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Objectives, designs and populations of the European Asclepios study on occupational hazards to male reproductive capability

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The main objective of the Asclepios program was to examine occupational risk factors for the male reproductive system. The program focused on occupational exposure to *fungicides* (farmers, greenhouse workers, and vineyard workers), *styrene* (laminators in the reinforced plastics industry) and *inorganic lead* (battery workers, foundry workers, and lead smelters). Questionnaire studies of time to pregnancy were combined with longitudinal and cross-sectional studies of semen quality. The 8 data-collecting centers addressed 6553 male workers and contributed time-to-pregnancy values on the 3077 most recent pregnancies. Data collection was by interview or self-collection. The average response rate across all exposures and centers was 69.8%. The Asclepios project is the first international multicenter research project on environmental risks to male reproductive function. A protocol for epidemiologic research on occupational risk factors to the male reproductive system was developed, and links between epidemiologic and experimental units were established. The majority, but not all, of the studies was completed within the given time frame.

Key terms fecundity, fertility, germ cell genotoxicity, occupation, reproductive toxicity, semen quality, testis, time to pregnancy, workplace exposure.

Environmental and occupational hazards to human reproduction have become a prominent public health issue in several countries during the past 25 years (1—4). From focusing on teratogenic exposures following the Minamata and thalidomide disasters, attention has been directed towards the reproductive failure of men and women. Infertility, defined as the inability to conceive within 1 year, occurs in about 15% of cohabiting

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couples (5). It is assumed that approximately 40% of couple infertility is caused by male inability to present semen of adequate quality.

However, the identification of hazards to male fecundity, including occupational hazards to the male reproductive function, may have importance that reaches further than the prevention of infertility. Genetic damage of male germ cells induced by toxicants present at the workplace may not only cause early embryonal loss, but also emanation of a number of disorders in the offspring, for example, cancer, inherited diseases, and certain malformations (6—8). Such hazards may even bring about an insidious rise in the frequency of adverse recessive genes (carrier status).

The realm of male reproductive toxicology therefore also includes some negative pregnancy outcomes and certain disorders in the offspring. Besides, research involving sensitive biological markers may detect hazards early in the continuum between exposure and disease and enhance the possibility of control and prevention.

The male reproductive system in humans is known to be highly vulnerable to certain physical and chemical exposures. Testis is, for example, more sensitive to ionizing radiation and heat than any other tissue of the organism (1, 2, 4). Small amounts of some chemicals may impair the male reproductive function. The example *par excellence* is the brominated organochlorine nematocide DBCP (2,3-dibromo-3-chloropropane), with its pronounced toxic effect on human germinal epithelium even at exposure levels not associated with signs of clinical intoxication (9, 10). This pesticide causes severe oligospermia after a few months of low-level exposure (11). Compared with several animal species, the amount of sperm-germinating epithelium that can be destroyed without fecundity impairment is limited in man. Therefore environmental risks to male reproductive function may be of greater significance than recognized until now.

During the past 20 years more than 60 field studies have been conducted to unravel the effects of various occupational exposures on testicular function (1, 2, 4). By far most of these studies have taken place in the United States. Although no workplace exposure has been associated with effects comparable to that of DBCP, several studies indicate that certain chlorinated pesticides, inorganic lead, carbon disulfide, metal welding, and a great variety of other substances constitute hazards to male reproductive capacity [for a review, see reference 2].

In general it is not possible to derive strong causal inferences from these studies because of several design limitations. The cross-sectional approach is most often associated with considerable differential dropout and uncertainty with respect to the comparability of exposed and unexposed groups. Exposure assessments seldom allow for exposure-response relationships or details with respect to the nature of the relation to be accurately determined between exposure and outcome. Moreover, information on the effects of short-term peak exposure and long-term cumulative exposure is often lacking. It may be possible to overcome some of the major limitations of previous studies by choosing a longitudinal design with sampling before and during exposure and with an examination of intraindividual changes. The spermatogenic cycle lasts 72 days, and reactions to deleterious exposures are expected to be detectable within a period spanning from a few weeks to 12 months (12).

In recent years there have been several innovations in the laboratory tests available for the examination of semen samples (13, 14). A standardized examination of semen samples collected at different geographic locations is now feasible. With the use of computerized video-imaging techniques all *conventional semen quality parameters* can be determined from fresh smears prepared and video-recorded at a local center (15). Besides standardized quantification of the number of spermatozoa, it is possible to obtain an approximated objective description of motility as the percentages of immotile, locally motile, and progressively motile spermatozoa. Motility patterns can also be characterized in terms of velocity and linearity. Sperm density and motility are considered the most important semen parameters used to predict fecundity.

The *flow-cytometric sperm-chromatin-structure assay* reflects alteration in the chromatin packaging process and sperm maturation (16). Poor-quality sperm-chromatin structure appears to be highly indicative of male subfecundity and may provide information which cannot be obtained by conventional sperm analysis.

The existing knowledge on the genotoxic effects of occupational hazards to the human germ-cell line is very limited, possibly due to the lack of suitable assays. The *spermatid micronucleus test* has been developed to detect chromosome breakage and chromosome-lagging events in meiosis (17). A novel approach is chromosome-specific *fluorescence in situ hybridization (FISH)*, which enables decondensed sperm nuclei to be analyzed (18). Aneuploidy can be studied simultaneously for 2, 3, or more selected chromosomes using multiple FISH and different fluorochromes. Another approach for evaluating sperm-cell genotoxic load is to examine the *DNA adduct pattern by the* P^{32}*-postlabeling technique* (19). The analysis measures electrophilic chemical components binding to nucleotides. With the development of adequate standards for different pesticide adducts, it may be possible to describe potential genotoxic exposure of the sperm cells at the molecular level.

