalate exposure, specific systemic effects are scarcely described in animal models, except for adjuvant effects on total or allergen specific IgE in serum\(^76,167\). Several studies have assessed the effects of the phthalates per se, but generally limited or no effects have been observed in the absence of a model allergen\(^24\).

The inflammatory response, triggered by infection or tissue injury, is driven by a complex network of cells, mediators and signaling pathways\(^168\). Deregulation of cytokines and chemokines may be linked to the onset and exacerbation of pathologies like asthma and other allergic diseases\(^54\), while allergic diseases like rhinitis and asthma may alter the levels of
circulatory cells, and inflammatory mediators in serum\textsuperscript{169,170}. Likewise, a controlled allergen inhalation, a research tool that induces several clinical and pathophysiologic features of asthma in sensitized individuals\textsuperscript{126}, has been reported to induce systemic effects\textsuperscript{171,172}.

While experimental studies in animal models focus mainly on adjuvant effects of phthalates, the cell culture studies mainly focus on effects in the absence of an allergen\textsuperscript{24,25,65}. Phthalates may enhance the production and release of inflammatory cytokines and chemokines like tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), IL-1\(\beta\), IL-6, and IL-8 from monocytes and macrophages\textsuperscript{25,173}. In addition, phthalates can significantly increase IL-4 production from activated CD4+ T cells in a concentration dependent manner, suggesting an enhancement of allergic responses\textsuperscript{79}. Phthalates may also promote a Th2 and Th17 immune response, facilitating the development of a mixed granulocytic airway inflammation, in the presence of a cockroach allergen extract\textsuperscript{74}. Moreover, immunosuppressive effects have also been reported for phthalates, for instance in terms of reduced cytokine production, phagocytosis and antigen-presenting capacity\textsuperscript{76}.

The whole blood assay (WBA) system provides a robust and physiologically relevant environment compared to other \textit{in vitro} designs, and through the use of inflammatory stimuli like lipopolysaccharide (LPS), Resiquimod (R848), and phorbol 12-myristate 13-acetate/Ionomycin (PMA/Ionomycin), a wide range of inflammatory responses can be assessed\textsuperscript{153}. WBA has been used to study immune cell function \textit{in vitro} in epidemiological and clinical studies\textsuperscript{118,119}. We previously reported that DBP dampened the innate immune response to inflammatory stimuli and had immunosuppressive effects \textit{in vitro}, demonstrating the value of the WBA for the assessment of interactions between environmental pollutants and immune cells\textsuperscript{153}.
The Phthalate-Allergen Immune Response (PAIR) study, presented here, is the first study to investigate systemic biological effects in humans due to inhalation of a known concentration of a single phthalate, DBP. Using a randomized, controlled, crossover design to enhance power, we tested the hypothesis that DBP inhalation, immediately prior to allergen inhalation, enhances the allergen-induced systemic inflammation state and promotes immune cell recruitment and activation in peripheral blood.

Since in vitro literature suggest altered functionality of innate immune cells independent of an allergen challenge, measurements reflecting innate immune responses were also included, such as pro-inflammatory cytokines in serum and bacterial or viral stimuli in the WBA.

4.3 Methods

4.3.1 Ethics statement

The study conforms to the standards of the Declaration of Helsinki and no deviations were made from the protocol approved by the research ethics board at the University of British Columbia (H14-01119), the Norwegian Regional Committees for Medical and Health Research Ethics (2014/1217) and registered at ClinicalTrials.gov (NCT02688478).

4.3.2 Study design and participant characteristics

Written informed consent was signed by study participants who enrolled in a double-blind, randomized, crossover study that was counter-balanced to order between two exposure conditions (DBP or control air, Figure 4.1). Sixteen allergen-sensitized (determined by skin prick test to birch, house dust mite or grass), healthy or mildly asthmatic participants between the ages of 19 to 49 years were recruited (Figure 4.1) following the inclusion/exclusion criteria.
The three-hour inhalation and dermal exposure took place at the Air Pollution Exposure Laboratory (APEL). The room’s air exchange rate, temperature, volatile organic compounds and humidity were measured and controlled throughout the duration of exposure.

The chosen target level of 150 (mean 147.5, standard deviation 63.4) µg/m³ of DBP was approximately 2 to 3 magnitudes greater than the background level reported in indoor air (average is 0.1 - 2.9 µg/m³), and 10 times the maximum reported (15 µg/m³) indoor level, to ensure a gradient by which to observe a potential effect⁷. A well-characterized doped latex paint exposure method¹²⁸ used previously in a human exposure study¹⁴ was followed, adapted to the APEL room specifications using paint doped with 10% DBP (Sigma Aldrich #18281) by weight.

Figure 4.1 Overview of study design, participant characteristics and endpoints. Consenting participants were screened and randomly assigned to two groups for a double-blinded crossover exposure to DBP and control. AHR: Airway hyper-responsiveness. HDM: House dust mite.
4.3.3 Sample analysis

Peripheral blood was collected into sodium heparin tubes (Vacutainer, BD, New Jersey, USA), before (~4h), as well as 3h and 20h post-exposure and processed immediately. 0h reflects the allergen inhalation time point. Whole blood was used to analyse the cellular fraction, while inflammatory mediators were analysed in serum samples achieved by a 10-minute centrifugation at 500g, stored at -80 °C until analysis.

For flow cytometry, cells were stained with fluorochrome-conjugated antibodies against surface and intracellular proteins (Table 4.1), following supplier’s instructions (BD Biosciences, New Jersey, USA), and collected on a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Conventional flow cytometry analysis was performed using FCS Express version 6.04.0034 (De Novo Software, Los Angeles, CA, USA), following a manual gating strategy (Figure 4.2).

Table 4.1 Standardized antibody panels for immunophenotyping of human whole blood samples in the PAIR study. Monoclonal antibody and clone (italicized) used to detect surface markers. Catalog number from BD Biosciences. *Fixable Viability Dye eFluor® 450.

