TECHNISCHE UNIVERSITÄT MÜNCHEN

Fakultät für Medizin

Effect of whole birch pollen grains on human bronchial epithelial at the air-liquid interface, with or without pre-exposure to fresh combustion aerosol

Joana Rita Gürth Candeias

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften (Dr. rer. nat.) genehmigten Dissertation.

Vorsitz: Prof. Dr. Susanne Kossatz

Prüfer*innen der Dissertation:

1. apl. Prof. Dr. Jeroen Buters
2. Prof. Dr. Jörg Durner

Die Dissertation wurde am 04.04.2022 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 11.10.2022 angenommen.

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List of Acronyms and Abbreviations

- %: percentage
- °C: degree celsius
- µl: microliter
- µm: micrometer
- μ_s: coefficient of static friction
- 3D: three dimensional
- ALI: air-liquid interface
- APC: antigen presenting cells
- ATCC: American Type Culture Collection
- ATI: alveolar type I cells
- ATII: alveolar type II cells
- ATP: adenosine triphosphate
- BC: black carbon
- CAST: Combustion Aerosol Standard
- cDNA: complementary DNA
- cm: centimetre
- cm/s: centimetres per second
- cm^2: square centimetre
- CMA: Comprehensive Molecular Analytics
- CO: carbon oxide
- CO_2: carbon dioxide
- CPC: Condensation Particle Counter
- Ct: cycle threshold
- DC: dendritic cells
- DEG: differentially expressed genes
- DEP: diesel exhaust particles
- DMEM: Dulbecco's Modified Eagle Medium
- DPBS: Dulbecco's phosphate-buffered saline

- DSTD: Derivatization Standard
- EC: Elemental carbon
- eV: electron Volt
- FBS: fetal bovine serum
- gDNA: genomic DNA
- GEO: Gene Expression Omnibus
- GO: gene ontology
- H: hour
- HC: hydrocarbon
- HDMS: hexamethyldisilazane
- HV: high voltage
- IDTD: In-situ derivatization and thermal desorption
- IgE: Immunoglobulin E
- IgG1: Immunoglobulin G1
- IL: interleukin
- ILC2: type 2 innate lymphoid cells
- ISTD: internal standard
- LDH: lactate dehydrogenase
- LPG: liquid petroleum gas
- LPM: litre per minute
- m: meter
- m^3: cubic meter
- mg: milligram
- min: minute
- ml: millilitre
- mm: millimetre
- mRNA: messenger RNA
- MSTFA: N-Methyl-N-(trimethylsilyl)trifluoroacetamide
- NCBI: National Center for Biotechnology Information
- nm: nanometer
- NO_x: nitrogen oxides

- PAH: polycyclic aromatic hydrocarbons
- pg: picogram
- PM: particulate matter
- PR: pathogen related
- PSC: pollen sedimentation chamber
- PTV: programmable temperature vaporization
- PTFE: Polytetrafluoroethylene
- PVC: polyvinyl chloride
- QFFs: quartz fibre filters
- RH: relative humidity
- RIN: RNA integrity number
- RNA: Ribonucleic acid
- ROS: reactive oxygen species
- RT-qPCR: Real-Time Quantitative Reverse Transcription PCR
- SVOCs: semi-volatile organic compounds
- Th2: T Helper Cell Type 2
- Tregs: regulatory T cell
- UFP: ultrafine particle
- UV-Vis: Ultraviolet–visible
- V: Volt
- VOCs: volatile organic compounds
- WAO: World Allergy Organization

Abstract

Allergic diseases affect about 20% of the population in developed countries, of which pollen is one of the main triggers. In vitro studies, however, use allergen extracts, missing important triggers of allergic sensitization due to whole pollen. Also environmental pollutants, such as the combustion aerosol from fossil fuels seem to be correlated with the increases of allergic sensitizations. The effect of a cleaner fuel, such as LPG, on the onset of allergic sensitization has not been studied in detail before.

To study pollen sensitization in a setting closer to real-life exposure, a whole pollen sedimentation chamber was developed. Three main aspects were important for the development of the chamber: the loading tube, the coating and the air pressure. A reproducible and even distribution was demonstrated using different doses of whole birch pollen, with a pollen loss to the system of 42.6% (*SD* = 3.5). In vitro exposures using whole pollen grains were performed with the technique created in this study.

BEAS-2B cells exposed to two different doses of birch pollen (4 mg and 10 mg) showed different responses. Different post-exposure transcriptome analysis showed that the lower doses express a lasting immune and inflammatory response, while for the higher doses the cells showed a faster but more limited response.

The priming exposure of the BEAS-2B cells to a diesel model aerosol showed an adjuvant effect on the birch pollen response. With priming the reaction of the cells to pollen occurred faster, with an earlier expression of genes related to asthma and inflammation. Exposure to the propane-CAST exhaust (a model for LPG) like diesel shifted also the reaction to earlier times, however with unspecific stress and inflammation-related genes compared to the combine diesel and pollen exposure.

This study demonstrated a valuable application of the pollen sedimentation chamber. BEAS-2B cells showed an immune related response when exposed to (real-life, very low) whole birch pollen doses and an adjuvant effect was observed when the cells were primed with combustion aerosols.

Zusammenfassung

In den entwickelten Ländern sind etwa 20 % der Bevölkerung von allergischen Erkrankungen betroffen, bei denen Pollen einer der Hauptauslöser sind. Bei In-vitro-Studien werden bisher jedoch Allergenextrakte verwendet, so dass wichtige Auslöser der allergischen Sensibilisierung durch einheimische Pollen fehlen. Umweltschadstoffe wie das Verbrennungsaerosol fossiler Brennstoffe scheinen mit der Zunahme der allergischen Sensibilisierung in der Bevölkerung in Zusammenhang zu stehen. Die Auswirkungen eines saubereren Kraftstoffs wie Flüssiggas auf das Auftreten einer allergischen Sensibilisierung wurden bisher noch nicht im Detail untersucht.

Um die Pollensensibilisierung in einer Umgebung zu untersuchen, die der realen Exposition näherkommt als die bisheriger Studien unter Verwendung von Allergenextrakten, wurde eine Pollensedimentationskammer für ganze Pollen entwickelt. Drei Hauptaspekte waren für die Entwicklung der Pollensedimentationskammer wichtig: die Beladung, die Beschichtung der Kammer und der Luftdruck. Es wurde eine reproduzierbare und gleichmäßige Verteilung der Pollen in der Kammer unter Verwendung verschiedener Dosen von einheimischen Birkenpollen nachgewiesen, wobei der Pollenverlust im System 42,6 % (SD = 3,5) betrug. Mit der in dieser Studie entwickelten Technik wurden In-vitro-Expositionen mit ganzen Pollenkörnern durchgeführt.

BEAS-2B-Zellen, die zwei verschiedenen Dosen von Birkenpollen (4 mg und 10 mg) ausgesetzt waren, reagierten unterschiedlich. Bei den niedrigeren Dosen zeigte sich eine längere Immun- und Entzündungsreaktion, während die Zellen bei den höheren Dosen eine schnellere, aber begrenztere Reaktion zeigten.

Die Vorexposition der BEAS-2B-Zellen gegenüber einem Dieselmodell-Aerosol vor der Exposition gegenüber Birkenpollen zeigte eine adjuvante Wirkung des Diesel-Aerosols. Nach der Vorexposition mit Diesel erfolgte die Reaktion der Zellen auf die Pollen schneller, einschließlich der Expression von Genen, die mit Asthma und Entzündungen in Zusammenhang stehen. Die Exposition gegenüber Propan-CAST-Abgasen (ein Modell für Flüssiggas)

verschoben wie Diesel-Vorexposition auch die Reaktion zeitlich nach vorne, hier jedoch mit unspezifischen Stress- und entzündungsbezogenen Genen.

Diese Studie demonstriert eine wertvolle Anwendung der entwickelten Pollensedimentationskammer, welche die adjuvanten Effekte von Verbrennungsabgasen mit der Induktion von "Gefahrensignalen" auf BEAS-2B-Zellen zeigt, wenn ganze Pollen in niedriger Konzentration (wie im wirklichen Leben) verwendet werden.

1. Introduction

1.1. Allergic diseases

Allergic diseases, on the rise since the 1960s, have reached an epidemic level (Platts-Mills, 2015). The World Allergy Organization (WAO) reported that more than 20% of the people in the most developed countries have some kind of allergic disease (Pawankar et al., 2013) and the trend is expected to further increase in the following decades. Allergens themselves and environmental pollutants are two of the main drivers for allergic diseases, such as asthma, food allergies, rhinosinusitis and allergic rhinitis. The same trend is observed in Germany with about 20% of the adult population suffering from at least one type of allergy (K.-C. Bergmann et al., 2016; Langen et al., 2013). In recent years, a stabilization – at a high level – of the prevalence of allergic sensitization in Germany was observed (Heidemann et al., 2021).

1.1.1. Allergies to pollen: pollen structure and allergic compounds

Allergic respiratory diseases, such as asthma and hay fever, can be triggered by several components from the atmospheric environment. One of them is pollen from certain plants and trees, or other airborne allergens, such as animal dander, house dust mites and bacteria.

Pollen grains are large particles (10 to 45 µm in diameter (Brown & Irving, 1973)) and comprise the male element of sexual reproduction of plants. Their main function is to transport the male gametes to the female structures and make fertilization possible. Pollen grain transportation depends on the type of plants. Entomophilous plants need the help of pollinators, such as bees or other insects, to enable this transport. Because of that, the surface of these pollen grains is covered by a viscous fluid with high content of lipids and sugars – called "pollenkit" –, which facilitate the adherence of pollen grains to the hairs of insects (Amador et al., 2017; Pacini & Hesse, 2005). Anemophilous plants reproduce themselves through wind pollination, thus have less adherent characteristics (Niklas, 1985).

Pollen from grass and birch trees, in focus in the present study, are generated from anemophilous plants and both plant species are classified as angiosperms, in which pollen grains have commonly a globular shape. The surface of a mature pollen grain can be divided in three different structures: the outer wall – exine –, composed mainly of sporopollenin; an inner wall – intine – , mainly constituted of cellulose; and the pollen coat, an extracellular matrix made of proteins, lipids, pigments and aromatic compounds (El-Ghazaly & Grafström, 1995; Hesse, 2000; Muller, 1979). Depending on the pollen type, the exine has different pores and apertures to initiate tube formation (Knox & Heslop-Harrison, 1970; Piffanelli et al., 1998). The pollen cytoplasm contains the nucleus, starch granules and polysaccharide-particles (Heidrun Behrendt & Becker, 2001). Allergens, the main triggers of pollen allergies, can be found in different parts inside the pollen grains, from the exine to the cytoplasmic matrix and also in the mitochondria (H. Behrendt et al., 1997; Heidrun Behrendt et al., 1999; Grote et al., 1994; Taylor et al., 1994). Allergens are any molecule that can bind to IgE antibodies (Aalberse, 2000). Not all allergens are sensitizing, but those that are able to induce a Th2 response and, consequently, induce the production of specific IgE, and thus an allergic disease (European Academy of Allergy and Clinical Immunology, 2016).

1.1.2. Birch pollen characteristics and distribution

Birch pollen has a diameter of 19 to 27 µm, depending on the method used to determine the aerodynamic size of the species (Brown & Irving, 1973; Caseldine, 2001; Eneroth, 1951; Karlsdóttir et al., 2008; Pohl, 1937). In 1874, Hubert Airy described birch pollen as triangular pollen (Airy, 1874a) and, later in the same year, he corrected to “spherical with three large protuberances“ (Airy, 1874b). Nowadays, birch pollen is characterized as a triporate pollen. Those 3 pores may differ in size depending on the species (Karlsdóttir et al., 2008).

Juhlin-Dannfelt reported in 1948 the existence of a birch pollen allergy season in Sweden (Juhlin- Dannfelt, 1948). At that time, it was believed that only one pollen type could induce allergy: grass pollen. Birch pollen allergy of one patient was also reported for the first time in Denmark, in the same year, by

Egon Bruun (Brunn, 1948). The explanation was the wind coming from Sweden, where concentrations of birch pollen trees and their pollen were higher. The allergic person had lived in Sweden for some time, where he probably was sensitized.

In Europe, birch trees are most concentrated North of the Alps with *Betula pendula* and *Betula pubescens* being the most abundant species (San-Miguel-Ayanz et al., 2016). In Russia, birch trees are found all over the country, but are more abundant in northern and southern west areas (Zyryanova et al., 2010). Birch pollen in those area´s are consequently the most abundant in the world. The birch pollen season occurs between March and May in Western Europe (see pollenscience.eu, accessed August 2021), and some weeks later in northern countries.

In Germany, Munich (Station Biederstein), between 2003–2018, the season peak of birch pollen reached concentrations of 3,500 pollen/m^3 (24 h average). Pollen data from Moscow show concentrations that can reach 10,000 to 20,000 pollen/m^3 (Buters et al., 2018). Concentration and length of pollen season has been reported to be increasing due to climate change (Anderegg et al., 2021; Biedermann et al., 2019; Rojo et al., 2021). It should also be noted that birch pollen in countries with low concentration of birch trees are not absent and high concentrations of pollen may occur, as the transport of pollens through wind may be very important for allergic sensitization (Biedermann et al., 2019; Buters et al., 2012).

1.1.3. Allergic diseases and birch pollen

Allergy to birch pollen is one of the most common allergies in Europe, with at least 8% of the population sensitized (Biedermann et al., 2019). The clinically most relevant allergen of birch pollen is Bet v 1, with about 14% of children and adolescents in Germany showing sensitization to this allergen (Schmitz et al., 2013). Due to the high homology with other allergens, such as Cor a 1 and Aln g 1 (Lorenz et al., 2008), most patients sensitized to pollen from trees show sensitization to this allergen (Biedermann et al., 2019). Studies previously conducted across all Europe showed that one pollen grain releases

about 3.2 pg of Bet v 1 and that the release of the allergen can vary more than 10-fold between days or locations (Buters et al., 2012).

Being sensitized does not directly mean that an individual will have allergic symptoms. Still, sensitization to Bet v 1 is considered a predictor of allergic rhinitis in children (Westman et al., 2015) and a study performed in Germany showed that concentrations of more than 100 pollen/m^3 induces severe nasal symptoms (Karatzas et al., 2018).

1.2. Human airways and the route of particles and pollen

The human airways are a complex system and can be divided in two different regions: the upper airways (includes the nose, mouth, nasopharynx and larynx) and the lower airways (comprising the trachea, the bronchi, the bronchioles, and the alveoli) (Itoh et al., 2004), see Figure 1.

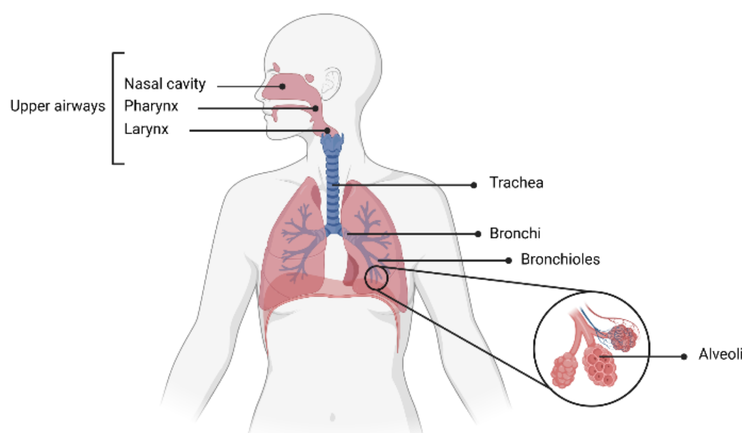


Figure 1: Human airways: representative scheme of the upper and lower airways. (created with BioRender.com)

1.2.1. Cells in human airways

The epithelial tissue of the human airways is the physical barrier to the environment. Airway epithelium has several functions, e.g., antibacterial and particulate clearance by cilia, mucus, and muscle contraction. Its integrity has extreme importance for allergic sensitization by prevention of penetration of allergens (Pothoven & Schleimer, 2017).

A pseudostratified column of ciliated epithelium is observed from the nose to the bronchi, constituted of ciliated epithelial cells, mucus producing goblet cells, neuroendocrine and basal cells and the recently discovered ionocytes (Hiemstra et al., 2015, 2018; Montoro et al., 2018; Plasschaert et al., 2018). Together, they are essential to maintain airway homeostasis. In contrast, terminal bronchioles are layered with a cuboidal epithelium leading to the alveolar region, with a thinner epithelial layer of squamous and cuboidal cells, including the alveolar type I and type II cells (ATI and ATII), allowing gas exchange (Fahy & Dickey, 2010; Hewitt & Lloyd, 2021; Hiemstra et al., 2018).

The composition of the airway epithelium differs depending on the location in the airway and can change with diseases (Chilosi et al., 2012; Hiemstra et al., 2015).

Epithelial cells form a tight barrier as they are in general bounded to each other with strong integrin bounds and cell tight junctions, together with the extracellular matrix (Kechagia et al., 2019). This complex tight junctions create the epithelial barrier, avoiding the entrance of external organisms and particles into the cellular space (Shigetomi & Ikenouchi, 2018; Vasileva & Citi, 2018).

1.2.2. Route of pollen and other particles

Pollen is considered a big particle, and therefore it is assumed that pollen grains would only reach the nasal mucosa. In 1973, Wilson showed that whole grass pollen reaches only the oropharynx (Wilson et al., 1973). However, allergens could be transported freely in the atmosphere and reach easier intrathoracic airways, triggering asthmatic reactions (Gilles-Stein et al., 2016; Schäppi et al., 1997), or bind to smaller particles and reach this area (Knox et al., 1997; Ormstad et al., 1998; Wood et al., 1993).

Besides allergens also other compounds from pollen grains can trigger allergic responses: mainly lipids from the extracellular pollen matrix (Bashir et al., 2013; Traidl-Hoffmann et al., 2002), but also proteases (McKenna et al., 2017). The hydrophobicity of the pollen is maintained with very-long-chain lipids from the pollen coat, important for fertilization (Preuss et al., 1993). The work of Bashir et al., 2013 showed that different compounds, mainly saturated lipids,

increase the immunogenicity and allergenicity of pollen. Another recent study showed that the allergen Bet v 1 alone is not enough to activate dendritic cell maturation, but the whole pollen extract could (Aglas et al., 2018).

1.3. Allergic disease and sensitization

The reaction created by allergens triggers a type 2 immune response, mediated by IgE. Allergens induce the differentiation of Th2 cells derived from antigen-presenting cells (APC). Th2 cells are differentiated by the activation of *IL4*, which induce the secretion of more cytokines, such as *IL5*, *IL9* and *IL13*, relevant in later stages of the allergic immune response (Kumar et al., 2019). B cells, activated by Th2 cells, then produce antigen-specific IgE. This IgE then binds to the FcƐRI receptor on mast cells. In a second contact with the allergen, mast cell degranulation occurs, releasing pro-inflammatory and chemokine mediators, such as histamine and others. These mediators lead to the recruitment of other effector cells, triggering the allergic symptoms. This is considered the early phase of allergen-induced airway inflammation, which occurs in the first hours after contact with the allergen (Galli et al., 2008).

The allergic reaction starts in the nasal and oral mucosa, where the first contact with pollen occurs. When pollen gets in contact with the mucosa it hydrates, releasing several substances as described above. Epithelial cells – one of the first cells that get in contact with pollen – can then release pro-inflammatory cytokines (e.g., *IL6*, *IL8*, *TNF-α*, *GM-CSF* and *TGFB1*) and alarmins such as *TSLP*, *IL33* and *IL25*, activating the recruitment of other effector cells and promoting allergic sensitization (Hackett et al., 2009; Hong et al., 2020; Kaur et al., 2015; Kouzaki et al., 2011, 2013, 2018; Redington et al., 1997; K. Röschmann et al., 2009; Tomee et al., 1998; Zhu et al., 2009) - Figure 2. Pollen grains also contain NADPH oxidases, increasing oxidative stress (Boldogh et al., 2005; Qu et al., 2017). This reaction takes more time than the reaction produced by mast cells, starting 2 – 6 hours after allergen exposure and peaks after 6 – 9 hours. This reaction is usually called the late phase allergy-induced inflammation and has common features with the early phase. Dendritic cells, besides having a possible direct contact with pollen, can also be

activated by epithelial cells. They are responsible to initiate Th2 differentiation in naive T cells, where *OX40L* is vital for this process, and can secrete chemokines such as *CCL17*, *CCL22* and *CXCL13*. Th2 cells, mast cells, basophils and natural killer T cells, are known to produce *IL4*, but the source of *IL4* in this process is still not well understood (Zhong et al., 2014). Eosinophils, as part of the Th2 response, are also important in allergic reaction being activated by *IL5* and *IL13*, secreted from the ILC2 (type 2 innate lymphoid cells) and Th2 cells (Lambrecht et al., 2019) - Figure 2.

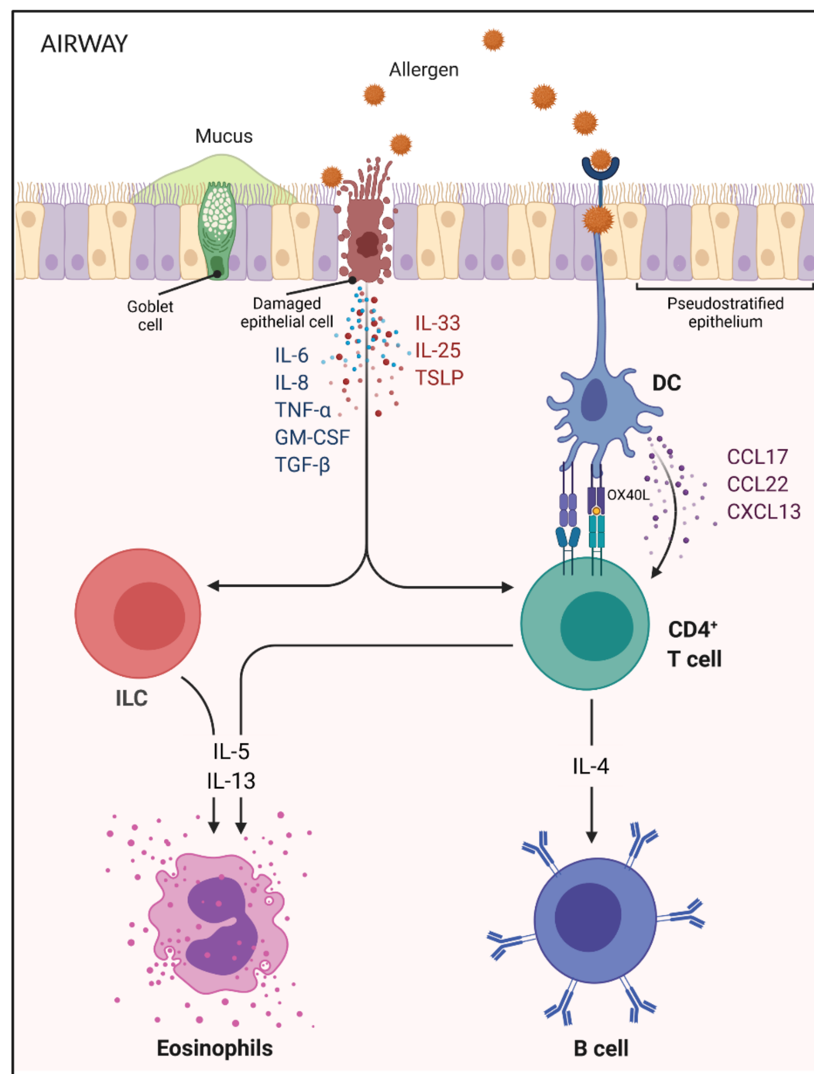


Figure 2: Airway epithelium and allergic response. When an allergen encounters the epithelium, epithelial cells produce cytokines (*IL6, IL8, TNF-α, GM-CSF* and *TGFB1*) and alarmins (*IL33, IL25* and *TSLP*) that induce dendritic cells (DC) to promote Th2 cell-based immunity by the up-regulation of *OX40L*. The same cytokines promote an innate response to allergens by the activation of ILC2s, basophils (not in the figure) and eosinophils. Dendritic cells

release important cytokines such as *CCL17*, *CCL22* and *CXCL13*. B cells are activated by the release of *IL4* from Th2 cells, which will drive into the synthesis of IgE and IgG1. Adapted from "Allergic Airway Inflammation", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

It is believed that different allergens could initiate allergic reactions in several ways by binding to different receptors, so that each allergen or group of allergens could result in diverse signals for allergenicity (Hsu et al., 2010; Radauer et al., 2008; M. Wolf et al., 2017). Protease activity of allergens has been linked with the potency of an allergen to induce allergic reactions inducing the disruption of the epithelial barrier which facilitates the entrance of allergens in the epithelium and consequently sensitization (Gough et al., 2001; Kikuchi et al., 2006; Lambrecht & Hammad, 2014; Matsumura, 2012; Wan et al., 1999). The destruction of the epithelial barrier with down-regulated tight junctions proteins and increased permeability was demonstrated (Xian et al., 2020; Zhao et al., 2018), as well as an antioxidant and inflammatory response caused by the reactive oxygen species (ROS) (Harmon et al., 2018; Liu et al., 2019), which could trigger occupational asthma (Roscioli et al., 2018). Bet v 1 belongs to the pathogen related (PR)-10 family of proteins (non-protease) and is able to bind to phospholipids and permeabilize cell membranes, which also can facilitate uptake of allergen by APC (Mogensen et al., 2007). This potential to enter the nasal epithelium seems to occur in allergic patients, but not in healthy (Joenväärä et al., 2009; Renkonen et al., 2010).

While the allergic reaction is well characterized, the allergic sensitization – the effect of first contact of a person to pollen/allergens – and how a person reaches the allergic phase, is not well known. Pollen reaches the airway epithelium, releases the allergens, and dendritic cells – as APCs – bind to the allergens and travel to the lymph node, inducing the production of T-helper cells. When an individual is tolerant to the allergens, IgG1 and IgG4 antibodies are produced and specific Tregs are differentiated. In a susceptible individual, this tolerant immune response does not happen. Pollen allergens trigger in this case a Th2-drive immune response, by signals not yet well known.

Non-allergic individuals do react to pollen exposure, inducing a non-specific, pro-inflammatory response. Up-regulation of factors such as *CXCL6*,

CXCL10, *CXCR2*, *IL8* and *IL1*, known to be involved in granulocyte chemotaxis and activation, are involved (Mattila et al., 2010). It is also not clarified why some individuals sensitized to specific allergens never have symptoms and others just develop allergies later in life. It seems that several factors can induce or inhibit this signal, such as genetic and epigenetic factors, allergen dose and co-exposure to adjuvant factors. Protective environments were identified, which were related to diversified and increased exposure to environmental commensals, especially found in farming environments (Schuijs et al., 2015; Stein et al., 2016; von Mutius & Vercelli, 2010).

1.4. Pollution and combustion aerosol - adjuvant effect on allergic diseases

Anthropogenic produced environmental exposure (car-derived combustion aerosol) was included in this study, as it seems to be correlated with the increase of allergic sensitization and diseases triggered by allergens. Various studies already showed the correlation of climate change, traffic, industrialization, and cleaning products with the onset of allergic diseases (Agache et al., 2019; Akdis, 2021; Atkinson et al., 2001; D'Amato et al., 2020; Eguiluz- Gracia et al., 2020; Friedman et al., 2001).

Air pollution, especially pollutants derived from the exhaust of fossil fuels, are known to be an adjuvant effect of allergic sensitization (H. Behrendt et al., 1997; Devalia et al., 1994; S. I. Lee et al., 2014; Mazzarella et al., 2012; Schäppi et al., 1997). A study from Jin et al., 2013 showed that the actual pollen extract, compared to the same 10 years ago, showed more allergenic potency, and Ziemianin et al., 2021 showed the same in southern Poland, between 2017 and 2019. Similar studies showed that diesel exhaust particles (DEP) can disrupt pollen and cause the release of the allergens, which were also shown to induce more allergic symptoms (Armentia et al., 2002; Cortegano et al., 2004; Ghiani et al., 2012; Motta et al., 2006).

One metric by which air pollution is assessed is particulate matter (PM), associated with about 9 million deaths per year in the world (Burnett et al., 2018). PM 10 (particles with a diameter of 10 µm and smaller) and PM 2.5 (2.5 µm and

smaller) have been studied for a long time, while ultrafine particles (UFP, smaller than 0.1µm) still need more studies to understand their effect. Because of their small aerodynamic diameter, UFP can reach deep into the human lung and even the circulatory system damaging cells and tissues (Cho et al., 2018). Studies have shown the effect of PM also on the human epithelial barrier in vitro. The induction of *TLSP* and *IL17A*, caused by the presence of PM, was also demonstrate on the exacerbation of chronic respiratory diseases (Brandt et al., 2015; Huff et al., 2019).

1.4.1. Diesel and propane combustion

Diesel fuel is one of the most used fuels and their exhaust represents an important anthropogenic source of PM, emitting about 40% more PM than gasoline cars (https://www.eea.europa.eu – data accessed on October 2021).

Diesel exhaust particles are small (PM 2.5 and smaller) and can thus penetrate deep in the lungs. DEPs have a carbonaceous core with a large surface area where chemicals can adhere. These chemicals can be grouped in organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), oxygenate species – derived from PAHs – heterocyclic compounds, aldehydes, etc.; and inorganic compounds. PAHs and oxygenated compounds are probably the most relevant adhering compounds to DEP as they are able to generate ROS and seem to play a detrimental role in human health by the increase of inflammatory responses, for example (Boland et al., 1999; D. Diaz-Sanchez, 1997; Durga et al., 2014; Vattanasit et al., 2014). Semi-volatile PAHs, such as phenanthrene, can be also part of the gaseous phase of air pollutants and are not only attached to PM (Cao et al., 2017; Khalili et al., 1995). Elemental carbon (EC), carbon monoxide (CO) and black carbon (BC) are also important emission components, the latter known to be associated with adverse respiratory effects, stronger than PM 2.5 (Janssen et al., 2012; Janssen et al., 2011).

The effect of DEP in human allergenicity has been studied. In vitro studies show that DEP increases Th2 cytokines, eotaxin and eosinophil activities (Ichinose et al., 2002; Takizawa et al., 2003). Limited in vivo studies

showed an augmentation of allergic inflammation after pre-exposure with fresh diesel exhaust (Carlsten et al., 2016; David Diaz-Sanchez et al., 1994; Mookherjee et al., 2018). Even though new regulations were made in the last years to reduce the pollutant emissions of diesel fuel (Martin & Ray, 2016; Parliament & Union, 2009), Euro 4 and Euro 5 cars still show important adverse effects in in vitro studies (Colasanti et al., 2018). Besides the improvements in composition of diesel exhaust to reduce environmental pollutants in modern cities (Hesterberg et al., 2011; C.-W. Lee et al., 2019; Milando et al., 2016; Santos et al., 2019), health effects are still observed with pollution levels in agreement with the legal limits (K. Wolf et al., 2021). It is important to note that heavy-duty traffic, building and agricultural machinery have a long lifetime. Old-fashioned heavy-duty diesel engines are not equipped with any filter type, and consequently emit higher concentration of pollutants (Parliament & Union, 2009).

Propane combustion aerosol can be produced indoors by cooking activities, for example (Pathak et al., 2019; Stapleton et al., 2020), but it can also be used as car fuel, usually called LPG – liquified petroleum gas. LPG is an alternative fuel for gasoline for spark ignition engines (Beroun & Martins, 2001; EU, 2018; Parliament & Union, 2009; Yeom et al., 2007), being extracted from crude oil, in the refining process of petroleum and natural gas (Bae & Kim, 2017; Beer et al., 2002; Chala et al., 2018). In Europe, less than 4% of people use cars running with LPG (European Commission, 2018, data accessed on November 2021).

LPG is composed of high amounts of propane but also propylene, butane and ethane, plus other hydrocarbons (HC) in smaller concentrations (Baron et al., 2009; Morganti et al., 2013). Earlier studies showed that cars running on LPG show a reduced fuel consumption and produce less HC and CO emissions but higher nitrogen oxides (NOx) than gasoline fuels (Fleming et al., 1972; Murillo et al., 2005). Bayraktar & Durgun, 2005 demonstrated that an LPG fuel with 100% propane is cleaner than gasoline, for example. Other studies showed that NOx emissions in LPG can be almost reduced to zero if a specific amount of dimethyl ether is added to the fuel composition Z. Chen et al., 2001, and Saleh, 2008 demonstrated that NOx and CO emissions depend on the

proportion of propane/butane. There is no specific regulation for the proportion of propane and butane in LPG in Europe, thus different countries have different compositions. The United Kingdom is the only country that uses 100% propane in LPG fuel (Saleh, 2008).

Studies to understand the health effects of LPG combustion aerosols derived from traffic are very few, with most of the studies being related with indoor pollution (cooking stove aerosol exposure). In countries where mostly biomass fuels are used for cooking, indoor levels of PM 2.5, CO and other pollutants are much higher than the WHO guidelines (C. Chen et al., 2016; Chowdhury et al., 2012; Naeher et al., 2007; WHO, 2014). Studies comparing biomass and LPG fuels for cooking purposes show that LPG produces significantly lower levels of pollutants (including PAHs) and induces less pro-inflammatory effects in human lung tissue (KC et al., 2020; Tiwari et al., 2013). A recent study from Tolis et al., 2021 showed that one car running on either gasoline or LPG, compared to other cars running on diesel, seem to increase volatile organic compounds (VOCs) concentrations in the cabin air when windows are closed, however the study is limited as authors also acknowledge. A related study was performed with minibuses in Mexico City where a trend for more harmful self-pollution, i.e. vehicle's own emissions entering the cabin, of LPG fuel for commuters using these minibuses is shown (Wöhrnschimmel et al., 2008). The effects observed in these studies should not be considered representative because of the limited number of cars studied, thus more studies are needed to understand the health effects on humans of LPG emissions.

1.5. Techniques used to study allergic sensitization

To study allergic diseases, submerged cultures using pollen extracts has been used in the majority of studies. In the last years, studies using cells at air-liquid interface (ALI), in organoids, spheroids and 3D cultures (with one cell culture or co-cultures) are increasing, as they mimic real-life exposure to allergen/pollen better (Chary et al., 2019). Lung-on-a-chip techniques are also applicable, as they mimic the breathing movements, but until now were only used for aerosol delivery of nanoparticles (Benam et al., 2015; Ding et al., 2020;

Hiemstra et al., 2018; Huh et al., 2010, 2012). A 3D basolateral microfluidic ALI in vitro model, used to expose human primary cells to grass pollen extract, was performed to study the release of *IL8* (Blume, Reale, et al., 2015). The cytokine release seems to be higher in the microfluidic system, but no further related studies using this technique are known.

In almost all studies, purified allergen solutions or pollen extracts are used. Comparing both, pollen extracts give a higher immune response than allergen solutions (K. I. L. Röschmann, van Kuijen, et al., 2012; K. I. L. Röschmann, Van Kuijen, et al., 2012). Besides that, different pollen extraction techniques were shown to give different immune responses of cells (Boldogh et al., 2005; Shalaby et al., 2013). Pollen extracts are then closer to real-life exposure, but the use of whole pollen would be an even better option. Such method is still not available, except for one technique developed in the last years (see below), of which no further studies are known (Metz et al., 2018).

BEAS-2B cells – an immortalized bronchial epithelial cell line (Reddel et al., 1988) – are used in our study. Besides the stability and absence of inter-donor variability, the BEAS-2B cell line has limited tight junctions (Hiemstra et al., 2018; Reddel et al., 1988; Stewart et al., 2012). Primary lung epithelial cells are usually obtained from bronchial brushes or biopsies from human subjects and can maintain more characteristics from human bronchial cells, but they are costly, have limited life span and show a high inter-individual variability (Zarcone et al., 2016, 2017). One advantage of primary epithelial cells is the possibility to focus on specific cells derived from donors with different diseases such as COPD and asthma (Amatngalim et al., 2017; Gras et al., 2012). 3D co-cultures with macrophages (THP-1), epithelial cells (A549) and endothelial cells (EA.hy 926) were developed and tested for respiratory sensitization to different substances (Klein et al., 2013) and could be a better model than single cell lines, however gene expression can only be measured from one cell type. In this case, secreted proteins could give a more general reaction than the use of genome analysis of only one cell type.

1.6. Techniques used to study combustion aerosol effects

The majority of the studies used submerged cultures and either expose cells to a control gas or to DEP or PM particles (Colasanti et al., 2018; Niu et al., 2020; Ritter et al., 2001). The particles are mainly collected on filters, thus not using fresh particles. Besides that, mouse models are used to mimic human exposure (Alessandrini et al., 2006; Brandt et al., 2020; Kim et al., 2016; Ouyang et al., 2018) when human studies are ethically problematic.

Another disadvantage of working with pre-collected DEP particles is the omission of the gaseous phase, which was shown to be biologically active with the up-regulation of important metabolites and pathways related with inflammation (Sapcariu et al., 2016). In order to make exposure closer to real-life situations, ALI exposures were developed in the last years (Aufderheide et al., 2017, 2003; Hakkarainen, 2018). A commercially available exposure system, from VITROCELL®, is able to expose cells at ALI to fresh combustion aerosol (and gaseous phase) with several studies showing the capabilities of this system (Ihantola et al., 2020; Oeder et al., 2015; Sapcariu et al., 2016).

Studies using fresh combustion aerosols from cars, ships, and wood stoves were performed (Bisig et al., 2016; Ihantola et al., 2020; Mülhopt et al., 2016; Oeder et al., 2015; Sapcariu et al., 2016), but they require special rooms and regulations, thus an easier access technique was created using a Combustion Aerosol Standard (CAST) generator. This generator is able to produce a stable aerosol from liquid fuel, by the use of a diffusion flame (Moore et al., 2014). Different fuels can be used in the instrument and different aerosol conditions can be created by the adjustment of physical and chemical properties, such as gas dilution and oxidant gas, which gives the possibility to create different real-life exposure settings. This technique has been used before and was shown to mimic the physical and chemical properties of a real-life fuel exposure (Mason et al., 2020; Mueller et al., 2016). There are, however, limitations when using the CAST system. In diesel engines the fuel is injected with pressure to an engine cylinder with compressed air, which ignites and

produces the exhaust, while the CAST exhaust is produced by use of a diffusion flame (Mason et al., 2020; Mueller et al., 2016).

2. Aims of the Project

2.1. Aims

- To study the effect of whole pollen with an air-liquid interface system, on human bronchial epithelial cells, enabling a closer to real-life exposure;
- To study the priming effect of a model fresh diesel exhaust on whole pollen exposure, at the air-liquid interface.

2.2. Interim aims

- Development of a pollen sedimentation chamber (PSC) for whole pollen in vitro exposure (Pollen-ALI);
- In vitro exposure to real-life doses of pollen;
- The effect of pre-exposure to different combustion emissions on whole pollen exposure of BEAS-2B cells.