Advances have also been made in the development and validation of questionnaire-based methods of studying subfertility (20—26). Such studies are acceptable in an occupational context, and the resulting data give stable and valid estimates of the fertility distribution (time to pregnancy), in groups of the order of 350 pregnancies.

The 2 approaches are complementary. Laboratory tests can reveal a biological abnormality, but without indicating what the consequences are to function; whereas questionnaires can detect functional impairments, but without distinguishing the biological mechanism involved.

Many of the new tests are designed to detect genetic damage (mutation), and the heterogeneous nature of the effects makes detection difficult. As many fertilized ova have lethal mutations, causing embryonal loss at an early stage, they are not recognized by the woman and are therefore registered as a nonconception-producing, apparent decrease in the level of fertility (longer times to pregnancy) in the exposed population.

The Asclepios project combined the use of biological and questionnaire-based methods in groups with comparable exposures, but from different countries or disparate regions within a country. This approach effectively increases the possibility of detecting adverse effects. Reference groups were chosen within the same country or location.

Objectives

The *overall objective* of the Asclepios project was to identify, characterize, and quantify selected widespread occupational risk factors for the male reproductive system. The goal was to obtain scientific knowledge that can serve as a basis for rational occupational health programs aimed at preventing infertility and other adverse reproductive outcomes, especially transmission of heritable disease to the offspring.

The *specific objectives* were to test the hypotheses that occupational exposure to fungicides, styrene, and lead causes decreased male fecundity in terms of (i) increased time to pregnancy, according to worker interviews, and (ii) reduced semen quality, as measured by conventional and new methods. The project was expected to result in knowledge on no-adverse-effect levels (NOAEL) and, if appropriate, the lowest observed-effect levels (LOEL) of the examined agents.

Refined methodologies for the study of environmental effects on the male reproductive function were to be achieved by enhancements of the time-to-pregnancy approach and by evaluation of the feasibility of conducting longitudinal field studies of semen quality, which have much higher internal validity than cross-sectional studies.

Valid measures of exposure and outcome and the control of timing between exposure and effect was expected to enhance the possibility of making valid causal inferences. The successful completion of the Asclepios project may have implications for research in related areas, for example, studies of genotoxic effects on somatic cell lines, and it has provided *comparable data* on semen quality in different regions of Europe, spanning contrasts of climate and of genetic, social, and environmental conditions. Such data were nonexistent until Asclepios, but are of considerable importance when reports on possible regional and temporal differences in semen quality are evaluated.

Selection of occupational exposures

Among occupational exposures with experimental or epidemiologic evidence indicating an impairment of human male reproductive capability (for a review, see references 2 and 4), the following were rated as having the highest priority when existing knowledge, future technology, use, and study feasibility are taken into consideration:

1. *Fungicides.* Workers involved in applying pesticides in agriculture and gardening are exposed to mixtures of several pesticides. In addition, manual handling of cultures and fruit sprayed with pesticides may add exposure by the dermal route. Some evidence indicates toxic effects of such pesticides as ethylene dibromide and carbaryl on the human testis, and several experiments have indicated male reproductive toxicity for several other pesticides. As the regulation of pesticides is based on animal models, it is important that the persons in contact with applicators or the spray fluid, often containing a mixture of pesticides, be followed carefully in order to recognize even the faintest signs of intoxication as, for example, a reduction in male fecundity. Appropriate study groups include workers that handle the applicators and also their attendants.

2. *Styrene.* An increased proportion of malformed spermatozoa has been reported among reinforced plastics workers exposed to high levels of styrene (2). Experimentally reduced sperm count and testicular degeneration have been found in rats exposed to styrene (2). Epidemiologic and experimental studies on a possible connection between fertility and styrene exposure are still scarce. Appropriate study groups include reinforced plastics workers.

3. *Inorganic lead.* High lead exposure (blood lead levels exceeding 70 µg/dl; 0.34 µmol/dl) still exists among battery assemblers and lead smelters, and relatively low exposures (40—50 µg/dl; 0.19—0.24 µmol/dl) are found for large groups in the metal manufacturing and consuming industries. While there is rather strong evidence that high exposure deteriorates spermatogenesis, the lowest observed effect level has not yet been established. Recent results indicate effects even below the recommended biological exposure limits (in Denmark 50 µg/l; 0.24 µmol/dl). Appropriate study groups include battery workers and lead smelters.

The list of agents was compiled so as to include both mutagenic (some fungicides and metabolites of styrene) and nonmutagenic (inorganic lead) types of occupational exposures. *Styrene* was included as a representative of an aromatic organic solvent and is particularly suitable for epidemiologic studies because concomitant exposure to other substances is limited and because reliable biological monitoring methods are available.

The evidence for male reproductive toxicity is by far the most convincing for *inorganic lead*, and the metal was included in the project also to ensure a kind of positive control of the entire program's ability to detect detrimental exposures. Several other exposures are of interest from an occupational health point of view, but they were not included in the Asclepios project because of anticipated difficulties in identifying exposed populations, simultaneous exposure to other compounds, or lack of methods to document historical exposure levels (certain glycol ethers, mild-steel welding, stainless steel welding, high-frequency electromagnetic fields).

Study designs and eligibility criteria

The Asclepios project combined separate questionnaire studies of time to pregnancy with separate longitudinal studies of semen quality and male germ-cell genotoxicity. The target groups were male employees with the aforementioned high-priority workplace exposures and appropriate reference groups.

