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LEAD EXPOSURE IN MOTHERS AND CHILDREN FROM AN INDUSTRIAL REGION OF POLAND: AN ASSESSMENT OF MICRONUCLEI USING FLUORESCENCE IN SITU HYBRIDISATION.

DOES LEAD CONTRIBUTE TO INCREASED MICRONUCLEI LEVELS?

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Abstract
Aims. To investigate the susceptibility to micronuclei in lead-exposed mother and child groups employing fluorescence in situ hybridisation on micronuclei using pan-centromeric DNA probes (MN-FISH assay).
Materials and Methods. DOP-PCR (degenerate oligonucleotide primed polymerase chain reaction)was used to generate a pan-centromeric DNA probe. After labelling, the probe was applied to slides with micronuclei smears, allowing the micronuclei to be examined for the presence of centromeric signals.
Results. Nine year–old children (n=11) had a significantly lower ($p<0.01$) level of micronuclei (both with, and without centromeric signals) in comparison with mothers (n=11), and a significantly lower level of centromere negative micronuclei was observed in the children ($p<0.01$). There was also a lower non-significant level of centromere positive micronuclei induced in comparison with centromere negative micronuclei.
Conclusions. The higher rate of micronuclei induced in mothers may arise through lead-exposure and increasing age, and the micronuclei induced arise more from chromosomal fragmentation, than chromosomal malsegregation. Key words: Lead, micronuclei, FISH, mother-children pairs, susceptibility

Rezumat.
Scop. Scopul lucrării de față a fost de a investiga susceptibilitatea de apariție a micronucleilor la grupuri de mame şi copii expuşi la plumb, folosind hibridizarea prin fluorescentă *in situ* asupra micronucleilor şi probe de ADN pan-centromere (metoda MN-FISH).
Material şi metodă
Pentru a genera probe de ADN pan-centromere s-a folosit tehnica DOP-PCR (Degenerate Oligonucleotide Primed-Polymerase Chain Reaction). După marcare, proba a fost aplicată lamelor cu frotiuri cu micronuclei, ceea ce a permis examinarea prezenței de semnale centromere. Rezultate
Copiii de 9 ani (n = 11) au avut un nivel de micronuclei (cu şi fără semnale centromere) semnificativ mai mic ($p< 0,01$) în comparație cu mamele (n =11) şi un nivel semnificativ mai mic de micronuclei cu centromere negative s-a observat la copii ($p< 0,01$). De asemenea, a fost un nivel ne-semnificativ mai mic de micronuclei induşi cu centromere positive în comparație cu micronuclei cu centromere negative. Concluzii
Rata mai mare de micronuclei induşi la mame poate creşte prin expunere la plumb şi cu creşterea vârstei, iar numărul de micronuclei induşi creşte mai mult prin fragmentarea cromozomilor decât prin malsegregare.

Cuvinte cheie: plumb, micronuclei, hibridizare prin fluorescență *in situ,* pereche mamă-copil, susceptibilitate

Abbreviations: BLL, blood lead level; MN, micronuclei; FISH, fluorescent *in situ* hybridization; C+MN, centromere-positive micronuclei; C-MN, centromere-negative micronuclei

INTRODUCTION

Lead is a divalent cation, which binds strongly to the sulfhydryl groups found on proteins. Its main targets within the body are the central nervous system (CNS) causing long term impaired neurocognitive development and behaviour of children (1). Lead is also capable of catalytically cleaving the phosphate sugar backbone of tRNA, at specific sites, and interfering with the protein transcription (2). Children are more susceptible to lead toxicity for a variety of reasons; the immature CNS is more susceptible to toxic agents than a mature CNS (2). Children have a far greater hand to mouth activity, so that more lead reaches the digestive system, where the gut absorbs it more readily (2). Lead then moves into the circulatory system, and may affect it in a number of ways. (3) δ-Aminolevulinic acid dehydratase (ALAD) is an enzyme

involved in the haem biosynthesis pathway (4), and because it contains thiol groups it has an increased affinity for lead. Lead then inhibits ALAD, causing an accumulation of δ-Aminolevulinic acid (ALA), which then contributes to lead-induced oxidative damage in erythrocytes (4). As shown in mice, it may be that lead inhibits a number of haematological parameters (erythrocyte count, haemoglobin etc), even when at background levels (5). It has also been suggested that lead may contribute to cardiovascular dysfunction, particularly in adults (6).