3. Materials and methods

3.1. Materials

3.1.1. Material for pollen sedimentation chamber (PSC) development

Consumable	Manufacturer
Aluminium tubes for PSC	Workshop of Klinikum rechts der Isar – Dermatology department
15 µm mesh	Utah Biodiesel Supply, USA
No-escape tube	Cassette housing, 25mm, Styrene; Zefon International, USA
Absorber tube/ test tubes	50 mL Falcon® conical centrifuge tubes
Cover slip (12 mm diameter)	Carl Roth, Germany
Cover slip (24 mm diameter)	Neolab, Germany
Microscope slides	Menzel-Gläser, Thermo Fisher Scientific, Germany
Vaseline	Bombastus-Werke, Germany
Melinex® tape	Burkard Manufacturing Co Ltd, England
Aluminium plates	Hagebaumarkt, Germany
Air Compressor	Jun-Air model-6 Compressor, UK
Grass pollen	Allergopharma, collected in 2018

3.1.2. Material and equipment for model diesel/propane exhaust exposure

Consumable/ Equipment	Type	Manufacturer
Magee Scientific Model A3E33-7	Aethalometer®	Magee Scientific, Slovenia
PALAS® VKL 10E	Flow diluter	PALAS, Germany
CPC Model 3022A	Condensation Particulate Counter	TSI, USA
Quartz fiber filters	Quartz-Microfibre Discs	Ahlstrom-Munksjö, Finland
Desert Research Institute, Model 2001A	Thermal-optical carbon analyser	Atmoslytic Inc. Calabasas, USA
Trimethylchlorosilane	-	Fisher Scientific, Germany
Derivatization Standard (DSTD)	Derivatized Alkanes and PAHs	Sigma-Aldrich, Germany

N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)	Silylation reagent	Fisher Scientific, Germany
Shimadzu AOC-5000 Plus	Autosampler	Shimadzu, Germany
Agilent 6890 Gas Chromatograph, LECO Pegasus 4D ToF Ms	GCxGC-ToFMS	Agilent Technologies, Germany
Optic 3	Injection system	ATAS GL, Netherlands
BPX50	Polar Column	SGE, Australia
BPX1	Non-Polar Column	SGE, Australia
Mini-CAST 5201D	CAST for diesel combustion	Jing, Switzerland
CAST 6204B	CAST for propane combustion	Jing, Switzerland
ALI Exposure Station	ALI exposure system	Vitrocell®, Germany
Diesel Fuel	Fuel	Gas station ARAL, Munich, Germany
Propane Fuel	Fuel	Linde, Germany

3.1.3. Medium and solutions – made in-house

Media/ Solution	Ingredients and manufacturer	Details
A549 cell culture medium	- Advanced DMEM (Thermo-Fischer Scientific) - GlutaMAX® (Thermo-Fischer Scientific) - FBS (GE Life Sciences) - Penicillin-Streptomycin (Thermo-Fischer Scientific)	For 500mL medium: 2% FBS, 1% GlutaMAX® and 1% Penicillin-Streptomycin solution
Coating solution for BEAS-2B cell culture	- Fibronectin - bovine collagen type I - bovine serum albumin all from Sigma-Aldrich®, Germany	According to ATCC recommendations: 0.01mg/mL fibronectin, 0.03 mg/mL bovine collagen type I and 0.01 mg/mL bovine serum albumin

3.1.4. Material for cell biology

Substance	Manufacturer
BEGM™ medium with SingleQuots™	Lonza, Basel, Switzerland
Trypan Blue (0.4%)	Thermo-Fischer Scientific, USA

ß-Mercaptoethanol	Carl Roth, Germany
Trypsin-EDTA (0.05%)	Thermo-Fischer Scientific, USA
DPBS	
Alamar Blue	Thermo Scientific, UK
Buffer RPE	QIAGEN, Germany
Triton-X 100 (2%)	Carl Roth, Germany

3.1.5. Commercial kits and assays

Kit	Manufacturer
LDH-Cytotoxicity Detection Kit assay	Roche Diagnostics GmbH, Germany
Fast Start SYBR Green Mastermix	Roche, Switzerland
High Capacity cDNA kit	Applied Biosystems, USA
RNeasy Plus Mini Kit	QIAGEN, Germany
RNA NanoChip	Agilent Technologies, Germany
One-Color RNA Spike-in Kit	
Low Input Quick Amp Labelling Kit, one-color	
One-Color SurePrint G3 8x60K Human gene expression arrays	

3.1.6. QIAGEN human primers

Target	Catalog number	QuantiTect Primer assay
18S	QT00199367	Hs_RRN18S_1_SG
ALDH8A1	QT00087864	Hs_ALDH8A1_1_SG
BACE1	QT00084777	Hs_BACE1_1_SG
CCL20	QT00012971	Hs_CCL20_1_SG
CXCL2	QT00013104	Hs_CXCL2_1_SG
ICAM1	QT00074900	Hs_ICAM1_1_SG
IL1B	QT00021385	Hs_IL1B_1_SG
IL24	QT00059059	Hs_IL24_1_SG
IL4R	QT00090013	Hs_IL4R_1_SG
IL6	QT00083720	Hs_IL6_1_SG
IL9R	QT00057722	Hs_IL9R_1_SG
ITGA4	QT00060627	Hs_ITGA4_1_SG
MADCAM1	QT00017367	Hs_MADCAM1_1_SG
MUC5B	QT01322818	Hs_MUC5B_2_SG
SOCS3	QT00244580	Hs_SOCS3_1_SG
ß-actin	QT01680476	Hs_ACTB_2_SG
TGFB2	QT00025718	Hs_TGFB2_1_SG
TNFAIP3	QT00041853	Hs_TNFAIP3_1_SG

3.1.7. General material and equipment

Consumable	Type	Manufacturer
Cell culture inserts	Costar #3450, 6-well with 0.4 µm pore polyester membrane	Corning, USA
6-/96-well plate	BD Falcon (non-) tissue culture plate treated, flat	BD Biosciences, USA
384-well plate	for RT-qPCR assay	Applied Biosystems, USA
Serological pipettes	2, 5, 10, 25 mL graduated	Greiner Bio-One, Germany
Reaction Tube	SafeSeal tube 1.5mL	Sarstedt, Germany
Pipette tips	1250 µL, 200 µL, 100 µL, 10 µL, 2.5 µL and 1 µL (SurPhob Low Binding tips and SafeSeal SurPhob tips)	Biozym Scientific, Germany
Hybridization backing slides, for microarrays	Pack of 20 Backings, 8 HD Arrays/Slide	Agilent Technologies, Germany

Equipment	Model and Manufacturer
Analytical Balance – for pollen	XS105, Mettler-Toledo, Germany
Microcentrifuge	MICRO STAR 17, VWR, Germany
Cell culture centrifuge	- Universal 32 R, Hettich, Germany - Universal 320 R, Hettich, Germany
ThermoMixer	F2.0, Eppendorf, Germany
Heating plates	- IKAMAG REO, Drehzahl Electronic, Germany - MR3001, Heidolph, Germany
Heating Block	
Light microscope – for pollen counting	DMRB, Leica Microsystems GmbH, Germany
Light microscope – for cell culture	- Axiovert 40 C, Carl Zeiss, Germany - Nikon Eclipse TS100, Germany
Microscope – Auto Imaging system	EVOS® FL Auto, Life Technologies, Thermo Fisher Scientific, Germany
Neubauer improved counting chamber	Marienfield Superior, Germany
Cell culture incubators	Heraeus, HERA Cell, Thermo Fisher Scientific, USA
Lab cycler gradient	SensoQuest, Germany
NanoPhotometer® N60	IMPLEN, Germany

Agilent 2100 Bioanalyzer RNA Nano chip	Agilent Technologies, Germany
Pipettes	1250 µL, 200 µL, 100 µL, 10 µL, 2.5 µL and 1 µL - Eppendorf
Microplate reader	Epoch, Biotek Instruments, Inc., USA
Viia 7 PCR System	Thermo Fisher Scientific, USA
Microarray Scanner	Agilent Technologies, Germany
Microarray Hybridization oven	Agilent Technologies, Germany
Vacuum sealing machine	VC10, Caso, Germany

3.1.8. Software

Software	Manufacturer
Microsoft Office 365	Microsoft, USA
R (v. 4.0.2) programming environment	R Foundation for Statistical Computing, Austria
ImageJ	National Institute of Health, USA
ViiA 7™ Software	Thermo Fisher Scientific, USA
Feature Extraction (v. 11.0.1.1)	Agilent Technologies, Germany
ChromaTOF (v. 4.50.8.0, optimized for Pegasus)	LECO, USA
Affinity Designer and Affinity Photo (v 1.9.2.1035)	Serif, UK

3.2. Methods

3.2.1. Cell culture

3.2.1.1 A549 cell line

Adenocarcinoma alveolar basal epithelial cells (A549) were used in the study for the preliminary tests. Cells were obtained from ATCC (CCL-185™) and cultured in submerged conditions in Advanced DMEM medium, with 2% FBS, 1% GlutaMAX® and 1% of Penicillin-Streptomycin solution. The confluence of cells was checked, medium was changed every other day and cells were sub-cultured (with 0.5% Trypsin-EDTA) when 80-90% confluence was reached. For the preliminary experiments, cells were seeded in submerged conditions in 6-well Transwells® ($0.4x10^6$ cells each well) and both basal and apical medium was changed after 24 hours. On the next day, cells showing 85-90% confluence were exposed to the whole pollen.

3.2.1.2 BEAS-2B cell line

The immortalized human bronchial epithelium cell line, BEAS-2B (ATCC® CRL-9609™) was cultivated as described previously (Oeder et al., 2015). In summary, cells were cultured in BEGM™ medium with supplements (gentamicin was not used, as ATCC® recommendations). Coating of flasks and inserts was performed at least one hour before cell culture seeding (see Materials) and left in the cell culture incubator until use. Confluence of the cells was checked by light microscope every day, medium was changed every other day and cells were sub-cultured before reaching 85% confluence.

For experiments, BEAS-2B were seeded on Transwells® ($0.4x10^6$ cells each well) and kept in submerged conditions for 24 hours. Over that time, basal medium was changed and the apical medium was removed to reach air-liquid interface conditions. Only inserts with more than 85% confluence were used for experiments. Cells were kept in ALI conditions for 24 hours before exposure.

3.2.2. Pollen-ALI: pollen sedimentation chamber exposure to BEAS-2B at the air-liquid interface

The pollen sedimentation chamber was developed and used for in vitro exposures, as described in (Candeias et al., 2021). For developmental experiments mostly birch, but also grass pollen was used. For in vitro exposures – "Pollen-ALI" –, only birch pollen was used. Birch pollen was collected fresh from multiple trees in 2018, in Anzing (surrounding of Munich), sieved through 100 and 70 µm meshes and stored at -70 °C in several aliquots. The grass pollen used was obtained from Allergopharma (see Materials).

For in vitro experiments, before each Pollen-ALI exposure, an aliquot of the birch pollen was thawed and allowed to reach room temperature 2 hours before opening the vial. Relative humidity and temperature of the room were recorded before weighing the pollen. The specific pollen dose was weighed inside the "No-escape tube"; the Falcon® tube (used as a shock absorber) was attached to the loading tube and placed on a transporter box.

Figure 3 illustrates the exposure set-up summary. One incubator was used only for Pollen-ALI exposure (Incubator 1), while for incubation of cells

after exposure, a second incubator was used. Incubator negative controls (only incubator) were always kept in Incubator 2. Control cover slips and culture plates with cells were placed inside the PSC rapidly to avoid changes in incubator conditions. The pollen chamber was closed and the “No-escape tube” was placed on the loading port. Pressurized air (0.5 bar) was injected on the loading tube for 5 seconds, the air pressure gun was removed and the cell culture incubator was closed. Temperature and CO_2 levels were recorded and cells were kept for 10 minutes in Incubator 1 to allow pollen to sediment. BEAS-2B cells were exposed for different times to whole birch pollen: 10 min, 55 min, 2 h 25 min, 6 h 50 min and 24 h. The same conditions were applied for mock exposures (devoid of pollen).

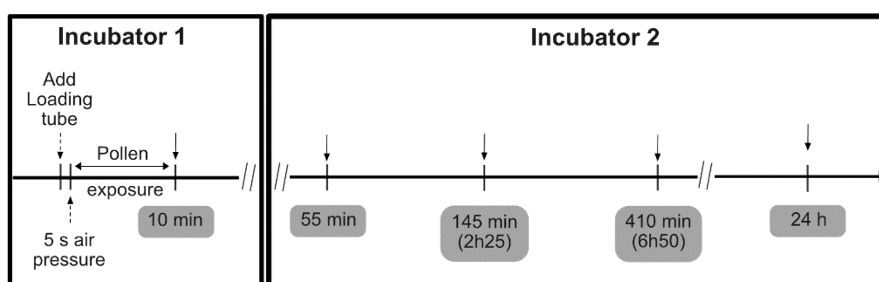


Figure 3: Pollen-ALI experimental design. Incubator 1 was used only for Pollen-ALI where pollen was dosed to the cells. Air was pressured for 5 seconds and after 10 min of pollen sedimentation, cells were transferred into the second incubator (Incubator 2) and kept in ALI conditions for the indicated times. Both incubators were kept at 37 °C and 5% CO_2.

For each exposure, exposed and control cells were placed in the PSC in the same cell culture plate. Three inserts – “Exposed Transwells” – are cells exposed to pollen, while the remaining three inserts – “Control Transwells” – did not receive pollen, by placing a 15 µm mesh over each well (allowing air exchange). Three extra controls – “Incubator controls” – were kept always at the Incubator 2, to be used as negative controls. After each exposure, cells were checked for their viability and cytotoxicity, using the Alamar Blue and LDH cytotoxicity assay, respectively. Some wells were used for RNA isolation, for genome wide analysis of transcriptional changes, see Figure 4.

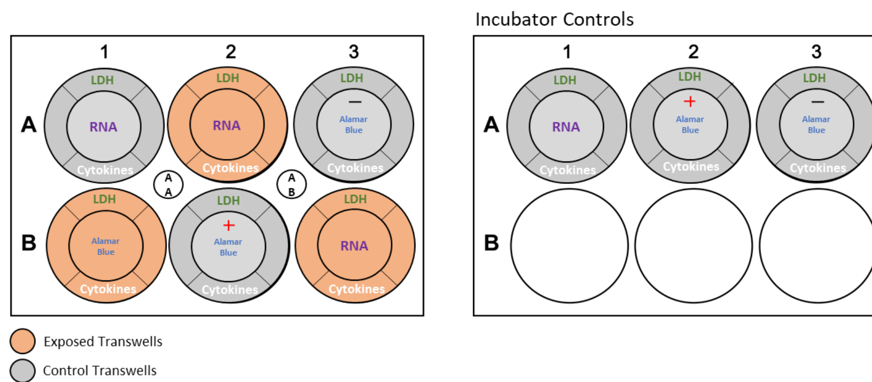


Figure 4: Pollen-ALI: samples and controls overview. Representation of two 6-well cell culture plates, used for one Pollen-ALI exposure. The wells in orange represent the cells exposed to pollen – "Exposed Transwells" –, while the wells in grey (on the left plate) represent the control cells, covered with a 15 µm mesh on top. The cell culture plate on the left is placed inside the PSC. The plate on the right is kept at Incubator 2 at all time and the three wells represent the negative controls of the exposure.

3.2.3. Pollen sedimentation chamber – pollen doses assessment and pollen loss to the chamber

Pollen deposition on the bottom of the PSC was checked by using Vaseline coated cover slips. Four circular cover slips, with 24 mm of diameter, were placed on the bottom, close to the cell culture plate. In addition, two smaller cover slips, with 12 mm of diameter, were placed inside the cell culture plate to be used for dose assessment inside the plate. The place of the cell culture wells were replaced with six cover slips for pollen deposition studies.

After each experiment, cover slips were placed in a group of two on a microscope slide with a droplet of water beneath each cover slip, for storage purposes. Pollen doses were quantitated with a light microscope, using 100x magnification and manual counting.

For pollen doses determination, two lines crossing each cover slip were counted, covering an area of 0.48 cm^2 and pollen recovered was calculated in relation to the area of the bottom of the PSC – 283.5 cm^2, using a pollen weight of 7 ng/pollen grain (Brown & Irving, 1973; Pohl, 1937; Schäppi et al., 1997). Pollen loss to the chamber was calculated by relating weighed pollen on the loading tube to the recovered pollen on the bottom of the chamber.

3.2.4. Pollen doses of the PSC – correlation with real-life exposure

We aimed to sediment pollen in the PSC in a realistic dose, encountered in real-life, at the human upper airways. Birch pollen, used in the study, is more abundant in Northern and Central Europe, but it is in northern Russia where the highest amount of birch trees (*Betula sp.*) is observed (San-Miguel-Ayanz et al., 2016).

The dose used in the PSC was estimated as follows: in Central Europe, Germany included, birch pollen is one of the main causes of allergies (Biedermann et al., 2019). Between 2003 and 2018 in Munich (Station Biederstein), the daily mean of birch pollen counts at their peak was 3,500 pollen/m^3 averaged over 24 hours. An adult can breathe in a day around 14 m^3 (Stifelman, 2007), while the area of the human nasal cavity is around 160 cm^2 (Gizurarson, 2012). This results in a dose in Munich, on high peak days, of 306 pollen/cm^2. In Russia, peaks of 10,000 – 20,000 birch pollen/m^3 are common (Buters et al., 2018), resulting in a dose of 1,000 – 2,100 pollen/cm^2.

3.2.5. Adjuvant effect exposure: exposure of BEAS-2B, at ALI, to fresh diesel/propane aerosol and to whole birch pollen

3.2.5.1 Generation of the combustion aerosol and their physical and chemical characterization

For the creation of the combustion aerosol, different CAST generators were used in the study, depending on the fuel.

For the diesel model aerosol, diesel fuel (low sulphur, no added biodiesel, according to DIN EN 590) was obtained from a local gas station in Munich. The diesel exhaust (gas and particulate phase) was obtained as described in previous studies (Mason et al., 2020; Mueller et al., 2016). For cell exposure, the diesel model exhaust was diluted about 1:200 with filtered air. The same dilution settings were used for the propane exhaust. To create the propane exhaust, the flow of the fuel was set to 60 ml/min, oxidation air was injected with 1.4 LPM (litres per minute) and cleaned (filtered) air was added with 180 ml/min.

Regarding the physical composition of each model exhaust, online and offline measurements were performed as described in (Candeias et al., 2022). For that, the aerosol was 10 times more diluted than for the in vitro exposure using an ejector diluter. For the online measurements, an Aethalometer® was used to measure black carbon and total PM. A Condensation Particle Counter (CPC) together with an electrostatic classifier were used to determine size distribution. Regarding the offline measurements, quartz fibre filters (QFFs) were used to collect the elemental and organic carbon (EC and OC, respectively). The filters were analysed using a thermal-optical carbon analyser, according to the IMPROVE_A protocol (Chow et al., 2007).

For the analysis of chemical organic composition of the diesel model exhaust, QFFs samples were used. Small pieces of 6 mm in diameter were placed into deactivated glass inserts (with trimethylchlorosilane) to be subjected to thermal desorption. An Autosampler was used to add first 1 µL of an internal standard (ISTD) to the QFFs and 1 µL of a derivatization standard (DSTD), and then 10 µL of the silylation reagent MN-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), see Table A- 1. In-situ derivatization and thermal desorption (IDTD) was performed as in (Orasche et al., 2011; Weggler et al., 2016).

For the analysis of particle-bound semi-volatile organic compounds (SVOCs), a GGxGC-ToFMS system was used. For programmable temperature vaporization (PTV) desorption an Optic 3 injection system was used (see Table A- 2 and Table A- 3. SVOCs were first separated on a polar column, focused and then released by a modulator onto a second non-polar column. SVOCs were later transferred to a mass spectrum, ionized with 70 eV and analysed. The sample was measured in triplicates. Table A- 4, Table A- 5 and Table A- 6 show more detailed information on the chromatography and mass spectral parameters. Pre-processing of the data was conducted with ChromaTOF, where baseline correction, peak find, library search and area calculation were performed. Column bleed, contaminations and unknown compounds were manually excluded. After this selection, only compounds present in at least 2

out of 3 technical replicates were included for further data inspection (see Table A- 7).

3.2.5.2 BEAS-2B ALI exposure to the diesel and propane model combustion aerosol

The pre-exposure to diesel and propane exhaust was achieved as described before (Mason et al., 2020; Sapcariu et al., 2016). In brief: a VITROCELL® Automated Exposure Station system was used to expose BEAS-2B cells to the model fresh combustion aerosol (Mülhopt et al., 2016; Oeder et al., 2015). The aerosol flow in the system was 100 ml/min for each insert and the system was conditioned to 85% relative humidity and 37 °C. If these minimum conditions were not achieved, cells were not used for further analysis. Control cells (named "CA") were exposed to sterile filtered ambient air in the VITROCELL® system and later in the PSC to 4 mg birch pollen. Cells were exposed for 2 hours to the diluted aerosol as in (Oeder et al., 2015) and then placed in a cell culture incubator, with fresh basal medium, until pollen exposure. One cell insert was used for cell toxicity and viability assessment of the exhaust exposure. After 24 hours, cells were exposed to 4 mg birch whole pollen as explained above (Pollen-ALI exposure). The difference in this exposure is that all cells were exposed to birch pollen (no mesh control cells were used). For the combined exposure, three different endpoints were analysed (2 h 25 min, 6 h 50 min and 24 h) – see Figure 5. Viability and toxicity levels were measured for all post-exposure times.

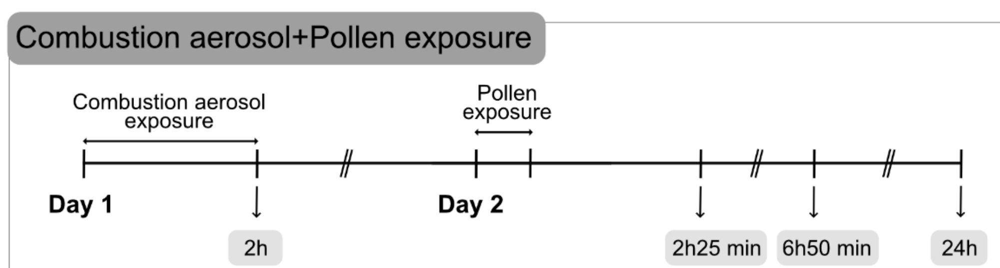


Figure 5: Exposure set-up of the combined aerosol and pollen exposure. BEAS-2B cells were exposed to the exhaust aerosol (diesel-CAST or propane-CAST) for 2 hours, at the ALI, in the Vitrocell® Automated Exposure system. After that, cells were placed in a cell culture incubator (with fresh medium). 24 hours after the combustion aerosol exposure, cells were

exposed to 4 mg birch pollen (Pollen-ALI), for 10 min. The cells were kept in ALI conditions for the indicated times.

3.2.6. Cell viability determination with the Alamar Blue® assay

Cell viability of in vitro exposures was assessed using the Alamar Blue® assay, according to manufacturer's instructions. For controls, incubator cells were used as negative controls and the results were normalized to these cells. Cells lysed with 2% Triton-X 100 were used as positive controls.

After the end of each exposure, 1.5 ml of the Alamar Blue® solution was applied on Transwells® for cell viability determination (see Figure 4) and incubated for 1 h 30 min to 2 hours. After that, absorbance was measured at 600 nm in a 96-well plate.

3.2.7. Cell cytotoxicity determination with the LDH assay

Cell toxicity was in all cases determined using the LDH-Cytotoxicity Detection Kit, according to manufacturer's instructions. Negative and positive control cells were obtained as for viability assay, explained above.

After the end of each exposure, an aliquot of basal medium of each insert was placed on a 96-well plate. The mixed solution (catalyst and dye solution) was placed on each well and absorbance was measured at 490 nm.

3.2.8. RNA isolation

Immediately after the end of each exposure, inserts selected for RNA isolation were lysed using Buffer RLT from RNeasy Plus Mini Kit, with an additional 1% ß-Mercaptoethanol, and stored at -80 °C for later RNA isolation. The possible genomic DNA (gDNA) was removed with gDNA eliminator columns and total RNA was extracted according to the QIAGEN protocol. After isolation, RNA was quantitated and the RNA-quality was assessed with a UV-Vis spectrophotometer and the Bioanalyzer, respectively. Only samples with a RNA integrity number (RIN) higher than 8 were used for further analysis.

3.2.9. Transcriptome analysis using gene expression microarrays

Whole genome expression analysis was performed for the highest doses of Pollen-ALI exposure (4 mg and 10 mg pollen) and for the combined exposure (combustion aerosol + pollen), as explained above.

RNA samples were spiked (One-Color RNA Spike-in Kit), Cy3-labelled (Low Input Quick Amp Labeling Kit, One-Color) and purified on RNeasy mini spin columns. Generated Cy3-labelled cRNA was hybridized on One-Color SurePrint G3 8x60K Human gene expression arrays, according to the manufacturer's protocol. Microarray slides were scanned and data were extracted using the Feature Extraction Software.

All transcriptome data were analysed using the statistical programming environment R (R Core Team, 2020). In detail, pre-processing of the microarrays was performed using the Limma package (Ritchie et al., 2015). Differentially expressed genes (DEG) were compared with the respective control cells for each exposure, using the eBayes method. Cutoffs were $p<0.05$ (adjusted *p*-value with Benjamin-Hochberg) and ≧ 1.1-fold up- or down-regulated.

Microarray raw data discussed in this study were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and can be accessed through GEO Series accession number GSE179942 (for 10 mg pollen) and GSE185399 (for 4 mg pollen and the combined diesel + pollen exposure).

3.2.10. Validation of selected genes with qRT-PCR

Selected genes from different enriched GO terms (related to allergy and inflammation response), differentially expressed by microarray analysis in different exposures, were validated by quantitative real-time PCR. Reverse transcription of total RNA samples used for microarray analysis was performed with the High Capacity cDNA kit, according to manufacturer’s instructions. The qRT-PCR was performed with ViiA 7 Real-Time PCR System using the FastStart Universal SYBR Green Mastermix. QIAGEN human primer Assays were used, including endogenous controls (ß-actin and 18S) – see Materials for

more details. The mRNA expression of the selected genes was normalized to the endogenous controls and relative quantification was calculated using the comparative Ct method ($2^{-\Delta\Delta CT}$), as described in (Schmittgen & Livak, 2008). All amplifications were carried out in duplicate and fold-changes in mRNA expression of exposed BEAS-2B cells were compared with control cells (according to each exposure-type).

3.2.11. Statistic methods

Independent sample T-test was performed for data with two groups. ANOVA was used for multiple groups, with the adequate Post-Hoc tests for each hypothesis. Benjamin-Hochberg p-value correction was used for all tests. Unless stated, standard deviation (*SD*) was used to report the variability of the mean.

All tests and cell exposure experiments were reported from at least 3 independent repetitions on different days, except for the diesel control exposure where only one replicate was reported (diesel vs. non-exposed was not the focus of the study).

The enrichment analysis of the transcriptome data was performed using the Metascape web-based portal (Zhou et al., 2019). Enriched terms were selected based on the GO (Gene Ontology) Biological Processes and pathways from different databases (Canonical, Hallmark, Reactome, KEGG, WikiPathways and BioCarta), with the cutoff of minimum three genes, fold change > 1.2 and *p*-value < 0.05.

4. Results

The aim of this study was to perform whole pollen exposure for which we developed the Pollen Sedimentation Chamber (PSC). As this technique was established during this study and is not addressed anywhere else, the detailed conception and validation is explained in Chapter 4.1 of this dissertation. We

believe that this could help the scientific community to use, improve and/or reproduce this technique for future research.

4.1. Development of the pollen sedimentation chamber

4.1.1. Conceptualization

The development of the pollen sedimentation chamber started by defining the main requirements needed to create a feasible sedimentation of whole pollen on ALI cell culture:

- Small enough to fit inside a cell culture incubator;
- Closed cylinder, but with possibility of air exchange (no escape of pollen);
- One “loading port” where whole pollen can be loaded into the chamber;
- Possibility to fit at least one 6-well culture plate inside the chamber;
- Easy access at the bottom of the chamber to add and remove the cells.

Figure 6 shows the first sketch idea for the pollen sedimentation chamber. The idea was adapted from (Golovko et al., 2013). Whole pollen would be loaded into the chamber through a small opening (tube clamp) where a tube containing the pollen should fit. By using pressurized air, the dispersion of pollen inside the PSC would be possible. Two movable plates were designed inside the chamber: the upper one would serve to stabilize the turbulence of the pollen, due to pressured air, before sedimentation at the bottom of the chamber. After stabilization, the plate would be removed and pollen could sediment on the bottom of the chamber, resulting in an even distribution. The bottom movable plate was thought to be closed once the desired exposure dose was reached, blocking more pollen to reach the cells. The movable plate could also be closed in steps so that different doses could be loaded on cells in different wells; or to block pollen deposition on control cells. On the top of the PSC, a 10 µm mesh closed the chamber, allowing air exchange – important for the cells –, but blocking pollen escaping the chamber (almost all pollen have a diameter larger than 10 µm). On the bottom of the chamber, a polyvinyl chloride (PVC)

plate would permit the complete closing of the PSC by introducing four pins. When the exposure was over, the pins could be opened, allowing to move the PSC away and access the cell culture plate. Finally, the pollen sedimentation chamber should fit inside a cell culture incubator, thus the complete chamber structure could not be higher than 50 cm or have more than 20 cm of diameter. That would allow one cell culture plate inside the chamber.

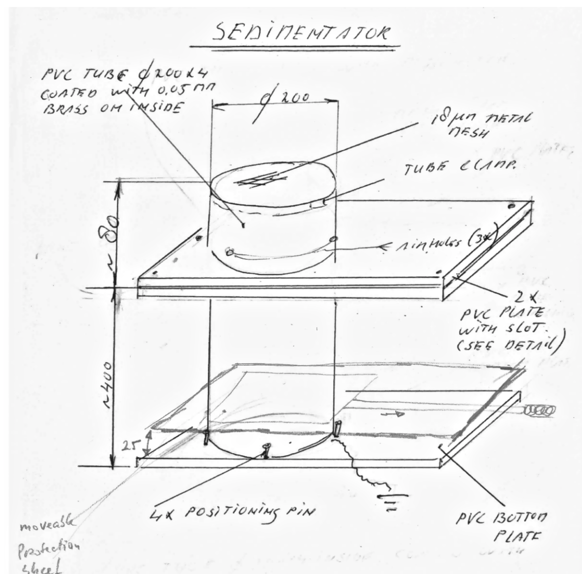


Figure 6: First sketch of the pollen sedimentation chamber. A three-stage tube was designed in which pollen grains would get dispersed, by pressurized air, inside the chamber. The top movable plate would be used to stabilize the turbulence of the pollen. After stabilization, plate opens and pollen can sediment at the bottom of the chamber, where cells are placed. The bottom movable plate was designed to dose different amount of pollen to the cells within the same exposure. Four positioning pins at the bottom of the chamber would create a closed environment. A 15 µm mesh would be placed at the top of chamber to allow air exchange but block the pollen.

Before building this design, an evaluation of the chamber, as designed, was crucial to know if it could be used for the purpose intended. One of the main issues when working with whole pollen is their ability to stick to any surface and form clusters when deposited. Thus, we expected pollen clusters on the bottom of the chamber and little pollen, as reported by (Golovko et al., 2013).

For the first tests, an aluminium tube was used to study the deposition of whole pollen. The tube had 100 cm height and 20 cm of diameter. The cylinder was closed attaching a Chemvol® stage with the same diameter as the

aluminium tube on the upper part. The aperture of the Chemvol® stage was closed with a 20 µm sieve, so that pollen could not escape. The area not covered with the sieve was closed with tape. For the safety of the experimenter (who could be allergic) an air cleaner was placed in the room, to clean possible pollen flying in the air.

A glass plate was added at the bottom of the aluminium tube, see Figure 7. This plate had two purposes: to close the chamber at the bottom and to be used as a "pollen deposition plate". Glass was used as it is known to be more hydrophilic than polystyrene (Mitchell, 2004), which would have an effect on the homogeneity of the pollen deposition. On the glass plate, six cover slips with a diameter of 24 mm (same as the inserts used for cell culture) and four microscope slides, all covered with Vaseline, were placed in specific positions as can be seen in Figure 7. Vaseline traps the pollen that deposits on the deposition plate directly after dosing, enabling to check the number and spread of the pollen by microscope. Slides and cover slips were not mounted, as is customary in the usual pollen preparation slides (Galán et al., 2007), to prevent pollen to move out of their initial deposition place ("Tsunami effect").

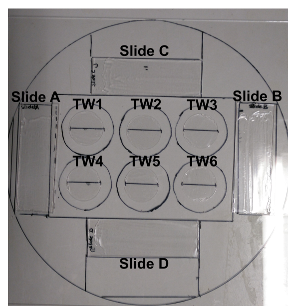


Figure 7: Glass plate used as deposition plate for the chamber prototype. Six cover slips and four microscope slides, covered with Vaseline, were placed in specific positions, for pollen counting purposes. The cover slips with 24 mm of diameter, here named from TW1 to TW6 (TW = Transwell), have the intent to count the pollen that would sediment on the cell culture transwells. With the four microscope slides we counted the pollen that sediments around the cell culture plate and known if sedimentation is even over the whole sedimentation area.

The aluminium tube had a 2-3 cm aperture on the top that was used as "loading port". To load the pollen inside the chamber, by pressurized air, an adapted 50 ml Falcon® conical centrifuge tube was used, together with a small round aluminium foil – Figure 8. This single-use foil had two functions: weigh the

pollen and transport it inside the chamber. The foil was placed on the balance and pollen was weighed directly on it and placed in the 50 ml Falcon® tube. The bottom of the tube had a small round aperture (about 5 mm diameter) to allow pollen to fly away, but not the aluminium foil on which pollen was weighed. To blow pollen inside the chamber an air compressor was used. An about 5 mm aperture on the lid of the loading tube enabled the air compressor pistol to blow the pollen out of the tube. Both apertures were closed with Parafilm before the exposure to prevent pollen to fly away during transport. This Parafilm was removed briefly before dosing.

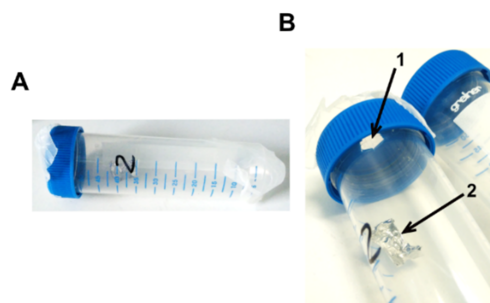


Figure 8: First prototype of the loading tube. (A) Adapted Falcon® conical centrifuge tube. The bottom of the tube was cut out to allow pollen to fly away. (B) Detail of the pollen loading tube. (1) Middle aperture of the lid to allow blowing pollen away, (2) aluminium foil used to weigh the pollen.

4.1.2. First pollen chamber prototype

Whole birch pollen was used for exposure. In the first experiment, the goal was to check how the birch pollen behaves and sediments on the chamber created. Two different doses of birch pollen were used to test the prototype chamber: 0.28 mg (35,264 pollen grains) and 4.41 mg (555,415 pollen grains). Pollen was allowed to reach room temperature for 2 – 3 hours before the experiment. Three microscope slides covered with Vaseline were placed at the centre of the deposition plate. The pressurized air used to blow the pollen inside the chamber was 3 bar. Each microscope slide was taken out of the plate at different times (5 minutes, 25 minutes and 1 hour) to evaluate if all pollen had sedimented as it is known that pollen has a fast sedimentation rate – 1.5 cm/s (Pohl, 1937). However, we did not know how pollen behaves in our chamber. The PSC was kept closed at all times, besides for these three moments.

Looking at the slides with a microscope, single birch pollen was obtained in the deposition plate (Figure 9). Thus, clusters of pollen on the bottom of the chamber were prevented with the technique established. We observed that the sample slides taken at 25 min and 1 hour after pollen loading did not show more pollen than the slide taken earlier, which confirms the fast sedimentation rate of pollen (about 1 min in a tube of 1 m when 1.5 cm/s sedimentation speed is assumed). The small doses of pollen used (0.28 mg) showed almost no pollen sedimented on the bottom of the chamber. This means that small doses are difficult to use for cell culture exposure or that within the procedure pollen was lost and did not reach the deposition plate.

Figure 9: Microscope picture of birch pollen deposited on the bottom of the prototype chamber. (400X magnification). The birch pollen looks wrinkled due to natural drying of water containing pollen (i.e. this is how pollen flies in ambient air: wrinkled).

4.1.2.1 Deposition of pollen – first settings

To study the deposition of the pollen on the bottom of the chamber, the same experiment procedure was performed as before, using 2.1 mg (*SD* = 0.62) birch pollen. Some differences were made in the procedure: 1 bar pressurized air was used, for 5 seconds (3 bar might be too strong), pollen was let to sediment in the chamber for 10 min and all cover slips and microscope slides were placed in the deposition plate as in Figure 7.

To address the question if all pollen goes from the loading tube into the chamber, the adapted loading tube, including lid and Parafilm, was weighed, before and after the experiment. The difference was the amount of pollen that was resuspended and had left the loading tube (i.e., inside the chamber). The same for the aluminium foil used to weigh and load the pollen - Table 4- 1. Some pollen was lost in the loading tube – 16.5% (*SD* = 8.5) which was visible by eye due to a yellow coating on the walls of the loading tube.

Table 4- 1: Characteristics of first pollen experiment settings – determination of pollen loss to the loading tube. Three pollen sedimentation replicates were performed and the pollen loss to the tube was determined by the weighing of the loading tube and aluminium foil, before and after the pollen loading inside the chamber.

Exposure	Birch pollen weight (mg)	Aluminium foil, before pollen load (mg)	Loading tube, before exposure (mg)	Loading tube + aluminium foil, after exposure (mg)	Final pollen load (mg)	Pollen loss in tube (%)
Replicate 1	2.03	66.91	13335.01	13402.21	1.74	14.29
Replicate 2	2.12	43.56	13644.99	13689.10	1.57	25.94
Replicate 3	2.15	53.29	13495.23	13548.72	1.95	9.30
Mean (*SD*)	2.1 (*SD* = 0.06)	-	-	-	1.75 (*SD* = 0.19)	16.51 (*SD* = 8.54)

In order to assess the amount of deposition of the pollen, pollen deposited on the cover slips and microscope slides needed to be counted. For the first experiments, pollen was counted as follows: different areas of the slides and cover slips were inspected with a light microscope, with 100X magnification, and the pollen seen in the area was counted, as seen in Figure 10.

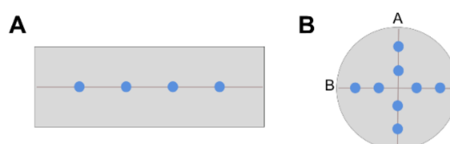


Figure 10: Pollen counting procedure for the first experiments. (A) method for slides and (B) method for cover slips. A and B are used to distinguish the two lines counted. Areas coloured in blue were used to count the pollen.

The area counted corresponds to the grid used in the eyepiece – 0.01cm^2 – when using 100X magnification. Figure 11 shows the distribution of the pollen at the bottom of the chamber (area of deposition plate: 90.76 cm^2). No significant differences were seen in the amount of pollen deposited (p = 0.155), per cm^2, in between the different methods used to count the pollen. The experiment showed an even distribution of the pollen in the prototype chamber, however, with a high standard deviation between locations (i.e., not reproducible).

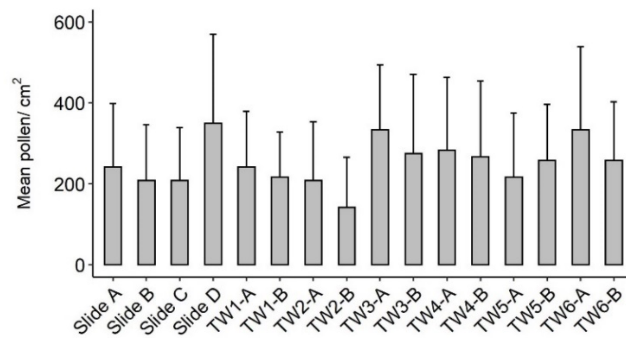


Figure 11: Deposition of pollen in the chamber, on the first test experiment. "Slide-A" to "Slide-D" correspond to the control slides used to count the pollen outside of the cell culture plate. "TW1-A" to "TW6-B" correspond to the different cover slips that replace the six cell culture inserts and, for each cover glass, several areas of two perpendicular lines were counted (Line A and Line B). No significant differences were seen between samples (N=3, ANOVA, p-value=0.155, $p < 0.05$).

4.1.3. Creation of the pollen sedimentation chamber

The preliminary experiments showed single pollen deposition and an even distribution in the prototype chamber.

Three aluminium tubes with different heights were built so that they fit inside a cell culture incubator. These tubes are movable and can be attached to each other in a complete closed fit using a built-in attachment to a laboratory stand. Each stage has a different function according to the pollen sedimentation process. The first stage is used to load the pollen inside the chamber and let the pollen dispersed into the chamber (Dispersion stage). The long stage lets pollen sediment to the bottom of the chamber (Sedimentation stage) while the last stage had the purpose to open and access the cells after loading the pollen on the cells (Loading stage) - Figure 12. In total, the Pollen Sedimentation Chamber had a height of 45 cm and 19 cm in diameter. The three stages were built to be attached to a laboratory stand to make moving of the chamber easier and to be able to reach the cells in the loading stage.

In the conceptualization of the chamber, a movable plate was created to stabilize the turbulence of the pollen before sedimentation. The aluminium plate used had about 1 mm thickness. A temporary mesh on the top of the chamber was used for the first experiments, made of aluminium, with a large diameter and blocked on the top with a thick layer of cotton tissues.

Regarding the loading stage, a mesh similar to the one used on the top of the chamber was used to block the loading port. This allowed the pollen to enter the chamber but blocks the aluminium foil used to load the pollen, so that it does not sediment on the cells.

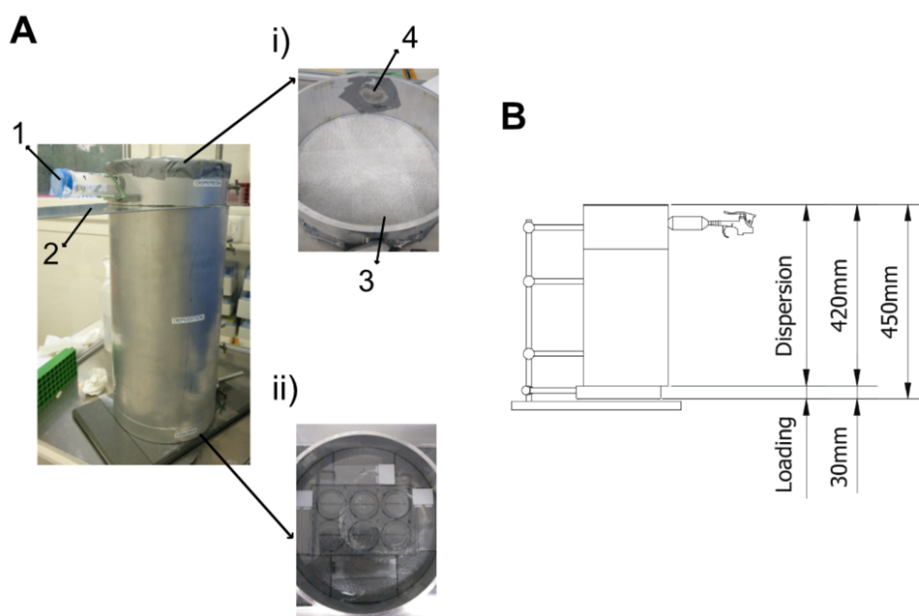


Figure 12: Pollen Sedimentation Chamber - first settings. (A) Pollen Sedimentation Chamber with (1) loading tube and (2) dispersion plate. (i) detail of the dispersion chamber with (3) temporary mesh and (4) mesh at the entrance port. (ii) pollen deposition plate with control cover slips and slides covered with Vaseline. (B) Sketch of the Pollen Sedimentation Chamber with the height details of the two main stages.

4.1.3.1 Counting methods

Different counting methods to determine pollen doses in the loading stage were used, but a technique which would be reproducible and not very tedious to achieve was required.

The first method used – count of specific areas – besides being efficient and quick, did not reproduce all areas of the sample. The areas counted were small (0.01 cm^2) and could result in a high standard deviation in the counting procedure. A second counting method was used – count different continuous lines – which gave a more realistic pollen count but, besides being very tedious, the counting method would have to be normalized to the area counted in each cover slip and microscope slide.

For that, two settings on the counting procedure were changed: all the samples would be collected using 24 mm cover slips, to easier normalize pollen counting, and a new counting procedure was implemented. Only two perpendicular lines at the middle of the cover slip covering the entire diameter were used for counting procedures (Figure 13). This was supported with images taken of the full cover slip and analysed with the ImageJ Software, which showed a uniform distribution of the pollen in all samples (data not shown).