The *time-to-pregnancy studies* compare the time taken to conceive the most recent live born child in couples in which the man was exposed or unexposed during the months preceding the time of conception. Comparisons are based on external referents (ie, men from other plants) or internal referents (ie, currently unexposed men from the same plants). Men were eligible if ever married or living as married.

The *longitudinal semen studies* examine the change of biological markers of male fecundity during a follow-up of newly hired workers or workers with seasonal exposure. The first approach uses internal control (1 year of follow-up), whereas the last approach needs an internal or external comparison group. Men between the ages of 18 and 55 years were eligible if not exposed to the agents of interest at least 6 months before entry into the study and if several specified conditions could be excluded (azoospermia, vasectomy, medical treatment with cytostatic drugs, ionizing radiation, salazopyrin or anabolic steroids, occupational exposure to ionizing radiation, microwaves, metal welding, mercury and cadmium).

During the project it became evident that longitudinal semen studies of workers exposed to lead were infeasible for several reasons. Therefore, this design was substituted by cross-sectional semen studies.

Creation of cohorts, enrollment of participants, and data collection were undertaken in 8 European countries in accordance with uniform protocols, whereas each analysis of biological specimens was assigned to 1 of 7 specialized laboratories.

Research methodology

Time-to-pregnancy studies. A time-to-pregnancy questionnaire to be completed by men was designed on the basis of experience drawn from earlier studies of couple fertility. The questionnaire was structured to obtain information about the time taken to conceive the most recent child (or current pregnancy if the spouse was at least 6 months pregnant). The key question was phrased as "How long did it take your wife or partner to become pregnant?" Questions on potential confounding factors (parental age, parity, last method of contraception, and smoking) and occupational exposures were related to the time exactly 12 months before the child was born to allow for gestation (9 months) and duration of spermatogenesis (3 months). However, data were also collected for all other pregnancies. The section of the questionnaire dealing with occupational exposure was adapted to each particular type of exposure (3 questionnaires). The questionnaire was elaborated in English and updated according to the results of pilot studies of the translated versions at the centers. The questionnaire was also translated into Arab and Turkish to allow the participation of men not speaking the national language. The appendix provides the structure of the questionnaire and the phrasing of the key questions. A comprehensive description of the questionnaire method has been given elsewhere (27).

Semen studies. For each of the 3 types of exposure a questionnaire was elaborated based on interviews with managers and foremen (type of production, raw materials, chemicals, departments, job tasks, exposure levels) and piloted to provide uniform data about plants and to obtain background information on participants with respect to demographic, occupational, medical, reproductive, and life-style factors. One comprehensive questionnaire was used when the men were enrolled into the longitudinal studies, and another brief questionnaire was used to record information during the follow-up. These questionnaires were later adapted for use in the cross-sectional studies. Finally, a questionnaire on data related to the sampling of semen was elaborated (date and time, spillage, period of sexual continence, febrile disease). All the questionnaires were designed for self completion. A detailed description of logistics, as well as the design and analysis of semen studies has been given elsewhere (28).

Semen sampling and initial processing. Six laboratory units were established for the field work. The essential equipment consisted of a microscope, a counting chamber, a heater, a video camera, a recorder and a visual display unit. The protocol requested that semen samples be preferably collected by masturbation after 2—5 days of sexual abstinence. The initial semen analysis was ideally undertaken within 1 hour after ejaculation, but samples delivered within 3 hours were accepted. The samples were either collected at the private residence or at a clinic. Semen volume and sperm concentration were measured on site. Video recordings of diluted and undiluted fresh samples were performed for later computer-assisted semen analysis for sperm motility and velocity. Fixed and unfixed air-dried slides were prepared for centralized morphology scoring, and finally the semen was frozen and stored.

Centralized semen analyses. The centralized semen analyses were undertaken in accordance with the schedule outlined in table 1. They included the following examinations: (i) morphology scoring based on fixed smears according to the World Health Organization and to strict guidelines (13), (ii) computer-assisted video analysis (CASA) of sperm motility and velocity (1), (iii) denaturation of sperm chromatin as measured by the flow cytometric sperm chromatin structure assay (SCSA) (16, 29), (iv) male germ-cell genotoxic damage as measured by FISH (18), (v) germ-cell genotoxicity after exposure to fungicides as measured by P^{32}-postlabeling techniques (18).

Analyses of follicular-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and sex-hormone-binding globulin were undertaken as independent measures of male fecundity with the potential to shed light on the mechanism of changes in semen quality — if any.

Exposure assessment. The Asclepios project aimed at independent and objective measures of cumulative and current occupational exposure during the relevant time periods. This goal proved impossible to achieve in all cases.

