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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.

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

55 1. Introduction

56 The Poaceae family is composed of about 12 000 wind-pollinated species and their pollen grains
57 (PGs) are considered as the major airborne biological threat to human health (García-Mozo,
58 2017). About 20% of the general population in the United States and Europe are sensitized to
59 grass pollen allergens (García-Mozo, 2017). Main allergenic species for the Poaceae family are:
60 *Alopecurus pratensis*, *Dactylis glomerata*, *Lolium perene*, *Cynodon Dactylon*, *Phleum pratense*, and *Poa annua*
61 (Guerin, 1993). Timothy grass, *Phleum Pratense* L., is one among the Poaceae family and it is
62 widely used in agriculture as a pasture and forage crop. Due to similarity and cross-reactivity of
63 *Phleum Pratense* pollen grains (PPPGs) with those from other species from Poaceae, allergens
64 extracted from PPPGs are used as a model in skin prick tests and PPPGs emerged as a model for
65 studies related to allergy to grass pollen (Andersson and Lidholm, 2003; White and Bernstein,
66 2003).



67
68 *Figure 1. (a) Inflorescence of timothy grass. (b) Close-up views of anthers. A single inflorescence can produce 1.2*
69 *million pollen grains (Albertine et al., 2014) (photography provided by authors).*

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

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

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

85 Despite sustained research efforts, the biochemical composition of allergenic PG is however not
86 yet fully elucidated neither is the causal relationship between variation in PGs composition and
87 pollen allergy risk (ie. the capacity of a PG, alone or with other factors such as air pollution, to
88 induce health impact (Galán et al., 2017)). First of all, research efforts are spread over different
89 allergenic species, the most studied being for trees *Betulaceae*, *Cupressaceae* and *Taxodiaceae*, for
90 grasses *Lolium* and *Phleum* and for weeds *Ambrosia* (ANSES, 2014). Pollen grains of allergenic
91 species do share common characteristics but they have also many differences including first and
92 foremost the nature and concentration of allergens. Secondly, the biochemical composition of
93 pollen grains depends on both intrinsic and extrinsic factors. It may vary among species, but also
94 among conspecific individuals, due to genetic determinants; it may also vary in space and time
95 due to plant age or environmental variations (composition of soil, meteorological and climatic
96 parameters...). Finally, the age of Anthropocene is characterized by significant levels of
97 environmental perturbations, such as pollutions in soil, atmosphere, water. Such perturbations
98 could influence the biochemical composition of plants and ultimately PGs. In an emblematic
99 way, pollination period as well as airborne pollen concentrations are affected by climate change
100 (Besancenot and Thibaudon, 2012; Lake, 2017). Increased concentrations of CO₂ may induce on
101 certain species changes in production of pollen grains (increasing the amount of PGs) and
102 allergenic protein contents (Albertine et al., 2014; Kim et al., 2018). Interestingly, a more severe
103 immunological response can also be induced by air pollution, either by direct effects on allergic
104 patients or by alterations of PGs (directly on the PGs or indirectly via alterations of plants). For
105 example, the worsening effects of soot particles and NO₂ are well established but the specific
106 biochemical reactions and molecular processes responsible for their effects are still not clearly
107 identified (Shiraiwa et al., 2012).