In Poland, children are exposed to lead from industrial processes (in the past also from the combustion of leaded gasoline, the disposal of which was forbidden approximately six months ago (7). The Silesia province and its surrounding areas, where ore is extracted and processed is a specific area of concern, and it is from this region that we have investigated mothers and children. The beginning of lead exploitation and smelting in Bukowno was in the 15th century, and the main industrial plant in this region is the mining and metallurgic plant "Bolesławiec" which is one of the greatest producers of zinc and lead concentrates in Poland. The activities of the plant involve mining of zinc and lead ores, the processing of these ores to concentrates, and the production of non-ferrous metals. This is a specific area of concern, and it has been shown that 13% of children living in the central part of the region have blood lead levels (BLL) near to or exceeding 100 μg/l, which is the current threshold of concern issued by the Centre for Disease Control and Prevention (7). However, the Scientific Committee on Occupational Exposure Limits (SCOEL) recommends the higher maximum exposure value of 300 μg/l in European countries (8).

It has been reported that the use of Giemsa strain to detect chromosomal aberrations does not provide for the detection of certain types of chromosomal damage, whereas the use of fluorescence in situ hybridisation (FISH) allows more detailed information of the range of aberrations available (9). In our investigation, we utilised fluorescence in situ hybridisation to detect the chromatin pan-centromeric signals within the micronuclei (MN). The use of a pan-centromeric probe with MN-FISH allows the classification of MN as being centromere positive (presence of fluorescent centromeres within a micronucleus) or centromere negative (no apparent centromeres are highlighted within a micronucleus). Accordingly, this characterises the origin of MN either due to chromosome breakage (C-8

MN), or as arising from chromosomal malsegregation (C+MN), including, but not limited to damage of the karyokinetic spindle (9).

In this study, we have investigated peripheral lymphocytes from this lead polluted area in Poland, as characterised by the levels of blood lead recorded. This was to determine whether or not children were more susceptible to lead induced chromosomal damage, by comparison with their mothers using the MN-FISH techniques. Employing a pan-centromeric probe, which detects all human centromeres, we were able to determine the number of MN with centromeres (aneugenicity), and without centromeres (clastogenicity) in the mothers and children.

MATERIALS AND METHODS

Population studied The population studied included 11 mothers of various ages, and 11 children of nine years of age, residing in the town of Bukowno, located in the neighbourhood of the Silesia province. The aims of the study were explained to parents, who provided consent, and completed a self-administered questionnaire. The parents then informed their children, and the children were further informed via their tutor and school nurse. The parents provided consent for the blood sampling of the children.

Blood sampling and slide preparation
Blood samples were collected into sodium-heparin Vacuette tubes for the blood cultures, and Vacutainers for determining BLL. The samples were collected and processed within 2 hours of collection. Levels of blood lead were determined using electrothermal atomic-absorption spectrophotometry (AAS), according to Stoeppler and Brandt (10). Slides with micronuclei spreads were prepared according to Fenech et al (11). Blood sampling, BLL determination and the slide preparation were carried out at the Institute of Occupational Medicine and Environmental Health, Department of Genetic Toxicology, Sosnowiec (Poland). Prepared sides were frozen at -20 °C and stored at that temperature. Frozen slides were shipped to the University of Bradford on ice, and immediately transferred to -20 °C.