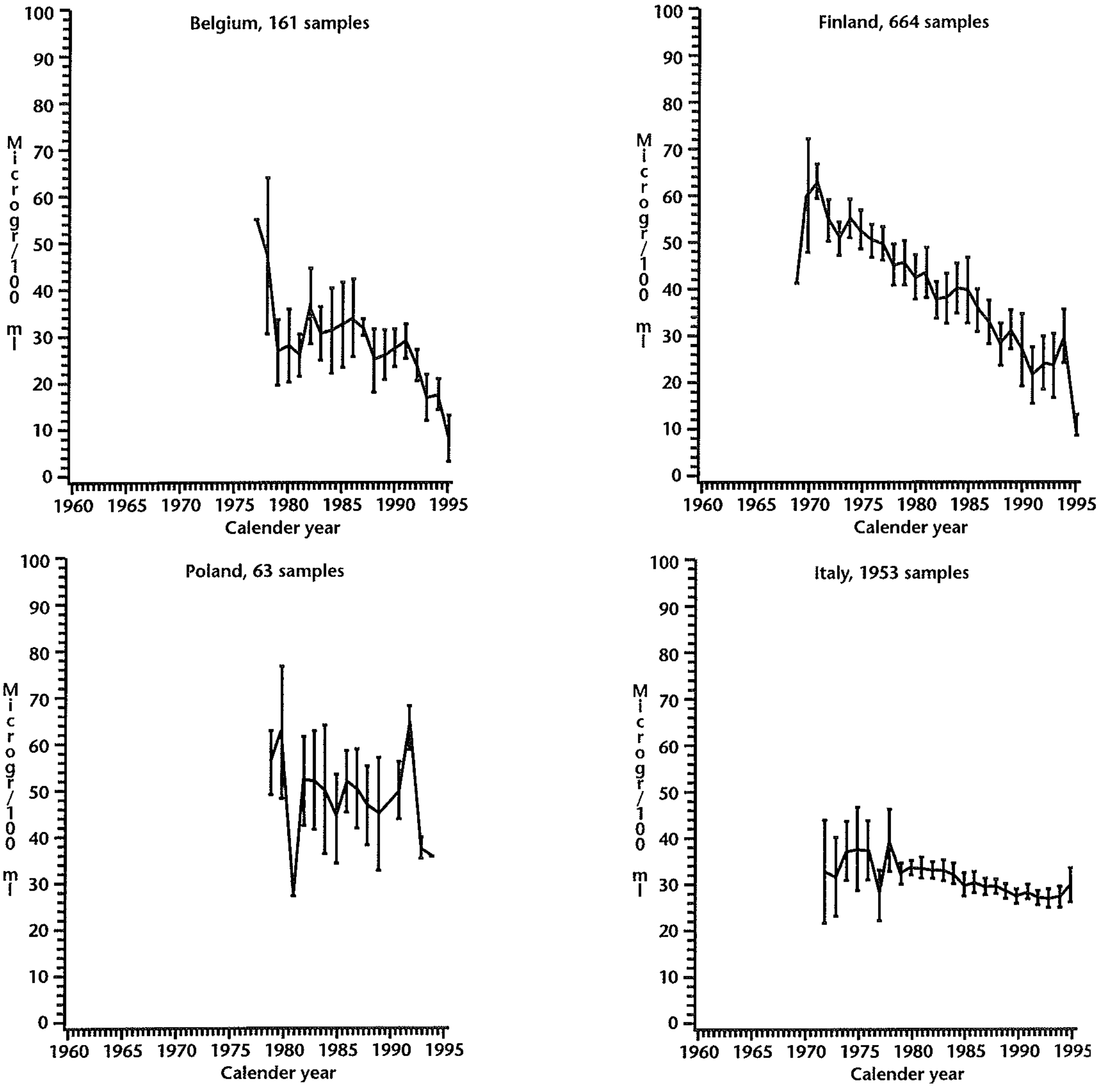
The studies concerned with inorganic lead took advantage of the monitoring of blood lead, which has been routine for decades in most European countries. The majority of time-to-pregnancy values could be identified with at least 1 individually matched blood lead value within 6 months of the time of conception if the worker was exposed to lead at that time. If the value was missing, it could reliably be estimated by expert

Table 1. Biological sampling schedule of the Asclepios project, 1994-1998. (+ = yes, # = optional, - = no, CASA = computer-assisted video analysis, SCSA = sperm chromatin structure assay, FISH = fluorescence in situ hydridization)

Test	Schedule			Group				Sample	Product	Laboratory
	Entry	Mid-term	Final	Lead	Styrene	Fungi-cides	Refe-rents			
Lead in blood	+	+	+	+	#	-	-	Blood, 5 ml	1 tube	Institute of Occupational Medicine, Erlangen-Nürnberg
Porphyrin in blood	+	+	+	+	#	-	-	Blood, 5 ml		Institute of Occupational Medicine, Erlangen-Nürnberg
Styrene metabolites[a]	-	+	+	-	+	-	-	Urine, 10 ml	1 tube	Institute of Occupational Medicine, Erlangen-Nürnberg
Fungicides on gloves	-	(+)	(+)	-	-	+	-	Skin	Gloves	Department of Occupational Toxicology, TNO, Rijswijk
Sex hormones	+	+	+	+	#	#	#	Serum, 1 ml	1 tube	Department of Growth and Reproduction, Copenhagen
Semen analysis										Local
Volume	+	+	+	+	+	+	+	Semen		
pH	+	+	+	+	+	+	+	Semen		
Density	+	+	+	+	+	+	+	Semen, 0.01 ml		
Motility	#	#	#	#	#	#	#	Semen, 0.01 ml		
CASA	+	+	+	+	+	+	+	Semen	Tape	Department of Growth and Reproduction, Copenhagen
Morphology scoring	+	+	+	+	+	+	+	Semen, 0.02 ml	2 slides	Department of Growth and Reproduction, Copenhagen; Reproduction Humaine, Epidemiologie, Paris; Institute of Neurotoxicology, Aarhus
SCSA	+	-	+	+	+	+	+	Semen, 0.4 ml	2 tubes	ENEA CRE Casaccia, Rome
FISH	+	-	+	-	-	+	+	Semen, 0.3 ml	1 tube	Department of Medical Genetics, Turku
P^{32}-postlabeling	+	-	+	-	-	+	+	Semen, 0.4 ml	1 tube	Institute of Environmental Medicine, Aarhus
Lead semen	+	+	+	+	+	-	-	Semen, 0.1 ml	1 tube	Institute of Occupational Health, Brescia

[a] Sampling of urine for the assessment of styrene exposure may take place earlier in the follow-up period, in order to ensure sampling among workers who quickly skip the job, but sampling during the first month should be avoided.

