

# Transplacental Chemical Exposure and Risk of Infant Leukemia with *MLL* Gene Fusion<sup>1</sup>

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## ABSTRACT

Infant acute leukemia (IAL) frequently involves breakage and recombination of the *MLL* gene with one of several potential partner genes. These gene fusions arise *in utero* and are similar to those found in leukemias secondary to chemotherapy with inhibitors of topoisomerase II (topo-II). This has led to the hypothesis that *in utero* exposures to chemicals may cause IAL via an effect on topo-II. We report a pilot case-control study of IAL across different countries and ethnic groups. Cases ( $n = 136$ ) were population-based in most centers. Controls ( $n = 266$ ) were selected from inpatients and outpatients at hospitals serving the same populations. *MLL* rearrangement status was derived by Southern blot analysis, and maternal exposure data were obtained by interviews using a structured questionnaire.

Apart from the use of cigarettes and alcohol, very few mothers reported exposure to known topo-II inhibitors. Significant case-control differences were apparent for ingestion of several groups of drugs, including herbal medicines and drugs classified as "DNA-damaging," and for exposure to pesticides with the last two being largely attributable, respectively, to one nonsteroidal anti-inflammatory drug, dipyrone, and mosquitocidals (including Baygon). Elevated odds ratios were observed for *MLL*<sup>+ve</sup> (but not *MLL*<sup>-ve</sup>) leukemias (2.31 for DNA-damaging drugs,  $P = 0.03$ ; 5.84 for dipyrone,  $P = 0.001$ ; and 9.68 for mosquitocidals,  $P = 0.003$ ). Although it is unclear at present whether these particular exposures operate via an effect on topo-II, the data suggest that specific chemical exposures of the fetus during pregnancy may cause *MLL* gene fusions. Given the widespread use of dipyrone, Baygon, and other carbamate-based insecticides in certain settings, confirmation of these apparent associations is urgently required.

## INTRODUCTION

In most cases of IAL<sup>3</sup>, both ALL and AML have rearrangements of the *MLL* gene at 11q23, which are referred to as *MLL*<sup>+ve</sup> (1–4).

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<sup>3</sup> The abbreviations used are: IAL, infant acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; topo-II, topoisomerase II; NQO1, NADPH:quinone oxidoreductase; OR, odds ratio; CI, confidence interval.

Investigation of identical twin pairs, concordant for infant leukemia (5), has shown that this genetic lesion was acquired *in utero* by one twin and transferred to the other, and *in utero* origin has been confirmed by retrospective analyses of neonatal blood spots (Guthrie cards) of affected infants (6). The high concordance rate for leukemia in monozygotic twins of this age and the very short latency suggests that an *MLL* fusion in the appropriate fetal hematopoietic stem cell is sufficient to cause leukemia (7). *MLL* rearrangements are rare in older cases of *de novo* leukemia but frequent in cases arising subsequent to chemotherapy with inhibitors of topo-II. This suggested that similar exposures *in utero* might be relevant to infant leukemia (5) and has led to the specific biologically based hypothesis that *MLL*<sup>+ve</sup> infant leukemia is caused by transplacental exposure to substances that form cleavable complexes with topo-II (8, 9).

Although the hypothesis is very precise in terms of case biological subgroups, timing of exposure, and biological mechanism, it is difficult to test because many inhibitors of topo-II remain unidentified. An additional problem is that the magnitude of the effect of different substances is unknown, so that computation of total exposure is not possible.

topo-II inhibitors include chemotherapeutic agents (10), benzene metabolites (such as benzoquinone and, hence, smoking; Refs. 11, 12), isoflavones, flavonoids, lignans (*e.g.*, Ref. 13), some herbal medicines (14), anthraquinone laxatives such as senna (15), podophyllin resin, quinolone antibiotics, some pesticides including certain fungicides and mosquitocidals (16, 17), some culinary herbs (*e.g.*, turmeric; Ref. 18), and many, though not all, phenolic substances or their metabolites (19).<sup>4</sup> Herbal medicines are frequently potent (20, 21), and an association with *MLL*<sup>+ve</sup> AIL was a prior hypothesis.

