Biochemical Composition of Phleum pratense Pollen Grains: a review

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Abstract

The Poaceae family is composed of 12 000 plant species. Some of these species produce highly allergenic anemophilous pollen grains (PGs). *Phleum pratense* pollen grains (PPPGs) emerged as a model for studies related to grass allergy. The biochemical composition of allergenic PGs has not yet been fully described despite potential health effects of PG constituents other than allergenic proteins. This review brings together the information available in literature aiming at creating a comprehensive picture of the current knowledge about composition of allergenic PGs from timothy grass. PPPGs have an average diameter between 30 to 35 μm and the mass of a single PG was reported between 11 and 26 ng. Pollen cytoplasm is filled with two types of pollen cytoplasmic granules (PCGs): the starch granules and the polysaccharide-particles (p-particles). Starch granules have a size between 0.6 to 2.5 μm with an average diameter of 1.1 μm (estimated number of 1 000 granules per PG) while p-particles have a size ranging around 0.3 to 0.4 μm (estimated number between 61 000 to 230 000 p-particles per PG). Rupture of PG induce the release of PCGs and the dispersion of allergens in the inhalable fraction of atmospheric aerosol.

PPPGs are composed of sporopollenin, sugars, polysaccharides, starch, glycoproteins (including allergens), amino-acids, lipids, flavonoids (including isorhamnetin), diverse elements (the more abundant being Si, Mg and Ca), phenolic compounds, phytoprostanoids, carotenoids (pigments) metals and adsorbed pollutants. PPPG contains about a hundred different proteins with molecular masses ranging from 10 to 94 kDa, with isoelectric points from 3.5 to 10.6. Among these proteins, allergens are classified in eleven groups from 1 to 13 with allergens from groups 1 and 5 being the major contributors to Phl p pollen allergy. Major allergen Phl p 5 was quantified in PPPGs by several studies with concentration ranging from 2.7 and 3.5 $\mu g.mg^{-1}$ in unpolluted environment. Values for other allergens are scarce in literature; only one quantitative assessment exists for allergen group Phl p 1, 2 and 4. The extractible lipidic fraction of PPPGs is estimated between 1.7 to 2.2% of the total PG mass. The main chemical families of lipids reported in PPPGs are: alkanes, alkenes, alcohols, saturated and unsaturated fatty acids, di- and tri-hydroxylated fatty acids, aldehydes and sterols. Several lipidic compounds with potential adjuvant effects on allergy have been specifically quantified in PPPGs: E2-like prostaglandin (PGE2), B4-like leukotriene (LTB4), unsaturated fatty acids (linoleic and linolenic acids and their hydroxylated derivatives), adenosine, vitamins and phenolic compounds. Some other biochemical characteristics such as NAD(P)H oxidase, protease activity and pollen microbiome were described in the literature. The bioaccessibility in physiological conditions has not been described for most biochemicals transported by allergenic PPPGs. There is also a considerable lack of knowledge about the potential health effects of pollen constituents other than allergens. The variability of pollen composition remains also largely unknown despite its importance for plant reproduction and allergy in an environment characterized by chemical pollution, climate change and loss of biodiversity.

1. Introduction

The Poaceae family is composed of about 12 000 wind-pollinated species and their pollen grains (PGs) are considered as the major airborne biological threat to human health (García-Mozo, 2017). About 20% of the general population in the United States and Europe are sensitized to grass pollen allergens (García-Mozo, 2017). Main allergenic species for the Poaceae family are: *Alopecurus pratensis*, *Dactylis glomerata*, *Lolium perene*, *Cynodon Dactylon*, *Phleum pratense*, and *Poa annua* (Guerin, 1993). Timothy grass, *Phleum Pratense* L., is one among the Poaceae family and it is widely used in agriculture as a pasture and forage crop. Due to similarity and cross-reactivity of *Phleum Pratense* pollen grains (PPPGs) with those from other species from Poaceae, allergens extracted from PPPGs are used as a model in skin prick tests and PPPGs emerged as a model for studies related to allergy to grass pollen (Andersson and Lidholm, 2003; White and Bernstein, 2003).

Figure 1. (a) Inflorescence of timothy grass. (b) Close-up views of anthers. A single inflorescence can produce 1.2 million pollen grains (Albertine et al., 2014) (photography provided by authors).

The main grass pollen season is observed in Europe from May to July (Guerin, 1993). A single inflorescence of timothy grass can indeed produce up to 1.2 million PGs (Figure 1) (Albertine et al., 2014). Atmospheric concentrations of grass pollen frequently reach more than a thousand of pollen grains per cubic meter (Mueller et al., 2016). Exposure to allergenic PGs is also effective in indoor air with persistence of allergens observed after the pollen season (Dybendal et al., 1989; Fahlbusch et al., 2001, 2001; Holmquist et al., 1999).

Pollen grains have historically been described as sole carriers of allergenic proteins as stated for example by Stull et al. in 1932: « *The allergen of timothy pollen is of protein nature and it is the only active*

substance in timothy pollen » (Stull et al., 1932). It is now clearly established that PGs and the pollen cytoplasmic granules (PCGs) are vectors of both allergenic proteins and other bioactive molecules (Pointner et al., 2020; Suphioglu et al., 1992). Pollen grains also transport lipids that are essential for plant reproductive functions (Rejón et al., 2016). Lipids are also bioactive mediators capable of activating the cells of the immune system (González Roldán et al., 2019). Other contributors to allergic inflammation also transported by PGs are pollen protease, NADPH oxidase and adsorbed gaseous or particulate pollutants (Sénéchal et al., 2015; Visez et al., 2020).

Despite sustained research efforts, the biochemical composition of allergenic PG is however not yet fully elucidated neither is the causal relationship between variation in PGs composition and pollen allergy risk (ie. the capacity of a PG, alone or with other factors such as air pollution, to induce health impact (Galán et al., 2017)). First of all, research efforts are spread over different allergenic species, the most studied being for trees *Betulaceae*, C*upressaceae and Taxodiaceae,* for grasses *Lolium* and *Phleum* and for weeds *Ambrosia* (ANSES, 2014). Pollen grains of allergenic species do share common characteristics but they have also many differences including first and foremost the nature and concentration of allergens. Secondly, the biochemical composition of pollen grains depends on both intrinsic and extrinsic factors. It may vary among species, but also among conspecific individuals, due to genetic determinants; it may also vary in space and time due to plant age or environmental variations (composition of soil, meteorological and climatic parameters…). Finally, the age of Anthropocene is characterized by significant levels of envrionmental perturbations, such as pollutions in soil, atmosphere, water. Such perturbations could influence the biochemical composition of plants and ultimately PGs. In an emblematic way, pollination period as well as airborne pollen concentrations are affected by climate change (Besancenot and Thibaudon, 2012; Lake, 2017). Increased concentrations of CO_2 may induce on certain species changes in production of pollen grains (increasing the amount of PGs) and allergenic protein contents (Albertine et al., 2014; Kim et al., 2018). Interestingly, a more severe immunological response can also be induced by air pollution, either by direct effects on allergic patients or by alterations of PGs (directly on the PGs or indirectly via alterations of plants). For example, the worsening effects of soot particles and NO_2 are well established but the specific biochemical reactions and molecular processes responsible for their effects are still not clearly identified (Shiraiwa et al., 2012).

