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Use of metabolomics in refining the effect of an organic food intervention on biomarkers of exposure to pesticides and biomarkers of oxidative damage in primary school children in Cyprus: A cluster-randomized cross-over trial

Corina Konstantinou, Stephanie Gaengler, Stavros Oikonomou, Thibaut Delplancke, Pantelis Charisiadis, Konstantinos C. Makris *

Cyprus International Institute for Environmental and Public Health, Cyprus University of Technology, Cyprus

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ABSTRACT

Background: Exposure to pesticides has been associated with oxidative stress in animals and humans. Previously, we showed that an organic food intervention reduced pesticide exposure and oxidative damage (OD) biomarkers over time; however associated metabolic changes are not fully understood yet.

Objectives: We assessed perturbations of the urine metabolome in response to an organic food intervention for children and its association with pesticides biomarkers [3-phenoxybenzoic acid (3-PBA) and 6-chloronicotinic acid (6-CN)]. We also evaluated the molecular signatures of metabolites associated with biomarkers of OD (8-*iso*-PGF2a and 8-OHdG) and related biological pathways.

Methods: We used data from the ORGANIKO LIFE + trial (NCT02998203), a cluster-randomized cross-over trial conducted among primary school children in Cyprus. Participants (n = 149) were asked to follow an organic food intervention for 40 days and their usual food habits for another 40 days, providing up to six first morning urine samples (>850 samples in total). Untargeted GC–MS metabolomics analysis was performed. Metabolites with RSD $\leq$ 20% and D-ratio $\leq$ 50% were retained for analysis. Associations were examined using mixed-effect regression models and corrected for false-discovery rate of 0.05. Pathway analysis followed.

Results: Following strict quality checks, 156 features remained out of a total of 610. D-glucose was associated with the organic food intervention ($\beta = -0.23$, 95% CI: −0.37,−0.10), aminomalonic acid showed a time-dependent increase during the intervention period ($\beta_{int} = 0.012$; 95% CI:0.002, 0.022) and was associated with the two OD biomarkers ($\beta = -0.27$, 95% CI:−0.34,−0.20 for 8-*iso*-PGF2a and $\beta = 0.19$, 95% CI:0.11,0.28 for 8-OHdG) and uric acid with 8-OHdG ($\beta = 0.19$, 95% CI:0.11,0.26). Metabolites were involved in pathways such as the starch and sucrose metabolism and pentose and glucuronate interconversions.

Discussion: This is the first metabolomics study providing evidence of differential expression of metabolites by an organic food intervention, corroborating the reduction in biomarkers of OD. Further mechanistic evidence is warranted to better understand the biological plausibility of an organic food treatment on children's health outcomes.

1. Introduction

The organic food market is growing and the recent European farm to fork strategy set a target for increasing organic food production, as it is considered a sustainable agriculture system, by positively affecting biodiversity and by reducing pesticides dependency (European Commision, 2020). Exposure to pesticides has been associated with oxidative stress and inflammatory response in animals (Ge et al., 2015; Gargouri et al., 2018) and humans (Prakasam et al., 2001; Lee et al., 2007, 2017).

Untargeted metabolomics offer a dynamic overview of the metabolic features within a system and may provide insight how the metabolome responds to external stimuli (Gertsman and Barshop, 2018). Gas-chromatography mass-spectrometry (GC–MS) is considered a "gold standard" in metabolomics with many advantages like high

* Corresponding author at: Cyprus International Institute for Environmental and Public Health, Cyprus University of Technology, Limassol, Cyprus.
E-mail address: konstantinos.makris@cut.ac.cy (K.C. Makris).

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chromatographic resolution, large databases of identified peaks and good sensitivity (Lu et al., 2008; Papadimitropoulos et al., 2018). As such, to better understand the metabolic changes associated with an organic food intervention, GC–MS metabolomic profiling may prove an important tool to gain new insights into the biochemistry of underlying alterations for people consuming systematically organic food. However, the use of metabolomics technologies in disentangling exposure outcome associations has only recently begun to aid in improving causality inference in environmental health sciences (Cadiou et al., 2021). Some have used the metabolome as an intermediary biological layer that improves estimates of causality in an exposome-outcome context (Cadiou et al., 2021).

Several human and animal metabolomic studies on pesticide exposures and their associated metabolic perturbations have already been published (Yang et al., 2011, 2020; Bonvallot et al., 2013; Liang et al., 2013, 2020; Du et al., 2014; Maitre et al., 2018; Yan et al., 2021). Moreover, targeted and untargeted metabolomics studies have been performed to elucidate the association between various dietary patterns and metabolic signatures (Altmaier et al., 2011; O'Sullivan et al., 2011; Andersen et al., 2014; Playdon et al., 2017; Acar et al., 2019). The use of metabolomics tools has been limited to facilitating the discrimination of organic food products from conventional foods (Vallverdú-Queralt et al., 2011; Novotná et al., 2012; Mie et al., 2014; Shepherd et al., 2014; Cubero-Leon et al., 2018; Xiao et al., 2018; Zhang et al., 2020) and they have not yet been used to examine the association between organic food consumption and metabolites.