Limited epidemiological support is available. Maternal dietary consumption of topo-II inhibitors has been associated specifically with infant acute myeloid leukemia in one United States case-control study ( $n = 84$  cases; Ref. 22). The metabolism of quinones, as exemplified by benzene detoxification, is critically controlled by the enzyme NQO1 (19). Two polymorphic variants of NQO1 have been identified (23). The first effectively inactivates the enzyme (24). This has been associated with chemotherapy-related leukemia (25) and *MLL*<sup>+ve</sup> infant leukemia (26).

The objectives of the present study were: (a) to test the hypothesis that exposure *in utero* known to inhibitors of topo-II increased risk of *MLL*<sup>+ve</sup> infant leukemia; (b) to conduct a preliminary evaluation of

<sup>4</sup> M. Smith, personal communication.

the more general hypothesis that exposures *in utero* to pharmaceutical drugs, solvents, pesticides, and herbal medicines were associated with IAL and specifically MLL<sup>+ve</sup> disease; and (c) to generate hypotheses that specific substances were associated with MLL<sup>+ve</sup> infant leukemia.

## MATERIALS AND METHODS

**Cases and Controls.** An international collaboration has permitted a pilot case-control study of IAL to be conducted in regions within countries in Europe and the Middle East (Italy, Greece, and Egypt), South America (Brazil and Chile), and Asia (mainland China, Hong Kong, and Japan).<sup>5</sup> Case ascertainment was based on referrals to treating hospitals but was population based<sup>6</sup> (*i.e.*, included all of or almost all of the cases of acute leukemia in infants <18 months of age diagnosed in defined time periods and geographical areas). Informed consent was requested from mothers, and 136 were recruited. Controls ( $n = 266$ ) were selected from inpatients and outpatients at the same or similar hospitals serving the same populations. Appropriate diagnostic inclusion and/or exclusion criteria were applied to the controls.<sup>7</sup> The case:control ratio was approximately 1:2 in each center, and to give similar distributions of the time elapsed from the birth index to the mother's interview, two controls were selected with the same gender and similar dates of birth (normally,  $\pm 1$  month) as soon as each case mother had been interviewed (normally shortly after diagnosis). Details provided by some centers (believed to be representative) indicate high percentages of mothers agreeing to be interviewed (cases, >98%; controls, >90%).

**MLL Gene Rearrangement Status.** A number of methods are available for evaluation of MLL gene rearrangement including Southern blot, reverse transcription-PCR, and fluorescence *in situ* hybridization. At the time of the initiation of this study, 4 years ago, we considered Southern blot to be the most practical. The procedure was as described previously (27) using a PCR-generated cDNA probe (exons 5 to 7 of MLL) and *Bam*HI digest. A standardized protocol was established, and individual centers either performed their own tests (Italy, China, Japan, and Hong Kong) or referred extracted DNA samples to the reference laboratory (M. F. G.) in London. In some instances (China, Japan, and Hong Kong), confirmation of the result was carried out in the reference laboratory. DNA from a MLL-*AF4* positive cell line (RS4:11) and a leukemic cell line (KG1) without a MLL gene rearrangement was available as a control. A germ-line (nonrearranged) MLL gene allele served as an internal control for the quality of DNA preparation. Either blood (with adequate blast cell counts) or bone marrow was used. For a proportion of cases, no suitable diagnostic specimen was available for analysis, or referred DNA samples (*e.g.*, on slides) were degraded and, in consequence, their MLL status is unknown. This includes all of the cases from Greece. This limitation restricted the power of the study but does not introduce any bias. In subsequent studies, we would recommend using fluorescence *in situ* hybridization methods on stored (frozen) unstained bone marrow smears (28).

