

# Cholinesterase activity in blood and pesticide presence in sweat as biomarkers of children's environmental exposure to crop protection chemicals

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## Abstract

**Introduction.** On the contrary to the adult population exposed to pesticides, mostly on occupational basis, rural children are mostly exposed to pesticides deposited in the environment. However, even this constant, distributed in time exposure to low concentrations of pesticides may lead to permanent health disorders and limit children's harmonious development.

**Objective.** The main objective of the study was to evaluate the usefulness of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity determination as a marker of children's environmental exposure to pesticides. An additional aim was to evaluate the usefulness of sweat patches as a novel, non-invasive method of detection of pesticides in sweat as a measure of pesticide exposure.

**Materials and method.** A total of 108 children living in areas of intense pesticide use, and as a control group, 92 children living in an agro-tourist area were enrolled in the study. The AChE and BuChE activity was assayed colorimetrically in diluted whole blood or plasma, respectively. In addition, selected pesticides were measured by GC/MS analysis in samples of the subject's sweat absorbed onto a sorbent.

**Results.** The study demonstrated significantly lower AChE and BuChE activity, respectively, in the diluted whole blood and plasma of children exposed to pesticides, compared to the control group ( $p < 0.001$  and  $p = 0.003$ , respectively). The measured mean level of AChE activity was  $241.63 \pm 26.76$  and  $348.0 \pm 46.95$  mU/ $\mu$ molHb in the exposed and the control group, respectively, whereas the mean activity of BuChE was  $424.1 \pm 81.1$  and  $458.6 \pm 86.5$  mmol/L/min. In addition, pesticide metabolites were detected in 19 (17.6%) sweat samples collected from exposed children.

**Conclusions.** Altogether, the study indicated that cholinesterase activity is a sensitive marker of the children's environmental exposure to pesticides, whereas sweat patches are useful devices for collecting samples to be analysed for the presence of the pesticides.

## Key words

pesticides, environmental exposure, children, biomarkers, esterase activity, sweat patch

## INTRODUCTION

Intensive crop cultivation requires seasonal applications of plant protection products – pesticides. The migration and persistence of pesticides in the local environment results in the unintentional exposure of biota inhabiting the surrounding areas. Exposure to environmental pesticides leads to complex, long-lasting adverse effects on human health through multifactorial chemical toxicity, and thereby poses a substantial risk to those living in areas devoted to agriculture; the most vulnerable being children and adolescents [1, 2]. Even exposure to low concentrations of pesticides may lead to chronic health problems and developmental disorders if the exposure is constant and long-lasting. Many cases of poisoning go unreported, the

adverse effects of pesticides therefore constitute a more serious health threat than is recognised. Long-term and unrecognized pesticide exposure in childhood can result in the delay of appropriate diagnosis, preventative and/or remedial treatment [3].

A given child's exposure to pesticides, in fact, starts during pre-natal development, when pesticides and/or their metabolites cross the placental barrier and adversely affect foetal development leading to premature birth and low neonatal body mass. Neonatal and infant metabolism substantially differs from that of adults. It is known that children more readily absorb toxins via the respiratory and gastrointestinal systems than adults, and accumulated xenobiotics are more slowly removed despite higher metabolic rates in children. Thus, children constitute a population group that is particularly vulnerable to the environmental exposure to pesticides. Therefore, it is advisable to evaluate the effect of environmental exposure to pesticides in children living in areas of intensive use of plant protection products [3].

The most common types of insecticides used in crop

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protection, such as organophosphorous compounds (OPs) and carbamates, are cholinesterase inhibitors, including human acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). AChE enzyme is present in human red blood cells (RBCs), nervous tissues and skeletal muscles, while BuChE is synthesized in the liver and is present in serum [4]. Thus, changes in the enzymatic activity of these proteins due to exposure to pesticides may be used as an internal marker of exposure.

In occupational exposure assessment, it is usually recommended to assess the presence of pesticides and/or their metabolites in urine or blood samples. However, for assessment of environmental exposure, when doses of xenobiotics are relatively low, this sources of biological material are not recommended. It is therefore important to find another biological material enabling assessment of biomarkers of environmental exposure with better accuracy.

