



Prenatal Exposure to Organophosphate Pesticides in Vojvodina

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Organophosphate pesticides (OPs) are commonly used pesticides which are metabolized and excreted in urine. There are many studies reporting the levels of OP metabolites in urine of pregnant women from different countries, but there is no published data on this problem in Serbia.

Aims: The aim of this study was to determine the level of OP pesticide exposure of pregnant women in the Vojvodina region, Serbia.

Methods: Sixty healthy pregnant women were recruited to participate in this study. A trained interviewer administered a questionnaire to each woman. The concentrations of five

dialkylphosphate metabolites of the organophosphorus pesticides were determined in urine samples that were taken on the third postpartum day. Fifty-eight maternal urine samples were collected.

Results: Approximately two thirds (65.5%) of pregnant women reported living in urban areas and rest living in rural areas. The number of positive urine samples varied from 65.5% for dimethylphosphate (DMP) to 34.5% for diethyldithiophosphate (DEDTP). The mean urine concentrations were highest for DMP (5.714 µg/L cre). Other metabolite concentrations averaged around 1 µg/L cre.

Conclusions: This study confirms prenatal exposure to OP pesticides in the Vojvodina region, Serbia. Levels of dialkylphosphate metabolites in pregnant women from Serbia are comparable with those reported for Caribbean countries and the Palestinian region.

Keywords: Organophosphate pesticides; exposure; pregnancy.

1. INTRODUCTION

A pesticide is defined as “any substance or combination of substances used to prevent or eradicate unwanted insects, including vectors of diseases in human-beings and animals, weeds, fungi, or animals in order to enhance food production and help production, processing, storage, transport or marketing of the food and agricultural commodities” [1].

Organophosphate pesticides (OPs) are the most commonly used pesticides [2]. OPs are absorbed by all routes, including ingestion, inhalation, and dermal absorption and humans are commonly exposed to OPs via ingested food and drink and inhalation of contaminated air, as well as being exposed through occupational contact and proximity to farms [3].

Once in the body, OP pesticides are rapidly metabolized and excreted in urine [4]. Hydrolysis of ester linkages in the parent compounds yields dialkylphosphate metabolites, which are not considered toxic, such as dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DETP), and diethylphosphate (DEP), diethylthiophosphate (DETP) and diethyldithiophosphate (DEDTP).

Several studies have examined the association between prenatal exposure to OPs and a child's health from the time of conception through later on in life. Although some studies did not establish a link between prenatal OP pesticide exposure and postnatal health problems [5], prenatal exposure to these pesticides has been associated with preterm labour and umbilical cord cholinesterase activity, as well as with a decrease in intellectual and social abilities [6-8].

There are many studies reporting on the levels of OP metabolites in urine of pregnant women from

different regions and countries (Jerusalem, Caribbean, China). To the best of our knowledge there is no published data on pesticide exposure of pregnant women in Serbia. Therefore the primary objective of this study was to determine the level of OP pesticide metabolites (DMP, DMTP, DEP, DETP, DEDTP) in urine of women shortly after delivery in Vojvodina, the northern agricultural region of Serbia. Secondary objective of this study was to gain sociodemographic data and to detect exposure to risk factors through interviewed questionnaire.

2. MATERIALS AND METHODS

2.1 Study Participants

From January 2015 to January 2016, 60 healthy pregnant women were recruited to participate in this study from the obstetric ward of the clinic for gynecology and obstetrics, clinical centre of Vojvodina. Eligible women were between 18 and 45 years of age, and reported no gestational or preexisting diabetes, hypertension, human immunodeficiency virus (HIV) infection or acquired immune deficiency syndrome (AIDS), and no use of illegal drugs in the preceding year. Women with multiple pregnancies were excluded from the study as well as women giving birth to babies with severe congenital anomalies after the physical exam of the baby by the pediatrician. All women were fully informed about the study and they have signed a consent form.

The study protocol was approved by the Medical Ethics Committee of the Clinical Center of Vojvodina No. 00-01/306.

2.2 Questionnaire

A trained interviewer administered a brief questionnaire to each woman after delivery.

