

Life-time carcinogenicity bioassays of toluene given by stomach tube to Sprague-Dawley rats

Studi di cancerogenicità a lungo termine del toluene somministrato mediante sonda gastrica a ratti Sprague-Dawley

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Summary

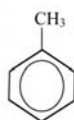
Toluene was administered by stomach tube, in olive oil solution, to male and female Sprague-Dawley rats, 7 weeks old at the start of the experiment, at a concentration of 0, 500, 800 mg/kg b.w., 4 days weekly, for 104 weeks; the animals were kept under observation until natural death or 130 weeks (Experiment BT 903 and BT 910). Overall, in our experimental conditions, toluene has been shown to cause an increase in: 1) total malignant tumours; 2) malignant tumours of the subcutaneous tissue; 3) malignant mammary tumours; 4) carcinomas of the oral cavity, lips and tongue; and 5) haemolymphoreticular neoplasias. On the basis of these data, toluene should be considered a potential carcinogenic agent for exposed populations. Eur. J. Oncol., 9 (2), 91-102, 2004

Key words: toluene, carcinogenicity, life-time bioassay, rat

Introduction

Toluene (methyl-benzene), produced during petroleum refining operations, is a clear, colourless, volatile and flammable liquid with a sweet, pungent, benzene-like odour.

Toluene (C₇H₈) has a molecular weight of 92.15 and its structural formula is:



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Riassunto

Il toluene è stato somministrato per sonda gastrica, in una soluzione di olio d'oliva, a ratti Sprague-Dawley, maschi e femmine, di 7 settimane di età all'inizio dell'esperimento, alle concentrazioni di 0, 500, 800 mg/kg p.c., 4 giorni alla settimana per 104 settimane; gli animali sono stati tenuti sotto osservazione fino a morte spontanea o 130 settimane (Esperimento BT 903 e BT 910). Complessivamente nelle nostre condizioni sperimentali, è stato dimostrato che il toluene provoca un aumento di: 1) tumori maligni totali; 2) tumori maligni del tessuto sottocutaneo; 3) tumori mammari maligni; 4) carcinomi del cavo orale, labbra e lingua; e 5) neoplasie emolinforeticolari. Sulla base di questi dati, il toluene dovrebbe essere considerato un potenziale agente cancerogeno per le popolazioni esposte. Eur. J. Oncol., 9 (2), 91-102, 2004

Parole chiave: toluene, cancerogenicità, studio a lungo termine, ratto

Toluene was discovered in 1835 by Pelletier and Walter as a degradation product obtained from heating the natural resin, balsam of Tolu, named from a small town in Colombia, South America. Until the Second World War, toluene was obtained primarily as a by-product of coke production. The need for large quantities of toluene in the production of trinitrotoluene (a constituent of many explosives) during the First World War, fostered its production from petroleum for the first time; the production was further increased during the Second World War¹.

In 1996 worldwide production of toluene was nearly 18 million tonnes, of which nearly 7 million tonnes were produced in the USA². Production capacity for toluene in Italy in 1994 was reported to be more than 495,000 tonnes³.

However, additional quantities of toluene are used with benzene and xylene to enhance the octane number of gasoline. It has been reported that in the 1980s more than 30 million tonnes of

toluene were consumed annually as a constituent of motor fuels⁴. Due to the declining use of methyl-tertiary-butyl ether (MTBE) as a fuel oxygenated additive, because of its known groundwater contamination and rodent carcinogenicity⁵⁻⁸, the production of toluene, blended with benzene and xylene to enhance gasoline octane numbers, will very likely increase. Data on other uses and the occurrence of toluene are given in Tables 1 and 2, respectively.

When human volunteers were exposed by inhalation to low levels of toluene, approximately 50% of the inhaled toluene was absorbed¹⁸. When toluene is administered orally, it is virtually completely absorbed from the gastrointestinal tract¹⁹.

Toluene is metabolized to benzyl alcohol, which in turn is oxidised to benzaldehyde and subsequently to benzoic acid. Most benzoic acid is converted to hippuric acid, which is excreted in urine at a concentration of several grammes per litre²⁰. Hippuric acid in urine is often used as a biomarker of exposure²⁰.

In cytogenetic studies, performed in occupationally exposed populations, increases in chromosomal aberrations, micronuclei and DNA strand breaks, have been described²¹. These effects have also been observed in rats and mice in some studies, and in cultured mammalian cells²¹.

In an inhalation study, Fisher-344 rats (F344) were exposed to 0, 30, 100 or 300 ppm of toluene for 24 months. After that, the animals still alive were killed and necropsied. The results of this experiment did not show any evidence that toluene causes carcinogenic effects in F344 rats at the concentrations used²². As these industry exposures were quite low, NTP continued with their higher exposure studies²³.

Long-term inhalation carcinogenicity bioassays on toluene were performed by the National Toxicology Program on rats and mice. Groups of 60 male and 60 female Fisher-344/N rats were exposed at 0, 600 or 1200 ppm of toluene, 6.5 hours/day, 5 days/week. Groups of 60 male and 60 female B6C3F1 mice were exposed at 0, 120, 600 or 1200 ppm with the same schedule. In both experiments the exposure to toluene lasted 103 weeks. After that, animals still alive were killed and necropsied. No significant increase in the incidence of neoplasms was observed in either experiment^{23, 24}.

Epidemiological studies conducted on industrial groups exposed to toluene have been reported. Consistent findings have been reported regarding the increased risk of cancers of the gastrointestinal tract, namely oesophagus, colon and rectum^{25, 26}. Increased mortality for cancer of the bone and connective tissue has been reported among rotogravure printing plant workers²⁷. A sig-

Table 1 - Toluene: uses^a

- Most toluene, mixed with benzene and xylene, is used in gasoline blending.
- The largest single use of isolated toluene is in the production of benzene.
- The second largest use of toluene is in solvent applications, especially in the paint and coating industry and also in inks, adhesives and the leather industry.
- Isolated toluene is also used in several consumer products, such as sanitizing agents, household aerosols, paints and varnishes, paint thinner and antirust preservatives.
- It is also used as salt for food preservatives and cosmetic articles such as soaps, perfume flavors, creams and lotions.
- Other important chemical products made from toluene include: trinitrotoluene and related explosives, benzaldehyde and saccharin.

^a From IARC⁹

Table 2 - Toluene: occurrence

Natural sources
Toluene occurs in nature in crude oil¹⁰, natural gas deposits and volatile emissions from volcanoes and forest fires¹¹.

Workplaces
Occupational exposures to toluene may occur in printing plants, trapezoid belt manufacturing, waste incinerators, plastic processing factories, rubber tyre vulcanisation, leather finishing, laboratories of histopathology and cytopathology, lithography, factories producing photographic albums and tarpaulins, fibrous glasswood plants, rubber coating plants; laminated kitchen counter, bathroom top and rubber sheet manufacturing, as well as among parquet floorers and shoemakers⁹.