Determination of pollen biochemical composition is also important for estimating properties of pollen regarding condensation and glaciation nucleus and for estimating the role of PGs and their sub–particles in the atmospheric water cycle (Diehl et al., 2002, 2001). Moreover, a better description of biochemical composition of pollen is essential for understanding the biological

mechanisms of respiratory allergic diseases and for the search of new therapeutic targets for the prevention and treatment of allergy. The past three decades have been fruitful in publications regarding PPPGs both on the characterization of allergens, lipids and other constituents. This review of the literature is in search of a detailed biochemical description of PPPGs.

2. Biochemical composition of *Phleum pratense* pollen grains

To the best of our knowledge, all biochemical compounds quantified in PPPGs and reported in literature are listed in Table 1. PPPGs are constituted of sugars, polysaccharides, starch, glycoproteins (including allergens), amino-acids, lipids, flavonoids (including isorhamnetin), diverse elements (the more abundant being Si, Mg and Ca), phenolic compounds, phytoprostanoids, carotenoids (pigments) metals and adsorbed pollutants.

Diameter and mass of a single pollen grain

There is a general agreement in literature for an average diameter between 30 to 35 µm for PPPG (Table 2). In contrast, two ranges of values emerge from literature for the mass of a single PPPG: values between 11.1 and 16.4 ng.grain^{-1} on the one hand, and values between 23.7 and 26 ng.grain^{-1} on the other hand (Table 3). Those two sets may distinguish different cultivars with different diameters and/or densities, such as the WP Barpenta and the WP Rubato, for which reported masses were 16.4 ng.grain^{-1} and 23.7 ng.grain^{-1} respectively (Jung et al., 2018). Those two sets may indeed correspond to two varieties of *Phleum* with different diameters and/or densities. The historical value of Brown and Irving (13.7 ng) is the most frequently used in literature. In the absence of a consensus on the mass of a grain, this value was then used also in this review for the mass fraction calculations (conversion from ng.grain^{-1} to ng.mg^{-1}). Settling velocity for PPPG has also been estimated in literature between 10 to 14 cm.s^{-1} (Raynor et al., 1971).

135 Table 1. Current state of knowledge on biochemical constituents of *Phleum pratense* pollen grains.
136 Abbreviations: PAHs polycyclic aromatic hydrocarbons, PALMS pollen-associated lipid
137 mediators.

Constituents	**Mass μg.mg^{-1}**	**Details**	**References**
Sugars	384	Fructose, sucrose, myo-inositol	(Mueller et al., 2016)
P-particles	100-246	61000-230000 particles, spherical, d=1.5 g.cm^{-3}	(Heslop-Harrison and Heslop-Harrison, 1982)
Starch granules	76	1000 granules, spherical, d=1.5 g.cm^{-3}	(Abou Chakra, 2009; Abou Chakra et al., 2011a)
Total proteins	50	Average from different extraction methods	(Aloisi et al., 2018)
Amino-acids	40	Extracted with aqueous solvent	(Mueller et al., 2016)
Internal lipids	26	Extracted with organic solvent	(Farah et al., 2020a)
External lipids	22	Extracted with organic solvent	(Farah et al., 2020b)
Flavonoids	14.4	Extracted with organic solvent	(Kolesnikov and Gins, 1999)
Silicium (element)	7.3	Sum of free-, hydrolyzed- and tightly bound-Si	(Kolesnikov and Gins, 1999)
Phenolic compounds	5	Extracted with organic solvent	(Smiljanic et al., 2019)
Phytoprostanoids	3.6	Extracted with organic solvent	(González Roldán et al., 2019)
Isorhamnetin	3.1	Extracted with organic solvent	(Kolesnikov and Gins, 1999)
Allergen Phl p 5	3.2	Average from different sources	Cf. table 4 for references
Magnesium (element)	1.7		(Smiljanic et al., 2019)
Adenosine	1.3		(Mueller et al., 2016)
Calcium (element)	1.2		(Smiljanic et al., 2019)
Folic acid	1.1	Vitamin B9	(Nielsen and Holmström, 1957)
Allergens group 2	0.59	1172 ng per 100 μg of total proteins	(Marth et al., 2004)
Allergen Phl p 4	0.2		(Fahlbusch et al., 1998)
Iron (element)	0.16		(Smiljanic et al., 2019)
Sum of metals	0.06	Cr, Ni, Cu, Zn, As, Cd, Hg, Pb	(Smiljanic et al., 2019)
Zinc (element)	0.04		(Smiljanic et al., 2019)
Adsorbed NO_2	0.004	Calculated for 10 h at 40 μg.m^{-3} NO_2	(Chassard et al., 2015)
PAHs	0.0004	Pollen from low pollution area	(Smiljanic et al., 2019)
PALMs	0.0004	PGE2+LTB4	(Behrendt et al., 2001)
Hg (element)	0.00004	Pollen from low pollution area	(Smiljanic et al., 2019)

138

139 Table 2. Average diameter (μm) reported in literature for *Phleum pratense* pollen grains.

Average diameter (μm)	Reference
30-35	(Brown and Irving, 1973)
34	(Raynor et al., 1971)
32±2	(Joly et al., 2007)
31±2	(Abou Chakra, 2009)

140

141 Table 3. Average mass (ng per pollen grain) reported in literature for *Phleum pratense* pollen grains.

Mass of a single grain (ng per grain)	Reference
11.1	(Abou Chakra, 2009)
13.7	(Brown and Irving, 1973)
16.4	Variety WP Barpenta (Jung et al., 2018)
23.7	Variety WL Rubato (Jung et al., 2018)
24±3	(Murphy and Aarssen, 1995)
26	(Guerin, 1993)

142 ## Pollen grain structure and sporopollenin

143 Sporopollenin protects the plant gametes against a wide range of environmental attacks such as
144 drying out and solar radiation (Li et al., 2019). Despite its importance, the chemical structure of
145 plant sporopollenin remained poorly documented and totally unexplored for *Phleum pratense*
146 pollen (Julier et al., 2016). A cross-sectional view of a PPPG showing details of the pollen wall
147 structure is presented in figure 2. The overall thickness of the pollen wall varies between 0.8 and
148 1.6 μm. In general, the pollen wall is composed of an outer envelope called exine (or exospore)
149 and an inner cellulosic membrane called intine (or endospore) (Halbritter et al., 2018). The exine
150 prevents the genetic material from damages due to pollen desiccation and is composed of two
151 layers: the tectum and the foot layer, just above the intine (Hesse, 2009). The tectum and the foot
152 layer are separated by a region called the columella composed of reinforcing rods. The exine of
153 PPPG is sculptured with spines (scabrae) and a microechinate ornamentation (Figure 3). The
154 pollen pore allows shrinking (harmomegathy) and swelling of the grain caused by changes in
155 moisture content. The circular aperture (pore) of PPPG has a lid (operculum).