Information was obtained on occupation, education, reproductive history, smoking habits, housing and diet. Women were specifically asked about presence of pests in their home/garden and use of household/garden pesticide products during pregnancy. In addition, women were asked about frequency of eating specific fruits, vegetables, and additional food items. Additional information on paternal effects such as OP exposure and occupation was also collected through personal interview. Each questionnaire was labelled with number and letter which was later used while analysing urine samples and in further statistical analyses to protect patient privacy.

2.3 Sample Collections

Urine samples were collected from each participant on the third postpartum day. Twenty milliliters of urine specimens were stored at -80°C . Analyses of dialkylphosphate metabolites were conducted at the Department for Pharmacology and Toxicology of Medical Faculty.

2.4 Chemicals and Materials

Dimethylphosphate (DMP) (certified assay: 98.1%) was purchased from AccuStandard Inc. (New Haven, CT). Diethylphosphate (DEP) (98.1%) was obtained from Supelco (Deisenhofen, Germany). O,O-dimethyldithiophosphate (DMDTP) (88.4%) were supplied as their sodium salts by AppliChem (Darmstadt, Germany), O,O-diethyldithiophosphate (DEDTP) ammonium salt (95%), sodium borohydride (98%, pellets), and 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr, 99%) were purchased from Aldrich (Steinheim, Germany). Dibutylphosphate (DBP, 97%) was obtained from Fluka (Deisenhofen, Germany). All other reagents used were of the best analytical grade commercially available with respect to pesticide analysis and supplied by Merck (Darmstadt, Germany). The stock solution of the internal standard (IS) was prepared by dissolving 50 mg dibutylphosphate in 50 mL methanol (1 g/L). This stock solution was diluted with water to yield a concentration of 10 mg/L. The resulting IS solution was used for spiking urine samples during sample preparation. Pool urine for preparation of standard solutions was collected from laboratory personnel without known exposure to organophosphates. It was stored at -18°C . After thawing and mixing it was filtered once before usage.

2.5 Sample Preparation

The urine samples were collected in polypropylene bottles and stored at -18°C until sample preparation was carried out. After thawing and mixing, 5 mL of urine was pipetted into a screw-top vial that already contained 4 g sodium chloride and was spiked with 100 μL IS solution (10 mg/L DBP). Five milliliters of diethylether/acetonitrile (1+1, v/v) was added, and the sample was acidified with 1 mL hydrochloric acid (6 mol/L). After being shaken in a laboratory shaker for 5 min, the vial was centrifuged for 5 min at 1500 x g. The organic phase was transferred into a new screw-top vial, which contained 10 mg potassium carbonate.

Afterwards, the extraction was repeated with 5 mL diethylether/acetonitrile (1+1, v/v), and the second extract was added to the first. The vials with the combined organic phases were sealed and mixed thoroughly on a vortex mixer. The solution was evaporated to dryness in a gentle stream of nitrogen. 750 μL acetonitrile were added to the residue and evaporated to dryness again in a gentle stream of nitrogen. The dry residue was suspended in 1.5 mL acetonitrile. After adding 5 mg potassium carbonate and 150 μL solution of PFBBr in acetonitrile (1+2, v/v) derivatization was performed in sealed vials in an oven at 40°C overnight (15 h). After cooling to ambient temperature, 5 mL n-hexane and 5 mL water were added, and the pentafluorobenzyl esters were extracted by shaking for 5 min and centrifuging for 5 min at 1500 x g. The extraction was repeated with 5 mL n-hexane. The combined hexane phases were concentrated to approximately 1 mL using a gentle stream of nitrogen. Toluene (200 μL) was added, and the sample was transferred to a smaller vial and concentrated to a final volume of 150 μL for subsequent analysis by GC-MS [9,10].