**Exposure Classification.** Maternal exposure data were obtained by interviews using a structured questionnaire. This was translated into local languages, and the validity of the translations was checked by dual interviewing of bilingual mothers. Questions focused on lifestyle (alcohol and smoking use), medical drug ingestion (prescription and other), and occupational exposures. Medication use was obtained from questions concerning the use of certain types of drugs (*e.g.*, antibiotics and herbal medicines), infectious illnesses, and medical consultations. A pharmacist (blind to numbers of mothers and status) reviewed the list of medications reported by the mothers, but none were recognized as known topo-II inhibitors. Analysis categories for drugs were determined after examination of overall frequencies blind to case-control status. The first type of category was based on the medical problem (*e.g.*,

Table 1 Cases included in the study

	MLL <sup>+ve</sup> (% <sup>+ve</sup> ) <sup>a</sup>	MLL <sup>-ve</sup>	MLL status NK <sup>b</sup>	Total
Diagnosis				
ALL	19 (53%)	17	13	49
AML	29 (66%)	15	30	74
Other <sup>c</sup>	2 (40%)	3	8	13
Age				
0-5 mo	18 (72%)	7	18	43
6-11 mo	14 (58%)	10	18	42
12-17 mo	18 (50%)	18	25	87
Gender				
Female	28 (60%)	19	23	70
Male	22 (58%)	16	28	66
Region				
Orient	18 (60%)	12	2	32
South America	22 (63%)	13	17	52
Europe	10 (50%)	10	32	52

<sup>a</sup> Percentage of MLL<sup>+ve</sup> of those cases with known MLL status.

<sup>b</sup> NK, no diagnostic sample available or sample degraded.

<sup>c</sup> Includes biphenytoic.

urinary tract infection); the second was based on pharmacological action (*e.g.*, nonsteroidal anti-inflammatory drugs); the third considered "DNA-damaging" drugs (*i.e.*, those for whom genotoxic effects had been reported for some organ in some species).<sup>8</sup> Finally, individual drugs with frequent reported use were to be analyzed if members of the groups associated with the case status and/or harmful effects were reported by the pharmacist. Occupational exposure of the mother to pesticides, solvents, organic dusts, and ionizing radiation was also sought in the interview.<sup>9</sup> Interviewers in some countries, notably Brazil, included nonoccupational exposures of mothers in the interview. Because these were qualitatively similar to many occupational exposures (*e.g.*, housewife/housemaid), they were combined for analysis. A policy for selection of specific substances for analyses similar to that for drugs was formulated.

**Statistical Analysis.** Exposures of case and control mothers during the year before the birth of index child have been compared using logistic regression (29) and exact methods (30) implemented by the packages SAS and EGRET. All of the analyses have adjusted for sex and region of residence (South America, Asia and Europe and the Middle East), and important results have been checked with further adjustment by country. All of the controls have been included in each analysis.<sup>10</sup> Results are expressed as ORs with 95% CIs. All of the *P*s are two-sided.

## RESULTS

Details of cases are shown in Table 1. Apart from the use of cigarettes and alcohol and a few instances where solvent exposure was specified as benzene or gasoline, no mothers reported exposure to known topo-II inhibitors. The ORs for "ever" compared with "never" for alcohol consumption and cigarette use were slightly elevated in the total series but <1 for MLL<sup>+ve</sup> cases (Table 2), although they are consistent with approximately 2-fold elevation of risk. Several drug groups determined by reason for use (vaginal infection, anemia or anemia prophylaxis, pain, and digestive problems) showed statistically significant ( $0.01 < P < 0.05$ ) positive associations with infant leukemia in the total series but reduced or similar ORs when MLL<sup>+ve</sup> cases and MLL<sup>-ve</sup> cases were compared with the controls. Only four mothers (three cases with two MLL<sup>-ve</sup> and one MLL<sup>+ve</sup> and one