We have previously shown that a 40-day organic food intervention was effective in lowering biomarkers of exposure to pesticides, as well as biomarkers of oxidative damage (OD) in primary school children in Cyprus (Makris et al., 2019). Specifically, a cluster-randomized crossover trial was conducted in 2017 and it was shown that an organic food intervention program followed for up to 40 days by 149 healthy children with mean age of 11 years old, reduced the body burden of biomarkers of exposure to pyrethroids [3-phenoxybenzoic acid (3-PBA)] and neonicotinoids [6-chloronicotinic acid (6-CN)] and reduced the levels of OD biomarkers, 8-OHdG and 8-*iso*-PGF2a. However, knowledge about possible changes in the human metabolome and response to organic food treatment (lower exposure to pesticides) and concomitant effects on outcomes of OD, is currently lacking.

In this study, we applied untargeted GC–MS metabolomics to better understand the effect of an organic food intervention on biomarkers of exposure to pesticides and oxidative lipid and DNA damage in primary school children participating in the ORGANIKO trial (NCT02998203). To our knowledge, this is the first study investigating the metabolomic profiles associated with an organic food intervention and OD biomarkers. The objectives of this study were to (i) assess the urinary metabolome changes in response to an organic food intervention of children and its association with biomarkers of exposure to pesticides (3-PBA and 6-CN), and to (ii) evaluate the molecular signatures of specific metabolites associated with biomarkers of OD (8-*iso*-PGF2a and 8-OHdG) and their downstream biological pathways.

2. Methods

2.1. Population study

This study involved participants from the ORGANIKO LIFE+ cluster-randomized crossover trial (NCT02998203). The primary and secondary objectives of this non-pharmacological trial were assessed previously (Makris et al., 2019); these were to evaluate the effect of an organic food intervention on pyrethroid and neonicotinoid pesticide metabolites and on OD biomarkers, respectively, in primary school children in Cyprus. The trial protocol was approved by the Cyprus National Bioethics Committee (ΕΕΒΚ/ΕΠ/2016/25) and the Cyprus Ministry of Education and Culture (7.15.06.15/2). Informed consent was provided by the school headmasters and children's parents or legal guardians, and a verbal assent was obtained from the children. The trial was performed in accordance with the principles of the Declaration of Helsinki. The trial included 191 healthy 10–12-year-old children, recruited from 6 schools in Limassol, in January 2017, for an 80-day intervention trial with two periods (40 days of organic diet and 40 days of conventional diet).

Briefly, six public primary schools in Limassol were randomized a priori to two groups that differed in the sequence of the treatments; organic food period followed by the conventional food period (Group 1) or the opposite (Group 2) (Fig. 1). During the conventional food period, participants were asked to maintain their usual dietary choices (>80% conventional diet) for a total of 40 days. During the organic food period, participants were asked to follow strictly the two ~20-day sequential organic dietary menus provided to them for 40 ± 3 days. Participants crossed over to the other treatment on the day after the first period was completed.

Each participant provided up to six first-morning urine samples during the whole duration of the study; one baseline sample and two samples in the conventional period, and three samples in the organic food period. Urine samples were collected in polypropylene vials and were temporarily stored in a school/home fridge (4 °C) until transferred to laboratory facilities for storage at −80 °C. Anthropometric measurements (weight, height, and waist circumference) were taken at the beginning of the study, at the end of the organic food period, and at the end of the study (for Group 2, the end of study and end of organic food period was the same time point) by trained researchers.

Two pesticide metabolites were measured in urine samples: 3-PBA, a metabolite of pyrethroid pesticides, and 6-CN, a metabolite of neonicotinoid pesticides using a gas-chromatographic-tandem mass spectrometric (GC–MS/MS) (Charisiadis et al., 2019). Competitive ELISA kits were used to determine urinary concentrations of 8-*iso*-PGF2α (Catalog no: STA-337; Cell Biolabs, Inc, California, USA) and 8-OHdG (Catalog no: ABIN2964843; antibodies-online, Aachen, Germany). Creatinine was measured using the colorimetric Jaffé method (Angerer and Hartwig, 2010). More information about the methodology can be found in the original article (Makris et al., 2019).