2.6 GC-MS Conditions

Analysis was performed on an Agilent 7890A GC equipped with a split-splitless injector and an Agilent 7673A automatic liquid injection system and fitted with an 5975C VL MSD (Agilent Technologies, CA, USA) mass selective detector (MSD, quadrupole). The GC operating conditions were as follows: GC column, DB-5MS (J&W, CA, USA), 30 m \times 0.25 mm i.d., 0.25 μm film thickness; column temperatures, 70°C (1 min) and $5^{\circ}\text{C}/\text{min}$ to 220°C (0 min) and $15^{\circ}\text{C}/\text{min}$ to 280°C (5 min); injection port temperature, 250°C ; carrier gas, helium (99.9999 % purity); flow

rate, 1 mL/min; injection pressure, 82 kPa. The injection volume was 1 μ L splitless. The MS operating conditions were as follows: ionization source temperature, 250° C; electron ionization, 70 eV; interface temperature, 300° C. The carrier gas was helium (99.9999 % purity), inlet pressure: 143 kPa, splitless injection of 1 μ L. MS conditions were as follows: ionization by electron impact (70 eV); multiplier 2300 V; selected ion monitoring (SIM) mode.

Detected Masses of the Analytes

PFB-esters of the substances	Detected masses (m/z)
DMP	306(target), 307(qual.), 194(qual.)
DEP	334(target), 278(qual.), 258(qual.)
DETP	350(target), 274(qual.), 169(qual.)
DMDTP	338(target), 157(qual.)
DEDTP	366(target), 185(qual.), 157(qual.)
DBP (ISTD)	335(target), 279(qual.)

2.6.1 Calibration and quality assurance

Six starting solutions of the metabolites DMP, DEP, DETP, DMDTP, and DEDTP were prepared by dissolving 25 mg of each compound in 25 mL methanol using six separate flasks (1 g/L). As different salts were used as reference standard compounds, the concentrations had to be related to the molecular masses of the diesters in acid form. One milliliter of each starting solution was pipetted into one 100-mL flask, and water was added to the mark resulting in one stock solution containing all the six analytes in a concentration of 10 mg/L each. Eight calibration standards with spiked concentrations in the range from 0 to 500 pg/L were prepared from these stock solutions by diluting with pool urine. The calibration standards were stable at -18° C for at least 12 months. The urinary standards were subjected to the same treatment as the urine samples. Linear calibration curves were obtained by plotting the quotients of the peak areas of the metabolites (molecular ions) and the IS (m/z 335) as a function of the concentrations. The coefficients of correlation were higher than 0.995 for all analytes. For quality assurance, two different control samples were included in each analytical series.

2.7 Limit of Detection (LOD)

The limit of detection defined as three times the signal-to-noise ratio concerning the molecular ions was 3 μ g/L for DMP; 0.5 μ g/L for DEP; 1 μ g/L for DETP, 0.8 μ g/L for DMDTP, and 1 μ g/L for DEDTP.

3. RESULTS

From January 2015 to January 2016, 58 maternal urine samples were collected from pregnant women. The mean age of enrolled women 29.66 years (range 18–41) and the mean duration of gestation at recruitment was 39.28 (range 36-42). High school education was reported by 35 women (60.34%), while 23 women reported higher (bachelor's or higher) degree. Approximately two thirds (65.50%) of pregnant women reported living in urban areas, while the remaining number reported to be living in rural areas (villages, near agricultural fields). Most of the women reported to be employed and working during pregnancy. In 16 of the interviewed pregnant women, positive indirect exposure to pesticides was reported, while 2 women were using pesticides by themselves during pregnancy. The demographic characteristics of pregnant women enrolled in the study are presented in Table 1.

Table 1. Demographic characteristics of pregnant women enrolled in the study

Age (mean+SD; years)	29.66+5.44
BW (mean+SD; kg)	75.35+13.55
Smoking before pregnancy (%)	43.1
Smoking during pregnancy (%)	29.31

3.1 Urinary OP Pesticide Concentrations

The number of positive urine samples (OP metabolite concentrations above LOQ) varied from 65.50% for DMP to 34.50% for DEDTP.

The mean urine concentrations were highest for DMP (5.714 μ g/L cre). Other metabolite concentrations averaged around 1 μ g/L cre (Table 2).

4. DISCUSSION

The results of our study suggest that women giving birth in Vojvodina are exposed to organophosphate pesticides. It should be of no surprise since Vojvodina is an agricultural region.