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Granulocytes</th>
<th>TBNK</th>
<th>T helper/T regulatory</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cat. No</strong></td>
<td><strong>Marker</strong></td>
<td><strong>Cat. No</strong></td>
<td><strong>Marker</strong></td>
</tr>
<tr>
<td>PerCP-Cy5.5</td>
<td>560835 CD3 [UCHT1]</td>
<td>560835 CD3 [UCHT1]</td>
<td></td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>557833 CD45 [2D1]</td>
<td>557833 CD45 [2D1]</td>
<td>557833 CD45 [2D1]</td>
</tr>
<tr>
<td>V450</td>
<td>*</td>
<td>560347 CD8 [RP4-T8]</td>
<td>563241 CD196 [IIA9]</td>
</tr>
<tr>
<td>BV510</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

68
Figure 4.2 Gating strategy for immunophenotyping of human whole blood samples.

Additionally, unsupervised computational analysis was performed using Cytobank (http://www.cytobank.org/) and an algorithm designed for the fully automated discovery of statistically significant biological signatures within single cell datasets called CITRUS (cluster identification, characterization, and regression).
For analysis of inflammatory mediators in serum, samples were thawed immediately before analysis with a human cytokine/chemokine 65-plex panel (Eve Technologies, AB). The 65 mediators included chemokines, Th2-related cytokines, regulators of normal immune cell function and maturation and growth factors (Table 4.2).

Finally, cellular functionality was assessed by *ex vivo* stimulation of whole blood as previously described\textsuperscript{153}. Briefly, whole blood diluted 1:1 vol/vol with RPMI-1640 Medium (Millipore-Sigma, St. Louis, USA) plus penicillin-streptomycin (Thermo Fisher Scientific, MA, USA) was stimulated with LPS (10ng/ml, from E. coli 026:B6, Millipore-Sigma, St. Louis, USA), R848 (314 ng/ml, Enzo Life Sciences, NY, USA,) or PMA/Ionomycin (25/1000 ng/ml, Ann Arbor, USA/ Abcam, San Francisco, USA), as shown in Figure 4.1. After a 20-hour incubation at 37 °C, the supernatant was examined for the presence of 10 inflammatory mediators (Custom Human Cytokine 10-Plex Panel, Eve Technologies, AB), as noted in Table 4.2.

**Table 4.2 Discovery Assay® measuring 65 mediators from research areas like immune response, inflammation and allergy.** Human Cytokine/Chemokine 65-Plex Panel from Eve Technologies. * Indicates mediators used in the Custom Human Cytokine 10-Plex Panel, for the whole blood assay. # Indicates values below the detection limit for n = 7.

<table>
<thead>
<tr>
<th>Human Cytokine/Chemokine 65-Plex Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>6CKine</td>
</tr>
<tr>
<td>BCA-1</td>
</tr>
<tr>
<td>CTACK</td>
</tr>
<tr>
<td>EGF</td>
</tr>
<tr>
<td>ENA-78</td>
</tr>
<tr>
<td>Eotaxin</td>
</tr>
<tr>
<td>Eotaxin-2</td>
</tr>
<tr>
<td>Eotaxin-3*</td>
</tr>
<tr>
<td>FGF-2</td>
</tr>
<tr>
<td>Flt-3L</td>
</tr>
</tbody>
</table>
4.3.4 Statistical analysis

To evaluate the overall effect of DBP exposure, a linear mixed effects model was applied, with participant number as a random effect, and exposure condition (DBP or CA) and outcome (cell percentage, concentration of cytokines, etc.) as fixed effects. All linear mixed effects analyses were performed for delta values, calculated for 3h and 20h timepoints relative to the baseline (-4h), for each individual and each exposure condition. Three potential effect modifiers, AHR status (yes or no), sex (male or female) and the type of allergen inhaled (grass, house dust mite or birch), were included in models where exposure-by-modifier interaction was considered as an additional fixed effect.

Statistical analysis to assess effects of DBP and effect modifiers was done in RStudio (R version 3.4.1). Testing one specific hypothesis was not the objective of our study, therefore correction for multiple statistical comparisons was not pursued (in other words, we determined a priori that results would be hypothesis-generating and not definitive). Our approach is consistent with expert statistical guidance.

To evaluate whether the effects of DBP were adjuvant, identification of the effects of the allergen inhalation per se on the systemic cellular and humoral endpoints was required. Thus, data from the CA exposure condition were analysed in GraphPad (v.6.01) by repeated measures one-way ANOVA, with a Dunnet post-hoc test, to assess significant changes relative to the -4h time-point. In doing so, we recognize that due to the study design differences between 3h and -4h could be due to either allergen-induced or diurnal effects, while differences between 20h and -4h should reflect the effects of allergen inhalation (since these samples were collected at the same time of day).
4.4 Results

4.4.1 Cellular responses in blood are observed as early as 3h after DBP exposure

The 3h inhalation of DBP increased the CD4+ T helper cells \((p=0.02)\) and decreased T regulatory cells \((p=0.03)\) in blood 3h post-exposure, and these effects persisted at 20h post-exposure \((p=0.03, \text{ both})\). DBP also decreased non-classical monocytes \((p=0.05)\) at 20h \((\text{Figure 4.3a, Table 4.3})\). CITRUS analysis supported these findings from conventional gating, also showing an increased abundance of CD4+ T helper cells (clusters 3, 4 and 5). In addition, the CITRUS analysis also showed increased abundance of CD8+ T cytotoxic cells (cluster 2) and the CD4+ T helper activation state, by increased expression of CD69 (clusters 4 and 5), at 3h post-exposure \((\text{Figure 4.3b})\).

Allergen inhalation alone did not alter the numbers of CD4+ T helper cells or T regulatory cells, instead it increased the number of granulocytes and eosinophil activation (increased CD69) significantly and decreased the number of lymphocytes and Th17 cells decreased (see supplement for details).

Effect modification by the type of allergen inhaled was significant for the percentages of monocytes, neutrophils, eosinophils and T cells and their subsets and for some activation markers \((\text{Table 4.3, Figure 4.3a})\). Generally, the effects of DBP tended to be stronger for subjects exposed to grass in monocytes and eosinophils exhibiting reduced cell percentages and activation markers, while T cell subsets appeared to be more affected by DBP in the HDM exposed individuals with a general trend for increased cell numbers \((\text{Table 4.3})\).

The effect modification by sex and AHR-status was less consistent for both cell numbers and activation \((\text{Table 4.3})\). However, the decreased percentage of T regulatory cells was stronger
in male subjects at both time-points (Table 4.3, Figure 4.3a). Some activation markers were affected only in men at the 3h time-point (reduced CD24 expression on eosinophils and neutrophils and CD196 in Th17-cells), while CD196 in Th17-cells was significantly increased in females at both time-points (Table 4.3, Figure 4.3a).

