Occurrence of deoxynivalenol in an elderly cohort in the UK: a biomonitoring approach

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Occurrence of deoxynivalenol in an elderly cohort in the UK: a biomonitoring approach

Deoxynivalenol (DON) is a Fusarium toxin, to which humans are frequently exposed via diet. Despite elderly are speculated to be sensitive to the toxic effects of DON as a result of age-related conditions, disease and altered DON metabolism, there is lack of available data on DON biomarkers in this age group. This study characterised urinary DON concentrations and its metabolites in elderly aged ≥ 65years (n=20) residing in Hull, UK. Morning urinary specimens were collected over two consecutive days together with food records to assess dietary intake over a 24h-period prior to each urinary collection. Free DON (un-metabolised), total DON (sum of free DON and DON-glucuronides or DON-GlcA) and de-epoxy deoxynivalenol (DOM-1) were analysed using a validated LC-MS/MS methodology. Total DON was detected in 90% of elderly men and women on both days. Mean total DON concentrations on day 1 were not different from those on day 2 (elderly men, day 1: 22.2 ±26.3 ng/mg creat, day 2: 28.0±34.4 ng/mg creat, p=0.95; elderly women, day 1: 22.4 ±14.6 ng/mg creat, day 2: 29.1±22.8 ng/mg creat, p= 0.58). Free DON and DON-GlcA were detected in 60-70% and 90% of total urine samples respectively. DOM-1 was absent from all samples. Estimated dietary intake of DON suggested that 10% elderly exceeded the maximum provisional tolerable daily intake for DON. In this single-site, UK-based cohort, elderly were frequently exposed to DON, although mean total DON concentrations were reported at moderate levels. Future larger studies are required to investigate DON exposure in elderly from different regions of the UK, but also from different counties worldwide.

Keywords: mycotoxins; deoxynivalenol; *Fusarium graminearum*; biomonitoring; elderly

Introduction

The trichothecene deoxynivalenol (DON), also known as vomitoxin, is a secondary metabolite of *Fusarium graminearum* and *Fusarium culmorum*, to which humans and animals are predominantly exposed via food and feed (Pestka and Smolinski 2005; Pestka 2010; European Food Safety Authority 2013). As a result of its solubility in water and stability during cooking (temperatures 120 °C), storage conditions and milling processes, DON contaminates cereal grains and remains to a large extent unaffected during manufacture (Bretz et al. 2006; Scudamore et al. 2009). Thus, it can be also found in cereal-based derivative products including breakfast cereals, bread, confectionary, beer, infant formulas, and baby foods (Scudamore et al. 2009; European Food Safety Authority 2013). Several animal studies have shown that acute DON exposure cause anorexia, vomiting, abdominal pain and diarrhoea, while lifetime exposure to DON has also been associated with reproductive impairments, neuro- and immuno-toxicity (Pestka 2010). Acutely, humans appear to be affected in a similar way as animals following high DON intake, confirming that the gastrointestinal track is a main target for DON toxicity (Pestka and Smolinski 2005; Pestka 2010; European Food Safety Authority 2013). In contrast, the chronic effects of DON exposure in humans are uncertain, and their study is hampered by challenges in assessing risk exposure, together with difficulties in differentially identifying symptoms of DON toxicity from those due to other illnesses (Etzel 2006; Marin et al. 2013).

Advances in biomonitoring with the development of highly sensitive analytical procedures have allowed the assessment of DON and its main metabolites in urine (Turner et al. 2008). DON can be excreted in urine in its un-metabolised form (free DON) or after

being converted to its metabolites (Turner et al. 2008). In brief, DON can be conjugated to glucoronides (DON-GlcA) in the liver and possibly in the intestine and kidneys, with deoxynivalenol-3-glucuronide (DON-3-GlcA) and deoxynivalenol-15-glucuronide (DON-15-GlcA) being two of the main DON metabolites currently identified (Pestka and Smolinski 2005; Pestka 2010; European Food Safety Authority 2013). Deepoxy-DON (DOM-1) has been characterised as another major metabolite of DON produced by gut microbiota with deepoxidase activity in mammals (Pestka and Smolinski 2005; Pestka 2010; European Food Safety Authority 2013). DOM-1 has been inconsistently detected in human biological samples (Turner et al. 2010a; 2010b; Follmann et al. 2016; Wells et al. 2017), and remains uncertain, if human microbiota naturally possesses de-epoxidase activity, or whether rumen microbiota, which effectively converts DON to DOM-1, is transferred to humans during their contact with animals (Turner et al. 2010a; Wu et al. 2010). Biomonitoring studies in human adults have shown great variability in exposure to DON, as evaluated by the frequency of detection and biomarker concentrations in urine specimens (Turner, et al. 2010a; 2010b; Turner et al. 2011; Warth et al. 2012; Shephard et al. 2013; Heyndrickx et al. 2015; Gerding et al. 2014; Rodriguez-Carrasco et al. 2014; Solfrizzo et al. 2014; Gerding et al. 2015; Wallin et al. 2015; Follmann et al. 2016; Wells et al. 2017). These differences can be largely explained by geographical differences and eating patterns, whilst differences in population characteristics (e.g., age, physiological/disease status) may also contribute to these results.