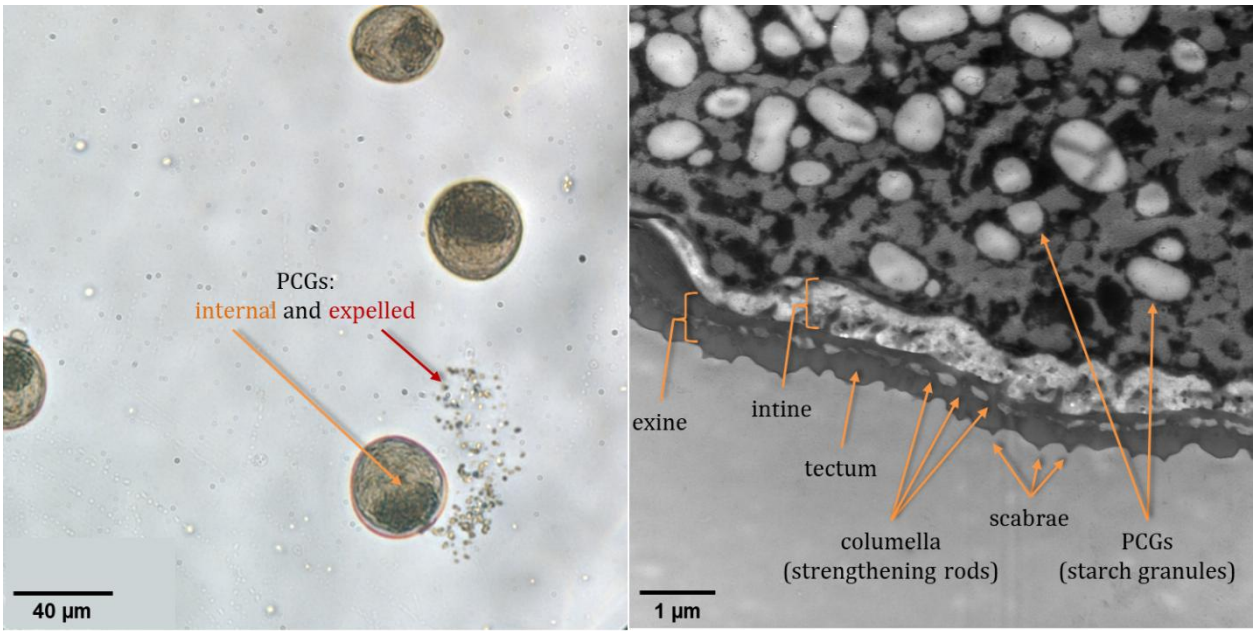

Figure 2. (left) *Phleum pratense* pollen grains in aqueous solution with internal and expelled pollen cytoplasmic granules (PCGs) and (right) close-up view of the cross-section of the pollen wall after glutaraldehyde fixation followed by osmium tetroxide treatment.

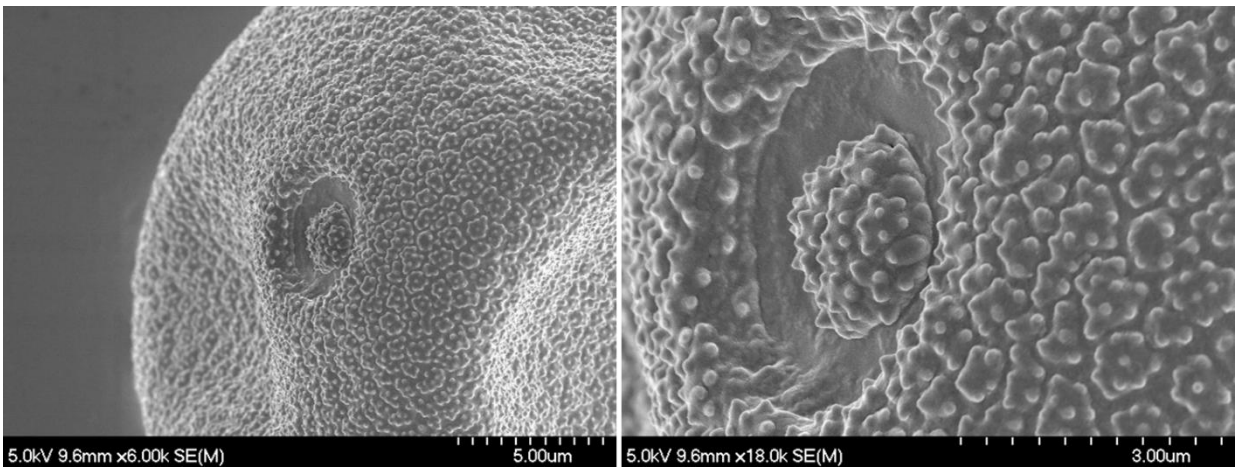

Figure 3. SEM images of a *Phleum pratense* pollen grain showing (left) the sculpturing of the wall and (right) a close-up view of the single operculate aperture.

Total proteins

The total amount of proteins extracted depends on the extraction protocol (Aloisi et al., 2018). Thus, when extraction was carried out with buffer lysis, by sonication with phosphate buffer saline solution (PBS) pH 7.5 or by continuous stirring with PBS, the total mass fraction of proteins on the same PPPG sample varied respectively from 45, 50 to 56 $\mu g.mg^{-1}$ (Aloisi et al., 2018). These values are in perfect agreement with the determination of 51 ± 4 $\mu g.mg^{-1}$ carried out by others with an extraction by water with continuous agitation (Rogerieux et al., 2007). Much lower values were subsequently determined at 13.2 $\mu g.mg^{-1}$ by extraction with PBS at 4°C with

constant agitation (Schäppi et al., 1999) and at 26 $\mu g.mg^{-1}$ by incubation at 37°C in vitro in phosphate-buffer pH 6.0 (Behrendt et al., 1999). Those lower values could be tentatively explained by the lower temperature (4°C) in one case and by the absence of agitation in the other one (incubation). These differences may be explained also by the quantification methods used or by biological differences between samples.

Allergens

Allergens from the Poaceae family are one of the most frequent cause of allergic symptoms worldwide (Mohapatra et al., 2005). They are classified in eleven groups from 1 to 13 (groups 8 and 9 are absent) based on their structural and biological properties (table 4). Allergens from groups 1 and 5 (1a and b, and 5a and b) are the major contributors to Phl p pollen allergy (Mohapatra et al., 2005). PPPGs extract analyzed by 2D gel electrophoresis silver staining exhibits about 100 different proteins with molecular masses from 10 to 94 kDa and isoelectric points (pI) from 3.5 to 10.6 (Abou Chakra et al., 2012; Rogerieux et al., 2007). Other allergens such as the water-insoluble types have been studied in Phl p pollen and in PCG (Abou Chakra et al., 2011b). Water-insoluble allergens seem to play a role in the centrally mediated inflammatory response, whereas water-soluble allergens may be involved in the peripheral humoral response.

