FRIEDRICH-ALEXANDER
UNIVERSITÄT
ERLANGEN-NÜRNBERG
MEDIZINISCHE FAKULTÄT

Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine
(Director: Prof. Dr. med. Hans Drexler)

Greenpeace-Japan-Study of the Effect of Nutrition Change on the Pesticide Exposure of Consumers

Final Report

Introduction

Since many decades pesticides are applied in agriculture to protect crops from fungal attacks, insects, and competing plants. In spite of food safety controls for pesticide residues, a conventional diet still leads to a noticeable exposure of the general population to several pesticides (Hamilton et al. 2004). The exposure situation may differ between the populations in different countries of the world, but exists in principle for both developed and developing countries. Particularly, residues in fruits and vegetables contribute to the daily pesticide intake via food (Hamilton et al. 2004; Zhang et al. 2008). Consequently, higher pesticide exposure was found in vegetarian communities (Berman et al. 2016). In contrast, lower pesticide exposure can be assumed in individuals who consume organic food due to refuse of synthetic pesticide application in organic agriculture (Johansson et al. 2014).

This hypothesis was supported by several studies on the relationship between dietary pesticide intake and the urinary concentrations of pesticides and their metabolites, respectively, in different populations (Kimata et al., 2009; McKelvey et al., 2013; Wielgomas, 2013; Morgan and Jones, 2013) as well as by studies on the effect of organic diet intervention (Macintosh et al. 2001; Lu et al., 2006; Oates et al. 2014; Bradman et al. 2015, Magnér et al. 2015). However, most of these studies focused on the exposure to organophosphate pesticides and pyrethroids only. Moreover, the determination of dialkylphosphates has been established for the human biomonitoring of organophosphate pesticides (Barr and Needham, 2002, Berman et al., 2013), but do only depict an exposure to this pesticide unspecifically. Nevertheless, the human biomonitoring (HBM) offers much more parameters than the determination of dialkylphosphates and pyrethoid metabolites in urine (Barr and Needham, 2002; Göen, 2016).

In the study the effect of dietary shift to organic food on the urinary levels of pesticide metabolites was investigated including HBM parameters for organophosphates, pyrethroids, carbamates, neonicotinoids, phenoxy herbicides and glyphosate.

Study design

For the study two Japanese families were recruited. Family A consists of an adult female and two female children. Family B consists of an adult male, an adult female and two male children. The members of the families were kept on a conventional diet for 5 days and afterwards switched to exclusively organic food intake for 10 days. For the analysis of the individual exposure, morning urine voids were collected from each participant at the first (family A) or second (family B) day, as well as at the sixth and sixteenth day. Directly after sampling the urine samples were stored in a refrigerator at 4-8°C not longer than 4 days. Then, urine samples were frozen at -20°C and kept frozen until analysis. In the urine samples six pyrethroide metabolites, six dialkylphosphates as metabolites of organophosphate pesticides, nine phenolic parameter for organophosphate pesticides and carbamates, 6-chloronicotinic acid as non-specific parameter for several neonicotinoid insecticides, seven phenoxy herbicides, glyphosate and its metabolite AMPA were quantified using gas chromatographic mass spectrometric methods. Moreover, the creatinine content of the samples was determined to verify the supply of appropriately concentrated specimen.

Analytical procedures

All analytical procedures were performed at the laboratory of the institute in Erlangen. The determination of TCPy, IPP, PNP, DMADMP, ADMP, DEAMP, THPI, 1-naphthol and 2-naphthol was carried out using a gas-chromatographic-tandem mass spectrometric (GC-MS/MS) method, which was described in detail elsewhere (Schmidt et al. 2013). For this procedure, 1 ml of the urine was spent. Briefly, the conjugates of the phenolic structure to glucuronic acid and sulfate were cleaved enzymatically at first. Afterwards, the phenolic compounds were extracted from the urinary matrix by solid phase extraction and underwent a derivatization with N-tert-butyldimethylsilyl-N-methyltrifluoro-acetamide (MTBSTFA). Isotope-labeled equivalent structures were used as internal standards for each of the analytes. Calibration

was carried out using standard solutions in pooled human urine. The limit of detection (LOD) and limit of quantification (LOQ) were determined by means of a seven equidistant point calibration near the proposed LOD, according to guideline DIN 32645 (Schmidt et al., 2013). The limits of quantification (LOQ) were 0.3 µg/L for TCPy, DMADMP, DEAMP, PNP, THPI, 1-naphthol and 2-naphthol, and 0.4 µg/L for IPP and ADMP.