Elderly may be an adult subpopulation at increased risk of experiencing toxicological effects when they are exposed to fungal toxins, including DON. DON toxic effects including anorexia, nausea, diarrhoea and stomach pain are similar to and may even exacerbate

97 symptoms associated with age-related conditions and diseases common amongst elderly
98 (Talley et al. 1992; Martone et al. 2013). Although data from human studies are currently
99 lacking, this notion is supported by findings in animal models suggesting increased
100 susceptibility of aged animals to the negative side effects (*i.e.*, anorexia) of DON exposure
101 compared to young adult animals (Clark et al. 2015). Ageing is accompanied by several
102 physiological and metabolic changes including a reduction in renal and hepatic clearance
103 (Mangoni and Jackson 2004), which in turn, may modify the way DON is absorbed,
104 distributed, metabolised and excreted. Alterations in intestinal microbiota composition with
105 increasing age have also been reported in humans (Claesson et al. 2011), albeit it remains
106 unknown if such changes would affect the detoxification of DON. Conversely, a significant
107 reduction in food intake has been reported with advancing age (Drewnowski 2000;
108 Wakimoto and Block 2001), which suggests a lower DON intake in elderly through this main
109 route of exposure. To our knowledge, elderly have not been previously considered as a
110 separate population group with distinct characteristics compared to those of their younger
111 counterparts within DON-related biomonitoring research.

112 This study aimed to characterise DON concentrations and its metabolites in urine of
113 elderly individuals aged ≥65 years residing in Hull, UK. To allow comparison with younger
114 adults, a dataset of UK adults aged 18-64 years residing in the same area, previously
115 published as part of our ongoing work in this area of research, was used (Wells et al. 2017).

Materials and Methods

Participants selection and recruitment

Elderly aged ≥65 years (n=20) and adults aged 18-64 years living in the Hull and East Yorkshire were recruited via word of mouth, by an announcement in the local newspapers and an email distributed by the University of Hull and Hull and East Yorkshire Hospitals NHS.

Inclusion criteria were being in general good health, not taking any current medication initiated within the last 3 months or were on stable medication (over a duration >3 months). Exclusion criteria were inability to provide informed consent, acute or chronic illness (chronic renal, hepatic or cardiac problems, cancer), chronic gastrointestinal conditions (*e.g.*, coeliac disease), gluten sensitivity, eating disorders, depression, psychosis or hospitalisation within the last three months prior to enrolment in the trial or participation in a weight loss programme. Individuals on stable medication, which may influence appetite such as oral steroids, were not included in the study.

Ethical approval was granted by the National Health Service (NHS), National Research Ethics Service (NRES) Committee Yorkshire & the Humber-Leeds West (IRAS project code: 147707).

Study design

This dataset analysed in the present study consists part of a more comprehensive investigation entitled “Experimental study of deoxynivalenol biomarkers in urine” performed for the European Food Safety Authority GP/EFSA/CONTAM/2013/04 (Brera et al. 2015), which

investigated the presence of DON and DON metabolites in urine samples collected from children (aged 3-9 years), adolescents (aged 10-17 years), adults (aged 18-64 years), pregnant women and elderly (aged ≥65 yeas) (total n=635) in the UK, Italy and Norway.

A validated, semi-quantitative food frequency questionnaire (FFQ) (previously used in (Brera et al. 2015; Wells et al. 2016; Wells et al. 2017), was designed to assess food consumption of cereal grains and cereal-based products that commonly contribute to DON dietary exposure over a month recall period, while a food record was used to collect detailed information about food items consumed 24hours preceding the collection of each urine sample. Age, height, weight and physical activity were self-reported as part of the FFQ. Participants were supplied with four urine collection containers of 50 mL each and written guidance for collecting their first morning urine samples at home, on two consecutive days. This timeframe was intended to cover potential between-day variability within participant and enhance the repeatability of our findings. Participants returned the urine samples at the Hull Royal Infirmary (Hull, UK) on the same day of collection. All samples were centrifuged at 2000 rpm for 10 minutes and stored at -80°C until further analysis. DON and its metabolites were tested using the validated HPLC-MS/MS methodology as previously described in (Turner et al. 2008).

Laboratory analysis

DON and its metabolites were analysed using ^{13}C labelled DON standard (Sigma, Saint Louis, MI, USA; product number: 34128, 1.2 mL), DON (Sigma, Saint Louis, MI, USA; product number: D0156, 1 mg), β-glucuronidase (Type IX-A from E. coli; Sigma, Saint

158 Louis, MI, USA; product number: G7396 - 2MU), DOM-1 (Sigma, Saint Louis, MI, USA;
159 product number: 34135, 2 mL), and DON test WB^{TM} immunoaffinity columns (Vicam,
160 Milford, MA, USA; product number: G1066). All analyses were conducted on a Waters 2795
161 HPC Separation Module (Waters Corp., Milford, MA, USA) with a Quattro Micro Triple
162 Quadrupole Mass Spectrometer (Micromass UK Ltd., Manchester, UK).

163 *Sample preparation*

164 Stored urine samples were centrifuged at 2000 rpm at -4 °C for 15 minutes. For each
165 participant, two aliquots (1 mL) were prepared by mixing ^{13}C-DON internal standard solution
166 to a final concentration of 20 ng/mL. In the first aliquot, total DON was determined as the
167 sum of DON-GlcAs and free DON. For measuring the combined DON-GlcAs (DON-3-GlcA
168 and DON-15-GlcA) and free DON, each sample was set to pH 6.8 and treated with β-
169 glucuronidase solution (23,000 units, in KH2PO4 75 mM) in a shaking water bath at 37 °C
170 for 18 hours. The samples were then centrifuged (2000 rpm; -4 °C; 15 min), and the
171 supernatant was diluted to a final 4 mL with phosphate buffered saline (PBS, pH 7.4), before
172 being passed through a wide bore DON immunoaffinity column. DON was removed from
173 columns with methanol (4 mL) and extracts were dried under vacuum using a SavantTM
174 SpeedVacTM (Thermo Fisher Scientific Inc., Waltham, MA, USA) or equivalent and
175 dissolved in 10% ethanol (250 µL) for LC-MS analysis. DOM-1 was quantified on the same
176 aliquot analysed for DON-GlcA. Free DON was assessed in the second aliquot using the
177 same procedures, but no β-glucuronidase treatment was performed.