Figure 4.3 Effect of DBP exposure on blood cells. a) Data indicate delta values for 3- and 20-hours post-exposure for all the participants, n=16. Top panel shows overall DBP effect compared to control air. Bottom panel shows type of allergen and sex modification of overall DBP effect, (*) p<0.05 for effect of DBP over
control. (#) p<0.05 for interaction effect. b) CITRUS analysis of blood lymphocytes 3 hours post-exposure from the TBNK panel (table 4.1). Clusters colored by MFI of surface markers CD3, CD4, CD8 and CD69 respectively. Bubbles indicate clusters where DBP exposure induced changes (p<0.05) in cellular abundance.

Table 4.3 Effect of DBP on blood cells. Data indicate delta values for 3- and 20-hours post-exposure for all the participants, n=16. Significant effect of DBP vs. control: bold p<0.1, bold* p<0.05. Significant interaction effect (#) p<0.05.
4.4.2 Serum levels of inflammatory mediators are altered by DBP exposure

The 3h inhalation exposure to DBP affected few of the 65 serum markers in the main analysis. However, the levels of the chemo-attractant MCP-4 increased in serum in DBP exposed individuals 3 hours post-exposure (p=0.09). The following morning, at 20h post-exposure, an increase in the chemo-attractant SDF-1α+β (p=0.03) was observed after DBP exposure, whereas mediators related to viral responses (IFNα2; p=0.06), growth factor regulator (PDGF-AA; p=0.08) and Th2-responses (TSLP; p=0.04) were reduced, Table 4.4).

In contrast to the markers affected by DBP inhalation, allergen inhalation alone significantly increased the levels of IL-5, IL-7, IL-10, TGFα, and I-309 while sCD40L and EGF levels in decreased (see supplement for details).

The effect modification by sex and AHR-status was significant for a range of mediators, while it was less pronounced for the type of allergen inhaled (Table 4.4). The DBP exposure generally had stronger effects in the male and non-AHR groups at both time-points. For example, DBP exposure increased the levels of chemo-attractant mediators in males but not females (Eotaxin-1, p=0.01; MCP-1, p=0.02; MCP-2, p=0.006 at 3h; MCP2, p=0.04; SDF-1α+β, p=0.06 at 20h; Table 4.4). In contrast, the levels of Th2-related cytokines were decreased in males after DBP exposure (IL-4, p=0.04; IL-13, p=0.02 and TSLP, p=0.02 at 3h; IL-13, p=0.009 and TSLP, p=0.007, at 20h), and so were the levels of pro-inflammatory cytokines (TNFα, p=0.05 at 3h; IL-1β, p=0.05; IL-6, p=0.06; IL-8, p=0.02 and IL-17A, p=0.07 at 20h; Table 4.4).
Table 4.4 Effect of DBP on inflammatory mediator levels in serum. Selection of mediators analysed in 65-plex assay. Data indicate delta values for 3- and 20-hours post-exposure for all the participants, n=16.

<table>
<thead>
<tr>
<th>Control</th>
<th>DBP Estimate (%)</th>
<th>SE</th>
<th>DBP Estimate (% change from control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intercept</td>
<td></td>
<td>Male (n=6)</td>
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</table>

### 3h post-exposure

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<tr>
<th></th>
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<tbody>
<tr>
<td>Eotaxin-1</td>
<td>-4.8</td>
<td>9.57</td>
<td>5.7</td>
<td>-10.7</td>
<td>-11.1</td>
<td>-111.5</td>
<td>-101.5</td>
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<td>3.2</td>
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<td>MCP-1</td>
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<td>31.902</td>
<td>29.2</td>
<td>-9.1</td>
<td>9.5</td>
<td>17.6</td>
<td>23.7</td>
<td>-3.4</td>
<td>9.4</td>
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<td>MCP-2</td>
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<td>1.4</td>
<td>-0.7</td>
<td>1.4</td>
<td>1.4</td>
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<td>-0.7</td>
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<td>MCP-4</td>
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<td>6.42</td>
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<td>17.7</td>
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<td>RANTES</td>
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<td>-16.2</td>
<td>-11.2</td>
<td>17.7</td>
<td>23.7</td>
<td>-3.4</td>
<td>9.4</td>
</tr>
<tr>
<td>IL-1b</td>
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<td>0.25</td>
<td>0.2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>TNFα</td>
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<td>0.7</td>
<td>-2.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>-2.1</td>
<td>1.1</td>
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<td>TGFβ1</td>
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<td>1633</td>
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<td>IL-20</td>
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### 20h post-exposure

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<tbody>
<tr>
<td>Eotaxin-1</td>
<td>15.08</td>
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<td>-10.7</td>
<td>-11.1</td>
<td>-111.5</td>
<td>-101.5</td>
<td>1.0</td>
<td>3.2</td>
</tr>
<tr>
<td>MCP-2</td>
<td>-0.514</td>
<td>1.8</td>
<td>1.4</td>
<td>-0.7</td>
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<td>1.4</td>
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<tr>
<td>SDF-1αβ</td>
<td>-158.6</td>
<td>245.1</td>
<td>154.5</td>
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<td>1.2</td>
<td>1.2</td>
<td>-4.9</td>
<td>1.2</td>
</tr>
<tr>
<td>IL-13</td>
<td>1.93</td>
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<td>1.2</td>
<td>-4.4</td>
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<td>2.0</td>
<td>2.0</td>
<td>-4.4</td>
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<td>TSLP</td>
<td>5.44</td>
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<td>1.4</td>
<td>1.4</td>
<td>-6.0</td>
<td>1.4</td>
</tr>
<tr>
<td>IL-1b</td>
<td>0.6</td>
<td>0.2</td>
<td>0.4</td>
<td>-1.1</td>
<td>-0.1</td>
<td>-0.1</td>
<td>-0.1</td>
<td>-1.1</td>
<td>-0.1</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.729</td>
<td>1.3</td>
<td>1.3</td>
<td>-0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>-0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>IL-17A</td>
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<td>1.6</td>
<td>-9.6</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>-9.6</td>
<td>2.3</td>
</tr>
<tr>
<td>IFNγ2</td>
<td>0.9</td>
<td>-4.83</td>
<td>2.4</td>
<td>-4.4</td>
<td>-2.3</td>
<td>-2.3</td>
<td>-2.3</td>
<td>-4.4</td>
<td>-2.3</td>
</tr>
<tr>
<td>PDGF-ΑΑ</td>
<td>83</td>
<td>-141.7</td>
<td>76.9</td>
<td>-37.1</td>
<td>-249.1</td>
<td>-249.1</td>
<td>-249.1</td>
<td>-37.1</td>
<td>-249.1</td>
</tr>
<tr>
<td>PDGF-ΒΒ</td>
<td>469.8</td>
<td>-7.59</td>
<td>2.65</td>
<td>-37.1</td>
<td>-249.1</td>
<td>-249.1</td>
<td>-249.1</td>
<td>-37.1</td>
<td>-249.1</td>
</tr>
</tbody>
</table>

Significant effect of DBP vs. control: bold p<0.1, bold* p<0.05. Significant interaction effect (#) p<0.05.