- Air*
- Worldwide atmospheric emissions of toluene have been estimated to be 6.2 million tonnes; and the major contributions include: loss from refineries (40%), automobile exhaust (32%), solvents (16%), petroleum losses at sea (8%)¹². According to the Environmental Protection Agency, in 2000, it was estimated that 38,000 tonnes of toluene were released into the environment in the USA, with most (37,000 tonnes) being released into the air¹³.
 - Toluene is transported rapidly from water, where it has low solubility, into the atmosphere.
 - In the vicinity of an automobile painting plant, levels of 0.06-0.6 mg/m³ have been detected 1.6-16.5 km downwind of the painting facilities, as compared with 0.006 mg/m³ upwind¹⁴.

- Water*
- The concentration of toluene in rainwater in the Federal Republic of Germany is reported to be 0.13-0.70 µg/l^{10,14}.
 - Levels of 42-100 µg/l have been reported in well water in the vicinity of landfill sites in the USA¹⁰.
 - It has been found at concentrations of 1-5 µg/l in water samples from a number of rivers in eastern and mid-western USA; concentrations ranging up to 12 µg/l have been found in the Mississippi River near New Orleans; a concentration of 0.8 µg/l has been reported in the Rhine River in the Federal Republic of Germany, and of 1.9 µg/l in Switzerland⁴.

- Other*
- Toluene exists in an absorbed state in soil; biodegradation by microorganisms in the soil ranged from 63-86% after 20 days^{10, 15, 16}.
 - Toluene concentrations of <1 mg/kg in 56 out of 59 samples of fish tested have been reported¹⁰.
 - Toluene was also detected at a concentration of 0.08-0.11 mg/kg in a few samples of maple syrup packaged in plastic containers¹⁷.

nificant increase in leukaemia was observed among workers of the chemical manufacturing industry in Shanghai²⁸. The authors noted that the risk of leukaemia could also be related to the co-existence of exposure to benzene as a contaminant or constituent of many industrial solvents²⁸. In an IARC summary of epidemiological findings, toluene exposure was mentioned in eight studies. However, considering the multiple exposure circumstances in most studies, and the weak consistency of findings, these results were not considered strong enough to conclude that there is an association between exposure to toluene and risk of cancer in humans²¹.

The available data on the toxicity and carcinogenicity of toluene have recently been reviewed and discussed extensively by Huff²⁴.

In 1981, in the framework of an integrated experimental project to evaluate the comparative carcinogenic effects of toluene and other similar aromatic solvents, namely benzene, xylenes,

and ethylbenzene, a long-term bioassay was started on toluene, administered by ingestion (stomach tube) to Sprague-Dawley rats, at a dose of 500 mg/kg b.w. (Experiment BT 903). Preliminary results, 92 weeks on from the start of the experiment, were published in 1983²⁹, and final results were summarized and published in 1985³⁰ and in 1997³¹.

In 1984, a second integrated project of long-term experiments was planned to measure the comparative carcinogenic effects between the same aromatic solvents and toluene, which was administered by ingestion to Sprague-Dawley rats at a dose of 800 mg/kg b.w. (Experiment BT 910). Some final results were published in 1997³¹.

The aim of this report is to refer more extensively all available information on both experiments performed in our laboratory on toluene, and to discuss the discrepancy with the results of other long-term bioassays on toluene as performed in other laboratories.

Materials and methods

Toluene was supplied by an Italian petrochemical industry. The analytical characterization of toluene resulted in percentages, w/w, as follows: toluene: 98.34; paraffin: 0.83; benzene: 0.28; ethylbenzene: 0.52; m-xylene and p-xylene: 0.029. Extra virgin olive oil was used as a carrier of the compound.

Male (M) and female (F) Sprague-Dawley rats were used from the colony of the Cancer Research Centre (CRC) of the Ramazzini Foundation (RF). This colony of rats has been employed for various experiments in the CRC/RF Laboratory for nearly 30 years. Historical data are available on about 15,000 historical controls, kept under observation for their life-span and submitted to systematic necropsies and standardized histopathological examinations. Data are thus available on the expected incidence of, and fluctuation in, the different types of tumour in control animals.

After weaning, at 4-5 weeks of age, the experimental animals were identified by ear punch, randomised in order to have no more than one male and one female of each litter in the same group, and housed in groups of 5 in makrolon cages (41 x 25 x 15 cm) with stainless-steel wire tops; a shallow layer of white woodshavings served as bedding. The animals were kept in one single room, at 23 ± 2°C and 50-60% relative humidity.

Both experiments were performed according to the Good Laboratory Practices (GLP) and Standard Operating Procedures (SOP) of the CRC/RF.

The plans of the experiments are shown in Tables 3 and 4.

In the first experiment, toluene was administered by gavage in extra virgin olive oil solution, 4 days weekly (Monday and Tuesday; Thursday and Friday), for 104 weeks, at a dose of 500 or 0 mg/kg b.w. (Exp. BT 903). Experiment BT 903 was started in January 1981 and the biophase ended after 141 weeks with the spontaneous death of the last animal at the age of 148 weeks.

In the second experiment, toluene was administered in the same way at doses of 800 or 0 mg/kg b.w. (Exp. BT 910). Experiment BT 910 started in May 1984 and the biophase ended after 123 weeks, with the sacrifice of the animals still alive. Experiment BT 910 was truncated at this stage because the treatment markedly decreased male and female survivals compared to the control group. The solutions of both experiments were prepared weekly and stored at 4 °C. Control animals were given 1 ml of

Table 3 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube to male (M) and female (F) Sprague-Dawley rats (Exp. BT 903): plan of the experiment

Group No.	Dose (mg/kg b.w. in olive oil)	Age at start (weeks)	Animals	
			Sex	No.
I	500	7	M	40
			F	40
			M+F	80
II	0 ^a (control)	7	M	50
			F	50
			M+F	100

^a Olive oil alone

Table 4 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube to male (M) and female (F) Sprague-Dawley rats (Exp. BT 910): plan of the experiment

Group No.	Dose (mg/kg b.w. in olive oil)	Age at start (weeks)	Animals	
			Sex	No.
I	800	7	M	50
			F	50
			M+F	100
II	0 ^a (control)	7	M	50
			F	50
			M+F	100

^a Olive oil alone

olive oil alone. The animals of both experiments were kept under observation until death, under highly standardized housing and diet conditions. Individual animal weight was measured every 2 weeks from 7 weeks until 111 weeks of age, and then every 8 weeks until the end of the experiments. Animal status and behaviour were examined 3 times daily and the animals were examined every 2 weeks throughout experiments in order to detect and register all gross lesions.