## OBJECTIVE

The main objective of the study was to evaluate the usefulness of the measurement of changes in AChE and BuChE activity as a biomarker of children's environmental exposure to pesticides. The study also exploited non-invasive sweat patches as a new device to collect sweat-excreted pesticide metabolites as a biomarker of children's environmental exposure to crop protection products. Currently, only a few studies have been published in which sweat patches were used to assess exposure to xenobiotics, mainly to demonstrate drugs consumption [5, 6].

## MATERIALS AND METHOD

The study was performed on 108 children aged 8–12, living in areas of intense use of pesticides for crops protection, and a matched control group of 92 children, aged 8–12, living in an area of agro-tourism. Detailed characteristics of the studied populations is given in Table 1. A detailed questionnaire containing information on socio-demographic characteristics, diet and residential use of pesticides in each family was completed by the children's parents.

**Table 1.** Characteristics of the studied population

	n	Weight [kg] (mean±SD)	Age (years)
Exposed group	108: 59 girls, 49 boys	38.95±11.76	10.78±1.55
Control group	92: 45 girls, 47 boys	38.74±11.01	10.59±1.63
Total	200 (104 girls and 96 boys)		

Blood samples (2 ml) were collected into sodium–heparin Vacutainers, delivered to the laboratory within 4 h and processed immediately. Sweat was collected with Pharmchek® sweat patches (PharmChem, Inc., Haltom City, Texas, USA). After cleaning the skin with isopropyl alcohol and drying, the patches were applied to the upper arm of each child. Sweat patches were worn for 5 days. Only patches that showed no signs of skin tampering were included in the study. After removal, the patches were stored at -20 °C until analysis.

The study was approved by the Bioethics Committee of the Institute of Rural Health in Lublin, Poland (Decision

No. 19/2008 of 21.10.2008). Parents of the children were fully informed about the purpose and scope of the research, and gave written consent for participation of their children in the study, in accordance with the ethical principles and organization of the research.

**Analysis of sweat-extraction procedure.** For the extraction of the analytes, the absorption part of the sorbent containing sweat was separated and extracted twice with 15 ml, and once with 10 ml of diethyl ether on a rotary shaker – 5 min each time, left for 30 min at room temperature and then centrifuged at 3,000 rpm for 5 min. After centrifugation, the organic layers were combined into the glass core. The extract was evaporated under reduced pressure, and the dry residue diluted in 2 ml of cyclohexane and analyzed by the gas chromatography–mass spectrometry (GC/MS) method.

**GC/MS analysis.** Analysis was performed using an Agilent 5975C inert XL EI/CI MSD with Triple Axis Detector gas chromatograph (Agilent Technologies, Santa Clara, California, USA). Chromatographic separation was achieved with a Agilent HP-5MS capillary column, 30 m in length, internal diameter 0.25 mm and 0.25 µm. Purified nitrogen was used as the carrier gas when MS detector was used or mixture of nitrogen and methane when a CI detector was used. The choice of temperature conditions throughout the analysis was dependent on the active substances which were measured.

**Determination of AChE and BuChE activity.** The activity of AChE and BuChE was determined using the Ellman's method with the Worek's modification [7]. All blood samples were prepared from freshly-drawn heparinized venous blood. In brief, 200 µL of blood was collected and dissolved in 20 mL of cold diluting reagent, then gently mixed and immediately frozen at -20 °C. To obtain plasma samples, blood samples were centrifuged at 500 ×g for 10 min, divided into 1 ml aliquots and frozen at -20 °C. Prior to analysis, the blood samples were thawed and stored on ice. Prior to the addition of enzyme's substrate, the samples were incubated for 10 min at 37 °C to stabilize their temperature, and to allow completion of the reaction of DTNB with sulfhydryl groups (-5,5'-dithio-bis- 2-nitrobenzoic acid, Ellman's reagent). Enzyme activity was subsequently corrected for the spontaneous hydrolysis of the substrate and degradation of DTNB (blank control). Enzyme activity (AChE, BuChE) was calculated as follows:

$$\begin{aligned} \text{Abs} &= \epsilon \times L \times c \\ c &= \Delta \text{Abs} / \epsilon \times L \\ \epsilon &= 10.6 \times 10^3 \times \text{M}^{-1} \times \text{cm}^{-1} \\ L &= 0.696 \text{ cm} \end{aligned}$$

The enzyme activity =  $\Delta \text{Abs} - \Delta \text{Abs}_b / 10.6 \times 0.696$ . The result was multiplied by 1,000 and divided by 316 (coefficient calculated adequately to the volume of the mixture in the hole). The AChE activity was standardized for haemoglobin content, read from morphological analysis performed for each sample.