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

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

116 **2. Biochemical composition of *Phleum pratense* pollen grains**

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

122 **Diameter and mass of a single pollen grain**

123 There is a general agreement in literature for an average diameter between 30 to 35 μm for PPPG
124 (Table 2). In contrast, two ranges of values emerge from literature for the mass of a single PPPG:
125 values between 11.1 and 16.4 ng.grain^{-1} on the one hand, and values between 23.7 and 26
126 ng.grain^{-1} on the other hand (Table 3). Those two sets may distinguish different cultivars with
127 different diameters and/or densities, such as the WP Barpenta and the WP Rubato, for which
128 reported masses were 16.4 ng.grain^{-1} and 23.7 ng.grain^{-1} respectively (Jung et al., 2018). Those
129 two sets may indeed correspond to two varieties of *Phleum* with different diameters and/or
130 densities. The historical value of Brown and Irving (13.7 ng) is the most frequently used in
131 literature. In the absence of a consensus on the mass of a grain, this value was then used also in
132 this review for the mass fraction calculations (conversion from ng.grain^{-1} to ng.mg^{-1}). Settling
133 velocity for PPPG has also been estimated in literature between 10 to 14 cm.s^{-1} (Raynor et al.,
134 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 $\mu\text{g}\cdot\text{mg}^{-1}$	Details	References
Sugars	384	Fructose, sucrose, myo-inositol	(Mueller et al., 2016)
P-particles	100-246	61000-230000 particles, spherical, $d=1.5\text{ g}\cdot\text{cm}^{-3}$	(Heslop-Harrison and Heslop-Harrison, 1982)
Starch granules	76	1000 granules, spherical, $d=1.5\text{ g}\cdot\text{cm}^{-3}$	(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)
Phytosteranoids	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 $40\text{ }\mu\text{g}\cdot\text{m}^{-3}$ 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)

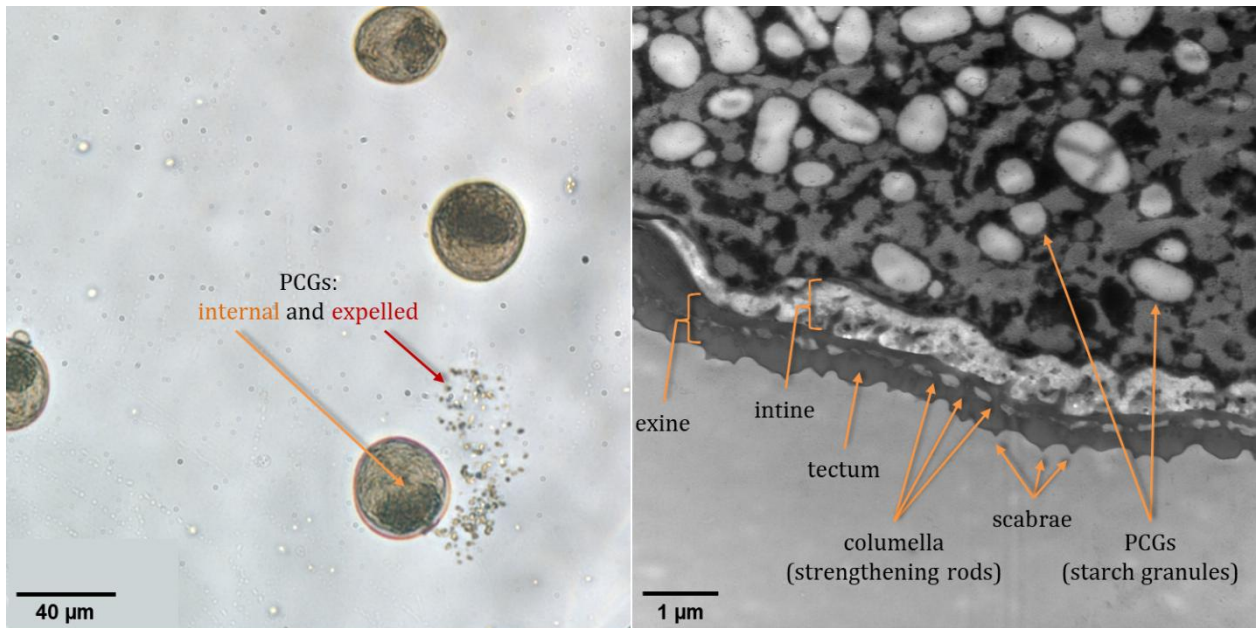
140

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)