judgement based on data concerning the plant, job task, and calendar period. The cumulative exposure to lead was computed from all available blood lead values from the start of employment. In the cross-sectional sperm studies the assessment of exposure was based on standardized measurements of lead in a blood sample



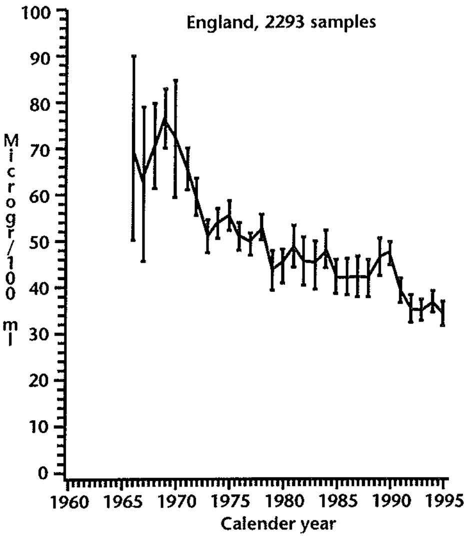


Figure 1. Levels of blood lead by country and calendar year in the occupational settings of the Asclepios project, 1994—1998. Mean (2 SEA). Altogether 5136 blood lead samples from 417 workers are included.

drawn at the same time as the semen sample was collected. In addition, inorganic lead was measured in seminal plasma and spermatozoa. The concentration of inorganic lead in these media differs from that in blood and may be better correlated with testicular toxicity (30).

In figure 1 the levels of lead in blood in the occupational settings of the Asclepios study are shown by country and calendar year. With the exception of Poland, the average blood lead level declined from 1970 to 1995 at all sites. The levels were higher in Poland and England — above 40 µg/100 ml during the entire period. In Italy and Belgium the levels were below 40 µg/100 ml during the entire period.

Exposure assessment in the studies concerned with exposure to *styrene* may have been more difficult since valid measures of the individual exposure were not available to the same extent. However, with the use of environmental data on styrene levels in ambient air, it proved possible to predict the exposure level by information on country, calendar time, and job task. In analogy with the lead studies it was possible to assign both a cumulative and a current exposure level to most of the time-to-pregnancy values. The level of styrene in ambient air has declined considerably during the past 25 years in the centers contributing data to the Asclepios studies (figure 2). In the longitudinal study of newly hired reinforced plastics workers the exposure to styrene was measured by repeated sampling of postshift spot urine samples taken during a period of 2 weeks. The creatinine-adjusted concentration of the styrene metabolite mandelic acid provided the basis for grouping the workers according to different levels of exposure. The exposure levels were well below the current German biological exposure index in the majority of cases.