Table 4. *Phleum pratense* (timothy grass) pollen allergens. Prevalence data (Mari, 2003; Niederberger et al., 1998). IUIS: International Union of Immunological Societies.

	Allergen	Biochemical name	MW /kDa (SDS-PAGE)	pI	Glyco-sylation	Prevalence (%)*	References
IUIS	Phl p 1a Phl p 1b	Beta-expansin	31-38	5.1-8.0	Yes	83	(Halim et al., 2015; Laffer et al., 1996; Petersen et al., 1998, 1995; Valenta et al., 1992; Wicklein et al., 2004)
IUIS	Phl p 2	Beta-expansin (fragment)	10-11	4.5-5.5	No	55	(Barre and Rougé, 2002; Dolecek et al., 1993; Laffer et al., 1996)
	Phl p 3	Beta-expansin (fragment)	10-11	8.9-9.3	No	37-70	(Devanaboyina et al., 2014; Petersen et al., 2006; Schweimer et al., 2008)
IUIS	Phl p 4	Berberine bridge enzyme	59-67	9.0-10.5	Yes	70	(Fischer et al., 1996; Leduc-Brodard et al., 1996; Nandy et al., 2005; Stumvoll et al., 2002; Zafred et al., 2013)
IUIS	Phl p 5a Phl p 5b	Ribonuclease	25-34	5.0-8.5	Yes	50	(Becker et al., 1995; Halim et al., 2015; Laffer et al., 1996; Valenta et al., 1992)

IUIS	Phl p 6	Iso-flavone reductase	13	4.3-5.5	No	44	(Blume et al., 2004; Petersen et al., 1995; Vrtala et al., 1999)
IUIS	Phl p 7	Ca-binding protein 2-EF hand	6 (8.6 kda calc mass)	4.0-4.2	No	7	(Niederberger et al., 1999; Verdino et al., 2002)
	Phl p 10	Cytochrome C	12	10-10.6	No	Unkown	(Ekramoddoullah et al., 1984, 1982; Sharma et al., 2008)
IUIS	Phl p 11	Ole e-1 related protein (trypsin inhibitor)	20	5.0	Yes	43	(Cipriani et al., 2018; DeWitt et al., 2002)
IUIS	Phl p 12	Profilin	14	4.4-5.0	No	15	(Cipriani et al., 2018; Valenta et al., 1994)
IUIS	Phl p 13	Polygalacturonase	55-60	6.5-8.0	Yes	25-75	(Suck et al., 2000a, 2000b; Wicklein et al., 2004)

Quantification of allergens

Phl p 5 was extensively quantified in PPPGs (Table 5); the values from the literature are in very good agreement. Values between 2.7 and 3.5 $\mu g.mg^{-1}$ were reported for unpolluted PPPGs of various origins and over a time scale of about 20 years (first measurements in 1999). The amount of proteins extracted depends on the pH of the extraction medium: total proteins increased from 13.2 to 17.1 $\mu g.mg^{-1}$ when the pH increased from 6.0 to 9.0 (Behrendt et al., 1999). An average value of 3.2 ± 0.5 $\mu g.mg^{-1}$ was calculated for Phl p 5 from literature data mentioned with an asterisk in Table . PPPGs exposed to air pollution exhibit a lower mass concentration of Phl p 5 compared to unexposed pollens (table 5).

Some information is available in literature on the quantification of allergens other than Phl p 5. Allergens from group 2 were quantified at 1172 ng per 100 µg of total proteins (Marth et al., 2004). The Phl p 4 amount in pollen extract was determined at 0.2 $\mu g.mg^{-1}$ quantified by an ELISA method. The absolute content of group 4 was in <1% relative to the total proteins of the Phl p pollen extract (Fahlbusch et al., 1998). Moreover, the quantification of *Phleum pratense* allergens Phl p 1 and Phl p 5 in pollen extract was performed by mass spectrometry. In the extract Phl p 1b (Phl p 1 basic, 150 $fmole.\mu L^{-1}$) was shown to be about 5 times less abundant than its variant Phl p 1a (Phl p 1 acid, 724 $fmole.\mu L^{-1}$) and Phl p 5b (Phl p 5 basic, 4393 $fmole.\mu L^{-1}$) was shown to be 9 times more abundant than the Phl p 5a (Phl p 5 acid, 499 $fmole.\mu L^{-1}$) (Seppälä et al., 2011). The allergen concentration derived from this work (between 15-21 $\mu g.mg^{-1}$ of Phl p 5) was not used for the calculation of the averaged Phl p 5 mass because of the discrepancy observed with other studies (average value of 3.2 ± 0.5 $\mu g.mg^{-1}$).

The amount of Phl p 5 in the atmosphere has a strong impact on the symptom load of allergenic patients (Bastl et al., 2015). For the purpose of assessing individual exposure to allergens, pollen allergen potency (amount of allergen per mass of PG (Galán et al., 2017)) are calculated for a given location by simultaneous analysis of concentration of pollen grains and of allergens (Galán et al., 2017). Comparison between the relative constancy of Phl p 5 per mg of pollen (or per pollen grain) and the pollen allergen potency reported in literature from grass species is questioning. Indeed, grass pollen allergen potency were reported as low as 0.2 $\mu g.mg^{-1}$ and with a high variability from 0.05 to 2.3 $\mu g.mg^{-1}$ (Buters, 2015; Jochner et al., 2015; Plaza et al., 2016). Decrease of Phl p 5 release by pollution is a possible explanation as well as discrepancies between allergen content between grass species.

Table 5. Quantification of Phl p 5 in *Phleum pratense* pollen grains (*values used for our determination of average Phl p 5 release).

Note	Mass of Phl p 5 ($\mu g.mg^{-1}$)	Reference
No information on pollen source	15-21	(Seppälä et al., 2011)
Plant grown in a natural field	3.5*	(Aloisi et al., 2018)
Pollen from rural meadow	3.5*	(Behrendt et al., 1999)
Timothy cultivar WL Barpenta	3.2*	(Jung et al., 2018)
Plant grown with O_3 30 ppb / CO_2 400-800 ppm	3.2*	(Albertine et al., 2014)
No information on pollen source	3.2*	(Huss-Marp et al., 2008)
Commercial pollen	3.0*	(Schäppi et al., 1999)
Timothy cultivar WL Rubato	2.7*	(Jung et al., 2018)
Pollen collected near road-traffic	2.2	(Behrendt et al., 1999)
Pollen exposed to SO_2	1.9	(Huss-Marp et al., 2008)
Plant grown with O_3 80 ppb and CO_2 400 ppm	1.3	(Albertine et al., 2014)
Stored commercial pollen	0.9	(Behrendt et al., 1999)

Pollen cytoplasmic granules (PCGs) and dispersion of allergens in respirable aerosols

There are two types of pollen cytoplasmic granules (PCGs): the starch granules and the polysaccharide-particles (p-particles). PCGs were thoroughly investigated for their implications in pollen-related asthma.