Degenerate oligonucleotide primer polymerase chain reaction (DOP-PCR)
DOP-PCR uses a degenerate universal primer and low annealing temperature to generate a quantity of amplified material, and compared with other types of PCR for genome mapping studies, is rapid, efficient and species independent (12). Adult

male lymphocytes isolated (as per manufacturer's instructions) from whole blood with the use of Lymphoprep (GibcoBRL, UK), were used as a DNA template (10 µl cell suspension per 50 µl PCR reaction). The final PCR reaction mix contained 1x Taq polymerase buffer (Invitrogen, UK), 1.5 mM $MgCl^{2+}$ (Sigma, UK), 200 µM of each dNTP (Promega, UK), 0.6 µM of primers WA1 and WA2 (13) as well as 0.05U of Taq polymerase (Invitrogen, UK). The preceding initial PCR step incubated the reaction for 10 min at 94 °C. The following steps: denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min and primer extension at 72 °C for 1 min were repeated in a cycle 34 times. The final step included 10 min at 72 °C and subsequently room temperature overnight, to allow the amplified probe DNA to reform as double-stranded DNA. The success of the DOP-PCR was checked on a 1.5% ethidium bromide (Sigma, UK) agarose gel made with 1x TBE buffer. The running buffer was 1x TBE, and voltage was maintained at 100 V for 60 min. A low molecular weight ladder (New England Biolabs, UK) was used to identify the 175 bp product. The PCR product and the ladder were loaded with 10% bromophenol blue loading dye (Sigma, UK). The gel was viewed and captured on a UV transilluminator. Finally, the pan-centromeric probe DNA was biotinylated using a standard biotinylation kit (Invitrogen, UK) and stored at -20 °C until required.

Fluorescence in situ hybridisation
The pan-centromeric probe was added to an equal amount of Cot-1 DNA and salmon sperm DNA (Sigma, UK) and mixed. MasterMix 2.1 (78.6% formamide, 14.3% dextran sulphate, 1.43x SSC) was added in a ratio of 7:3, mixed and briefly spun. This hybridisation mixture was denatured at 78 °C for 10 min and incubated for pre-annealing in a 37 °C water-bath for 30 min. Denaturation of the binucleated and micronuclei DNA was achieved by incubating the slides in 70% formamide/2xSSC pH 7-7.5 (Sigma, UK) at 72 °C for 2 min. Subsequent dehydration was carried out by running the slides through an ethanol series of 70%, 90% and absolute for 2 min each. After allowing them to air-dry, the hybridisation mix was applied to the slides, which were covered with a 22x22 mm^2 cover-slip and sealed with Fixogum (Marabu, Germany). The slides were incubated in a humidity chamber for 72 h at 37 °C. After removing the rubber-cement, the cover-slips were allowed to detach in 2x SSC (Sigma, UK). The slides were quickly and stringently washed for 2 min in 0.1x SSC at 42 °C. For blocking, 400 µl of PNM

buffer (PN buffer containing 5% non-fat dry milk powder and 0.02% sodium azide, filtered) was added to each slide. The slides were left for 10 min at room temperature covered with a plastic cover-slip. For detection of the biotin on the probe, 1 mg/ml streptavidin conjugated with FITC (Vector, UK), and consecutively 5 mg/ml anti-streptavidin-FITC (both diluted in PNM buffer) were added to the slides and left to incubate for 30 min each at room temperature. The slides were washed for each detection step in PN buffer (phosphate buffer, pH 8, containing 0.05% Igepal CA-630, Sigma, UK) at room temperature for 10 min each. Vectashield (Vector, UK) with DAPI (0.5 µg/ml) was added and cover-slips placed on slides. The visualisation of the slides was carried out using a Leitz fluorescence microscope with an Optiscan Proscan unit, and filter wheel attached. The filter wheel was equipped with single bandpass filters for FITC and DAPI. Chromosomal centromeres appeared in green (FITC: emission max. 519 nm) and chromatin was observed in blue (DAPI: emission max. 455 nm).

