Health Council of the Netherlands

Methotrexate

Evaluation of the effects on reproduction, recommendation for classification

Gezondheidsraad
Health Council of the Netherlands

Aan de staatssecretaris van Sociale Zaken en Werkgelegenheid

Onderwerp : Aanbieding advies *Methotrexate*
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Geachte staatssecretaris,

Graag bied ik u hierbij het advies aan over de effecten van methotrexaat op de vruchtbaarheid en het nageslacht; het betreft ook effecten die optreden na blootstelling via de borstvoeding. Dit advies maakt deel uit van een uitgebreide reeks waarin voor de voortplanting giftige stoffen worden geclassificeerd volgens richtlijnen van de Europese Unie. Het gaat om stoffen waaraan mensen tijdens de beroepsuitoefening kunnen worden blootgesteld.

Dit advies is opgesteld door een vaste commissie van de Gezondheidsraad, de Subcommissie Classificatie reproductietoxische stoffen. Het is vervolgens getoetst door de Beraadsgroep Gezondheid en omgeving van de raad.

Ik heb dit advies vandaag ter kennisname toegezonden aan de staatssecretaris van Infrastructuur en Milieu en aan de minister van Volksgezondheid, Welzijn en Sport.

Met vriendelijke groet,

prof. dr. L.J. Gunning-Schepers,
voorzitter

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Methotrexate

Evaluation of the effects on reproduction, recommendation for classification

Subcommittee on the Classification of Reproduction Toxic Substances,
a Committee of the Health Council of the Netherlands

to:

the State Secretary of Social Affairs and Employment

No. 2011/24, The Hague, October 19, 2011

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Samenvatting

In het voorliggende advies heeft de Gezondheidsraad methotrexaat onder de loep genomen. Methotrexaat is een foliumzuurantagonist. Het wordt onder meer gebruikt voor het beëindigen van zeer vroege en van buitenbaarmoederlijke zwangerschappen, als cytostaticum en voor de behandeling van rheumatische aandoeningen en psoriasis. Dit advies past in een reeks adviezen waarin de Gezondheidsraad op verzoek van de minister van Sociale Zaken en Werkgelegenheid de effecten van stoffen op de voortplanting beoordeelt. Het gaat vooral om stoffen waaraan mensen tijdens de beroepsuitoefening kunnen worden blootgesteld. De Subcommissie Classificatie reproductietoxische stoffen van de Commissie Gezondheid en beroepsmatige blootstelling aan stoffen (GBBS) van de raad, hierna aangeduid als de commissie, kijkt zowel naar effecten op de vruchtbaarheid van mannen en vrouwen als naar effecten op de ontwikkeling van het nageslacht. Bovendien worden effecten van blootstelling van de zuigeling via de moedermelk beoordeeld.

Op basis van Verordening (EG) 1272/2008 van de Europese Unie doet de commissie een voorstel voor classificatie. Voor methotrexaat komt de commissie tot de volgende aanbevelingen:

- voor effecten op de fertiliteit adviseert de commissie om methotrexaat te classificeren in categorie 2 (*stoffen die ervan verdacht worden dat zij toxisch zijn voor de menselijke voortplanting*) en te kenmerken met H361f (*wordt ervan verdacht de vruchtbaarheid te schaden*).

- voor effecten op de ontwikkeling adviseert de commissie om methotrexaat te classificeren in categorie 1A (s*toffen waarvan bekend is dat zij toxisch zijn voor de menselijke voortplanting*) en te kenmerken met H360D (*kan het ongeboren kind schaden*).
- voor effecten tijdens lactatie adviseert de commissie om methotrexaat niet te kenmerken wegens onvoldoende geschikte gegevens.

Executive summary

In the present report, the Health Council of the Netherlands reviewed methotrexate. Methotrexate is used to induce (i.e. non-surgical) abortions in very early pregnancies and to treat ectopic pregnancies, various cancers, rheumatoid arthritis and other inflammatory rheumatic disorders, and psoriasis. This report is part of a series, in which the Health Council evaluates the effects of substances on reproduction, at the request of the Minister of Social Affairs and Employment. It mainly concerns substances to which man can be occupationally exposed. The Subcommittee on the Classification of Reproduction Toxic Substances of the Dutch Expert Committee on Occupational Safety (DECOS) of the Health Council, hereafter called the Committee, evaluates the effects on male and female fertility and on the development of the progeny. Moreover, the Committee considers the effects of a substance on lactation and on the progeny via lactation.