**Statistical analysis.** Data are shown as arithmetic means and standard deviations. Statistical comparisons were made between the various groups using the non-parametric Mann-Whitney *U* test or the Student's *t* test. Test results were considered statistically significant for the *p*-value <0.05. All

statistical tests were performed using STATISTICA ver. 8.0 (StatSoft).

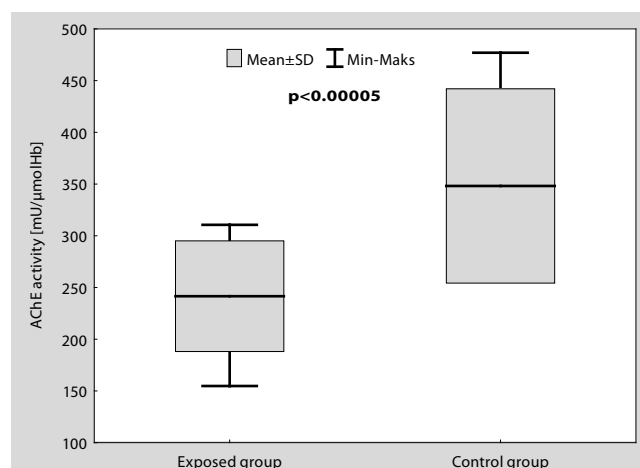
## RESULTS

**Sweat analysis.** Sweat analysis confirmed the information given in the questionnaires completed by the parents of the group of children exposed to pesticides. The presence of selected pesticides was demonstrated in 19 sweat sorbents (Tab. 2) from the group of children exposed to plant protection products. No pesticides were detected in the sweat of children from the control group.

**Table 2.** Characteristics of pesticides detected in sweat sorbents

Pesticide	CAS No.	Type of pesticide	No. of positive samples
1 Carbetamide, [1-(ethylamino)-1-oxopropan-2-yl] N-phenyl-carbamate	16118-49-3	herbicide	4
2 Carbofuran, (2,2-dimethyl-3H-1-benzofuran-7-yl) N-methylcarbamate	1563-66-2	insecticide	2
3 Chloridazon, 5-amino-4-chloro-2-phenylpyridazin-3-one	1698-60-8	herbicide	1
4 Dodemorph, 4-cyclododecyl-2,6-dimethylmorpholine	1593-77-7	fungicide	6
5 Cyclopropanecarboxamide, N-(5-(2-Methoxypyridinyl)) cyclopropane-carboxamide	112860-04-5	fungicide	3
6 Permethrin, [3-(phenoxy)phenyl]methyl (1R,3R)-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate	52645-53-1	insecticide	3

**Cholinesterase activity.** The activity of AChE and BuChE proved to be a valuable biomarker of environmental exposure to selected pesticides. The study demonstrated a significantly lower activity of AChE and BuChE in children exposed to the pesticides, compared to controls. The mean activity of AChE was  $241.63 \pm 26.76$  and  $348.21 \pm 46.95$  mU/ $\mu$ molHb ( $p < 0.00005$ ) in the exposed and control group, respectively (Fig. 1). Similar differences were observed between these 2 groups, when divided according to gender, but no differences were found between the genders (Tab. 3).