Another modification was made to the counting method. Besides the six cover slips that mimic the cell culture inserts and the four cover slips outside the cell culture area, two small 12 mm round cover slips were placed in the middle of the 6-well plate outside the cell culture wells. These smaller cover slips were strategically placed to be used in further in vitro exposures inside the cell culture plate, but outside the wells to check pollen doses inside the plate (Figure 13).

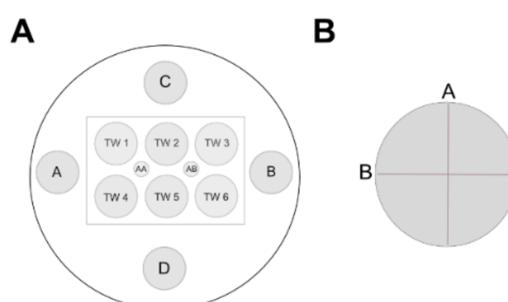


Figure 13: Counting procedure method. (A) position of each cover slip on the deposition plate. A-D are control cover slips; TW1-6 are the cover slips that mimic the cell culture insert, while AA and AB are control cover slips that can be placed inside the culture plate to control pollen doses inside the plate. (B) Counting method on each cover slips. Two perpendicular lines, A and B in brown, are counted.

4.1.3.2 Pollen deposition test with the PSC

For the first test, the aim was to evaluate the effect of the movable dispersion plate. Two preliminary experiments were performed using 2 mg of birch pollen with or without the dispersion plate. The dispersion plate of the PSC was opened 10 seconds after pollen was loaded inside the chamber. For both exposures, with and without the dispersion plate, the pollen deposited on the cover slips was counted (Figure 14).

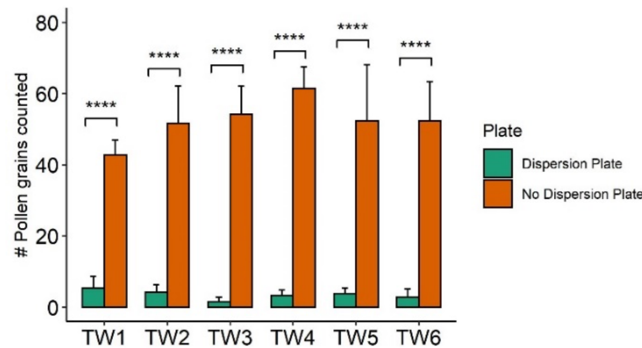


Figure 14: Pollen counts on different cover slips, in an exposure with or without the dispersion plate. Data are shown as mean and *SD*. (N=3). $p < 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \leq 0.0001$****, by ANOVA and Tukey´s Post Hoc test for multiple comparisons.

The dispersion plate significantly reduces the amount of pollen on the loading stage ($p \leq 0.0001$). Without the dispersion plate, about eight times more pollen was sedimented on the bottom of the chamber than with the plate. After the pollen experiment was possible to observe by eye a fine uniform layer of pollen covering all the area of the deposition plate that was inside the PSC (Figure 42). This can be explained by the fast sedimentation rate of the pollen. After loading the pollen inside the chamber, pollen is quickly dispersed and sediments a few seconds after. The dispersion stage, where the dispersion plate was placed, was 6 cm in height, which means that after 6 seconds all the birch pollen had sedimented on the dispersion plate (sedimentation rate of 1.5 cm/s - (Pohl, 1937)).

The goal of using movable plates in the Pollen Sedimentation Chamber – to let pollen disperse evenly before deposition or to block some wells to receive pollen, to be used as controls – would be difficult to implement. Almost no clusters of pollen were seen in the sample analysis of the microscope. The use of a second movable plate, between the deposition and loading stage, to have cells exposed to different times or different doses of pollen on the same experiment seemed now also difficult to accomplish. The use of movable plates was then abolished for further experiments. One of the movable plates was then used at the bottom of the chamber, instead of the glass plate. The plate was made of aluminium, helping to reduce electrostatic effect; is less dangerous than a glass plate and weighs less.

4.1.3.3 Influence of temperature, humidity, and culture plate on pollen sedimentation

One important factor that needed to be considered were the conditions planned for the future in vitro exposures. Up until now, all the pollen sedimentation experiments were performed at normal room temperature and humidity conditions. Exposures with in vitro cell culture should happen inside a cell culture incubator to avoid extra stress for the cells. It is known that pollen and the release of allergens depends on the temperature and humidity of the surrounding (Beck et al., 2013; Hughes et al., 2020). Thus, it was necessary to assess if pollen sedimentation changes in a normal cell culture condition environment.

Besides that, the cell culture plate is made out of polystyrene, which can disturb pollen deposition due to electrostatic effect. To study both environmental conditions and cell culture plate material effect, different experiments were performed: pollen was loaded inside the cell culture incubator (at 37 °C, 85% RH) or at normal room temperature and humidity conditions (19 °C and 70% RH), with or without a 6-well cell culture plate. For this experiment, no cell culture was used, but cover slips covered with Vaseline were placed inside each well, mimicking the cell culture insert. The same doses and pollen were used for all exposures: 2.05 mg (*SD* = 0.15) birch pollen.

Looking first at the material effect of the cell culture plate, we observed a reduction on the sedimentation of pollen either inside an incubator or at environmental (room) conditions, see Figure 15. However, the standard deviation between different replicates is lower compared to the experiments without a cell culture plate. Thus, using the cell culture plate increases the reproducibility but reduces the number of sedimented pollen. In addition, no significant differences were seen between different positions of the sedimentation plate, which reflects the uniform distribution of the pollen, as was already observed before. Interestingly, the material effect, which we thought to disturb the deposition of the pollen, had actually the opposite effect. A more reproducible exposure was observed with no effect on the distribution of the pollen at the bottom of the chamber.

Regarding the effect of the environment conditions for the pollen sedimentation, pollen sedimentation happening in cell culture conditions (37 °C and 85% RH) was higher than under normal room environment conditions (Figure 15). This difference was significant between the experiments in which a cell culture plate was present: we obtain 253 (SD = 24) pollen/cm^2 inside the incubator, compared to 131 (SD = 85) pollen/cm^2 ($p \leq 0.01$), obtained when experiment happened in normal room environment conditions (Figure 15C).

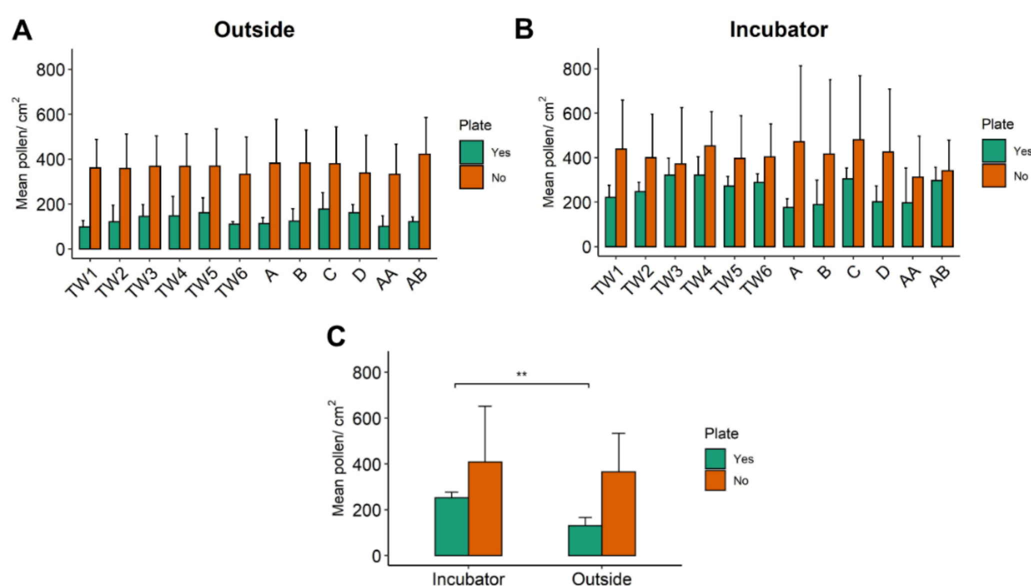


Figure 15: Effect of environmental conditions and polystyrene plate on pollen deposition in the PSC. (A) Effect of room environment conditions and (B) effect of cell culture incubator conditions for the different positions (cover slips) counted on the deposition plate. (C) Mean of the pollen deposition in all control cover slips, for room and incubator environment conditions. All experiments were done with (green) or without (orange) cell culture plate. Data are shown as mean and SD (N=3). $p < 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \leq 0.0001$****, Student's t-test.

4.1.3.4 Influence of the electrostatic effect

As the material of the culture plate influenced pollen doses, we evaluated if the appliance of high voltage (HV) at the bottom of the deposition plate would decrease the electrostatic effect from the culture plate and increase deposition efficiency.

A round aluminium plate, with the same diameter of the PSC, was built and adapted to receive an electric field. A direct voltage of 1000 V (adjustable to minus and plus polarity) was applied on the bottom plate. Birch pollen was dosed in the chamber – 2.06 mg (SD = 0.13) – and experiments were compared

to another one with or without cell culture plate. No significant differences were observed between the different exposures ($p < 0.05$), but a tendency for the HV positive (+1000 V) to help deposition efficiency was observed, comparing to the same experiment, without an electric field – “Plate” here named ($p = 0.78$) (Figure 16). However, comparing both experiments, the standard deviation of the experiment is higher when using high voltage, so it was decided to not use high voltage in further experiments.

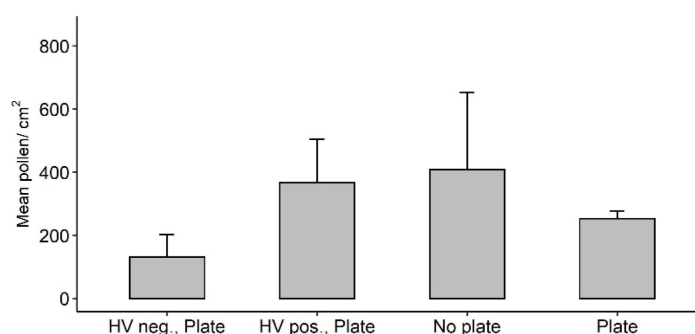


Figure 16: High voltage influence on pollen deposition in PSC. Two different voltages were applied to study pollen deposition, with polystyrene culture plate: “HV neg., Plate”: -1000 V applied and “HV pos., Plate”: +1000 V applied. “No plate”: pollen deposition without cell culture plate; “With plate”: pollen deposition with cell culture plate. All experiments were performed inside a cell culture incubator. Data are shown as mean and *SD*. (N=3). $p < 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \leq 0.0001$****, ANOVA with Tukey’s Post Hoc for multiple comparisons.

The same experiment was performed, but instead of using high voltage, an anti-static gun was applied directly on the pollen, before loading them in the chamber. Tests were not conclusive, and the technique was not further used, as the application of an anti-static gun could depend on the time and place where it was applied. Too many factors, not easy to control, could change the result and hamper experiment reproducibility.

We also observed that after loading the pollen inside the chamber some pollen remained in the aluminium foil. An extra cleaning of the aluminium foil with acetone was evaluated, as it could decrease (less adhesion to grease) the pollen loss in this step. Experiments were performed with aluminium foils washed with 100% acetone. Indeed, cleaning the foil with acetone decreased significantly the pollen loss at this step of the procedure (Figure 17), ($p \leq 0.01$). From this time on, aluminium foil pre-washed with acetone was always used.

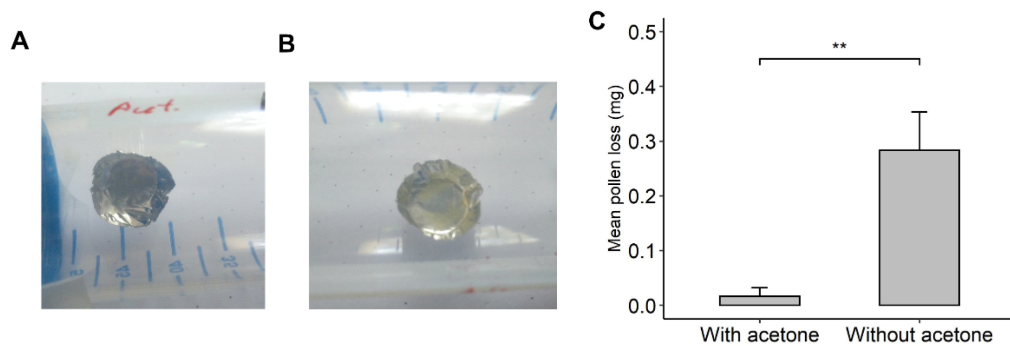


Figure 17: Effect of pre-washing of the aluminium foil with acetone for pollen loss on the loading tube. (A) Example of aluminium foil cleaned with 100% acetone and (B) not cleaned with acetone. Both pictures were taken after pollen were off . (C) Pollen loss, in mg, in the aluminium foil, after loading the pollen inside the PSC, with and without the acetone pre-washing. Data are shown as mean and *SD*. (N=3). $p < 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \leq 0.0001$****, Student's t-test.

4.1.3.5 Test of different birch pollen doses

In order to know if higher doses would give a more reproducible exposure, 1.98 mg (*SD* = 1.16) and 3.97 mg (*SD* = 0.13) birch pollen was dosed in the PSC. At this time, the temporary mesh on the top of the chamber was replaced with a 15 µm mesh and a small loading tube holding station was added to avoid instability at the loading tube - Figure 18A. When comparing the two different doses of pollen tested, the amount of pollen deposited on the bottom of the PSC is proportional to the pollen dosed (Figure 18B). The double amount of pollen dosed is not directly translated into a double amount of pollen sedimented, but the correlation is sufficient (Pearson correlation = 0.868).

One important aspect that needs to be quantified is the pollen loss inside the system. That means, the amount of pollen that does not reach the bottom of the chamber and is lost during the sedimentation process. No significant differences were observed for the different doses tested ($p > 0.05$) however, more than 70% of the pollen loaded in the system does not reach the bottom of the chamber (Figure 18C).

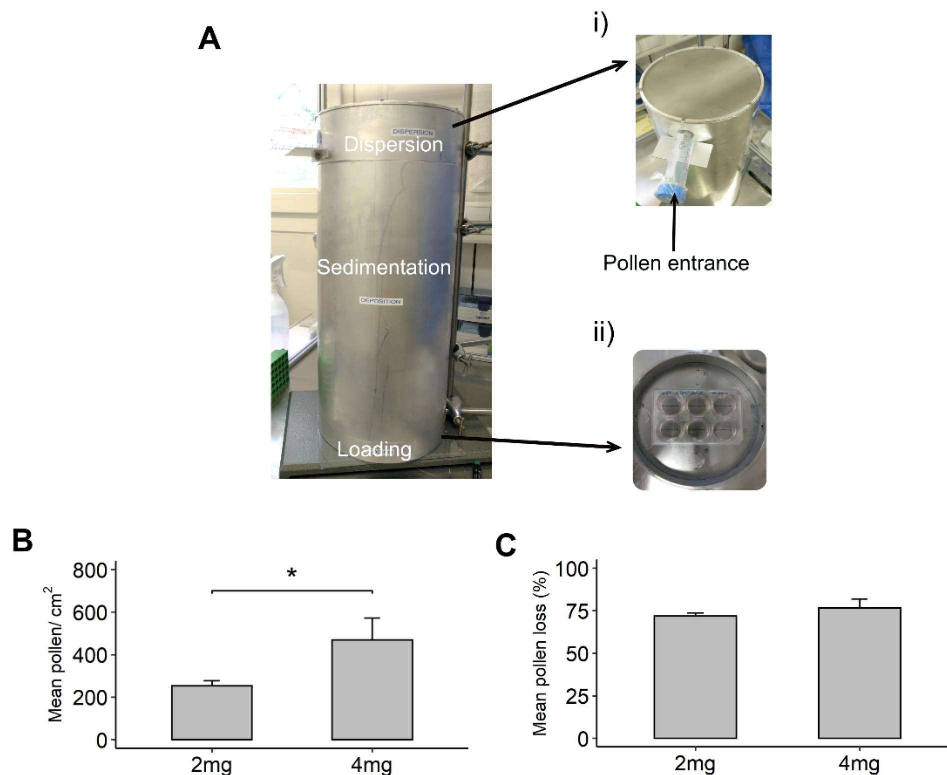


Figure 18: Pollen Sedimentation Chamber – technical improvements and pollen doses tested. (A) Pollen Sedimentation Chamber with three different stages (Dispersion, Sedimentation, and Loading); (i) detail of the dispersion stage with a 15 μm mesh and loading tube, (ii) detail of the loading stage with cell culture plate and control cover slips. (B) Different pollen doses in the PSC (2 mg and 4 mg birch pollen) and (C) pollen loss in the chamber, for the doses tested. Data are shown as mean and *SD*. (N=3). $p < 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \leq 0.0001$****, Student's t-test.

4.1.4. Improvements of the pollen sedimentation chamber

In order to minimize pollen loss and increase reproducibility of the pollen sedimentation, three important improvements were implemented in the PSC, described below.

4.1.4.1 Surface coating

One solution to increase exposure reproducibility would be to reduce the stickiness of the walls by coating the surface of the actual PSC. Several coating surfaces were tested. For that, an assay was established to check the adhesion of pollen to a surface: the "slide-friction-test". Different coating samples were placed in a 45° angle, about 2 mg of birch pollen were placed with a pointed

brush and, without any disturbance, the pollen was allowed to slide on the surface, as exemplified in Figure 19. The sample that lets pollen slide the furthest would be the coating surface that shows the least friction to the pollen. The surface with the least adhesion was a special PTFE-based formula, usually known as Teflon® (see Materials for details). The Polytetrafluoroethylene (PTFE) is a synthetic fluoropolymer with one of the lowest coefficients of friction (0.05-0.10), being also hydrophobic (Makison & Tabor, 1964).

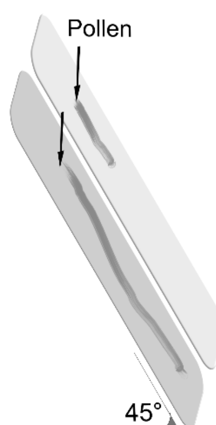


Figure 19: Surface coating test – "Slide-friction-test". An example of the test system to evaluate the effect of different coating surfaces on pollen stickiness. A specific amount of pollen is placed on different surfaces and let slide by itself. The surface that lets pollen slide the furthest is the one with the least friction to the pollen.

In order to test the efficacy of the coating surface on pollen sedimentation, one chamber was built and coated with a special Teflon® formula and pollen sedimentation was compared between a coated and non-coated chamber. A comparison was made before to evaluate the friction level of the pollen on the coated chamber. The "Slide-friction-test" was performed for both chambers as explained above. As can be seen in Figure 20A, the PSC coated with a special PTFE-based formula showed less friction to the pollen than the non-coated chamber. This test proved that using the coated PSC might reduce the amount of pollen that is stuck on the surface of the chamber. To confirm this hypothesis, pollen sedimentation exposures using 2.10 mg (*SD* = 0.19) birch pollen were performed in both chambers. Pollen was injected with 1 bar. Pollen loss was lower when using the coated chamber and, consequently,

pollen doses in the loading stage increased by 13% (p = 0.58) with the coated PSC. However, the differences were not significant between both chambers.

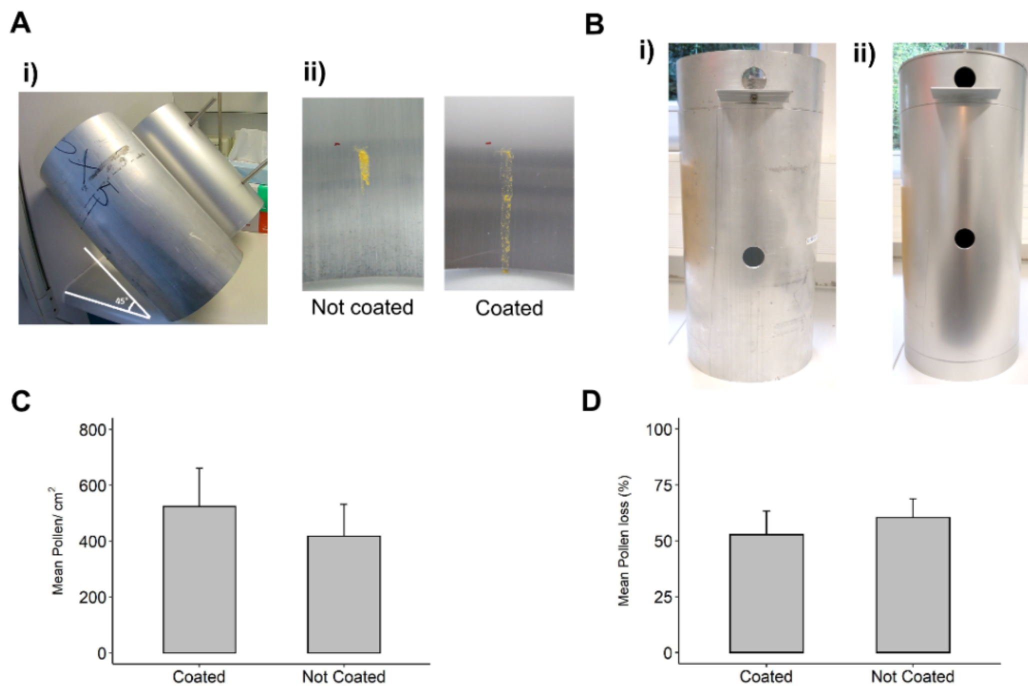


Figure 20: Chamber coating effect in pollen sedimentation. (A) "Slide-friction-test"; i) Non-coated PSC was placed together with the chamber coated with a special PTFE-based formula on a 45° angle to perform the sliding test, with birch pollen; ii) Result of the sliding test. (B) Different Pollen Sedimentation Chambers, where i) shows a PSC without surface coating and ii) with coating. (C) Pollen deposition with a coated or not coated PSC. (C) Pollen loss in a coated and not coated PSC. Data are shown as mean and *SD*. (N=3). $p < 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \leq 0.0001$****, Student's t-test.

Figure 20 shows that both prototype chambers have a second loading port in the middle of the sedimentation stage. This was created to check if different loading heights influence the deposition of pollen. No significant differences were observed between the two heights and, for the lower loading port, more clusters of pollen were seen at the loading stage samples (data not shown). Therefore, the highest loading port was used for all experiments and the second port was sealed using Teflon® tape.

4.1.4.2 Loading Tube

An essential step of the PSC is the loading tube used to disperse the pollen inside the chamber. As described before, pollen adheres easily to any

surface. Different loading tubes were investigated to choose the one that reduces the pollen loss most. Figure 21A shows the different tubes tested: two different versions of Falcon® tubes, mentioned above already; a copper tube together with a lid of a 50 ml Falcon® tube; a glass tube specially produced to resemble a 50 ml Falcon®, and the “No-escape tube”. The glass tube was additionally silazaned before with hexamethyldisilazane (HDMS) to increase hydrophobicity and, consequently, reduce pollen loss to the walls. The “No-escape” tube was created by adapting a cassette housing used for air sampling. The polystyrene tube is a 5 mm (on the top) by 50 mm (on the bottom) tube and the pollen is weighed directly on the small port with 5 mm of diameter. We named it the “No-escape tube” as the pollen is directly weighed in the tube, without aluminium foil, and the tube is placed on the loading port so that half of the tube is already inside the chamber.

Experiments with 2.14 mg (*SD* = 0.14) birch pollen were performed. The loss of pollen to the loading decreased significantly when using the “No-escape tube” (Figure 21B). Clusters were seen when the glass tube was used, which did not happen with the other loading tubes. From this time on, the No-escape tube was used as the loading tube for all exposures. The pollen loss to the loading tube was reduced when using the “No-escape tube” however, inside the chamber, a substantial loss remained and the effect of the loading tube on loss of pollen to the walls of the chamber was not significantly different (Figure 21C and D).

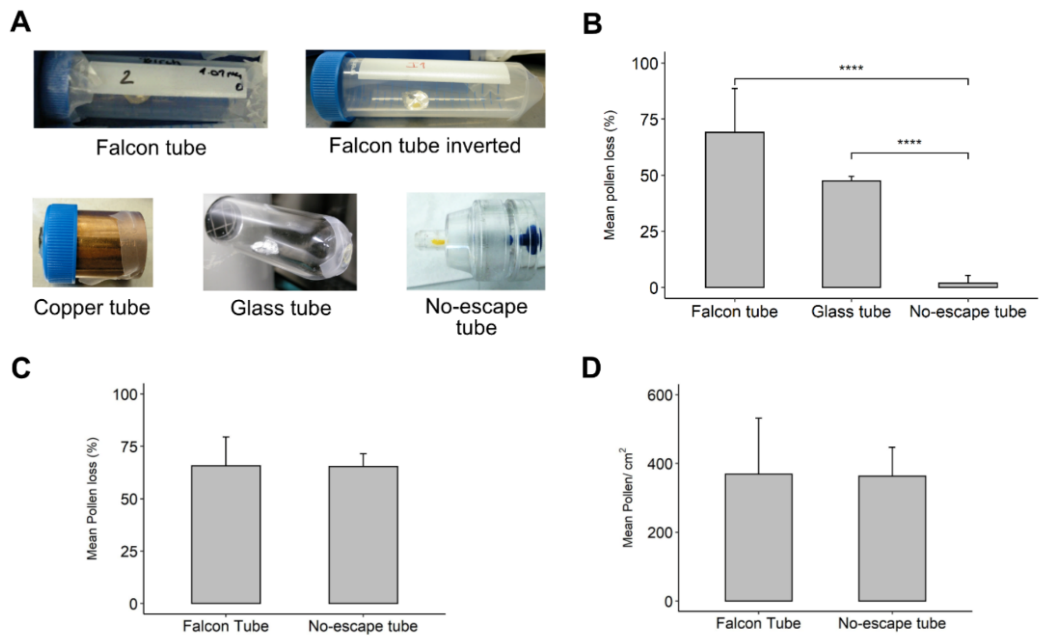


Figure 21: Effect of different loading tubes in pollen loss. (A) Different loading tubes tested. (B) Pollen loss to the loading tube for different loading tubes. (C) Pollen loss and (D) pollen deposition in the chamber when using the Falcon® and the No-escape tube. Data are shown as mean and *SD*. (N=4). $p < 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \leq 0.0001$****, ANOVA with Tukey's Post Hoc for multiple comparisons or Student's t-test.

4.1.4.3 Air pressure

The final setting adjusted was the pressure used to load the pollen inside the PSC. Until now, 1 bar of pressure was used, which seemed to be a good setting as single and intact pollen was obtained at the bottom of the chamber. However, even after coating the chamber and using the No-escape loading tube, a loss of about 65 % (*SD* = 6.10) of the pollen was observed.

One possibility was that the air pressure used was too high and some pollen, after loading, would be propelled against the opposite wall to which they stick. To test if the pressure used was too high, experiments with 2.05 mg (*SD* = 0.11) birch pollen, with different air pressures (0.5 and 1 bar) were performed. Melinex® tapes covered with Vaseline were placed inside the chamber on the walls of the dispersion stage to check pollen adherence at different places of the chamber (Figure 22A). The majority of the pollen was seen at the opposite site of loading and a difference in the amount of pollen was observed when different air pressure was applied. Figure 22B shows an area of the Melinex® tape from the opposite site of loading, observed in the microscope, for the two

air pressures applied. The lower air pressure shows less pollen adherence to the walls and this was confirmed when pollen deposition and loss was determined. Pollen loss decreases significantly by around 18.4% (*SD* = 6.1) (p=0.047), see Figure 22C. Consequently, lower pressure led to a significant increase of deposited pollen to about 62.7% (*SD* = 23.1) of pollen/cm^2 on the inserts (Figure 22D, p<0.05). Besides that, the variability between exposures was reduced. The experiment showed that pollen could be propelled against the opposite wall, increasing pollen loss and resulting in dosage variability.

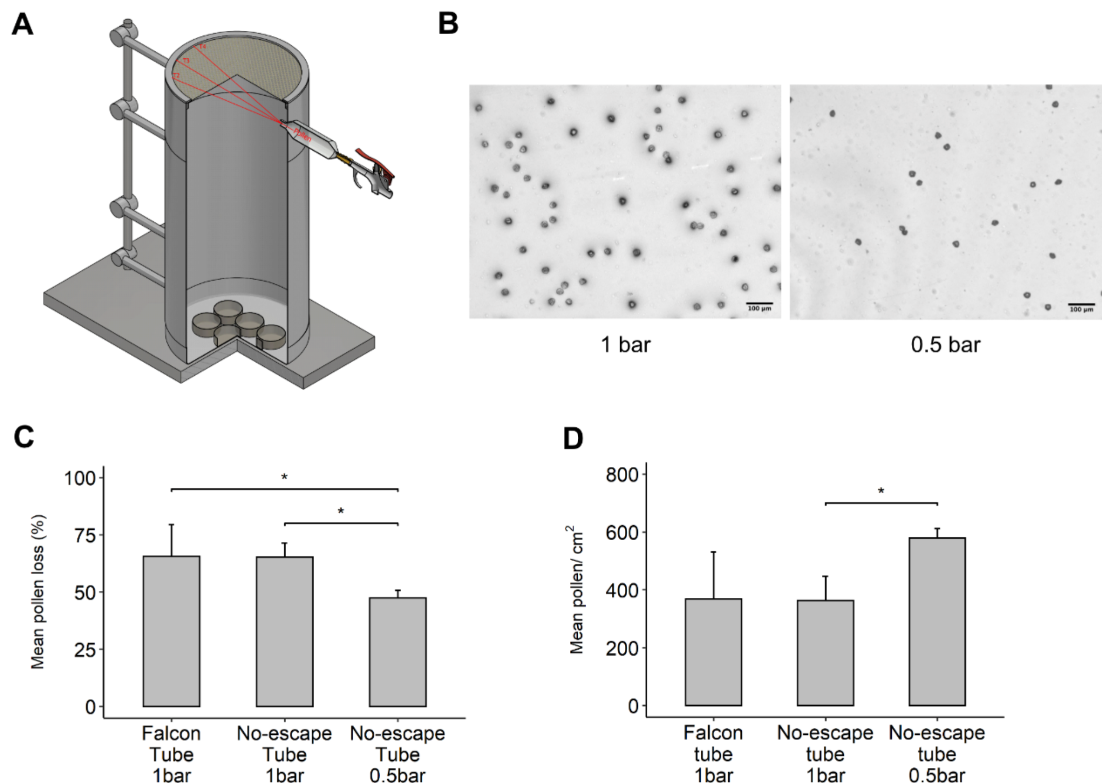


Figure 22: Effect of air pressure on pollen sedimentation at the PSC. (A) Set-up of PSC to test different air pressures. Red arrows show different areas where Melinex® tapes were placed. (B) Microscope pictures of sedimented birch pollen in the opposite position of loading, for the different air pressures tested. (C) Pollen loss and (D) deposition in the chamber, for different air pressures and loading tubes used. Data are shown as mean and *SD*. (N=3). p < 0.05*, p ≤ 0.01**, p ≤ 0.001***, p ≤ 0.0001****, ANOVA with Tukey's Post Hoc for multiple comparisons.

The final setting for cell exposures using the Pollen Sedimentation Chamber was thus obtained: a pollen chamber covered with special PTFE-based formula, fitting inside a cell culture incubator, was incorporated with a

loading tube where pollen can be directly applied and loaded inside the chamber, using 0.5 bar air pressure (Figure 23).

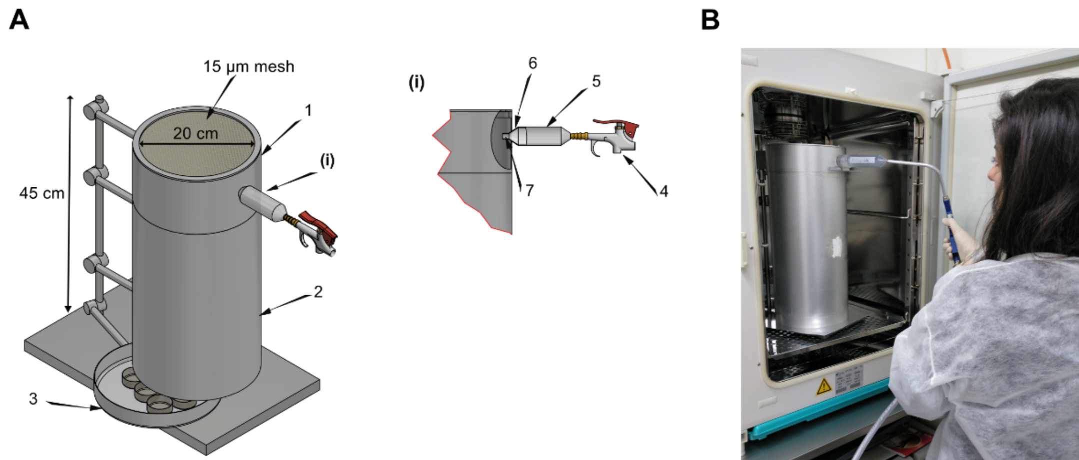


Figure 23: Pollen sedimentation chamber – final settings. (A) Pollen Sedimentation Chamber final sketch: dispersion (1), sedimentation (2), and cell loading chamber (3). i) Detail with a pressured air gun (4), pressure shock absorber (5), "No-escape" loading tube (6) and location of the "to be dosed" pollen (7). (B) Moment of pollen loading in PSC.

4.2. Exposure of different birch pollen doses in the PSC and correlation with human real-life doses

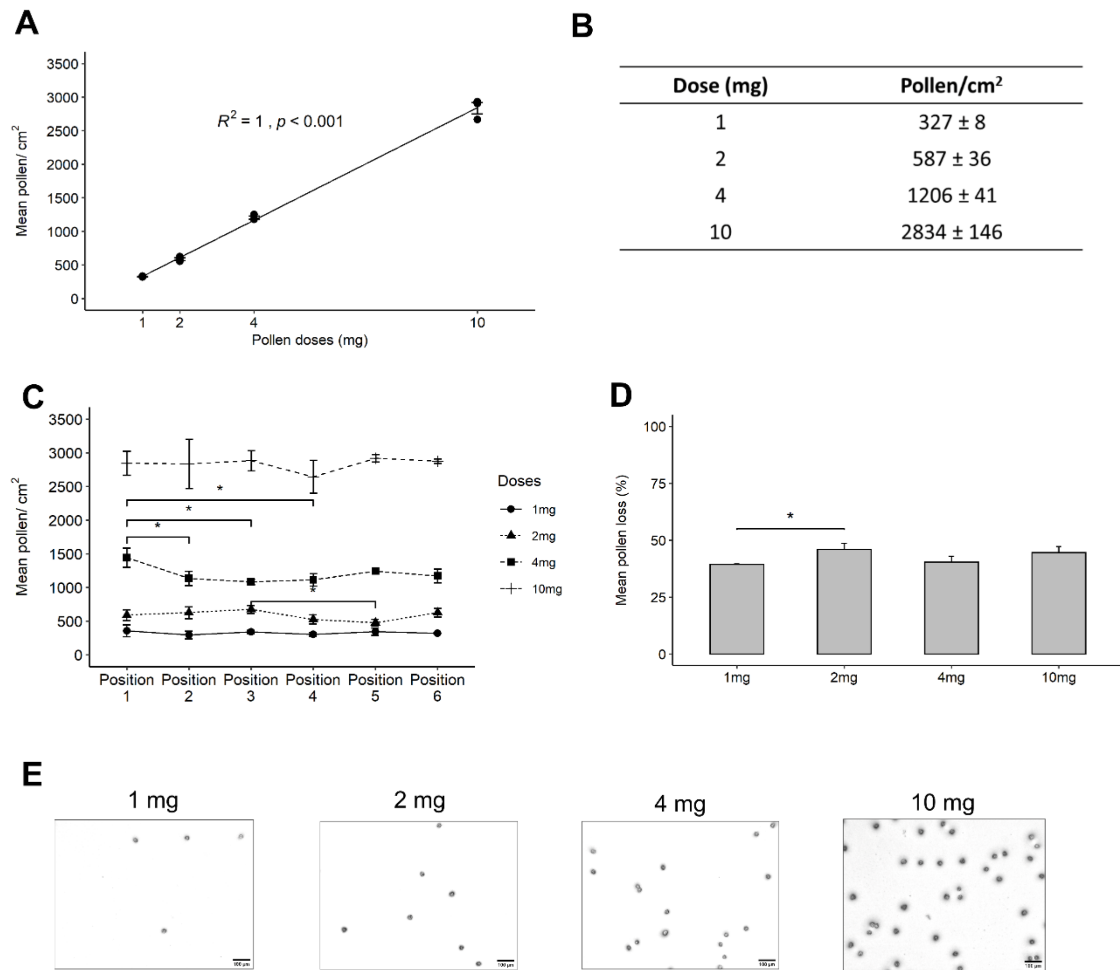
One of the aims of the project was to create a pollen exposure as close to real-life as possible. For that, experiments with different birch pollen doses were performed in order to find how low the chamber doses could be, and whether they correlated with human birch pollen exposure.

Figure 24A shows the distribution of birch pollen on the bottom of the chamber for different doses of loaded pollen: 1, 2, 4 and 10 mg. A dose-dependent curve with a high correlation was observed ($r = 0.99$, $p > 0.001$, Pearson correlation). Looking at the six positions of the cell culture inserts no major statistically significant differences were seen between the positions or between the different doses (Figure 24C).

The pollen loss to the chamber was reduced to the minimum, but still substantial, with low variability in between exposures: 42.6% ($SD = 3.5$) (Figure 24D). The pollen density for each of the doses is visualized in Figure 24E, which correlates with the doses obtained.

The doses used in our chamber correlate with a real-life human exposure to birch pollen (see Methods). From a high birch pollen season day in Central

Europe (1 mg) to peak days in areas where the concentration of birch pollen is the highest (4 to 10 mg), see Figure 24B.



Dose (mg)	Pollen/cm²
1	327 ± 8
2	587 ± 36
4	1206 ± 41
10	2834 ± 146

Figure 24: Birch pollen doses in the Pollen Sedimentation Chamber. (A) "Dose-curve" of the PSC, for different doses of whole birch pollen, and (B) table corresponding to the data shown. (C) Distribution of pollen sedimentation in different positions of a cell culture plate, for different doses of birch pollen. (D) Pollen loss to the PSC, for the different doses. (E) Microscope pictures of the surface distribution of pollen, for the different doses. Data are shown as mean and *SD*. (N=3). p < 0.05*, p ≤ 0.01**, p ≤ 0.001***, p ≤ 0.0001****, ANOVA with Tukey´s Post Hoc for multiple comparisons.

4.3. Test of different pollen in the PSC: whole grass pollen experiments

To evaluate if the PSC created could be used for other pollen types, grass pollen was used and different doses were loaded in the chamber: 2 and 4 mg. When about 4 mg pollen was loaded, 2.2 times more pollen was sedimented on the bottom of the chamber, compared with the 2 mg pollen

loaded (Table 4- 2). The same was observed in the birch pollen experiments. Thus, pollen deposition in the PSC seems to behave similarly for different pollen types. Grass pollen grains are twice the size as birch pollen and consequently the number of pollen per milligram is less with grass pollen. Thus, the pollen/cm^2 is lower with the grass than with birch pollen.

Table 4- 2: Deposition of grass pollen in the Pollen Sedimentation Chamber. Different doses of grass pollen were loaded in the PSC, as mean and *SD*. are given. N ≥ 4 for all time points.

Doses of grass pollen (mg)	Pollen deposition (pollen/cm^2)	Pollen loss (%)
1.98 (*SD* = 0.03)	194 (*SD* = 13)	56.13 (*SD* = 11.23)
4.00 (*SD* = 0.05)	423 (*SD* = 23)	58.04 (*SD* = 2.51)

4.4. First in vitro exposures in the PSC – use of the A549 cell line

The effect of whole pollen exposure on cells needed to be evaluated. For that, the A549 cell culture line was used for the first experiments.

For the first experiment, the aim was to evaluate how whole birch pollen behaves in cell culture conditions. A549 cells were cultured in submerged conditions, exposed to 2 mg birch pollen and incubated for different times after pollen exposure (10 min, 1, 2, 6 and 24 hours). Cells were microscopically inspected at different times to assess pollen and cell behaviour. No differences were observed either in cells or in pollen (Figure 25). Birch pollen looked intact even after 24 hours in cell culture medium, and we observed that pollen was “swimming” in the medium. In some areas, pollen seemed to form clusters, (not seen later at ALI conditions) - Figure 25C –, and interestingly, it was possible to observe one pollen releasing its contents (including the allergens) – Figure 25D.