Starch granules

Starch granules have a size between 0.6 to 2.5 μm with an average diameter of 1.1 μm (Abou Chakra et al., 2011a, 2011b; Suphioglu, 1998). Their number has been estimated to 1,000 starch granules per PPPG (Abou Chakra et al., 2011a). Starch granules were also been estimated between 700 and 1,000 per PG for ryegrass having a pollen size (and thus a volume) very close to the one of PPPG (Singh et al., 1991; Suphioglu, 1998).

P-particles

P-particles have a size ranging around 0.3 to 0.4 μm (Grote et al., 1994; Heslop-Harrison and Heslop-Harrison, 1982). The number of p-particles per pollen grains has not been directly measured. P-particles occupy between 20 to 30% of the PG volume corresponding to an estimate of 230,000 p-particles per PG (based on a spherical volume of pollen of 32 μm and spherical volume of p-particles of 0.35 μm) (Heslop-Harrison and Heslop-Harrison, 1982). Arabinogalactan polysaccharides have been quantified to about 10% of the mass of PPPG (Brecker et al., 2005). This polysaccharide fraction is composed by mass of 68% D-galactose and 32% L-arabinose. By considering that this mass of polysaccharide corresponds exclusively to p-particles, we obtain an estimate of 61,000 p-particles per pollen grain (density equals to 1.5 $g.cm^{-3}$ and spherical particles of 0.35 μm diameter). We retain as an estimate between 61,000 and 230,000 of p-particles per pollen grain which is lower than the literature value of more than one million p-particles per PG (Heslop-Harrison and Heslop-Harrison, 1982). Indeed, this value of one million would result in a mass of p-particles greater than that of a single pollen grain.

Localization of allergens

Visualization of allergen by immunogold-labelling technique has been widely used allowing localization of allergens between outer and inner parts of PPPGs. Allergens of group 1 and 5 were localized on exine and intine as well as in the cytoplasmic matrix between p-particles and cell organelles (mitochondria) (Behrendt et al., 1999; Grote et al., 2001, 1994). Only allergens of group 5 were localized on amyloplasts (starch granules) (Grote et al., 1994). Phl p 1 was found only in the intine (Behrendt et al., 1999). Phl p 4 were localized in the exine, cytoplasm and amyloplast (Fischer et al., 1996). Some allergens as Phl p 1 and Phl p 4 were also localized in pollen coat. (See below) (Bashir et al., 2013b; Rejón et al., 2016).

In a comprehensive study, allergens Phl p 1, 4, 5, 6 and 12 were detected both on whole PPPGs and PCGs extracts whereas Phl p 11 was only detected on PCGs extract and Phl p 2 and 13 only on PGs extract (Abou Chakra et al., 2012). Phl p 6 has been localized on p-particles (Vrtala et al.,

1999). Confirming those localizations of allergens, PCGs are recognized by IgE from pollen-sensitive rat sera and they trigger lymph node proliferation in these rats (Motta et al., 2004). Water soluble and water insoluble protein extracts from PCGs were recognized by IgE from rat sera and also from grass pollen allergic patient sera. The proteins recognized as allergens by IgE from sensitized rat may correspond to allergens also recognized by human IgE (Abou Chakra et al., 2011b). In a recent study, Phl p 2 and Phl p 5 are the allergens more concentrated in PPPG sub particles; Phl p 1 and Phl p 6 were also detected to a lesser extent (Cecchi et al., 2020).

The pollen coat also contains a number of proteins, most of which are also specifically synthesized in the tapetum layer. Many of these proteins are capable of triggering an IgE-mediated immune response in humans, thus highlighting the importance of the pollen coat as also a source of aeroallergens (Bashir et al., 2013b). Among these allergens, Phl p 1, Phl p 4 and Phl p cystein protease were identified (Rejón et al., 2016).

Expulsion of PCGs from the cytoplasm

Expulsion of PCGs from the cytoplasm when pollen is immersed into an aqueous solution is a well-documented mechanism common to all grass species (figure 2) (Grote et al., 2001). This cytoplasmic expulsion has been directly observed for PPPGs and it induces the release of hundreds of PCGs and allergens out of the PGs (Grote et al., 2001; Swoboda et al., 2004; Taylor et al., 2002). Figure 2 shows PCGs expelled from the aperture of a PPPG immersed in water and a close-up view of oblong PCGs. Cytoplasmic expulsion occurs much less frequently when the time between pollen collection from grass and immersion is prolonged (Grote et al., 2001; Taylor et al., 2002).

This expulsion of the cytoplasm is considered to be the major cause of allergen dispersion in the fine fraction of the atmospheric aerosol (Suphioglu et al., 1992). However, the mechanism by which allergens are transferred from the aqueous solution (in which the cytoplasm is expelled) to the small-sized respirable aerosol has never been studied. For this reason, work has been carried out to estimate the emission of fine particles charged with allergens directly from plant during pollination. An aerosol of particles smaller than 5 μm can be formed when grass and pollen is subjected to moisture-drying cycles and followed by exposure to an air flow (Taylor et al., 2002). This mechanism has been observed for grass species (*Lolium* and *Cynodon*) but not directly for *Phleum*. Other hypothesis for pollen rupture (not directly tested for PPPGs) are rupture induced by exposure to electrical fields and rupture induced by mechanical stress during wind transportation (Vaidyanathan et al., 2006; Visez et al., 2015). Rupture of grass pollen species are directly linked to thunderstorm asthma (Knox, 1993; Suphioglu, 1998; Taylor et al., 2002).

Atmospheric pollution is a facilitating agent in inducing pollen rupture (Motta et al., 2006; Smiljanic et al., 2019).

Detection of allergens in the fine fraction of atmospheric aerosols

PPPG allergens have been detected several times in the fine (respirable or less than 10 µm) fraction of atmospheric aerosol (Schäppi et al., 1996; Spieksma et al., 1995, 1990; Taylor et al., 2002). However, Phl p 5 allergen was most of the time detected predominantly in coarse particle mode (PM>10 µm) (Alan et al., 2018; Buters, 2015). After rainy events, grass pollen allergens were detected in the atmosphere while PGs were for their part not observed (Holmquist et al., 1999). Large quantities of starch granules have also been directly observed in the atmosphere after rainy episodes but not on dry days (Suphioglu, 1998). To the best of our knowledge, p-particles have never been directly observed in the atmosphere, probably because of their small size making their discrimination difficult among atmospheric particulate matter.