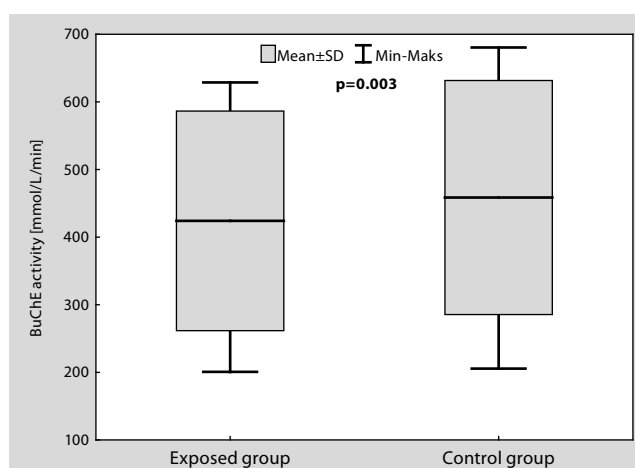


**Figure 1.** Average AChE activity in blood samples of exposed and control groups

**Table 3.** Influence of gender on activity of AChE enzyme in the studied populations

Sex	AChE activity (mU/ $\mu$ molHb), mean $\pm$ SD				p
	n	exposed	n	control	
Boys	49	243.40 $\pm$ 28.17	47	349.59 $\pm$ 50.19	<0.001
Girls	59	240.02 $\pm$ 25.52	45	346.91 $\pm$ 44.29	<0.001
p		0.956		0.989	
TOTAL	200		282.13 $\pm$ 62.95		

Similarly, BuChE activity showed a significant decrease in the exposed group, compared to the control group. The mean activity of BuChE was, respectively,  $424.1 \pm 81.2$  and  $458.6 \pm 86.5$  mmol/L/min ( $p = 0.003$ ) (Fig. 2); however, this difference was not maintained when the studied populations were differentiated by gender (Tab. 4).



**Figure 2.** Average BuChE activity in plasma samples of exposed and control groups

**Table 4.** Influence of gender on the activity of the BuChE enzyme in the studied populations

Sex	BuChE activity (mmol/L/min), mean $\pm$ SD				p
	n	exposed	n	control	
Boys	49	423.9 $\pm$ 77.9	47	470.6 $\pm$ 94.1	0.076
Girls	59	424.2 $\pm$ 84.6	45	447.3 $\pm$ 78.2	0.613
p		0.999		0.624	
TOTAL	200		437.2 $\pm$ 84.7		

## DISCUSSION

Biomarkers are commonly used to confirm human exposure to various hazardous substances [8]. The most widely used biomarkers include determination of the presence of xenobiotic metabolites, measurements of the activity of specific enzymes, and measurements of damage to the genetic material or changes in gene expression.

Organophosphorus compounds are the most commonly used pesticide products in agriculture [9]. All organophosphorus compounds share a chemical structure based on a phosphorus atom bound with oxygen or sulfur. Their toxicity depends largely on their ability to phosphorylate cholinesterase type enzymes that leads to the enzyme inhibition [10]. Carbamates are also widely used

for pest control, and also act primarily by inhibition of insect and mammalian acetylcholinesterases. Thus, decreased cholinesterase activity is commonly used as a biomarker of the occupational and environmental exposure to OPs and carbamates. This includes measurement of blood cell AChE and plasma BuChE activities. The advantages of these biomarkers include, among others, relatively easy measurement, sensitivity, and dependence on the pollutant exposure [11]. On the other hand, the method is relatively invasive and may cause discomfort, i.e. bruises. Furthermore, in the case of low level chronic exposure, the method is not fully consistent with the actual exposure. Therefore, an alternative or complementary method, e.g. measuring pesticide metabolites in urine, is often used to assure full reliability [12].

The results of the presented study show a significant reduction of AChE and BuChE activity in children exposed to pesticides in comparison to the control group. Similar findings were previously reported in a study performed on 134 farmers and 134 control subjects, aged 10–35 years, conducted by Singh et al. [13]. They observed that people exposed to organophosphorus compounds had considerably reduced activity of AChE and paraoxonase, compared to control. Furthermore, the AChE activity was significantly associated with age and the time of exposure to pesticides. In another study, comparing occupational and environmental exposure of rural residents to pesticides, a higher concentrations of pesticide metabolites in urine and saliva, and lower plasma BuChE activity was observed, compared to the control group. Interestingly, the BuChE activity was also decreased in the rural residents environmentally exposed (farmers not using pesticides), compared to the control group [14].