Urinary concentrations of the 6 dialkylphosphates were determined using a GC-MS/MS procedure (Barr et al., 2010; Berman et al. 2013). Isotope-labeled equivalent structures of the analytes were added to the urine, which was then freeze-dried. The lyophilized urine was extracted with diethyl ether and acetonitrile. Then the analytes were derivatized with pentafluorobenzyl bromide. After addition of water, liquid-liquid extraction was carried out twice with hexane to separate the derivatives from matrix components and excess of derivatization agent. Thereafter, GC-MS/MS analysis took place. Calibration was performed with standard solutions prepared in pooled urine. LOD and LOQ were estimated using a signal-to-noise ratio of 3:1 and 9:1, respectively. LOD ranged from 0.01µg/L for DEDTP and 0.05 for DMDTP to 0.1µg/L for DEP, DETP, DMP, and DMTP while the limit of quantification (LOQ) ranged from 0.03 µg/L for DEDTP and 0.15 for DMDTP to 0.3 µg/L for DEP, DETP, DMP, and DMTP.

The determination of pyrethroid metabolites were performed by a procedure described elsewhere (Schettgen et al. 2002). In brief, five milliliters of urine is pipetted into a 20-ml glass vial with teflon-lined screw top. Then, 25 µL of the working solution of the labelled internal standards ($^{13}C_6$-3-PBA and d6-trans-DCCA, 1 mg/L) is spiked. Hydrolysis of the conjugated carboxylic acids is performed by adding 1 ml of concentrated hydrochloric acid (37 %) and heating for 1 h at 90 °C in a water-bath. After cooling to room temperature, the samples are further processed. The acidic urine samples were then extracted two times with 5 ml of n-hexane by short vortexing and subsequent mechanically shaking for 10 min. After centrifugation for 5 min at 1500g, the organic layers were taken up and combined in a 20-ml glass vial with teflon-lined screw top. For further cleanup, 2 ml of aqueous 0.1 N NaOH was added to the organic phase and the carboxylic metabolites were re-extracted into the aqueous phase by mechanically shaking for 10 min. After centrifugation for 5 min at 1500g, the organic phase was discarded. The remaining aqueous phase was again acidified by adding 100 µL of concentrated hydrochloric acid (37 %) and once again

extracted with 1.8 ml n-hexane. Following centrifugation at 1500g for 5 min, the upper layer was transferred to a micro-vial. Fifty microliters of toluene is added as a keeper, and the extract was evaporated under a gentle stream of nitrogen to a volume of approximately 50 µL. Then, 10 µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) is pipetted into the glass vial, and the solution was transferred to a microvial and sealed tightly. For derivatisation, the vial was heated at 80 °C for 60 min in an oven. One microliter volume of this sample was then analyzed by GC-MS/MS in electron ionization mode. LOD and LOQ were estimated using a signal-to-noise ratio of 3:1 and 9:1, respectively. LOD was 0.03 µg/L and LOQ was 0.1 µg/L for all six parameters.

The determination of chlorinated phenoxycarboxylic acids and 6-chloronicotinic acid were performed by a GC-MS procedure. In brief, two milliliters of urine is pipetted into a 8-ml glass vial with teflon-lined screw top. Then, 25 µL of the working solution of the labelled internal standards ($^{13}C_4$-2,4-D, $^{13}C_4$-2,4,5-T and $^{13}C_4$-MCPA, 1 mg/L, or 13C6-ClNA, 1 mg/L) is spiked. Hydrolysis of the conjugated carboxylic acids is performed by adding 0.5 ml of concentrated hydrochloric acid (37 %) and heating for 2 h at 80 °C in a water-bath. After cooling to room temperature, the samples are further processed. The acidic urine samples were then extracted with 4 ml of tert.-butylmethylether by mechanically shaking for 10 min. After centrifugation for 5 min at 2200g, the organic layers were taken up and combined in a 8-ml glass vial. The extract was evaporated under a gentle stream of nitrogen to dryness (without heating). Then, 250 µgL acetonitrile, 30 µL of hexafluoroisopropanol and 15 µL diisopropylcarbondiimide were pipetted into the glass vial, and the solution was shaken for 10 min. Thereafter, subsequently 1 mL 1 M NaHCO3 solution and 500 µL isooctane was added and the extraction was performed by 10 min shaking. After centrifugation (2200 g) the organic layer was transferred in a 2 mL vial and the solution was reduced to 100 µL under a gentle stream of nitrogen. One microliter volume of this sample was then analyzed by GC-MS/MS in EI-mode. LOQ was determined by means of a ten equidistant point calibration near the proposed LOD, according to guideline DIN 32645. LOQ was 0.25 µg/L for MCPA, 2,4-D, 2,4,5-T, Mecoprop, Dichlorprop Fenoprop and Trichlopyr, and 0.3 µg/L for 6-chloronicotinic acid, respectively.