Scoring and statistical analysis The scoring of bi, tri and tetra-nucleate cells, and micronuclei was carried out according to Fenech et al (11) with the addition of the micronuclei being examined for the presence of centromeric signals. For FISH analysis, the slides were scored with a Leica DMLB epifluorescence microscope (Weztler, Germany). The micronuclei present in the binucleated lymphocytes with intact cytoplasm were examined for the presence, and the number of centromeric signals, and were classified as centromere positive (C+MN) or centromere negative (C-MN). A total of 1000 binucleate cells were analysed for each subject. The cytokinesis-block proliferation index (CBPI = number binucleate cells + 2 [number multinucleate cells]/total number of cells) was used to calculate cell cycle delay. For scoring, all slides were coded to exclude bias (double blind study). Differences in levels of micronuclei were examined for levels of significance using a one-way ANOVA test, as the data were normally distributed. Data analysis was carried out using the statistical package SPSS v.11 (Sigma, US)

RESULTS

Table 1 shows the age, smoking status, mean BLLs of the mothers studied, and the age, sex, and BLLs of the children studied.

In the lead-exposed mother and child population, the frequency of C-MN in mothers was 2.7 fold increased ($p<0.01$) when compared to their children, whereas the total frequency of C+MN was almost identical between children and

mothers (Figure 1). As a measure of cell cycle delay, the CBPI showed no differences in rates of cell cycling between mothers and their children.

When the total frequencies of C+MN were plotted against those of C-MN a linear upward trend was seen only for the children, suggesting that with a higher score of C-MN the number of C+MN with multiple centromere signals was increasing (Figure 2).

In Figure 3, the correlation of the total MN numbers with the levels of blood lead (μg/l) in children and mothers are shown. The observed gradient of the linear regression line is higher in children than in their mothers. Despite a significantly higher number ($p=0.005$) of total MN in the group of mothers, their BLLs were low in comparison with the children.

Figure 4 shows BLLs correlated with C-MN in mothers ($n=11$) and children ($n=11$), with a linear trend line for the two groups. Children have the higher correlations when the linear regression line is added.

DISCUSSION

Separate control groups of unexposed mothers and children were not included in this current study. However, a previous study showed micronucleus levels in such unexposed control groups to be approximately one-third of the value of the exposed groups (unpublished data). This present study was merely to determine any differences between mothers and children in an exposed environment.

The effects of lead on humans has been recognised since the time of the Ancient Greeks, but it was not until the 1970s that studies began to provide information on the abnormalities in neurocognitive development and protein transcription (2,14). Damage may arise throughout childhood development with chronic exposure to lead, particularly as a number of populations in the world still come into contact with high amounts, especially via leaded petrol, and as in this instance also through industrial processes (7). It has also been shown that lead exposed workers are more prone to DNA damage in comparison with non-exposed individuals, and the DNA repair processes in lead-exposed individuals are not as effective as in control individuals (15).

By employing the MN-FISH assay, the chromatin of the cells is stained in a similar manner to Giemsa staining, allowing the assessment of binucleate cells and, if present, micronuclei. Additionally, when utilising a pan-centromeric probe it is possible to distinguish between MN carrying one or more centromeric signals (C+MN) and MN without signals (C-MN). By

differentiating between these two categories, we are able to conclude the possible origin of the aberration, i.e. MN, which are showing centromeric signals most likely derive from non-disjunction leaving whole chromosomes, or at least the derivative of a chromosome within the MN. The presence of whole chromosomes in a micronucleus tend to arise via failure of the mitotic spindle, kinetechore, other parts of the mitotic apparatus, or by alterations in cellular physiology, mechanical disruption or damage to the chromosome sub-structures (9). However, C-MN originate from one or more acentric fragments residing after chromosomal damage, and these aberrations will not be included in either of the two daughter nuclei after mitosis.