HPLC-MS analysis: DON determination

The separation of DON was performed by utilising reversed phase chromatography using a Luna C_{18} column (150 × 4.6 mm, 5-µm particle size) (Phenomonex, Macclesfield, UK) with a mobile phase sequence of 27 minutes 20% methanol, reconstructed to a wash of 75% methanol after 10 minutes followed by 20% methanol after 16 minutes (flow rate 1 mL/min; injection volume 25µL). One fifth of the eluent was placed into the desolvation chamber of the MS. Selective ion recording (SIR) was used to quantify DON with respect to ^{13}C-DON internal standard. The following mass spectrometer conditions were kept stable: capillary voltage 3.5 kV, desolvation temperature: 300 °C, extraction cone voltage: 3.00 V, sampling cone voltage: 35.00 V, source temperature: 100 °C, cone gas flow: 50 L/h, collision energy: 1.0 and desolvation gas flow 500 L/h. Two masses of DON ([DON-H]+, m/z 297.2 and [DON-Na]+, m/z 319.2) and ^{13}C-DON ([^{13}C-DON-H]+, m/z 312.2 and [^{13}C-DON-Na]+, m/z 334.2) were monitored for 0.25 seconds (each mass) and were then summed to form a total ion current peak for the internal standard and each analyte. The calibration curve (range 2-250 ng/mL) was established by injecting DON and ^{13}C-DON standard solutions (prepared in 10% ethanol) DON-GlcA concentrations were estimated indirectly, by subtracting free DON from total DON values.

LC-MS analysis: DOM-1 determination

DOM-1 was separated by utilising the same chromatographic column used for DON separation combined with a mobile phase sequence of 35 minutes 20% methanol, changed to a wash of 75% methanol after 20 minutes and then to a phase of 20% methanol after 26

minutes (injection volume 25 L; flow rate 1 mL/min). Part of the eluent was driven into the MS desolvation chamber. Similar to DON analysis, DOM-1 was quantified by SIR with reference to the calibration curve (range of 2–200 ng/mL), which resulted from the injection of DOM-1 standard solutions (prepared in 10% ethanol). Each of two masses of DOM-1, [DOM-1-H]+, m/z 281.3 and [DOM-1-Na]+, m/z 303.3 were monitored (0.25 seconds) and summed to obtain a total ion current peak for DOM-1.

Analysis of creatinine

Adjustments of DON concentrations for creatinine were used to account for differences in dilution between individuals that may have resulted from the sole collection of a first morning urine samples. An in-house micro-titre plate assay was used to determine urinary creatinine. Samples were diluted in water (1:20) and 100 µL was added, in duplicate, to a 96-well plate. A duplicate standard curve of creatinine concentrations within the range 0-20 µg/mL was obtained for each plate. A further100 µL of alkaline picric acid solution was added to each well, incubated at 25 °C for 30 minutes and measured at 490 nm using a plate spectrophotometer. Urinary total DON concentrations are presented as unadjusted (ng/mL) and adjusted for creatinine (ng/mg creatinine).

Estimated dietary exposure of DON based on urinary analysis

The estimated dietary exposure of DON was calculated using formula 1 (Ezekiel et al. 2014; Heyndrickx et al. 2015):

$$\textit{Estimated dietary exposure of DON}\ (\mu g/kg\ b.w./day) = \frac{\text{total DON x V}}{\text{ER x b.w.}} * 1000 \quad (1)$$

Total DON = unadjusted total DON concentrations in urine

ER = urinary excretion rate of DON is 72% (Turner et al. 2010b).

V = daily urine output (mL/day) was estimated to be 1.5 L

b.w. = self-reported body weight (kg)

Based on the toxicological data in animals and humans, the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organisation (WHO) Expert Committee on Food Additives (JECFA) have set a provisional maximum tolerable daily intake (PMTDI) at 1 μg/kg body weight (b.w.) day for DON and its main metabolites (Joint Expert Committee on Food and Additives, 2010). As such, we compared the estimated dietary exposure of DON with PMTDI at μg/kg b.w./day and characterised exposure as below or exceeding PMTDI.

Dietary assessment and analysis

In this report, the results of the food records covering the dietary intake over a 24-h period prior to each urinary samples collection are only reported. The food records included the following food categories that are commonly contaminated with DON: breakfast cereals and snacks, bread, products alternative to bread, flakes, cereals, other breads, biscuits, bakery goods, pizza, pasta, wheat germ, beer, pancakes and pita bread. These categories are in line with the validated FFQ designed to assess DON exposure though diet and used in previous DON-related research (Brera et al. 2015; Wells 2016; 2017). Each of the two food records included three main meals (breakfast, lunch, dinner) and snacks. For each of these eating occasions, examples of food items belonging to the aforementioned food categories were included. To enhance consistency and enable participants to record intake in an easy way,

each category was tabulated with tick boxes as to whether the portion consumed was small, medium or large. Photographic examples of portion sizes were also used to help participants to accurately quantify their dietary intake. Importantly, participants completed food records at the presence of research dietitians, who provided clarification and asked supplementary information. Portion sizes reported in the food records were converted into grams by research dietitians prior to data analysis.