Values are in pg/ml. MCP: monocyte chemo-attractant protein, RANTES: regulated on activation, normal T cell.
expressed and secreted, MDC: macrophage-derived chemokine, TSLP: thymic stromal lymphopoietin, TNF: tumor necrosis factor, TGF: transforming growth factor, SDF-1α+β: stromal cell-derived factor-1α and β, IFNα: interferon α, PDGF-AA: platelet-derived growth factor subunits AA or BB. Dotted lines separate mediators into 4 main functionality groups (acknowledging that some have a wide range of biological activity and cross-function), chemo-attractants, Th2-related, pro-inflammatory, and regulatory.

In non-AHR participants, Th2-related cytokines were also reduced by DBP exposure, for instance IL-13, p=0.04 and TSLP, p=0.07 at 3h; while IL-13, p=0.03; IL-33, p=0.02 and TSLP, p=0.004 levels were reduced at 20h, opposite to AHR participants. Regulatory cytokines like TGFβ1 increased in non-AHR at both time-points (p=0.03), whereas anti-viral IFNα2 was reduced at 20h, p=0.01. The DBP-effect seemed to be stronger for grass-atopic participants, although results were less consistent (Table 4.4).

4.4.3 Functionality of blood cells are affected by DBP exposure

In the whole blood assay, the DBP inhalation reduced the R848-induced IL-1β (p=0.02) and IL-8 (p=0.09) release ex vivo, in blood collected at the 20h time-point (Table 4.5). Moreover, DBP increased the PMA/Ionomycin-induced release of IL-5 (p=0.06) at 3h. In contrast to these DBP-induced effects, the allergen inhalation alone increased the LPS-induced IL-1β and IL-5 release, as well as the R848-induced IL-5 release ex vivo (see supplement for details).

The effect modification was significant for the type of allergen inhaled for several ex vivo stimulants and mediators, but not for sex or AHR status (Table 4.5). The effect of DBP on reduced R848-induced IL-1β (p=0.03) and IL-8 (p=0.03) release was stronger in birch atopic individuals, and also for R848-induced IL-1α (p=0.09). In contrast, in participants who inhaled grass, the PMA/Ionomycin-induced mediator release was generally increased (IL-1α, p=0.02; IL-1β, p=0.02; IL-5, p=0.03; IL-10, p=0.03; IFNγ, p=0.05; TNFα, p=0.01 at 3h; IL-1α, p=0.02; IL-6, p=0.02; TNFα, p=0.02 at 20h). Although no clear pattern was found for
AHR status or sex, the R848-induced release of IL-1α, IL-1β and IL-8 was also significantly reduced in non-AHR subjects (p=0.03, p=0.01 and p=0.05 respectively).

Table 4.5 DBP effect on inflammatory mediator release from whole blood cells stimulated \textit{ex vivo}.
Significant effect of DBP vs. control: bold p<0.1, bold* p<0.05. Selection of mediators analysed in 10-plex assay, and selection of stimuli. Significant interaction effect (#) p<0.05. Values are in pg/ml and the delta calculated from unstimulated controls. IFNα or γ: Interferon alpha or gamma. TNFα: Tumour necrosis factor alpha.

<table>
<thead>
<tr>
<th>Controls</th>
<th>DBP Effect (% change from control)</th>
<th>SE</th>
<th>Control</th>
<th>DBP Effect (% change from control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα</td>
<td>8.8 (215.2)</td>
<td>9.6</td>
<td>-4.0</td>
<td>22.9 (124.5)</td>
</tr>
<tr>
<td>IFNγ</td>
<td>140.9 (158.7)</td>
<td>105.3</td>
<td>-1.1</td>
<td>174.3 (101.0)</td>
</tr>
<tr>
<td>TNFα</td>
<td>353.1 (312.2)</td>
<td>482.8</td>
<td>-0.4</td>
<td>525.7 (108.9)</td>
</tr>
<tr>
<td>IL-1α</td>
<td>185.4 (208.4)</td>
<td>113.8</td>
<td>-0.4</td>
<td>279.5 (162.3)</td>
</tr>
</tbody>
</table>

4.5 Discussion

Exposure to DBP followed by an allergen inhalation challenge, increased the presence of CD4+ T helper cells in peripheral blood and reduced the number of T regulatory and non-classical monocytes 20 hours post-exposure. Except for effect modifications, only minor effects were observed on inflammatory mediators and cellular functionality. More specifically, the effect of DBP on cellular endpoints was stronger for grass atopic individuals, while the effect on inflammatory mediators was stronger in males. In general, these systemic
effects of DBP were not a reflection of a promotion of the systemic effects due to allergen inhalation.

Epidemiological studies linking phthalate exposure to allergic diseases have been complemented by experimental animal studies, in which adjuvant effects have been presented as a possible mechanistic explanation\textsuperscript{24}. In contrast, the systemic DBP effects in the PAIR study did not appear to be adjuvant, since the significant effects of DBP on T helper subsets, inflammatory mediators and cellular functionality were not consistent with the systemic effects due to the allergen inhalation alone. Although, most studies report limited or no effects of phthalates in the absence of a model allergen, a recent study in mice reported effects of DEHP alone\textsuperscript{24,74}. Specifically, DEHP nasal instillation increased the DC, Th17 cells, IL-17A, IL-6 and MCP-1 inflammatory mediator levels\textsuperscript{74}. To determine whether the effects observed in the PAIR study are a result of DBP and allergen co-exposure, or DBP alone, further studies are required. However, the presence of a pattern of effect modification for the type of allergen inhaled on cellular populations and functionality, suggests that the DBP effect is indeed related to the allergen challenge. But since DBP exposure does not enhance the effect of the allergen alone we do not consider it to be an adjuvant effect.