Lipidomic

Total lipidomic analysis

Some lipids are bioactive compounds able to interfere in the cascade of biological reactions of the immune system. Some lipids can control early immune system response signals and amplify the response induced by allergens (Bashir et al., 2013a). This influence of lipids on immune response is of course directly ruled by their nature and biochemical characteristics.

Extractible lipidic fraction was determined as early as 1932 at 1.7% of the mass of PPPGs by Soxhlet extraction with an organic solvent (Stull et al., 1932). This value is very close to the one obtained by weighing of the dry extract (2.2%) obtained after pollen washing with methylene chloride (Farah et al., 2020b). However, use of organic solvents only allows to extract the external fraction of pollen lipids (Evans et al., 1991). The internal fraction of PPPGs was recently obtained by grinding the grains prior to extraction: the total extractable lipids are distributed between 22 $\mu g.mg^{-1}$ for the external fraction and 26 $\mu g.mg^{-1}$ for the internal fraction (Farah et al., 2020a). Complete analysis of the extractable lipid fraction is still missing: less than 20% of lipids have been identified and quantified (Farah et al., 2020a, 2020b).

PPPGs from various geographical origins in the Czech Republic and the USA and from different harvest years (from 2007 to 2018) underwent lipid extraction with an organic solvent followed by analysis by gas chromatography coupled to mass spectrometry (Farah et al., 2020b). The variability in total lipid quantities was shown to be low between samples, less than 9%, despite geographical and pollen age differences. A larger dataset would be needed to confirm that (i) the

lipids of pollen are stable despite storage and (ii) the lipid fraction is relatively constant in abundance in PPPGs from various geographical origins and years of collection.

Chemical composition of lipids

Main chemical families of lipids observed in PPPGs by extraction with an organic solvent are: alkanes, alkenes, alcohols, saturated and unsaturated fatty acids, di- and tri-hydroxylated fatty acids and aldehydes (Bashir et al., 2013a; Farah et al., 2020b; González Roldán et al., 2019). Several sterols have also been detected but not quantified in extractable fraction of PPPGs (Bashir et al., 2013a; Farah et al., 2020b). The comparison between the semi-quantitative analysis carried out by Bashir et al. (2013) and the quantitative analyses of Farah et al. (2020) highlights significant disparities between the main chemical families despite similar procedures of extraction and analyses between studies (figure 4) (Bashir et al., 2013a; Farah et al., 2020a, 2020b). These differences are in our opinion due to differences in the interpretation of some mass spectra and/or due to simultaneous extraction of external and internal lipids consecutively to (uncontrolled) rupture of PGs during laboratory extraction protocols (Farah et al., 2020a, 2020b).

The external and internal lipid fractions of PPPGs share the same lipids but with a deeply different partitioning (figure 4Figure). A large predominance of saturated and unsaturated fatty acids has been observed in the cytoplasmic fraction (Farah et al., 2020a). The potential health effects of the presence of these specific lipids in PCGs of PPPGs has not been yet investigated.

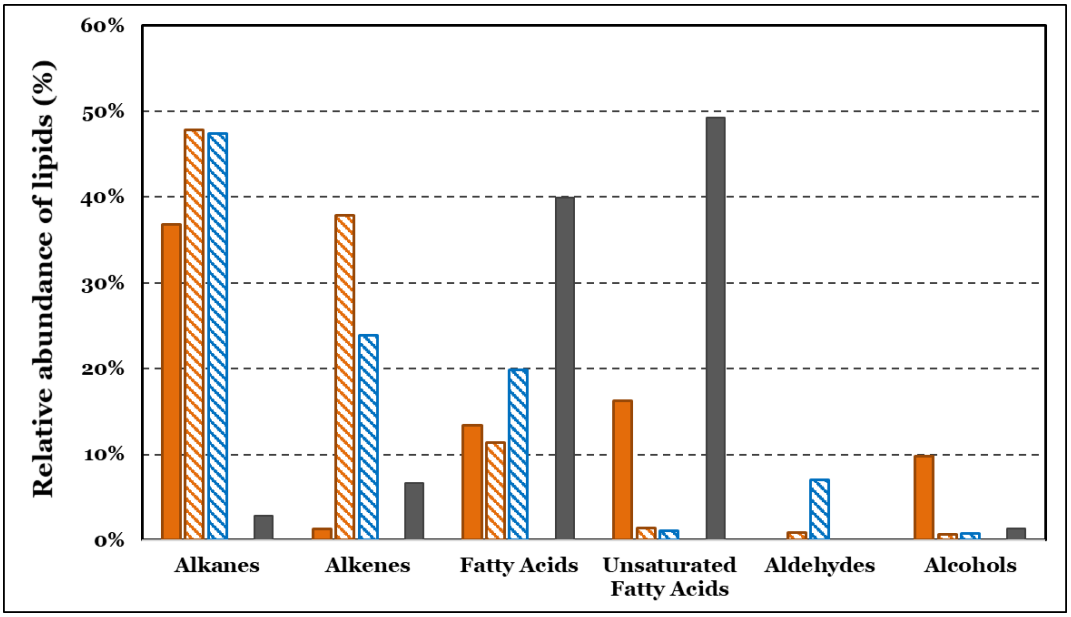

Figure 4. Relative abundances of main chemical families of lipids from *Phleum pratense* pollen grains. Orange: external lipids (Bashir et al., 2013a); hatched orange: external lipids (Farah et al.,

2020b); hatched blue: external lipids after exposure to ozone at 100 ppb for 16 hours (Farah et al., 2020b); grey: internal lipids (Farah et al., 2020a).

Prostaglandin and leukotriene

Pro-inflammatory substances, as E2-like prostaglandin (PGE2) and B4-like leukotriene (LTB4), have been extracted from PPPGs with aqueous solvent. Those so-called Pollen Associated Lipid Mediators (PALMs) were quantified in very low mass fractions of 0.00007 $\mu g.mg^{-1}$ for PGE2 and of 0.00004 $\mu g.mg^{-1}$ for LTB4 (Behrendt et al., 2001, 1999; Huss-Marp et al., 2008). Mass fractions of PALMs increased for PPPGs sampled in environment influenced by road-traffic compared to PPPGs sampled in rural environment (+100% for LTB4, +50% for PGE2) (Behrendt et al., 2001). The release of these substances occurred in less than 30 minutes after incubation of PPPGs in PBS (Behrendt et al., 2001).

Linoleic and linolenic acids and their derivatives

Discrepancies between studies exist for the quantification of linoleic and linolenic acids extracted from PPPGs: between 0.003 and 0.813 $\mu g.mg^{-1}$ and between 0.051 and 5.0 $\mu g.mg^{-1}$ respectively (Farah et al., 2020a, 2020b; Plötz et al., 2004; Traidl-Hoffmann et al., 2002). Those differences could be explained, at least in part, by uncontrolled rupture of PPPGs during extraction procedure (Farah et al., 2020a). Mono-hydroxylated derivatives of linoleic acids and linolenic acid were quantified in lipids of PPPGs in the range between 0.006 and 0.010 $\mu g.mg^{-1}$ (Traidl-Hoffmann et al., 2002).