2.2. Untargeted GC–MS metabolomics analysis

Urine samples were subjected to untargeted GC–MS metabolomics analysis (Chan et al., 2011). Details about GC–MS data acquisition and sample processing are described in S2 and have been previously published (Andrianou, Charisiadis and Makris, 2017). Briefly, analysis was performed in an Agilent INTUVO 9000 GC coupled to an Agilent 5977B mass spectrometer and urine samples, were subject to urea depletion, metabolite extraction and derivatization. Quality-control (QC) samples were prepared by pooling all study samples and by adding fatty acid methyl esters (FAME) markers. Five to seven QCs and one blank sample (absence of urine sample) were used in each batch.

2.3. Data pre-processing

Deconvolution of samples, QCs and blanks (n = 1310) was done automatically with Global Natural Products Social Molecular Networking (GNPS) (Aksenov et al., 2021) using the default settings of the GC–MS EI method (cluster_spectra = no; RT_tolerance = 2.0). Following the generation of the feature table, we kept only features with balance score > 60% (Aksenov et al., 2021) and excluded features missing in > 20% of samples.

We normalized data with the systematical error removal using random forest algorithm (SERRF - version 9.1.2020) (Fan et al., 2019) and included features with relative standard deviation (RSD) ≤ 20% and D-ratio ≤ 50% (Broadhurst et al., 2018). To assess the quality of the data (biological vs technical variability), clustering of the QCs in principal component analysis (PCA) was used. Following log-transformation, scaling and centering of features, we excluded outlier samples using PCA (contribution ≥ 4 SD). Only samples that were included in the