The quantitation of glyphosate and its metabolite AMPA in urine was performed by the Medizinisches Labor Bremen (Germany) with an analytical procedure using gas

chromatography (Krüger et al. 2014). The limit of quantification was 0.1µg/L for both parameters.

Creatinine in urine was determined according to the Jaffé method. Quality assessment of the analytical procedures was performed by analyzing of blank samples and quality control samples and recording in quality control charts as well as by successful participation in proficiency tests of the German External Quality Assessment Scheme (GEQUAS) for all of the analytes including creatinine (Göen et al., 2012). All urinary blank samples appeared to be free of any of the analytes.

Statistics

Results below the LOQ were included as half of LOQ for statistical analyses. Median values of the results of all seven members of the two families were calculated separately for the three sampling dates. Differences between the parameter levels of the three sampling dates were investigated by non-parametric comparison test of two dependent samples (Wilcoxon test), whereas statistical differences were assigned for error probabilities below 5 % ($p < 0.05$).

Results

Table 2 demonstrates the descriptive results of each parameter for the individuals of both families separated for the three sampling dates. Some HBM parameters were not found in any or hardly any of the samples, whereas other parameters were detected in each or almost each sample. Parameters of a high share of results above the LOQ at least in the two first sampling days were metabolites of organophosphate pesticides, e.g. DMP, DMTP, DEP, DETP, TCPy, PNP, DEAMP), pyrethroides (cis-Cl2CA, trans-Cl2CA, PBA) and glyphosate and its metabolite AMPA. Parameters which were not found in any of the samples are DEDTP, FPBA, ADMP, DMADMP, 2,4,5-T, Mecoprop, Fenoprop and Trichlorpyr. Generally, the comparative analyses revealed greater shares as well as higher levels of the parameters in the samples taken during the common diet period (first and second sampling) compared to the organic diet period (third sampling). The parameter levels did not diver between first and second sampling, whereas lower median values were found for almost all parameters in the samples of the organic diet period compared to the second sampling. The differences were statistical significant for DMP, DMTP, DMDTP, DETP, cis-Cl2CA, trans-Cl2CA, PBA, TCPy, glyphosate and AMPA.