Phytoprostanes and phytofurans

Phytoprostanoids are peroxidation products derived from α-linolenic acid and markers of oxidative stress among plants. Their concentrations in PPPGS was determined to 3.6 $\mu g.mg^{-1}$ (González Roldán et al., 2019). Phytoprostanes and phytofurans are rapidly released by PPPGs upon hydratation (González Roldán et al., 2019). Phytoprostanoids bound to glycerol are able to trigger mast cells degranulation (González Roldán et al., 2019).

Adenosine

Adenosine was identified as a potent immunoregulatory substance in PPPGs and detected to mass fraction between 0.65 and 1.29 $\mu g.mg^{-1}$; it is controversial whereas such low concentration of bioactive compound may or may not reach significant health-induced effects (Gilles et al., 2011; Mueller et al., 2016).

Vitamins

PPPGS are rich in B9 vitamin (folic acid 1.1 $\mu g.mg^{-1}$) and its conjugates (5.6 $\mu g.mg^{-1}$) (Nielsen and Holmström, 1957). Two other vitamins were also identified but not quantified in organic extracts of PPPGs: a-tocopherol and L-ascorbic acid (Bashir et al., 2013a).

Phenolic compounds

Phenolic compounds can provide an anti-oxidant capability to PPPGs by suppressing reactive oxidant response and by quenching free radicals (Shiraiwa et al., 2012; Smiljanic et al., 2019). The total phenol mass was estimated in PPPGs to 5 $\mu g.mg^{-1}$ (gallic acid equivalent) and the mass of flavonoids to 14.4 $\mu g.mg^{-1}$ (Kolesnikov and Gins, 1999; Smiljanic et al., 2019). Isorhamnetin was identified in PPPGs and quantified to 3.1 $\mu g.mg^{-1}$ (Kolesnikov and Gins, 1999). The flavonoids play a role in allergenicity of some proteins but its role on Phl p allergens was not yet described.

Water content

Release of proteins and allergens from PPPGs can be modified by an exposure of PGs to relative humidity (Fritzsche et al., 1997). There is however no measurement of water content specifically for PPPGs. The amount of water in pollen grains varies greatly at the time of pollination depending on the meteorological conditions: wind, humidity and temperature (Heslop-Harrison, 1979). The water content of fresh pollen ranges from 15 to 35% for angiosperms but most PGs used in laboratory are dried to a water concentration of a few percents before storage (Guerin, 1993; Heslop-Harrison, 1979).

Others biochemical characteristics

NAD(P)H oxidase

PPPGs and their extracts contained NAD(P)H oxidases (Boldogh et al., 2005) generates reactive oxygen species (ROS) and plays a prominent role in the pathogenesis of allergies in mouse models (Wang et al., 2009). ROS released by PGs has the ability to rapidly increase oxidative stress in respiratory system epithelium and as a consequence to augment allergic airway inflammation induced by PGs (Boldogh et al., 2005). Pollen NAD(P)H oxidase was located at the surface and in the cytoplasm of pollen grains from species of the Poaceae family (not directly observed for PPPGs) (Wang et al., 2009).

Protease activity

Protease activity was observed for grass PGs (*Secale cereale*, *Poa pratensis*, *Lolium perenne* and *Cynodon dactylon*) but not directly for PPPGs (Gunawan et al., 2008; Raftery et al., 2003).

Microbiome

DNA from bacteria and fungi was isolated from PPPGs. The microbial composition of PGs has been correlated with environmental exposure parameters. The microbial composition is also correlated with expression of allergens and quantities of PALMS in PPPGs (Obersteiner, 2017; Obersteiner et al., 2016). The potential effects of microbial stress on allergenicity of pollens are currently not well understood. Lipids originating from pollen microbiome may contribute to the sensitization process (Bublin et al., 2014).

Pollutants

Several pollutants have been detected in PPPGs (Table *1*) but they remain minor components on a mass basis of the whole pollen grains (Chassard et al., 2015; Farah et al., 2020b; Goschnick and Schuricht, 1996; Smiljanic et al., 2019). PAHs and metals could be contributors to oxidative stress both onto pollen grains and on human cells after contact with a PPPG (Shiraiwa et al., 2012; Smiljanic et al., 2019).

3. Discussion

Phleum pratense pollen grain (PPPG) is probably, with pollen from *Betula* and *Ambrosia,* one of the best described allergenic pollen grains. The biochemical description of PPPG was greatly improved during the last 30 years. Its main constituents have been very likely identified and some of them were quantified (Table *1*). There is a growing evidence of the importance of molecules co-delivered with allergens during allergenic sensitization (Pointner et al., 2020). PPPG is mainly composed of sporopollenin, oligosaccharides or polysaccharides, starch, proteins some of which are allergens, amino-acids, lipids and diverse elements, Si and Ca being the most abundant. Some important constituents of allergenic pollen like protease and NAD(P)H oxidase were not quantified in PPPGs. The way in which *a priori* incompatible substances (oxidants and anti-oxidants, protease and proteins) are distributed and segregated in pollen grains is also still rather poorly known (Taylor et al., 2007).

Data in table 1 could be considered as an average biochemical composition of PPPGs issued from various samples of pollen. The variability of pollen composition according to vegetal or environmental conditions remains largely unknown. Repeated quantification on a consequent number of samples is only available for Phl p 5 allergen: the agreement between studies is good with an average of 3.2±16% (table 4). On a limited number of samples, variability on lipids was estimated to 9% (Farah et al., 2020b). Most of others constituents quantified in PPPGs were determined for only one pollen sample (vitamins, extractible lipids, flavonoids, phenolic

compounds…). A better knowledge of this variability is important for estimating the composition of PPPGs in the future in the context of a dramatic increase in atmospheric CO_2 concentration and of climate disasters. The effects of CO_2 on PPPG has been reported by one research group only for allergens and the number of PGs produced by anthers (Albertine, 2013; Albertine et al., 2014). Nothing is known about variability in lipids and other constituents nor about the adaptation of *Phleum pratense* to future climate change. Air pollution can also bring an important additional variability on certain biochemical parameters of PPPG: allergens release, changes in pollen composition, pollen rupture, oxidative alterations, adsorption of gaseous and particulate pollutants (Behrendt et al., 2001, 1991; Behrendt and Becker, 2001; Chassard et al., 2015; Farah et al., 2020b; Goschnick and Schuricht, 1996; Huss-Marp et al., 2008; Motta et al., 2006; Risse et al., 2000; Rogerieux et al., 2007; Shiraiwa et al., 2012; Smiljanic et al., 2019).