Upon death, animals underwent systematic necropsy. Histopathology was routinely performed on the following organs and tissues: the brain, pituitary gland, Zymbal glands, submaxil-

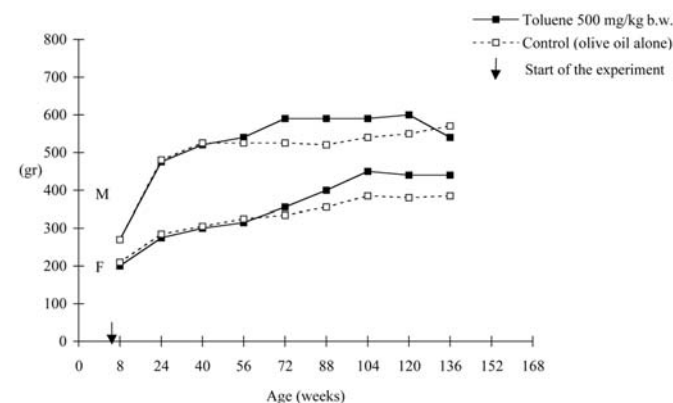


Fig. 1. Mean body weight in male (M) and female (F) Sprague-Dawley rats (Exp. BT 903)

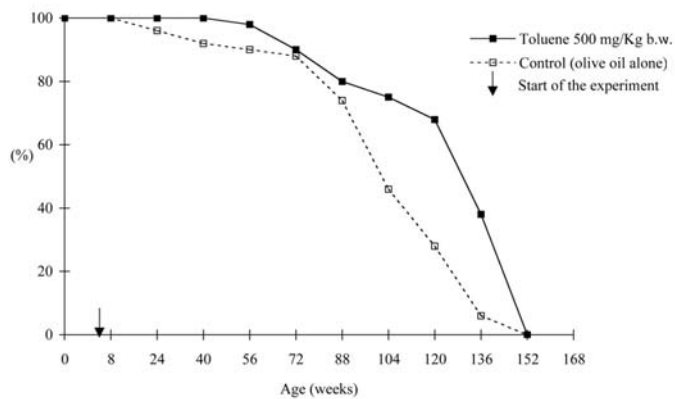


Fig. 2. Survival in male Sprague-Dawley rats (Exp. BT 903)

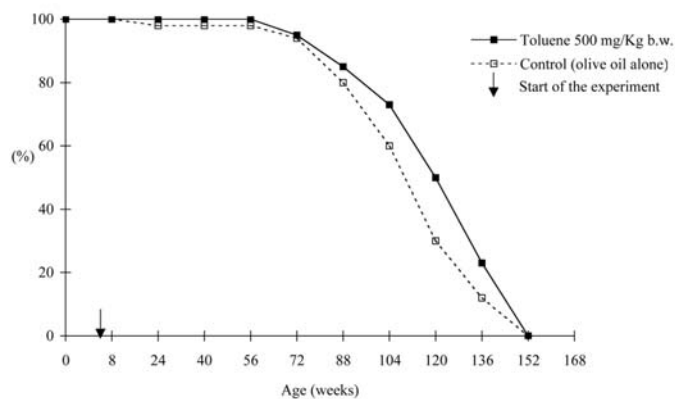


Fig. 3. Survival in female Sprague-Dawley rats (Exp. BT 903)

Table 5 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube, to male (M) and female (F) Sprague-Dawley rats (Exp. BT 903). Results: number and percentage of male and female Sprague-Dawley rats bearing various types of benign and malignant tumours ^a

Site	Histotype	Groups								
		I: 500 mg/kg b.w.				II: 0				
		Male		Female		Male		Female		
No.	%	No.	%	No.	%	No.	%			
Skin	Acanthoma	1	2.5	0	-	0	-	0	-	
	Dermatofibroma	0	-	0	-	1	2.0	0	-	
	Papilloma	0	-	0	-	0	-	1	2.0	
	Squamous cell carcinoma	1	2.5	0	-	0	-	1	2.0	
Subcutaneous tissue ^b	Fibroma	2	5.0	0	-	1	2.0	0	-	
	Lipoma and fibrolipoma	3	7.5	1	2.5	1	2.0	0	-	
	Liposarcoma	2	5.0	0	-	0	-	0	-	
	Rhabdomyosarcoma	1	2.5	0	-	0	-	0	-	
Mammary glands ^c	Adenoma	0	-	1	2.5	0	-	0	-	
	Fibroma and fibroadenoma	2	5.0	27 (47)	67.5	2 (3)	4.0	17 (29)	34.0	
	Adenocarcinoma	1	2.5	7 (9)	17.5	0	-	7	14.0	
	Fibrosarcoma	0	-	3	7.5	0	-	0	-	
Zymbal glands	Carcinoma	1	2.5	0	-	1	2.0	0	-	
Nasal cavities	Carcinoma	0	-	1	2.5	0	-	0	-	
Oral cavity and lips	Acanthoma	2	5.0	1	2.5	2	4.0	0	-	
	Carcinoma	0	-	1	2.5	0	-	0	-	
Lung	Adenoma	1	2.5	1	2.5	0	-	0	-	
Stomach	- Forestomach	Acanthoma	1	2.5	0	-	0	-	0	-
Liver	Hepatoma	0	-	1	2.5	0	-	1	2.0	
	Cholangioma	0	-	1	2.5	1	2.0	0	-	
	Hepatocarcinoma	2	5.0	0	-	3	6.0	0	-	
Pancreas	Islet cell adenoma	5	12.5	2	5.0	0	-	0	-	
Kidneys	Nephroblastoma	0	-	0	-	1	2.0	0	-	
Pelvis	Squamous cell carcinoma	0	-	1	2.5	0	-	0	-	
Prostate	Squamous cell carcinoma	1	2.5			0	-			
Testes	Interstitial cell adenoma	2	5.0			1	2.0			
	Embryonal carcinoma	1	2.5			0	-			

(Table 5 continued)

Table 5 cont.