Statistical Analysis

All numerical data were checked for normality using the Shapiro-Wilk test. Urinary DON levels between day 1 and day 2 within adults and elderly were compared using a paired *t-test* and a Wilcoxon signed-rank test for normally and non-normally distributed respectively. Comparisons between groups were performed using an independent *t-test* or Mann-Whitney test for normally and non-normally distributed, respectively. Fisher's exact test was used for comparing categorical variables. The association between urinary DON levels and its metabolites and sex, weight, BMI and dietary intake of commonly foods contaminated with DON were assessed using the Spearman's correlation coefficient (two-tailed). All analyses were carried out using IBM SPSS Statistics Version 24.0. Results were deemed significant at a p-value ≤ 0.05.

Results

Baseline characteristics

A total of 50% of the 20 elderly (aged ≥65 years) and 52% of the 31 adults (aged 18-64 years)

were men. Baseline characteristics of elderly and adults by sex are shown in Table 1. Men were significantly taller (elderly, p=0.15; adults, p<0.001) and heavier (elderly, p=0.04; adults, p<0.001) than women in both the elderly and adult groups. There was a trend towards a higher BMI in elderly men than women (p=0.051) and BMI was not different between adult men than women (p=0.10). Elderly men were significantly shorter than adult men (p=0.03), but there were no further differences in weight (p=0.28) and BMI (p=0.63). Height (p=0.34), weight (p=0.22) or BMI (p=0.36) did not differ between adult and elderly women. In all groups, most of the participants reported moderate physical activity (elderly men, 80%; elderly women, 70%; adult men, 50%; adult women, 80%), and there were no significant differences for physical activity categories among groups (p-values from 0.14 to 0.49). Elderly women had significantly lower urinary creatinine levels (mg/dl) than elderly men (p=0.003) and adult women (p=0.004), which is in line with sex and age differences shown previously (Barr et al., 2005).

Total DON Concentrations in Urine Samples

Table 2 presents unadjusted and creatinine-adjusted DON concentrations in urine samples on day 1 and 2 in elderly (n = 20) and adults (n = 31). Total DON was detected in 90% of elderly men and women on both days and 100% of adult men and women on days 1 and 2. Mean total creatinine-adjusted DON concentrations (ng/mg creat) on day 1 were not different from total DON concentrations on day 2 in elderly (men, day 1: 22.2 ±26.3, day 2: 28.0±34.4, p=0.95; women, day 1: 22.4 ±14.6, day 2: 29.1±22.8, p= 0.58) or adults (men, day 1: 24.3 ±38.2, day 2: 21.3±19.1, p=0.41; women, day 1: 12.7 ±7.9, day 2: 18.2±18.0, p=0.82) (Table

2). Mean total creatinine-adjusted DON concentrations (ng/mg creat) for both days (pooled data for day 1 and day 2) were 25.1 for elderly men (n =10) and 22.8 for adult men (n = 16), with no differences found between the two groups (p=0.70). Although greater mean total DON concentrations (pooled data for day 1 and 2) were reported in elderly women (25.7±17.4 ng/mg creat) compared to those in adult women (15.5±11.2 ng/mg creat), these did not reach statistical significance (p=0.079).

DON metabolite concentration in urine samples

Free DON (>limit of quantification or LOQ 0.25 ng/mL) was detected in 60-70% of elderly men and women, and in all urine samples of adults. DON-GlcA (>LOQ 0.50 ng/mL) was present in elderly (90%) and adults (100%) samples. In contrast, DOM-1 was absent from all samples of both age groups. In elderly subjects, DON-GlcA made significant contribution ranging from 83 to 91% of the total DON and free DON represented 9-17% of total DON; with these contributions being very similar among elderly and adults (Table 2).

Mean free DON concentrations (ng/mg creat) were 3.8 and 3.2 on day 1 and day 2 in urinary specimens of elderly men and 2.3 and 2.8 for days 1 and 2 in samples of elderly women (Table 2). In adult men, mean urinary levels of free DON were reported to be 4.2 and 3.2 ng/mg creat on days 1 and 2, while the respective values in adult women were 1.9 and 2.8 ng/mg creat (Table 2). There were no differences in mean free DON concentrations between day 1 and day 2 in elderly (men, p=0.50; women, p=0.58) or adults (men, p=0.50; women, p=0.45). Furthermore, mean free DON concentrations (pooled data for days 1 and 2), were not significantly different between elderly and adult men (elderly men: 3.5±4.3

303 ng/mg creat *vs.* adult men: 3.7 ±4.8 ng /mg creat; p=0.52) or women (elderly women: 2.3±1.6
304 ng/mg creat *vs.* adult women: 2.6±1.6 ng /mg creat; p=0.74).

305 Amongst elderly individuals, mean DON-GlcA (ng/mg creat) concentrations were
306 18.5 and 21.8 on days 1 and 2 in men and 20.1 and 26.3 for days 1 and 2 in women (Table
307 2). Among adults, mean urinary levels of DON-GlcA (ng/mg creat) for days 1 and 2 were
308 20.0 and 18.1 for men and 10.8 and 15.4 for women (Table 2). There were no differences
309 between days 1 and 2 in any group (elderly men, p=0.95; elderly women, p=0.21; adult men,
310 p=0.41; adult women, p=0.96). When DON-GlcA (ng/mg creat) data were pooled for both
311 days, no differences were seen between elderly and adult men (elderly men: 20.1±15.1 ng/mg
312 creat *vs.* adult men: 19.1±22.1 ng /mg creat; p=0.62) or elderly and adult women (elderly
313 women: 23.2±16.4ng/mg creat *vs.* adult women: 13.1±9.7 ng /mg creat; p=0.067).