Our results show (Fig. 1) that significantly more MN (2.7 fold; $p<0.01$) were found in mothers compared to their children. There was no effect on the proliferation index (CBPI) in both populations, i.e. no delay of the cell cycles was observed, although a previous study has suggested that there is a decrease. Focussing on the discrimination between C+MN and C-MN, it becomes clear that this difference is driven by the significantly increased number of C-MN in the mothers (2.7 fold higher than in children; $p<0.01$). Both mothers and children show approximately the same number of C+MN. When the total number of C-MN is plotted against the total of C+MN (Fig. 2) it becomes obvious that the children are clustered in the lower quadrant with a lower number of C-MN compared to their mothers, but, more C+MN with one or more centromeric signals. The trend line for the children also shows that with an increasing number of C-MN the C+MN is increasing in a linear fashion. However, these same assumptions cannot be made for the mothers. They have higher levels of C-MN, but not higher numbers of C+MN.

As the C-MN category arises from acentric fragments or chromosomal fragmentation, lead seems to act in this study mainly as a clastogen rather than an aneugen, as the number of C+MN is low suggesting that the mitotic spindle and the segregation of the chromosomes are not gravely affected. Even though the toxic effects on the body have been well documented, inconsistencies about the mutagenicity, clastogenicity and carcinogenic properties of lead and its compounds (15-17) still remain.

The levels of blood lead shown (Fig. 3) fall just below the Centre for Disease Control and Prevention threshold of 100 µg/l since the highest level of lead is approximately 92 µg/l (7). Children show higher levels of blood lead (BLL) when compared to their mothers even if they do have 2.7 fold

less detectable MN. This may attributed to a number of different causes. Children do play outside more, (contact with air, dust etc), and are more likely to have hand-to-mouth activity (2, 18). It may also be attributed to smoking parents/ environment, high lead content in the houses or even from being in contact with parents' work-clothes (19).

The higher levels of lead appear to account for lower levels of micronuclei, which suggests that the DNA damage due to lead toxicity may be being repaired more effectively, and an adaptive response (AR) may be occurring, although the mechanisms of this remain unclear. Studies which involve low levels of radiation and micronuclei induction have obtained conflicting results (20-22) about the mechanisms of an AR, and thus it cannot be concluded that an AR occurs regularly in lymphocytes. Our results also suggest that the mothers are more susceptible to higher levels of micronuclei with lower BLLs through age. It is intriguing that mothers have higher numbers of MN and that the nine-year-old children can more effectively cope with the induced damage of a higher BLL than their mothers. Figure 4 also shows this relationship between BLL and numbers of C-MN in the two groups, that higher BLL do not necessarily correlate with a higher number of C-MN. This suggests that the C-MN in the children arise through lead exposure in the children via clastogenic mechanisms.

Previous studies have concluded a relationship between age and an increase in levels of MN (23, 24), or, other factors such as smoking (25) or work exposures (15), which may contribute to higher levels of overall MN observed in the "older", adult population group (mothers). A recent pooled-analysis has shown that non-exposed children aged 5-9 yrs old have a mean MN frequency of 5.62, whereas the group of children in the present study (Table 1) have a mean MN level of 8.1 per 1000 BN (26). The study also showed that as the age of children increases, so do levels of MN, confirming that MN rates increase with age (26). It has also been reported that children have a higher level of susceptibility than adults, but a lack of available studies has prevented conclusive reporting (27). This current study has also shown that children do have the higher levels of blood lead, confirming the higher level of hand-to-mouth activities (7).

In conclusion, nine year-old children exposed to lead had a significantly lower level of micronuclei in comparison with their mothers, but the children themselves had much higher levels of blood lead than the adults. This highlights a

need for further investigations into the impact of lead in all individuals, and that preventative measures continue in lowering the levels of lead exposure in those populations most at risk.