Site	Histotype	Groups							
		I: 500 mg/kg b.w.				II: 0			
		Male		Female		Male		Female	
No.	%	No.	%	No.	%	No.	%		
Ovaries	Granulosa cell tumour			2	5.0			0	-
	Adenocarcinoma			1	2.5			0	-
Uterus	Polyp			5	12.5			3	6.0
	Squamous cell carcinoma			1	2.5			0	-
	Adenocarcinoma			1	2.5			0	-
Vagina	Fibrosarcoma			1	2.5			0	-
Peritoneum	Mesothelioma	1	2.5	0	-	0	-	0	-
Pituitary gland	Adenoma	2	5.0	13	32.5	0	-	6	12.0
Thyroid gland	Follicular carcinoma	2	5.0	1	2.5	0	-	1	2.0
	C-cell carcinoma	1	2.5	1	2.5	0	-	0	-
Adrenal glands	Cortical adenoma	1	2.5	5	12.5	0	-	2	4.0
	Pheochromocytoma	14 (19)	35.0	4 (6)	10.0	20 (28)	40.0	11 (14)	22.0
	Cortical adenocarcinoma	2	5.0	3 (4)	7.5	0	-	0	-
	Pheochromoblastoma	1	2.5	0	-	0	-	0	-
Nervous system									
	- Brain								
	- Meninges								
	Neuroblastoma	1	2.5	0	-	0	-	0	-
	Oligodendroglioma	1	2.5	1	2.5	1	2.0	1	2.0
	Benign meningioma	0	-	2	5.0	1	2.0	0	-
Bones									
- Head	Osteoma	0	-	0	-	1	2.0	0	-
- Other	Osteosarcoma	1	2.5	0	-	0	-	0	-
	Osteochondrosarcoma	0	-	0	-	1	2.0	0	-
Soft tissues	Myxoma	0	-	0	-	1	2.0	0	-
Thymus	Squamous cell carcinoma	0	-	0	-	1	2.0	0	-
Spleen	Fibroangioma	1	2.5	0	-	0	-	1	2.0
Mediastinal lymph nodes	Angioma	0	-	0	-	1	2.0	0	-
Mesenteric lymph nodes	Fibroangioma	1	2.5	0	-	0	-	0	-
Haemolympho- reticular tissues ^d	Benign histiocytoma	1	2.5	0	-	0	-	0	-
	Lymphomas and leukaemias	3	7.5	7	17.5	3	6.0	1	2.0

^a Between brackets the number of tumours (one animal can bear more than one tumour)

^b See table 7

^c See table 8

^d See table 9

lary glands, Harderian glands, cranium (with oral and nasal cavities and external and internal ear ducts), tongue, pharynx, larynx, thymus and mediastinal lymph nodes, lung and mainstem bronchi, diaphragm, liver, spleen, pancreas, kidneys, adrenal glands, oesophagus, stomach (fore and glandular), intestine (three levels), urinary bladder, prostate, gonads, interscapular fat pad, subcutaneous and mesenteric lymph nodes and any other organs or tissues with pathological lesions.

All organs and tissues were preserved in 70% ethyl alcohol, except for the bones which were fixed in 10% formalin and then decalcified with 10% formaldehyde and 20% formic acid in water solution. The normal specimens were trimmed, following SOP at the CRC/RF Laboratory: i.e. parenchymal organs were dissect-

ed through the hilus to expose the widest surface, and hollow organs were sectioned across the greatest diameter(s). Any pathological tissue was trimmed through the largest surface, including normal adjacent tissue. Trimmed specimens were processed as paraffin blocks, and 3-5 micron sections of every specimen were obtained. Sections were routinely stained with haematoxylin-eosin. Specific stainings were performed when needed. All slides were examined microscopically by the same group of pathologists; a senior pathologist reviewed all tumours and any other lesion of oncological interest. All pathologists followed the same criteria of histopathological evaluation and classification. Multiple tumours of different type and site, or of different type in the same site, or of the same type in bilateral organs, or at distant sites

Table 6 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube, to male (M) and female (F) Sprague-Dawley rats (Exp. BT 903). Results: total malignant tumours

Group No.	Dose (mg/kg b.w. in olive oil)	Animals		Malignant tumours			
		Sex	No.	Tumour-bearing animals		Tumours	
				No.	%	No.	Per 100 animals
I	500	M	40	18	45.0*	23	57.5**
		F	40	20	50.0**	33	82.5**
		M+F	80	38	47.5	56	70.0
II	0 ^a (control)	M	50	10	20.0	11	22.0
		F	50	10	20.0	11	22.0
		M+F	100	20	20.0	22	22.0

^a Olive oil alone* Statistically significant (p < 0.05) using χ^2 test** Statistically significant (p < 0.01) using χ^2 test

** Statistically significant (p < 0.01) using Fisher-Snedecor test, df = 89

Table 7 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube, to male (M) and female (F) Sprague-Dawley rats (Exp. BT 903). Results: tumours of the subcutaneous tissue

Group	Dose (mg/kg b.w. in olive oil)	Animals		Benign tumours				Malignant tumours			
		Sex	No.	Tumour-bearing animals		Tumours		Tumour-bearing animals		Tumours	
				No.	%	No.	Per 100 animals	No.	%	No.	Per 100 animals
I	500	M	40	5	12.5	5	12.5	3	7.5	3	7.5*
		F	40	1	2.5	1	2.5	0	-	0	-
		M+F	80	6	7.5	6	7.5	3	3.8	3	3.8
II	0 ^a (control)	M	50	2	4.0	2	4.0	0	-	0	-
		F	50	0	-	0	-	0	-	0	-
		M+F	100	2	2.0	2	2.0	0	-	0	-

^a Olive oil alone

* Statistically significant (p < 0.05) using Fisher-Snedecor test, df = 89

Table 8 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube, to male (M) and female (F) Sprague-Dawley rats (Exp. BT 903). Results: malignant mammary tumours

Group No.	Dose (mg/kg b.w. in olive oil)	Animals		Malignant mammary tumours			
		Sex	No.	Tumour-bearing animals		Tumours	
				No.	%	No.	Per 100 animals
I	500	M	40	1	2.5	1	2.5
		F	40	10	25.0	12	30.0
		M+F	80	11	13.8	13	16.3
II	0 ^a (control)	M	50	0	-	0	-
		F	50	7	14.0	7	14.0
		M+F	100	7	7.0	7	7.0

^a Olive oil alone

of diffuse tissue (i.e. bones, skeletal muscle, etc.), were plotted as single/independent tumours. Multiple tumours of the same type in the same tissue and organ (including those of the bilateral organs) were plotted only once.

Statistical analysis was performed using the χ^2 , Fisher-Snedecor and Log-rank tests, in order to evaluate the level of significance in tumour incidence differences, between treated and control group. In particular, the last method assumes lethality of

Table 9 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube, to male (M) and female (F) Sprague-Dawley rats (Exp. BT 903). Results: haemolymphoreticular neoplasias

Group No.	Dose (mg/kg b.w. in olive oil)	Animals			Animals with haemolymphoreticular neoplasias		
		Sex	No.	Corrected number	No.	% ^a	% ^b
I	500	M	40	34 ^c	3	7.5	8.8
		F	40	39 ^d	7	17.5*	17.9*
		M+F	80	73	10	12.5	13.7
II	0 ^e (control)	M	50	39 ^c	3	6.0	7.7
		F	50	49 ^d	1	2.0	2.0
		M+F	100	88	4	4.0	4.5

^a Percentages refer to the number at start

^b Percentages refer to the corrected number

^c Alive male rats at 75 weeks of age, when the first leukaemia was observed

^d Alive female rats at 60 weeks of age, when the first leukaemia was observed

^e Olive oil alone

* Statistically significant ($p < 0.05$) using χ^2 test

† Statistically significant ($p < 0.05$) using Log-Rank test

the tumour and the statistical method is a log-rank test as described in Mandel³² and Cox³³.