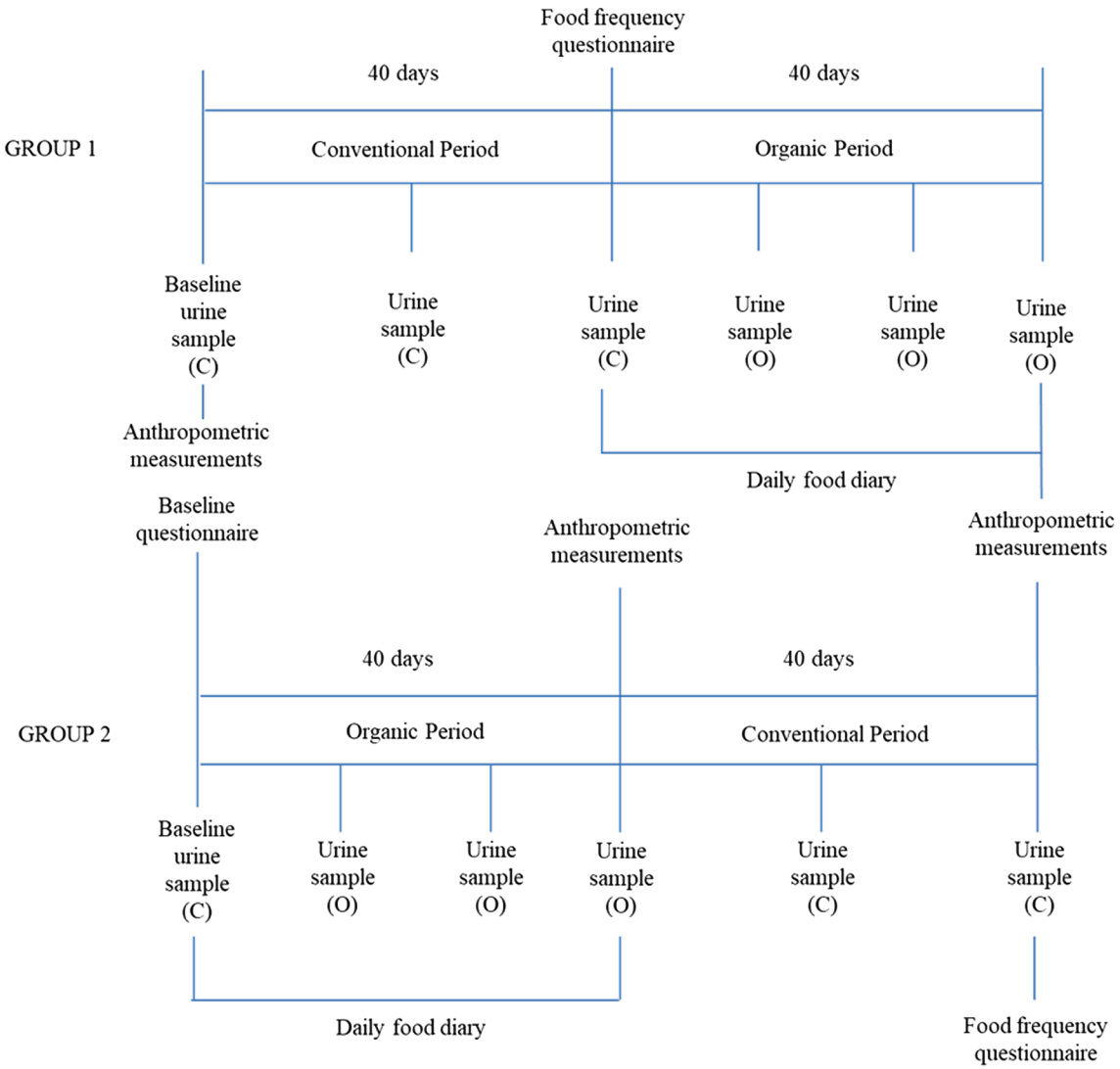

Fig. 1. Study timeline and data collection procedure for the two groups of the study. "C" denotes that the sample was taken when the participant was following his/her usual habits (conventional food consumption) whereas "O" when the participant was following the organic food intervention.

initial ORGANIKO LIFE + analysis (samples from participants with ≥ 11 days in the organic food period) were included in this analysis (n = 853).

In order to assess blank contribution, we flagged features with median peak area in blank samples >15% of median peak area of the same feature in the samples, as "potentially contaminated" (Broadhurst et al., 2018).

2.4. Data analysis

Step 1: Exposome-wide association study (ExWAS)

We performed an ExWAS to examine associations of each feature with the organic food intervention, the pesticide metabolites (3-PBA and 6-CN) and the OD biomarkers (8-*iso*-PGF2a and 8-OHdG).

Linear mixed-effect regression models with an unstructured covariance matrix and a random intercept for participant were used. Continuous variables, other than time, were scaled and centered. Features and biomarkers (OD biomarkers and PBA) were additionally log transformed. Three sets of models were prepared, as described in the next paragraphs. The models were adjusted for sex, baseline BMI (z-scores), creatinine and time (days of treatment), where time = 0 was used for the start of the treatment. The second and third set of models were further adjusted for the treatment condition (organic food vs conventional food as reference). The threshold for the significance of the association for the three sets of models (p-value ≤ 0.05) was adjusted for multiple testing by controlling the false-discovery rate (FDR) (Benjamini and Hochberg, 1995).

Statistical analysis was conducted in R (version 4.0.4) (R Core Team, 2017) with RStudio (version 1.4.1106) (RStudio Team, 2015). The input data, scripts, and output are available in S4.

Step 1A: Association of features with organic food intervention

A first set of models included each feature as outcome and treatment condition (organic food vs conventional food as reference) as fixed effect. An interaction term for time and treatment was considered and subsequently dropped if it did not meet the threshold of p-value ≤ 0.05.

Step 1B: Association of features with pesticide metabolites

A second set of models included each feature as outcome, and 3-PBA (log-transformed) or 6-CN (binary; below and above LOD because of their high % of values < LOD) as fixed effect. An interaction term for time and 3-PBA or 6-CN, respectively, was considered and subsequently dropped if it did not meet the threshold of p-value ≤ 0.05.

Step 1C: Association of features with OD biomarkers

A third set of models included each OD biomarker as outcome, and each feature, and the baseline value (first urine sample for all children) of the outcome as fixed effects. These models were adjusted for the baseline value of the OD biomarker, to account for the background participant levels. An interaction term for time and feature was considered and subsequently dropped if it did not meet the threshold of p-value ≤ 0.05.

Step 2: Identification of features

The significantly altered urinary features derived from the three sets of models were identified initially, by comparing their spectra with spectra of metabolites from NIST14 library (level 2) and secondly, by using authenticated standard compounds in urine (level 1). For level 1 identification, we acquired 13 out of the 20 differentially expressed metabolites. Following the exact same methodology as in the analysis of the study samples (QCs and participant samples), we analyzed urine samples spiked with each of the 13 differentially expressed compounds (13 samples in total) and one urine sample spiked with all the 13 compounds (mix std). The samples with the standard compounds (STDs) were processed with the exact same way as the study samples (deconvolution in GNPS and compound identification in NIST MS Search with NIST14 library). Metabolites were classified based on the metabolomics standards initiative (MSI) guidelines for metabolite identification (Sumner et al., 2007); level 1: Identified compounds with reference standards (*m/z*, and RT), level 2: putatively annotated compounds based on the use of spectral library (NIST14), and level 4: unknowns.

Step 3: Pathway analysis

The identified metabolites were processed for pathway analysis using the "Pathway Analysis" module and the KEGG pathway library in MetaboAnalyst 5.0 (Pang et al., 2021) in order to assess the biological pathways in which these metabolites are implicated.