314 ***Estimated dietary DON exposure using urinary total DON concentrations***

315 We carried out a risk assessment by comparing the estimated dietary intake of DON with the
316 PMTDI of 1 μg/kg b.w./day. The mean estimated dietary intake of DON was 0.43
317 μg/kg·b.w./day (minimum: 0, maximum: 2.33) for elderly and 0.37 μg/kg·b.w./day
318 (minimum: 0.07, maximum: 1.33) for adults. In total, 10% of elderly and <3% of adults were
319 estimated to exceed the PMTDI of 1 μg/kg b.w./day.

320 ***Correlation between urinary DON, demographic and anthropometric***
321 ***characteristics***

322 In elderly, no correlations were found between urinary mean total DON (ng/mg creat) (pooled
323 data for day 1 and day 2) and sex (r=-0.43; p=0.86), height (r= -0.128; p=0.59), weight (r=-

0.294; p=0.21) or BMI (r=-0.259, p=0.27).

Correlation between urinary total DON and Food Intake

Table 3 presents main food categories commonly consumed by elderly and adults, which are often contaminated with DON. One adult participant had a total intake of all food categories equal to 0 on both experimental days, which is unlikely given the characteristics of our cohorts (healthy individuals, not under energy restriction). Therefore, this participant was excluded from this correlation analysis between urinary total DON and main food categories contributing to DON exposure. Analysis was performed in 20 elderly and the remaining 30 adults with complete data. Elderly consumed significantly more flakes (p=0.016), biscuits (p=0.033) and baked good (p=0.044) compared to adults. There were no other differences for any other food category between the two age groups (p values from 0.066 to 0.57). Total intake of commonly contaminated foods with DON did not differ between the elderly compared to adults (p=0.28). In elderly, no significant correlations were found between urinary mean total DON (ng/mg creat) for both days and any food category that commonly contribute to dietary DON exposure (p values from 0.056-0.93). In a pooled analysis of elderly and adults, urinary mean total DON (ng/mg creat) for both days were positively correlated with flakes consumption (r=0.426, p=0.02), but not with any other food category or total intake of commonly contaminated foods with DON (p values from 0.13 to 0.95) (Table 3).

Discussion

Elderly individuals may be particularly susceptible to DON exposure due to age-related alterations in absorption, distribution, metabolism and excretion of toxins, but also as a result of diseases, which may resemble or increase sensitivity to the toxic effects of DON on bodily systems (Mangoni and Jackson 2004; Clark et al. 2015). Our study provides data on the concentrations of DON and its metabolites in urine samples of elderly individuals. A frequent detection of total DON (90%) at a mean concentration of 25.4 ng/mg creat, suggests DON exposure, although at relatively moderate levels in this small cohort in elderly. Compared to adults aged 18-64 years residing in the same area, elderly appeared to have similar urinary levels of DON, indicating no significant differences in DON exposure with age.

Comparative data in elderly are only available as part of our larger investigation, which in addition to the UK, included participants from Norway and Italy (Brera et al. 2015). Mean total DON concentrations in UK elderly (n=20, incidence=90%, mean levels=25.4 ng/mg creat) were almost 3-fold to those detected in the elderly cohorts in Norway (n=20, incidence=100%, mean levels=8.9 ng/mg creat) and Italy (n=20, incidence=100%, mean levels=8.3 ng/mg creat) (Brera et al. 2015). Most available studies in adult populations have not included elderly individuals (aged ≥65 years), whereas those, that have included participants ≥65 years, have treated them as part of a mixed group of adults (≥18 years) (Turner et al. 2010a; 2010b; Turner et al. 2011; Warth et al. 2012; Gerding et al. 2014; Heyndrickx et al. 2015; Follmann et al. 2016). Similarly to our study in elderly and adults based in a single urban area in the UK, current findings from European counties suggest that adults are frequently exposed to DON, however, the magnitude of exposure is highly variable

366 (Germany: n=30, incidence=100%, mean levels=6.0 ng/mg creat (Föllmann et al. 2016);
367 Belgium: n=239, incidence 100%, mean levels: 87.9 ng/mg creat (Heyndrickx et al. 2015);
368 Spain: n = 22; incidence: 73%, mean levels = 14.8 ng/mg creat (Rodriguez-Carrasco et al.
369 2014); Sweden: n=252, incidence: 63%, mean levels=7.0 ng/mg (Wallin et al. 2015); Italy:
370 n=52, incidence: 96%, mean levels=11.9 ng/mL (Solfrizzo et al. 2014); and Austria: n=27,
371 incidence=96%, mean levels =20.4 ng/mL (Warth et al. 2012). Similar findings have also
372 been reported in non-European countries including China (Turner et al. 2011), South Africa
373 (Shephard et al. 2013) and Haiti (Gerding et al. 2015). The variability in the results may
374 reflect differences in exposure as a result of dietary patterns, climate conditions, cultivating
375 and processing or may be due to differences in study design, characteristics of study
376 populations or analytical procedures and lower limits of quantification used for urinary DON
377 assessment.