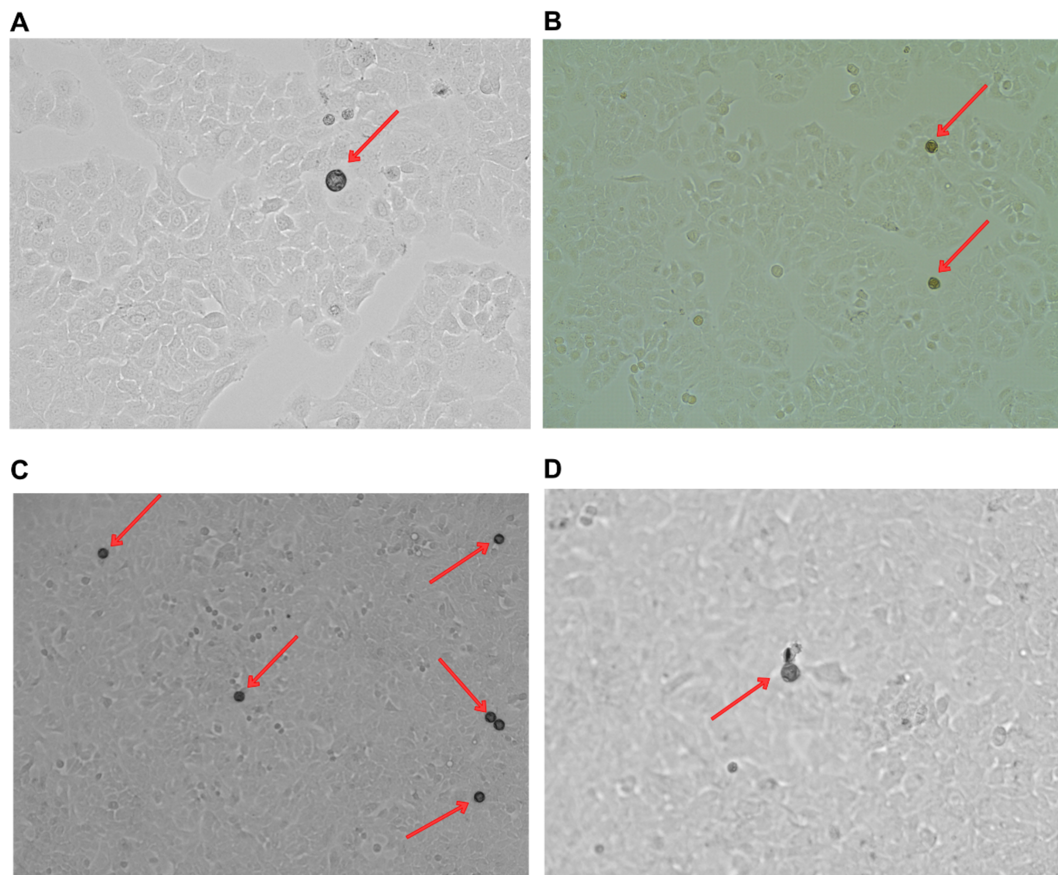


Figure 25: Microscope pictures of submerged A549 cells exposed to birch pollen. (A) 10 min and (B) and (C) 24 hours after pollen exposure. (D) Pollen releasing their contents. Red arrows indicate birch pollen.

Knowing that pollen stays intact after contact with cell culture, ALI-exposures with A549 were performed in order to assess cell toxicity after pollen exposure. Different doses of birch pollen were sedimented on A549 cells, from 2 to 16 mg, with the aim to see if higher doses show toxic levels. Cells were kept for different times (2, 4, 6, 8 and 24 hours) to evaluate if different post-exposure times affect cell cytotoxicity. Figure 26 shows the results of the LDH assay. No cytotoxicity was observed, even at the higher doses or higher post-exposure times. On the contrary, cells exposed to higher doses of pollen (8 and 16 mg) show negative cytotoxicity levels, which could mean that cells have higher reproduction rates, compared with the cells not exposed to pollen ("neg. Control"). The other theory is the possible interference of the pollen with the measurement.

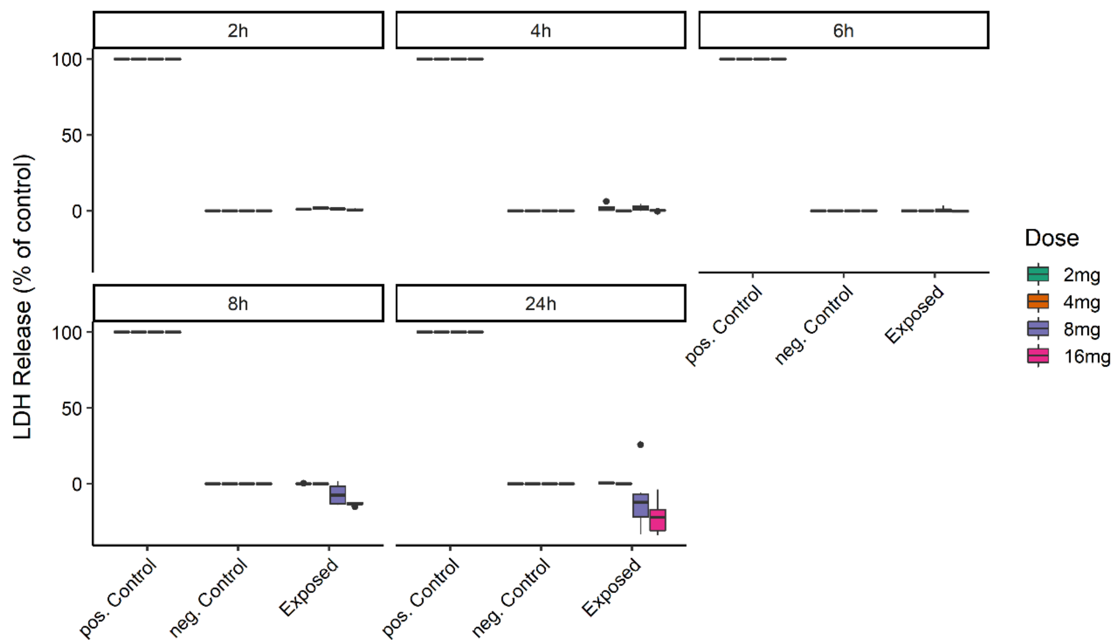


Figure 26: Cytotoxicity levels of A549 cells exposed to different doses of birch pollen, at ALI. Negative control are in the experiment cells kept at all times in a cell culture incubator, not being exposed to pollen (N = 6, technical replicates).

4.5. Whole pollen exposure of BEAS-2B cells

After being able to dose low whole pollen doses with good reproducibility and preliminary tests with submerged cells not showing toxic effect, the PSC could then be used to study the effect of pollen on human bronchial epithelial cells, at ALI. Two different birch pollen doses were evaluated in order to correlate with the real-life doses.

4.5.1. Pollen Counts

The human bronchial epithelial cell line BEAS-2B was exposed to two different doses of birch pollen (4 and 10 mg) and different post-exposure times: 10 min, 55 min, 2 h 25 min, 6 h 50 min and 24 hours. The doses of pollen were quantitated for each post-exposure time and doses – see Table 4- 3. For the lower dose (4 mg), 1247 pollen grains/cm^2 (*SD* = 232) had sedimented on the cells, while for the 10 mg dose 2830 birch pollen grains/cm^2 (*SD* = 158) were sedimented on the BEAS-2B. No significant difference between the different post-exposure times for either 4 mg or 10 mg pollen.

Table 4- 3: Dose of birch pollen to the cells, with higher (10 mg) and lower (4 mg) pollen doses, at the different post-exposure times. No significant differences were observed between the post-exposure times for each dose ($p > 0.05$, independent sample t-test). Mean and *SD* are given. N ≥ 3 for all time points.

Post-exposure time	4 mg Birch pollen (pollen/cm^2)	10 mg Birch pollen (pollen/cm^2)
10 min	1129 (*SD* = 184)	2819 (*SD* = 153)
55 min	1482 (*SD* = 208)	2860 (*SD* = 162)
2 h 25 min	1018 (*SD* = 182)	2703 (*SD* = 227)
6 h 50 min	1385 (*SD* = 215)	2865 (*SD* = 156)
24 h	1218 (*SD* = 81)	2904 (*SD* = 118)

4.5.2. Viability and cytotoxicity of BEAS-2B cells after birch pollen exposure

Before using the cells for further analysis, the viability and cytotoxicity were analysed. As seen in Figure 27, for the different doses and post-exposure times, cells had a viability higher than 88% and the cytotoxicity levels did not reach the toxic level (defined as no more than 15%), besides three samples that were excluded from further analysis. For all exposures, control, negative control and positive control cells were included (see Figure 4).

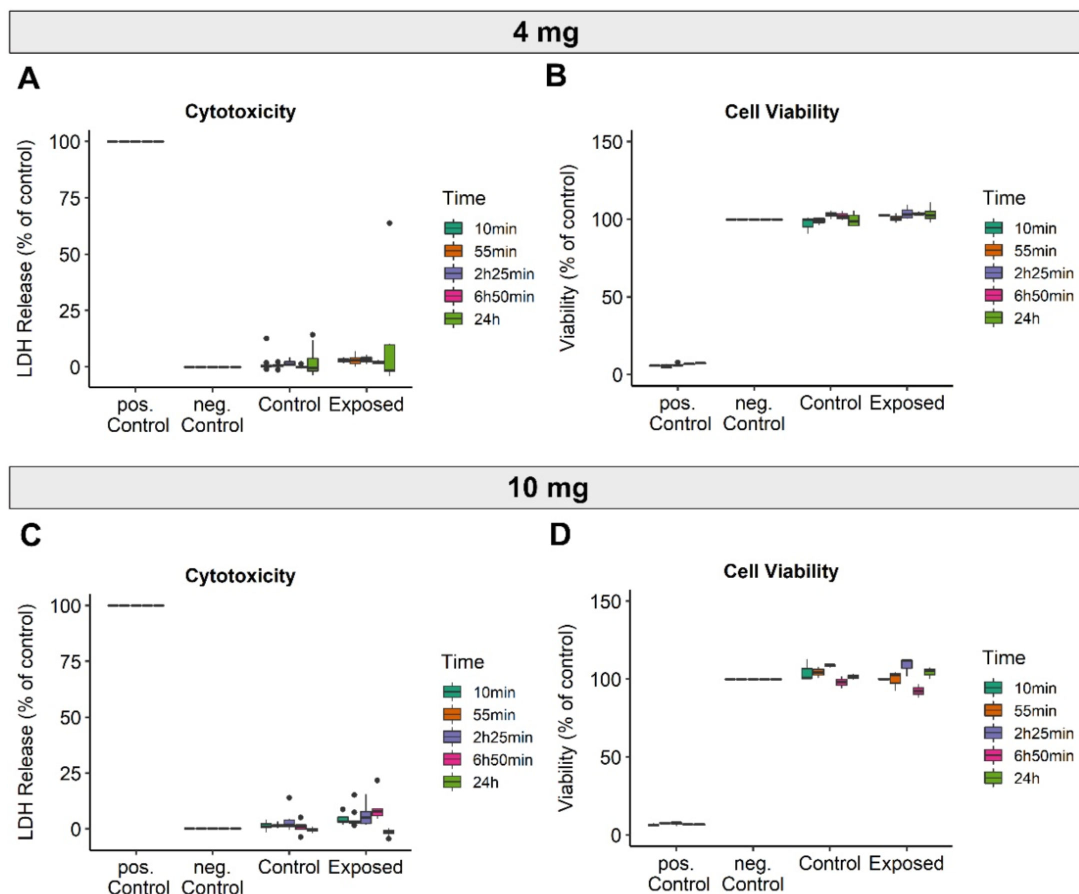


Figure 27: BEAS-2B cell cytotoxicity and viability after exposure to different doses of birch pollen, at ALI. (A) Cytotoxicity and (B) viability of the cells exposed to 4 mg birch pollen, for the different post-exposure times. (C) Cytotoxicity and (D) viability of the cells exposed to 10 mg birch pollen, for the different time points. Cell cytotoxicity and viability were performed with LDH and Alamar Blue® assay, respectively. Positive cells were lysed with 2% Triton-X 100, negative control cells are cells that were at all times in a cell culture incubator and control cells were mock exposed cells.

4.5.3. Manipulation stress

In addition to the high viability and low cytotoxicity of the cells, transcriptome analysis was performed to address possible stress manipulation on the cells.

The stress manipulation of the BEAS-2B was analysed performing a mock exposure in the Pollen-ALI set-up. Cells exposed to only pressurized air (named "Air" cells) were compared against cells that were at all times in a cell culture incubator and were not submitted to any exposure ("Incubator" cells). For this evaluation, transcriptome analysis were performed for only one post-

exposure time (2 h 25 min). Mock exposed cells were compared with incubator cells.

A limited number of genes were significantly expressed: 31 up- and 2 down-regulated. Enrichment terms of the up-regulated genes are related to the biological processes: protein phosphorylation (*DUSP6, DUSP5, SOCS3, IL6, RGCC, SERTAD1, EREG, TRIB1*) and response to stimulus (*DUSP6, DUSP5, MT1B, SOCS3, IL6, CSNK1G2, FOSB, CITED4, SNAI1, RGCC, C8G, ANGPTL4, EREG, TRIB1*) – see Figure 28.

The stress manipulation observed in the mock exposure is considered minimal and intrinsic to any exposure occurring out of normal cell culture conditions. Thus, the 10 minutes exposure manipulation and transport of cells had a minimal influence in cell stress and toxicity, substantiated also by LDH and Alamar Blue assays. Nevertheless, a mock control was subtracted from all experimental results in further exposures.

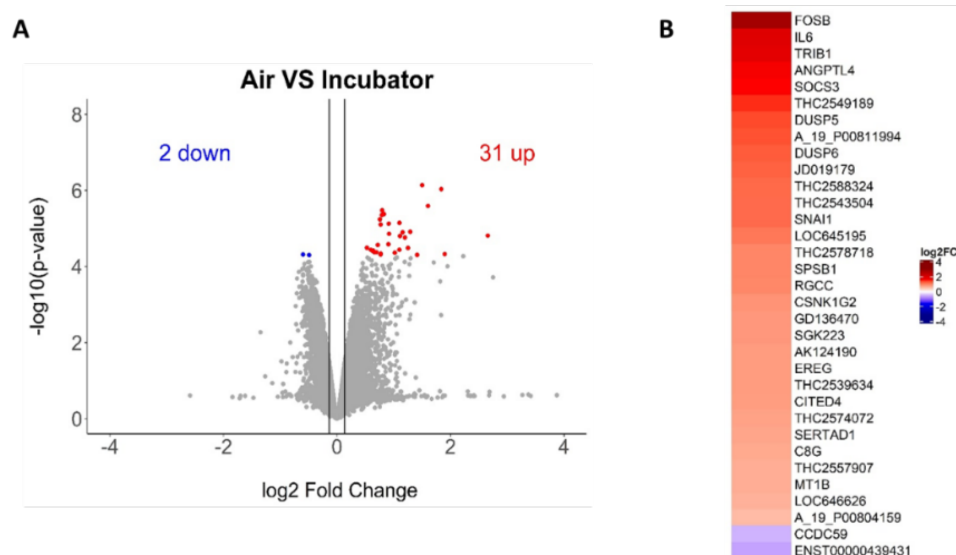


Figure 28: Effect of mock exposure in cellular stress of BEAS-2B cells. (A) Volcano plot and (B) heatmap of the effect of mock experiments (no pollen) on BEAS-2B, analysed by microarrays after 2 h 25 min. Significant regulated genes were marked in colour. N=3, threshold was set to *p* adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1.

4.5.4. Exposure to 4 mg whole birch pollen – Central Europe doses

Transcriptome analyses of BEAS-2B cells exposed to 4 mg birch pollen were performed for the different post-exposure times. A high significant differential regulation of genes at 55 min and 24 h was observed (Figure 29).

More than 1000 genes were up- or down-regulated in both post-exposure times. At 6 h 50 min, 379 genes were differential up-regulated and 547 were down-regulated. For the 10 min and 2 h 25 min after pollen exposure, just a few genes were differential regulated: 4 genes and 9 genes in total, for 55 min and 2 h 25 min, respectively.

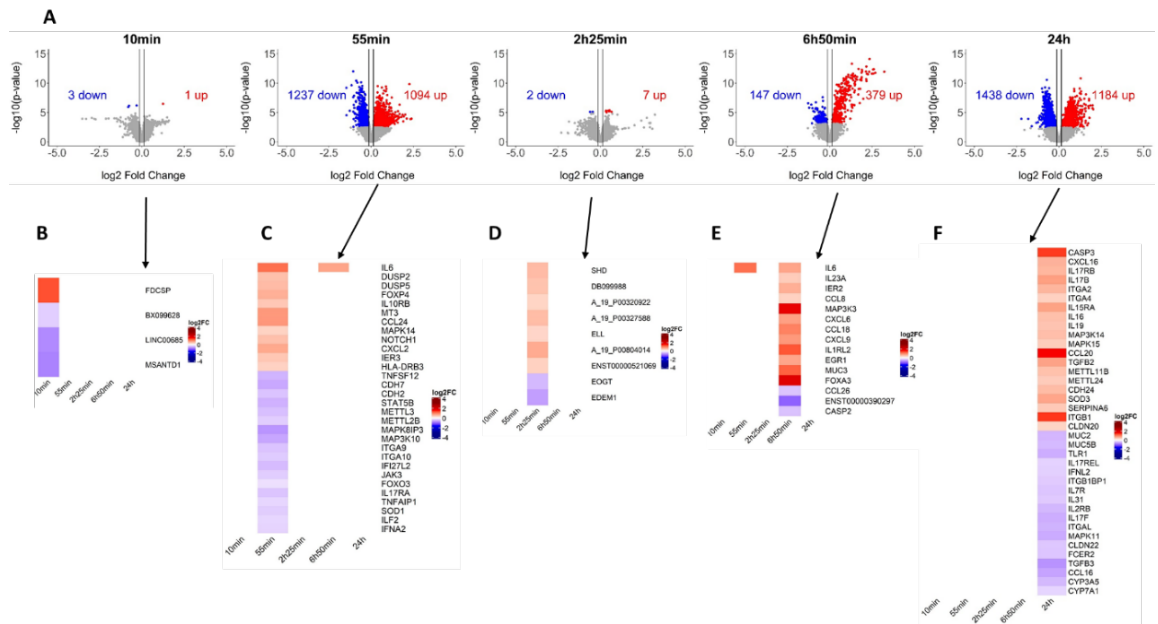


Figure 29: Transcriptome analysis of the effect of 4 mg whole birch pollen on BEAS-2B cells, at the air-liquid interface. (A) Volcano plots and (B, C, D, E, F) heatmaps of significant regulated genes for all the post-exposure times. For (C), (E) and (F), only the most relevant genes for immune and allergic response are shown. Significant regulated genes are marked in colour. N = 3 in all cases; threshold was set to *p* adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1. Data expressed against control cells (not exposed to pollen but to pressurized air).

For the 10 min post-exposure time just one gene was up-regulated: *FDCSP*, a secreted protein known to bind to B cells in antibody responses (Marshall et al., 2002). The down-regulated genes are protein-coding genes with unknown function (Figure 29B).

At 55 min after pollen exposure, 1094 genes we up- and 1237 down-regulated. The enrichment analysis of the up-regulated expression showed gene ontology (GO) terms belonging to cellular response to chemical stimulus and oxidative stress, to cellular component disassembly, IL-4 and IL-13 pathway, TNF signalling and to the Fc-receptor mediated stimulatory signalling pathway. Some relevant genes that are up-regulated are *IL6, DUSP5* and *DUSP2,*

FOXP4, IL10RB, MAPK14, MT3, NOTCH1, CCL24 (eotaxin-2), *CXCL2, IER3* and *IGBP1* (Figure 29C). Regarding the down-regulated genes, the most relevant regulated GO terms were: cilium assembly and oxidative phosphorylation, with the most relevant expressed genes being *STAT5B, TNFSF12, MAPK8IP3, MAPK3K10, CDH2* and CDH7, *JAK3, ITGA9* and *ITGA10*, *FOXO3, IL17RA, TNFAIP1, IFNA2, SOD1* and *ILF2* (Figure 29C).

At 2 h 25 min, a small differential regulation of genes was observed, as for 10 min, with no relevant up- or down-regulated genes (Figure 29D).

At 6 h 50 min after whole pollen exposure, less genes were differentially expressed compared with the 55 min post-exposure time, but more relevant genes, related to immune and allergic response, were expressed. An up-regulation of the following GO terms was observed: leucocyte migration, chemokine signalling pathway, cytokine-cytokine receptor interaction, T cell differentiation/activation, regulation of immune effector processes, regulation of cell adhesion, etc. The up-regulated genes included in those enrichment terms were: *IL6, IL23A, IER2, CCL8, MAP3K3, CXCL6, CCL18, CXCL9, IL1RL2, MUC3* and *FOXA3* (Figure 29E). Regarding the down-regulated genes, the enriched GO terms were related with leucocyte and T cell migration, receptor-mediated endocytosis and regulation of complement activation. The most relevant genes regulated were *CCL26, CASP2 and IGLV1-44* (in the heatmap as ENST00000390297), see Figure 29E.

At 24 hours, the highest gene differential regulation was observed, in which the up-regulated enriched GO terms were: tight junction, cellular response to lipid, intracellular receptor signalling pathway and TGF-beta signalling pathway. The most relevant up-regulated genes were *CASP3, CXCL16, IL17RB, IL17B, ITGA2, ITGA4, IL15RA, IL16, IL19, MAP3K14, MAPK15, CLDN20, CCL20, TGFB2, METTL11B, METTL24, SERPINA11, CDH24, SERPINA6*, and *SOD3*. Regarding the down-regulated expressed genes, most of them were related to integrin and cell-cell junction organization, oxidation by cytochrome P450, cellular protein disassembly and lymphocyte activation, such as *MUC5B, TLR1, IFNL2, IL7R, IL3R, IL2RB, IL17F, ITGAL, CLDN22, FCER2, TGFB3, CCL16, CYP3A5* and *CYP7A1*.

A total of 128 genes were differentially expressed in more than one post-exposure time. Most of the differential regulated genes do not have a relevant function, but some are related to apoptosis and the Wnt signalling pathway (*IL6, RARA, TRAF1, AKT2, AGER*). The *IL6* gene is up-regulated at 55 min and 6h50 min, while the other genes are mostly down-regulated at 55 min and later up-regulated at 24 hours (Figure 29).

Enrichment analysis between the different post-exposure times was performed to understand their correlation. Only time points with high differentially regulated genes were analysed (analysis of the 10 min and 2 h 15 min was not performed).

Between the 55 min and 6 h 50 min post-exposure time only a few correlation of pathways is observed, with the GO Biological processes “immune system development” and “response to cytokine” and also the pathway “TNFA signalling via NFKB” being enriched for both up-regulated lists. The 55 min post-exposure time shows significantly higher enriched terms expression than the 6 h 50 min time (Figure 43).

When comparing the 55 min and 24 h post-exposure times, the same trend was observed. Most of the common pathways are related with cell cycle, pathways such as “Cytokine Signalling in Immune system”, “regulation of MAPK cascade” and “response to cytokine” are up-regulated, while for the 24 hours they are for the down-regulated list significantly expressed (Figure 44).

The enrichment analysis between the 6 h 50 min and 24 hour post-exposure showed some interesting common pathways. Most of the relevant pathways and GO biological processes (related with immune and allergic response) are expressed in the up-regulated list for the 6 h 50 min and for the 24 hours at the down-regulated gene expression list (“positive regulation of interleukin-6 production”, “innate immune response”, “regulation of immune effector process” and “inflammatory response”, for example), see Figure 45. The “TNFA signalling via NFKB” pathway and the “cytokine-cytokine receptor interaction”, are expressed in both up-regulated lists, but the trend is observed for all post-exposure times: only a few pathways are common between the different time-points, for the same dose of pollen.

4.5.4.1 Validation of selected genes by RT-qPCR

Genes related to allergy and immune response were selected from different post-exposure time points and validated by RT-qPCR.

The results obtained confirmed the transcriptome data: a 4-fold expression was obtained for the *IL6* gene, at 55 min and a 1.5-fold expression at 6 h 50 min (Figure 30). The up-regulation of *CXCL2* and down-regulation of IL17RA for 55 min was confirmed, as well as the expression of the genes *BACE1, CCL20, ITGA4* and *TGFB2* for 24 hours after birch pollen exposure. The down-regulation of *MADCAM1* and *MUC5B* was confirmed, with about -2-fold expression (Figure 30).

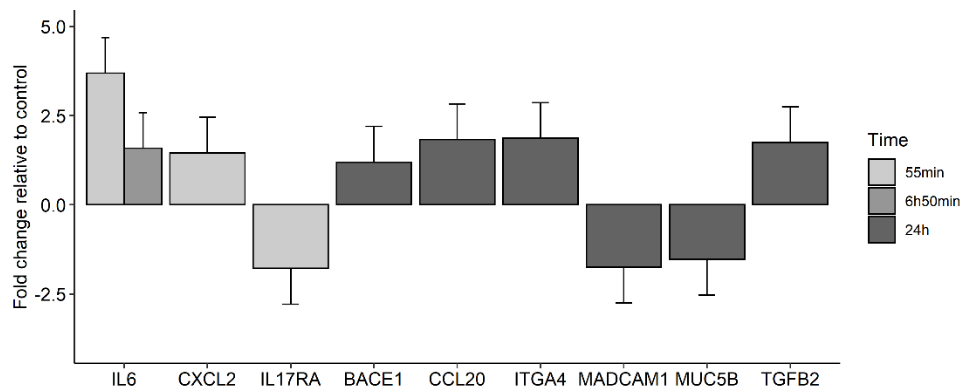


Figure 30: BEAS-2B mRNA expression of selected genes by qRT-PCR that were differentially expressed in transcriptome data, for different post-exposure times, with the 4 mg birch pollen doses. N ≥ 2 for all experiments. Data expressed against control cells (not exposed to pollen but to pressurized air).

4.5.5. Exposure to 10 mg whole birch pollen – "Russian doses"

For the higher doses of birch pollen exposure, a different gene expression was observed, compared to the lower doses. Early post-exposure time points showed a less significant differentially gene expression: nothing at 10 min, 11 up-regulated genes at 55 min, 20 up- and 1 gene down-regulated at 2 h 25 min (**Error! Reference source not found.**A). Only one time-point showed a high expression of genes: at 6 h 50 min, about 600 genes were up- and 76 down-regulated (**Error! Reference source not found.**A). After 24 hours, no

genes were differentially expressed anymore (**Error! Reference source not found.**A).

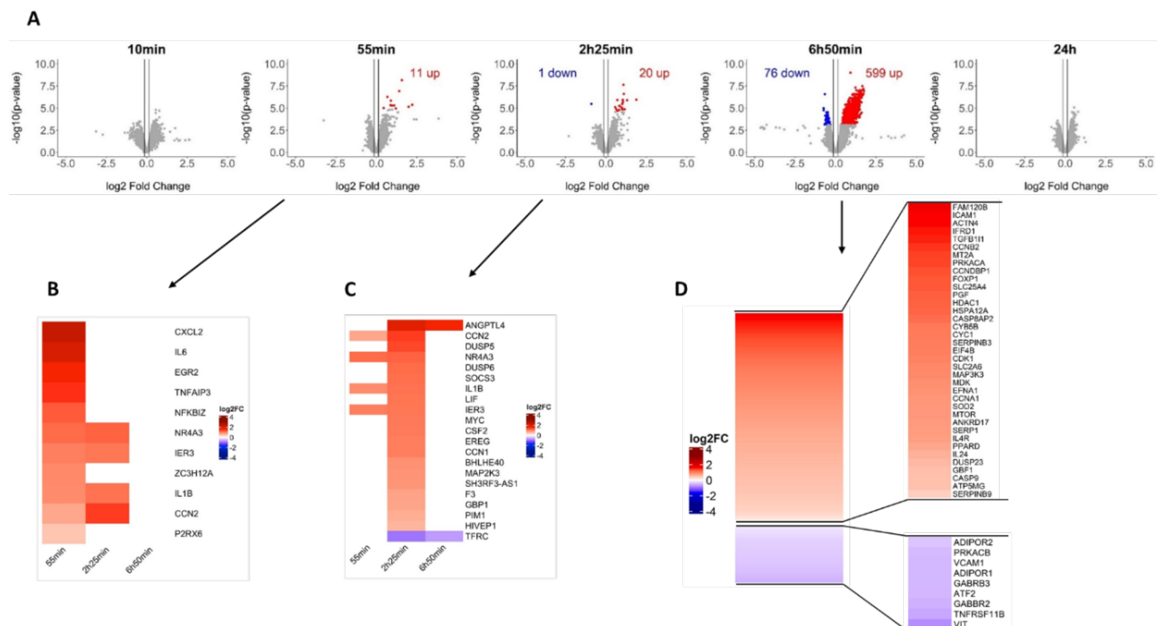


Figure 31: Transcriptome analysis of the effect of 10 mg whole birch pollen on BEAS-2B cells, at the air-liquid interface. (A) Volcano plots and (B, C) heatmaps of all significant regulated genes for the 55 min and 2 h 25 min post-exposure times, respectively. (D) Heatmap of the significant regulated genes for 6 h 50 min post-exposure time, with focus on the most relevant genes related to immune and allergic response. N ≥ 3 in all cases; threshold was set to *p* adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1. Data expressed against control cells (not exposed to pollen but to pressurized air).

At 55 min, the few genes expressed were related to inflammatory response (*TNFAIP3, IL-6, IL1B, CXCL2, ZC3H12A, IER3, EGR2* and *NFKBIZ*), see **Error! Reference source not found.**B.

The up-regulated genes at 2 h 25 min showed a similar response as at 55 min. The most enriched GO terms were related to cytokine signalling, inflammatory response and MAPK-pathway (*CCN2, CSF2, DUSP6, DUSP5, EREG, F3, GBP1, IL1B, LIF, MYC, PIM1, MAP2K3, SOCS3*), see **Error! Reference source not found.**C.

6 h 50 min after exposure to whole birch pollen, BEAS-2B cells showed the highest differential expression of genes. The Metascape analysis focused on enriched GO terms related to signalling, response to stimulus, adhesion and immune system. With a focus on these groups, a set of genes was looked into more detail. *CDK1, CEP* family genes, *ICAM1* and *ACTNA* are related to cell

junction, being up-regulated at 6 h 50 min (**Error! Reference source not found.**D). Pathways related to intracellular signalling pathway, stress response, VEGF, mTOR related and oxidative phosphorylation were also up-regulated. The genes *FOXP1, SERPIN* family genes, *SOD2*, caspase family genes, *CYB5B* and *SLC25A4* are the more relevant genes from these groups (**Error! Reference source not found.**D). Genes related to immune response, NOTCH signalling and TNF signalling via NFKB were in general up-regulated after about 7 hours exposure to birch pollen. The most relevant genes from this group were *IL24, IL4R, TGFB1I1, MAPK3K3, MTOR, PPARD* and *SLC2A6*, for example (**Error! Reference source not found.**D).

No enrichment analysis between the different post-exposure times was performed as only the 6 h 50 min showed a high differentially expression of genes.

4.5.5.1 Validation of selected genes by RT-qPCR

The transcriptome data analysis was focused on the enriched GO terms related to allergy and immune response in which selected genes were validated with RT-qPCR.

Gene expression of the selected genes was confirmed by RT-qPCR where *CXCL2, IL6*, and *TNFAIP3* showed an up-regulation with more than 2-fold expression, at 55 min after birch pollen exposure (Figure 32). The *IL1B* mRNA confirmed the trend shown by the transcriptome data: a 1.8-fold expression was observed for the 55 min incubation time, and a higher expression was observed for the 2 h 25 min (2.3-fold), see Figure 32. At 2 h 25 min, *SOCS3* was also confirmed by RT-qPCR, with about 2-fold expression, while for the 24 hours incubation time, the expression of *ICAM1, IL24* and *IL4* was confirmed, with all genes showing an up-regulation compared with control cells.

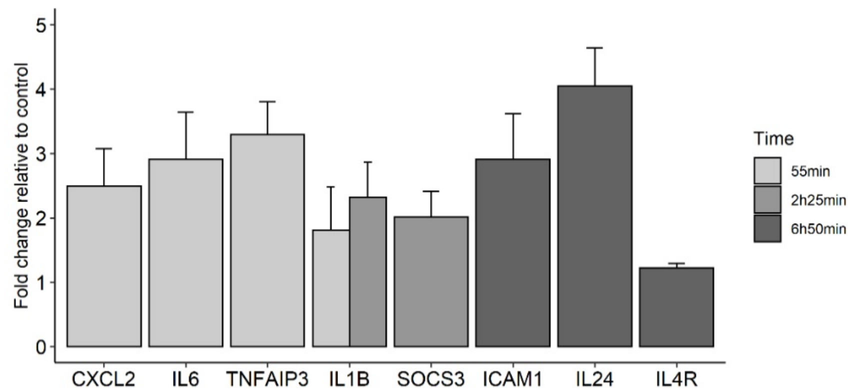


Figure 32: BEAS-2B mRNA expression of selected genes by qRT-PCR that were differentially expressed in transcriptome data, for different incubation times, with the 10 mg birch pollen doses. N ≥ 2 for all experiments. Data expressed against control cells (not exposed to pollen but to pressurized air).

4.5.6. Common differential expression of genes between different doses

The differential expression of genes for both doses was compared and 105 genes were commonly found at both exposures (Figure 33).

In general, the higher dose of pollen (10 mg) induced a higher fold-change in expression of the genes, that where already up-regulated at the lower dose (4 mg). We also observed that most of the genes that were down-regulated at the lower doses switched their regulation at the higher birch pollen dosed exposure.

The reaction of the BEAS-2B cells to whole birch pollen seems to be different for different exposure doses with only a few common genes. Those genes are related to cell cycle, VEGF pathway, response to wounding, stress, cell junction and response to hypoxia. Focusing on the most relevant genes, *IL6*, *CXCL2*, *IER3*, *DUSP5*, *MAP3K3* and *MAP3K10* were commonly expressed.

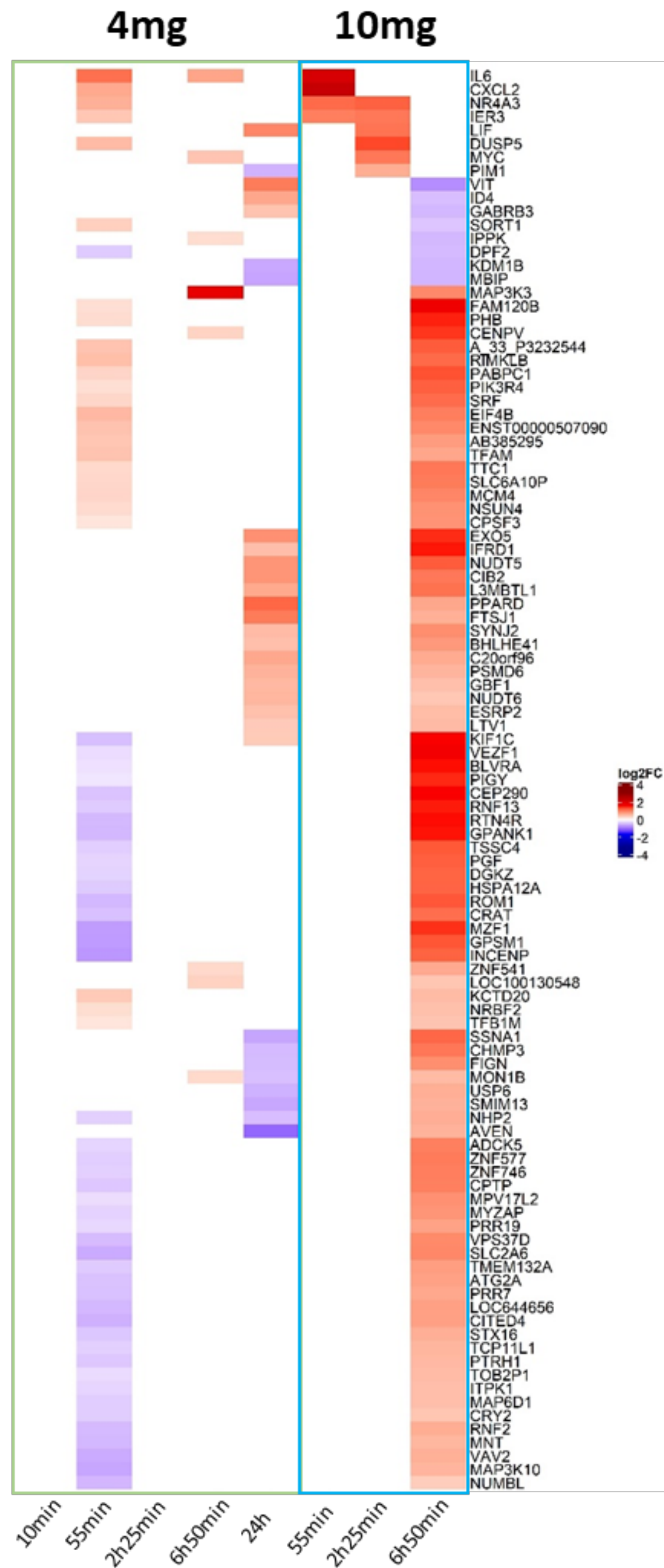


Figure 33: Heatmap of the common differentially expressed genes in the two pollen exposure doses. Only time-points with differential expressed genes are shown. N ≥ 3 in all

cases; threshold was set to *p* adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1. Data expressed against control cells (not exposed to pollen but to pressurized air).

4.6. Diesel-CAST and consequent birch pollen exposure of BEAS-2B cells

To address the adjuvant effect of diesel combustion aerosol on allergic sensitization, BEAS-2B cells at ALI were for 2 hours pre-exposed to a fresh diesel aerosol and, 24 hours later, to 4 mg birch pollen (see Methods).

The following results were obtained together with the CMA (Comprehensive Molecular Analytics) group from the Helmholtz Center Munich.

4.6.1. Physical and chemical characterization of the diesel-CAST combustion aerosol

The diesel-CAST exhaust was physically and chemically characterized. The physical analysis is summarized in Table 4- 4. During the 2 hours of pre-exposure to the fresh model diesel aerosol, the BEAS-2B cells received a concentration of particles of 240 ± 59 µg/m^3, with a geometric mean of 136 ± 8 nm. The concentration of black carbon particles was 207 ± 50 µg/m^3, and the chemical composition of the diesel-CAST showed organic compounds typical of a diesel exhaust aerosol.

Table 4- 4: Physical characterization of the model diesel-CAST combustion aerosol. Mean and *SD* are given for concentrations encountered by the BEAS-2B cells in the in vitro exposure system (N=3).

Parameters	Mean (*SD*)
Geometric mean (nm)	136 (*SD* = 8)
Particle number (#/cm^3)	46 301 (*SD* = 5424)
Total PM (µg/m^3)	240 (*SD* = 59)
BC (µg/m^3)	207.5 (*SD* = 49.9)
Total Carbon (µg C/m^3)	130.5 (*SD* = 5.9)
OC/EC ratio	0.01 (*SD* = 0.01)

The chemical characterization of the diesel model combustion aerosol is summarized in Table 4- 5. The most abundant oxygenated species detected were cyclopentanedione, butylated hydroxytoluene, hexadecanoic acid and phenol, for example. Regarding the PAHs, naphthalene, acenaphthylene,

pyrene, fluorine and fluoroanthene were detected, as well as other important compounds such as propanamide, benzenesulfonamide and N-butyl- and 2,6-ditert-butyl-4-nitrophenol. For the detailed GC×GC-ToFMS chromatogram see Figure 46.

Table 4- 5: Diesel-CAST chemical composition. Different compound classes were selected and the most abundant compounds found in the diesel-CAST aerosol are listed. For the PAHs, compounds known to be relevant in diesel exhaust, but with lower abundance in our exposure, were added. Mean of the area and unique mass is shown (* blank corrected). (N = 3)

Compound Class	NIST Hit/Name	Mean Area (DTIC)	Unique mass (m/z)
Oxygenated Species	2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-4a,5-dimethyl-3-(1-methylethylidene)-, (4ar-cis)-	6648540	161
	1,2-Cyclopentanedione	4130742	98
	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	3024325	219
	9-Octadecenoic acid, methyl ester, (Z)-	2365545	55
	Butylated Hydroxytoluene	1879953	205
	Hexadecanoic acid, methyl ester	1426061	74
	Heptadecanoic acid (derivatized with trimethylsilyl ester)	1259976	117
	Phenol, 2,6-bis(1,1-dimethylethyl)-	1207964	191
	Phenol	1153591	94
	9,19-Cyclolanostan-3-ol, acetate, (3á)-	1037367	135
PAHs	Naphthalene	774744*	128
	1H-Indene, 1-ethylidene-	288982	142
	Biphenyl	247602	154
	Acenaphthylene	153093	152
	Pyrene	127386	202
	Naphthalene, 2,6-dimethyl-	116680	156
	Fluorene	94139	166
	1,1'-Biphenyl, 4-methyl-	91557	168
	9H-Fluorene, 2-methyl-	76086	165
	Fluoranthene	63204	202
	Naphthalene, 1,4,6-trimethyl-	50824	155
	Naphthalene, 2-ethyl-	40294	141
Diverse	Propanamide, 3-[3,5-di(tert-butyl)-4-hydroxyphenyl]-N-(2-hydroxyethyl)-	3101757	147
	Benzenesulfonamide, N-butyl-	3067819	141
	2,6-ditert-butyl-4-nitrophenol	848617	236

4.6.2. Pollen Counts

BEAS-2B cells pre-exposed to the diesel-CAST exhaust aerosol (or to clean air) were exposed 24 hours later to 4 mg whole birch pollen. Pollen doses and loss of the pollen to the chamber are summarized in Table 4- 6 for all time

points. No significant differences were observed between all time points, with 1076 pollen/cm^2 (*SD* = 54) dosed onto the cells and a pollen loss to the chamber of 46.62 % (*SD* = 7.58).

Table 4- 6: Pollen doses and loss to the chamber for the combined exposure to diesel-CAST and birch pollen. No significant differences were observed between the exposures (p > 0.05, independent sample t-test). Mean and *SD* are shown, N = 3 for all time points.

Incubation time	Pollen deposition (pollen/cm^2)	Pollen loss (%)
2 h 25 min	1109 (*SD* = 218)	44.97 (*SD* = 10.84)
6 h 50 min	1105 (*SD* = 139)	45.16 (*SD* = 6.92)
24 h	1014 (*SD* = 132)	49.72 (*SD* = 6.55)

4.6.3. Viability and cytotoxicity levels of BEAS-2B cells after combined exposure

BEAS-2B cells exposed to the diesel-CAST and to whole birch pollen ("Diesel+Pollen") showed no significant cytotoxicity levels and cell viability was higher than 85% at all time points (Figure 34). Control cells ("CA+Pollen") also showed a low cytotoxicity of the treatment (Figure 34). Cell viability was not determined for control cells.