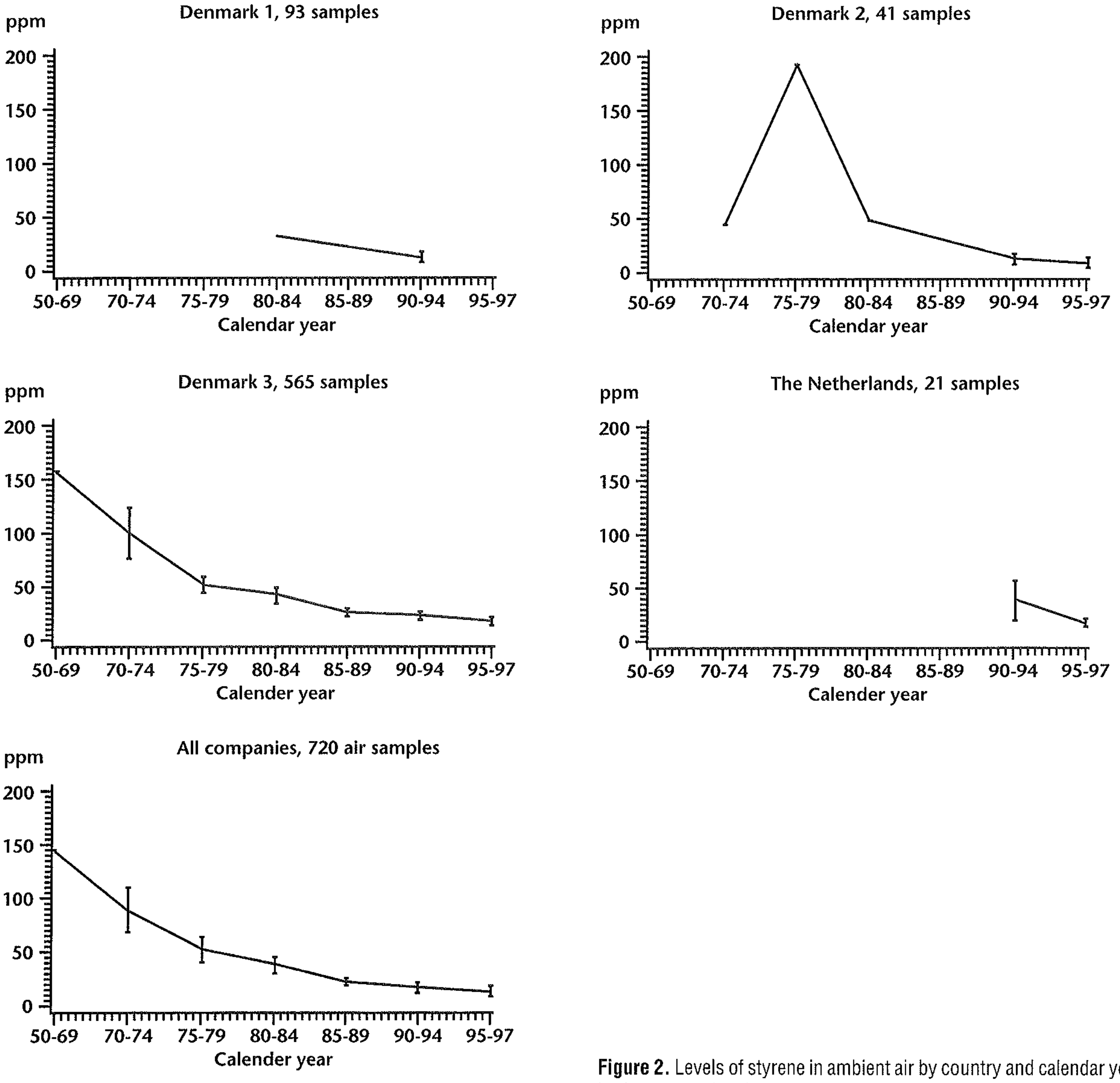


Figure 2. Levels of styrene in ambient air by country and calendar year in the occupational settings of the Asclepios project, 1994—1998.

The exposure situation was far more complex in the fungicide studies. Workers in greenhouses, vineyards, and agriculture are exposed to mixtures of several compounds, and the exposure profile probably differs significantly across the last decade. It is obvious that exposures in different occupational settings spanning different products, climates, and job tasks would be incomparable, and therefore this group of investigations was designed as stand-alone studies. Three different approaches were applied to identify and quantify current exposure. First, evaluation of dermal exposure by high-pressure liquid chromatography for 16 specified fungicides absorbed on cotton gloves used for 1 hour at the first entry after application [Dutch fruit growers (unpublished data: Tielemans E, Louwerse E, De Cock J, Thuis H, Brouwer D, de Cort W, et al. Exposure to fungicides in fruit growing: relationship between re-entry time and dermal exposure)] Second, the measurement of pesticides in hair (head) cut before and after the fungicide spraying season. Altogether 17 xenobiotics were measured by gas chromatography-mass spectrometry, and this approach was used to assess the exposure of French vineyard workers (31). No pesticides were measured in the preexposure samples, while 5 substances were recovered in nanogram concentrations for 4 workers in postexposure samples. Third, metabolites of maneb and mancozeb (ETU) were determined in the urine collected from potato growers within 24 hours after they had sprayed the fungicides (Danish farmers). The method has proved useful in earlier studies (32, 33), and a pilot study indicated measurable amounts of ETU in 24-hour urine samples collected after the application of maneb-mancozeb. Although specific exposures were documented in some of the studies, most of the results were based on crude information on job characteristics collected by interview and by log books in which details on type and amount of fungicides were recorded.

The execution of the studies was based on a protocol specifying eligibility criteria, sampling schedules, and procedures for semen, blood and urine preparation, storage, and shipment. A laboratory manual described in detail the semen examination that was to take place at the local centers, and several training courses were held to ensure compliance with the protocol. Finally, a quality assurance program was established to ascertain the local and central semen analytical procedures.

Data collection and populations

Time-to-pregnancy studies. Cohorts of current male workers were designated in each exposure-defined study population from rosters of employees at selected plants (lead and styrene) or from registries (farmers and vineyard workers). Reference groups of currently unexposed workers were established according to the same criteria from the same plants or comparable industries whenever appropriate. Data collection was by interview (telephone or face-to-face), which may be crucial to maximize the response rate and to obtain valid information. Nevertheless, self-administered questionnaires were accepted if funding for interviewers was not available. Men refusing to participate were asked a few questions (marital status and number of children) to decide whether they would have been eligible or not. At 1 center complete information on marital status and number of children could be obtained from a register.

Semen quality studies. Male workers were supposed to be consecutively enrolled into the study when newly appointed to jobs conferring exposure to styrene or lead. One semen sample and 1 blood sample were collected within the first week on the new job, and 2 additional samples were collected after 6 and 12 months. Each of 7 centers were supposed to enroll at least 10 men to each exposure category during a 2-year period. The following three different methods for enrollment adapted to country and particular exposure were suggested: (i) to pick up volunteers at preemployment health examinations, (ii) to address pupils at schools or job training centers, and (iii) to recruit newly hired men directly from work sites by arrangements with managers or on-site health personnel.

In occupational groups with exposure to fungicides during summer, longitudinal studies were based on the sampling of blood and semen before, during, or after the summer period conferring exposure to fungicides. In order to detect possible effects on early and late stages of the spermatogenesis, 2 semen samples were collected after the start of exposure, 1 sample at the end of exposure, and another 16 weeks after the first day of exposure.

Results

Time-to-pregnancy studies. The 8 data collection centers addressed 6553 male workers and contributed time-to-pregnancy values on the 3077 most recent pregnancies surviving at least 6 months (table 2). Men unable to read the questionnaire, unmarried men, and men never living as married were considered ineligible. After the exclusion of men with known ineligibility, the response rate was defined as men completing the questionnaire divided by the number of men who were invited to participate. The average response rate across all exposures and centers was 69.8%, and the site and study-specific response rate ranged from 22% to 99% (table 2). The number of workers addressed by each center ranged from 57 to 1733. The unexposed pregnancies were either contributed from reference populations from other industries or from currently exposed men who were unexposed 1 year before the birth of the most recent child. Data

Table 2. Populations for the time-to-pregnancy (TTP) studies in the European Asclepios project, 1993—1998 — preliminary data.