378 Understanding the metabolism of DON in elderly is important in characterizing the
379 risk of this population to the toxic effects of DON exposure. Towards this end, free DON
380 (unmetabolised form of DON) was found in 60-70% of the urine samples of the elderly
381 individuals. DON-GlcA was detected in 90% of all urine specimens of elderly individuals.
382 In the positive samples, free DON accounted for 9-17% of the total DON concentrations;
383 whilst DON-GlcA made the greatest contributions to total DON concentrations ranging from
384 83-91%. Our results suggest that conjugation to glucuronides is the main detoxification route
385 for DON in elderly humans. Similarly to our elderly cohort, contributions of DON-GlcA over
386 80% of total DON concentrations were evident in our adult cohort. Our findings in elderly
387 and adults individuals are in line with those of previous studies in adult populations (Turner

et al. 2011; Warth et al. 2012; Heyndrickx et al. 2015; Gerding et al. 2014). Few studies have also given insight into the different conjugates (Warth et al. 2012). For example, in a study in Austrian adults, DON-15GlcA accounted for about 75% of total DON-GlcA (Warth et al. 2012). Heyndrickx et al., also confirmed that DON-GlcA at the 15-position is preferential than glucuronidation of DON at the 3-position (Heyndrickx et al. 2015). In our work, we were not able to differentiate between DON-GlcA at different positions since an enzymatic hydrolysis with *β*-glucuronidase allowed the estimation of DON-GlcA indirectly, by subtracting free DON from total DON concentrations.

In contrast to free DON and DON-GlcA, DOM-1 was not detected in any sample of our elderly or adult population. These results are consistent with previous findings in elderly from Italy (Brera et al. 2015), but also other studies in adults (Turner et al. 2011; Rodriguez-Carrasco et al. 2014) and support the idea that conversion of DON to DOM-1 may not be a main metabolic pathway in humans. This may be due to the natural absence of microbiota with the enzymatic activity required for this conversion (Turner et al. 2010a). Alternatively, if DON was converted to DOM-1, this metabolite was not significantly excreted in urine samples in the present investigation. Our results are strengthened by the analytical approach we followed for DOM-1 determination; given that urine samples were treated with *β*-glucuronidase, identification of both DOM-1 and DOM-1–glucuronide would be possible. DOM-1 has been detected in some other adult cohorts across Europe. In the experimental study of DON biomarkers in urine (Brera et al. 2015) performed in Italy, Norway and UK, DOM-1 (>LoQ) was detected in 12% of Norwegian samples, probably due to a lower LoQ compared to those used for the analysis of the samples in Italy and the UK. Similarly, 34%

410 of the samples of French farm workers (Turner et al. 2010a) and 38–60% of the total samples
411 of German mil workers and controls (Föllmann et al. 2016) were positive for DOM-1. These
412 latter findings have been suggested to result from the transmission of rumen microbiota with
413 de-epoxidase activity to humans (Turner et al. 2010a). Given that there is evidence to suggest
414 changes in microbiota with increasing age, it will be useful to understand variations in the
415 ability to detoxify DON by deepoxidation to DOM-1 in larger studies in elderly from
416 different countries, and to consider differences by disease status.

417 Urinary biomarkers concentrations can be used to estimate the dietary intake of DON
418 at an individual level, which can be subsequently be compared with the PMTDI (1 μg/kg
419 b.w./day). By assuming a 72% urinary excretion ratio of DON and 1.5 L daily urine output
420 (Turner et al. 2010b), we showed that the estimated dietary intake of DON varied between 0
421 and 2.33 μg/kg b.w./day for elderly. In total 10% of the elderly were shown to exceed the
422 PMTDI, but, notably, the 75% of the elderly individuals were estimated to have dietary intake
423 of DON < 0.5 μg/kg b.w./day, which is below the half of the PMTDI. The percentage of the
424 elderly exceeding the PMTDI is higher than the <3% of adults aged 18-64 who exceeded the
425 PMTDI in this analysis, though the numbers in the study were low. Higher percentages
426 exceeding the PMTDI than those reported amongst elderly in the present study have also
427 been reported in other studies in adults in the UK (n=35, estimated dietary intake of DON:
428 0.008–1.24 μg/kg·b.w./day, 17% participants exceeded PMTDI) (Turner et al. 2010b),
429 Belgium (n=239, 16-29% participants exceeded PMTDI) (Heyndrickx et al. 2015), Italy
430 (n=52, 40% participants exceeded PMTDI) (Solfrizzo et al. 2014) and Austria (n=27, 33%
431 participants exceeded PMTDI) (Warth et al. 2012). In contrast, lower percentages were

shown in Spain (n=22; <4% exceeded PMTDI) (Rodriguez-Carrasco et al. 2014). Our results and their comparisons with the findings of previous studies should be interpreted after considering the limitations of current ways to estimate dietary intake of DON and the small number of participants in most available studies. For example, the uncertainty in these estimations was depicted by Heyndrickx and colleagues who showed 16 and 29% of total sample to exceed PMTDI depending on the formula used (Heyndrickx et al. 2015). In our previous work in children and adolescents, we also showed significant discrepancies in the percentages of participants exceeding PMTDI, when different assumption of daily urine output were tested (Papageorgiou et al. 2018). Assumptions of the urinary excretion of DON, reported to range 50-72% in previous human studies (Turner et al. 2010b; Shephard et al. 2013), may further influence the estimations of dietary intake of DON based on urinary biomarkers. Other reasons that may explain the variable estimations of the dietary intake of DON include differences in dietary habits of population residing in different countries or seasonal variations in dietary intake within a population, but also discrepancies in DON contamination of food sources as a result of environmental conditions, cultivation, handling and processing (Bullerman & Biachini, 2007; Oldenburg et al., 2012; Gratz et al., 2014). Taken together, the findings of the available studies and their comparison underpin the need for further research in the area and standardization of the methodology used for estimating dietary exposure to DON. Future investigations using larger sample sizes of elderly populations are also required to confirm our findings and allow comparisons in this age population group.