The overall increase in CD4+ T helper cells at 3h and 20 h post-DBP exposure, could be the result of an expansion of already present and previously primed memory T cells. T helper cell functions are considerably more complex and heterogeneous than originally thought, and the original characterization of the Th1 and Th2 pathways has now been expanded to include additional subsets, like Th9, Th17 and T regulatory cells, each with their own cytokine repertoire and transcription factors\textsuperscript{174}.
In addition, memory T cells are a long-lived subtype that provides an enhanced, faster and stronger immune response upon re-exposure to a pathogen or antigen\textsuperscript{175}. Since our study participants are atopic and previously exposed to the allergen, it is plausible that they have this population ready for expansion. Based on the current results, further characterization studies using specific memory T cells markers are recommended.

The reduction of T regulatory cells at 3h and 20h, and non-classical monocytes at 20h in peripheral blood, could be due to migration of these cells to the affected tissue, specifically the lungs. In support of this hypothesis, our group has also reported increased levels of MIP-1\textalpha and fractalkine, 20h post-DBP exposure in lung lavage samples, both implicated in the migration of leukocytes to the lung\textsuperscript{149,150}. At the same timepoint the percentage of macrophages was increased in BAL accompanied by an M2 skewing, possibly consistent with the migration of non-classical monocytes to the lung.

There were limited effects of the DBP exposure on inflammatory mediators in serum, except for increased levels of leukocyte chemoattractant MCP-4 and SDF-1\textalpha+\beta, supporting the interpretation of a possible leukocyte migration to the affected tissue. Additionally, MCP-4 has been presented as a serum biomarker that predicts susceptibility to asthma, associated with exacerbations, and systemic evidence of allergic inflammation\textsuperscript{176}. Independent of an inflammatory environment, SDF-1\textalpha+\beta participates in lymphocyte recirculation and monocyte recruitment during normal replenishment and turnover of tissue phagocytes\textsuperscript{177}.

DBP had suppressive effects on the anti-viral response marker IFN\textalpha2, potentially limiting the host’s ability to appropriately respond to environmental insults and prevent the development of viral infections\textsuperscript{178}. Similarly, the anti-viral response was compromised when blood from
participants exposed to DBP in vivo was stimulated with R848, a synthetic compound that activates anti-viral pathways via TLR7/TLR8 MyD88-dependent signaling\textsuperscript{103}. Immuno-suppressive effects of DBP in vitro have been reported before, for instance, decrease in cytokine production, immunogenicity and antigen-presenting capacity of murine macrophages\textsuperscript{76}. In addition, our group specifically described a dampening of the R848-induced release of IFNγ and TNFα from innate blood cells in the presence of DBP\textsuperscript{153}.

Interestingly, Th17 cells had a different pattern for males and females, suggesting sex differences in the effects of DBP. The effect on T regulatory cells was stronger in males, although without a significant modification effect. Consistently, a range of inflammatory mediators showed stronger effects in males, and also significant interaction effects.

Phthalates are endocrine disruptors that interfere with hormone pathways\textsuperscript{48} and androgen-receptor interactions have been suggested\textsuperscript{179}, providing a possible mechanism for the stronger effects observed in males. Several studies have reported sex differences regarding phthalate effects and allergen on airway diseases. For instance, an epidemiological study found that urinary levels of MBP were associated with reduced FEV\textsubscript{1}, and decreased pulmonary function in males but not females\textsuperscript{180}. Epidemiological studies in children reported that prenatal exposure to phthalates is associated with increased asthma occurrence, and development of eczema, in boys but not in girls\textsuperscript{181–183}.

Cellular endpoints, like percentages of cellular subtypes in peripheral blood, expression of surface markers and functionality of blood cells in the whole blood assay, showed consistently stronger DBP effects on grass-atopic individuals. Recently, mice studies have highlighted the importance of the applied model allergen, suggesting that cellular
mechanisms involved in experimental models may be allergen-dependent\textsuperscript{74,184}. For example, in a cockroach-allergen extract model, DEHP induced a skewing from eosinophilic to neutrophilic inflammation, promoting a Th2 and Th17 immune response\textsuperscript{74} whereas DEHP induced an eosinophilic Th2 response in the OVA model\textsuperscript{65}.

The PAIR study demonstrates that a 3h inhalation exposure to DBP induces systemic effects, specifically in T cell subsets. Moreover, effect modifications were observed for sex and type of allergen inhaled. The systemic effects due to DBP inhalation do not appear to be adjuvant and further studies are required to determine whether these effects were independent DBP effects or due to allergen co-exposure.

4.6 Supplemental material

Differences between the three time-points for CA exposure

Since the participants were exposed to allergen at the 3h time-point for both exposures, the study design only allows for limited separate assessment of allergen-induced and diurnal effects. More specifically, differences between 3h and -4h could be due to either allergen-induced or diurnal effects, while differences between 20h and -4h reflect effects of the allergen inhalation since these samples were collected at the same time of day.

Cell recruitment

The number of granulocytes in blood increased by 15\% and 13\% at 3h and 20h respectively (p < 0.01). The number of neutrophils also increased by 4\% (p < 0.01) at 3h, whereas eosinophils decreased by 49\% (p < 0.01) at this time. However, eosinophil activation, in terms of increased CD69 levels (MFI), increased by 121\% (p= 0.01) at 20h.
The number of lymphocytes decreased in blood by 21% at 3h and remained decreased at 20h (p < 0.01). More specifically, B cells decreased at 3h (31%, p < 0.01) while Th17 cells decreased at 20h (22% p= 0.04), and so did their expression of CD196 surface marker (11%, p < 0.01). Monocyte levels did not change after the allergen inhalation.

Overall, the allergen inhalation increased the number of granulocytes and activated eosinophils significantly in peripheral blood at 20h, while the number of lymphocytes and Th17 cells decreased. It is not possible to determine whether the effects on neutrophils, eosinophils and B-cells at the 3h time-point were due to diurnal effects or the allergen inhalation.