Site	Lead studies[a]				Styrene studies				Fungicide studies				All studies	
	Addressed	Eligible	Respondents	Pregnancies	Addressed	Eligible	Respondents	Pregnancies	Addressed	Eligible	Respondents	Pregnancies	Addressed	Pregnancies
Belgium	130	116	99	66									130	66
Denmark					1116	1002	700	456	1733	1328	972	897	2849	1353
Finland	355	264	191	136									355	136
France									1326	1161	811	748	1326	748
Italy	947	694	603	446	136	118	117	74					1083	520
The Netherlands	178	160	94	72	164	145	64	57					342	129
Poland	?	?	60	60										60
United Kingdom	408	391	85	65									408	65
All	2018	1681	1132	836	1416	1265	881	567	3059	2489	1783	1645	6553	3077

[a] Status 1 November 1998. Data collection continued as of 1998 at all sites.

collection in the lead studies continued during 1998 at 3 sites.

Semen quality studies. Longitudinal semen studies dealing with exposure to fungicides and based on the seasonal pattern of exposure during the summer period were conducted at 2 sites. Thirty vineyard workers and 171 farmers provided semen samples before and after the period during the summer when fungicides were applied. At both sites men were enrolled during 2 consecutive seasons. Although each subject acted as his own control, unexposed reference groups comprising 10 unexposed vineyard workers and 85 organic farmers were enrolled according to the same procedures and inclusion criteria in order to control for temporary shifts in sperm parameters due to seasonal or unknown factors. The semen study populations are outlined in table 3. The number of semen samples examined by the advanced methods are given in table 4.

Longitudinal studies of newly hired workers were only successful at 1 center, where 34 of 131 newly hired reinforced plastics workers were recruited for a longitudinal study of styrene exposure. Interview information and biological samples (semen, blood, and spot urine) were collected within 2 weeks of the start of employment and 6 and 12 months after the initial exposure. Cross-sectional semen studies in the lead industry were designed as the longitudinal approach failed, and 3 centers are expected to provide 1 semen sample from 100—200 men at each center. So far some 200 men have provided semen samples (table 3), and the collection of data continued throughout 1998.

Critical reappraisal of achievements

The Asclepios project provided data allowing the examination of possible male reproductive toxicity of a broad group of fungicides, styrene, and inorganic lead as measured by the time to pregnancy and by the semen quality approach. The total number of men enrolled into the time-to-pregnancy and semen studies were higher or close to the numbers outlined in the protocol (time to pregnancy: 3077 versus 2150; men providing semen sam-

Table 3. Populations for the semen studies in the European Asclepios project, 1993—1998 — preliminary data.

	Fungicide studies		Styrene study	Lead studies[a]					All studies
	Danish farmer study	French vineyard study	Danish reinforced plastics study	Italy		United Kingdom	Belgium		
				Longitudinal	Cross-sectional	Cross-sectional	Longitudinal	Cross-sectional	
Addressed men	789	?	131	6?	315	269[b]	6	0[c]	1516?
Respondents	256	40	34	5?	67	199	3	0	604?
1. Semen sample[b]	256	40	34	5?	19[d]	153	3	.	510?
2. Semen sample	248	40	23	?	.	.	2	.	313?
3. Semen sample	.	40	16	?	.	.	1	.	57?
All samples	504	120[e]	73	5?	19	153	6	0	880?

[a] Status as of 1 November 1997. Data collection continued in 1998.
[b] Only men offering to provide time to pregnancy were asked to donate semen samples (the total work force was 885 men).
[c] Data collection awaits the time-to-pregnancy studies to be finalized.
[d] Data collection continues and it is expected that samples will be collected from all respondents.
[e] The French semen sampling protocol included a 4th semen sample.

Table 4. Semen analyses in the European Asclepios project, 1993—1998 — preliminary data.

Semen analysis	Number of analysed samples
WHO analysis[a]	650
CASA analysis[b]	650
SCSA analysis[c]	504
FISH analysis[d]	65

[a] WHO, semen examination: conventional semen parameters according to the World Health Organization guidelines (13).
[b] CASA, computer assisted analysis of sperm motility (1).
[c] SCSA, sperm chromatin structure assay (16).
[d] FISH, florescence in situ hybridization assay of structural abnormalities in chromosome 1 and 7 (18).

ples: 354 versus 360). Nevertheless, the expiring concerted action has revealed several restraints and does not entirely provide the data predicted at the start of the program.

As originally planned, 7 centers contributed data to the *lead time-to-pregnancy studies*, but some contributions were very small, and 1 site failed to document the process of data collection and therefore made the exclusion of that subset necessary. Only 3 of 6 sites provided *styrene time-to-pregnancy* data from reinforced plastics workers, and only 3 of 5 stand-alone studies of time to pregnancy were carried out for men exposed to fungicides. A stand-alone study of *fruit growers* in The Netherlands was abolished at a very late stage because the National Trade Organization withdrew its consent to support the study.

A shortcoming of several of the time-to-pregnancy studies was difficulties in identifying adequate reference groups (ie, only 8 of the 12 time-to-pregnancy Asclepios studies had national external reference groups). For example, it proved impossible to define a suitable reference group for reinforced plastics workers in Denmark because of the characteristics of this group of workers (rapid workforce turnover, unskilled work, special social and life-style factors). However, the majority of the most recent children in this and other groups had unexposed fathers and were supposed to be an adequate internal reference.

Objective measurements of types and levels of exposure are cornerstones of high-quality occupational research. Objective assessment of historical and current exposure was possible in the lead and styrene studies but was difficult in the fungicide studies, for which an ideal solution was never identified. Therefore, the time-to-pregnancy studies concerned with fungicides essentially compared groups defined by occupation or work characteristics and accordingly contained less scientific information. The situation was not much different for the semen studies among farmers, greenhouse workers, and vineyard workers. The exposure assessment protocol based on measurements of several indicator compounds on gloves was appropriate for reentry activity in greenhouses and among fruit growers, but not among farmers mixing and applying fungicides.