Results

1. Experiment BT 903

Mean body weight was slightly increased in male and female treated rats compared to controls from 56 and 88 weeks of age, respectively (fig. 1).

A decreased survival was observed in control males and females from 88 weeks of age until the end of the experiment (figs. 2 and 3). The difference was more evident in males.

The occurrence of benign and malignant tumours is shown in Table 5. Differences observed between treated and control animals were: 1) an increase in total malignant tumours in male and female treated rats (Table 6); 2) an increase in subcutaneous malignant tumours among treated males (Table 7); 3) an increased incidence of malignant mammary tumours in treated females (Table 8); 4) an increased incidence of haemolymphoreticular neoplasias in treated females (Table 9).

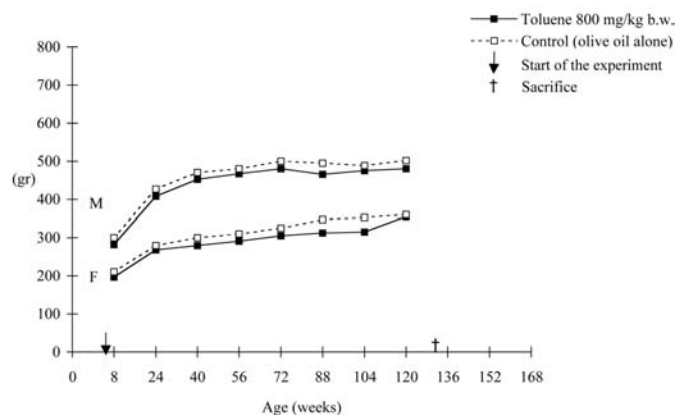


Fig. 4. Mean body weight in male (M) and female (F) Sprague-Dawley rats (Exp. BT 910)

2. Experiment BT 910

No substantial differences were observed in mean body weight between treated and control rats (fig. 4).

An increase in mortality among male and female treated rats was observed from 24 until 130 weeks of age (figs. 5 and 6), when it was decided to truncate the experiment.

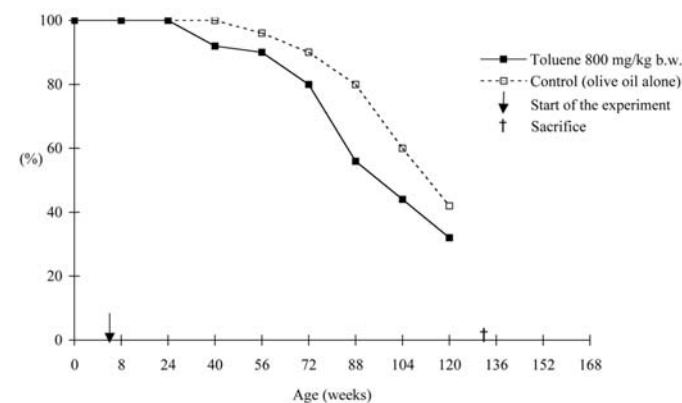


Fig. 5. Survival in male Sprague-Dawley rats (Exp. BT 910)

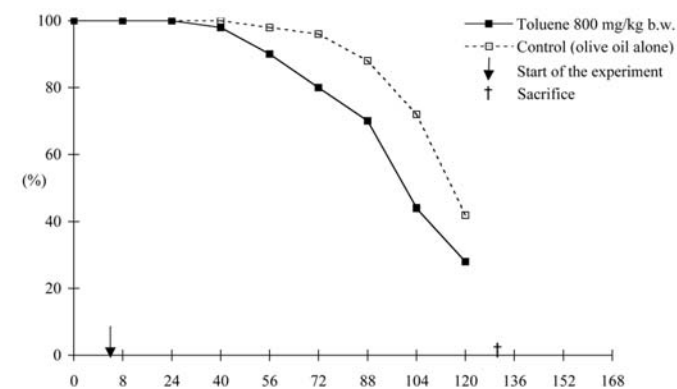


Fig. 6. Survival in female Sprague-Dawley rats (Exp. BT 910)

Table 10 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube, to male (M) and female (F) Sprague-Dawley rats (Exp. BT 910). Results: number and percentage of male and female Sprague-Dawley rats bearing various types of benign and malignant tumours ^a

Site	Histotype	Groups							
		I: 800 mg/kg b.w.				II: 0			
		Male		Female		Male		Female	
No.	%	No.	%	No.	%	No.	%		
Skin	Acanthoma	0	-	0	-	1	2.0	0	-
	Dermatofibroma	1	2.0	0	-	0	-	0	-
Subcutaneous tissue	Fibroma	0	-	1	2.0	0	-	0	-
	Fibroangioma	0	-	0	-	1	2.0	1	2.0
	Fibrosarcoma	1	2.0	0	-	0	-	0	-
	Leiomyosarcoma	0	-	0	-	1	2.0	0	-
Mammary glands	Fibroma and fibroadenoma	0	-	11 (15)	22.0	2	4.0	23 (31)	46.0
	Adenocarcinoma	0	-	2	4.0	0	-	2	4.0
	Carcinosarcoma	0	-	0	-	0	-	1	2.0
	Fibrosarcoma	0	-	2	4.0	0	-	0	-
Zymbal glands	Squamous cell carcinoma	0	-	0	-	1	2.0	1	2.0
Ear ducts	Acanthoma	0	-	0	-	1	2.0	0	-
	Squamous cell carcinoma	1	2.0	0	-	0	-	1	2.0
Nasal cavities	Papilloma	0	-	1	2.0	0	-	0	-
	Squamous cell carcinoma	1	2.0	0	-	0	-	0	-
Oral cavity, lips and tongue ^b	Squamous cell carcinoma	7	14.0	3	6.0	0	-	1	2.0
Stomach - Forestomach	Acanthoma	0	-	0	-	1	2.0	0	-
	Squamous cell carcinoma	0	-	1	2.0	0	-	0	-
Liver	Hepatoma	0	-	1	2.0	2	4.0	0	-
	Cholangioma	0	-	1	2.0	0	-	0	-
	Hepatocarcinoma	0	-	0	-	2	4.0	0	-
Pancreas	Islet cell adenoma	2	4.0	1	2.0	2	4.0	1	2.0
Kidneys	Adenoma	0	-	1	2.0	0	-	0	-
	Adenocarcinoma	0	-	0	-	1	2.0	0	-
Testes	Interstitial cell adenoma	2 (3)	4.0			4 (5)	8.0		
Ovaries	Theca cell tumour			1	2.0			0	-
	Sertoli cell tumour			2 (3)	4.0			1	2.0
	Carcinosarcoma			0	-			1	2.0
	Malignant granulosa cell tumour			1	2.0			0	-
Uterus	Polyp			7	14.0			2	4.0
	Leiomyosarcoma			0	-			1	2.0
	Angiosarcoma			1	2.0			0	-
Pituitary gland	Adenoma	1	2.0	11	22.0	6	12.0	24	48.0
Thyroid gland	Adenoma	0	-	0	-	0	-	2	4.0
	C-cell carcinoma	0	-	2	4.0	1	2.0	0	-
Adrenal glands	Cortical adenoma	0	-	2	4.0	0	-	3	6.0
	Pheochromocytoma	15 (23)	30.0	7 (10)	14.0	11 (18)	22.0	9(14)	18.0
	Cortical adenocarcinoma	0	-	2	4.0	0	-	0	-
	Pheochromoblastoma	2	4.0	1	2.0	0	-	0	-
Nervous system - Brain - Meninges	Oligodendroglioma	0	-	2	4.0	0	-	0	-
	Fibrosarcoma	0	-	1	2.0	0	-	0	-