*Serum mediator levels*

The BCA levels in serum increased by 19% (p < 0.01), 3h after allergen inhalation, and so did IL-7, by 59% at 3h and remained increased by 36% (p < 0.01) the following morning. Also, at 20h post-allergen inhalation, increased serum levels of IL-5 (42175%, p < 0.01), IL-10 (140%, p < 0.01), TGFα (51%, p < 0.01), and I-309 (47%, p < 0.01) were observed. In contrast, MIP-1d levels decreased at 3h by 15% (p < 0.01), while sCD40L decreased by 24% at 3h and by 17% at 20h (p < 0.01). The EGF levels decreased by 29% and 24% at 3h and 20h respectively (p < 0.01) in serum.

Overall, allergen inhalation increased the levels of IL-5, IL-7, IL-10, TGFα, and I-309 while sCD40L and EGF levels decreased. The increased BCA level and decreased MIP-1d at 3h could be due to allergen-induced or diurnal effects.
Cell functionality

Whole blood cells collected 3h post-allergen inhalation and stimulated ex vivo with LPS, increased the IFNγ release by 156% (p= 0.05) and IL-10 by 48% (p=0.02), while R848-induced IFNγ levels increased by 306% (p= 0.04), and IL-12p40 levels by 114% (p=0.04). Blood cells collected 20h post-allergen inhalation and stimulated with LPS, increased the release of IL-1β by 33% (p= 0.02) and IL-5 by 4726% (p= 0.01), whereas R848-induced IL-5 release increased by 7127% (p= 0.02).

Allergen inhalation alone increased the LPS-induced IL-1β and IL-5 release, and the R848-induced IL-5 release.
Chapter 5: Conclusions

5.1 Overview and significance of main findings

The work described in this thesis represents a significant expansion of the epidemiological and experimental evidence regarding airway and systemic effects of human exposure to phthalates. The inhalation exposure route, a considerable source of exposure to phthalates and directly linked to airway effects, has been neglected in both experimental and epidemiological studies. Since the inhalation exposure route is common for both aeroallergens and phthalates, and there is an increasing number of associations between phthalate exposure and respiratory and allergic diseases reported in epidemiological studies, this is an astounding gap, and the motivation for conducting our research.

Chapter 2 described the following hypothesis:

- DBP in the presence of an inflammatory stimuli (IS) in vitro, will downregulate the surface marker expression and increase the activation state of innate immune cells from peripheral blood, and decrease the production of pro-inflammatory cytokines.

We found that DBP had a dampening effect upon the IS-induced expression of surface markers on granulocytes and upon the IS-induced cytokine release. These immuno-suppressive effects are indicative of impaired functionality of the innate immune cells from peripheral blood in the presence of DBP. Moreover, we demonstrated the value of the WBA as a tool for the assessment of interactions between environmental pollutants and immune cells in vitro.

Chapter 3 and 4 described the results from the PAIR study, testing the following hypotheses:
• Inhalation of DBP prior to allergen will
  
  o enhance airway inflammation and responsiveness and promote immune cell recruitment and activation in the lower airway.

  o enhance allergen-induced systemic inflammation and promote immune cell recruitment and activation in peripheral blood.

The lung function of human study participants was affected by DBP exposure, exemplified by an augmented allergen-induced lung function decline after DBP exposure compared to control air. The allergen challenge is a long-standing exacerbation model of allergic asthma that can induce several clinical and pathophysiologic features of asthma in sensitized individuals\textsuperscript{126}. Although, there was no effect upon FeNO used as a marker of airway inflammation or the commonly used test for airway hyper responsiveness, methacholine PC\textsuperscript{20} measurements, both FEV\textsubscript{1} and methacholine PC\textsubscript{20} were significantly reduced in AHR-participants. This is the first study utilizing an allergen inhalation challenge as a research tool to provide evidence of a direct negative impact on lung function due to phthalate inhalation. The current data provide a proof of principle for the inhalation exposure route, in demonstrating that phthalate inhalation can indeed affect the airways. Based on these results we would like to emphasize the importance of including measures of airway exposure in future epidemiological studies\textsuperscript{71} and assessing airway exposure to phthalates in animal models\textsuperscript{74}.

Recruitment of macrophages, specially M2, to the lungs, accompanied by an increased expression of activation marker CD206 in the cellular surface was observed after DBP exposure. Moreover, DBP tended to stimulate the migration of human peripheral blood
monocyte-derived M2 macrophages and not M1, and significantly increased M2 derived IL-1β release\textsuperscript{77}. Increased M2 macrophages have been associated with decreased lung function measures\textsuperscript{136}. Since the observed M2 skewing after inhalation of DBP was not associated with the measures of declined lung-function in our study participants, the clinical implications of this finding are still not determined.

The animal studies generally use OVA for allergic sensitization\textsuperscript{65,72}, and only one recent study used nasal instillation of a naturally occurring aeroallergen, in terms of a cockroach allergen extract\textsuperscript{74}. In that study, DEHP nasal instillation skewed cellular infiltration from eosinophilic to neutrophilic, in the presence of cockroach allergen extract\textsuperscript{74}, in contrast to the pure eosinophilic effects of DEHP reported in the other animal models\textsuperscript{65}. The authors hypothesized that the type of cellular infiltration depends upon the allergen model used, and also showed that DEHP alone had an effect\textsuperscript{74}. The observed effect modification of the type of allergen inhaled on both lung and systemic effects suggest that the type of allergen is of importance also in humans. Further studies are warranted in terms of the role of the type of allergen in the effects of co-exposure to phthalate and allergen, in human, animal and cell culture models.

Systemically, the levels of CD4+ T cells in peripheral blood increased and became activated, while T regulatory cells and non-classical monocytes decreased after DBP exposure. The decrease of T regulatory cells and non-classical monocytes in blood could be reflective of migration to the lungs, supported by increased leukocyte chemoattractant inflammatory mediators in the lung (MIP-1α, fractalkine) and in blood (MCP-4 and SDF 1α+β) at the same timepoints.
Inflammatory mediators in lung samples and serum were modestly altered by DBP overall. Nonetheless, levels of chemo-attractant mediators generally increased in both lung lavage and serum, whereas for other types of makers a decrease was observed. For example, growth factor regulators, Th2-related cytokines, and mediators of anti-viral responses were dampened by DBP exposure. The functionality of alveolar macrophages (reported in chapter 3) and innate immune blood cells (reported in chapter 4) was also moderately altered by DBP exposure \textit{ex vivo}.