The Committee recommends classification according to Regulation (EC) 1272/2008 of the European Union. For methotrexate, these recommendations are:

- for effects on fertility, the Committee recommends classifying methotrexate in category 2 (*suspected human reproductive toxicant*) and labelling with H361f (*suspected of damaging fertility).*
- for effects on development, the Committee recommends classifying methotrexate in category 1A (*known human reproductive toxicant*) and labelling with H360D (*may damage the unborn child*).

- for effects during lactation, the Committee recommends not labelling methotrexate due to a lack of appropriate data.

Chapter 1

Scope

1.1 Background

As a result of the Dutch regulation on registration of compounds toxic to reproduction that came into force on 1 April 1995, the Minister of Social Affairs and Employment requested the Health Council of the Netherlands to classify compounds toxic to reproduction. This classification is performed by the Health Council's Subcommittee on the Classification of Reproduction Toxic Substances of the Dutch Expert Committee on Occupational Safety (DECOS). The classification is performed according to European Union Regulation (EC) 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures. The CLP guideline is based on the Globally Harmonised System of Classification and Labelling of Chemicals (GHS). The subcommittee's advice on the classification will be applied by the Ministry of Social Affairs and Employment to extend the existing list of compounds classified as reproductive toxicant (category 1A and B and 2) or compounds with effects on or via lactation.

1.2 Committee and procedure

This document contains the classification of methotrexate by the Health Council's Subcommittee on the Classification of Reproduction Toxic Substances, hereafter called the Committee. The members of the Committee are listed in Annex A. The classification is based on the evaluation of published human and

animal studies concerning adverse effects with respect to fertility and development as well as lactation of the above mentioned compound.

Classification for reproduction (fertility (F) and development (D)):	
Category 1	Known or presumed human reproductive toxicant (H360(F/D))
Category 1A	Known human reproductive toxicant
Category 1B	Presumed human reproductive toxicant
Category 2	Suspected human reproductive toxicant (H361(f/d))
No classification for effects on fertility or development	
Classification for lactation:	
	Effects on or via lactation (H362)
	No labelling for lactation

The classification and labelling of substances is performed according to the guidelines of the European Union (Regulation (EC) 1272/2008) presented in Annex B. The classification of compounds is ultimately dependent on an integrated assessment of the nature of all parental and developmental effects observed, their specificity and adversity, and the dosages at which the various effects occur. The guideline necessarily leaves room for interpretation, dependent on the specific data set under consideration. In the process of using the regulation, the Committee has agreed upon a number of additional considerations (see Annex C).

In 2011, the President of the Health Council released a draft of the report for public review. The individuals and organizations that commented on the draft report are listed in Annex D. The Committee has taken these comments into account in deciding on the final version of the report.

1.3 Labelling for lactation

The recommendation for classifying substances for effects on or via lactation is also based on Regulation (EC) 1272/2008. The guideline defines that substances which are absorbed by women and have been shown to interfere with lactation or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child, shall be classified and labelled. Unlike the classification of substances for fertility and developmental effects, which is based on hazard identification only (largely independent of dosage), the labelling for effects during lactation is based on risk characterization and therefore, it also includes consideration of the level of exposure of the breastfed child.

Consequently, a substance should be labelled for effects during lactation when it is likely that the substance would be present in breast milk at potentially toxic levels. The Committee considers a concentration of a compound as potentially toxic to the breastfed child when this concentration leads to exceeding the exposure limit for the general population, e.g. the acceptable daily intake (ADI).

1.4 Data

Literature searches were conducted in the on-line databases Current Contents and Medline, starting from 1966 up to 2006 and by searches on internet. A final search was performed in November 2010 in PubMed. Publications cited in the selected articles, but not selected during the primary search, were reviewed if considered appropriate. In addition, handbooks and a collection of most recent reviews were consulted. References are divided into literature cited and literature consulted but not cited.

The Committee describes both human and animal studies in the text. The animal data are described in more detail in Annex E as well. Of each study, the quality of the study design (performed according to internationally acknowledged guidelines) and the quality of documentation are considered.

1.5 Presentation of conclusions

The classification is given with key effects, species, and references specified. In case a substance is not classified as toxic to reproduction, one of two reasons is given:

Lack of appropriate data precludes the assessment of the compound for reproductive toxicity.

Sufficient data show that no classification for reproductive toxicity is indicated.