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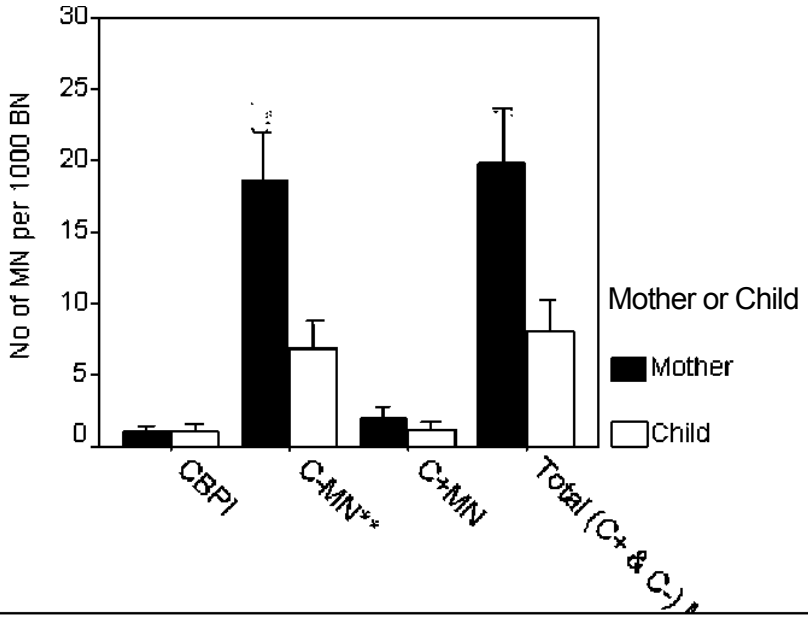

Fig. 1 – The overall numbers of micronuclei with (C+MN) and without signals (C-MN), the total number of micronuclei, and the CBPI of mothers (n=11) and their children (n=11), standard error of the mean also shown.

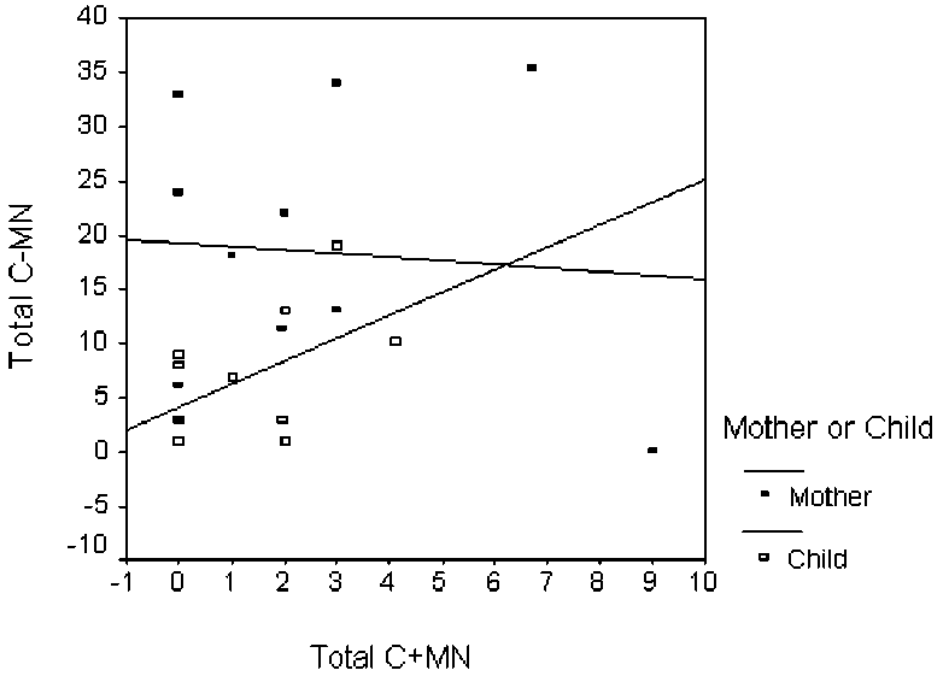

Fig. 2 - Total micronuclei numbers without signals (C-MN) plotted against the total micronuclei with signals (C+MN) in mothers (n=11) and children (n=11), with a linear trend line for the two groups.