3. Results

3.1. Demographics and other characteristics

The demographics and other characteristics of the participants are presented in Table 1 of Makris et al. (2019). Children who followed the organic food intervention for at least 12 days and provided at least one urine sample during the organic food period were included in the analysis, hence a total of 149 children were included, with 43 children in Group 1 and 106 children in Group 2. Their mean age was 11 years old and 89% of the children completed 29–40 days of organic food intervention. The majority of the participants' parents had a high educational level with 82% of mothers and 65% of fathers holding at least a university/college degree. At baseline, more than half of the children (61%) had a normal weight, 38% were overweight or obese and 1% belonged in the thinness group.

3.2. Quality of untargeted metabolomics spectra

Deconvolution of samples, QCs and blanks generated 610 features and of those, 229 features had balance score $\geq$ 60% (S1: Figure S1), while 64 features were excluded due to missing values. Following SERRF normalization, the RSDs of the features improved (S1: Table S1, Figure S2) and features with RSD > 20% and D-ratio > 50% were excluded (n = 9). QCs were adequately clustered (S1: Figure S3). A total of 156 features and 853 samples remained following pre-processing (S1: Figure S1).

3.3. Metabolome alterations

A total of 42 features were significantly associated (FDR p-value $\leq$ 0.05) with either organic food intervention or/and pesticide metabolites or/and OD biomarkers (Fig. 2, S1: Table S2). Five features were significantly associated with the organic food intervention, ten features with 3-PBA, one feature with 6-CN and 15 features with 8-*iso*-PGF2a and 8-OHdG, respectively. Significant (p-value $\leq$ 0.05) interactions between variables of interest with duration of treatment (in days) can be found in the supplementary (S1: Table S2). Out of these 42 features, 20 (two of them were the same compound) were putatively annotated (level 2) based on spectra comparison (S1: Table S3, S3). Following acquisition of authenticated standard compounds for 13 metabolites, 11 were identified at level 1 (S1: Table S4).

A significant positive interaction between days of treatment and the organic food intervention was observed for aminomalonic acid (β_{int} = 0.012; 95% CI:0.002, 0.022; p = 0.019), indicating a time-dependent increase during the intervention period (S1: Table S2). Aminomalonic acid was also negatively associated with 8-*iso*-PGF2a ($\beta = -0.27$, 95% CI:−0.34, −0.20; FDR p-value < 0.001) and positively associated with 8-OHdG ($\beta = 0.19$, 95% CI:0.11, 0.28; FDR p-value = 0.001) (Table 1). D-glucose was negatively associated with the organic food intervention ($\beta = -0.23$, 95% CI:-0.37, −0.10; FDR p-value = 0.019) and gluconic acid was negatively associated with 3-PBA ($\beta = -0.11$, 95% CI:−0.18, −0.04; FDR p-value = 0.036) and positively associated with 8-OHdG ($\beta = 0.18$, 95% CI:0.09, 0.26; FDR p-value = 0.003). Two metabolites - tartaric acid ($\beta = -0.12$, 95% CI:-0.20, −0.05; FDR p-value = 0.016) and D-psicose ($\beta = -0.18$, 95% CI:−0.25, −0.11; FDR p-value < 0.001) were negatively associated with 3-PBA.

Six metabolites – 2,3-dihydroxybutanoic acid ($\beta = 0.16$, 95% CI:0.08, 0.23; FDR p-value = 0.003), erythritol ($\beta = 0.11$, 95% CI:0.04, 0.18; FDR p-value = 0.049), threonic acid ($\beta = 0.19$, 95% CI:0.11, 0.27; FDR p-value < 0.001), 7-methylxanthine ($\beta = 0.14$, 95% CI:0.07, 0.21; FDR p-value = 0.005), N-acetyl-D-glucosamine ($\beta = 0.11$, 95% CI:0.04, 0.18; FDR p-value = 0.040) and galactosylglycerol ($\beta = 0.13$, 95% CI:0.05, 0.20; FDR p-value = 0.017) were positively associated with 8-*iso*-PGF2a, while phosphoric acid ($\beta = -0.12$, 95% CI:-0.20, −0.05; FDR p-value = 0.036) and cellobiose ($\beta = -0.13$, 95% CI:−0.20, −0.06; FDR p-value = 0.009) were negatively associated with 8-*iso*-PGF2a. Seven metabolites - 1,2,3-butanetriol ($\beta = 0.20$, 95% CI:0.11, 0.28; FDR p-

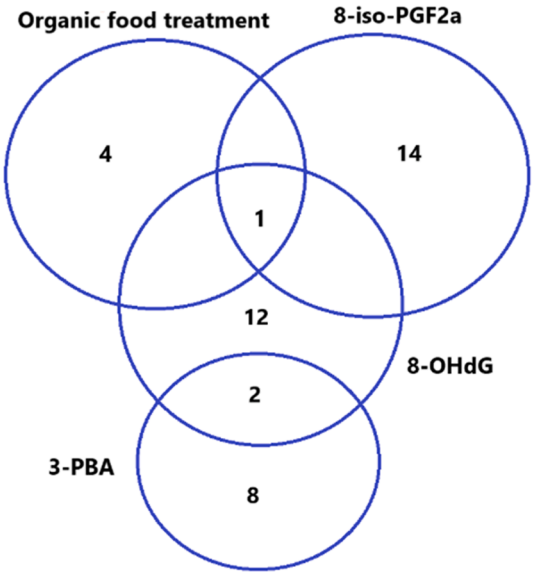

Fig. 2. FDR-adjusted significant associations (FDR p-value $\leq$ 0:05) based on the three sets of linear mixed effect models (one association with 6-CN not shown) – unknowns features (Level 4) are included (S1: Table S2).