In this assessment, we assumed that 100% of the absorbed maternal OP pesticides dose is expressed in urine as diethyl and dimethyl phosphate metabolites. Although the kinetics of elimination vary among the dimethyl and diethyl phosphate metabolites, toxicologic evidence suggest that the metabolites of many OP

Table 2. OP metabolite concentrations

Metabolite	%>LOQ	Mean	SE	Min	Median	90 th percentile	95 th percentile	Max
DMP (µg/L)	65.5	7.509	0.971	0	5.640	16.613	19.679	32.57
DMP (µg/L cre)	65.5	5.714	0.810	0	4.640	13.278	17.866	30.625
DEP (µg/L)	53.4	0.496	0.072	0	0.535	1.362	1.655	1.760
DEP (µg/L cre)	53.4	0.415	0.063	0	0.342	1.105	1.289	2.066
DMDTP (µg/L)	56.9	0.566	0.073	0	0.680	1.255	1.353	2.070
DMDTP (µg/L cre)	56.9	0.469	0.071	0	0.476	1.176	1.751	2.393
DETP (µg/L)	43.1	0.569	0.091	0	0	1.478	1.893	2.360
DETP (µg/L cre)	43.1	0.401	0.065	0	0	1.034	1.353	1.562
DEDTP (µg/L)	34.5	0.439	0.082	0	0	1.426	1.571	1.810
DEDTP (µg/L cre)	34.5	0.284	0.053	0	0	0.867	0.983	1.204

compounds are excreted primarily, but not exclusively in the urine [11,12]. Furthermore, total OP pesticides exposure may be underestimated because several OP pesticides, such as acephate, do not metabolize to any of the urinary dialkylphosphate metabolites and are therefore not included in our exposure-dose estimate. These OP pesticides, which do not devolve into dialkylphosphate metabolites, represent approximately 20% of total OP pesticide use [13].

While direct exposure to dialkylphosphate metabolites is possible, they are primarily considered to be biomarkers of recent OP pesticide exposure [14]. In an attempt to design a study in which we could assess the extent of fetal exposure to OP pesticides we considered the fact that a single urine sample may not adequately reflect the overall extent of the exposure. There are authors who found a high degree of within person variability, high enough to present a challenge for designing well powered epidemiological studies [15]. Since in the above-mentioned study samples were taken at intervals of at least seven weeks apart, it is arguable if the seasonal variations in the level of pesticides are responsible for the change in the level of pesticides. Another important factor that must be taken into consideration is the fact that a single interview regarding the use of pesticides increased awareness of the patients that also inevitably affects the results [16].

There are studies showing that metabolite levels from a single urine sample taken from each subject reflect that subject's exposure over several months. Although nondifferential exposure misclassification is likely to occur to some extent, a single measure may adequately represent true average exposure over a longer period of time [17]. Therefore we decided to use a single urine sample, and to compare our

results with the results of the studies with a similar epidemiological design.

The results of the current study show that pregnant women in the Novi Sad, Vojvodina region are exposed to OP pesticides during pregnancy. When compared to exposure of Palestinian women [18], the median metabolite levels are similar or lower compared to concentrations measured in Palestinian women. However the 95th percentile values and maximum values of metabolites are much lower in most of the metabolites in pregnant women from Vojvodina (Table 3).

Some other studies report lower exposure of pregnant women. In the study by Forde et al., 2015 published data on OP metabolite concentration show lower median values for DMP, but higher for DEP compared to concentrations determined in the urine of pregnant women in Vojvodina (Tables 4 and 5).

DMDTP was not detected in nine of the sampled Caribbean countries with this OP metabolite being detected in 60% of the samples from Bermuda with a geometric mean of 0.42 mg/L. All of the Caribbean samples tested below the LOD for the OP metabolite DEDTP.

Urine samples of pregnant women in Novi Sad showed lower concentrations of DEP and DETP while the level of DMDTP was almost the same as in study conducted in Palestine [18]. Interestingly, the level of DEDTP was higher in the urine samples of pregnant women in Serbia than in Palestine. An important difference in exposure is the fact that the percent of samples positive to the presence of OP metabolites was lower in Vojvodina compared to Palestine, although LOQ in our study was lower for all of the analyzed metabolites [18].