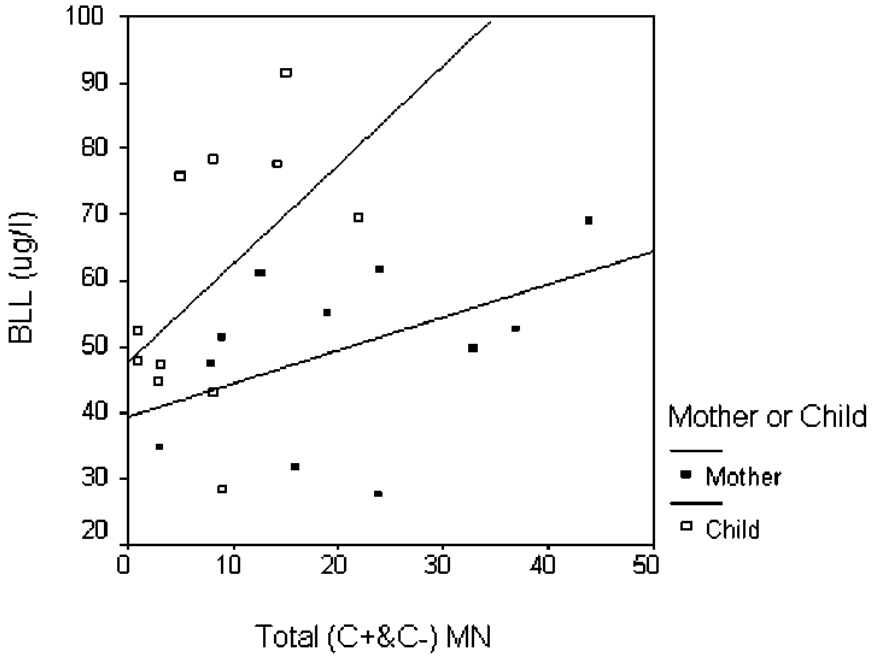

Fig. 3 – Total micronuclei numbers (C+ and C-) plotted against BLL (ug/l) in mothers (n=11) and children (n=11), with a linear trend line for the two groups.

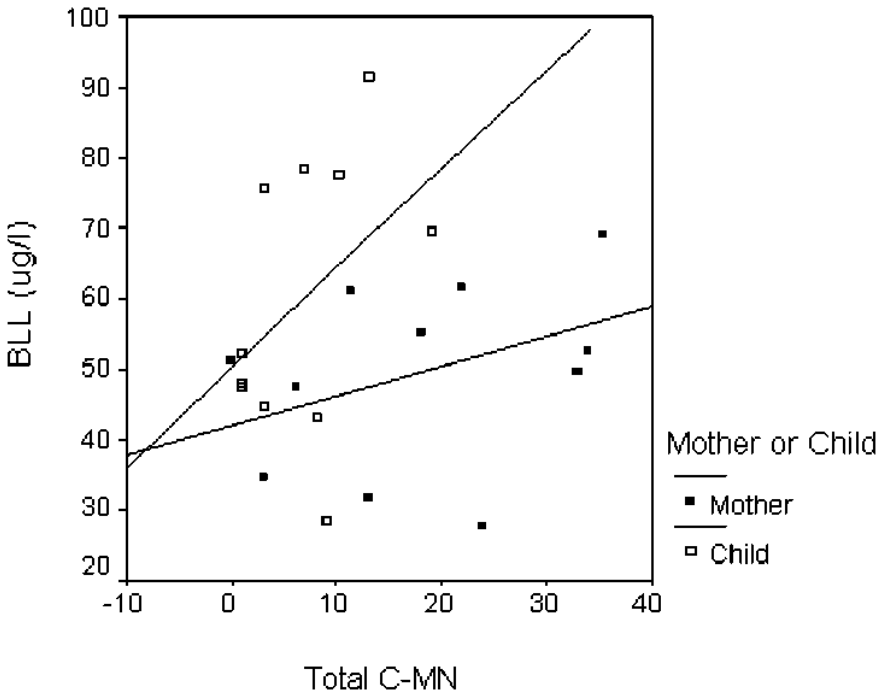

Fig. 4 – C-MN plotted against BLL (ug/l) in mothers (n=11) and children (n=11), with a linear trend line for the two groups.

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