The DBP effect in the lungs and systemically, was modified by sex, AHR status and the type of allergen inhaled. The effect was consistently stronger in male participants. Generally, cellular effects seemed to be modified by the type of allergen inhaled, whereas inflammatory mediators were modified by sex and AHR status. Interestingly, the effect of DBP exposure in male, grass-atopic participants \textit{in vivo} (chapter 4) shared similarities to \textit{in vitro} (chapter 2) DBP exposure. The expression of DAMP costimulatory receptor CD24 on blood neutrophils and eosinophils, was reduced by DBP. A possible implication could be a defective innate immune response. In the case of grass-atopic individuals, they may represent a susceptible group.

In our data, anti-viral pathways were affected by DBP exposure, both in terms of cellular activation and functionality and inflammatory mediators. In the whole blood assay, R848 was used to mimic viral responses. R848 is a synthetic compound with the ability to bind to TLR7/TLR8 and trigger anti-viral response pathways\textsuperscript{103,104}. Moreover, CD169+ macrophages participate in the induction of CD8+ T cell-mediated anti-viral responses\textsuperscript{140}. The R848-induced release of IFNγ and TNFα was dampened by DBP \textit{in vitro} (Chapter 2). Moreover, DBP inhalation reduced the percentage of CD169+ macrophages in the lungs (Chapter 3) and
the levels of IFNα in serum (Chapter 4). In addition, R848-induced release of IL-1β and IL-8 from blood cells and TNFα from alveolar macrophages was reduced *ex vivo* (Chapter 3 and 4). Thus, the immuno-suppression of the response to an allergen, or inflammatory stimuli (*in vitro* or *ex vivo*), observed in the presence of DBP, suggests an impairment of the associated anti-viral response pathways. An impaired anti-viral response in susceptible populations can lead to respiratory infections, and acute asthma symptoms have been correlated with a variety of viral pathogens\(^{141}\). Interestingly, the T cell stimulatory function of plasmacytoid DC (pDCs) pre-treated with DEHP and inflammatory stimuli was impaired *in vitro*, providing further support for interference of phthalates with immunity against infection\(^{78}\).

DBP inhalation generally enhanced the effects of allergen inhalation on various lung function measures. Therefore, the effect of DBP on lung function was considered to be adjuvant. In contrast, the effects of DBP inhalation on the cellular and inflammatory mediator endpoints did not overlap with the effects of allergen inhalation in the lung and systemically. More specifically, the effect of DBP over macrophages in the lungs and T cell subsets systemically was not an enhancement of allergen-induced responses. While it was impossible to determine the effect of the allergen inhalation alone over cells and mediators in the lungs in the PAIR study (no bronchoscopy before allergen challenge), a Th2 response is generally expected. Meanwhile, exposure to DBP did not have an effect over Th2-associated cells or mediators in the lungs. Systemically, the DBP effect over T cell subsets was not connected to the observed effects of an allergen inhalation alone like increased number of granulocytes and eosinophil activation. Thus, DBP did not appear to have an adjuvant effect on these endpoints. Even though the cellular and mediator effects of DBP inhalation did not appear to be adjuvant, we cannot exclude that they contributed to the observed lung-function decline. Further studies
are recommended to determine to what extent the observed effects of DBP on cells and mediators contribute to the lung-function decline.

5.2 Strengths and limitations

The PAIR study is unprecedented. This is the first human exposure study to assess adjuvant effects of phthalates inhalation and demonstrate that phthalate inhalation can indeed induce effects in the airways. Consequently, we are expanding the existing literature and bridging the knowledge from exposure studies, epidemiological and experimental studies. The randomized controlled design is widely recognized as the gold standard in generating robust experimental evidence. Likewise, the crossover design is very powerful as each person serves as their own control such that modifiers including baseline genetic variants remain stable, while dynamic changes can be examined at a high-resolution. We tested the exposure order effect in an additional interaction model and concluded that the 4-week washout period and randomization were appropriate. The order of exposure was not a confounding factor.

At the same time, we acknowledge some limitations in the design and implementation. The short duration of exposure is not representative of the continuous nature of an actual environmental exposure. Also, the concentration applied is higher than the reported home environment, to assure that the controlled exposure exceeded the naturally occurring background levels. A bigger sample size would have allowed the analysis of group interactions in bronchoscopy-related samples like BW. Moreover, our study only allows for assessment of allergen-induced DBP effects since the conditions tested always included an allergen inhalation component. Lastly, the influence of the total phthalate exposure, in terms of urinary metabolite levels, on the observed airway and systemic effects has not been
assessed. Since epidemiological and experimental animal studies report associations with the total or oral phthalate intake, it would be appropriate to test whether the total phthalate intake causes effect modifications.

5.3 Lessons learned from the process

A study of this magnitude usually takes several years to complete. From the initial stages of idea conceptualization, ethical and regulatory approvals, development of study protocols, to the sample collection and analysis that occurs years later, the encompassing field of study is also continuously evolving.

In addition to the DBP and CA exposures followed by an allergen inhalation challenge, it would have been beneficial to include DBP and CA exposures without the allergen inhalation component. Although the majority of the animal studies report effects of phthalates only in presence of a model allergen\textsuperscript{65,72}, a more recent study using airway exposures reports effects of the phthalate DEHP alone\textsuperscript{74}. Accordingly, the data in PAIR also point towards effects of DBP in the airways and systemically that are not simply enhanced effects of the allergen-induced effects. PAIR was a novel exploratory study, and the experimental data at the time of study design could not justify the inclusion of an additional non-allergen arm of the study. However, the PAIR study will serve as a basis for more optimal design of future controlled phthalate exposure studies in humans.

At a logistic and strategic level, we also learned about the challenges of recruiting for a study with multiple visits and relatively large time commitment. Figure 5.1 shows the number of participants enrolled at each stage of the study.
Figure 5.1 PAIR study participant enrollment. Participants that responded to advertising material were contacted by email. After a series of screenings, by phone and in our center, those meeting the inclusion criteria were enrolled in the PAIR study (Diads).