<sup>8</sup> This category was derived by one of us (F.E.A.) by record linkage searching abstracts of papers published in English from 1995-1999 and using the following key words: genotoxic, carcinogenic, highly reactive, single strand breaks, and sister chromatid. Any evidence of genotoxic behavior of the drug and/or a metabolite in any organ of any species was taken as a defining entry to this category. Frequently, this was based on the Comet or similar assays. This process was conducted blind to the numbers and the status of mothers reporting drug use, and it was suggested by the observation of Prof. Martyn Smith (University of Berkeley, CA) that many of the drugs in the list were capable of causing DNA damage.

<sup>9</sup> Except in Greece.

<sup>10</sup> Except when, for analyses of case subgroups, those in countries with no cases in the subgroup have been excluded. Specifically, controls in Egypt and Greece were excluded for analyses of MLL<sup>+ve</sup> and <sup>-ve</sup> subgroups.

Table 2 *In utero* chemical exposures and infant leukemia (OR,<sup>a</sup> 95% CI)

Exposure <sup>b</sup>	Total series	MLL <sup>+ve</sup> subgroup	MLL <sup>-ve</sup> subgroup	ALL	AML
Smoking (ca = 36, ct = 53)	1.43 (0.86–2.39) P = 0.17	0.98 (0.46–2.09) P = 0.97	1.72 (0.74–3.99) P = 0.21	1.59 (0.82–3.07) P = 0.17	1.33 (0.63–2.80) P = 0.45
Alcohol (ca = 21, ct = 33)	1.23 (0.68–2.23) P = 0.50	0.74 (0.29–1.90) P = 0.54	2.09 (0.91–4.84) P = 0.08	0.63 (0.25–1.60) P = 0.33	1.92 (0.90–4.10) P = 0.09
DNA damaging drugs (ca = 37, ct = 47)	1.71 (1.03–2.84) P = 0.039	2.31 (1.06–5.06) P = 0.036	0.56 (0.18–1.80) P = 0.33	1.78 (0.95–3.34) P = 0.07	2.28 (1.10–4.71) P = 0.026
Herbal medicines (ca = 27, ct = 22)	2.93 (1.57–5.48) P = 0.001	3.00 (1.38–6.54) P = 0.006	2.64 (1.04–6.67) P = 0.04	4.45 (2.06–9.63) P < 0.001	2.09 (0.89–4.92) P = 0.09
Maternal pesticide (ca = 15, ct = 10)	3.67 (1.54–8.74) P = 0.003	4.96 (1.71–14.43) P = 0.003	1.87 (0.36–9.61) P = 0.45	2.53 (0.71–8.97) P = 0.15	5.08 (1.84–14.04) P = 0.002
Dipyrrone (ca = 12, ct = 9)	2.83 (1.15–6.99) P = 0.02	5.84 (2.09–16.30) P = 0.001	0.64 (0.08–5.41) P = 0.68	3.13 (1.02–9.57) P = 0.046	3.01 (0.93–9.79) P = 0.07
Baygon/mosquitocidal (ca = 7, ct = 3)	5.14 (1.27–20.85) P = 0.02	9.68 (2.11–44.40) P = 0.003	0 (0–15.19) P = 1.0	4.30 (0.66–28.08) P = 0.13	7.82 (1.73–35.39) P = 0.008

<sup>a</sup> ORs are for exposed compared with nonexposed.

<sup>b</sup> Numbers are for cases (ca) and controls (ct) exposed in the total series.

control) reported exposure to gasoline or benzene. More convincing evidence, including significant associations for both the total series and MLL<sup>+ve</sup> cases compared with controls, was found for DNA-damaging drugs, herbal medicines, and maternal pesticide exposure (Table 2).