Table 1
Annotated features being statistically significantly associated (FDR p-value ≤ 0.05) in the linear mixed-effect models described under Data Analysis - Step 1. *In the model with aminomalonic acid as outcome and organic food intervention as predictor, the interaction term between organic food intervention and time was significant (β_{int} = 0.012; 95% CI:0.002, 0.022; p = 0.019).

Feature	Estimate	CI (95%)	Raw p-value	FDR p-value	Model
Phosphoric acid	−0.12	−0.20, −0.05	0.002	0.036	8-*iso*-PGF2a
1,2,3-Butanetriol	0.20	0.11, 0.28	<0.001	0.001	8-OHdG
Succinic acid	0.17	0.09, 0.25	<0.001	0.004	8-OHdG
2,3-Dihydroxybutanoic acid	0.16	0.08, 0.23	<0.001	0.003	8-*iso*-PGF2a
2,4-Dihydroxybutanoic acid	0.22	0.11, 0.32	<0.001	0.003	8-OHdG
3,4-Dihydroxybutanoic acid	0.14	0.05, 0.23	0.002	0.039	8-OHdG
Aminomalonic acid*	−0.27	−0.34, −0.20	<0.001	<0.001	8-*iso*-PGF2a
	−0.44	−0.68, −0.19	<0.001	0.013	Organic food
	0.19	0.11, 0.28	<0.001	0.001	8-OHdG
Erythritol	0.11	0.04, 0.18	0.003	0.049	8-*iso*-PGF2a
Threonic acid	0.19	0.11, 0.27	<0.001	<0.001	8-*iso*-PGF2a
Tartaric acid	−0.12	−0.20, −0.05	0.001	0.016	3-PBA
D-Xylitol	0.17	0.08, 0.26	<0.001	0.005	8-OHdG
Ribonic acid	0.15	0.05, 0.24	0.003	0.047	8-OHdG
D-Psicose	−0.18	−0.25, −0.11	<0.001	<0.001	3-PBA
D-Glucose	−0.23	−0.37, −0.10	0.001	0.019	Organic food
Gluconic acid	0.18	0.09, 0.26	<0.001	0.003	8-OHdG
	−0.11	−0.18, −0.04	0.002	0.036	3-PBA
7-Methylxanthine	0.14	0.07, 0.21	<0.001	0.005	8-*iso*-PGF2a
N-Acetyl-D-glucosamine	0.11	0.04, 0.18	0.002	0.040	8-*iso*-PGF2a
Uric acid	0.19	0.11, 0.26	<0.001	<0.001	8-OHdG
Galactosylglycerol	0.13	0.05, 0.20	0.001	0.017	8-*iso*-PGF2a
Cellobiose	−0.13	−0.20, −0.06	<0.001	0.009	8-*iso*-PGF2a

value = 0.001), succinic acid (β = 0.17, 95% CI:0.09, 0.25; FDR p-value = 0.004), 2,4-dihydroxybutanoic acid (β = 0.22, 95% CI:0.11, 0.32; FDR p-value = 0.003), 3,4-dihydroxybutanoic acid (β = 0.14, 95% CI:0.05, 0.23; FDR p-value = 0.039), D-xylitol (β = 0.17, 95% CI:0.08, 0.26; FDR p-value = 0.005), ribonic acid (β = 0.15, 95% CI:0.045, 0.24; FDR p-value = 0.047) and uric acid (β = 0.19, 95% CI:0.11, 0.26; FDR p-value < 0.001) - were positively associated with 8-OHdG. The masses and the retention times (RT) of the unidentified compounds (level 4) are presented in the supplementary (S1: Table S5).

Assessment of the blank contribution showed that for two out of the 20 metabolites (phosphoric acid and cellobiose) included in Table 1, their median peak area in blanks was higher compared to the corresponding area in samples (S1: Table S6) and hence the peaks of these metabolites were flagged as "potentially contaminated". For 7-methylxanthine, the blank contribution was marginally higher (15.7%) than the criterion set (15%), so we did not flag it.