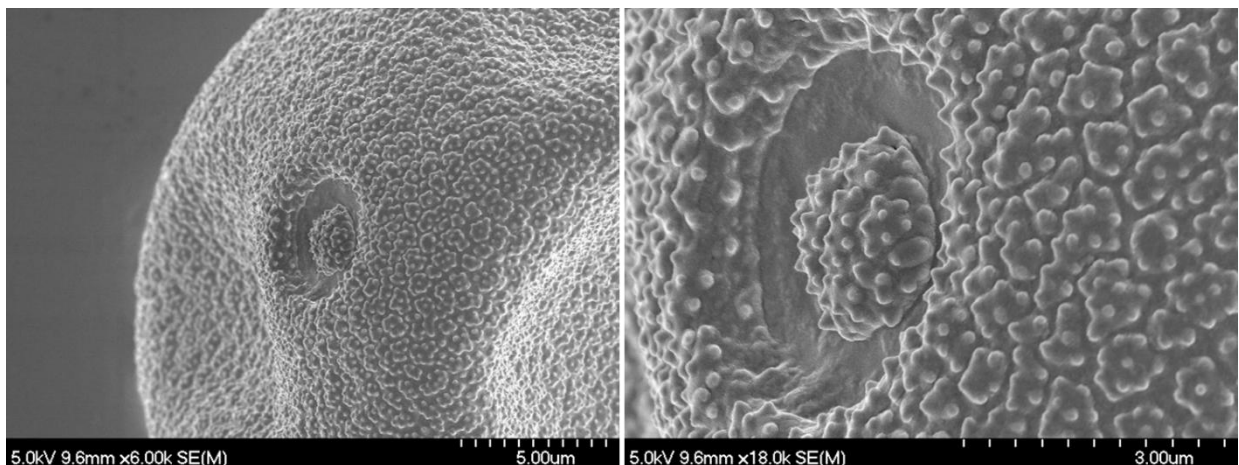
142 Pollen grain structure and sporopollenin

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).



156

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



160

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

163 Total proteins

164 The total amount of proteins extracted depends on the extraction protocol (Aloisi et al., 2018).
 165 Thus, when extraction was carried out with buffer lysis, by sonication with phosphate buffer
 166 saline solution (PBS) pH 7.5 or by continuous stirring with PBS, the total mass fraction of
 167 proteins on the same PPPG sample varied respectively from 45, 50 to 56 $\mu\text{g}\cdot\text{mg}^{-1}$ (Aloisi et al.,
 168 2018). These values are in perfect agreement with the determination of $51 \pm 4 \mu\text{g}\cdot\text{mg}^{-1}$ carried out
 169 by others with an extraction by water with continuous agitation (Rogerieux et al., 2007). Much
 170 lower values were subsequently determined at $13.2 \mu\text{g}\cdot\text{mg}^{-1}$ by extraction with PBS at 4°C with

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

176 Allergens

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

187 Table 4. *Phleum pratense* (timothy grass) pollen allergens. Prevalence data (Mari, 2003;
 188 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)

<i>IUIS</i>	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)
<i>IUIS</i>	Phl p 7	Ca-binding protein 2-EF hand (8.6 kda calc mass)	6	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)
<i>IUIS</i>	Phl p 11	Ole e-1 related protein (trypsin inhibitor)	20	5.0	Yes	43	(Cipriani et al., 2018; DeWitt et al., 2002)
<i>IUIS</i>	Phl p 12	Profilin	14	4.4-5.0	No	15	(Cipriani et al., 2018; Valenta et al., 1994)
<i>IUIS</i>	Phl p 13	Polygalacturonase	55-60	6.5-8.0	Yes	25-75	(Suck et al., 2000a, 2000b; Wicklein et al., 2004)

189 Quantification of allergens

190 Phl p 5 was extensively quantified in PPPGs (Table 5); the values from the literature are in very
 191 good agreement. Values between 2.7 and 3.5 $\mu\text{g}\cdot\text{mg}^{-1}$ were reported for unpolluted PPPGs of
 192 various origins and over a time scale of about 20 years (first measurements in 1999). The amount
 193 of proteins extracted depends on the pH of the extraction medium: total proteins increased from
 194 13.2 to 17.1 $\mu\text{g}\cdot\text{mg}^{-1}$ when the pH increased from 6.0 to 9.0 (Behrendt et al., 1999). An average
 195 value of $3.2\pm 0.5 \mu\text{g}\cdot\text{mg}^{-1}$ was calculated for Phl p 5 from literature data mentioned with an
 196 asterisk in Table . PPPGs exposed to air pollution exhibit a lower mass concentration of Phl p 5
 197 compared to unexposed pollens (table 5).