(Table 10 continued)

The occurrence of benign and malignant tumours is shown in Table 10. Differences observed between treated and control animals were: 1) an increased incidence of total malignant tumours in

treated male and female rats (Table 11); 2) an increased incidence of carcinomas of the oral cavity, lips and tongue in male and female treated animals compared to controls (Table 12).

Table 10 cont.

Site	Histotype	Groups							
		I: 800 mg/kg b.w.				II: 0			
		Male		Female		Male		Female	
No.	%	No.	%	No.	%	No.	%		
Bones									
- Head	Osteosarcoma	1	2,0	1	2,0	0	-	0	-
Soft tissues	Lipoma	0	-	0	-	0	-	1	2,0
	Liposarcoma	0	-	0	-	2	4,0	0	-
Spleen	Fibroangioma	0	-	0	-	1	2,0	0	-
Mesenteric lymph nodes	Fibroangioma	0	-	0	-	0	-	1	2,0
Haemolymphoreticular tissues	Lymphomas and leukaemias	8	16,0	5	10,0	5	10,0	3	6,0

^a Between brackets the number of tumours (one animal can bear more than one tumour)

^b See table 12

Table 11 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube, to male (M) and female (F) Sprague-Dawley rats (Exp. BT 910). Results: total malignant tumours

Group No.	Dose (mg/kg b.w. in olive oil)	Animals		Malignant tumours			
		Sex	No.	Tumour-bearing animals		Tumours	
				No.	%	No.	Per 100 animals
I	800	M	50	20	40.0	21	42.0
		F	50	15	30.0	24	48.0
		M+F	100	35	35.0	45	45.0
II	0 ^a (control)	M	50	12	24.0	13	26.0
		F	50	11	22.0	11	22.0
		M+F	100	23	23.0	24	24.0

^a Olive oil alone

Table 12 - Long-term carcinogenicity bioassay on toluene, administered by stomach tube, to male (M) and female (F) Sprague-Dawley rats (Exp. BT 910). Results: carcinomas of the oral cavity, lips and tongue

Group No.	Dose (mg/kg b.w. in olive oil)	Animals			Animals with carcinoma		
		Sex	No.	Corrected number	No.	% ^a	% ^b
I	800	M	50	32 ^c	7	14.0*	21.9**
		F	50	27 ^d	3	6.0	11.1
		M+F	100	59	10	10.0	16.9
II	0 ^e (control)	M	50	42 ^c	0	-	-
		F	50	39 ^d	1	2.0	2.6
		M+F	100	83	1	1.0	1.2

^a Percentages refer to the number at start

^b Percentages refer to the corrected number

^c Alive male rats at 78 weeks of age, when the first carcinoma was observed

^d Alive female rats at 90 weeks of age, when the first carcinoma was observed

^e Olive oil alone

* Statistically significant ($p < 0.05$) using χ^2 test

** Statistically significant ($p < 0.01$) using Log-Rank test

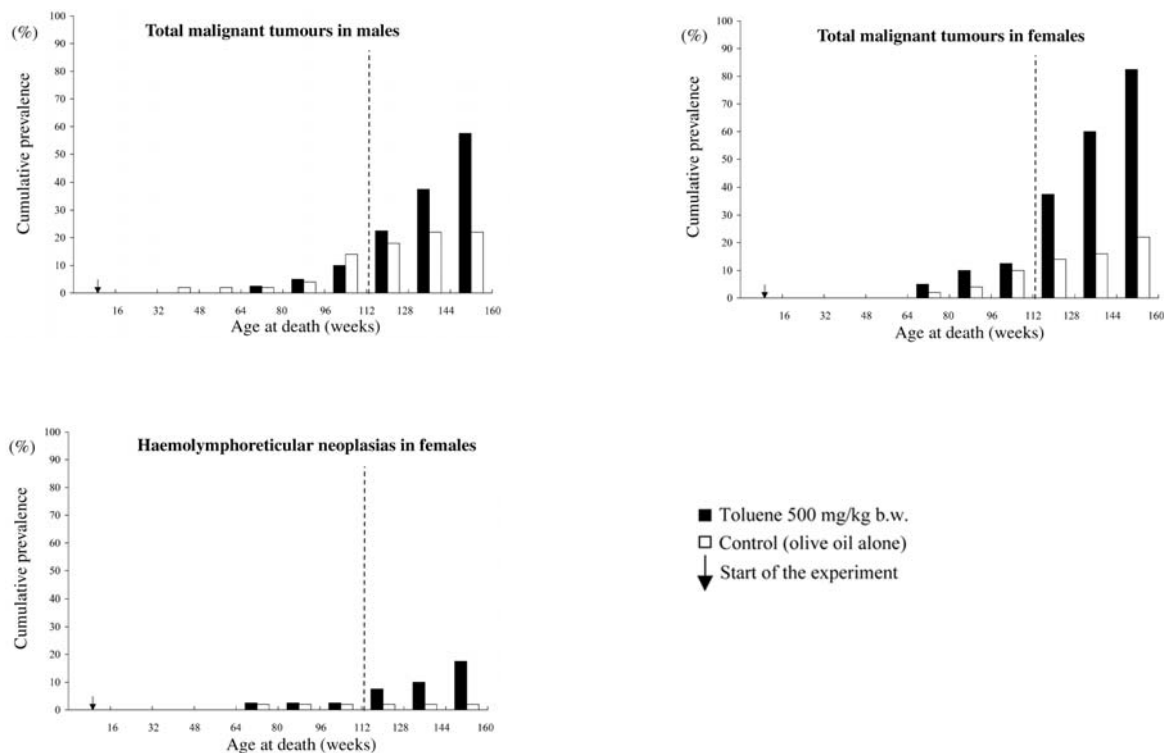


Fig. 7. Cumulative prevalence of total malignant tumours in males and females, and haemolymphoreticular neoplasias in females, histopathologically observed, by age at death, among the Sprague-Dawley rats treated with 500 mg/kg b.w. of toluene for 104 weeks, and then kept under control until spontaneous death (Exp. BT 903)