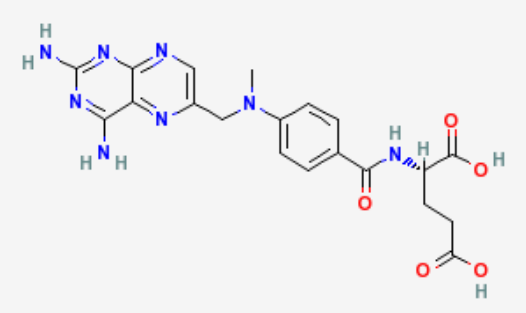
1.6 Final remark

The classification of compounds is based on hazard evaluation only (Niesink *et al*., 1995)[35], which is one of a series of elements guiding the risk evaluation process. The Committee emphasizes that for derivation of health-based occupational exposure limits these classifications should be placed in a wider context. For a comprehensive risk evaluation, hazard evaluation should be combined with dose-response assessment, human risk characterization, human exposure assessment, and recommendations of other organizations.

Chapter 2

Methotrexate

2.1 Introduction

name	:	methotrexate
IUPAC name	:	(2*S*)-2-[(4-{[(2,4-diamino-7,8-dihydropteridin-6-yl)methyl](methyl)amino}phenyl) formamido]pentanedioic acid
CAS name	:	L-glutamic acid, *N*-(4{[(2,4-diamino-6-pteridinyl)methyl]methyl-amino}benzoyl-
CAS registry number	:	59-05-2
synonyms	:	amethopterin; 4-amino-10-methylfolic acid; 4-amino-N^{10}-methyl-pteroylglutamic acid; *N*-{*para* [(2,4-diamino-6-pteridinyl)methyl] methylamino}-benzoyl L-(+)-glutamic acid; *N*-(4-{[(2,4-diamino-6-pteridinyl)methyl]methylamino}-benzoyl)-L-glutamic acid; metho-trexatum; *N*-{*para*-[(2,4-diaminopteridin-6-yl-methyl)methyl-amino]benzoyl}-L-glutamic acid; α-methopterin; methylaminopterin
molecular formula	:	$C_{20}H_{22}N_8O_5$
structural formula	:	

molecular weight	:	454.4
melting point	:	185-204°C (monohydrate)
vapour pressure	:	not found
solubility in water	:	(practically) insoluble
Log $P_{octanol/water}$	:	-1.85 (experimental); -1.28 (estimated)
appearance	:	orange-brown, crystalline powder
use	:	to induce (i.e. non-surgical) abortions in very early pregnancies[7]; to treat ectopic pregnancies (where the fertilized egg is embedded in the fallopian tube or cervix instead of the uterus)[44], various cancers, rheumatoid arthritis and other inflammatory rheumatic disorders, and psoriasis. Doses may range from a single dose of 1.4 mg/kg bw to induce abortions[7] to cumulative doses of more than 300 mg/kg bw to treat certain tumours15, given in single daily doses of 0.625-2.5 mg/kg bw[2].

data from NLM[33], IARC[1], and http://www.srcinc.com/what-we-do/databaseforms.aspx?id385, unless otherwise indicated

Methotrexate, an anti-metabolite, is a folic acid antagonist. Methotrexate and its polyglutamate metabolites inhibit dihydrofolate reductase, an enzyme reducing folic acid to tetrahydrofolic acid. Tetrahydrofolates are utilized as carriers of one-carbon fragments necessary for synthesis of purine nucleotides and thymidylate. Inhibition of the folic acid reduction thus interferes with DNA synthesis and repair and cellular replication.[2]

Oral absorption of methotrexate appears to be highly variable and dose dependent. At doses <0.8 mg/kg bw, methotrexate is generally well absorbed with a mean bioavailabilty of about 60%. At doses of >2 mg/kg bw, absorption is significantly less probably due to saturation. Peak serum levels are generally reached within one-two hours or 30-60 minutes after oral or intramuscular administration, respectively.[2]

Methotrexate is actively transported across cell membranes; at serum concentrations >0.1 mmol/L (i.e. 45 mg/L), passive diffusion becomes the major transport mechanism. Approximately 50% is bound to plasma proteins. Methotrexate is widely distributed into body tissues and extracellular fluids with a steady-state volume of distribution of 0.4-0.8 L/kg bw. The highest concentrations are found in the kidneys, gall bladder, spleen, liver, and skin. Methotrexate crosses the placenta; it has been detected in breast milk (see Section 2.2).[2,30]