3.4. Pathway analysis

Out of the 20 annotated metabolites found statistically significant in the linear mixed-effect models, 16 of them had a KEGG ID and could be used for pathway analysis (S1: Table S3). Even though D-psicose had a KEGG ID (C06468), there was no match with the KEGG human pathway library, and it was excluded from the pathway analysis (S1: Table S7). Metabolites were found in 12 pathways with the impact in half of them being zero (S1: Table S8) and with a maximum number of 2 hits (matched number from the 15 uploaded metabolites). Metabolites were involved in pathways such as the starch and sucrose metabolism, the pentose and glucuronate interconversions, the galactose metabolism, the amino sugar and nucleotide sugar metabolism and the pentose phosphate pathway (S1: Table S8).

4. Discussion

In this study, we used untargeted metabolomics to investigate the associations between an organic food intervention, the endogenous response on the human metabolome and classical biomarkers of pesticide exposure (3-PBA and 6-CN) and OD (8-*iso*-PGF2α and 8-OHdG); these specific biomarkers of exposure and effect were associated with the organic food intervention in the same study population, as shown earlier (Makris et al., 2019). In the present analysis, significant differences in the urinary metabolomics profile of primary school children following an organic food intervention were observed. Following annotation, pathway analysis of significantly altered metabolites showed that pathways relevant to the metabolites were the starch and sucrose metabolism and the pentose and glucuronate interconversions.

Two metabolites were significantly associated with the organic food intervention, two with the pyrethroids' metabolite (3-PBA) and nine with each of the OD biomarkers (8-OHdG and 8-*iso*-PGF2a). Aminomalonic acid, which was positively associated with the organic food intervention has been associated with radical mediated protein oxidation, as isolated from *Escherichia coli* cultures and from human atherosclerotic plaques (Dean et al., 1997). In a metabolomics study with 35 neonates, the urine metabolite profiles of two nutrition regimens were examined for seven days and it was shown that aminomalonic acid was up-regulated in breast milk fed neonates (Dessì et al., 2016). A type II diabetes nested case-control study (n = 197) in China, showed that aminomalonic acid was among the metabolites with high potential to predict type 2 diabetes in high-risk individuals (Lu et al., 2016).

Glucose, which was negatively associated with the organic food intervention, was previously inversely associated with vegetable, fruit and nut intake for 300 children aged 7–10 years from four primary schools in Verona, Italy (Giontella et al., 2019). D-glucose is involved in the pentose phosphate pathway (S1: Figure S4), which is the main contributor of NADPH, and has oxidative and non-oxidative branches (Ge et al., 2020). This pathway is considered to regulate cellular reduction–oxidation homeostasis and biosynthesis and has been associated with metabolic diseases, such as type 2 diabetes. Also, D-glucose is involved in other pathways, like the starch and sucrose metabolism, along with cellobiose, which was negatively associated with 8-*iso*-PGF2a, and the galactose metabolism along with galactosylglycerol, which was positively associated with 8-*iso*-PGF2a. Moreover, gluconic acid, which was significantly associated with 8-OHdG and 3-PBA is the oxidation product of D-glucose (Bankar et al., 2009) and is a component of the pentose phosphate pathway.

Uric acid, which was positively associated with 8-OHdG, is the final oxidation product of purine metabolism and can be produced in the body via two pathways with xanthine oxidase converting xanthine to uric acid (Maiuolo et al., 2016). Recent epidemiological studies in children have shown association of uric acid with hypertension starting

in childhood and continuing in adulthood (Jr et al., 2004; Kubota, 2019), metabolic syndrome (Bussler et al., 2017; Kubota, 2019), chronic kidney disease, obesity, insulin resistance and dyslipidemia (Kubota, 2019).

Threonic acid, a catabolite of antioxidant ascorbic acid (vitamin C) (Chazot and Kopple, 2013) was found to be positively associated with 8-*iso*-PGF2a. Similar association was observed for 2,3-dihydroxybutanoic acid, which is a product of threonine catabolism (Appiah-Amponsah et al., 2009) and was previously associated with diabetes mellitus in rats (Jing and Chengji, 2019). Three metabolites that were positively associated with 8-OHdG − 2,4-dihydroxybutanoic acid, 3,4-dihydroxybutanoic acid and ribonic acid - were previously associated with macroalbuminuria in an untargeted serum metabolomics analysis from 637 persons with type 1 diabetes (Tofte et al., 2019). Succinic acid which was positively associated with the OD biomarker, 8-OHdG, has a significant role in innate immunity regulation via activation of the pro-inflammatory cytokine IL-1β, leading to inflammation (Martínez-Reyes and Chandel, 2020) and is a metabolite of the citrate cycle (S1: Figure S4), which is involved in energy-production.