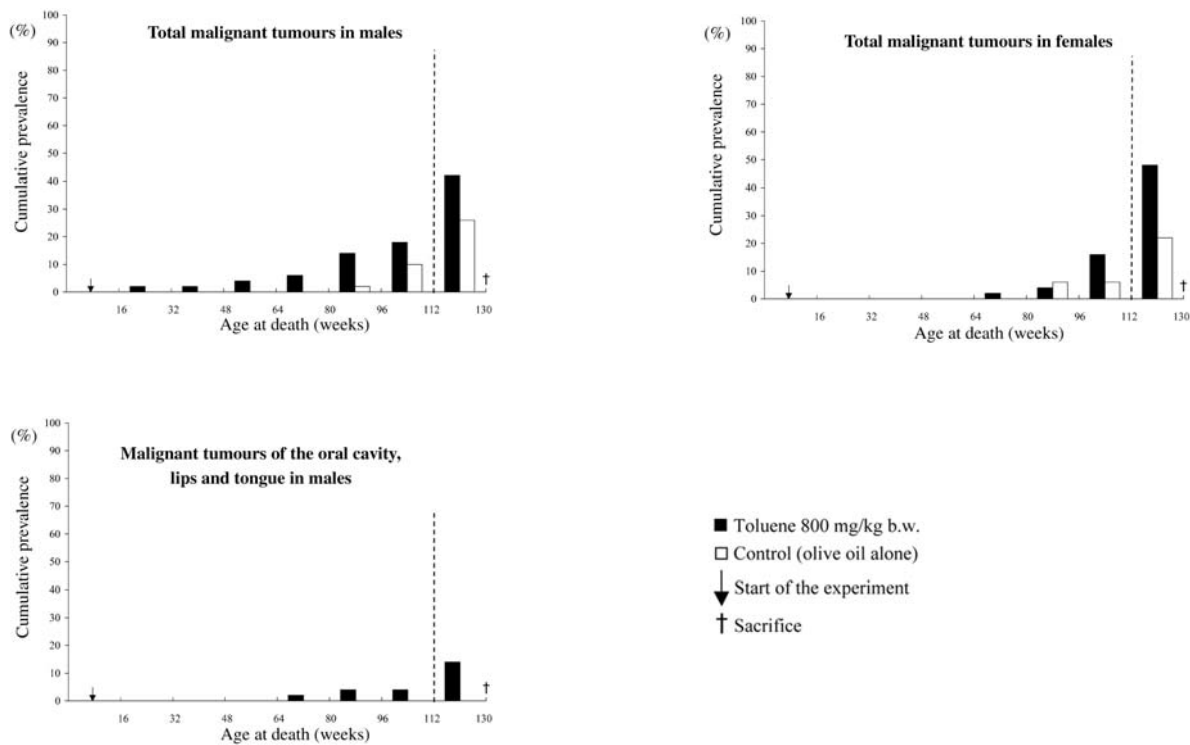


Fig. 8. Cumulative prevalence of total malignant tumours in males and females, and malignant tumours of the oral cavity, lips and tongue in males, histopathologically observed, by age at death, among the Sprague-Dawley rats treated with 800 mg/kg b.w. of toluene for 104 weeks, and kept alive until 130 weeks of age; then the animals still alive were killed (Exp. BT 910)

Conclusions

In our tested experimental conditions, toluene has shown carcinogenic effects in both experiments, performed sequentially at different periods and dose levels.

In the first experiment treatment with 500 mg/kg b.w. of toluene for 104 weeks (Exp. BT 903), and observing animals until spontaneous death (the last animal died at the age of 148 weeks), caused an increase in total malignant tumours in males and females, and in malignant mammary tumours and haemolymphoreticular neoplasias in females. An increase in malignant tumours of the subcutaneous tissue was also observed in males. Higher mortality was observed among male and female Sprague-Dawley rats of the control group.

In the second experiment (Exp. BT 910), toluene was administered at 800 mg/kg b.w. for 104 weeks and the animals were observed until 130 weeks of age, when the experiment was truncated because of the higher mortality observed in males and females of the treated group. In this experiment, toluene induced an increase in the incidence of total malignant tumours and carcinomas of the oral cavity, lips and tongue, in male and female treated rats.

The two long-term inhalation bioassays on toluene performed by the industry²² and the National Toxicology Program²³ did not show any carcinogenic effect of toluene. As Huff pointed out in his paper²⁴, there are several differences between the experiment performed by these studies and that of the Ramazzini Foundation, namely:

1. different species;
2. differences in routes of exposure: oral versus inhalation;
3. differences in "bolus" oral dosing versus more extended inhalation exposure;
4. different exposure concentrations of 500 and 800 mg/kg b.w. versus up to 1200 ppm (estimated as 1350 mg/kg b.w. for rats over a longer period);
5. differences in length of experiment; although in all these studies animals were exposed for 104 weeks, the Ramazzini Foundation studies continued without exposure for roughly another six months.

This last factor is in our opinion crucial to reveal all the carcinogenic potential of an agent. As was pointed out by Huff²⁴ and by us²⁵, the distinctive characteristic of the Ramazzini Foundation long-term carcinogenicity bioassays, namely to keep experimental animals under observation until natural death, could be the most influential factor in the difference between results. Had we truncated our toluene experiments at 112 weeks, as was done in the inhalation studies, we would never have appreciated the difference in tumour incidence between treated and control animals, and so failed to demonstrate the carcinogenicity of toluene. In fact, as shown in figures 7 and 8, the higher incidence of malignancies in treated animals was appreciated after 112 weeks of age.