Table 3. Comparison of results obtained in Vojvodina and Palestine*

Metabolite	Region	%>LOQ	Geometric mean	Percentile		
				50th	95th	Maximum
DMP (µg/L)	NS	65.5	5.714*	4.640	17.866	30.625
	PA	89.7	3.7	4.9	41.5	203.3
DMTP (µg/L)	NS	-	-	-	-	-
	PA	97.2	8.6	8.6	84.9	1523.1
DMDTP (µg/L)	NS	56.9	0.469*	0.476	1.751	2.393
	PA	69	0.4	0.4	9.3	314.3
DEP (µg/L)	NS	53.4	0.415*	0.342	1.289	2.066
	PA	98.6	2.9	2.70	37.9	205.6
DETP (µg/L)	NS	43.1	0.401*	0	1.353	1.562
	PA	71.7	0.9	0.8	12.7	52.1
DEDTP (µg/L)	NS	34.5	0.284*	0	0.983	1.204
	PA	42.8	0.02	0.01	0.2	2.8

* Abdeen Z, Berman T, Azmi K, Abu Seir R, Agha H, Ein-Mor E, et al. Urinary organophosphate metabolite levels in Palestinian pregnant women: results of the Middle East Regional Cooperation Project. *Int J Environ Health Res* 2016;26:254-66.

Table 4. Comparison of values of DEP obtained in Vojvodina and Caribbean Islands**

Region	Diethylphosphate (DEP) (µg/L)				
	Geometric mean	Median	90th	Minimum	Maximum
Serbia – Novi Sad	0.496*	0.535	1.362	0	1.760
Antigua and Barbuda	4.47	4.30	16.00	0.50	17.00
Belize		1.40	19.00	0.50	27.00
Bermuda	1.95	2.30	5.40	0.50	9.10
Dominica	1.31	1.10	4.50	0.50	5.40
Grenada	1.72	1.40	3.90	0.50	9.10
Jamaica	2.07	1.50	14.00	0.50	29.00
Montserrat			3.10	0.50	6.60
St Lucia		1.30	3.90	0.50	5.40
St Kitts and Nevis	2.52	2.00	6.40	0.50	41.00
St Vincent and the Grenadines			6.00	0.50	8.00
Caribbean islands	1.65	1.50	7.20	0.50	10.00

*arithmetic mean; ** Forde M, Robertson L, Laouan Sidi E, Cote S, Gaudreau E, Drescher O et al. 2015. Evaluation of exposure to; organophosphate, carbamate, phenoxy acid, and chlorphenol pesticides in pregnant women from 10 Caribbean; countries. *Environ Sci Process Impacts*.17:1661-71.

Table 5. Comparison of values of DMP obtained in Vojvodina and Caribbean Islands**

Region	Dimethylphosphate (DMP) (µg/L)				
	Geometric mean	Median	90th	Minimum	Maximum
Serbia – Novi Sad	7.509*	5.640	16.61	0	32.57
Antigua and Barbuda	3.30	3.00	14.00	0.50	20.00
Belize			2.00	0.50	2.60
Bermuda	3.84	4.60	11.00	0.50	11.00
Dominica		1.00	8.90	0.50	12.00
Grenada	1.29	1.20	4.50	0.50	13.00
Jamaica			2.60	0.50	7.50
Montserrat			14.00	0.50	34.00
St Lucia	1.28	1.00	5.90	0.50	12.00
St Kitts and Nevis	3.17	2.40	29.00	0.50	49.00
St Vincent and the Grenadines	1.51	1.40	7.70	0.50	11.00
Caribbean islands	1.60	1.40	11.00	0.50	49.00

*arithmetic mean; ** Forde M, Robertson L, Laouan Sidi E, Cote S, Gaudreau E, Drescher O et al. 2015. Evaluation of exposure to; organophosphate, carbamate, phenoxy acid, and chlorphenol pesticides in pregnant women from 10 Caribbean; countries. *Environ Sci Process Impacts*.17:1661-71.

When comparing our results with the results of the Caribbean islands [19], the geometric mean and median of the DMP were higher in Vojvodina than in all of the Caribbean islands, while DEP was less commonly detected in Serbia than in the Caribbean islands. The level of DETP showed similar results in these two studies, but with a difference between different regions. Therefore, pregnant women in St. Vincent and the Grenadines showed a lower median level of DETP than in Vojvodina, while higher median concentrations of DETP were detected in Jamaica compared to Vojvodina [19].