Methotrexate is metabolized hepatically and intracellularly to polyglutamate conjugates; this process is reversible. Small amounts of the polyglutamates may be converted to 7-hydroxymethotrexate; due to its low solubility, this hydroxy metabolite may accumulate substantially following administration of high doses

of methotrexate. In addition, partial intestinal metabolism may occur following oral administration.[2,30]

Excretion occurs primarily in the urine and to a small extent in the faeces, probably via the bile; enterohepatic recirculation has been proposed. Excretion depends upon dose and route of administration. Following intravenous doses of 0.1-10 mg/kg bw and oral doses of 0.1 mg/kg bw, about 60-90% was excreted in the urine within 24 hours, and 2-5% and 7-9%, respectively, in the faeces. Following an oral dose of 10 mg/kg bw, 15% and 45% were excreted in the urine within one and five days, respectively, and 39% in the faeces. Terminal half-lives are 3-10 hours at therapeutic doses <0.8 mg/kg bw and 8-15 hours at high doses.[2,30]

2.2 Human studies

Fertility studies

No studies are available regarding the effects of occupational exposure to methotrexate on human fertility.

Ectopic pregnancies

Buster and Krotz evaluated the impact of treatment choice for unruptured ectopic pregnancy on reproductive performance by reviewing the literature available through PubMed in the period 1975-2006. Twelve studies concerning variable-dose methotrexate treatment in 338 patients and seven studies concerning single-dose methotrexate treatment in 393 patients were obtained. Subsequent pregnancy rates in these two treatment groups were 52% (67/129) and 61% (39/64), respectively, and were comparable with rates following expectant management or laparoscopic salpingostomy.[4]

In a prospective study, Oriol *et al*. (2008) evaluated whether methotrexate treatment for ectopic pregnancy compromised ovarian reserve and future reproductive outcome in a group of 25 women undergoing assisted reproductive technology. Oriol *et al*. concluded that single-dose methotrexate treatment did not affect ovarian reserve in terms of anti-Müllerian hormone (AHM) levels and subsequent *in vitro* fertilization – intra-cytoplasmatic sperm injection (IVF-ICSI) cycle outcomes such as cycle duration, gonadotropin requirement, peak E_2 levels, number of oocytes retrieved, and total number of embryos obtained.[36]

Results of a retrospective cohort study in 2009 suggested a time-limited and reversible impact of methotrexate on oocyte yield in 48 women with a history of infertility undergoing ovarian stimulation after being treated with methotrexate for ectopic pregnancy.[31]

Kung *et al.* (1997) evaluated the subsequent reproductive and obstetric outcome in 22 case reports regarding cervical pregnancy (a rare form of ectopic pregnancy) treated with methotrexate, that were published in the period 1983-1995. Of the 13 women who wished to conceive (and could be followed for at least three years), nine (69%) succeeded in having live births without congenital malformations, one (8%) spontaneously aborted, and three (23%) suffered from infertility.[26]

Cancer

Rustin *et al*. (1984) assessed possible infertility based on the obstetric histories of 445 long-term surviving women treated with chemotherapy for gestational trophoblastic tumours for a mean of four months between 1958 and the end of 1978. Of the 217 women wishing to conceive, 118 were treated with methotrexate alone and 81 with methotrexate in combination with one or more other cytotoxic drugs. Of the women treated with methotrexate alone, 116 (98%) conceived and 106 (90%) had at least one live birth.[39]

A similar investigation among women treated in the same centre between 1957 and October 1990 yielded 392 women treated for gestational trophoblastic disease with methotrexate alone and wanting to get pregnant. Of them, 365 (93%) conceived and 327 (83%) had at least one live birth.[47]

Shamberger *et al*. (1981) studied the gonadal function of two women and three men 30-36 months after they were treated with methotrexate alone (with leucovorin rescue) as postoperative adjuvant therapy for osteosarcoma. In the women, no effects on serum FSH or LH levels were seen. Cyclic menses were normal and none of the women had developed amenorrhea or menstrual irregularities even while receiving treatment. In men, sperm concentrations (data for only two men) and serum testosterone, FSH, and LH levels were not affected. Evaluation of samples obtained during therapy and frozen showed normal serum FSH and LH levels in two men while, in the third man, there was an increased FSH level ($p<0.01$) returning to normal after completion of treatment.[41]

Due to the small numbers, the Committee considers this study as inconclusive with respect to the effects of methotrexate on human fertility.