198 Some information is available in literature on the quantification of allergens other than Phl p 5.
 199 Allergens from group 2 were quantified at 1172 ng per 100 μg of total proteins (Marth et al.,
 200 2004). The Phl p 4 amount in pollen extract was determined at 0.2 $\mu\text{g}\cdot\text{mg}^{-1}$ quantified by an
 201 ELISA method. The absolute content of group 4 was in <1% relative to the total proteins of the
 202 Phl p pollen extract (Fahlbusch et al., 1998). Moreover, the quantification of *Phleum pratense*
 203 allergens Phl p 1 and Phl p 5 in pollen extract was performed by mass spectrometry. In the
 204 extract Phl p 1b (Phl p 1 basic, 150 fmole. μL^{-1}) was shown to be about 5 times less abundant than
 205 its variant Phl p 1a (Phl p 1 acid, 724 fmole. μL^{-1}) and Phl p 5b (Phl p 5 basic, 4393 fmole. μL^{-1})
 206 was shown to be 9 times more abundant than the Phl p 5a (Phl p 5 acid, 499 fmole. μL^{-1}) (Seppälä
 207 et al., 2011). The allergen concentration derived from this work (between 15-21 $\mu\text{g}\cdot\text{mg}^{-1}$ of Phl p
 208 5) was not used for the calculation of the averaged Phl p 5 mass because of the discrepancy
 209 observed with other studies (average value of $3.2\pm 0.5 \mu\text{g}\cdot\text{mg}^{-1}$).

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

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

Note	Mass of Phl p 5 ($\mu\text{g}\cdot\text{mg}^{-1}$)	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)

222 Pollen cytoplasmic granules (PCGs) and dispersion of allergens in respirable 223 aerosols

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

227 Starch granules

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

233 P-particles

234 P-particles have a size ranging around 0.3 to 0.4 μm (Grote et al., 1994; Heslop-Harrison and
235 Heslop-Harrison, 1982). The number of p-particles per pollen grains has not been directly
236 measured. P-particles occupy between 20 to 30% of the PG volume corresponding to an estimate
237 of 230,000 p-particles per PG (based on a spherical volume of pollen of 32 μm and spherical
238 volume of p-particles of 0.35 μm) (Heslop-Harrison and Heslop-Harrison, 1982).
239 Arabinogalactan polysaccharides have been quantified to about 10% of the mass of PPPG
240 (Brecker et al., 2005). This polysaccharide fraction is composed by mass of 68% D-galactose and
241 32% L-arabinose. By considering that this mass of polysaccharide corresponds exclusively to p-
242 particles, we obtain an estimate of 61,000 p-particles per pollen grain (density equals to 1.5 $\text{g}\cdot\text{cm}^{-3}$
243 and spherical particles of 0.35 μm diameter). We retain as an estimate between 61,000 and
244 230,000 of p-particles per pollen grain which is lower than the literature value of more than one
245 million p-particles per PG (Heslop-Harrison and Heslop-Harrison, 1982). Indeed, this value of
246 one million would result in a mass of p-particles greater than that of a single pollen grain.

247 Localization of allergens

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

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

259 1999). Confirming those localizations of allergens, PCGs are recognized by IgE from pollen-
260 sensitive rat sera and they trigger lymph node proliferation in these rats (Motta et al., 2004).
261 Water soluble and water insoluble protein extracts from PCGs were recognized by IgE from rat
262 sera and also from grass pollen allergic patient sera. The proteins recognized as allergens by IgE
263 from sensitized rat may correspond to allergens also recognized by human IgE (Abou Chakra et
264 al., 2011b). In a recent study, Phl p 2 and Phl p 5 are the allergens more concentrated in PPPG
265 sub particles; Phl p 1 and Phl p 6 were also detected to a lesser extent (Cecchi et al., 2020).