We examined the registered OP pesticides in Vojvodina and we found that the OP pesticides most commonly used are those containing the following active substances: dimethoate, chlorpyrifos, malathion and pirimiphos-methyl. Although dialkylphosphate metabolites are not specific biomarkers, it is possible to assume the most probable source (parent pesticide) of a specific metabolite [20]. Seven products containing dimethoate are currently registered in Vojvodina, and dimethoate is the most probable source of DMDTP. Chlorpyrifos, the active ingredient in 16 of the registered products, is the most probable precursor of DEP. Malathion can be found in 4 registered pesticides, and is the probable precursor of DMP [19]. The most concerning conclusion is that DETP, which was also determined in relatively high levels can be assigned as a metabolite of highly toxic and banned OPs such as Diazinon and Parathion [20].

5. CONCLUSION

This study on the concentrations of OP pesticide metabolites in maternal urine samples confirms prenatal exposure to these pesticides in Vojvodina region, Serbia. In general, levels of dialkylphosphate metabolites in mothers shortly after delivery from Serbia are comparable with those reported for Caribbean countries and Palestinian region. Since data on pesticide exposure in Serbia are scarce, results of this study are good starting point for future biomonitoring studies in this region. Use of pesticide in agricultural regions is inevitable, but data on pesticide use and exposure can be useful in improving the use of pesticides. By that manner potential health consequences can be prevented, especially in vulnerable population such are pregnant women.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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INFORMATION FOR THE PARTICIPANTS

Dear Ms.,

You have been kindly asked to participate in the study which investigates the exposure of pregnant women to pesticides and related substances on the territory of Vojvodina, Serbia and the impact of these substances on the development of the fetus and newborn. Pesticides and related substances affect the development of a number of organs, and above all, can damage reproductive organs and nervous system which can lead to consequences in the form of infertility and hindered the development of the child.

On our clinic we are conducting a study that will track pregnant women's exposure to pesticides. You have to be aged 18 to 45 to be included in this study.

After talking with a doctor about testing, if you choose to participate in a study you will sign the consent, and your responsibility would be to, while in the hospital, fill in a questionnaire about habits (questions related to cigarette smoking, intake of coffee and other beverages containing caffeine etc.) and give a sample of urine after delivery. You will be asked to give 100ml of urine which will be used for pesticides analysis. In addition to the aforementioned requirements we might need to use your medical records during this hospitalization.

You are not obligated to participate in this study and that is your free decision. If you do not participate, your decision will not affect any current or future medical care that your doctor can provide.

If you agree to participate in a study, you can withdraw from it at any time, without giving any explanation.

Your right is to request any additional information before you make a decision about joining the study. If you do not want to access this study (or in the case to agree, and that over time would give up) you will not bear any consequences and your doctor will continue to treat you in the usual way.

Your name and surname, as well as all information about your participation in a study are confidential and remain a secret.

In case you agree with participation in the study, you will receive a form, which you will sign.

Addendum No. 2

CONSENT

I'm familiar with medical and scientific significance of study about exposure of pregnant women to pesticides in the territory of Vojvodina, Serbia and the impact of these substances on the development of the newborn. I'm pleased with the answers to my questions about the study plan and its execution. I do not expect any material gain from this study and I can withdraw from the study at any time with no fear for my future medical treatment. I have read the attached information about the study, I fully understand the terms of my participation and I voluntarily decided to participate in the study.

THE SIGNATURE OF THE SUBJECT

SIGNATURE OF WITNESS

I confirm that I have explained to the participant the nature, purpose and possible risks of this study and all the specified tests:

SIGNATURE OF EXAMINER

DATE

Annex No. 3

THE QUESTIONNAIRE

Dear Ms.,

You have been asked to join a study about exposure of pregnant women to pesticides and related substances on the territory of Vojvodina, Serbia and the impact of these substances on the development of the fetus and newborn. You do not have to participate in this study and that is your free decision. If you agree to participate in the study, please answer the questions that follow. Your name and surname, as well as all information about your participation in the survey are confidential and remain a secret. The data obtained by this questionnaire will be used solely for scientific purposes.