453 To further explore the relationship between dietary exposure of DON and DON
454 biomarkers, correlation analysis between the consumption of common foods contaminated
455 with DON (based on food records over 24h prior to each urine sample collection) and total
456 DON concentrations (pooled data for the 2 experimental days) in urine was performed. For
457 our total samples of elderly and adults, this analysis revealed a significant positive association
458 between flakes consumption and urinary mean total DON (ng/mg creat), but no significant
459 correlations for any other food category were shown. The lack of pronounced associations
460 may at least partially be explained by the fact that overall, DON exposure was relatively low
461 amongst both elderly and adult participants and in this small sample. Limitations in the
462 dietary assessment method (i.e., food records over a 24h period), challenges in estimating
463 portion sizes or reporting bias may have also contributed to these non-significant
464 associations. Nevertheless, given that urinary DON reflects to a large extent the DON dietary
465 intake of the previous days (Turner et al. 2010b), food records over 24h prior to urine sample
466 collection may more accurately reflect DON dietary exposure compared to FFQs, which
467 typically capture dietary intake over a more prolonged period of time (e.g., last month)
468 (Marin et al. 2013). For future studies investigating DON exposure, we recommend a longer
469 recording period to get information on habitual dietary intake or intake of less frequently
470 consumed foods. Inclusion of actual measurements of DON and metabolites in the recorded
471 foods would be also useful in ascertaining associations between DON dietary exposure and
472 DON biomarkers in biological samples. To minimize reporting bias and increase accuracy of
473 portion sizes, research dietitians with experience in performing semi-structured interviews
474 asked supplementary questions on the foods records and pictures with portion sizes were used

475 respectively. The food categories and example of food items used as the basis of the food
476 records were comprehensive and previously used in DON research (Brera et al. 2015; Wells
477 et al. 2017; Papageorgiou et al. 2018), however, some dietary sources contributing to the
478 exposure of DON, which may be consumed less frequently at a local level, may have still
479 been omitted (e.g., soups and sauces).

480 We provided a detailed analysis of DON exposure in elderly, by determining DON
481 and its main metabolites in urinary specimens and assessed intake of main food sources
482 contaminated to DON. It is important to note that other factors including defence mechanisms
483 and altered metabolism due to illness not investigated as part of this work may influence
484 elderly's sensitivity to the toxic effects of DON. Our elderly population were relatively
485 healthy (as indicated by inclusion and exclusion criteria), implying no major impairments in
486 gastrointestinal, renal, hepatic or immune functions. As such, our results may be less
487 generalizable in elderly individuals with disease (e.g., cancer, renal and liver disease,
488 gastrointestinal disorders, or immunosuppression). Furthermore, it remains unknown how
489 DON exposure (e.g., low *vs.* high) affects elderly individuals who experience symptoms that
490 resemble DON toxicity such as anorexia of ageing or unintended weight loss and whether
491 DON biomarkers can capture such effects.

492 **Conclusions**

493 Despite their potential sensitivity to DON toxicity, the elderly are an understudied population
494 in mycotoxin research. Although elderly individuals in this single-site, UK-based cohort were
495 commonly exposed to DON, mean total DON concentrations were reported at moderate

levels and PMTDI for DON (1 μg/kg b.w./day) was exceeded by 10% of the population studied. Furthermore, our findings support no significant differences in DON and its metabolites between elderly (aged ≥65 years) and younger adults (aged 18-64 years). Future larger studies are needed to explore DON exposure in elderly from different regions of the UK, but also from different counties worldwide. Simultaneous assessment of urinary DON biomarkers, dietary patterns, gastrointestinal distress (as a result of ageing, disease or DON toxicity) and disease status would be important to understand DON exposure, susceptibility and toxicity in this population.

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Declaration of interest statement:

The authors declare no conflict of interest. EFSA disclaimer: EFSA is not responsible for the views displayed in the publications and/or in conjunction with the activities for which the grant is used. The funding sponsors had a role in the design of the study; Clear information regarding the required design of the study was given in EFSAs call for proposal. Further details can be found at: http://www.efsa.europa.eu/en/art36grants/article36/gpefsacontam201304.

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637

638

639 **Table 1.** Baseline characteristics of elderly (≥65 years) and adults (18-64 years) by sex

	Elderly (≥65 years)		Adults (18-64 years)	
	Men (n=10)	**Women (n=10)**	**Men (n=16)**	**Women(n=15)**
Height (cm)	170.1 (5.9)*,†	163.1 (6.6)	177.6 (5.3)*	165.5 (5.7)
Weight (kg)	85.3 (13.3)*	67.1 (11.2)	91.1 (12.8)*	73.1 (12.1)
BMI (kg/m^2)	29.6 (5.6)	25.2 (3.6)	28.8 (3.2)*	26.7 (3.9)
Physical activity				
Sedentary (n, %)	1 (10)	1(10)	0 (0)	0 (0)
Light (n, %)	0 (0)	2 (20)	6 (38)	2 (13)
Moderate (n, %)	8 (80)	7 (70)	8 (50)	12 (80)
Heavy (n, %)	1 (10)	0 (0)	1 (6)	1 (7)
Exceptional (n, %)	0 (0)	0 (0)	1 (6)	0 (0)
Urine creatinine (mg/dl)	110 (57)*	38 (24)‡	124 (88)	114 (84)

640 The Table includes adult data previously presented in Wells et al. 2017. *, $p<0.05$, significant different from women in the
641 same age group; †, $p<0.05$, significant different from adult men; ‡, $p<0.05$, significant different from adult women.
642

643 **Table 2.** Unadjusted and creatinine-adjusted DON and metabolites concentrations by day
644 and sex in elderly and adults.