Discussion

Former studies on the exposure of individuals of the Japanese general populations exist for the human biomonitoring parameters of organophosphates, e.g. DMP, DMTP, DEP and DETP in urine (Ueyama et al. 2015; Osaka et al. 2016), and pyrethroids, e.g. PBA in urine (Kimata et al. 2009; Osaka et al. 2016), cis- and trans-Cl2CA in urine (Osaka et al. 2016), as well as for the urinary excretion of several neonicotinoids (Ueyama et al. 2015; Osaka et al. 2016). Osaka and coworkers (2016) reported for a study group of 223 three-year-old children (recruited in 2012-2013) median levels of 14.32 µg/L for DMP, 5.45 µg/L for DMTP, 5.27 µg/L for DEP, 0.55 µg/L for DETP, 0.72 µg/L for cis-/trans-Cl2CA and 1.01 µg/L for PBA. Ueyama and coworkers (2005) found in a group of 18 elderly Japanese women (aged 68 ± 5 years), which was recruited in 2011, median levels of 13.8 µg/L for DMP, 8.6 µg/L for DMTP, 6.6 µg/L for DEP and 0.2 µg/L for DETP. Kimata and coworkers (2009) investigated the urinary level of PBA in 619 residents of a rural area of Hokkaido and found a median level of 0.31 µg/L. The parameter levels found in the present study for the conventional diet period are in the same magnitudes as the results of the former studies, which may indicate that during conventional diet the exposure of the two families to organophosphates and pyrethroids was comparable with the Japanese general population. Moreover, the former studies (Osaka et al. 2016; Ueyama et al. 2015) demonstrated considerable exposure only to the neonicotinoid dinotefuran, a representative of this pesticide group which do not metabolize to 6-chloronictotinic acid. Thus, the former study results do also match with the unfrequent and low 6-chlornicotinic acid levels found in the present study. Experiences of the exposure of Japanese individuals to other pesticides do only exist for accidental exposure to glyphosate and MCPA (Hori et al. 2003; Takayasu et al. 2008), which are unsuitable for the verification of the common exposure to pesticides. However, a recent publication reported on the urinary levels of glyphosate and AMPA in young adults of the German general population (Conrad et al. 2017). For the year 2015 they reported medians and maxima of 0.16 and 0.57µg/L, respectively, for glyphosate and < 0.1 and 0.41µg/L, respectively, for AMPA, which indicates a somewhat higher glyphosate exposure of the Japanese general population or at least of the two investigated families compared to the German population.

In the present study, the highest levels were found for human biomonitoring parameter of exposure to organophosphate (DMP, DMTP, DEP, DETP, TCPy, PNP, DEAMP), the carbamate propoxur (2-isopropoxyphenol), pyrethoids (3-phenoxy-

benzoic acid) as well as 1-naphthol and 2-naphthol. During conventional diet DMTP was the most prominent parameter, indicating exposure particularly to organophosphorus compounds of dimethylthiophosphate structure, as chlorpyrifos-methyl and pirimphos-methyl. This result is supported by the high urinary concentrations of TCPy and DEAMP. Moreover, DEP showed considerable urinary levels in the conventional diet period, indicating an exposure to organophosphorus compounds of diethylphosphate structure, as paraoxon. This result fits also with the high levels of para-nitrophenol. Interestingly, the effect of diet switch was low for DEP as well as PNP and did not reach statistical significance, which confirm the connection between the two parameters. In contrast, the effect of diet switch was significant and most pronounced for DMTP and TCPy, which also confirm the connection between these parameters.

Conclusions

The results of the study demonstrate a beneficial effect of switching from conventional diet to organic diet for individuals of the Japanese general population regarding the exposure to pesticides. This effect was most pronounced for parameters of exposure to organophosphate pesticides, pyrethroids and glyphosate. Nevertheless considerable exposure to some pesticides still existed in the individuals in the case of organic food consumption, which may indicate an unreliable protection of the organic food market against pesticide pollution.

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Prof. Dr. rer. nat. Thomas Göen

Principal investigator

Institute and Outpatient Clinic of Occuaptional, Social and Environmental Medicine