In their current study, AChE was more susceptible to inhibition caused by OPs compounds than BuChE (30.61% and 7.53% decrease, respectively). In contrast, Ellison et al. [15] reported that plasma BuChE was more than AChE susceptible to inhibition by oxone CHP in a group of Egyptian farmers aged 14–69 years. Therefore, the authors concluded that BuChE appeared to be a more sensitive biomarker of exposure to OPs compounds. Also, Dhananjayan et al. [16] observed a statistically significant reduction in enzyme activities (AChE 14%, BuChE 56%) in a group of vegetables and grape cultivation workers exposed to plant protection compounds. However, in view of presented results, it seems that the high inhibition of BuChE may be compound specific.

Once reliable and sensitive biomarkers of exposure are found, an easy and non-invasive methods of their acquirement must be provided. Blood and urine collection, being probably the most commonly used, are relatively invasive, inconvenient and uncomfortable, and generates some ethical problems especially, if sampling from is planned. Many reports in the literature prove the effectiveness of the method of detecting xenobiotics in sweat, avoiding many of the problems observed, for example, in the blood or urine analysis.

Kacinko et al. [17] showed that the analysis of sweat is also a non-invasive method for monitoring exposure to drugs. Xenobiotics were detected in 70% of patches worn for one week by persons taking drugs at low doses and 100% worn by persons taking drugs at high doses. Also, Liberty et al. [18] demonstrated that sweat absorbents are a convenient and effective method for the detection of drug metabolites. In addition, it was found that the patches are easy to use,

quick to apply and readily accepted by patients. The use of sweat in toxicological studies shows the benefits, such as non-invasive sampling, high reproducibility of results, reduced discomfort for children, sick, and the elderly persons, and significantly reduced risk of infection. In addition, allowing for long-time application, the sweat sorbents also increase the diagnostic window [19].

However, to the best of the knowledge of the authors of the presented study, knowledge, only one study has been published, designed to determine if sweat could be used for monitoring pesticide levels in exposed farm workers [20]. Therefore, in the current study, sweat sorbents were applied as an individual collecting device to measure children exposure to pesticides. Chromatographic analysis revealed the presence of various compounds used in plant protection products, such as Carbetamide, Carbofuran, Chloridazon, Dodemorph, ICIA0858, and Permethrin, in 19 sweat sorbents (Tab. 2). Thus, it was demonstrated that the sweat sorbents and sweat analysis can be a valuable tool for determining the biological markers of pesticide exposure.

However, this method also has some shortcomings:

- 1) the much lower concentration of the analyte in the sample in comparison to commonly used sources, such as urine and blood, requires high analytical skills and adequate instrumentation;
- 2) there is a risk of contamination of the sample by other exogenous compounds;
- 3) loss of the original concentration of the analyte by spontaneous decomposition and reabsorption;
- 4) side-effects, such as skin allergies.

The comparison of different sources of samples, including urine and sweat sorbents, has shown that sweat sorbents can give false positive results of exposure to a xenobiotic. Additionally, the risk of contamination of the sweat patch should be taken into consideration during interpretation of the results of the patch. Therefore, it is necessary to carry out the analysis properly, and compare the results with other biomarkers, such as metabolites from urine [21, 22]. Moreover, the site of the sorbent placement is very important for the amount of collected analyte in the patch [23].

Analysis of sweat is effective and reliable; however, differences in individual sweat suggest that the results should be interpreted qualitatively rather than quantitatively [24]. Moreover, so far only a few laboratories are involved in the toxicological analysis of sweat [5, 6].

## CONCLUSIONS

The last decade has brought significant progress in understanding the molecular toxicology of xenobiotics and their health effects on children. Nevertheless, any new data that can expand the knowledge in this area is vital to formulate effective prevention measures and an early diagnosis of the occult and long-term environmental exposure to xenobiotics in the most vulnerable populations.

Research conducted within the framework of this study allowed the gaining of valuable knowledge on the applicability of sweat sorbents as an innovative tool for the assessment of exposure to pesticides. Sweat sorbents applied correctly have proved to be an effective marker of exposure to pesticides. Analysis of sweat patches will provide a non-



invasive alternative to the analysis of urine or blood samples analysis. However, for a comprehensive assessment of the degree of exposure, additional analysis is needed which will take into account not only the content of the active substances contained in pesticides, but also their metabolites excreted in sweat. Additional validation of used sweat sorbents, based on a larger study population is also necessary.

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