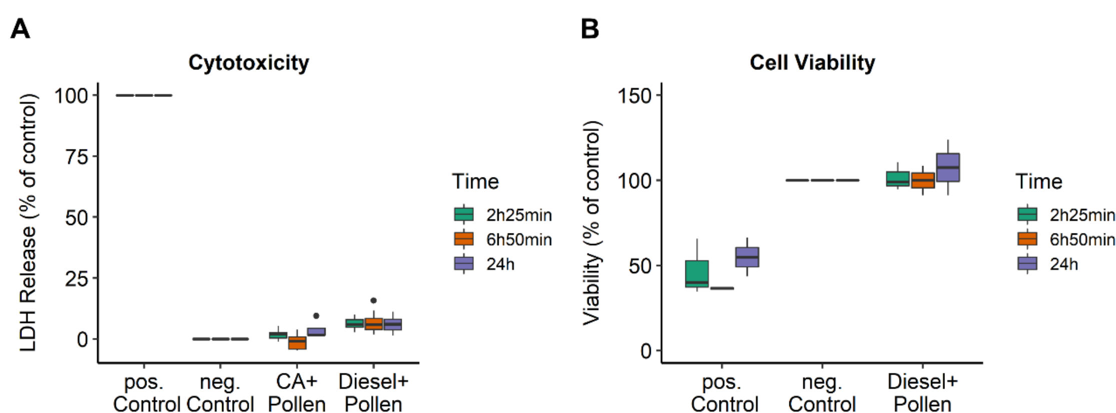


Figure 34: BEAS-2B cell cytotoxicity and viability after the combined exposure to fresh diesel and whole birch pollen, at ALI. (A) Cytotoxicity and (B) viability of the cells exposed ("Diesel+Pollen"), for the different post-exposure times. "CA+Pollen" were cells exposed to filtered clean air and later to pollen. For all exposures, negative and positive controls were performed. Positive cells were lysed with 2% Triton-X 100, negative control cells are cells that were at all times in a cell culture incubator. (N ≥ 3 for all time points).

4.6.4. Transcriptome analysis of the combined exposure

The pre-exposure to the diesel-CAST combustion aerosol in BEAS-2B cells exposed to 4 mg whole birch pollen showed the highest differential gene expression at 2 h 25 min after the exposure: 214 genes up- and 262 down-regulated (Figure 35A). A few genes were differentially regulated for the later time points: 14 genes up- and 12 down-regulated for the 6 h 50 min; and 7 up- and 4 down-regulated genes at 24 hours after the combined exposure.

At the 2 h 25 min post-exposure time, the enriched GO Terms such as cell adhesion, response to extracellular stimulus, detoxification and organic hydroxy compound metabolism were up-regulated. The most relevant genes included in these GO terms were *ITGA4*, *CD34*, *SERPINH1*, *HGB1*, *SOX1* and *GNGT2* (Figure 35B). Regarding the down-regulated genes, the enriched GO Terms were related to regulation of protein glycosylation, amine transport, negative regulation of G protein-coupled receptor signalling pathway and cellular response to organic cyclic and nitrogen compounds. Genes such as *IL9R*, *CYP11A1*, *MAPK11*, *GALNT13*, *CCK* and *CCNA2* were included in the gene list (Figure 35B).

Regarding the cells incubated for 6 h 50 min after the combined exposure, most of the regulated genes have an unknown function. However, some genes are known, such as *MED1* (up-regulated), which is included in the enriched GO term “regulation of interferon gamma” and “response to extracellular stimulus”. Besides *MED1*, also the *FBP1* gene was up-regulated and both are known to be associated with asthma (Hu et al., 2021; Schoettler et al., 2019; Valette et al., 2021), see Figure 35C.

24 hours after the combined exposure, only 7 genes were up-regulated, and *ALDH8A1*, an aldehyde dehydrogenase gene, was included; and 49 genes were down-regulated, with *CCNA2*, *CCK* AND *PPEF1* being the more relevant genes (Figure 35D).

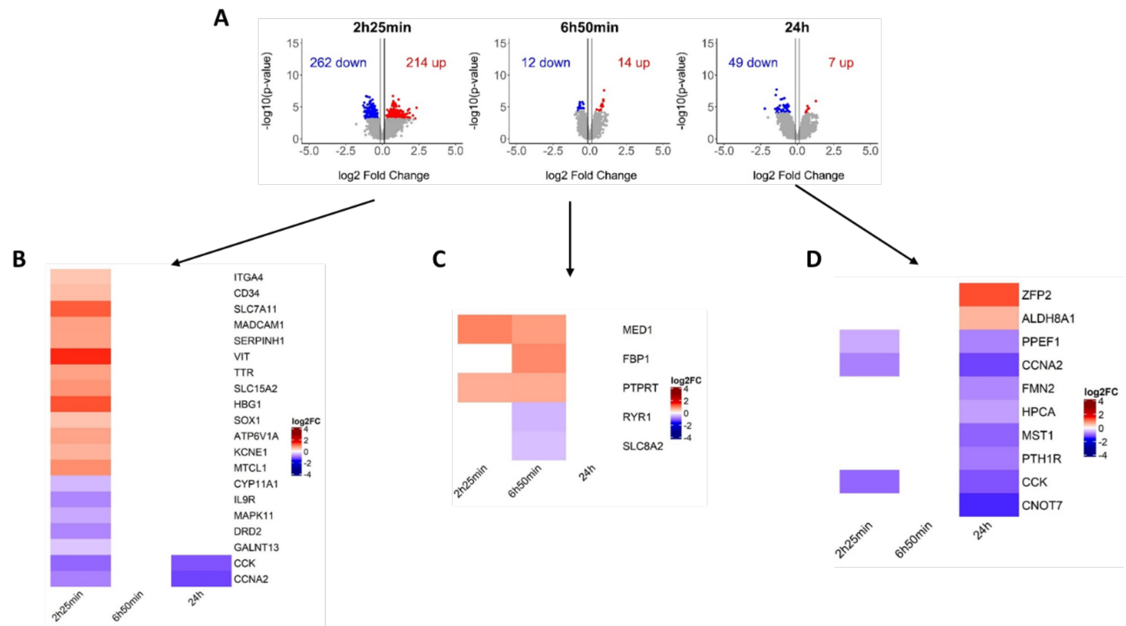


Figure 35: Transcriptome analysis of the effect of combined diesel and 4 mg pollen exposure on BEAS-2B cells, at the air-liquid interface for the different post-exposure times. (A) Volcano plots and (B, C, D) heatmaps of the most relevant significant genes, related to allergic and anthropogenic responses, for all the post-exposure times analysed after the combined exposure. Significant regulated genes are marked in colour. N ≥ 3 per time point; threshold was set to p adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1. Data expressed against control cells (cells exposed to filtered air and pollen – “CA+Pollen” cells).

4.6.5. Validation of selected genes by RT-qPCR

Selected genes, differentially expressed in the transcriptome data, were validated by RT-qPCR. The genes selected are known to be related to immune response and diesel exposure, at the highest differential expression incubation time – 2 h 25 min (Figure 36). The results obtained for *ALDH8A1* and *IL9R* confirmed the transcriptome data, for example. *ALDH8A1* was over-expressed by more than 2-fold, compared to control cells and *IL9R* was down-regulated by 2-fold. The genes *MADCAM1*, *BACE1* and *ITGA4* also confirmed the microarray data, with the last two showing about 1.5-fold expression and *MADCAM1*, with a 4-fold expression (Figure 36).

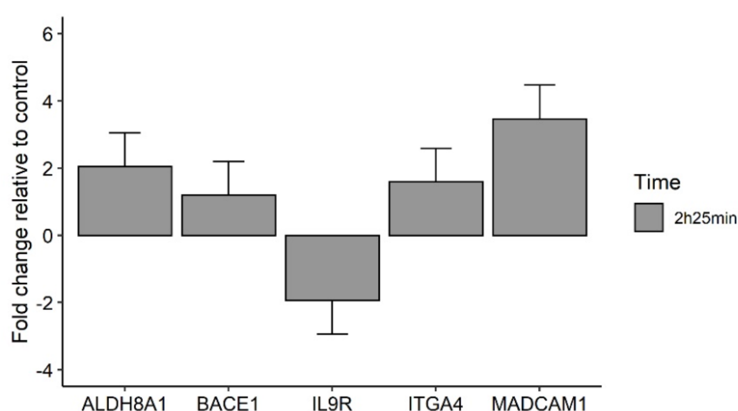


Figure 36: BEAS-2B cells mRNA expression of selected genes by qRT-PCR that were differentially expressed in the transcriptome data, at the combined diesel + 4 mg pollen exposure, for the 2 h 25 min post-exposure time. Data expressed against control cells (cells exposed to filtered air and pollen – "CA+Pollen" cells). N ≥ 2 for all experiments.

4.6.6. Comparison between pre-exposed cells to diesel-CAST and the pollen exposure alone

As one of the aims of this study was to investigate the priming effect of the fresh diesel combustion aerosol on the whole pollen exposure in BEAS-2B cells, the expression of genes that were in both exposures differentially regulated was evaluated. For that, the DEG lists of the two sets for all time points were overlapped. The combined exposure affected 104 genes of the genes that were also affected by pollen alone (Figure 37A). Figure 37B shows a heatmap of all common differentially expressed genes. For the pollen exposure alone, at 10 min and 2 h 25 min, no overlap of genes was found in the cells primed with the diesel-CAST. At 55 min, a few genes overlap, but most of them with unknown function. Most of the genes that overlap with the combined exposure are expressed at the later time-point at 24 hours. We observed in general an earlier up- and down-regulation of the genes when BEAS-2B cells were primed with the diesel model aerosol, compared with the pollen exposure alone.

An enrichment analysis with the genes in common was performed and, focusing on the relevant enriched GO terms previously described, a set of genes was selected. Genes correlated with Class B/2 and adenylate cyclase-activating G protein-coupled receptor signalling pathway (*CRH*, *GIPR*, *GLP1R*,

GNGT2, *PTH1R*, *ADGRB3*); detection of external stimulus (*GNGT2*, *BACE1*, *GRK7*), response to toxic substance/detoxification (*BMP7*, *HBG1*, *SLC15A2*, *EHMT1*), cell adhesion related genes, such as *ITGA4* and *MADCAM1*; and cell response to nitrogen compounds (*CRH*, *GLP1R*, *HRH1*, *ITGA4*, *RYR1*, *BACE1*, *LYPD1*) were differentially expressed in both exposures (Figure 37C). Most of the genes were up-regulated by both exposures and the diesel-CAST pre-exposure enhanced their expression.

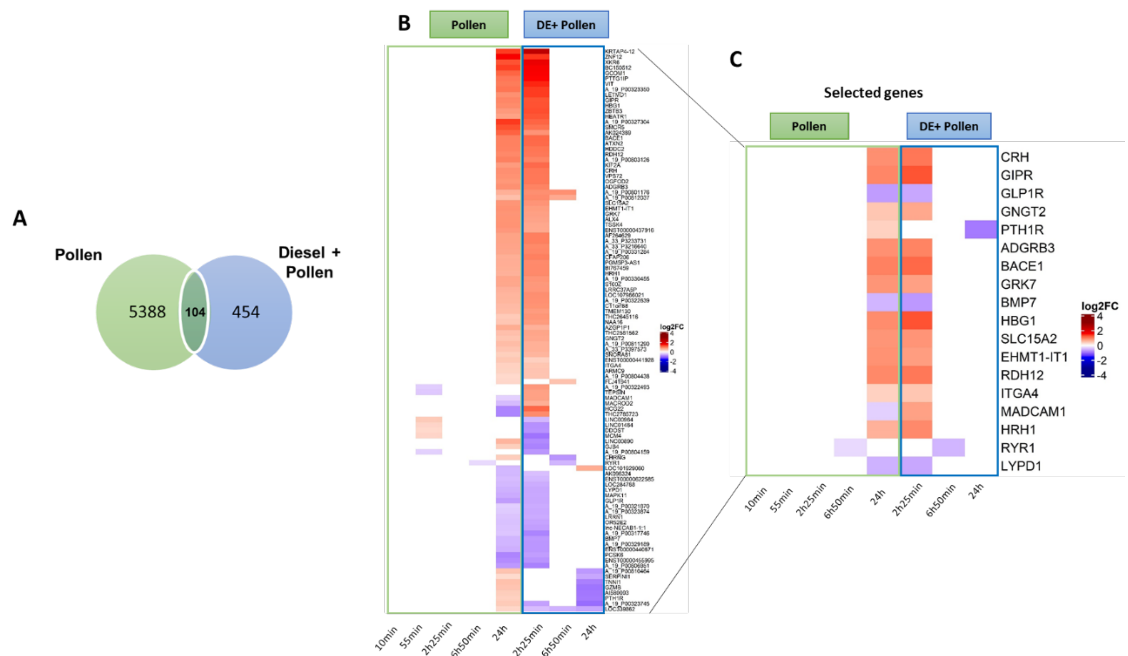


Figure 37: Comparison of the differential regulated genes between the combined diesel + pollen exposure and the pollen exposure alone. (A) Venn diagram summarizing the total number of differentially expressed genes in both pollen exposure alone and combined exposure. (B) Heatmap of the significantly expressed common genes between both exposures (n = 104). (C) Heatmap of the most relevant expressed genes in both exposures, related to immune and allergic responses. N ≥ 3 per time point; threshold was set to *p* adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1. Data expressed against the control cells of each exposure.

Enrichment analysis between the different post-exposure times of the two exposures was performed to analyse the correlations.

For the 55 min after pollen exposure alone and the combined exposure 2 h 15 min time we observe that the pollen exposure alone has more pathways significantly expressed. There is almost no correlation between the two exposures, also observed above with the common related genes (Figure 47).

Between the 6 h 50 min pollen exposure alone and the combined exposure 2 h 15 min post-exposure we observed that the combined exposure showed the expression of pathways related with cell cycle and cellular response to nitrogen compounds. The pollen exposure alone shows clearly the expression of pathways related with immune response (already mentioned above). Only a few common pathways were expressed in both exposures, such as “positive regulation of leukocyte cell-cell adhesion”, leucocyte migration related pathways and “regulation of response to extracellular stimulus”, all up-regulated in both exposures, see Figure 48.

The same trend was observed in the enrichment analysis between the 24 hours post-exposure alone and the 2 h 15 min after the combined exposure (Figure 49). The expression of pathways related with immune response are highly regulated at the pollen exposure alone (up-regulated list) and a few are common with the combined exposure such as: “cellular response to organic cyclic compound”, “cell-cell adhesion” and “inflammatory response”, for example.

4.7. Propane-CAST and consequent birch pollen exposure of BEAS-2B

The following results were obtained together with the CMA (Comprehensive Molecular Analytics) group, from the Helmholtz Center Munich.

4.7.1. Physical characterization of the propane-CAST combustion aerosol

The propane-CAST exhaust was physically characterized, see Table 4- 7. The BEAS-2B cells exposed to propane-CAST for 2 hours received a concentration of particles of 417.4 ± 121.5 μg/m^3, with a geometric mean of 26 ± 1 nm. The concentration of black carbon particles was 220.2 ± 72.4 μg/m^3 and, in contrary to the diesel-CAST, the propane-CAST combustion aerosol had a significant amount of organics, with a ratio between organic and elemental carbon of 0.67 ± 0.06.

Table 4- 7: Physical characterization of the model propane-CAST combustion aerosol. Mean and *SD* are given for concentrations encountered by the BEAS-2B cells in the in vitro exposure system (N = 3).

Parameters	Mean (*SD*)
Geometric mean (nm)	26 (*SD* = 1)
Particle number (#/cm^3)	3 186 318 (*SD* = 550 790)
Total PM (µg/m^3)	417.4 (*SD* = 121.5)
BC (µg/m^3)	220.3 (*SD* = 72.4)
Total Carbon (µg C/m^3)	266.4 (*SD* = 72.5)
OC/EC ratio	0.67 (*SD* = 0.06)

The chemical characterization of the propane model combustion aerosol was unfortunately not possible due to technical problems.

4.7.2. Pollen Counts

For the propane-CAST, exposure was set as in the diesel-CAST: BEAS-2B cells were primed with the propane model exhaust aerosol (or to clean air) for 2 hours and, 24 hours later, to 4 mg whole birch pollen. Pollen doses and loss of the pollen to the chamber are summarized in Table 4- 8, for all time points. No significant differences were observed between the different time points, with 1090 pollen/cm^2 (*SD* = 158) dosed onto the cells and a pollen loss to the chamber of 45.41 % (*SD* = 4.76).

Table 4- 8: Pollen doses and loss to the chamber for the combined exposure to propane-CAST and birch pollen for the different post-exposure time points. No significant differences were observed between the exposures ($p > 0.05$, independent sample t-test). Mean and *SD* are shown, N = 3 for all time points.

Incubation time	Pollen deposition (pollen/cm^2)	Pollen loss (%)
2 h 25 min	1151 (*SD* = 164)	43.67 (*SD* = 4.06)
6 h 50 min	1158 (*SD* = 144)	49.92 (*SD* = 2.72)
24 h	960 (*SD* = 120)	42.62 (*SD* = 4.49)

4.7.3. Viability and cytotoxicity of BEAS-2B after propane-CAST and birch pollen exposure

Cells exposed to the propane-CAST and later to whole birch pollen ("Propane+Pollen") showed cytotoxicity levels lower than 25% and cell viability was higher than 90% at all time points (Figure 38). Control cells ("CA+Pollen") showed low cytotoxicity levels (Figure 38A). As for the diesel combined exposure, cell viability was not determined for control cells.

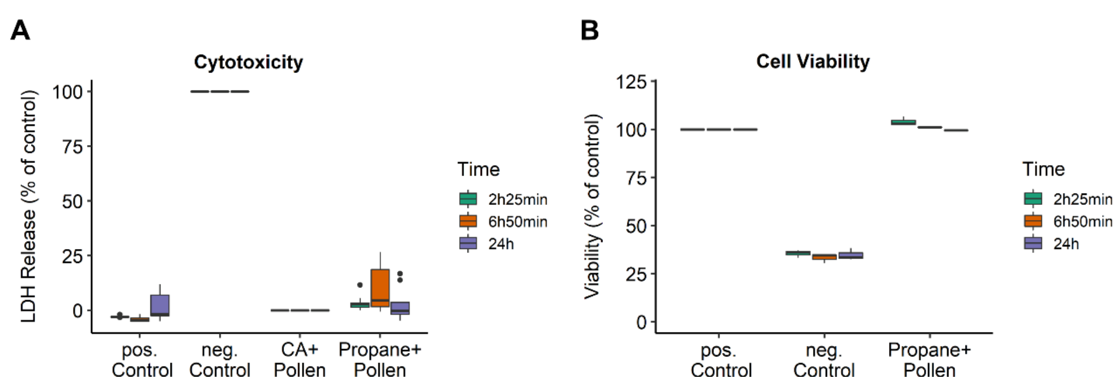


Figure 38: BEAS-2B cell cytotoxicity and viability after the combined exposure of BEAS-2B to fresh propane and birch pollen, at ALI. (A) Cytotoxicity and (B) viability of the cells exposed, for the different post-exposure times. "Propane+Pollen" samples were cells exposed to Propane CAST and to 4 mg birch pollen. "CA+Pollen" were cells exposed to filtered clean air and later to pollen. Positive cells were lysed with 2% Triton-X 100, negative control cells are cells that were at all times in a cell culture incubator. For all exposures, negative and positive controls were performed (N = 3 for all cases).

4.7.4. Transcriptome analysis

For this study, we focused on the priming effect of this fuel on the birch pollen exposure to BEAS-2B cells. Transcriptome analysis was performed for all post-exposure times, as described before.

The same trend was observed as for the pre-exposure to diesel-CAST: the higher differential expression was observed at the 2 h 25 min post-exposure time (310 genes up- and 120 down-regulated), see Figure 39A. The later time points showed a few regulated genes: 5 genes up- and 4 down-regulated for the 6 h 50 min; and 25 up- and 21 down-regulated genes for the 24 hours after the combined exposure (Figure 39A).

At 2 h 25 min incubation time, the most relevant enriched GO terms were related to response to inorganic substance, degradation of the extracellular matrix, chemokine signalling pathway, organic hydroxy compound transport, positive regulation of epithelial cell apoptotic process, cellular response to IL6, and regulation of inflammatory response, for example. The most relevant expressed genes included in these terms are *ALDH1A3*, *CYB5A*, *SERPING1*, *FOXO3*, *CXCR6*, *JAK1*, *MAPK8IP3* and *CASP12* (Figure 39B).

At 6 h 50 min after the combined exposure only 9 genes were differentially regulated, either with an unknown- or not immune related function (Figure 39C). Regarding the 24 hours post-exposure time, the enriched GO terms were related to signalling by WNT, lipid catabolic process and negative regulation of hydrolase activity. The most relevant genes are *GNGT2*, *DLL4* and *PROK2* (Figure 39D).

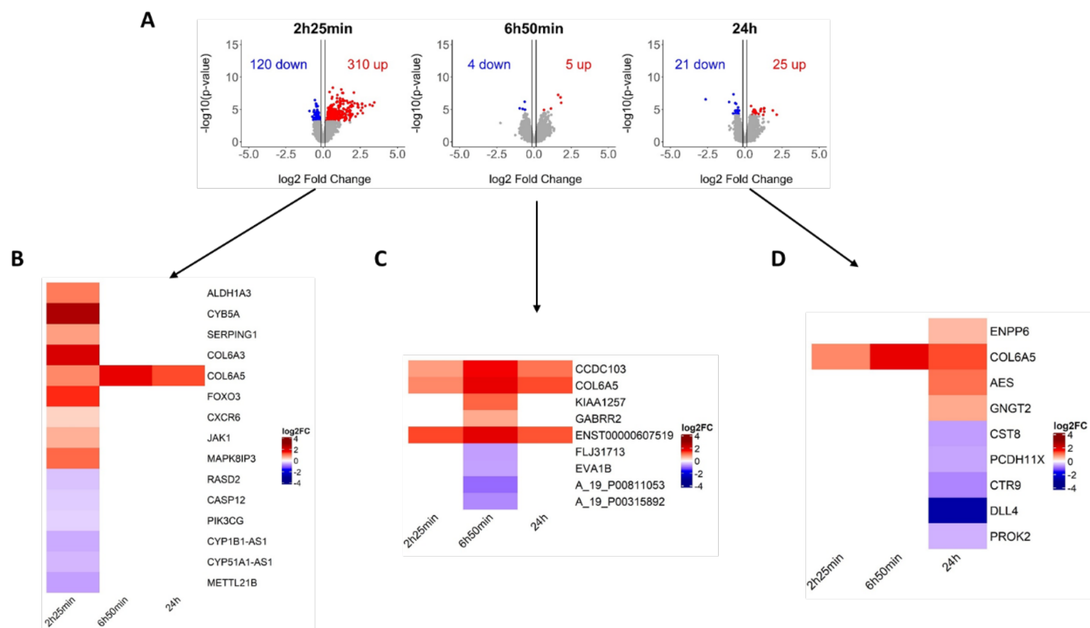


Figure 39: Transcriptome analysis of the effect of combined propane + pollen exposure on BEAS-2B cells, at the ALI. (A) Volcano plots and (B, C, D) heatmaps of the most relevant significant genes, related to allergic and anthropogenic responses, for all the post-exposure times analysed after the combined exposure. Significant regulated genes are marked in colour. N ≥ 3 per time point; threshold was set to *p* adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1. Data expressed against control cells (exposed to filtered ambient air and to birch pollen – “CA+Pollen” cells).

4.7.5. Comparison between primed cells to propane-CAST and the pollen exposure alone

As performed for the combined diesel-CAST/ pollen exposure, an analysis to find the genes expressed in both propane + pollen exposure and the pollen alone was executed. In total, 49 genes were differentially expressed in both exposures (Figure 40A).

As for the combined diesel + pollen exposure, genes were expressed earlier at the combined propane + pollen, compared with the pollen exposure alone (Figure 40B), with no significant change in the intensity of the fold change. The direction of the gene expression regulation changed for some genes: a few genes that were down-regulated in the pollen exposure alone were up-regulated at the combined exposure. *DLL4* was the only gene with the opposite change of direction (Figure 40B). Of most of these commonly expressed genes we are not aware of function in immunology or allergy. However, 6 are related to the following enriched GO terms: chemokine signalling pathway, regulation of oxidative stress, cellular response to nitrogen compounds and MAPK cascade related terms. The most relevant genes included in the list are *GNGT2, INS*, *PIK3CG*, *FOXO3*, *MAPK8IP3* and *DLL4*; the last three with a change of regulation between exposures, as explained above (Figure 40B).

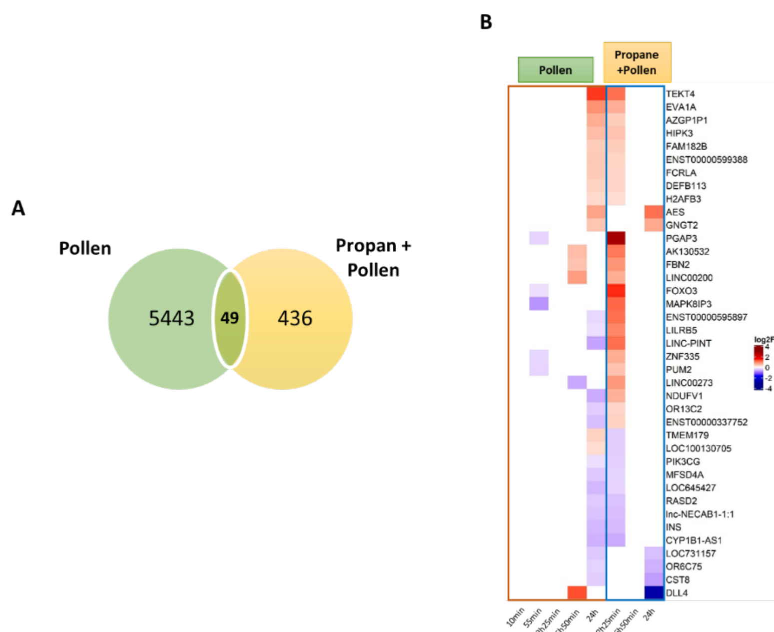


Figure 40: Common differential regulated genes between the combined propane + pollen exposure and the pollen exposure alone. N ≥ 3 per time point; threshold were set to *p* adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1. Data expressed against control cells of each exposure.

Enrichment analysis between the different post-exposure times was performed, as for the diesel combined exposure and the pollen exposure alone. In the case of propane-CAST, only the 2 h 25 min time-point was analysed has the other time point have fewer significantly differential regulated genes.

When comparing the 55 min pollen exposure time with the 2 h 15 min after the combined exposure, almost no correlation was observed between the two different exposures, with a clear significant enrichment of pathways for the pollen exposure alone (Figure 50). Pathways related with immune response are enriched at the 55 min after exposure to birch pollen, however this trend is not observed for the combined exposure, as mentioned above. A negative regulation of immune response is observed at the up-regulated list for the combined exposure, enriched at the down-regulated list for the exposure alone, see Figure 50.

When comparing the 6 h 50 min after pollen exposure alone and the 2 h 15 min post-combined exposure, we observe that the pollen exposure alone shows, for the up-regulated lists, an enrichment of pathways related with immune response, not correlating with the combined exposure (Figure 51). A clear difference is also observed for this two time-points, with the combined exposure showing an enrichment of pathways related with the response to inorganic substance, not enriched at the pollen exposure alone.

The same trend was observed when comparing the enrichment analysis of the 24 h after pollen exposure and the 2 h 15 min after the combined exposure, see Figure 52. Some correlations can be observed between the two exposures: the GO biological process “regulation of immune effector process” and “organic hydroxyl compound metabolic process”, for example, are up-regulated at the combined exposure, but down-regulated at 24 hours after the pollen exposure.

4.7.6. Comparison between the propane-CAST and the diesel-CAST combined exposure

The comparison of the differentially gene expression for both combustion aerosol exposures (with birch pollen) was performed. Looking at the common genes between both exposures only 15 genes are differentially expressed in BEAS-2B cells (Figure 41). This shows that not only the exposures are different in their physical characterization but also in the response from the BEAS-2B cells.

Looking at the genes in detail most of them have unknown function, but the majority is expressed in both exposures at the same post-exposure time, with the exception of *GNGT2*. The *GNGT2* gene – known to be related with the detection of external stimulus – is up-regulated in both exposures, but at the earlier time for the diesel-combined exposure (2 h 15 min) and for the propane-combined exposure is only expressed at the 24 hours post-exposure time.

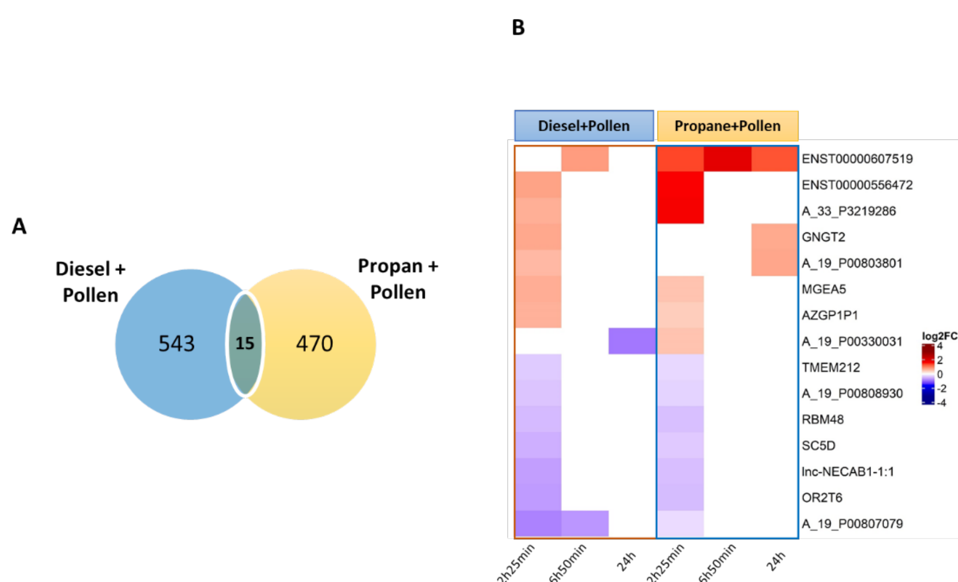


Figure 41: Common differential regulated genes between the combined diesel + pollen and propane + pollen exposure. N ≥ 3 per time point; threshold was set to p adj. < 0.05 and log2 Fold Change < -1.1 and > +1.1. Data expressed against control cells of each exposure.

The enrichment analysis between the two exposures shows that BEAS-2B cells react differently to each combustion aerosol.

For the 2 h 15 min post-exposure, for both exposures, a few pathways are enriched for both exposures (Figure 53). The propane-CAST seems to enrich pathways more related with response to inorganic substance and signalling to EGFR pathway and the diesel-combine exposure with cell-cell adhesion and response to organic cyclic compounds, for example.

For the other post-exposure times no enrichment analysis was performed as the number of differentially regulated genes is very low.

5. Discussion

The aim of this doctoral thesis was to develop a whole pollen sedimentation chamber (PSC) and, with the new system, study the effect of whole pollen on epithelial cells and the priming effect of anthropogenic aerosols on the pollen exposure. The creation of this new in vitro system enables a closer to real-life exposure, which can help to better understand the human reaction to allergens and combustion products.

5.1. Development of the pollen sedimentation chamber

The first step of the study was the development of the PSC. One of the main problems of working with whole pollen is the high adherence of the pollen to any surface. Pollen sticks everywhere, but depending on the temperature and humidity conditions, their dynamic and morphology also changes. Figure 9 and Figure 25 show the same pollen type – birch pollen – in different conditions. When pollen is dehydrated it wrinkles, which is as pollen usually looks like in the ambient air. When pollen is in contact with water or a solution, as in case of Figure 25 (in contact with cell culture medium), they take up the water and change their morphology (hydration). The high dynamic of pollen makes it difficult to use whole pollen on in vitro systems. Besides that, we anticipated that pollen would form clusters upon resuspension, as described by others (Golovko et al., 2013), which would decrease the reproducibility of the exposure. As the sedimentation rate of pollen is about 1.5 cm/s (Pohl, 1937) we set the height of the PSC as high as possible to fit inside a cell culture incubator but to enable pollen to fly and sediment as single particles. Despite the restriction of height, we obtained single pollen in all experiments and seldom encountered clusters of pollen. Some systems for powder dispersion units use vibration meshes, which in our case could have increased the formation of clusters. A possible drawback we expected of using pressurized air was the rupture of birch pollen, as reported by (Visez et al., 2015), but we did not observe this using our experimental design.

Avoiding or reducing the adherence of the pollen to the surfaces of the instrument was the major difficulty we had to overcome. To reduce the number of pollen collisions to the walls of the chamber a cylinder form was chosen. The material of the pollen chamber was also an important aspect. To reduce static electricity, known to affect pollen aerodynamics (Matsuyama et al., 2003; Niklas, 1985), aluminium was used. The use of pressurized air on the highest stage of the PSC to disperse the pollen allowed a vertical straight-line gentle sedimentation onto cells. We could not prove that the contact of the pollen with cells was gentle, although, when we compare the impacting speed of birch pollen – 1.5 cm/s – with the human nose inhalation speed – 750 cm/s (Ogden & Birkett, 1975) –, the system compares favourably. The vertical deposition does not mimic human real-life settings as deposition occurs horizontally. However, the human nasal epithelial cells are covered with mucus, which our cell line does not produce (see Discussion – section 5.8). Mucus reduces the high impact on epithelial cells, thus the gentle gravimetric sedimentation used in the system can reproduce better a real-life pollen contact with nasal epithelial cells.

The influence of the ambient conditions also interferes with the pollen behaviour, as explained above. In our PSC, we obtained more pollen sedimented when experiments happened at 37 °C and 85% RH i.e., inside the cell culture incubator, compared to ambient conditions. Besides that, the cell culture plate also enhanced pollen sedimentation. This was contrary to expected, as the plate is made out of polystyrene which we thought would disturb pollen sedimentation. We obtained more pollen when the culture plate was placed inside the chamber and inside the cell culture incubator a significant increase of 52% more pollen was observed, in addition to a decrease of the standard deviation for all exposures using a plate.

The final PSC settings were improved with three critical changes in the experimental design: the surface coating, the loading tube and the air pressure. Regarding the surface coating, the "slide-friction test" was an important tool to check the best surface i.e., the one to which pollen adheres less. A Teflon® based formula showed the least adherence, which can be explained by the low coefficient of friction: between 0.05 and 0.10 (coefficient of static friction - μ_s).

The coated chamber increased pollen doses on the bottom of the chamber by 13% compared to the not coated chamber, however the difference was not significant ($p = 0.58$, probably due to lack of repeats).

How to load the pollen inside the chamber was also an important setting of the experimental design. The use of aluminium foil on the first experiments decreased the pollen loaded inside the chamber by 17 % (*SD* = 8.5). After testing different loading tubes and materials, the “No-escape tube” reduced the pollen loss to the tube. As the pollen is weighed directly on a small port of the tube, this setting removed the need of an aluminium foil, thus removing an important disturbance of the system.

The air pressure is another important settings of the experimental design. When we decreased the air pressure to blow the pollen inside the chamber from 1 bar to 0.5 bar, we observed a significantly reduced pollen loss by 18% (*SD* = 6.1), a higher pollen deposition – about 63% (*SD* = 23) and a decrease of variability between experiments. Higher pressures propel pollen to the opposite wall site of the loading tube, where they stick to the wall and are lost. We believe that this setting needs to be changed accordingly to the pollen type used in the experiment.

Our efforts in improving the PSC resulted in a homogenous and reproducible distribution of the pollen at the bottom of the chamber and on the cells.

5.2. Different pollen doses used and correlation with human exposure

After setting the final PSC improvements, different birch pollen doses were tested to evaluate the pollen sedimentation. With our final experimental design, a linear “dose-effect” curve was obtained, with an even distribution of the deposited pollen. However, the pollen loss to the chamber was about 40%, but with a low variability between experiments. The same amount of pollen loss was obtained in Visez et al., 2015 for a wind-induced birch pollen rupture system. As the pollen loss was reproducible and linear, we can obtain any pre-set dose of pollen on cells using our PSC.

Specific doses were used to expose BEAS-2B cells to whole birch pollen. Such doses were calculated to be comparable to the real-life human birch pollen exposure on a high peak day. We demonstrate that the PSC enables the exposure to real-life pollen doses: with 1 mg mimicking a birch pollen season day in Central Europe (about 300 pollen/cm^2), and by using between 4 and 10 mg we were able to mimic the birch pollen concentrations in Russia, where concentrations are the highest known (about 1,000-2,100 pollen/cm^2). Cells were exposed to two different doses of birch pollen: 4 mg – corresponding to 1206 pollen/cm^2 (SD = 41), and 10 mg – corresponding to 2834 pollen/cm^2 (SD = 146). Working with a cell line can give a different reaction than if we would have worked with primary cells that create a pseudo-stratified epithelium and have more interactions, mimicking closer the allergic human reaction. Calculating that on one cm^2, when dosing 2 mg of pollen, only around 0.7% of the cells will have direct contact with a birch pollen, we decided to set 4 mg pollen as the main dose to test. For the 10 mg exposure we have around 1.4% of cells in direct contact to pollen. The doses used are not unrealistic and still resulted in few cells being exposed to pollen. Besides that, our experimental design compares favourably with submerged exposure studies, where doses up to 10 mg/ml of pollen, or higher, are used (Blume, Swindle, et al., 2015; Papazian et al., 2015). If we would compare directly with submerged exposures (assuming 100 µl/cm^2), in our PSC we would dose around 0.2 mg/ml for the higher doses.

The concentration of the main birch pollen allergen – Bet v 1 – in our study (about 3.2 pg), is comparable with previous studies that evaluate the concentration of the allergen through Europe (Buters et al., 2012).

Grass pollen was loaded into the pollen sedimentation chamber to assess the ability to use the chamber with other pollen types. We also obtained a reproducible and even distribution with different grass pollen doses. Thus, the PSC can be used with possible small adjustments for diverse pollen types.

5.3. Preliminary exposure of A549 cells to whole birch pollen

In order to evaluate how cells react to the whole birch pollen, preliminary exposures using submerged A549 cells were performed. A549 cells have a lower cost and are easier to work with, compared with BEAS-2B cells. The first submerged exposures showed that whole birch pollen does not induce cytotoxicity to the A549 cells, even at high pollen doses (16 mg) and longer incubation time-points (24 hours). Microscopic pictures of the submerged A549 cells with birch pollen show that the pollen grains “swim” together with the cell culture medium and we even observed the content release of a pollen grain – see Figure 25D. In submerged cultures, we do not observe the creation of “burn holes” caused by the birch pollen or any change in the epithelial layer of these cells. However, one of the goals of this thesis was to study the effect of whole pollen on the more close to real-life possible exposure – at ALI. Thus, exposure of A549 cells at ALI was performed, also for different doses of birch pollen. When cells were checked microscopically, after different post-exposure times (2 hours, 6 hours and 24 hours), no difference on the epithelial layer was observed (no “burn holes”, data not shown). Pollen grains did not move away from the initial deposition place, however no further details were observed for the ALI cultures. As cell cultures at ALI are difficult to observe with a light microscope, different fixing techniques were used in order to study pollen deposition and their effect on the epithelial layer, but with no success. As pollen grains “swim” in contact with any liquid (because of their density), when the fixing solution was used, pollen grains moved out of the deposition place and only a few pollen were seen at the microscope, which does not show the real deposition situation (data not shown).

5.4. Manipulation stress of the PSC

Before exposing BEAS-2B cells at ALI to different pollen doses and for different post-exposure times, the manipulation stress of the PSC was evaluated. At the 2 h 25 min post-exposure, mock exposed BEAS-2B cells were analysed at the transcriptome level, compared to never manipulated cells.

The Pollen-ALI manipulations resulted in an up-regulation of 31 genes and down-regulation of 2 genes. *FOSB, IL6, ANGPTL4* and *SOCS3* were the highest up-regulated genes; all related to cellular stress. We considered this response minimal and unspecific, in comparison with the pollen exposed cells. This stress probably derives from the manipulation of the cell culture plate and not from the "pressurized air puff" generated, as that would never reach the cells (15 µm mesh was placed on top of those cells). In our pollen exposures, mock exposed cells – negative controls – were placed together with the exposed cells in the PSC. These cells were used to compare with exposed cells, thus this effect was deleted. Besides that, genes differentially regulated in mock exposed cells are later also regulated in cells exposed to pollen, eliminating the unspecific response during analysis.

5.5. BEAS-2B cells exposure to different doses of whole birch pollen

BEAS-2B air-liquid interface cells exposed to birch pollen at different post-exposure times and doses, showed no cytotoxicity (from cellular wall permeability) or loss of viability. Transcriptome analysis of the cells showed that different birch pollen doses give different responses in BEAS-2B cells.

5.5.1. BEAS-2B cells exposure to 4 mg birch pollen

BEAS-2B cells exposed to the lower birch pollen doses (4 mg) showed at the transcriptome level an up-regulation of genes related with the disturbance on the epithelial barrier and inflammation response, from the early to the later post-exposure times. A higher "immune response" relevant genes was observed, with higher expression of cytokines at the later times – 6 h 50 min and 24 h. At 6 h 50 min, cytokines known to attract other cells to regulate the immune response are up-regulated, such as *IL6*, *IL23A*, *CXCL9* and *CXCL6*.

At 24 h, the up-regulation of *IL17B* – a low affinity ligand of *IL17RB*, also up-regulated at 24 h – indicates that there could have been an induction of *IL25*. *IL25* is an important allergic reaction regulator (which we could not detect). *IL17B* plays an anti-inflammatory role by blocking the expression of *IL25* and,

consequently, Th2 response (Reynolds et al., 2015; Tworek et al., 2016). However, at 24 hours we still observed an up-regulation of genes related to a Th2 response, such as *IL19* and *CCL20*, known to be involved in allergic asthma (Liao et al., 2004; Pichavant et al., 2005; Vroling et al., 2008; Weng et al., 2019).