Rheumatoid arthritis, other inflammatory rheumatic disorders, and psoriasis
The results on sperm characteristics (count, motility, morphology) determined before during, and after methotrexate treatment presented in several reports on single cases or case series of psoriatic patients indicated that methotrexate caused reduced sperm quality compared with pre-treatment values. The reductions in counts varied from less than 10% to more than 50%.[16,17,40,45] The Committee notes that in many cases values were still within normal limits (as defined by Grunnet *et al*.[16]) and that in none of these studies, statistical analyses were presented.

In one of these case reports, decreased sperm counts were reported in a man who was treated for severe psoriasis for eight years. Restarting methotrexate therapy after a treatment-free period of seven months during which the sperm characteristics normalized again resulted in severe oligospermia (sperm counts fell from 51 to 1.8 million/mL within three weeks).[42]

El Beheiry *et al*. (1979) did not find changes in semen obtained from 26 male psoriatic patients (age 33-52) or in testicular biopsies and spermatogenic activity in five of these patients. Analyses were performed 70 days after ten-week treatments with weekly oral methotrexate doses of 25 mg.[13]

Developmental toxicity studies

No studies are available regarding the effects of occupational exposure to methotrexate on development in humans.

Induced abortions
Three cases were reported of multiply malformed infants born to women following failed treatment with methotrexate during pregnancy to induce abortion. The congenital defects observed included malformations of the skull, face, and fingers as well as growth retardation.[3,32]

Cancer
Matsui *et al*. (2003) investigated the outcome of the first pregnancy in patients who achieved remission after completing chemotherapy for gestational trophoblastic tumours . Out of 39 patients receiving methotrexate as a single agent, 29 had a normal pregnancy outcome with live birth (mean total doses: 423±304 mg), four an abnormal pregnancy (not further specified; mean total doses: 500±216 mg), and six an elective (‘therapeutic’) abortion (doses unknown).[29]

Two cases were reported of malformed infants born to women who were treated with methotrexate for trophoblastic disease during pregnancy. The effects observed included malformations of the skull, face, and toes, clitoral hypertrophy, and growth retardation.[11,14]

Rheumatoid arthritis, other inflammatory rheumatic disorders, and psoriasis
A systematic review of six articles performed by Martínez Lopéz *et al*.[28] was aimed at the potential effects of methotrexate in the treatment of rheumatoid arthritis on pregnancy outcome. The Committee summarized these studies in Annex E and listed only data on women who used methotrexate only (as far as could be inferred from the articles) during pregnancy.

The studies are all descriptions of cases from retrospectively searched clinical records of patients followed at individual centres or from surveys. The studies included a total of 91 methotrexate-treated pregnant women with the following outcomes: 17 elective abortions (19%); 17 miscarriages (19% of all pregnancies; 23% of pregnancies in which abortion was not induced); 54 live births (59 and 73%, respectively); and three unknown outcomes. Four malformations (one metatarsus varus and eyelid angioma; the other three unknown) were reported (4 and 5%, respectively).

Martinez Lopéz *et al*.[28] stated that figures in the general population are approximately 12-15% for miscarriages before 20 weeks and 3-5% for birth defects. The Committee notes that the percentages of miscarriages and birth defects in the general population depend on the level of ascertainment, which may vary.

Four cases were reported of malformed infants born to women who were treated with methotrexate during pregnancy[9,6,25,34] and one case of a spontaneous abortion (no malformations seen at autopsy)[18]. The malformations included craniofacial, skeletal, cardiopulmonary, gastrointestinal, and genital abnormalities.[9,6,25,34]

Lactation

Johns *et al.* (1972) published data on the secretion of methotrexate into human milk. A 25-year-old woman was treated for choriocarcinoma with oral methotrexate doses of 22.5 mg/day (duration not mentioned) one month post-partum. Milk, blood, and urine samples were obtained at two-hour intervals on the first day of treatment and less frequently on subsequent days. Methotrexate was readily detectable in milk two hours after administration, reaching a peak milk level

(5 x 10^{-9} M or ca. 2.3 µg/L)) and a highest peak milk:plasma ratio (0.08:1) at ten hours. On days 2 and 3, peak milk levels were 6 x 10^{-9} M or ca. 2.7 µg/L.[19]

2.3 Animal studies

Fertility and developmental toxicity studies in laboratory animals are summarized in Annex E.