Age Group		Total DON		Free DON		DON-GlcA		DOM-1	
	Day	1	2	1	2	1	2	1	2
Elderly Men (n=10)	Incidence	90	90	70	60	90	90	nd	nd
	%Total DON	N/A	N/A	17	9	83	91	nd	nd
	Unadjusted (ng/mL)								
	Mean	26.7	20.1	5.0	2.2	21.7	17.8	nd	nd
	Median	7.2	13.9	0.6	1.0	5.5	12.0	nd	nd
	Min-Max	0-186	0-65.1	0-42.0	0-11.8	0-144	0-53.3	nd	nd
	Adjusted (ng/mg creatinine)								
	Mean	22.2	28.0	3.8	3.2	18.5	21.8	nd	nd
	Median	11.2	11.6	1.6	0.7	8.8	10.3	nd	nd
	Min-Max	0-82.9	0-97.0	0.0-18.7	0.0-12.5	0-64.1	0-56.8	nd	nd
Elderly Women (n=10)	Incidence	90	90	90	70	90	90	nd	nd
	%Total DON	N/A	N/A	11	11	89	89	nd	nd
	Unadjusted (ng/mL)								
	Mean	8.0	8.8	0.8	1.0	7.3	7.8	nd	nd
	Median	6.2	8.1	0.9	0.8	5.7	7.8	nd	nd
	Min-Max	0-28.8	0-17.5	0.1-1.8	0-3.3	0-27.0	0.0-14.2	nd	nd
	Adjusted (ng/mg creatinine)								
	Mean	22.4	29.1	2.3	2.8	20.1	26.3	nd	nd
	Median	15.7	24.3	2.0	2.5	13.8	22.7	nd	nd
	Min-Max	0.0-42.5	0-81.0	1.1-5.7	0.0-7.8	0-39.9	0-76.3	nd	nd
Adults Men (n=16)	Incidence	100	100	100	100	100	100	nd	nd
	%Total DON	N/A	N/A	16	15	84	85	nd	nd
	Unadjusted (ng/mL)								
	Mean	13.1	18.1	2.1	2.9	11.0	15.2	nd	nd
	Median	11.9	12.6	1.8	1.9	7.1	11.0	nd	nd
	Min-Max	2.2-19.4	5.1-58.8	0.3-4.2	0.5-13.8	1.8-26.8	4.3-45.0	nd	nd
	Adjusted (ng/mg creatinine)								
	Mean	24.3	21.3	4.2	3.2	20.0	18.1	nd	nd
	Median	10.9	14.4	1.8	2.1	9.8	12.2	nd	nd
	Min-Max	0.5-153	2.5-62.3	0.1-31.5	0.4-12.7	0.4-122	2.1-55.4	nd	nd
Adult Women (n=15)	Incidence	100	100	87	87	100	100	nd	nd
	%Total DON	N/A	N/A	15	16	85	84	nd	nd
	Unadjusted (ng/mL)								
	Mean	12.4	14.1	2.0	2.4	10.4	11.8	nd	nd
	Median	10.7	10.1	1.8	1.3	9.0	9.6	nd	nd
	Min-Max	3.3-40.6	0.9-36.0	0.3-4.2	0.1-8.6	2.9-31.9	0.7-27.4	nd	nd
	Adjusted (ng/mg creatinine)								
	Mean	12.7	18.2	1.9	2.8	10.8	15.4	nd	nd
	Median	11.3	11.3	1.6	2.5	10.0	10.5	nd	nd
	Min-Max	3.9-27.8	1.0-66.2	0.2-4.7	0.2-9.3	3.3-23.1	0.8-56.8	nd	nd

The Table includes adult data previously presented in Wells et al. 2017. Data are presented as mean, medium and min (minimum) - max (maximum); DON: deoxynivalenol; DON: deoxynivalenol; DON-GlcA: deoxynivalenol glucuronide; DOM-1: deepoxy-deoxynivalenol; min: minimum; max: maximum; nd: not detected. LOQ for free DON was 0.25 ng/mL and for DON-GlcA and DOM-1 0.50 ng/mL.

Table 3. Consumption of food categories that commonly contribute to dietary DON exposure in elderly (n=20) and adults (n=30).

Food category	Elderly (*n* = 20)	Adults (n=30)
Bread (g/d)	101±49 (0-203)	89±60 (0-203)
Flakes (g/d)	22±16 (0–60)*	12±14 (0-50)
Breakfast cereals (g/d)	4±8 (0–23)	5±8 (0–30)
Other breads (g/d)	17±30 (0–90)	34±45 (0–135)
Biscuits (g/d)	15±15 (0-53)*	8±13 (0-60)
Baked goods (g/d)	28±32 (0–112)*	15±32 (0–112)
Pizza (g/d)	9±30 (0-128)	17±41 (0-128)
Pasta (g/d)	23±47 (0–115)	60±77 (0–230)
Wheat germ (g/d)	2±5 (0-15)	1±3 (0-15)
Beer (g/d)	43±105 (0-431)	91±153 (0-431)
Pancakes (g/d)	6±28 (0-124)	7±23 (0-93)
Total (g/d)	269±148 (65–625)	339±180 (45-658)

Data are presented as mean±1SD (range: minimum-maximum). Analysis was performed in 20 elderly and 30 adults with complete data. *, $p<0.05$, significant different from adults.