For a better knowledge of variability of pollen composition, the location and exact date of pollen harvesting should be systematically mentioned in published works. Lack of this information is a major gap in literature making it more difficult to discriminate potential effects linked to pollen ageing during storage or to specificity in composition linked to regional or seasonal variability. A striking example is the discrepancy in the mass of a single PPPG reported in various studies (table 3). It was also mentioned by Stull in 1932 that 10-year-old aqueous pollen extracts were less rich in protein than recently harvested pollen (Stull et al., 1932). Occurrence of pollen rupture drastically decreased after only several weeks of pollen storage (Grote et al., 2001). A good practice for pollen-related publications would be to systematize the following information: date and place of harvest, storage conditions, defatted or not, supplier (if any) and water content.

PPPGs are rich in several biochemical compounds in concentrations ranging from 0.00004 to 384 $\mu g.mg^{-1}$ (table 1). Human exposure to these compounds transported by pollen requires both their bioaccessibility (extraction from pollen in physiological conditions) and their bioactivity (inhibitory or promoting effect in the allergic reaction). Bioactivity presumes its bioaccessibility. Bioaccessibility requires knowledge of the kinetic of release of the biochemical constituents of PG but also of their ability to diffuse into the mucus (Mueller et al., 2016). Such information on bioaccessibility remains scarce for PPPG constituents in particular and for allergenic pollens in general. Bioaccessibility also questions the distribution of biochemical constituents in the pollen grain structure and the state (intact or broken) of the PGs being inhaled. As example, the partitioning of lipids between the outer and cytoplasmic regions of PPPG has only been quantified in one study (Farah et al., 2020a). Rupture of PGs is a factor that facilitates the release of specific compounds as observed with lipids preferentially extracted by PBS on broken (ruptured) PPPGs (Farah et al., 2020a). However, this partitioning raises the question of the use

of defatted pollens for the production of allergen extracts for skin prick tests. A pollen defatted with an organic solvent will always carry its internal lipids, which can also be released if the pollen is broken during an allergen extraction protocol.

Besides bioavailability, bioactivity requires that compounds extracted from PPPGs reach high enough concentrations for activities in physiological conditions. Mueller et al. (2016) have for example calculated concentrations of adenosine in nasal conditions based on assumptions on airborne pollen concentrations and of amount of nasal fluid. They conclude that, most of the time, nasal level of adenosine brought by PGs are too low to reach physiological levels (Mueller et al., 2016). Their work illustrates the difficulties in estimating nasal concentrations of pollen constituents after their extraction *in vivo* conditions. To estimate bioactivities, there are clear lacks in our knowledge of both kinetic of pollen clearance and in the volume of diffusion of its constituents (i.e. physiological concentrations reached in close vicinity of deposited pollen in airways) (Wang et al., 2009).

Significant work has been carried out to characterize the eleven groups from 1 to 13 (groups 8 and 9 are absent) of allergens in PPPGs. However, only the major allergen Phl p 5 was repeatedly assayed with a mean value calculated at 3.2 ± 0.5 $\mu g.mg^{-1}$. Phl p 5 and allergens from group 2 were the only allergens to be quantified in PPPGs and representing together 7-8% of total proteins. The mass distribution of allergens between the different groups is not known, nor is the quantitative distribution of allergens between the outer and inner parts of the PG. Besides the 11 allergens described in *Phleum pratense* pollen (table 4) and in other grass pollen grains, other allergens were identified as Phl p CP a cysteine protease, Phl p EXY an endoxylanase (Bashir ME et al 2013) (www.allergome.org) and Phl p Fe/Mn SOD a superoxide dismutase (Conti et al 2014). Six allergens (Phl p 1, 2, 4, 5, 6 and 12) are present in PPPG and PCG extracts whereas Phl p 11 was only detected only PCG extract (Abou Chakra et al., 2012)

The atmospheric concentrations of PGs do not necessarily coincide with the observations of symptom peaks (Bastl et al., 2016; Jochner et al., 2015). Furthermore, strong correlations were reported between symptom severity and air pollution but the biochemical mechanisms at work are largely unknown (Berger et al., 2020; Naclerio et al., 2020; Shiraiwa et al., 2012). Biochemical alterations induced by pollution to PGs may be at least in part responsible of aggravation of symptoms in polluted environment. Establishing a relationship between symptom intensity and pollution-altered PGs is made difficult by the need to characterize the degree of pollution at the PG level with a time-resolved sampling. More generally, information about inhaled PGs are lacking: releasable quantities of allergens and bioactive compounds, water content, fraction of

ruptured PGs, degree of alterations induced by pollution, number and nature of adhered atmospheric particles on the pollen surface… These biochemical parameters influence the biological micro-environment around a sedimented PG in human airways and contribute to modulate allergenic reactions.

Atmospheric pollen counts are considered relevant for public health and these samplings are widely used in routine across the world (Lehtimäki, 2020). The presence of allergens in the atmosphere in the absence of pollen grains and the direct link between symptoms and allergen load made several authors to conclude on the importance of additional allergen analysis in the atmosphere in addition to pollen counts (Bastl et al., 2015; Buters, 2015; Jochner et al., 2015). More generally, more information is needed about PGs (pollution, rupture of pollen grains) and sub-pollen particles (atmospheric concentrations) sampled directly in the atmosphere.

4. Conclusion

PPPGs are composed of polysaccharides, starch, allergenic and non-allergenic proteins, amino-acids, lipids, phenolic compounds, diverse elements (the more abundant being Si, Mg and Ca), phytoprostanoids, and adsorbed pollutants including metals and polycyclic aromatic hydrocarbons (PAH). The cytoplasm of one single PPPG is filled with about 1000 starch granules (average diameter 1.1 μm) and between 61,000 to 230,000 p-particles (0.3-0.4 μm). Much remains to be discovered about bioavailability and bioactivity of these constituents of PPPG and more generally of allergenic PGs. The lipid fraction of PPPG has, for example, not yet been fully described qualitatively and quantitatively with only 20% of extractible lipids being identified and quantified. Similarly, only 7-8% of total proteins have been quantified as allergens of group 2 and 5 allergen concentrations in other groups are unknown for PPPGs. The distribution of the constituents between the external and internal parts is also relatively poorly described in literature.

Phl p 5 is the only allergen that has been assayed by several research groups in PPPGs. A mean concentration of 3.2 ± 0.5 $\mu g.mg^{-1}$ has been determined for this allergen; this is the only quantitative estimation available for the variability of allergens over a heterogeneous set of PPPG samples. The variability of the biochemical composition of pollen is indeed not well known, including its determinants (vegetal, microbial, meteorological, geographical, or environmental causes). The existence of such variability could complicate our understanding of the biological effects of PPPG. Inspired on commercial batches of model particles used by atmospheric physicochemists, the aerobiology community should define a laboratory standard for PPPGs (and

other allergenic PGs as well). This would require the establishment of sufficient stocks of pollen from plants grown under controlled, or at least known, conditions and storage of PPPGs under optimal conditions. This could allow intercomparative studies between laboratories and could hopefully generate new knowledge on PPPGs.

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