Two specific drugs were identified by the pharmacist as being potentially harmful (dipyrrone, a nonsteroidal anti-inflammatory drug, and metronidazole, an antibiotic used for vaginal infections). These are members of the group of DNA-damaging drugs, and numbers of reports were adequate for analyses. Only one specific pesticide was used by more than two mothers (Baygon, reported by seven mothers; this has been combined with mosquitocidals for analysis). Analyses of these exposure subgroups show highly significant and selective associations with MLL<sup>+ve</sup> leukemia for dipyrrone and for Baygon/other mosquitocidals. There is a suggestion of a stronger association with mosquitocidals for AML than for ALL. The associations of infant leukemia with DNA-damaging drugs and maternal pesticide exposure have reduced ORs and no longer approach statistical significance if dipyrrone and mosquitocidals are excluded. The numbers of mothers exposed are shown in Table 3. The highest percentages exposed are for MLL<sup>+ve</sup> AMLs.

Only seven mothers (five cases and two controls) reported use of metronidazole, and the association with cases in the total series was not statistically significant (OR, 3.59; 95% CI, 0.83–15.41; P = 0.09). The OR for MLL<sup>-ve</sup> cases was higher than for MLL<sup>+ve</sup> with neither approaching statistical significance. There was a suggestion of specific association with AML (OR, 4.66; 95% CI, 0.89–24.28; P = 0.07).

The questionnaire included questions that provided the opportunity to monitor for evidence of recall bias. These included two other occupational exposures (ionizing radiation and organic dust), infectious illness, and several drug groups. These showed no evidence that mothers of the cases were over-reporting.

**DISCUSSION**

This is the first case-control study of infant leukemia to include MLL status and, hence, report analyses specific to the biologically

distinct subset of MLL<sup>+ve</sup> cases. It includes children in different geographical settings and ethnic groups and has the potential to identify a larger spread of exposure than would be possible in one region. Its conduct and design have two weaknesses: (a) the small numbers of cases, especially those molecularly classified; and (b) the controls may not be representative. We cannot be certain that confounding by factors relating to selection of controls has not artificially generated the results we present. Recall bias does not appear to be influential; it is unlikely that parents would specifically focus on exposures relating to chemical exposure, but we cannot be certain of this. The best argument against recall and interviewer bias is when positive results relate selectively to MLL<sup>+ve</sup> disease, because interviewers and mothers of cases were unaware of MLL status. Case selection bias is unlikely but impossible to quantify because cancer registries do not operate in the study regions. Any such bias, either at ascertainment or at recruitment, would be independent of MLL status. Because the same control set was used for analyses of MLL<sup>+ve</sup> and MLL<sup>-ve</sup> cases, the selective associations that we have observed are very unlikely to be attributable to confounding by deprivation or other features that may have influenced control selection. Those associations, which we have found to be of similar strength across case subgroups (e.g., that for herbal medicines), must be treated cautiously.

Testing the hypothesis of exposure to known topo-II inhibitors was not possible because few case and control mothers reported their use. The weakness of the association of infant leukemia with maternal alcohol use is surprising, because most previous studies (31) have reported significant positive associations. The present data suggest that previous results may not have been attributable to exposures of MLL<sup>+ve</sup> cases. Previous studies (31) of tobacco use have been inconsistent and included negative results similar to ours.

However, this study has found evidence that *in utero* exposure to chemicals is associated with risk of IAL, in general, and MLL<sup>+ve</sup> disease in particular. The “DNA-damaging drug” category effect will be diluted by the inclusion of drugs that could not harm human hematopoietic cells and the exclusion of others that could do so. The significant association in the presence of the diluting effect would be consistent with a much stronger association with a more restricted