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

271 Expulsion of PCGs from the cytoplasm

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

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

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

294 Detection of allergens in the fine fraction of atmospheric aerosols

295 PPPG allergens have been detected several times in the fine (respirable or less than 10 μm)
296 fraction of atmospheric aerosol (Schäppi et al., 1996; Spieksma et al., 1995, 1990; Taylor et al.,
297 2002). However, Phl p 5 allergen was most of the time detected predominantly in coarse particle
298 mode ($\text{PM}>10 \mu\text{m}$) (Alan et al., 2018; Buters, 2015). After rainy events, grass pollen allergens
299 were detected in the atmosphere while PGs were for their part not observed (Holmquist et al.,
300 1999). Large quantities of starch granules have also been directly observed in the atmosphere
301 after rainy episodes but not on dry days (Suphioglu, 1998). To the best of our knowledge, p-
302 particles have never been directly observed in the atmosphere, probably because of their small
303 size making their discrimination difficult among atmospheric particulate matter.

304 Lipidomic

305 Total lipidomic analysis

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

310 Extractible lipidic fraction was determined as early as 1932 at 1.7% of the mass of PPPGs by
311 Soxhlet extraction with an organic solvent (Stull et al., 1932). This value is very close to the one
312 obtained by weighing of the dry extract (2.2%) obtained after pollen washing with methylene
313 chloride (Farah et al., 2020b). However, use of organic solvents only allows to extract the external
314 fraction of pollen lipids (Evans et al., 1991). The internal fraction of PPPGs was recently
315 obtained by grinding the grains prior to extraction: the total extractable lipids are distributed
316 between $22 \mu\text{g}\cdot\text{mg}^{-1}$ for the external fraction and $26 \mu\text{g}\cdot\text{mg}^{-1}$ for the internal fraction (Farah et al.,
317 2020a). Complete analysis of the extractable lipid fraction is still missing: less than 20% of lipids
318 have been identified and quantified (Farah et al., 2020a, 2020b).

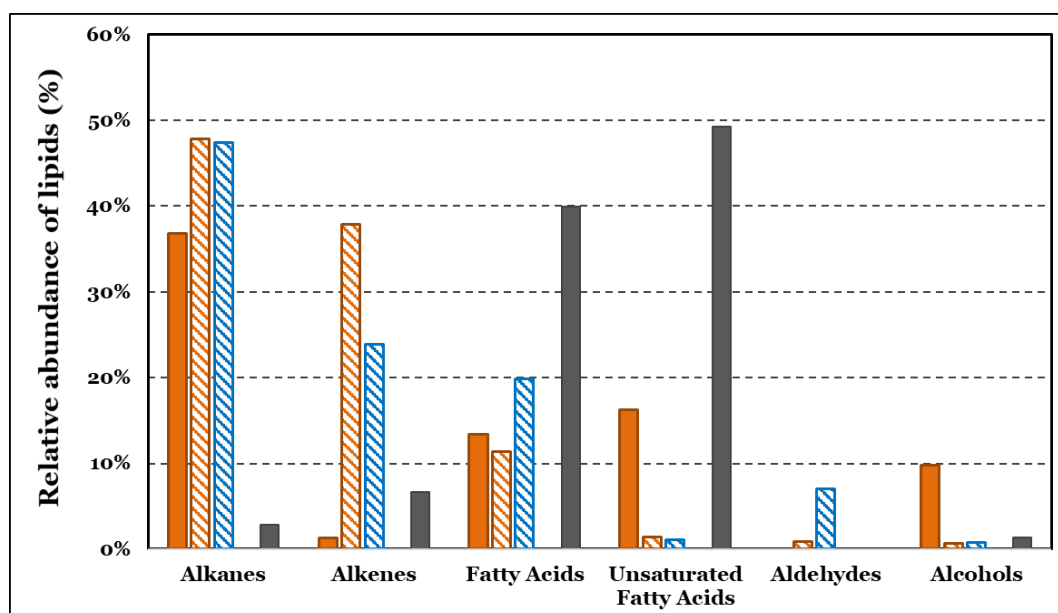
319 PPPGs from various geographical origins in the Czech Republic and the USA and from different
320 harvest years (from 2007 to 2018) underwent lipid extraction with an organic solvent followed by
321 analysis by gas chromatography coupled to mass spectrometry (Farah et al., 2020b). The
322 variability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