1. How old are you?

2. Do you live in the city or in the village?

- 1) City
- 2) Village

3. Do you live in a house or apartment?

- 1) House
- 2) Apartment

4. What is your profession?

5. Where do you work?

6. Does anyone smoke in your working place?

- 1) YES
- 2) NO

7. Are pesticides used in your workplace?

- 1) YES
- 2) NO

8. If used, what kind of pesticides (it is possible to round up more of the responses).

- 1) Acaricides (agents for suppression of mites),
- 2) Herbicides (agents for weed control)
- 3) Fungicides (agents for suppression of fungus),
- 4) Insecticides (agents for controlling harmful insects),
- 5) Rodenticides (agents for suppression of harmful rodents)
- 6) Other: _____(please state).

9. Have you been smoker before pregnancy?

- 1) YES
- 2) NO

10. If you answered YES to previous question, how many cigarettes did you smoke a day?

- 1) 1-4,
- 2) 5-9,
- 3) 10-14,
- 4) 15-19,
- 5) 20 and more.

11. Have you been smoking during the pregnancy?

- 1) YES
- 2) NO

12. If your answer to the previous answer is YES, how many cigarettes did you smoke a day during pregnancy?

- 1) 1-4 cigarettes,
- 2) 5-9 cigarettes,
- 3) 10-14 cigarettes,
- 4) 15-19 cigarettes,
- 5) 20 or more cigarettes.

13. Are there smokers in your home (before and/or during pregnancy)?

- 1) YES
- 2) NO

14. If there are, how many cigarettes they smoke per day?

- 1) 1-4 cigarettes,
- 2) 5-9 cigarettes,
- 3) 10-14 cigarettes,
- 4) 15-19 cigarettes,
- 5) 20 or more cigarettes.

15. Did you drink alcohol for three months before and/or during pregnancy?

	every day	Once a week	More than once a week	Once per month	In rare circumstances	Never
	1	2	3	4	5	6
a. beer						
b. wine						
c. strong drinks						

16. Have you been drinking coffee during and a month before pregnancy?

- 1) YES
- 2) NO

17. Did you drink a coke or pepsi during pregnancy or in the month before pregnancy?

- 1) YES
- 2) NO

18. Did you take any medication during pregnancy and six months before?

- 1) YES
- 2) NO

19. If you answered YES to the previous question, did you take any of these medications during pregnancy or six months before pregnancy:

	No	Yes, once	Yes, more than once
	1	2	3
a. Headache			
b. Abdominal pain			
c. Sleeping problems			
d. Anxiety			
e. Vomiting			
f. _____ (other, please state)			

20. Have you been in contact with any kind of pesticides during pregnancy (personally, anyone in your home, someone from deration)?

- 1) YES
- 2) NO

21. If yes, in which form did you use the pesticides (it is possible to choose multiple answers)?

- 1) Spray
- 2) Tape
- 3) Glue
- 4) Other: _____ (please state which)

22. Which type of pesticides did you use (it is possible to choose multiple answers)?

- 1) Acaricides (agents for suppression of mites),
- 2) Herbicides (agents for weed control)
- 3) Fungicides (agents for suppression of fungus),
- 4) Insecticides (agents for controlling harmful insects),
- 5) Rodenticides (agents for suppression of harmful rodents)
- 6) Other: _____ (please state which).

23. How often did you eat fruit during pregnancy?

- 1) Every day,
- 2) 4-6 times a week,
- 3) 1-3 times a week,
- 4) 1-4 times per month,
- 5) Less than once per month,
- 6) I did not eat fruit during pregnancy.

24. What kind of fruit did you eat most during pregnancy?

- 1) Apples,
- 2) Oranges, lemons, kiwi,
- 3) Peaches, apricots
- 4) Strawberries, raspberries, blackberries,
- 5) Watermelon, melon
- 6) Other: _____ (specify).

25. Where do you buy your fruit?

- 1) In the market,
- 2) In the supermarket,
- 3) From my own gardens,
- 4) Local small shops,
- 5) Other: _____ (specify).

26. Do you have pets?

- 1) YES
- 2) NO

27. If you answered YES to the previous question please state which pet do you have?

Thank you for your time.

If you believe that there is any other important information that is not mentioned in this questionnaire or if you have any suggestions please state those here:

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