The gene *TGFB2* was up-regulated 24 hours after birch pollen exposure. *TGFB2* is known to regulate allergen induced inflammation in asthma patients (Bottoms et al., 2010; Lopez-Guisa et al., 2012), which could suggest that pollen initiates the bronchial epithelium remodelling 24 hours after birch pollen exposure. Genes involved in epithelial barrier regulation, such as *CLDN20* and *MUC5B* (Cremades-Jimeno et al., 2021; Lachowicz-Scroggins et al., 2016; Ordoñez et al., 2001), are also differentially up-regulated at this time-point.

Regarding the down-regulated genes, genes related to both Th1 and Th2 response and immune effector cells recruitment were expressed, such as I*FNL2*/*IL28A*, *IL7R*, *IL2RB*, *FCER2*, *TGFB3*, *CCL16*, and *IL31*. The expression of these genes upholds the idea of a decreased inflammatory response and increased homeostasis 24 hours after exposure to birch pollen.

The most relevant genes of the exposure (*IL6*, *CXCL2*, *IL17RA*, *CCL20*, *ITGA4*, *MADCAM1*, *MUC5B* and *TGFB2*) were validated by RT-qPCR, showing that the mRNA expression of those genes is significantly expressed in BEAS-2B cells after 4 mg pollen exposure

5.5.2. BEAS-2B cells exposure to 10 mg birch pollen

Bronchial epithelial cells exposed to the higher dose of whole birch pollen (10 mg) showed a faster but more limited response. At 55 min, an up-regulation of genes related with inflammatory response was observed and this response continued at 2 h 25 min post-pollen exposure. Besides that, cytokine signalling and MAPK-related genes were also up-regulated. The highest response was observed at 6 h 50 min, with a differential up regulation of genes related to Th2 response and to the remodelling of the epithelial barrier. At the same time, a down-regulation of genes related to cell homeostasis and TNF signalling was expressed.

The most relevant enriched GO terms involved in the allergic response were related to inflammation response, cellular stress and cell junction. At 55 min after pollen exposure, *IL6* but also *IL1B* are up-regulated. *IL1B*, also up-regulated at 2 h 25 min, is known to be an important mediator of the inflammatory response and is involved in cytokine and T-cell activation, inducing mediators, such as *TNF* and *IL6* (Cahill & Rogers, 2008; Stylianou, 2006; Tanaka et al., 2014). *IL6* was shown (Badorrek et al., 2017) to be induced after allergen challenge. Still at 55 min after pollen exposure, *TNFAIP3* and *CXCL2* were up-regulated. Both genes are related to inflammatory responses and were shown in other studies to be regulated in allergic diseases (Krusche et al., 2019; K. I. L. Röschmann et al., 2011; K. I. L. Röschmann, Van Kuijen, et al., 2012; Vroman et al., 2018; Wagener et al., 2013).

The expression of genes related to cell junction was observed at 6 h 50 min after pollen exposure occurred, such as *ICAM1*. This gene was induced in other studies where BEAS-2B cells were exposed to house dust mite and pollen allergen and is known to play an important role in epithelial transmigration of neutrophils (Ciprandi et al., 1994; Österlund et al., 2009; Shaw et al., 2004; Wegner et al., 1990; Yang et al., 2005). Inflammation related genes were also up-regulated at about 7 hours after exposure. *IL24* is known to be associated with inflammation (U M Zissler et al., 2016), as well as *IL4R* and *SOCS3*. The IL4-receptor can respond to *IL4* but also to *IL13*, inducing a type 2 inflammation response (Floc'h et al., 2020). Genes from the *SOCS* family were shown to be expressed in the nasal epithelium of patients with allergic rhinitis, modulating inflammation and cytokine signalling (S. H. Lee et al., 2012).

The most relevant genes of the exposure were validated by RT-qPCR – *CXCL2*, *IL6*, *TNFAIP3*, *IL1B*, *SOCS3*, *ICAM1*, *IL24* and *IL4R* – with all of them showing more than 1.2-fold expression, supporting the microarray data.

Contrary to the 4 mg birch pollen exposure, BEAS-2B do not show any differential gene expression 24 hours after exposure – see Figure 29 and Figure 31. One day after pollen exposure, BEAS-2B showed no changes compared to control cells, thus a restoration of the cells was observed.

This doctoral thesis shows for the first time the exposure of bronchial epithelial cells to whole birch pollen grains. We can conclude that BEAS-2B cells have a different response to different doses of birch pollen. This was shown also when performing enrichment analysis between the different post-exposure times and only a few pathways were common between them. Comparing the genes that were expressed in both exposures (common genes); a few genes were commonly regulated. We observed a general higher expression of the regulated genes at the higher doses, such as *IL6* and *CXCL2* and an up-regulation of genes that were down-regulated at 4 mg. The genes are mainly related to stress, response to wounding and hypoxia with the most relevant expressed genes: *IL6*, *CXCL2*, *IER3*, *DUSP5*, *MAP3K3* and *MAP3K10*.

In general, transcriptome changes of the BEAS-2B cells were limited. It is known that pollen releases their mediators within minutes of hydration, but in our study, we observed a limited effect, especially for the higher doses. Exposing BEAS-2B cells to 10 mg whole birch pollen induced a faster but more limited reaction than dosing 4 mg. We observe that the lower dose – more comparable to human real-life exposure – show more differentially regulated genes than the high dose, at the transcriptome level. BEAS-2B cells exposed to 4 mg whole pollen seem to show, at 55 min, a typical allergy acute-phase response and, at a later time (6 h 50 min and 24 h), a more late-phase response, not observed at the higher doses.

When comparing the doses used in this study with other studies (submerged conditions), our doses are still very low. The use of whole pollen and such low doses have not been used until now, neither in in vitro or in animal studies. In this study, we show that low doses of whole pollen are enough to induce a pro-allergic reaction, as in a human real-life situation.

5.6. BEAS-2B cells exposure to the combined diesel-CAST and whole birch pollen

We are not aware of a specific technique that combines whole pollen exposure (i.e. exposure to whole pollen) with chemical air pollutants (PM10,

PM2.5, UFP etc.), at the air-liquid-interface due to technical difficulties. Human studies however, have been previously performed (Carlsten et al., 2016; David Diaz-Sanchez et al., 1997; Mookherjee et al., 2018; Rider et al., 2016) showing an allergy adjuvant effect of diesel and propane combustion products. Thus, one of the aims of this project was to develop and provide an ALI-exposure technology for both pollen and chemical particles simultaneously, necessary to test their mutual influence on epithelial cells, as we think these cells are involved in the first steps of becoming allergic.

To study the priming effect of combustion aerosols in allergic reaction, we exposed bronchial epithelial cells, at ALI, to the anthropogenic source before and, later, to the whole birch pollen. Pre-exposure mimics a real-life situation, where the seasonal pollen encounter an airway epithelium already exposed to omnipresent combustion aerosols. In this part of the study, we used a diesel-CAST model aerosol to expose the BEAS-2B cells. The effect of the model diesel exhaust alone was not carried out in this study as it has been amply described elsewhere (Klein et al., 2017; Oeder et al., 2015; Sapcariu et al., 2016) and was not the focus of this thesis.

Regarding the physical and chemistry characterization of the fresh diesel model exhaust, our combustion aerosol is comparable with previous studies. Human chamber exposures studies used similar particle concentration and size distribution of a diesel aerosol (Birger et al., 2011; Carlsten et al., 2016). The diesel-CAST aerosol produced in our study contains aromatic hydrocarbon compounds, such as acenaphthylene, fluoranthene, fluorene and pyrene, which are typically produced by diesel exhaust (Corrêa & Arbilla, 2006). High amounts of 1,2-Cyclopentanedione, 7-Hexadecenoic acid, methyl ester were also identified, as well as 2,6-ditert-butyl-4-nitrophenol (DBNP, also known as Bayer 28,589), an alkylphenolic antioxidant usually added to fuels to prevent degradation (Jensen et al., 2014). The diesel-CAST generated also black carbon, an important element of diesel combustion aerosols and known to induce adverse respiratory effects (N. A. Janssen et al., 2012; N. A. H. Janssen et al., 2011).

The priming exposure of BEAS-2B to the model diesel exhaust and then to pollen showed the highest differential regulation of genes at 2 h 25 min. Most up-regulated genes were related to cell adhesion, response to extracellular stimulus, detoxification and organic hydroxy compound metabolism. The down-regulation showed the expression of processes related with cellular response to organic cyclic- and nitrogen-containing compounds.

Several of the regulated relevant genes are known to be induced in allergic and/or severe asthma. *ITGA4*, for example, is known to bind to *MADCAM1* and recruit lymphocytes to the mucosa (Pacheco et al., 1998). Both genes were amplified in asthma and chronic inflammation (Briskin et al., 1997; Wang et al., 2018). *BACE1*, *FBP1* and *GNGT2*, also up-regulated at 2 h 25 min after the combined exposure, were correlated with asthma in other studies (Dong et al., 2020; Hu et al., 2021; Madore et al., 2016; Madore & Laprise, 2010).

Regarding the down-regulated genes expressed 2 h 25 min after the combined exposure, genes related to the regulation of immune response and Th2 differentiation were observed, such as *IL9R* – a pathway that is of particular importance for asthma (Musiol et al., 2021).

5.6.1. Comparison of the combined exposure with pollen exposure alone

BEAS-2B cells pre-exposed to the model diesel exhaust showed diminished differentially regulated genes in a subsequent whole pollen exposure. This effect was also observed in other studies (Li et al., 2012; Rider et al., 2016).

Regarding the regulated genes expressed in both exposures (combined and pollen alone – 4 mg doses of pollen), BEAS-2B cells primed with the diesel exhaust reacted faster to birch pollen, compared to the cells exposed only to birch pollen. As follows, the enrichment of pathways related to inflammation were expressed at earlier times, with the most relevant genes in common being *ITGA4*, *MADCAM1*, *BACE1* and *GNGT2*.

The expression of genes towards a regulation of immune response, observed at the combined exposure, was seen for pollen exposure alone, but at

a later time-point (24 hours), confirming the adjuvant effect of the diesel-CAST exposure to the allergic response.

The validation of selected genes was performed by RT-qPCR. The genes ALDH8A1, BACE1, ITGA4 and MADCAM1 were more than 1.5-fold expressed and IL9R was down regulated – about -2-fold; supporting the transcriptome data.

The enrichment analysis of the combined exposure with the pollen exposure alone shows that the BEAS-2B cells react different to the exposures. In general, the pollen exposure alone promotes a stronger immune reaction of the cells – more genes differentially expressed and more pathways enriched. The pathways in common in both exposures are limited. The pollen exposure shows a distinguish immune response while the BEAS-2B cells primed with the diesel showed a limited response, which could mean the exhaust of the cells from the pre-exposure to the combustion aerosol. To note that the control cells of the exposure are cells exposed to filtered air and pollen. The reaction of the pollen exposure alone is numbed and we observe with this setting the priming effect of the diesel to the pollen response. Thus, the genes here expressed are valid to understand the adjuvant effect of the combustion aerosol to the pollen sensitization.

5.7. BEAS-2B cells exposure to the combined propane-CAST and whole birch pollen

In this thesis, the diesel fuel was designated as the “dirty fuel”, to simulate a usual exposure to diesel exhaust aerosol from passenger cars; while the purpose of the propane fuel was to evaluate the effect of a “cleaner fuel” in bronchial epithelial cells and their priming effect on the pollen exposure.

Regarding the physical characterization and comparing with the diesel-CAST, the propane-CAST combustion aerosol showed a different composition. The particles produced were smaller but the concentration of particles was higher than with the diesel-CAST. The black carbon concentration was similar, but in contrast with the diesel-CAST, the combustion of propane fuel produced

substantial more organics, with a more balanced ratio between organic and elemental carbon. This data comes in agreement with the studies of Tolis et al., 2021; Wöhrnschimmel et al., 2008 which showed more organic compounds in cars running on LPG, compared with diesel fuel. However, a more profound study is needed to understand better the composition of propane combustion aerosol. One important difference in the propane-CAST is the production of very small particles (26 nm against 136 nm in diesel-CAST), which can travel easier to the deeper lungs and have severe effects on respiratory diseases (HEI Review Panel on Ultrafine Particles, 2013; Jakobsson et al., 2018; Riva et al., 2011). Thus, when BEAS-2B cells were exposed to the propane-CAST this mimicked an exposure to UFP with substantial organics in their composition. Contrary to expected, propane was not the “cleaner fuel”. This is due to the deliberate setting to a more incomplete combustion in our model to the generate a dirty aerosol (with more smaller particles and more organics than with diesel exposure).

Regarding to the transcriptome analysis, the propane-CAST model aerosol showed the highest differential expression at the 2 h 25 min after the combined exposure, as observed for the diesel-combined exposure. Cells seem to recognize the external particles, but no exacerbation of specific allergic/immune response was observed when BEAS-2B cells were exposed to the propane-CAST, contrary to diesel exposure. Furthermore, we observe an expression of genes related to stress and inflammation response, as *FOXO3*, *CXCR6*, *MAP8IP3* and *ALDH1A3*.

A recent study from (Juarez-Facio et al., 2022) showed an increase of oxidative stress and inflammation markers (*NQO1* and *IL8*) in NHBEs exposed to a propane-CAST (no pollen) with a high content of organics. However, the study exposed the cells only to 35 minutes of the aerosol and cells were let to incubate for 3 hours until RNA isolation. Our study shows that the combined exposure of UFP with whole pollen seem to induce stress and inflammation response in BEAS-2B cells, but no direct exacerbation of the allergic reaction was observed compared with the diesel-CAST exposure.

5.7.1. Comparison of the combine exposure with the pollen exposure alone

To evaluate the priming effect of the propane-CAST on the common genes differentially expressed in both combined and pollen exposure alone, DEG lists were compared. Only 49 genes were commonly expressed at the combined exposure and the whole birch pollen exposure alone. In general, the response occurred faster in the combined exposure, as observed for the combined diesel exposure, but besides the expression of *FOXO3* and *MAPK8IP3*, the remaining genes are not known to be relevant for the immune and/or allergic response. The change of direction (up-regulation in the combined exposure, of *FOXO3* and *MAPK8IP3*, both related with stress and chemokine signalling, could be related with the high organics present in the propane-CAST, as seen in (Juarez-Facio et al., 2022).

Besides that, both combined exposures were compared between each other. Only 15 genes are shown to be in common for both exposures, with the most of them not having a known function. Regarding the enrichment analysis, BEAS-2B cells exposed to diesel and pollen seem to react more, with the enrichment of pathways related to the response to organic substances and to immune response, while the combined propane and pollen exposure seems to react to inorganic substances with no specific immune response.

Few studies were performed to analyse the allergic sensitization using pollen grains in human environmental exposure chambers (Badorrek et al., 2017; K. Bergmann et al., 2021; Gherasim et al., 2021; Hohlfeld et al., 2010; Krug et al., 2003; Werfel et al., 2015). The study of Badorrek et al., 2017 shows the increase of important cytokines in allergic patients, but not in healthy ones. We used in our study BEAS-2B cells that, by itself, are not from an allergic individual and thus can reproduce the effect of healthy patients (we analyse here pollen sensitization). Other studies are known that used diesel exhaust particles to understand their effect in humans (Drizik et al., 2020; Mookherjee et al., 2018; Salvi et al., 2000). Studies that combine both exposures, as they happen in real-life – pollen grains with diesel exhaust particles – are to our knowledge not yet

performed. The work of (Rider et al., 2016) and (David Diaz-Sanchez et al., 1999) used DEP particles and/or allergen extract solutions (or comparable) to understand the effect of combustion aerosols in allergic reaction. No study was performed until now using UFP. We observe, as in (Rider et al., 2016), that the combined DEP+ allergen extract shows a lower reaction of the cells but the miRNA-expression of inflammation markers is observed, with clear effects from the allergen to the exposure. The same result was obtained by Carlsten et al., 2016 and an important condition is that both studies used participants with a known sensitization to the allergen, in contrary to our study.

We can unfortunately not make a direct comparison of the present study with others as such methods (or the combination of them) were, to our knowledge, never performed before.

5.8. Drawbacks of the study

This doctoral thesis has some drawbacks, which, at the same time, can be used as outlook for further studies using the Pollen Sedimentation Chamber.

One of the drawbacks of the study is the use of the bronchial epithelial cell line BEAS-2B. It was used before as a model to study the reaction of the airway epithelium to pollen (Chan et al., 2017; Ge et al., 2021; Honda et al., 2013; Österlund et al., 2009). The upper and lower airways have different characteristics but they share a valid correlation in the expression of biomarkers, thus a concept named as “united airways” has been studied (Ulrich M Zissler et al., 2018). Besides that, BEAS-2B are absent of cilium and mucus, and have limited tight functions (Stewart et al., 2012). This could have limited the initial response of the cells to pollen as, in a real-life exposure, cells are protected by a epithelial mucus layer, which prevents or lowers the first contact with the pollen particles. However, studies using epithelial cell lines or primary cells (from equine respiratory epithelial cells) showed a disturbance of the epithelial barrier (Runswick et al., 2007; Van Cleemput et al., 2019; Vinhas et al., 2011). We believe that for the proof-of-concept of the Pollen Sedimentation Chamber the use of the BEAS-2B cell line to study the effect of whole pollen was a cost effective cell model. The use of human primary cells to validate this study is

being established in another project (see https://eithealth.eu/product-service/adapt/).

IL25, *TSLP* and *IL33*, commonly thought to be important in allergic disease, were not differentially regulated in any exposure of this study. A point to consider in this study is the low pollen dose used to expose the BEAS-2B cells at ALI. This could lead to reactions different than the ones obtained in previous studies where higher doses were used. However, our study is not far from the real-life exposure and can help to understand allergic sensitization better. We also studied here the first contact of bronchial epithelial cells to pollen; the cells were not primed with any cytokine (such as *IL4*, for example) in order to prime the allergic reaction. We evaluated here the early factors that could prompt individuals to become allergic. Repeated exposures, however, using the same experimental design, could induce the regulation of those relevant allergic regulation genes and should be performed in future studies.

An important evaluation missing in this study is the release of relevant cytokines from the BEAS-2B cells exposed to whole birch pollen, as we analysed transcriptome (and RT-qPCR mRNA expression) only. Transcriptome analysis does not catch release of mediators only. Important regulators, such as *IL8* and *IL33* for example, missed in the genome-wide analysis could have been evaluated by ELISA. This technique allows the measurement of important cytokines that are released from the cells, using the basal medium, and other studies showed an important release of cytokine mediators from the cells (Blume, Swindle, et al., 2015; Kouzaki et al., 2011; Steerenberg et al., 1998; Willart et al., 2012). The medium was collected from each exposure performed however, during the time of this doctoral thesis, the measurement of those markers was not possible.

Regarding the combustion aerosol exposures, the space limitation of the pollen-ALI and the VITROCELL® systems did not allow to perform the exposure of BEAS-2B cells to the combustion aerosol only. Besides not being the goal of this study and amply performed by others, it would have been important to guarantee that the genes that were differentially regulated at the combined

exposed are caused only by the adjuvant effect of the combustion aerosol and are not a later response to the aerosol itself.

Both combustion aerosols used are only model aerosols. They are generated by the use of a diffusion flame and not by combustion, therefore they lack important compounds such as nitrogen oxides and nitrated PAHs, for example. A point to consider for both exposures is that the complete aerosol exhaust is used, thus the biological response observed can be attributed not only to the particles but also to the gas phase produced from the combustion aerosol.

Regarding the propane-CAST exposure, the chemical characterization was not performed, which would have given important information of which organic compounds contributed to the oxidative stress and inflammation factors observed in the exposure. That would have helped to better understand the effect of this UFP-produced aerosol on the bronchial epithelial cells.

6. Conclusion

This thesis describes the development and successful use of a pollen sedimentation chamber (PSC) to dose whole pollen to cells at the air-liquid interface.

Three factors were important for the performance of the PSC to reduce the pollen loss and variability between exposures: the use of a "No-escape" loading port, air pressure and coating of the chamber with a Teflon based formula. Despite a 40% of pollen loss to the system, we were able to obtain a reproducible, "dose dependent" and even distribution of birch and grass pollen in the PSC.

The bronchial epithelial cell line BEAS-2B was exposed to different doses of whole birch pollen that mimic (higher) real-life exposures. The lower doses showed at the transcriptome level mainly an inflammatory response, with the highest expression of cytokines happening at later times (6 h 50 min and 24 h), such as *IL6*, *IL23A*, *CXCL9*, *CXCL6*, *IL19* and *CCL20*. At 24 hours after birch pollen exposure, a down-regulation of genes related to inflammatory response was observed (*IL28A*, *CCL16* and *IL31*), which could mean a decrease of the immune response and increase of homeostasis after the pollen exposure. The high doses showed a limited but faster response. Inflammatory response-related genes were up-regulated at earlier times (*IL6*, *IL1B*, *TNFAIP3* and *CXCL2*), with the highest differential regulation observed at 6 h 50 min after pollen exposure. Here, *ICAM1*, *IL24*, *IL4R* and *SOCS3* were up-regulated. A complete restoration of the cells seems to happen at the higher doses as no genes were differentially regulated 24 hours after exposure. In conclusion, a low dose, corresponding to the real-life exposure, is enough to induce allergy promoting (inflammatory) genes in BEAS-2B cells.

BEAS-2B cells primed with the diesel-CAST and then exposed to birch pollen showed a faster reaction at the transcriptome level, compared to the pollen exposure alone, demonstrating the adjuvant effect of the diesel exhaust. Genes related to severe asthma and inflammation were expressed (*ITGA4*,

MADCAM1, *BACE1* and *GNGT2*) at earlier time-points. After 6 hours, the reaction of cells ceased.

The pre-exposure of BEAS-2B cells to the propane-CAST and then to birch pollen resembled an exposure to UFP particles, which can reach the deep lower airways (more than the larger diesel particles). The reaction of BEAS-2B cells was also shifted to earlier time-points. No exacerbation of specific genes related to allergy or immune response was observed. However, expression of genes related to degradation of matrix, stress and inflammatory response was observed, with the more relevant genes being *FOXO3*, *CXCR6*, *MAP8IP3* and *ALDH1A3*.

In conclusion, our experiments show that pollen induced a limited reaction of the BEAS-2B cells, but we could demonstrate that even a low birch pollen dose induces a “danger-signal” in the cells. Danger signals are often needed when animals are intentionally made allergic or when desensitizing humans. Prior exposure to diesel- or LPG-exhaust accelerated this process. The results of this thesis can be used as a guide for future human studies, where the finding for biomarkers is important. The PSC can be now used in studies to investigate the synergistic effects of environmental pollutants on pollen sensitization.

7. References

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8. Acknowledgments

First of all, I would like to thank my supervisor Prof. Dr. Jeroen Buters. Before being my supervisor, he gave me the opportunity 8 years ago to be part of his group. Even after countless “no´s” from my part, he kept convincing me to stay and I cannot be more thankful for what all he taught me. I can only thank you for the countless professional and personal skills I have gained during all these years. Thank you for all your support during my thesis, for trusting me and giving me the freedom I needed to grow my ideas. Thank you for giving me the opportunity to conduct my doctoral thesis with you, I do not imagine a better place to do it, besides at ZAUM with you!

Second, I would like to thank the head of the ZAUM institute, Prof. Dr. Carsten Schmidt-Weber, for giving me the opportunity to work at the institute and all the support and guidance during the last years. I would also like to thank my supervisor Prof. Dr. Jörg Durner and mentor Dr. Stefanie Bauer, for all the critical support during my thesis, special at the thesis committee meetings.

I would also like to thank my cooperation partners that made my doctoral thesis possible: the CMA group from the Helmholtz Center Munich, specially Dr. Sebastian Öder, Elias Zimmermann, Nadine Gawlitta, Dr. Christoph Bisig and Anja Huber. Thank you for all the support during the time I was working with you at CMA, but also through all these years. I would also like to thank the CPC group, especially Dr. Tobias Stöger, Dr. Ali Önder Yildirim for all the critical input during our Task Force meetings and Verena Häfner, that besides not having the opportunity to directly cooperate with, was always supportive and tried to help in what she could.

A special thanks to Dr. Erwin Karg, for the all support when we needed; N. Ertl, for making the creation of the pollen sedimentation chamber possible and being always so helpful. Also Butraco, for external technical support in refining the chamber and Dr. Matt Smith for both manuscript reviews.

I would also like to thank all the ZAUM members. First of all, my group (Cordula, Gudrun, Christine and Dr. José Manzano), for all the support during the last years, and the past members Danijel Kupresanini and Dr. José Oteros,

which indirectly made my doctoral thesis easier with what I learned with you the years before. To all the ZAUM/IAF members and past members: thank you for all the critical input, support and all the nice moments I had with you in the last 8 years. Will never forget the nice wedding cake I never expected to have! A special thanks to Jana, since the beginning there ready to help, Madlen and Myriam for all the support at ZAUM but also outside the "walls" of the institute.

To Juliana, Cristiana and Sofia, the friends I got during the university and keep being there. Thank you for the kind words, even with more than 2000 km apart most of the times.

To my parents, thank you for always being so supportive and understand the countless times I was in front of the computer during my vacation. To my sister, I have to thank you for being an example for me, to be crazy enough to do a 360° professional change and make me able to teach you how a PCR works and how wonderful biochemistry is! Thank you for always celebrating my success as yours. To the Gürth family, for all the support and care during the last years.

To Ferdi, the German I found at ZAUM and did not let go. Thank you for all the support, the countless nice dinners you prepared during the most stressful times, for giving me calm and marry me between a thesis and a pandemic!

A. Supplemental Data

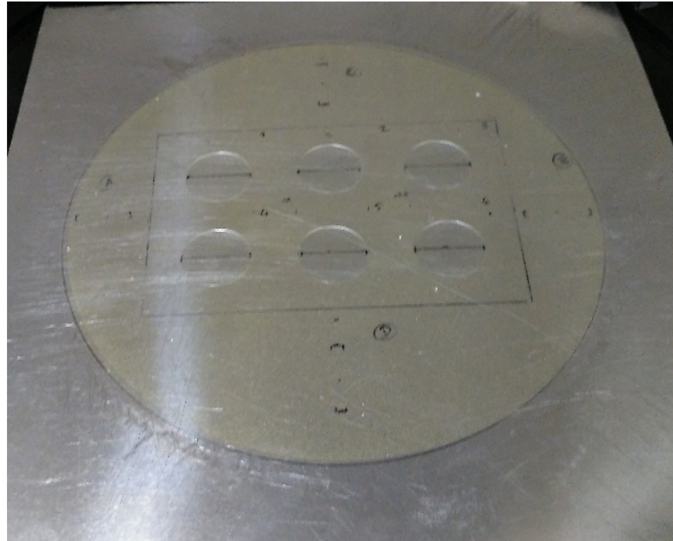

Figure 42: Picture of the deposition plate after pollen exposure.A uniform distribution of the pollen through all the area covered by the PSC can be observed.

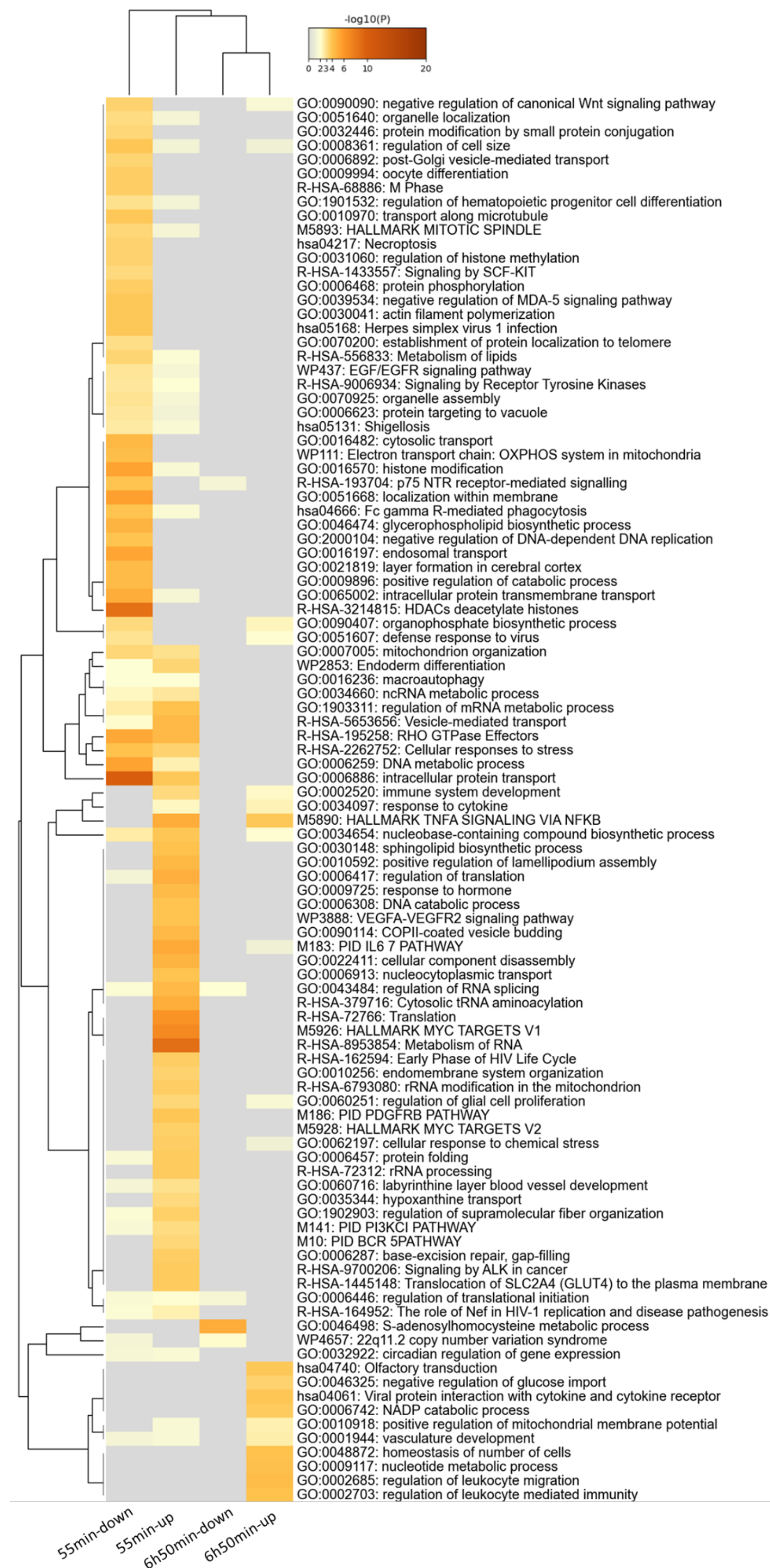

Figure 43: Enrichment heatmap analysis of the 55 min and 6 h 50 min post-exposure times of BEAS-2B exposed to whole birch pollen. The top 100 of the enriched GO Terms

and pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10(p-value).

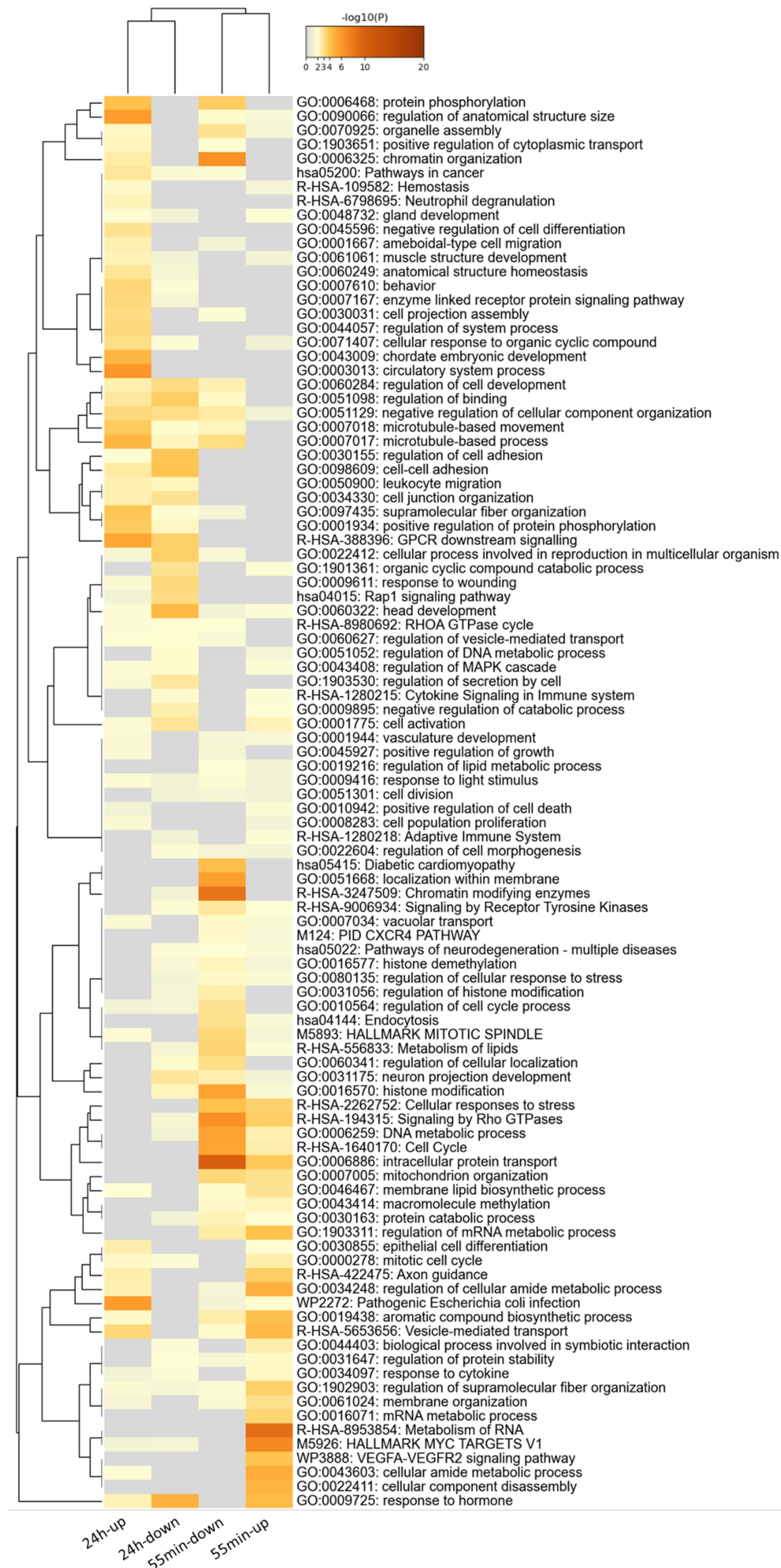

Figure 44: Enrichment heatmap analysis of the 55 min and 24 h post-exposure times of BEAS-2B exposed to whole birch pollen. The top 100 of the enriched GO Terms and

pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10(p-value).

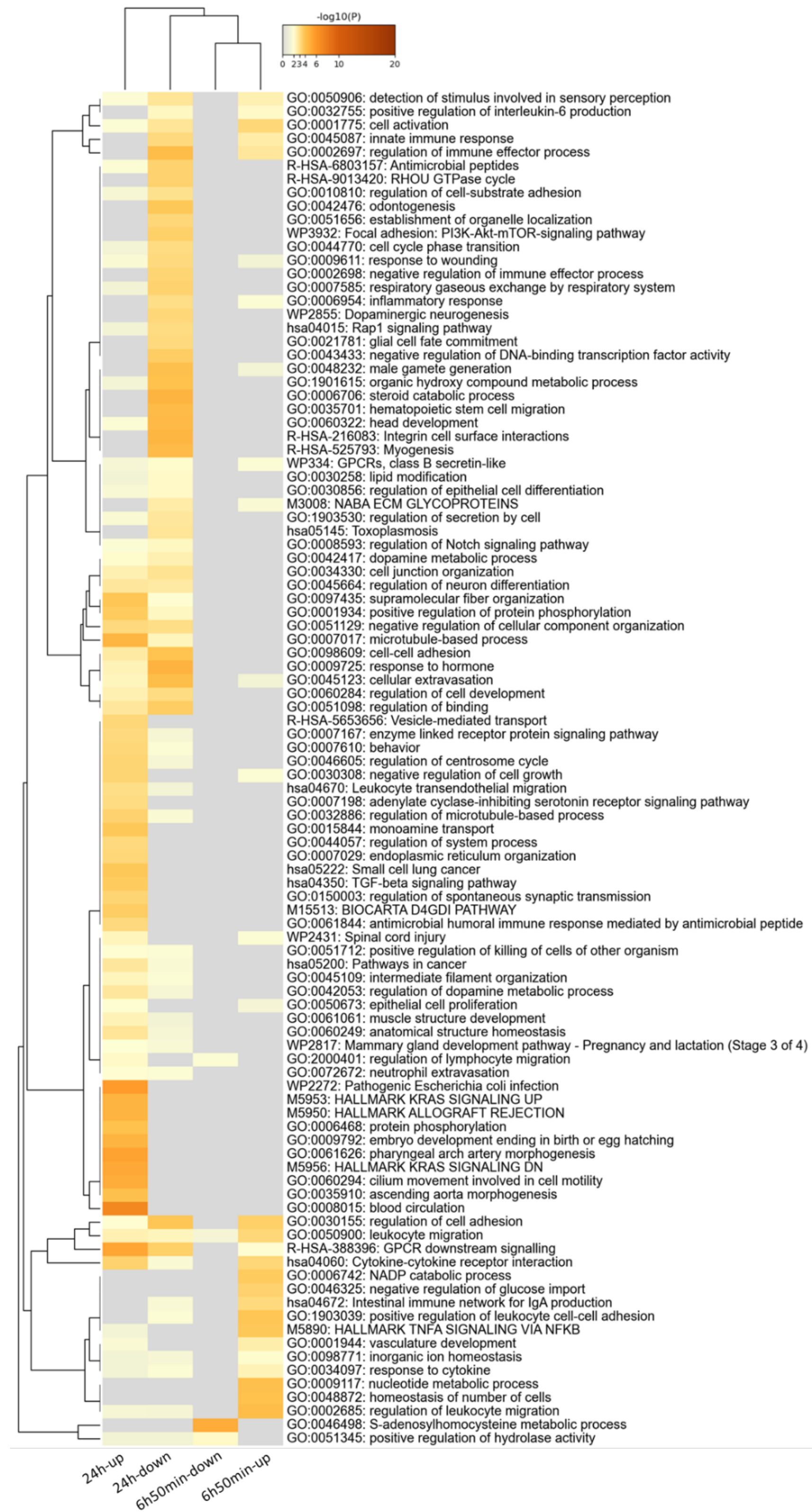

Figure 45: Enrichment heatmap analysis of the 6 h 50 min and 24 h post-exposure times of BEAS-2B exposed to whole birch pollen. The top 100 of the enriched GO Terms and

pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10(p-value).

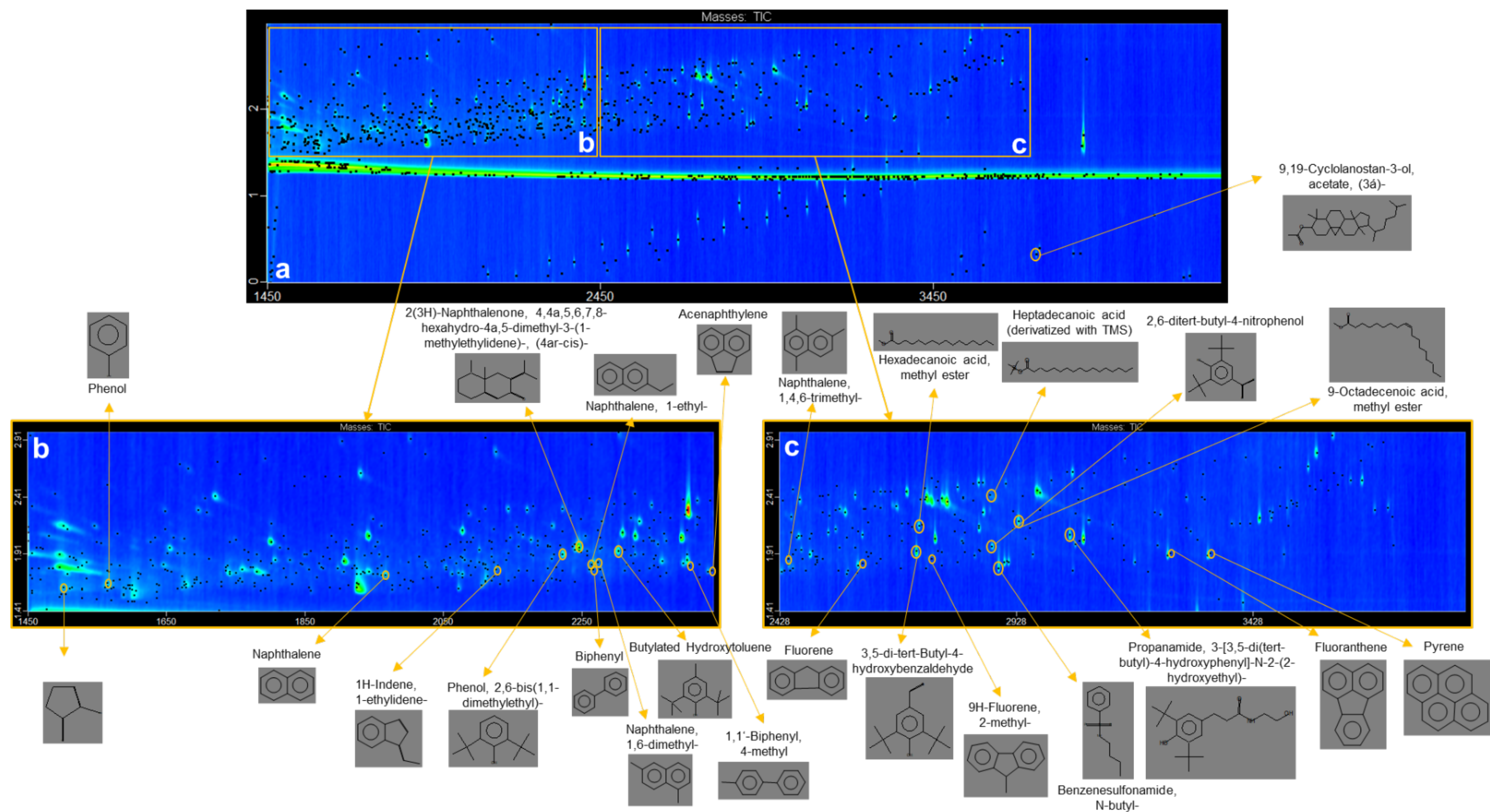

Figure 46: Chemical composition of Diesel exhaust (CAST) by GC×GC-ToFMS. (a) Full GC×GC-ToFMS chromatogram. (b) and (c) Selection of specific areas of the chromatogram with the most abundant compounds highlighted. For methodology see supplemental information.