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

326 Chemical composition of lipids

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

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



342
343 Figure 4. Relative abundances of main chemical families of lipids from *Phleum pratense* pollen
344 grains. Orange: external lipids (Bashir et al., 2013a); hatched orange: external lipids (Farah et al.,

345 2020b); hatched blue: external lipids after exposure to ozone at 100 ppb for 16 hours (Farah et
346 al., 2020b); grey: internal lipids (Farah et al., 2020a).

347 *Prostaglandin and leukotriene*

348 Pro-inflammatory substances, as E2-like prostaglandin (PGE2) and B4-like leukotriene (LTB4),
349 have been extracted from PPPGs with aqueous solvent. Those so-called Pollen Associated Lipid
350 Mediators (PALMs) were quantified in very low mass fractions of 0.00007 $\mu\text{g}\cdot\text{mg}^{-1}$ for PGE2 and
351 of 0.00004 $\mu\text{g}\cdot\text{mg}^{-1}$ for LTB4 (Behrendt et al., 2001, 1999; Huss-Marp et al., 2008). Mass fractions
352 of PALMs increased for PPPGs sampled in environment influenced by road-traffic compared to
353 PPPGs sampled in rural environment (+100% for LTB4, +50% for PGE2) (Behrendt et al.,
354 2001). The release of these substances occurred in less than 30 minutes after incubation of
355 PPPGs in PBS (Behrendt et al., 2001).

356 *Linoleic and linolenic acids and their derivatives*

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

364 *Phytosteranes and phytofurans*

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

370 *Adenosine*

371 Adenosine was identified as a potent immunoregulatory substance in PPPGs and detected to
372 mass fraction between 0.65 and 1.29 $\mu\text{g}\cdot\text{mg}^{-1}$; it is controversial whereas such low concentration
373 of bioactive compound may or may not reach significant health-induced effects (Gilles et al.,
374 2011; Mueller et al., 2016).

375 *Vitamins*

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

379 *Phenolic compounds*

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

386 *Water content*

387 Release of proteins and allergens from PPPGs can be modified by an exposure of PGs to relative
388 humidity (Fritzsche et al., 1997). There is however no measurement of water content specifically
389 for PPPGs. The amount of water in pollen grains varies greatly at the time of pollination
390 depending on the meteorological conditions: wind, humidity and temperature (Heslop-Harrison,
391 1979). The water content of fresh pollen ranges from 15 to 35% for angiosperms but most PGs
392 used in laboratory are dried to a water concentration of a few percents before storage (Guerin,
393 1993; Heslop-Harrison, 1979).

394 *Others biochemical characteristics*

395 *NAD(P)H oxidase*

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

403 *Protease activity*

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

406 Microbiome

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

413 Pollutants

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

419 3. Discussion

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

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

438 compounds...). A better knowledge of this variability is important for estimating the
439 composition of PPPGs in the future in the context of a dramatic increase in atmospheric CO₂
440 concentration and of climate disasters. The effects of CO₂ on PPPG has been reported by one
441 research group only for allergens and the number of PGs produced by anthers (Albertine, 2013;
442 Albertine et al., 2014). Nothing is known about variability in lipids and other constituents nor
443 about the adaptation of *Pbleum pratense* to future climate change. Air pollution can also bring an
444 important additional variability on certain biochemical parameters of PPPG: allergens release,
445 changes in pollen composition, pollen rupture, oxidative alterations, adsorption of gaseous and
446 particulate pollutants (Behrendt et al., 2001, 1991; Behrendt and Becker, 2001; Chassard et al.,
447 2015; Farah et al., 2020b; Goschnick and Schuricht, 1996; Huss-Marp et al., 2008; Motta et al.,
448 2006; Risse et al., 2000; Rogerieux et al., 2007; Shiraiwa et al., 2012; Smiljanic et al., 2019).