Table 3 *Numbers of cases exposed to dipyrrone and Baygon/mosquitocidals*

	Dipyrrone (No. and percentages of cell exposed)				Baygon/mosquitocidals (No. and percentages of cell exposed)			
	ALL	AML	Other	Total	ALL	AML	Other	Total
MLL +ve	4 (14%)	5 (26%)	0 (/2)	9 (18%)	1 (3%)	4 (21%)	0 (/2)	5 (10%)
MLL -ve	1 (7%)	0 (/17)	0 (/3)	1 (3%)	0 (/15)	0 (/17)	0 (/3)	0
MLL status NK <sup>a</sup>	1 (3%)	0 (/13)	1 (12%)	1 (2%)	1 (7%)	1 (11%)	0 (/8)	2 (6%)
total	6 (8%)	5 (10%)	1 (8%)	10 (7%)	2 (3%)	5 (11%)	0 (/13)	7 (6%)

<sup>a</sup> NK, no diagnostic sample available or sample degraded.



class of drugs. A similar comment applies to pesticides and herbal medicines. Pesticides have frequently been associated with childhood leukemia and specifically with infants (either as occupational or home exposure and involving both parents and children; see Ref. 32 for review). The literature shows the same concentration of risk in AML cases as we have found. The reason for use was not often provided for herbal medicines, and substances were frequently "herbal teas." Camomile and many other herbal teas contain polyphenols (33). Three subjects who used herbal teas (all of them mothers of MLL<sup>+ve</sup> cases) indicated weight loss as their objective. Many herbal teas used to reduce weight contain a topo-II inhibitor, senna, in pharmacologically important quantities (34).

We found two statistically highly significant associations that may be of etiologic importance. These are for dipyrone and Baygon (or another usually unnamed substance used against mosquitoes). Dipyrone (or "Mexican aspirin") was widely used as an analgesic before it was found to cause agranulocytosis and is weakly mutagenic in mice (35). It is a pyrazolone derivative (35) not presently licensed for use in the United Kingdom or the United States. Other pyrazolone derivatives include phenylbutazone, which has been classified by the IARC as a possible human carcinogen (36). Dipyrone use in pregnant women in Brazil has been associated with Wilms' tumor (37). Mothers in Brazil and Chile as well as in Italy and Egypt reported dipyrone use. Baygon is the brand name for the carbamate pesticide, propoxur. Another carbamate pesticide has been shown to be detrimental to pregnancy outcome in rats when administered at the beginning or in the middle of pregnancy (38, 39). The developmental role of the *MLL* gene implies that exposures causing MLL<sup>+ve</sup> leukemia would also be likely to cause adverse pregnancy outcome. It is known that some mosquitocidals inhibit topo-II. Propoxur is a phenol-liberating substance (40) and mosquitocidal. Because some mosquitocidals and many phenolics inhibit topo-II (see "Introduction"), it is plausible that any leukemogenic action of Baygon operates via topo-II.

Ross (4) has identified two important questions concerning the etiology of infant leukemia: (a) can topo-II inhibitors cause other *in utero* genetic damage and, hence, MLL<sup>-ve</sup> leukemia; and (b) for MLL<sup>+ve</sup> infant leukemia, is the etiology of ALL and AML the same? The present data support the hypothesis that MLL<sup>+ve</sup> infant leukemia has a distinct etiology, but the exposures on which we have focused appear to have stronger associations with AML; however, the numbers are extremely small.

This pilot study evaluated a broad range of exposures in diverse settings. We did not consider diet, although this is likely to be important (4, 41). Our study has supported the hypothesis that *in utero* exposure to chemicals causes MLL<sup>+ve</sup> infant leukemia and has generated specific hypotheses that require further testing. Exposure to dipyrone is widespread, particularly in Central and South America where it is available as an inexpensive, nonprescription drug. Mosquitocidals are similarly in general use in these same settings. Propoxur is also widely used against cockroaches, fleas, and similar pests. Therefore, it is important that the associations observed in this study are reevaluated in an extended case-control study. In view of the association we have reported recently (26) between MLL<sup>+ve</sup> infant leukemia and lack of function NQO1 alleles, screening for metabolic polymorphisms involved in carcinogen metabolism should also be included in subsequent studies.

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