References

1. Cier HE. Toluene. In Kirk-Othmer encyclopaedia of chemical technology. New York, Interscience Publishers, 1969.
2. Ozokwelu ED. Toluene. In Kir-Othmer encyclopaedia of chemical technology. New York, John Wiley & Sons, 1997.
3. Fabri R, Graeser U, Simo TA. Toluene. In Gerhartz W and Yamamoto YS, eds, Ullmann's encyclopedia of industrial chemistry, 5th rev. Ed. Weinheim VCH Publishers, 1996, A27: 147-57.
4. Merian E, Zander M. Volatile aromatics. In: Hutzinger O. Handbook of environmental chemistry, Vol. 3, Part B. Anthropogenic compounds. Berlin: Springer, 1982, 117-61.
5. United States National Library of Medicine. Hazardous substances data bank (HSDB), Bethesda, MD, record No. 131, 1997.
6. Belpoggi F, Soffritti M, Maltoni C. Methyl-tertiary-butyl ether (MTBE). A gasoline additive causes testicular and haematopoietic cancers in rats. Toxicol Ind Health 1995; 11: 119-49.
7. Belpoggi F, Soffritti M, Filippini F, *et al.* Results of long-term carcinogenicity of methyl-tertiary-butyl ether. Ann NY Acad Sci 1997; 837: 77-95.
8. Bird MG, Burleigh-Flayer HD, Chun JS, *et al.* Oncogenicity studies of inhaled methyl-tertiary-butyl ether (MTBE) in CD-1 mice and F-344 rats. J Appl Toxicol 1997; 17 (S1): S45-S55.
9. International Agency for Research on Cancer (IARC). Monographs on the evaluation of the carcinogenic risk of chemical to humans. Vol. 47. Some organic solvents, resin monomers and related compounds, pigments and occupational exposures in paint manufacture and painting. Lyon: IARC, 1989.
10. United States Environmental Protection Agency (US EPA). Health assessment document for toluene (Publ. No. PB84-100056). Washington, DC: US Department of Commerce, National Technical Information Service, 1983.
11. National Research Council. Vapor-phase organic pollutants. Washington, DC: National Academy of Sciences, 1976.
12. Merian E. The environmental chemistry of volatile hydrocarbons. Toxicol Environ Chem 1982; 5: 167-75.
13. United States Environmental Protection Agency (US EPA). Toluene. In Toxic Release Inventory (TRI) Chemicals. TRI on-site and off-site reported releases (in pounds), for facilities in all industries, for all chemicals, US 2000, available on <http://www.epa.gov/cgi-bin/broker>, 2003.
14. International Programme on Chemical Safety. Toluene (Environmental Health Criteria 52). Geneva: World Health Organization, 1985.
15. Wilson JT, Enfield CG, Dunlap WJ, *et al.* Transport and fate of selected organic pollutants in a sandy soil. J Environ Qual 1981; 10: 501-6.
16. Wilson JT, McNabb JF, Wilson RH, *et al.* Biotransformation of selected organic pollutants in ground water. Dev Ind Microbiol 1983; 24: 225-33.
17. Hollifield HC, Breder CV, Dannison JL, *et al.* Container-derived contamination of maple syrup with methyl methacrylate, toluene, and styrene as determined by headspace gas-liquid chromatography. J Assoc Off Anal Chem 1980; 63: 173-7.
18. Löf A, Wigaeus Hjelm E, Colmsjö A, *et al.* Toxicokinetics of toluene and urinary excretion of hippuric acid after human exposure to ²H₆-toluene. Br J Ind Med 1993; 50: 55-9.
19. Baelum J, Molhave L, Honoe-Hansen S, *et al.* Hepatic metabolism of toluene after gastrointestinal uptake in humans. Scand J Work Environ Health 1993; 19: 55-62.
20. Henderson HF. Aromatic hydrocarbons-benzene and other alkylbenzenes. Toluene. In Bingham E, Cohorsen B, Powell CH, Eds. Patty's toxicology. New York: John Wiley & Sons, 2001.
21. International Agency for Research on Cancer (IARC). Monographs on the evaluation of the carcinogenic risk of chemical to humans. Vol. 71. Part II. Re-evaluation of some organic chemicals, hydrazine and hydrogenperoxide. Lyon: IARC, 1999.
22. Gibson JE, Hardisty JF. Chronic toxicity and oncogenicity bioassay of inhaled toluene in Fisher-344 rats. Fundam Appl Toxicol 1983; 3: 315-9.
23. United States National Toxicology Program. Toxicology and carcinogenesis studies of toluene (CAS No. 108-88-3) in F344/N rats and B6C3F1 mice (inhalation studies) (NTP TR 371); NIH Publ. No. 90-2826, Research Triangle Park, NC, 1990.
24. Huff J. Absence of carcinogenic activity in Fisher rats and B6C3F1 mice following 103-week inhalation exposure to toluene. Int J Occup Environ Health 2003; 9: 138-46.
25. Gerin M, Semiatycki J, Desy M, *et al.* Associations between several sites of cancer and occupational exposure to benzene, toluene, xylene, and styrene: results of a case-control study in Montreal. Am J Ind Med 1998; 34: 144-56.
26. Goldberg MS, Parent ME, Semiatycki J, *et al.* A case-control study of the relationship between the risk of colon cancer in men and exposures to occupational agents. Am J Ind Med 2001; 39: 531-46.

27. Wiebelt H, Becker N. Mortality in a cohort of toluene exposed employees (rotogravure printing plant workers). *J Occup Environ Med* 1999; 41:1134-9.
28. Linet MS, Yin SN, Travis LB, *et al.* Clinical features of Haematopoietic malignancies and related disorders among benzene-exposed workers in China. *Environ Health Perspect* 1996; 104 (Suppl 6): 1353-1364.
29. Maltoni C, Conti B, Cotti G. Benzene: a multipotential carcinogen. Results of long-term bioassays performed at the Bologna Institute of Oncology. *Am J Ind Med* 1983; 4: 589-630.
30. Maltoni C, Conti B, Cotti G, *et al.* Experimental studies on benzene carcinogenicity at the Bologna Institute of Oncology: current results and ongoing research. *Am J Ind Med* 1985; 7: 415-46.
31. Maltoni C, Ciliberti A, Pinto C, *et al.* Results of long-term experimental carcinogenicity of the effects of gasoline, correlated fuels, and major gasoline aromatics on rats. In Bingham E & Rall DP, Eds. Preventive strategies for living in a chemical world. *Ann NY Acad Sci* 1997; 837: 15-52.
32. Mandel N. Evaluation of survival data on two new rank order statistics arising in its consideration. *Cancer Chemother* 1966; 50: 163-70.
33. Cox DR. Regression models and life-tables (with discussion). *J R Stat Soc* 1972; 24: 187-220.
34. Huff J. Chemicals studied and evaluated in long-term carcinogenesis bioassays by both the Ramazzini Foundation and the National Toxicology Program: in tribute to Cesare Maltoni and David Rall. *Ann N Y Acad Sci* 2002; 982: 208-30.
35. Soffritti M, Belpoggi F, Minardi F, Maltoni C. Ramazzini Foundation cancer program: history and major projects, life-span carcinogenicity bioassay design, chemicals studied, and results. *Ann N Y Acad Sci* 2002; 982: 26-45.