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

459 PPPGs are rich in several biochemical compounds in concentrations ranging from 0.00004 to
460 384 µg.mg⁻¹ (table 1). Human exposure to these compounds transported by pollen requires both
461 their bioaccessibility (extraction from pollen in physiological conditions) and their bioactivity
462 (inhibitory or promoting effect in the allergic reaction). Bioactivity presumes its bioaccessibility.
463 Bioaccessibility requires knowledge of the kinetic of release of the biochemical constituents of
464 PG but also of their ability to diffuse into the mucus (Mueller et al., 2016). Such information on
465 bioaccessibility remains scarce for PPPG constituents in particular and for allergenic pollens in
466 general. Bioaccessibility also questions the distribution of biochemical constituents in the pollen
467 grain structure and the state (intact or broken) of the PGs being inhaled. As example, the
468 partitioning of lipids between the outer and cytoplasmic regions of PPPG has only been
469 quantified in one study (Farah et al., 2020a). Rupture of PGs is a factor that facilitates the release
470 of specific compounds as observed with lipids preferentially extracted by PBS on broken
471 (ruptured) PPPGs (Farah et al., 2020a). However, this partitioning raises the question of the use

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

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

485 Significant work has been carried out to characterize the eleven groups from 1 to 13 (groups 8
486 and 9 are absent) of allergens in PPPGs. However, only the major allergen Phl p 5 was repeatedly
487 assayed with a mean value calculated at $3.2 \pm 0.5 \mu\text{g}\cdot\text{mg}^{-1}$. Phl p 5 and allergens from group 2 were
488 the only allergens to be quantified in PPPGs and representing together 7-8% of total proteins.
489 The mass distribution of allergens between the different groups is not known, nor is the
490 quantitative distribution of allergens between the outer and inner parts of the PG. Besides the 11
491 allergens described in *Phleum pratense* pollen (table 4) and in other grass pollen grains, other
492 allergens were identified as Phl p CP a cysteine protease, Phl p EXY an endoxylanase (Bashir ME
493 et al 2013) (www.allergome.org) and Phl p Fe/Mn SOD a superoxide dismutase (Conti et al
494 2014). Six allergens (Phl p 1, 2, 4, 5, 6 and 12) are present in PPPG and PCG extracts whereas
495 Phl p 11 was only detected only PCG extract (Abou Chakra et al., 2012)

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

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

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

516 **4. Conclusion**

517 PPPGs are composed of polysaccharides, starch, allergenic and non-allergenic proteins, amino-
518 acids, lipids, phenolic compounds, diverse elements (the more abundant being Si, Mg and Ca),
519 phytoprostanoids, and adsorbed pollutants including metals and polycyclic aromatic
520 hydrocarbons (PAH). The cytoplasm of one single PPPG is filled with about 1000 starch
521 granules (average diameter 1.1 μm) and between 61,000 to 230,000 p-particles (0.3-0.4 μm). Much
522 remains to be discovered about bioavailability and bioactivity of these constituents of PPPG and
523 more generally of allergenic PGs. The lipid fraction of PPPG has, for example, not yet been fully
524 described qualitatively and quantitatively with only 20% of extractible lipids being identified and
525 quantified. Similarly, only 7-8% of total proteins have been quantified as allergens of group 2 and
526 5 allergen concentrations in other groups are unknown for PPPGs. The distribution of the
527 constituents between the external and internal parts is also relatively poorly described in
528 literature.

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

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

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