Longitudinal semen studies were attempted as a solution to some of the major constraints of most earlier studies in this field. Although the entire organizational framework, including the establishment of field laboratory units, was established at 6 sites, it became evident — except at 1 site — that the design was unfeasible in the lead and reinforced plastics industry studies. The main problem was the economic recession and low workforce turnover — not refusal to provide semen samples. In addition, several centers failed to obtain national funding, and the ability to maintain attention on the recruitment of newly hired workers over prolonged time periods probably also became a critical issue due to the shortness of resources. Fortunately 1 site managed to set-up a longitudinal study of stand-alone size among reinforced plastics workers. However, this national study did not have the strength of an international study with respect to variation of exposure levels, and, although the study was large from the national point of view, the study size was less than 50% of the planned international study. The longitudinal lead studies were abandoned for the aforementioned reasons and substituted by cross-sectional studies at 3 centers. Since the fertility of the source population was characterized by the time-to-pregnancy questionnaire, it will be possible to describe the magnitude and direction of the selection bias, which is almost invariably associated with cross-sectional studies with rather low response rates. It is an advantage that the cross-sectional approach enables the study of effects of long-term exposure, which may be important for lead-exposed workmen. The data collection continued through 1998, and the results will not be available for some time. If successfully completed, the lead studies may provide comparable data on semen quality in different regions of Europe. Such data are not existent and have not been provided by the Asclepios project so far. The longitudinal semen studies on men with seasonal exposure to fungicides provided by far the majority of the semen samples and proved to be a valid approach even when a reference group is essential to control for inherent seasonal changes of some measures of semen quality.

Concluding remarks

The Asclepios project is the first international multicenter research project on environmental risks to male reproductive function. The project has developed, described, and standardized epidemiologic and laboratory research tools, and useful links between epidemiologic, andrological, and experimental researchers within the field have been established. It is expected that the Asclepios project will provide clues to the identified main scientific questions, but the community-added value of a part of the program is limited since the longitudinal semen studies at the international level failed. The majority, but not all, of the studies were completed within the given time frame. The preliminary findings are presented in brief communications in this supplement.

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Appendix

Structure of the time-of-pregnancy questionnaire

Have you ever been married/living as married?

❑$_1$ NO
❑$_2$ YES

Only men ever married or living as married were asked about time to pregnancy.

If the answer to the last question is "NO", <u>STOP HERE</u>.
For most of the questionnaire we ask questions about your children. We also ask about other pregnancies of which you have been the father. However, we are not interested in abortions (medically induced terminations of pregnancy).

Questions to establish whether the man had fathered a pregnancy of at least 6 months' duration.

Is your wife/partner pregnant now?

❑$_1$ NO   ❑$_2$ YES

If YES -
Is she at least 6 months pregnant?

❑$_1$ NO   ❑$_2$ YES

If not the men are guided to a section of the questionnaire dealing with infertility (not included here).

Have you been the father of any children (NOT counting a current pregnancy)?
Please count all pregnancies that reached the point where your wife/partner was at least 6 months pregnant (within 3 months of the expected date of delivery), no matter what happened after that, even if the child was stillborn or did not survive.

❑$_1$ NO   ❑$_2$ YES

The next set of questions is about your **most recent child**.
If your wife/partner is pregnant now, as long as she is at least 6 months pregnant (within 3 months of delivery), please tell us about this one. If this is not the case, *and* you have not had any children, please go straight to question...

Focus is on most recent child/pregnancy of at least 6 months' duration.

What was the date of birth of your most recent child?

.... / .... / .... (day/month/year)
If your wife/partner is pregnant now, please give the expected date of delivery (as well as you can).

At around the time this pregnancy was conceived, were you or your wife/partner using any method of birth control?

❑$_1$ NO   ❑$_2$ YES

Eligible pregnancies: time to pregnancy is only defined for couples not using birth control.

If YES -
Were you using this all the time, nearly all the time, or only sometimes?

a.	Only sometimes	❑$_1$
b.	Nearly all the time	❑$_2$
c.	All the time	❑$_3$

If NO or ONLY SOMETIMES -
How long did it take your wife/partner to get pregnant?
- please write in how many months and/or years
...... (months) ..... (years)

The key question on time taken to conceive from the start of unprotected intercource to pregnancy.

During this time, was your wife/partner breastfeeding any of the time?

❑$_1$ NO   ❑$_2$ YES

Breastfeeding acts as a partial contraceptive.

Did you stop contraception before the pregnancy started, or did the pregnancy result from a birth control failure?

❑ 1 NO ❑ 2 YES

A check of the eligibility of the pregnancy.

The next set of questions is about your **work**, concentrating on the time **exactly 12 months before the baby was born.** (If your wife/partner is pregnant now, please focus on the point 12 months before the baby is expected).

The time window for occupational exposures and possible determinants of fecundity such as tobacco smoking was the time 12 months before the baby was born to account for duration of pregnancy (9 months) and spermatogenesis (3 months).

Exposure-specific questions (example):
As far as you are aware, were you exposed to lead, or to any substance that contains lead?

❑ 1 NO ❑ 2 YES

About how many cigarettes a day, if any, did you smoke on average at that time?

a.	None/didn't smoke	❑ 1
b.	1-10 cigarettes a day	❑ 2
c.	11-20 cigarettes a day	❑ 3
d.	Over 20 cigarettes a day	❑ 4