Friedrich-Alexander Universität Erlangen-Nürnberg

Schillerstrasse 25

91054 Erlangen (GERMANY)

Tel. +49 / 9131 / 85 26121

Fax. +49 / 9131 / 85 22317

E-Mail: Thomas.Goeen@fau.de

Table 1: Overview on the HBM parameter investigated

Abbreviation or Synonym	IUPAC nomenclature	Parent compounds
Dialkylphosphates		
- DMP	dimethylphosphate	many; e.g. bomyl, crotoxyphos, dichlorvos; naled
- DMTP	O,O-dimethylthiophosphate	many; e.g. chlorpyrifos-methyl, pirimiphos-methyl
- DMDTP	O,O-dimethyldithiophosphate	many; e.g. azinphos-methyl, malathion, phosmet
- DEP	diethylphosphate	many; e.g. chlorfenvinphos, paraoxon, TEPP
- DETP	O,O-diethylthiophosphate	many; e.g. chlorpyrifos, parathion, pirimiphos-ethyl
- DEDTP	O,O-diethyldithiophosphate	many, e.g. azinphos-ethyl, dialifos, disulfoton, ethion
Pyrethroid metabolites		
- Br_2CA	cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxyl acid	deltamethrin
- cis-Cl_2CA	cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxyl acid	ß-cyfluthrin, cypermethrin, permethrin
- trans-Cl_2CA	trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxyl acid	trans-cyfluthrin, cypermethrin, permethrin
- CTFCA	3-(2-chloro-3,3,3-trifluoropropen-1-yl)-2,2-dimethylcyclopropane-carboxyl acid	cyhalothrin
- PBA	3-phenoxybenzoic acid	many; e.g. detamethrin, cypermethrin, permethrin
- FPBA	4-fluoro-3-phenoxybenzoic acid	ß-cyfluthrin
Phenolic metabolites		
- TCPy	3,5,6-trichloro-2-pyridinol	chlorpyrifos, chlorpyrifos-methyl
- PNP	4-nitrophenol	parathion, paraoxon
- IPP	2-isopropoxyphenol	propoxur
- 1NAP	1-naphthol	carbaryl, naphthalene
- 2NAP	2-naphthol	naphthalene
- DMADMP	2-(dimethylamino)-5,6-dimethylpyrimidin-4-ol	pirimicarb
- ADMP	2-amino-5,6-dimethylpyrimidin-4-ol	pirimicarb
- DEAMP	2-(diethylamino)-6-methylpyrimidin-4-ol	pirimiphos-ethyl, pirimiphos-methyl
- THPI	cis-1,2,3,6-tetrahydrophthalimide	tetralin

Table 1 (continued)

Abbreviation or Synonym	IUPAC nomenclature	Parent compounds
6-Chloronicotinic acid (ClNA)	6-chloropyridine-3-carboxylic acid	many, e.g. acetamiprid, imidacloprid, thiacloprid
Phenoxy carboxylic acids		
- MCPA	4-chloro-2-methylphenoxyacetic acid	itself
- 2,4-D	2,4-dichlorophenoxyacetic acid	itself
- 2,4,5-T	2,4,5-trichlorophenoxyacetic acid	itself
- Mecoprop	2-(4-chloro-2-methylphenoxy) propionic acid	itself
- Dichlorprop	2-(2,4-dichlorophenoxy) propionic acid	itself
- Fenoprop	2-(2,4,5-trichlorophenoxy) propionic acid	itself
-Trichlopyr	[(3,5,6-Trichloropyrdin-2-yl)-oxy]acetic acid	itself
Glyphosate and AMPA		
- Glyphosate	N-(phosphonomethyl)glycine	itself
- AMPA	(aminomethyl)phosphonic acid	glyphosate

Table 2: Urinary concentrations (in µg/L) of pesticide biomonitoring parameters separated for the sampling dates (median (min-max; N > LOQ))