Table A- 1: Internal standard (ISTD) and derivatization standard (DSTD) used for the analysis of Diesel-CAST samples.

ISTD	DSTD
Acenaphthylene-D8	Fumaric Acid-D2
Acenaphthene-D10	Adipic Acid-D10
Biphenyl-D10	P-Nitrophenol-D4
Fluorene-D10	Glycerol-D8
Anthracene-D10	Levoglucosane 13C6
Phenanthrene-D10	Vanillin 13C6
Fluoranthene-D10	Palmitic Acid-D31
Pyrene-D10	Dodecanol-D25
Benz[a]anthracene-D12	Cholesterol-D6
Chrysene-D12	
Benz[b]fluoranthene-D12	
Benz[k]fluoranthene-D12	
Benz[e]pyrene-D12	
Benz[a]pyrene-D12	
Perylene-D12	
Indeno[1,2,3-cd]pyrene-D12	
Dibenz[a,h]anthracene-D14	
Benz[ghi]perylene-D12	
Coronene-D12	
n-Hexadecane-D34	
n-Docosane-D46	
n-Tetracosane-D50	
n-Triacontane-D62	
Benz[a]anthracene-7,10-dione-D10	
9,10-Anthraquinone 13C6	

Table A- 2: Flow parameters. Carrier gas used: Helium.

Column flow [mL min^{-1}]	Split flow [mL min^{-1}]	t [s]
0.1	10	1200
1	100	90
2.6	0	600
1	100	2510

Table A- 3: Injection temperature profile.

T rate [°C s^{-1}]	T [°C]	t [s]
-	70	40
2	300	1350
-	250	3010

Table A- 4: Column setup.

Column setup	Type	Length [m]
Pre-column	BPX5 (0.25 mm i.d. x 0.25 µm df)	2
1st Dimension	BPX50 (0.25 mm i.d. x 0.25 µm df)	58
2nd Dimension	BPX1 (0.1 mm i.d. x 0.1 µm df)	1.4

Table A- 5: GC oven temperature profile. The secondary oven had a temperature offset of 5 °C relative to the GC oven. The modulator temperature offset was 15 °C relative to the secondary oven. The modulation time was 3 s.

T rate [°C min^{-1}]	T [°C]	Holding time [min]
-	40	5
5	300	-
-	300	15

Table A- 6: Mass spectral parameters.

MS Parameters	Settings
Transferline temperature	280 °C
Acquisition rate	100 Hz
Acquisition range	29 – 750 m/z
Optimized Voltage Offset	100
Electron ionization	70 eV
Ion source temperature	250 °C

Table A- 7: Processing parameters of Leco ChromaTOF. Version 4.50.8.0 optimized for Pegasus.

Baseline Correction		
Baseline Offset	1	auto no. of data points for smoothing
S/N	50	
Peak Find	**1st dimension**	**2nd dimension**
Peak Width [s]	12	0.1
Match required to combine Peaks	-	800
Subpeak	min. S/N	5
Library Search Settings		
Identity/Search Mode	normal/forward	
No. of Hits	10	
Molecular Weight Min./Max.	29/800	
Mass Threshold	50	
Similarity Match Min.	650	
Library	NIST	
Mass for Area Calculation	DTIC	
Export	.csv-files	

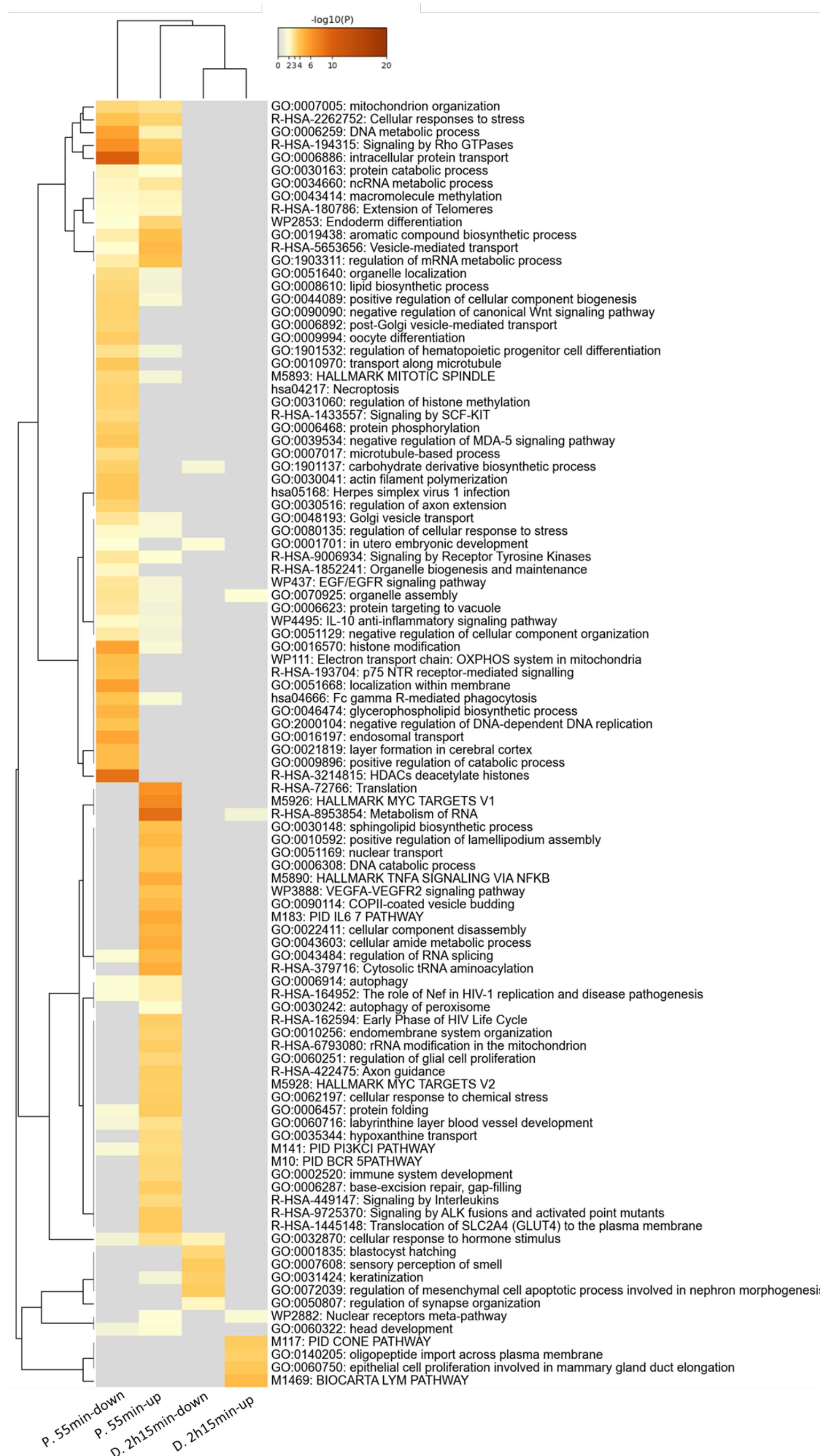

Figure 47: Enrichment heatmap analysis of the 55 min after pollen exposure alone (P.) and 2 h 15 min post-exposure time of BEAS-2B cells exposed to diesel and pollen (D.).

The top 100 of the enriched GO Terms and pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10 (p-value).

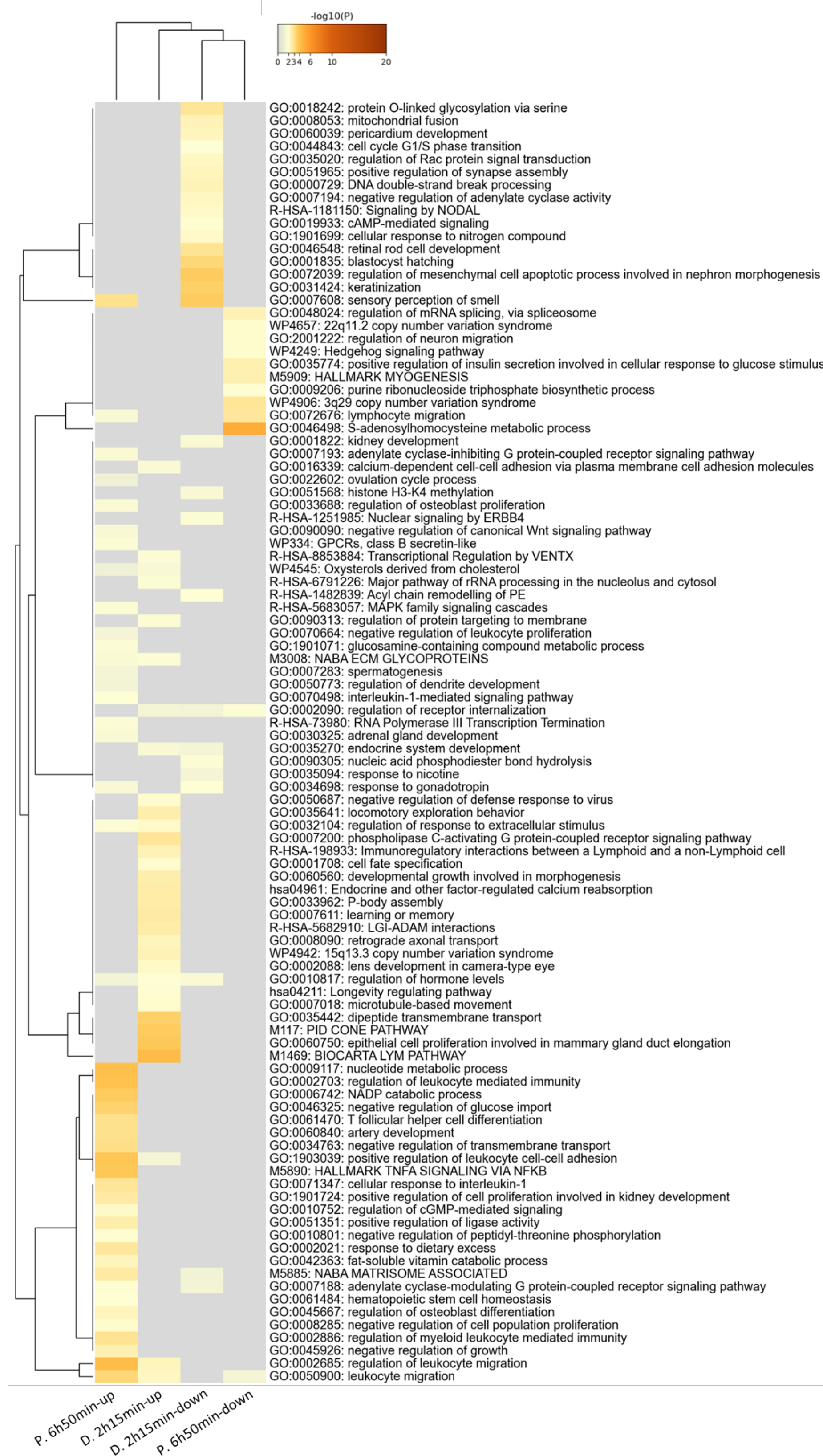

Figure 48: Enrichment heatmap analysis of the 6 h 50 min after pollen exposure alone (P) and 2 h 15 min post-exposure time of BEAS-2B cells exposed to diesel and pollen (D).

The top 100 of the enriched GO Terms and pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10 (p-value).

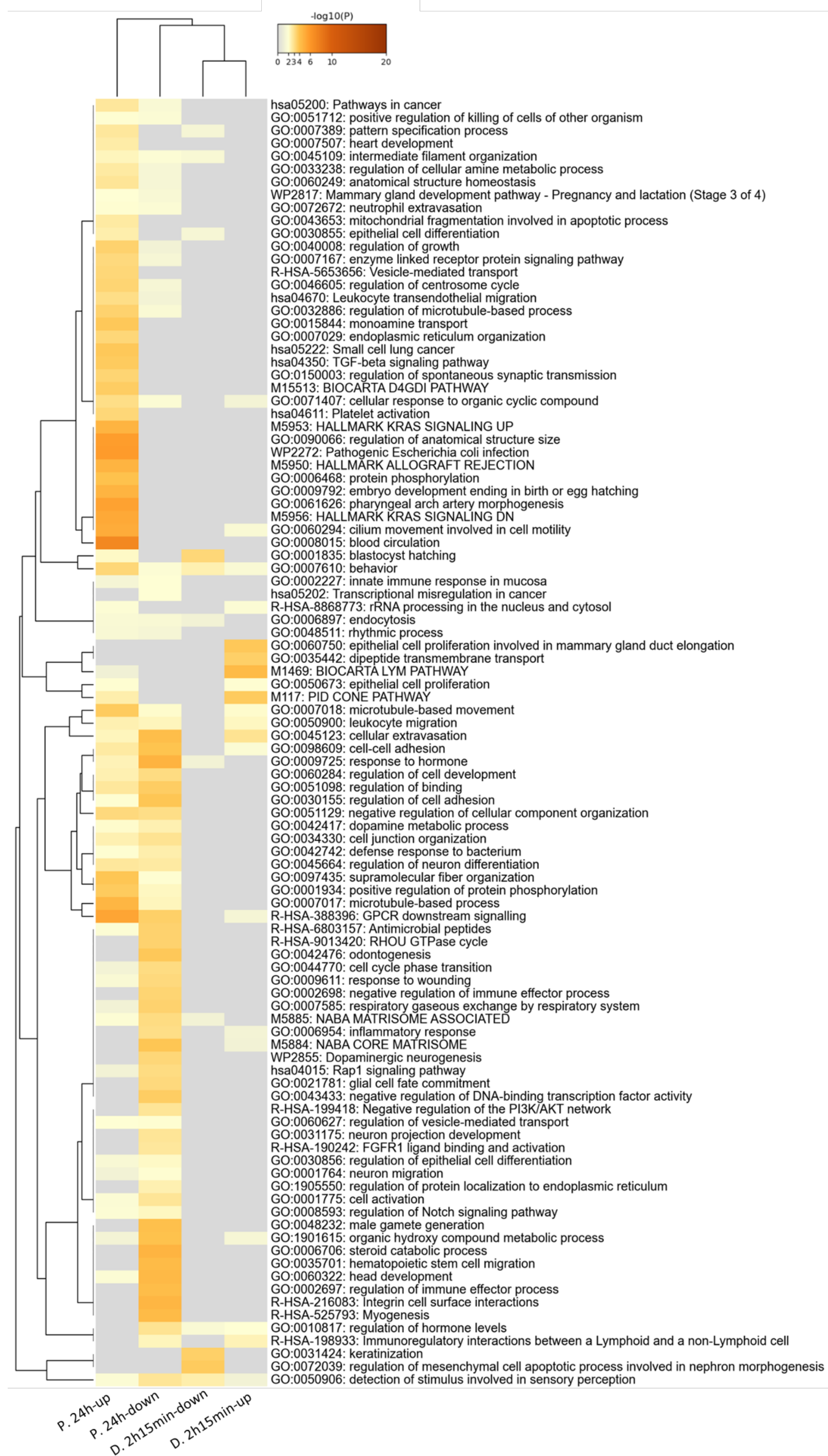

Figure 49: Enrichment heatmap analysis of the 24 h after pollen exposure alone (P) and 2 h 15 min post-exposure time of BEAS-2B cells exposed to diesel and pollen (D). The top

100 of the enriched GO Terms and pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10 (p-value).

Figure 50: Enrichment heatmap analysis of the 55 min after pollen exposure alone (P.) and 2 h 15 min post-exposure time of BEAS-2B cells exposed to propane and pollen

(Prop.). The top 100 of the enriched GO Terms and pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10 (p-value).

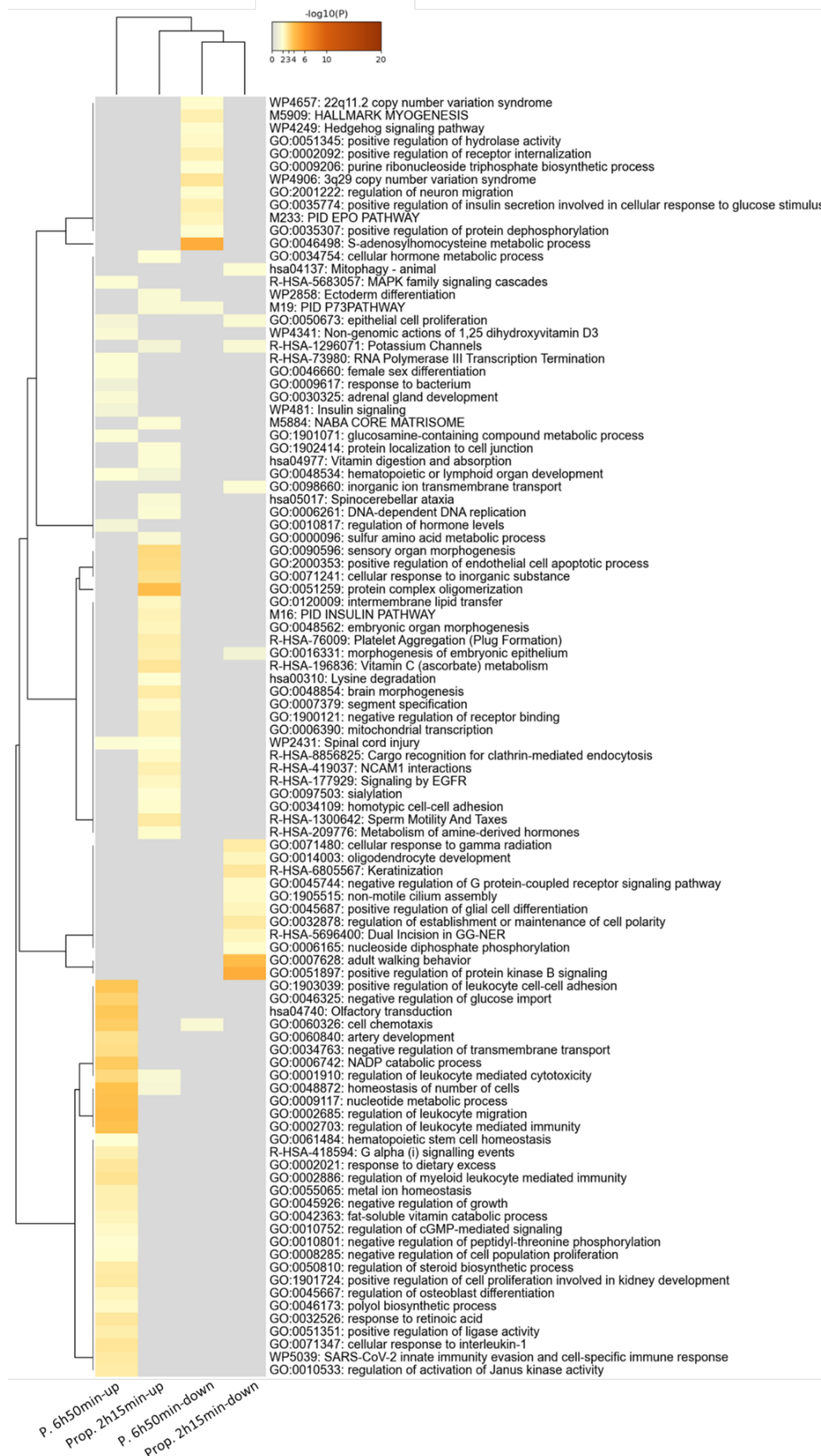

Figure 51: Enrichment heatmap analysis of the 6 h 50 min after pollen exposure alone (P.) and 2 h 15 min post-exposure time of BEAS-2B cells exposed to propane and pollen

(Prop.). The top 100 of the enriched GO Terms and pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10 (p-value).

Figure 52: Enrichment heatmap analysis of the 24 h after pollen exposure alone (P.) and 2 h 15 min post-exposure time of BEAS-2B cells exposed to propane and pollen (Prop.).

The top 100 of the enriched GO Terms and pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10 (p-value).

Figure 53: Enrichment heatmap analysis of the 2 h 15min combined diesel exposure (D.) and 2 h 15 min post-exposure time of BEAS-2B cells exposed to propane and pollen

(Prop.). The top 100 of the enriched GO Terms and pathways is shown. Up- and down-regulated lists are shown for both exposures. Data is expressed as –log10 (p-value).

Table A- 8: Metascape enrichment pathway analysis for the up-regulated genes, at 55 min after 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0062197	cellular response to chemical stress	-5,198	-1,663	23/349	APEX1,DNAJA1,IL6,MCL1,MT3,NR4A2,PCNA,TPM1,TRPC6,XRCC5,NR4A3,KLF4,BAG5,TRAP1,PRDX3,CAB39,ERRFI1,ADPRS,ANKZF1,SELENOS,DIABLO,VKORC1L1,GPX8
GO Biological Processes	GO:0034599	cellular response to oxidative stress	-4,150	-1,098	19/301	APEX1,IL6,MCL1,MT3,NR4A2,PCNA,TPM1,TRPC6,NR4A3,KLF4,BAG5,TRAP1,PRDX3,ADPRS,ANKZF1,SELENOS,DIABLO,VKORC1L1,GPX8
GO Biological Processes	GO:0030148	sphingolipid biosynthetic process	-5,128	-1,663	12/109	CCN1,SMPD1,UGCG,B4GALT3,VAPA,CERS2,ELOVL5,ST6GALNAC5,TLCD3B,HACD2,CERS3,ENPP7
GO Biological Processes	GO:0022411	cellular component disassembly	-5,125	-1,663	31/565	DNASE1L3,DNASE2,GLE1,GSPT1,HSPA8,IL6,KLC1,LRP1,MMP14,PIK3R1,PPP1CA,RDX,MRPL12,SMARCC1,TOP2A,SQSTM1,WDR1,GABARAPL2,AKAP8L,MRPS7,MRPL35,TEX264,VTA1,MAP1S,SCAF4,ATG9A,MTPN,CARMIL2,RETREG3,ARID2,CTRB2
GO Biological Processes	GO:0032984	protein-containing complex disassembly	-3,136	-0,687	18/334	GLE1,GSPT1,HSPA8,KLC1,PPP1CA,RDX,MRPL12,SMARCC1,WDR1,GABARAPL2,MRPS7,MRPL35,VTA1,MAP1S,SCAF4,MTPN,CARMIL2,ARID2
Canonical Pathways	M183	PID IL6 7 PATHWAY	-4,999	-1,588	8/47	CEBPD,MAPK14,IL6,JUNB,MCL1,PIK3R1,RAC1,HSP90B1,DYNC1LI2,CXCL2,KLC1,RPSA,ARPC1B,MYH14,POTEF,CBFB,DUSP5,HES1,HSPA8,ARF1,GSDME,PHB,MMP14,PCNA,SQSTM1,GABARAPL2,PLCD3
Canonical Pathways	M166	PID ATF2 PATHWAY	-2,654	-0,548	6/59	CBFB,MAPK14,DUSP5,HES1,IL6,JUNB
Reactome Gene Sets	R-HSA-6785807	Interleukin-4 and Interleukin-13 signaling	-1,923	-0,219	7/108	CEBPD,HSPA8,IL6,JUNB,MCL1,PIK3R1,HSP90B1
WikiPathways	WP364	IL-6 signaling pathway	-1,786	-0,172	4/43	IL6,JUNB,PIK3R1,RAC1
Reactome Gene Sets	R-HSA-9662834	CD163 mediating an anti-inflammatory response	-1,775	-0,172	2/9	MAPK14,IL6
KEGG Pathway	ko04668	TNF signaling pathway	-1,428	-0,030	6/108	MAPK14,CXCL2,IL6,JUNB,MMP14,PIK3R1
GO Biological Processes	GO:0006418	tRNA aminoacylation for protein translation	-3,280	-0,742	6/45	AARS1,CARS1,GARS1,TARS1,YARS1,FARSB
Hallmark Gene Sets	M5890	HALLMARK TNFA SIGNALING VIA NFKB	-4,808	-1,485	16/200	CEBPD,DUSP2,DUSP5,CXCL2,HES1,CCN1,IL6,JUNB,MCL1,NR4A2,KLF10,NR4A3,IER3,SQSTM1,KLF4,TNIP2
GO Biological Processes	GO:0110053	regulation of actin filament organization	-3,706	-0,914	17/273	ARF1,CCN2,ARHGAP35,PIK3R1,RAC1,RDX,CCL24,TPM1,WDR1,ARPC1B,ACTR3,ABI2,SORBS3,CYFIP1,MTPN,CARMIL2,CDC42EP5
GO Biological Processes	GO:0002431	Fc receptor mediated stimulatory signaling pathway	-1,736	-0,150	8/145	LYN,PIK3R1,PTPRC,RAC1,NR4A3,ARPC1B,ACTR3,CYFIP1
GO Biological Processes	GO:0002433	immune response-regulating cell surface receptor signaling pathway involved in phagocytosis	-1,393	-0,020	7/139	LYN,PIK3R1,PTPRC,RAC1,ARPC1B,ACTR3,CYFIP1
Reactome Gene Sets	R-HSA-5653656	Vesicle-mediated transport	-4,060	-1,098	32/673	ARF1,CD4,DNM1,DNASE2,DYNC1LI2,HSPA8,IGF2R,KLC1,LRP1,PRKAG1,RAB13,RAC1,SORT1,SLC18A3,HSP90B1,YWHAZ,TUBA1A,APOL1,CYTH2,SEC16A,ACTR3,RABEPK,TFG,YWHAQ,GABARAPL2,SEC31A,COPS7A,VTA1,EXOC1,SYT8,SC

						GB3A2,TUBB8
GO Biological Processes	GO:0006986	response to unfolded protein	-2,935	-0,631	12/185	GFPT1,DNAJA1,HSPA4,HSPA8,PIK3R1,HSP90B1,BAG3,PDIA6,SEC31A,FKBP14,SELENOS,DERL1
GO Biological Processes	GO:0044270	cellular nitrogen compound catabolic process	-2,805	-0,608	26/602	APEX1,MAPK14,DAZ1,DNASE1L3,DNASE2,FEN1,GSPT1,HSPA8,IL6,RPSA,YBX1,PSMD11,RPL18,RPL22,RPS3A,YWHAZ,CSDE1,ANP32A,SYNCRIP,XRN2,NT5C2,PABPC1,SAMD4B,SMG8,FBH1,NT5C3B

Table A- 9: Metascape enrichment pathway analysis for the down-regulated genes, at 55 min after 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
Reactome Gene Sets	R-HSA-5334118	DNA methylation	-6,388	-3,136	13/65	DNMT3B,H2AC8,H2AC7,H2BC5,H2AC14,H2BC13,H2BC15,H2BC14,H2BC9,H2BC17,H2BC11,H2BU1,H3C15
GO Biological Processes	GO:0060271	cilium assembly	-2,983	-0,813	28/415	CFAP410,CSNK1E,GAS8,RAB8A,RFX2,CDKL5,CDK10,CEP135,ENTR1,NINL,CEP131,HAUS5,NUDCD3,B9D1,EHD4,TBC1D13,ODAD2,TRAPPC14,CENPJ,WDR19,TXNDC15,CEP290,B9D2,TBC1D10A,WDR90,NPHP4,TMEM80,DNAJB13
KEGG Pathway	hsa00190	Oxidative phosphorylation	-3,017	-0,836	13/133	ATP5MC2,ATP6V0C,ATP6V1C1,ATP5PO,COX6C,COX7C,ND3,ND6,NDUFA3,NDUFB4,NDUFB9,NDUFC1,UQCR11
GO Biological Processes	GO:0040029	regulation of gene expression, epigenetic	-3,831	-1,433	19/205	DNMT3B,EZH1,GSK3A,H1-2,H2AC8,H2AC7,JARID2,POLR2E,H2AC15,H2AC17,TAF1C,MTA1,PCGF3,POLR1A,SERTAD1,METTL3,ZNF335,H2AC12,H3C15

Table A- 10: Metascape enrichment pathway analysis for the up-regulated genes, at 6 h 50 min after 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0050900	leukocyte migration	-4,178	-0,155	14/511	CX3CR1,GPR183,IL6,LEP,CXCL9,CCN3,CCL8,CCL18,CXCL6,RIPK3,CD300A,IGLV2-14,IGKV2D-30,IL23A
KEGG Pathway	hsa04062	Chemokine signaling pathway	-3,180	-0,152	7/182	CX3CR1,CXCL9,PLCB4,CCL8,CCL18,CXCL6,SHC3
GO Biological Processes	GO:1990868	response to chemokine	-2,965	-0,126	5/97	CX3CR1,CXCL9,CCL8,CCL18,CXCL6
KEGG Pathway	hsa04060	Cytokine-cytokine receptor interaction	-2,822	-0,124	8/270	CX3CR1,IL6,LEP,CXCL9,CCL8,CCL18,CXCL6,IL23A
GO Biological Processes	GO:0009593	detection of chemical stimulus	-2,545	-0,090	11/513	OR10A3,OR4K15,OR6N1,OR52B4,OR51D1,OR9G1,OR10V1,OR6C76,OR14I1,OR2T27,OR4M1
GO Biological Processes	GO:0030217	T cell differentiation	-3,777	-0,155	9/246	CR1,GPR183,EGR1,IL6,LEP,IL1RL2,RIPK3,IL23A,DLL4

GO Biological Processes	GO:0046427	positive regulation of receptor signaling pathway via JAK-STAT	-3,385	-0,152	4/44	IL6,LEP,CD300A,IL23A
GO Biological Processes	GO:0042110	T cell activation	-3,341	-0,152	12/475	CR1,GPR183,EGR1,IL6,LEP,IL1RL2,RIPK3,CD300A,ICOS,IL23A,APBB1IP,DLL4
GO Biological Processes	GO:0002697	regulation of immune effector process	-2,355	-0,028	10/467	A2M,CR1,IL6,LEP,CXCL6,RIPK3,CD300A,IGLV2-14,IGKV2D-30,IL23A
GO Biological Processes	GO:0030155	regulation of cell adhesion	-1,824	0,000	12/734	CDSN,CR1,IL6,LEP,IL1RL2,CD300A,ICOS,IL23A,APBB1IP,FERMT1,TRPV4,FRMD 5
GO Biological Processes	GO:0001817	regulation of cytokine production	-1,308	0,000	11/782	CR1,CX3CR1,EGR1,IL6,LEP,CXCL6,IL1RL2,RIPK3,IL23A,FERMT1,POLR3H
GO Biological Processes	GO:0006742	NADP catabolic process	-3,726	-0,155	2/3	NUDT13,NUDT12
GO Biological Processes	GO:0009117	nucleotide metabolic process	-3,391	-0,152	13/538	AMPD1,ATP5MC1,NPR1,PDE2A,PPAT,TK2,NUDT13,ACOT11,ACSM5,MFN1,ANT KMT,DCAKD,NUDT12
GO Biological Processes	GO:0034341	response to interferon-gamma	-1,679	0,000	5/197	CYP27B1,HLA-DQA1,HLA-DQA2,CCL8,CCL18
KEGG Pathway	hsa05310	Asthma	-1,599	0,000	2/31	HLA-DQA1,HLA-DQA2
KEGG Pathway	ko04658	Th1 and Th2 cell differentiation	-1,429	0,000	3/92	HLA-DQA1,HLA-DQA2,DLL4
GO Biological Processes	GO:0006968	cellular defense response	-3,045	-0,126	4/54	CX3CR1,CXCL9,NCF2,IL1RL2
WikiPathways	WP3929	Chemokine signaling pathway	-1,365	0,000	4/165	CX3CR1,CXCL9,PLCB4,SHC3
GO Biological Processes	GO:0071347	cellular response to interleukin-1	-2,486	-0,074	6/180	EGR1,IL6,MAP3K3,CCL8,CCL18,IL1RL2
GO Biological Processes	GO:0071496	cellular response to external stimulus	-1,458	0,000	6/303	CYP27B1,FOXA3,LEP,PDE2A,NPRL3,WDR59
WikiPathways	WP619	Type II interferon signaling (IFNG)	-1,457	0,000	2/37	IFIT2,CXCL9
GO Biological Processes	GO:0045916	negative regulation of complement activation	-2,660	-0,102	2/9	A2M,CR1
GO Biological Processes	GO:0022617	extracellular matrix disassembly	-1,543	0,000	3/83	A2M,IL6,TLL2
GO Biological Processes	GO:0051133	regulation of NK T cell activation	-2,660	-0,102	2/9	CD300A,IL23A

Table A- 11: Metascape enrichment pathway analysis for the down-regulated genes, at 6 h 50 min after 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological	GO:0072678	T cell migration	-2,946	0,000	3/66	CRKL,CCL26,C10orf99

Processes						
GO Biological Processes	GO:0050900	leukocyte migration	-2,295	0,000	6/511	CRKL,IGKC,MAG,CCL26,IGLV1-44,C10orf99
GO Biological Processes	GO:0006898	receptor-mediated endocytosis	-2,425	0,000	5/333	FMR1,IGKC,MAGI2,RABEPK,IGLV1-44
GO Biological Processes	GO:0043255	regulation of carbohydrate biosynthetic process	-2,480	0,000	3/96	GNMT,PPP1R3E,C1QTNF12
GO Biological Processes	GO:0030449	regulation of complement activation	-2,251	0,000	3/116	A2M,IGKC,IGLV1-44

Table A- 12: Metascape enrichment pathway analysis for the up-regulated genes, at 24 h after 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
KEGG Pathway	hsa04530	Tight junction	-2,584	-0,352	12/170	CD1A,ITGB1,MYH2,ROCK1,TUBA3C,EZR,MAGI1,ROCK2,CLDN20,TUBAL3,IGSF5,AMOT
GO Biological Processes	GO:1901889	negative regulation of cell junction assembly	-1,962	-0,194	4/32	ACVRL1,ROCK1,SLIT1,ROCK2
GO Biological Processes	GO:0001820	serotonin secretion	-5,467	-1,800	5/10	CRH,HTR1A,HTR1B,P2RX1,SYK
GO Biological Processes	GO:0071868	cellular response to monoamine stimulus	-2,106	-0,242	8/104	APP,HRH1,HTR1A,HTR1B,HTR1D,KCNQ1,PRKN,VPS35
GO Biological Processes	GO:0071870	cellular response to catecholamine stimulus	-2,106	-0,242	8/104	APP,HRH1,HTR1A,HTR1B,HTR1D,KCNQ1,PRKN,VPS35
GO Biological Processes	GO:0071407	cellular response to organic cyclic compound	-5,242	-1,655	35/558	APP,CASP3,CCNB1,CRH,EIF4E,ESR2,FOLR2,GABRB3,GJB2,GNAI1,HRH1,HTR1A,HTR1B,HTR1D,ITGA2,KCNQ1,MN1,NPM1,P2RY1,PRKN,PPARD,RXRB,RXRG,SLC8A3,SMARCA4,EZR,TRIM24,ROCK2,DNM1L,PGRMC2,CIB2,WBP2,VPS35,LMO3,GRAMD1A
GO Biological Processes	GO:0071396	cellular response to lipid	-1,701	-0,103	24/556	CCNB1,CRH,EIF4E,ESR2,GJB2,GNAI1,ITGA2,MN1,PF4,PPARD,RXRB,RXRG,SMARCA4,TNFSF4,TRIM24,ROCK2,DNM1L,PGRMC2,CXCL13,WBP2,LMO3,GRAMD1A,SGMS1,BRINP3
GO Biological Processes	GO:0030522	intracellular receptor signaling pathway	-1,698	-0,102	14/275	ESR2,MN1,PPARD,PRCP,RARA,RXRB,RXRG,SLC15A2,SMARCA4,TRIM24,ZNF536,WBP2,LMO3,DCBLD2
GO Biological Processes	GO:0060294	cilium movement involved in cell motility	-4,824	-1,444	13/114	AKAP4,DNAAF11,CCDC40,QRICH2,IQCF1,TEKT4,CFAP206,TTLL9,TSSK4,EFCAB9,CFAP157,CATSPER4,HOATZ
KEGG Pathway	hsa04350	TGF-beta signaling pathway	-4,004	-0,960	10/84	ACVR2A,BMPR1A,BMPR2,E2F5,ID4,SMAD9,ROCK1,TGFB2,TGIF1,ACVR1C

Table A- 13: Metascape enrichment pathway analysis for the down-regulated genes, at 24 h after 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0045123	cellular extravasation	-5,249	-0,883	12/71	ADD2,AGER,BST1,ITGAL,ITGB7,PIK3CG,PRTN3,MADCAM1,OLFM4,PLCB1,PODXL2,JAM2
GO Biological Processes	GO:0061756	leukocyte adhesion to vascular endothelial cell	-1,407	0,000	5/54	ADD2,ITGB7,MADCAM1,PODXL2,JAM2
GO Biological Processes	GO:1901615	organic hydroxy compound metabolic process	-4,189	-0,796	38/555	ACADL,AGTR2,ALOX15,APOA2,APOA4,BMP6,AKR1C4,CYP3A4,CYP3A5,CYP7A1,CYP11B2,CYP19A1,DDC,CYB5R3,FGF1,GOT1,GPD2,GRIN2A,HPRT1,KIT,LDHA,NR4A2,RAN,RDH5,AKR1D1,FGF23,NR0B2,SOAT2,SCARF1,SYNJ1,ABCG1,EBP,CYP46A1,PLCB1,CYP4V2,RTL4,DUOXA2,MOXD2P
WikiPathways	WP43	Oxidation by Cytochrome P450	-2,831	-0,334	8/63	CYP3A4,CYP3A5,CYP7A1,CYP11B2,CYP19A1,CYB5R3,CYP46A1,CYP4V2
Reactome Gene Sets	R-HSA-211897	Cytochrome P450 - arranged by substrate type	-2,699	-0,325	8/66	CYP3A4,CYP3A5,CYP7A1,CYP11B2,CYP19A1,POMC,CYP46A1,CYP4V2
GO Biological Processes	GO:1901616	organic hydroxy compound catabolic process	-2,280	-0,229	8/77	CYP7A1,GPD2,AKR1D1,FGF23,SCARF1,SYNJ1,CYP46A1,MOXD2P
Reactome Gene Sets	R-HSA-211859	Biological oxidations	-1,325	0,000	13/222	CBR3,CYP3A4,CYP3A5,CYP7A1,CYP11B2,CYP19A1,CYB5R3,POMC,CYP46A1,SULT4A1,PODXL2,CYP4V2,GLYATL3
GO Biological Processes	GO:0043624	cellular protein complex disassembly	-4,455	-0,796	21/222	ADD2,FLII,MAP1B,PEX14,ATXN7,SPAST,SYNJ1,ARHGEF2,NES,MRPL3,MRPS28,MRPL51,MRPL30,MRPS10,CRACD,MICAL3,MRPS15,MRPL32,KIF2B,MRRF,NCKAP5
Reactome Gene Sets	R-HSA-216083	Integrin cell surface interactions	-4,431	-0,796	12/85	COL2A1,COL7A1,COL9A3,COL13A1,COL16A1,COMP,IBSP,ITGAL,ITGB3,ITGB7,MADCAM1,JAM2
GO Biological Processes	GO:0034332	adherens junction organization	-4,051	-0,796	10/66	BMP6,CDC42,CDH5,CDH10,CDH15,CTNNA1,CTNND1,DSP,CADM1,CDH20
GO Biological Processes	GO:0045216	cell-cell junction organization	-2,520	-0,287	16/211	BMP6,CDC42,CDH5,CDH10,CDH15,CTNNA1,CTNND1,DSP,PKHD1,PRTN3,TGFB3,CADM1,CDH20,CLDN22,PARD3,MARVELD3
GO Biological Processes	GO:0046649	lymphocyte activation	-4,028	-0,796	47/754	AGER,AIF1,BST1,CDC42,MSH6,HLX,HPRT1,IGHA1,IL7R,IDO1,INS,ITGAL,KIT,LCK,LGALS9,CD180,MFNG,MLH1,PAK3,PIK3CG,PRNP,SLAMF1,SOX12,WAS,WNT1,YY1,FOXN1,TNFSF11,TESPA1,CCR9,PAXIP1,JMJD6,CADM1,IGHV4-34,IGHV3-72,IGHV3-66,IGHV1-18,ZBTB7B,MZB1,MARCHF7,BCL11B,NKAP,TNFRSF13C,PIK3R6,TICAM1,UNC13D,TARM1
Reactome Gene Sets	R-HSA-6803157	Antimicrobial peptides	-3,336	-0,408	11/95	DEFA5,REG3A,PRTN3,TLR1,BPIFB1,RNASE8,DEFB108B,DEFB114,DEFB128,DEFB132,DEFB131A
GO Biological Processes	GO:0051223	regulation of protein transport	-3,327	-0,408	35/549	AGTR2,APOD,BMP6,CDC42,GLP1R,GPLD1,FFAR1,DNAJA1,HSPA1L,INS,ITGB3,NNAT,NOS2,PAM,CDK16,PPID,MAPK1,PRNP,RAN,TGFB3,TP53BP2,BAP1,NR0B2,BRSK2,ITGB1BP1,ABCG1,WWP2,TARDBP,NUP62,KRT20,ANO1,VPS11,RHOU,SYT9,BMP8A
WikiPathways	WP3932	Focal Adhesion-PI3K-Akt-mTOR-s	-3,257	-0,408	23/309	COL2A1,COMP,CSH1,EFNA2,FGF1,GNG4,IBSP,IL2RB,IL7R,INS,ITGAL,ITGB3,ITGB7,KIT,NOS2,PFKFB1,PIK3CG,MAPK1,PRLR,PHLPP2,FGF22,CAB39L,GNG8