Erythritol and xylitol were positively associated with 8-*iso*-PGF2a and 8-OHdG, respectively; they are sugar alcohols that occur naturally in fruits, vegetables and fermented foods (only erythritol) (Bond and Dunning, 2006; Sreenath and Venkatesh, 2016). Moreover, erythritol and D-xylitol are used as sweeteners and have potential anti-hyperglycemic properties (Wölnerhanssen et al., 2020). Erythritol has been shown to act as an antioxidant in vivo by being a free radical scavenger (den Hartog et al., 2010; Regnat, Mach and Mach-Aigner, 2018) and xylitol, to improve peripheral glucose utilization mainly based on animal models, a finding that needs to be investigated further in human studies (Regnat, Mach and Mach-Aigner, 2018; Salli et al., 2019). In a cohort study of 246 young adults (18–19 years old), metabolomics analysis showed that *meso*-erythritol concentration was higher in the group of participants with incident central adiposity compared to the group with stable adiposity and in participants with higher glycemia compared to lower glycemia (Hootman et al., 2017). In the same study, further in-vivo analysis showed that erythritol is produced endogenously by glucose via the pentose phosphate pathway, contrary to previous studies reporting no erythritol synthesis in humans.

As far as we know, this is the first study assessing children's metabolomic profiles following a systematic consumption of organic food. The metabolomic profile is an integral part of the human exposome, the term used to describe the totality of exposures and associated endogenous response throughout a person's lifetime (Wild, 2005; Miller and Jones, 2014). As suggested in a recent commentary (Dennis et al., 2017), in order to effectively characterize the exposome, a combination of traditional biomonitoring approaches and untargeted discovery of metabolites should be used; in our study, we coupled data from targeted measurements of pesticide metabolites and OD biomarkers with untargeted metabolomics analysis with the aim to evaluate biological alterations associated with organic food consumption.

Strengths of this study include the prospective and randomized study design, including its decent sample size, high repeated measures sampling frequency and the long intervention duration (40 days). Selection bias in this study was low, as all samples from the original study were included in this study (>850 samples). The quality of such exposome studies (selection bias or study design) and their issues are touched upon in a recent scoping review of all exposome studies (Haddad, Andrianou and Makris, 2019). About half of the exposome studies published until March 2019 used omics tools as part of their methodology and more specifically, metabolomics was used as an exposure assessment tool in 22 out of 78 studies and as an outcome metric in 12 out of 48 studies (Haddad, Andrianou and Makris, 2019). Similarly, in our study, we used metabolomics as an *a priori* intermediary biological layer, based on a notion of biological plausibility. The temporality of exposures and metabolomics (intermediary data) should precede onset of disease outcome so prospective studies, like this work, should be always preferred, albeit not always feasible. Differences in the performance of these metabolomics-based approaches in unraveling associations between exposures and an outcome should be anticipated between studies with repeated measures and studies using only two time points for measurements.

We acknowledge some limitations of the study. First, due to the repeated cross-over nature of the study, we could not perform routine analysis often performed in metabolomics studies, such as (ortho)partial least squares analyses - discriminatory analyses (PLS-DA) or pathway analysis using concentration data (peak area) since these types of analysis require that samples have discrete classification and samples may be paired as derivatives of two. In our study, each participant provided two to six urine samples in the form of repeated measures; such complex study designs require specialized biostatistical methods. The high dimensionality of exposome study designs and their datasets is a burden in the quest of causality inference in exposome studies. Also, the use of a single mass spectrometry platform for a single biospecimen matrix may only capture a small percentage of the total human metabolome (>110 K compounds). Moreover, some of the significantly associated features were categorized as unknowns (level 4), meaning no knowledge about their role and the pathways involved could be derived. Due to the low number of metabolites used in pathway analysis (n = 15), the pathways impact values were low as the maximum number of hits observed in all pathways was two. Furthermore, there was a difference in the educational attainment status between the study participants' parents (72%, 61% fathers and 82% mothers) and the Cyprus population aged 30–34 years (57%, 49% men and 64% women) (CYSTAT, 2019).

Future studies are needed to determine whether these findings can be replicated in other populations. In summary, changes in the metabolomic profile of primary school children were observed following a 40-day long systematic organic food intervention. This is the first metabolomics study providing evidence of differentially expressed metabolites in an organic food intervention corroborating the reduction in biomarkers of OD in primary school children.

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CRediT authorship contribution statement

Corina Konstantinou: Data curation, Investigation, Methodology, Writing – review & editing. **Stephanie Gaengler:** Data curation, Investigation, Methodology, Writing – review & editing. **Stavros Oikonomou:** Data curation, Writing – review & editing. **Thibaut Delplancke:** Writing – review & editing. **Pantelis Charisiadis:** Writing – review & editing. **Konstantinos C. Makris:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.107008.

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