We learned that online advertisement was the best route for our participant’s demographic, followed by posters around the university campus and hospital areas. One of the main reasons limiting participant enrollment was the need for the study to occur during weekdays. Interested participants with full-time jobs were typically unable to join. Also, having an open communication with study participants and sharing with them the importance of their contribution was key to facilitate the multiple schedule re-arrangements, natural occurrence in this type of study. It was important to have several participants ready for exposure (passed screening) in case we needed to re-schedule due to a cold, a personal emergency or any other reason. The resources already in place, special rooms booked and the entire team, including bronchoscopy personnel had to be cancelled otherwise.

We learned about the benefits of a well organized and structured document database, including standard operating procedures, forms for screening, clinical tests and laboratory analysis. Keeping the sample inventory up to date and readily available for collaborative
work. Before the study began, we conducted a pilot diad (without an actual exposure or bronchoscopy) with one of our team members as the study participant. This allowed us to revise and optimize the organization and training of study personnel, as well as the study protocols, forms and checklists.

Additionally, knowledge from in vitro or animal models that was not available during the early stages of study design can emerge. This new information could support or contradict established protocols or could also facilitate the discovery of answers to pre-set hypothesis. In our case, markers for the identification and characterization of cellular subtypes, like different macrophage populations in the lung, is a key area that developed substantially during the last 5 years. In light of this new literature, inclusion of other macrophage markers would have been feasible. Likewise, the use of mass cytometry technology (for example, cytometry by time of flight or CyTOF) to characterize the cellular environment in the lungs in response to DBP or CA exposure, would have allowed us to test up to 30 different markers in a single cell. Instead we were able to measure up to 22 markers distributed across 5 flow cytometry panels. By adding this endpoint and the technology to our protocol we would have been able to more accurately define cell populations using not only surface or intracellular markers but also functional ones.

Furthermore, with the use of Seahorse analyzer technology, additional metabolic parameters like bioenergetic profiles (mitochondrial vs glycolytic energy production) of immune cells in the lungs could have been measured. By simply measuring oxygen consumption rate and extracellular acidification rate of live cells, this analysis would have provided an insight into mechanisms potentially altered by DBP exposure, and a functional characterization of macrophage populations.
5.4 Future directions and potential applications

Further studies of the molecular mechanisms involved in the observed effects of DBP inhalation are warranted. This is possible to achieve through further analyses of already collected material from the PAIR study. Several potential molecular mechanisms involved in the asthma promoting effect of phthalates have been suggested. For instance, epigenetic changes, like DNA hypermethylation of GATA-3 repressor zinc finger protein 1 is a highly relevant candidate for mediating the enhanced susceptibility for Th2-driven allergic asthma\(^9\). In a murine transgenerational asthma model, pre-natal exposure to phthalate, specifically butyl benzyl phthalate (BBzP), was associated with increased airway inflammation and linked to asthma severity in the offspring\(^9\). In that study, the authors reported that the disease-promoting effect was mediated by BBzP-induced global DNA hypermethylation in CD4+ T cells in the offspring\(^9\).

Additionally, PPARs have also been identified as phthalate targets and suggested as a possible mechanistic link through partial antagonist effects\(^7\). DBP has been known to interact, although weakly, with all three PPAR subtypes\(^1\). More specifically, in recent \textit{in vitro} studies, DBP modified monocyte-to-macrophage differentiation through interaction with PPAR\(\gamma\)\(^1\). Dysregulation of PPAR\(\gamma\) by DBP inhalation could lead to interruption of PPAR\(\gamma\) anti-inflammatory function and thus indirectly promote inflammation.

Another proposed mechanism by which phthalate exposure has been associated to allergic diseases is through increased oxidative stress\(^1\). In a mice asthma model, DEHP-induced adjuvant effects were linked to increased reactive oxygen species and malonaldehyde levels, common oxidative stress markers\(^1\). Moreover, DBP and MBP specifically have been
reported to interact with anti-oxidant enzyme superoxide dismutase, and in a concentration-dependent manner, inhibit the free-radical scavenger capacity\textsuperscript{186}.

Moving forward, DNA methylation analysis and RNA sequencing of bronchial and blood cells collected in the PAIR study, will facilitate the discovery of the potential mechanisms involved. Specifically, the expression of Th2-associated markers (GATA-3 and Stat6) in relation to Th1-associated markers (T-bet and Stat4) will be assessed. At the same time, more specific M2 macrophage markers like IRF4 and the presence of Arginase-1 and Ym1 genes could be detected and compared to M1 macrophage markers IRF5 and iNOS.

Moreover, PPAR nuclear receptor associated genes and oxidative stress marker expression can be determined. In addition to blood and bronchial cells, the oxidative stress marker content in samples including urine, nasal, serum and bronchial lavage can be detected. Furthermore, measurement of IgE levels in serum and lung lavage samples will determine DBP’s potential for enhancing allergic responses.

Lastly, the potential effect modification of the total phthalate body burden on the airway and systemic endpoints should be addressed in the PAIR study. The presence of phthalate urine metabolites has been determined for metabolites of metabolites of DBP, DEHP, BBzP and DINP. A separate analysis addressing associations between urinary phthalate metabolites and the airway and systemic endpoints would also be feasible and supported by the epidemiological and animal study literature\textsuperscript{24}.

The effect modification observed in both the airway and systemic data emphasize how future studies should account for the type of allergen applied (grass, birch or HDM) as well as sex differences. The animal literature is completely dominated by studies using OVA mice.
models, except for a recent study using an aeroallergen. The current data support further investigation of the importance of the type allergen in the phthalate-induced effects. Similarly, the epidemiological studies report stronger effects in males in several studies, while the sex differences in phthalate-induced effects on allergy have not been addressed in animal models. Overall, both the role of the allergen and sex differences should be more clearly addressed in the future in epidemiological and animal studies.

The results from the PAIR study should be interpreted with caution and used as a starting point for further, more targeted investigations. The PAIR study is currently the only study that investigates airway and systemic effects in humans due to inhalation of a known concentration of a single phthalate, DBP. Our findings represent a substantial expansion of the reported associations between phthalate exposure and airway and allergic disease, demonstrating that phthalate inhalation can indeed induce effects in the airways. This new knowledge regarding a neglected exposure pathway is an important contribution to the field of environmental pollutants and health effects. Moreover, the current data provides further support for a plausible link between phthalate exposure and adverse health effects. We hope that these data will inspire further studies and assist policy makers and stakeholders in the development or regulation of healthcare and environmental policies.
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