Table A- 14: Metascape enrichment pathway analysis for the up-regulated genes, at 55 min after 10 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
Hallmark Gene Sets	M5890	HALLMARK TNFA SIGNALING VIA NFKB	-15,037	-10,673	8/200	EGR2,CXCL2,IL1B,IL6,TNFAIP3,NR4A3,IER3,ZC3H12A
WikiPathways	WP4754	IL-18 signaling pathway	-11,621	-7,557	7/273	CXCL2,IL1B,IL6,TNFAIP3,IER3,NFKBIZ,ZC3H12A
GO Biological Processes	GO:0071216	cellular response to biotic stimulus	-7,781	-4,417	5/233	CXCL2,IL1B,IL6,TNFAIP3,ZC3H12A
GO Biological Processes	GO:0071396	cellular response to lipid	-7,567	-4,244	6/565	CXCL2,IL1B,IL6,TNFAIP3,NR4A3,ZC3H12A
KEGG Pathway	ko04657	IL-17 signaling pathway	-7,440	-4,185	4/93	CXCL2,IL1B,IL6,TNFAIP3
WikiPathways	WP530	Cytokines and Inflammatory Response	-6,939	-3,862	3/26	CXCL2,IL1B,IL6
GO Biological Processes	GO:0050865	regulation of cell activation	-9,050	-5,163	7/636	CCN2,IL1B,IL6,TNFAIP3,NR4A3,NFKBIZ,ZC3H12A
GO Biological Processes	GO:0002822	regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	-8,670	-4,907	5/155	IL1B,IL6,TNFAIP3,NFKBIZ,ZC3H12A
GO Biological Processes	GO:0002819	regulation of adaptive immune response	-8,468	-4,802	5/170	IL1B,IL6,TNFAIP3,NFKBIZ,ZC3H12A
GO Biological Processes	GO:0002697	regulation of immune effector process	-8,021	-4,607	6/474	IL1B,IL6,TNFAIP3,NR4A3,NFKBIZ,ZC3H12A
GO Biological Processes	GO:0072539	T-helper 17 cell differentiation	-6,792	-3,802	3/29	IL6,NFKBIZ,ZC3H12A
GO Biological Processes	GO:0097237	cellular response to toxic substance	-5,753	-3,090	4/245	IL6,TNFAIP3,NR4A3,ZC3H12A
GO Biological Processes	GO:0034599	cellular response to oxidative stress	-5,370	-2,807	4/306	IL6,TNFAIP3,NR4A3,ZC3H12A
GO Biological Processes	GO:0032651	regulation of interleukin-1 beta production	-5,101	-2,666	3/104	IL6,TNFAIP3,ZC3H12A
GO Biological Processes	GO:0050867	positive regulation of cell activation	-6,533	-3,616	5/415	CCN2,IL1B,IL6,NR4A3,NFKBIZ
GO Biological Processes	GO:0002720	positive regulation of cytokine production involved in immune response	-5,987	-3,235	3/53	IL1B,IL6,NR4A3
GO Biological Processes	GO:0002699	positive regulation of immune effector process	-5,908	-3,187	4/224	IL1B,IL6,NR4A3,NFKBIZ
GO Biological Processes	GO:0022409	positive regulation of cell-cell adhesion	-5,548	-2,946	4/276	IL1B,IL6,NR4A3,NFKBIZ
GO Biological Processes	GO:0032675	regulation of interleukin-6 production	-6,514	-3,612	4/158	IL1B,IL6,TNFAIP3,ZC3H12A
GO Biological Processes	GO:0032635	interleukin-6 production	-6,470	-3,583	4/162	IL1B,IL6,TNFAIP3,ZC3H12A
GO Biological Processes	GO:0071347	cellular response to interleukin-1	-4,388	-2,211	3/180	IL1B,IL6,ZC3H12A

GO Biological Processes	GO:1901652	response to peptide	-6,025	-3,259	5/526	CCN2,EGR2,IL1B,TNFAIP3,NR4A3
GO Biological Processes	GO:0080135	regulation of cellular response to stress	-5,239	-2,744	5/761	CCN2,IL1B,NR4A3,IER3,ZC3H12A
Hallmark Gene Sets	M5891	HALLMARK HYPOXIA	-6,104	-3,311	4/200	CCN2,IL6,TNFAIP3,IER3
GO Biological Processes	GO:0002366	leukocyte activation involved in immune response	-3,915	-1,859	4/720	IL6,NR4A3,NFKBIZ,ZC3H12A
GO Biological Processes	GO:0009611	response to wounding	-3,960	-1,890	4/701	CCN2,IL6,TNFAIP3,P2RX6
GO Biological Processes	GO:0042060	wound healing	-2,881	-1,151	3/587	IL6,TNFAIP3,P2RX6
GO Biological Processes	GO:0006913	nucleocytoplasmic transport	-3,571	-1,613	3/340	EGR2,IL1B,ZC3H12A
GO Biological Processes	GO:1990868	response to chemokine	-3,198	-1,352	2/97	CXCL2,ZC3H12A

Table A- 15: Metascape enrichment pathway analysis for the up-regulated genes, at 2 h 50 min after 10 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
Hallmark Gene Sets	M5890	HALLMARK TNFA SIGNALING VIA NFKB	-20,845	-16,539	12/200	CSF2,DUSP5,F3,CCN1,IL1B,LIF,MYC,MAP2K3,NR4A3,BHLHE40,IER3,SOCS3
Hallmark Gene Sets	M5891	HALLMARK HYPOXIA	-10,228	-6,223	7/200	CCN2,F3,CCN1,PIM1,BHLHE40,IER3,ANGPTL4
Reactome Gene Sets	R-HSA-1280215	Cytokine Signaling in Immune system	-9,302	-5,582	9/707	CSF2,DUSP6,GBP1,IL1B,LIF,MYC,PIM1,MAP2K3,SOCS3
GO Biological Processes	GO:0043408	regulation of MAPK cascade	-9,128	-5,522	9/740	CCN2,DUSP5,DUSP6,GBP1,CCN1,IL1B,LIF,MYC,MAP2K3
Hallmark Gene Sets	M5953	HALLMARK KRAS SIGNALING UP	-8,369	-5,018	6/200	CSF2,DUSP6,EREG,IL1B,LIF,ANGPTL4
Hallmark Gene Sets	M5932	HALLMARK INFLAMMATORY RESPONSE	-6,610	-3,720	5/200	EREG,F3,IL1B,LIF,MYC
Reactome Gene Sets	R-HSA-6783783	Interleukin-10 signaling	-5,311	-2,696	3/47	CSF2,IL1B,LIF
KEGG Pathway	hsa04010	MAPK signaling pathway	-7,155	-4,121	6/320	DUSP5,DUSP6,EREG,IL1B,MYC,MAP2K3
Reactome Gene Sets	R-HSA-5683057	MAPK family signaling cascades	-5,571	-2,889	5/325	CSF2,DUSP5,DUSP6,EREG,MYC
Hallmark Gene Sets	M5897	HALLMARK IL6 JAK STAT3 SIGNALING	-6,400	-3,557	4/87	CSF2,IL1B,PIM1,SOCS3
Hallmark Gene Sets	M5921	HALLMARK COMPLEMENT	-4,959	-2,439	4/200	DUSP5,DUSP6,F3,PIM1
WikiPathways	WP3888	VEGFA-VEGFR2 Signaling Pathway	-6,371	-3,542	6/435	CCN2,DUSP5,F3,CCN1,MAP2K3,NR4A3
GO Biological Processes	GO:0001817	regulation of cytokine production	-6,104	-3,317	7/795	CSF2,EREG,F3,GBP1,IL1B,MAP2K3,NR4A3

WikiPathways	WP619	Type II interferon signaling (IFNG)	-5,629	-2,909	3/37	GBP1,IL1B,SOCS3
GO Biological Processes	GO:0032663	regulation of interleukin-2 production	-3,096	-1,133	2/59	GBP1,IL1B
GO Biological Processes	GO:0043280	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	-5,598	-2,905	4/138	CCN2,F3,CCN1,MYC
GO Biological Processes	GO:0045862	positive regulation of proteolysis	-5,252	-2,671	5/378	CCN2,F3,CCN1,IL1B,MYC
WikiPathways	WP49	IL-2 Signaling Pathway	-3,391	-1,324	2/42	MYC,SOCS3
GO Biological Processes	GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	-5,034	-2,492	6/741	CCN2,EREG,F3,IL1B,NR4A3,SOCS3
GO Biological Processes	GO:0051403	stress-activated MAPK cascade	-4,393	-2,047	4/279	CCN2,IL1B,MYC,MAP2K3
GO Biological Processes	GO:0080135	regulation of cellular response to stress	-3,801	-1,615	5/761	CCN2,IL1B,MYC,NR4A3,IER3
BioCarta Gene Sets	M12095	BIOCARTA IL1R PATHWAY	-3,657	-1,510	2/31	IL1B,MAP2K3
WikiPathways	WP4496	Signal transduction through IL1R	-3,602	-1,478	2/33	IL1B,MAP2K3
GO Biological Processes	GO:0038066	p38MAPK cascade	-3,239	-1,213	2/50	IL1B,MAP2K3
WikiPathways	WP195	IL-1 signaling pathway	-3,157	-1,173	2/55	IL1B,MAP2K3
GO Biological Processes	GO:0045766	positive regulation of angiogenesis	-3,439	-1,357	3/199	F3,IL1B,ANGPTL4
GO Biological Processes	GO:0032757	positive regulation of interleukin-8 production	-3,126	-1,157	2/57	F3,IL1B
Hallmark Gene Sets	M5902	HALLMARK APOPTOSIS	-3,709	-1,541	3/161	EREG,IL1B,IER3
Canonical Pathways	M196	PID IL23 PATHWAY	-3,502	-1,403	2/37	IL1B,SOCS3

Table A- 16: Metascape enrichment pathway analysis for the up-regulated genes, at 6 h 50 min after 10 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
Reactome Gene Sets	R-HSA-8953854	Metabolism of RNA	-8,299	-3,934	37/673	ADARB1,EIF4B,FUS,SNU13,RPL8,RPS14,RPS15A,SNRPF,U2AF1,YWHAB,APOBEC3B,PSMD6,PDCD7,HNRNPR,POP7,PPIH,SNRNP35,FTSJ1,PRPF6,DCAF13,GNL3,PABPC1,TFB1M,CPSF3,XRN1,TEX10,NHP2,CCAR1,ELAC2,WDR77,PUS1,FIP1L1,UTP15,LTV1,TRMT61A,NSUN4,

						GTF2H5
GO Biological Processes	GO:0042254	ribosome biogenesis	-7,091	-3,152	22/307	SNU13,NVL,RPS14,POP7,GTPBP4,DCAF13,ZNF658,TFB1M,MRTO4,TEX10,HEATR3,NHP2,DDX28,DDX54,EFL1,UTP15,MPV17L2,LTV1,FAM207A,RPL7L1,NSUN4,GTF2H5
Reactome Gene Sets	R-HSA-1640170	Cell Cycle	-6,989	-3,152	35/692	CDK1,CDC6,CKS1B,H2BC3,HDAC1,INCENP,MCM4,MDM4,PRKACA,RAD51,RB1,UBE2V2,YWHAB,H2BC14,H2BC10,SSNA1,CCNA1,CCNB2,KNTC1,PSMD6,OPTN,PLK4,MAU2,ITGB3BP,NIPBL,GMNN,CHMP3,ERCC6L,NHP2,CENPH,CEP290,CEP78,GINS4,CHMP4B,SKA2
GO Biological Processes	GO:0065004	protein-DNA complex assembly	-5,502	-2,179	17/242	H2BC3,MCM4,RAD51,RB1,TAF7,TAF12,H2BC14,H2BC10,HAT1,CHAF1A,ITGB3BP,GMNN,ATF7IP,CENPH,NAA60,CENPV,GTF2H5
GO Biological Processes	GO:0051301	cell division	-5,048	-1,830	28/600	CCNT1,CDK1,CDC6,CKS1B,INCENP,MDK,NEK3,PGF,RB1,CCNA1,CCNB2,NUMBL,KIF20B,KNTC1,PLK4,KATNA1,MAU2,ITGB3BP,GNL3,PIK3R4,CHMP3,ERCC6L,FIGN,CHMP4B,CDCA2,CENPV,REEP3,SKA2
Reactome Gene Sets	R-HSA-196757	Metabolism of folate and pterines	-4,959	-1,825	5/17	SLC19A1,MTHFS,MTHFD1L,DHFR2,MTHFD2L
Reactome Gene Sets	R-HSA-5653656	Vesicle-mediated transport	-4,164	-1,247	28/673	VPS51,CAPZB,CHML,AP1S1,COPA,CPD,CUX1,KIF22,YWHAB,STX16,GBF1,SYNJ2,AP1G2,RAB9A,KIF20B,DENND4B,GJC1,OPTN,PLIN3,KIF1C,STON1,MON1B,CHMP3,TRAPPC2L,SYS1,CHMP4B,VPS37D,DENND6A
GO Biological Processes	GO:0016569	covalent chromatin modification	-4,179	-1,247	22/465	APBB1,CDK1,CTBP1,HDAC1,PRMT2,INCENP,PHB,RNF2,TAF7,TAF12,HAT1,LDB1,PER2,SETDB1,NIPBL,RESF1,ATF7IP,RIOX1,NAA60,ZNF541,TBL1Y,C6orf89
GO Biological Processes	GO:0016575	histone deacetylation	-3,966	-1,093	8/79	CTBP1,HDAC1,PHB,PER2,NIPBL,ZNF541,TBL1Y,C6orf89
GO Biological Processes	GO:0030522	intracellular receptor signaling pathway	-3,643	-0,907	15/277	ACTN4,CRY2,HDAC1,PRMT2,IRF3,PHB,PPARD,TAF7,UBE3A,UFD1,ANKRD17,FOXP1,DDX54,CLPB,FAM120B
WikiPathways	WP4521	Glycosylation and related congenital defects	-2,967	-0,676	4/25	MOGS,ALG3,ALG1,ALG14
GO Biological Processes	GO:0009100	glycoprotein metabolic process	-2,226	-0,385	16/423	FUT4,STT3A,PPARD,ST3GAL2,ST3GAL4,MOGS,ALG3,MARCHF6,CHP1,SDF2L1,SERP1,DSE,CHST15,ALG1,EGFLAM,ALG14
Reactome Gene Sets	R-HSA-5617833	Cilium Assembly	-1,886	-0,222	9/201	CDK1,PRKACA,SSNA1,GBF1,PLK4,IFT172,CEP83,CEP290,CEP78

Table A- 17: Metascape enrichment pathway analysis for the down-regulated genes, at 6 h 50 min after 10 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
KEGG Pathway	hsa04211	Longevity regulating pathway	-4,174	-0,256	4/109	ATF2,PRKACB,ADIPOR1,ADIPOR2
GO Biological Processes	GO:0048871	multicellular organismal homeostasis	-3,371	-0,152	6/491	TNFRSF11B,PRKACB,TFRC,KDM1A,ADIPOR1,ADIPOR2
GO Biological Processes	GO:0009636	response to toxic substance	-1,740	0,000	4/506	VCAM1,KDM1A,HCN3,ADIPOR2
Reactome Gene Sets	R-HSA-9024446	NR1H2 and NR1H3-mediated signaling	-3,944	-0,256	3/47	KDM1A,MYLIP,KDM1B

KEGG Pathway	hsa04310	Wnt signaling pathway	-2,330	0,000	3/169	DVL2,GPI,PRKACB
GO Biological Processes	GO:0016570	histone modification	-2,690	0,000	5/452	ATF2,TRRAP,KDM1A,MBIP,KDM1B
GO Biological Processes	GO:1901699	cellular response to nitrogen compound	-3,529	-0,196	7/650	GABRB3,PRKACB,VCAM1,KDM1A,ADIPOR1,HCN3,DDI1
KEGG Pathway	ko04727	GABAergic synapse	-3,138	-0,018	3/88	GABRB3,PRKACB,GABBR2
Reactome Gene Sets	R-HSA-9031628	NGF-stimulated transcription	-2,561	0,000	2/39	ATF2,ID4
GO Biological Processes	GO:0002639	positive regulation of immunoglobulin production	-2,438	0,000	2/45	GPI,TFRC
GO Biological Processes	GO:0002637	regulation of immunoglobulin production	-2,089	0,000	2/68	GPI,TFRC
GO Biological Processes	GO:0071320	cellular response to cAMP	-2,283	0,000	2/54	KDM1A,HCN3
WikiPathways	WP2018	RANKL/RANK (Receptor activator of NFKB (ligand)) Signaling Pathway	-2,268	0,000	2/55	TNFRSF11B,VCAM1
KEGG Pathway	ko04668	TNF signaling pathway	-1,707	0,000	2/108	ATF2,VCAM1
GO Biological Processes	GO:0010035	response to inorganic substance	-1,640	0,000	4/543	ATF2,TNFRSF11B,VCAM1,DDI1
GO Biological Processes	GO:0060191	regulation of lipase activity	-1,786	0,000	2/98	SORT1,NMUR1
GO Biological Processes	GO:0005975	carbohydrate metabolic process	-1,417	0,000	4/639	GPI,ADIPOR1,EDEM2,IPPK

Table A- 18: Metascape enrichment pathway analysis for the up-regulated genes, at 2 h 25 min after diesel + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0033631	cell-cell adhesion mediated by integrin	-2,486	0,000	2/17	DPP4,ITGA4
WikiPathways	WP4493	Cells and Molecules involved in local acute inflammatory response	-2,486	0,000	2/17	ITGA4,SELP
GO Biological Processes	GO:0050900	leukocyte migration	-2,347	0,000	8/511	CD34,CMKLR1,DPP4,HRH1,ITGA4,SELP,MADCAM1,SLC7A11
KEGG Pathway	hsa04514	Cell adhesion molecules (CAMs)	-2,209	0,000	4/144	CD34,ITGA4,SELP,MADCAM1
GO Biological Processes	GO:0030198	extracellular matrix organization	-1,817	0,000	6/395	SERPINH1,DPP4,ITGA4,VIT,TTR,MADCAM1
GO Biological Processes	GO:0034121	regulation of toll-like receptor signaling pathway	-2,140	0,000	3/78	ESR1,SMPDL3B,RTN4
GO Biological Processes	GO:0032104	regulation of response to extracellular stimulus	-2,120	0,000	2/26	MED1,PRKCG

GO Biological Processes	GO:0030522	intracellular receptor signaling pathway	-1,886	0,000	5/275	ESR1,MED1,SLC15A2,NR1I2,RNF125
GO Biological Processes	GO:0043124	negative regulation of I-kappaB kinase/NF-kappaB signaling	-1,595	0,000	2/49	ESR1,OTUD7A
GO Biological Processes	GO:0002224	toll-like receptor signaling pathway	-1,324	0,000	3/160	ESR1,SMPDL3B,RTN4
GO Biological Processes	GO:0009581	detection of external stimulus	-1,452	0,000	3/142	GNGT2,BACE1,GRK7
GO Biological Processes	GO:0009582	detection of abiotic stimulus	-1,429	0,000	3/145	GNGT2,BACE1,GRK7
GO Biological Processes	GO:0140205	oligopeptide import across plasma membrane	-3,602	-0,191	2/5	SLC15A2,SLC7A11
GO Biological Processes	GO:1990961	xenobiotic detoxification by transmembrane export across the plasma membrane	-3,159	0,000	2/8	SLC15A2,RALBP1
GO Biological Processes	GO:0009636	response to toxic substance	-2,862	0,000	6/239	HBG1,SLC15A2,RALBP1,SLC7A11,EHMT1,RDH12
GO Biological Processes	GO:0098754	detoxification	-2,274	0,000	4/138	HBG1,SLC15A2,RALBP1,RDH12
Reactome Gene Sets	R-HSA-418597	G alpha (z) signalling events	-1,611	0,000	2/48	GNGT2,PRKCG
WikiPathways	WP363	Wnt Signaling Pathway	-1,531	0,000	2/53	PRKCG,SOX1
GO Biological Processes	GO:2000300	regulation of synaptic vesicle exocytosis	-1,502	0,000	2/55	PRKCG,BACE1
GO Biological Processes	GO:0007200	phospholipase C-activating G protein-coupled receptor signaling pathway	-2,730	0,000	4/103	CMKLR1,ESR1,HRH1,TRHR
GO Biological Processes	GO:0043950	positive regulation of cAMP-mediated signaling	-2,656	0,000	2/14	CRH,GIPR
Reactome Gene Sets	R-HSA-9660821	ADORA2B mediated anti-inflammatory cytokines production	-2,319	0,000	4/134	CRH,GIPR,GNGT2,GPR25
GO Biological Processes	GO:0033555	multicellular organismal response to stress	-2,305	0,000	3/68	CRH,DPP4,PRKCG
Reactome Gene Sets	R-HSA-418555	G alpha (s) signalling events	-2,199	0,000	4/145	CRH,GIPR,GNGT2,GPR25
GO Biological Processes	GO:1901615	organic hydroxy compound metabolic process	-1,643	0,000	7/555	HRH1,MED1,TTR,DPM1,SLC7A11,RDH12,LDHD
GO Biological Processes	GO:0060294	cilium movement involved in cell motility	-1,697	0,000	3/114	CFAP206,TSSK4,HOATZ
GO Biological Processes	GO:0006898	receptor-mediated endocytosis	-1,567	0,000	5/333	ITGA4,MSR1,ATXN2,TMEM108,RSPO1
GO Biological Processes	GO:0071417	cellular response to organonitrogen compound	-2,243	0,000	9/648	ATP6V1A,CRH,HRH1,ITGA4,KCNE1,IRS4,KL,BACE1,MBD5
GO Biological Processes	GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	-1,465	0,000	8/740	ARF4,ATP6V1A,IRS4,KL,PTPRT,MBD5,RTN4,TMEM108
GO Biological Processes	GO:0032102	negative regulation of response to external stimulus	-1,822	0,000	6/394	CD34,DPP4,FGL2,BACE1,SMPDL3B,RNF125

GO Biological Processes	GO:0051606	detection of stimulus	-1,534	0,000	8/717	GNGT2,BACE1,OR5K1,GRK7,OR13F1,OR1N1,OR5AP2,OR6C65
GO Biological Processes	GO:0045197	establishment or maintenance of epithelial cell apical/basal polarity	-1,720	0,000	2/42	ARF4,MTCL1

Table A- 19: Metascape enrichment pathway analysis for the down-regulated genes, at 2 h 25 min after diesel + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0007608	sensory perception of smell	-3,589	-0,047	10/454	DRD2,OR52E2,OR2T12,GJB4,OR5M8,OR2T6,OR8D1,OR4N4,OR51I1,OR51A4
GO Biological Processes	GO:0007606	sensory perception of chemical stimulus	-3,068	0,000	10/530	DRD2,OR52E2,OR2T12,GJB4,OR5M8,OR2T6,OR8D1,OR4N4,OR51I1,OR51A4
GO Biological Processes	GO:0045109	intermediate filament organization	-2,099	0,000	2/24	KRT2,TCHH
GO Biological Processes	GO:0006486	protein glycosylation	-1,846	0,000	5/253	DDOST,FKTN,POGLUT1,COG7,GALNT13
GO Biological Processes	GO:0048512	circadian behavior	-2,684	0,000	3/45	DRD2,NAGLU,C3orf70
GO Biological Processes	GO:0007194	negative regulation of adenylate cyclase activity	-2,299	0,000	2/19	DRD2,GALR1
GO Biological Processes	GO:0051954	positive regulation of amine transport	-1,832	0,000	2/33	CCK,DRD2
GO Biological Processes	GO:0045744	negative regulation of G protein-coupled receptor signaling pathway	-1,477	0,000	2/51	DRD2,RPGRIP1L
GO Biological Processes	GO:0071371	cellular response to gonadotropin stimulus	-2,504	0,000	2/15	CCNA2,PAX8
Reactome Gene Sets	R-HSA-6804756	Regulation of TP53 Activity through Phosphorylation	-1,820	0,000	3/92	CCNA2,MAPK11,RBBP8
GO Biological Processes	GO:0036152	phosphatidylethanolamine acyl-chain remodeling	-2,031	0,000	2/26	PLA2G1B,PLAAT2
GO Biological Processes	GO:0071407	cellular response to organic cyclic compound	-1,415	0,000	7/558	BMP7,CCNA2,DRD2,PGR,LARP1,XRN1,SRARP
GO Biological Processes	GO:1901699	cellular response to nitrogen compound	-1,345	0,000	8/703	CCNA2,CYP11A1,DRD2,GLP1R,PLA2G1B,LARP1,XRN1,LYPD1

Table A- 20: Metascape enrichment pathway analysis for the down-regulated genes, at 24 h min after diesel + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0051606	detection of stimulus	-2,280	0,000	4/717	PPEF1,OR2T12,OR5D14,OR5M8
GO Biological Processes	GO:0016572	histone phosphorylation	-3,161	0,000	2/39	CCNA2,MST1
GO Biological Processes	GO:0071456	cellular response to hypoxia	-1,750	0,000	2/206	CCNA2,FMN2
GO Biological Processes	GO:0036294	cellular response to decreased oxygen levels	-1,719	0,000	2/214	CCNA2,FMN2
GO Biological Processes	GO:0104004	cellular response to environmental stimulus	-1,368	0,000	2/332	HPCA,PPEF1
GO Biological Processes	GO:0043255	regulation of carbohydrate biosynthetic process	-2,387	0,000	2/96	MST1,PTH1R
GO Biological Processes	GO:0018108	peptidyl-tyrosine phosphorylation	-2,238	0,000	3/370	CCK,EPHA5,CNOT7
GO Biological Processes	GO:0031348	negative regulation of defense response	-1,640	0,000	2/236	CCK,CNOT7
GO Biological Processes	GO:0008285	negative regulation of cell population proliferation	-1,420	0,000	3/753	FKTN,PTH1R,CNOT7

Table A- 21: Metascape enrichment pathway analysis for commonly regulated genes, between the pollen exposure alone (4 mg) and the diesel + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
Reactome Gene Sets	R-HSA-373080	Class B/2 (Secretin family receptors)	-5,732	-1,424	5/95	CRH,GIPR,GLP1R,GNGT2,PTH1R
Reactome Gene Sets	R-HSA-9660821	ADORA2B mediated anti-inflammatory cytokines production	-4,999	-1,135	5/134	CRH,GIPR,GLP1R,GNGT2,PTH1R
Reactome Gene Sets	R-HSA-418555	G alpha (s) signalling events	-4,833	-1,135	5/145	CRH,GIPR,GLP1R,GNGT2,PTH1R
GO Biological Processes	GO:0009581	detection of external stimulus	-2,520	0,000	3/135	GNGT2,BACE1,GRK7
GO Biological Processes	GO:0009582	detection of abiotic stimulus	-2,493	0,000	3/138	GNGT2,BACE1,GRK7
GO Biological Processes	GO:0008277	regulation of G protein-coupled receptor signaling pathway	-1,472	0,000	2/135	GIPR,GRK7
GO Biological Processes	GO:0009636	response to toxic substance	-3,617	-0,393	5/262	BMP7,HBG1,SLC15A2,EHMT1,RDH12
GO Biological Processes	GO:0098754	detoxification	-3,502	-0,393	4/152	BMP7,HBG1,SLC15A2,RDH12
GO Biological Processes	GO:1990748	cellular detoxification	-2,707	0,000	3/116	BMP7,HBG1,RDH12

GO Biological Processes	GO:0097237	cellular response to toxic substance	-2,624	0,000	3/124	BMP7,HBG1,RDH12
GO Biological Processes	GO:1902742	apoptotic process involved in development	-2,481	0,000	2/40	BMP7,XKR6
GO Biological Processes	GO:0034113	heterotypic cell-cell adhesion	-3,498	-0,393	3/62	BMP7,ITGA4,MADCAM1
Canonical Pathways	M212	PID INTEGRIN5 PATHWAY	-3,226	-0,260	2/17	ITGA4,MADCAM1
GO Biological Processes	GO:2000403	positive regulation of lymphocyte migration	-2,595	0,000	2/35	ITGA4,MADCAM1
GO Biological Processes	GO:2000401	regulation of lymphocyte migration	-2,123	0,000	2/61	ITGA4,MADCAM1
Reactome Gene Sets	R-HSA-216083	Integrin cell surface interactions	-1,847	0,000	2/85	ITGA4,MADCAM1
GO Biological Processes	GO:0007229	integrin-mediated signaling pathway	-1,644	0,000	2/109	ITGA4,MADCAM1
GO Biological Processes	GO:0002687	positive regulation of leukocyte migration	-1,472	0,000	2/135	ITGA4,MADCAM1
KEGG Pathway	hsa04514	Cell adhesion molecules (CAMs)	-1,421	0,000	2/144	ITGA4,MADCAM1
KEGG Pathway	ko04514	Cell adhesion molecules (CAMs)	-1,421	0,000	2/144	ITGA4,MADCAM1
GO Biological Processes	GO:0031589	cell-substrate adhesion	-1,370	0,000	3/364	ITGA4,VIT,MADCAM1
GO Biological Processes	GO:0007159	leukocyte cell-cell adhesion	-1,349	0,000	3/371	BMP7,ITGA4,MADCAM1
GO Biological Processes	GO:0050900	leukocyte migration	-1,346	0,000	3/372	HRH1,ITGA4,MADCAM1
GO Biological Processes	GO:1901699	cellular response to nitrogen compound	-3,184	-0,237	7/692	CRH,GLP1R,HRH1,ITGA4,RYR1,BACE1,LYPD1
GO Biological Processes	GO:0071417	cellular response to organonitrogen compound	-2,633	0,000	6/637	CRH,GLP1R,HRH1,ITGA4,RYR1,BACE1
GO Biological Processes	GO:1904646	cellular response to amyloid-beta	-2,325	0,000	2/48	ITGA4,BACE1
GO Biological Processes	GO:1904645	response to amyloid-beta	-2,151	0,000	2/59	ITGA4,BACE1
GO Biological Processes	GO:0099536	synaptic signaling	-1,660	0,000	5/752	CHRNG,CRH,HRH1,BACE1,LYPD1
GO Biological Processes	GO:1901652	response to peptide	-1,637	0,000	4/508	BMP7,GLP1R,ITGA4,BACE1
GO Biological Processes	GO:1901653	cellular response to peptide	-1,373	0,000	3/363	GLP1R,ITGA4,BACE1
GO Biological Processes	GO:0010942	positive regulation of cell death	-1,370	0,000	4/618	BMP7,CRH,ITGA4,BACE1
WikiPathways	WP395	IL-4 signaling pathway	-2,210	0,000	2/55	HRH1,MAPK11

Table A- 22: Metascape enrichment pathway analysis for the up-regulated genes, at 2 h 25 min after propane + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0051259	protein complex oligomerization	-4,323	0,000	9/229	ALDH1A3,B2M,KCND3,MAT1A,RS1,TP63,USP16,TWNK,WDCP
GO Biological Processes	GO:0071241	cellular response to inorganic substance	-2,982	0,000	7/216	B2M,CRK,ITPKB,HVCN1,TPH2,SYT10,DDI1
GO Biological Processes	GO:0010035	response to inorganic substance	-1,750	0,000	9/542	B2M,CRK,CYB5A,ITPKB,MTTP,HVCN1,TPH2,SYT10,DDI1
GO Biological Processes	GO:0003341	cilium movement	-1,499	0,000	4/164	TEKT4,CATSPER3,SPEM1,CCDC103
GO Biological Processes	GO:0001867	complement activation, lectin pathway	-2,483	0,000	2/12	SERPING1,MASP1
GO Biological Processes	GO:0034109	homotypic cell-cell adhesion	-2,447	0,000	4/86	GP1BA,JAK1,MPL,TSPAN32
GO Biological Processes	GO:1903035	negative regulation of response to wounding	-2,376	0,000	4/90	SERPING1,CRK,GP1BA,RGMA
GO Biological Processes	GO:1903034	regulation of response to wounding	-1,499	0,000	4/164	SERPING1,CRK,GP1BA,RGMA
Reactome Gene Sets	R-HSA-1474228	Degradation of the extracellular matrix	-1,719	0,000	4/140	COL6A3,FBN2,TMPRSS6,COL6A5
KEGG Pathway	ko04974	Protein digestion and absorption	-1,556	0,000	3/90	COL6A3,CTRL,COL6A5
WikiPathways	WP3929	Chemokine signaling pathway	-1,490	0,000	4/165	CRK,FOXO3,GNB2,CXCR6
GO Biological Processes	GO:0120009	intermembrane lipid transfer	-2,345	0,000	3/46	MTTP,STARD3,GRAMD1B
GO Biological Processes	GO:0015850	organic hydroxy compound transport	-1,842	0,000	6/271	TOR1A,MTTP,NCOA2,STARD3,GRAMD1B,SYT10
GO Biological Processes	GO:2000353	positive regulation of endothelial cell apoptotic process	-1,923	0,000	2/23	CD160,ECSCR
GO Biological Processes	GO:1904037	positive regulation of epithelial cell apoptotic process	-1,468	0,000	2/40	CD160,ECSCR
Reactome Gene Sets	R-HSA-177929	Signaling by EGFR	-2,243	0,000	3/50	SH3GL3,LRIG1,EPN1
Canonical Pathways	M143	PID IL2 PI3K PATHWAY	-1,599	0,000	2/34	FOXO3,JAK1
GO Biological Processes	GO:0071354	cellular response to interleukin-6	-1,358	0,000	2/46	JAK1,ST3GAL6
GO Biological Processes	GO:1902414	protein localization to cell junction	-1,347	0,000	3/109	JAK1,CLSTN1,MAPK8IP3
Reactome Gene Sets	R-HSA-512988	Interleukin-3, Interleukin-5 and GM-CSF signaling	-1,324	0,000	2/48	CRK,JAK1
Reactome Gene Sets	R-HSA-209776	Metabolism of amine-derived hormones	-2,131	0,000	2/18	TPH2,IYD
GO Biological Processes	GO:0006664	glycolipid metabolic process	-1,328	0,000	3/111	ST8SIA4,ST3GAL6,PGAP3

Table A- 23: Metascape enrichment pathway analysis for the down-regulated genes, at 2 h 25 min after propane + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0051897	positive regulation of protein kinase B signaling	-4,147	-0,383	5/175	INS,PIK3CG,TEK,RASD2,MYORG
GO Biological Processes	GO:0050995	negative regulation of lipid catabolic process	-2,767	0,000	2/25	INS,PIK3CG
GO Biological Processes	GO:0050727	regulation of inflammatory response	-2,672	0,000	5/369	INS,PIK3CG,TEK,TTBK1,CASP12
GO Biological Processes	GO:0002673	regulation of acute inflammatory response	-2,207	0,000	2/48	INS,PIK3CG
GO Biological Processes	GO:0031347	regulation of defense response	-2,142	0,000	6/697	INS,PIK3CG,TEK,ZMPSTE24,TTBK1,CASP12
GO Biological Processes	GO:0043410	positive regulation of MAPK cascade	-2,023	0,000	5/527	INS,PIK3CG,SSTR4,TEK,UBC
GO Biological Processes	GO:0002526	acute inflammatory response	-1,546	0,000	2/107	INS,PIK3CG
Reactome Gene Sets	R-HSA-983168	Antigen processing: Ubiquitination & Proteasome degradation	-2,153	0,000	4/309	UBC,HECTD1,LRR1,FBXL13
Reactome Gene Sets	R-HSA-983169	Class I MHC mediated antigen processing & presentation	-1,860	0,000	4/377	UBC,HECTD1,LRR1,FBXL13
GO Biological Processes	GO:0006165	nucleoside diphosphate phosphorylation	-2,376	0,000	3/132	HK3,INS,AK7
GO Biological Processes	GO:0034219	carbohydrate transmembrane transport	-1,482	0,000	2/116	INS,MFSD4A
KEGG Pathway	hsa04068	foxo signaling pathway	-1,351	0,000	2/137	INS,CCNB3
GO Biological Processes	GO:2001056	positive regulation of cysteine-type endopeptidase activity	-2,190	0,000	3/154	IFT57,TTBK1,CASP12
GO Biological Processes	GO:0045744	negative regulation of G protein-coupled receptor signaling pathway	-2,156	0,000	2/51	SSTR4,KLK14
GO Biological Processes	GO:1904029	regulation of cyclin-dependent protein kinase activity	-1,561	0,000	2/105	TTBK1,CCNB3

Table A- 24: Metascape enrichment pathway analysis for the up-regulated genes, at 24 h after propane + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
Reactome Gene Sets	R-HSA-195721	Signaling by WNT	-1,940	0,000	2/332	TLE5,GNGT2
KEGG Pathway	ko04151	PI3K-Akt signaling pathway	-1,915	0,000	2/342	GNGT2,COL6A5
WikiPathways	WP4172	PI3K-Akt Signaling Pathway	-1,908	0,000	2/345	GNGT2,COL6A5

KEGG Pathway	hsa04151	PI3K-Akt signaling pathway	-1,828	0,000	2/380	GNGT2,COL6A5
Reactome Gene Sets	R-HSA-372790	Signaling by GPCR	-1,332	0,000	2/704	GNGT2,RGS16
GO Biological Processes	GO:0051606	detection of stimulus	-1,318	0,000	2/717	GNGT2,OR9I1
GO Biological Processes	GO:0016042	lipid catabolic process	-1,918	0,000	2/341	ENPP6,CES5A

Table A- 25: Metascape enrichment pathway analysis for the up-regulated genes, at 24 h after propane + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
GO Biological Processes	GO:0050673	epithelial cell proliferation	-1,915	0,000	2/439	DLL4,PROK2
GO Biological Processes	GO:0045165	cell fate commitment	-2,384	0,000	2/251	CTR9,DLL4
GO Biological Processes	GO:0051346	negative regulation of hydrolase activity	-1,884	0,000	2/456	CST8,PCDH11X

Table A- 26: Metascape enrichment pathway analysis for commonly regulated genes, between the pollen exposure alone (4 mg) and the propane + 4 mg birch pollen exposure. Only relevant terms related with immune and allergic response and with p-value less than 0.05 are shown.

Category	Term	Description	LogP	Log(q-value)	InTerm_InList	Symbols
WikiPathways	WP3929	Chemokine signaling pathway	-3,036	0,000	3/165	FOXO3,GNGT2,PIK3CG
WikiPathways	WP127	IL-5 signaling pathway	-3,005	0,000	2/40	FOXO3,PIK3CG
GO Biological Processes	GO:1903426	regulation of reactive oxygen species biosynthetic process	-2,847	0,000	2/48	FOXO3,INS
KEGG Pathway	hsa04062	Chemokine signaling pathway	-2,847	0,000	3/192	FOXO3,GNGT2,PIK3CG
GO Biological Processes	GO:1903201	regulation of oxidative stress-induced cell death	-2,442	0,000	2/77	FOXO3,INS
GO Biological Processes	GO:1900407	regulation of cellular response to oxidative stress	-2,291	0,000	2/92	FOXO3,INS
GO Biological Processes	GO:1902882	regulation of response to oxidative stress	-2,213	0,000	2/101	FOXO3,INS
GO Biological Processes	GO:0080135	regulation of cellular response to stress	-2,090	0,000	4/692	FOXO3,INS,HIPK3,MAPK8IP3
GO Biological Processes	GO:0071417	cellular response to organonitrogen compound	-1,547	0,000	3/573	FOXO3,INS,PIK3CG
GO Biological Processes	GO:0031668	cellular response to extracellular stimulus	-1,512	0,000	2/237	TLE5,FOXO3
GO Biological Processes	GO:1901699	cellular response to nitrogen compound	-1,440	0,000	3/632	FOXO3,INS,PIK3CG

GO Biological Processes	GO:0071496	cellular response to external stimulus	-1,324	0,000	2/301	TLE5,FOXO3
GO Biological Processes	GO:0050995	negative regulation of lipid catabolic process	-3,380	-0,078	2/26	INS,PIK3CG
GO Biological Processes	GO:0002673	regulation of acute inflammatory response	-2,865	0,000	2/47	INS,PIK3CG
GO Biological Processes	GO:0050994	regulation of lipid catabolic process	-2,641	0,000	2/61	INS,PIK3CG
GO Biological Processes	GO:0042110	T cell activation	-2,497	0,000	3/255	INS,PIK3CG,DLL4
GO Biological Processes	GO:0045833	negative regulation of lipid metabolic process	-2,180	0,000	2/105	INS,PIK3CG
GO Biological Processes	GO:0043408	regulation of MAPK cascade	-2,103	0,000	4/686	INS,PIK3CG,HIPK3,MAPK8IP3
GO Biological Processes	GO:0009611	response to wounding	-1,874	0,000	3/430	INS,PIK3CG,MAPK8IP3
GO Biological Processes	GO:0043410	positive regulation of MAPK cascade	-1,724	0,000	3/490	INS,PIK3CG,MAPK8IP3
GO Biological Processes	GO:0043405	regulation of MAP kinase activity	-1,720	0,000	2/183	PIK3CG,HIPK3
GO Biological Processes	GO:0051606	detection of stimulus	-1,383	0,000	3/666	GNGT2,OR6C75,OR13C2