Parameter	Conventional diet		Organic diet	Wilcoxon test (p value) [#]	
	First sampling	Second sampling	Third sampling	1st vs 2nd	2nd vs 3rd
Dialkylphosphates					
- DMP	9.43 (3.04 – 14.71; 7)	6.04 (2.94 – 21.24; 7)	1.20 (0.68 – 3.33; 7)	0.866	**0.028**
- DMTP	11.84 (3.61 – 29.20; 7)	14.91 (3.74 – 169.6; 7)	1.18 (0.67 – 3.08; 7)	0.176	**0.018**
- DMDTP	0.17 (< 0.15 – 0.31; 4)	0.24 (0.19 – 3.43; 7)	< 0.15 (0)	0.063	**0.018**
- DEP	3.82 (0.86 – 10.49; 7)	3.30 (1.82 – 20.68; 7)	2.41 (0.95 – 16.95; 7)	0.398	0.150
- DETP	1.08 (< 0.30 – 5.25; 4)	1.40 (0.53 – 14.79; 7)	< 0.30 (0)	0.237	**0.018**
- DEDTP	< 0.03 (0)	< 0.03 (< 0.03 – 0.04; 1)	< 0.03 (0)	0.317	0.317
Pyrethroid metabolites					
- Br_2CA	0.20 (< 0.10 – 0.34; 4)	< 0.10 (< 0.10 – 0.13; 3)	< 0.10 (0)	0.068	0.109
- cis-Cl_2CA	0.37 (< 0.10 – 0.64; 4)	0.14 (< 0.10 – 0.97; 6)	< 0.10 (0)	0.753	**0.028**
- trans-Cl_2CA	0.91 (0.20 – 1.70; 7)	0.44 (0.30 – 1.00; 7)	0.24 (< 0.10 – 0.74; 5)	0.176	**0.043**
- CTFCA	< 0.10 (< 0.10 – 0.26; 1)	< 0.10 (< 0.10 – 0.20; 3)	< 0.10 (0)	0.715	0.109
- PBA	2.16 (0.20 – 6.98; 7)	0.77 (0.28 – 1.78; 7)	0.26 (< 0.10 – 0.61; 5)	0.063	**0.018**
- FPBA	< 0.10 (0)	< 0.10 (0)	< 0.10 (0)	1.000	1.000
Phenolic metabolites					
- TCPy	3.97 (0.73 – 12.89; 7)	1.66 (1.06 – 37.03; 7)	0.57 (< 0.30 – 1.27; 6)	0.866	**0.018**
- PNP	4.78 (2.67 – 20.99; 7)	3.91 (1.63 – 18.54; 7)	3.68 (1.44 – 6.64; 7)	0.237	0.612
- IPP	2.16 (< 0.40 – 6.09; 5)	0.52 (< 0.40 – 3.03.; 4)	< 0.40 (< 0.40 – 0.58; 3)	0.173	0.144
- 1NAP	4.91 (1.54 – 12.74; 7)	4.00 (2.21 – 17.47; 7)	2.83 (1.01 – 4.47; 7)	0.499	0.091
- 2NAP	3.41 (1.31 – 9.17; 7)	2.04 (1.00 – 14.39; 7)	2.25 (0.46 – 3.35; 7)	0.735	0.091
- DMADMP	< 0.30 (0)	< 0.30 (0)	< 0.30 (0)	1.000	1.000
- ADMP	< 0.40 (0)	< 0.40 (0)	< 0.40 (0)	1.000	1.000
- DEAMP	3.34 (0.90 – 3.76; 7)	1.18 (0.78 – 1.80; 7)	0.84 (0.41 – 2.32; 7)	0.063	0.310
- THPI	< 0.30 (< 0.30 - 0.48; 1)	< 0.30 (< 0.30 - 0.30; 2)	< 0.30 (0)	1.000	0.157

[#] t test for difference between the urinary levels at 1st and 2nd sampling and 2nd and 3rd sampling, respectively; p values below 0.05 were highlighted by bold letters.

Table 2 (continued)

Parameter	Conventional diet		Organic diet	Wilcoxon test (p value) [#]	
	First sampling	Second sampling	Third sampling	1st vs 2nd	2nd vs 3rd
6-Chloronicotinic acid	< 0.30 (< 0.30 – 0.43; 1)	< 0.30 (< 0.30 – 0.32; 1)	< 0.30 (0)	0.655	0.317
Chlorinated phenoxy carboxylic acids					
- MCPA	0.28 (< 0.25 - 0.57; 4)	< 0.25 (< 0.25 - 0.30; 2)	< 0.25 (< 0.25 - 0.36; 1)	0.068	1.000
- 2,4-D	< 0.25 (< 0.25 - 0.33; 2)	< 0.25 (0)	< 0.25 (< 0.25 - 0.29; 1)	0.180	0.260
- 2,4,5-T	< 0.25 (0)	< 0.25 (0)	< 0.25 (0)	1.000	1.000
- Mecoprop	< 0.25 (0)	< 0.25 (0)	< 0.25 (0)	1.000	1.000
- Dichlorprop	< 0.25 (< 0.25 – 2.83; 2)	< 0.25 (0)	< 0.25 (0)	0.180	1.000
- Fenoprop	< 0.25 (0)	< 0.25 (0)	< 0.25 (0)	1.000	1.000
-Trichlopyr	< 0.25 (0)	< 0.25 (0)	< 0.25 (0)	1.000	1.000
Glyphosate and AMPA					
- Glyphosate	0.47 (0.10 – 0.74; 7)	0.43 (0.17 – 1.09; 7)	< 0.10 (<0.10 – 0.15; 1)	0.176	**0.018**
- AMPA	0.48 (<0.10 – 1.23; 6)	0.75 (<0.10 – 2.02; 6)	< 0.10 (<0.10 – 0.16; 1)	0.075	**0.028**

[#] t test for difference between the urinary levels at 1st and 2nd sampling and 2nd and 3rd sampling, respectively; p values below 0.05 were highlighted by bold letters.