Fertility studies

Johnson *et al*. (1994) studied the effect of methotrexate on the testes in eight-week-old male post-pubertal Sprague-Dawley rats (n=8/group). Methotrexate was given intravenously at single doses of 100, 300, 500, and 700 mg/kg bw. Necropsy was scheduled 56 days later. Testicular toxicity was evaluated qualitatively by histology and quantitatively by testicular weight, sperm head count, modified Johnsen score*, repopulation index**, and epididymal index***. Fifty percent of the rats treated with 300 mg/kg bw and all rats treated with the higher doses died within five days after methotrexate administration. All other rats appeared healthy and no other delayed deaths occurred. At autopsy of the surviving animals, no effects were observed on body weight, or on kidney, liver, lung, and (absolute and relative) testis weight, nor gross (kidney, liver, lung, testes) or histological (kidney, liver, lung) abnormalities. There was a (not dose-related) reduction in sperm head count at 100 and 300 mg/kg bw. No effects were seen on histology, repopulation index, and epididymal index.[20]

Koehler *et al.* (1988) evaluated the changes in the morphology and function of the gonads in peripubertal male rabbits. Ten 35-45-days-old New Zealand white rabbits received intravenous doses of methotrexate of 6 mg/kg bw, once a week for 14 weeks. In the 15th week, the animals received 57.5 mg/kg bw methotrexate in 0.9% saline as a five-hour infusion while being kept anaesthetized. FSH, LH, testosterone, and androstendione were measured in plasma. Furthermore, the following parameters were measured: concentration methotrexate in testes, tubu-

* Johnsen score: any abnormality suggestive of injury is assigned a score of zero and normal findings are assigned a score of 1. The maximum score is 14.

** repopulation index: the number of tubules with signs of spermatogenic repopulation expressed as a fraction of the total number of tubular sections

*** epididymal index: the amount of sperm in the ductus epididymis semiquantitatively evaluated

lar fertility index (TFI)*, and mean spermatogonia number per tubule in 100 cross sections. The TFI (76.2±2.58) and mean spermatogonia number per tubule (2.55±0.16) were statistically significantly lower in the treated group compared to the controls (88.8±0.75 and 4.85±0.29, respectively). Spermatogonia showed cytoplasmic swelling and vacuolization. No changes were observed at the level of cellular junctions. Methotrexate concentrations in the testes were higher than in serum (37.1±4.22 nmol/L or ca. 17±2 µg/L vs. 13.4±4.95 nmol/L or ca. 6±2 µg/L; $p<0.001$). Compared to the control group, plasma FSH and androstendione levels were elevated, LH was unaltered, and testosterone levels were statistically significantly lower.[23]

Developmental toxicity studies

Oral

Khera (1976) studied the teratogenicity of methotrexate in domestic cats. Daily methotrexate doses of 0 and 0.5 mg/kg bw were given by gavage to pregnant short-haired European and Persian breeds of random origin (n=17-20/group) on gestational days 11-14, 14-17, or 17-20**. Cats were killed on gestational day 44. Live foetuses were processed for skeletal and visceral examination. Maternal toxicity was observed in 8/55 methotrexate-treated cats and included vomiting, leukopenia, decreased neutrophile/lymphocyte ratio, and progressive body weight loss. In the groups exposed during gestational days 11-14, 14-17, and 17-20, mortality occurred in 1/20, 4/17, and 3/18 cats, respectively (vs. 0/10 in controls) and abortion in 5/20, 2/17, and 6/18 cats, respectively (vs. 1/10 in controls). The number of live foetuses and foetal weights were not affected. The malformation rate was increased in all treated groups. Anomalies seen were umbilical hernia, retarded calvarial ossification, hydrocephalus, spina bifida, malformed limbs, cleft palate, talipes varus, bent tail, and subcutaeous oedema.[22]

Parenteral

Darab *et al*. (1987) examined the effects of methotrexate early in embryonic development. C57BL/6J mice (n=15 litters) were treated with a single intraperitoneal methotrexate dose of 20 mg/kg bw (vehicle: saline) on gestational day 9.

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* tubular fertilty index: percentage of seminifeous tubules containing identifiable spermatogonia (per 100 cross sections of tubules)

** comparative time periods of implantation: gestational day 13-14 in cats and 7-8 in humans

A group receiving the methotrexate-preservative benzyl alcohol (0.9% w/v in saline) (n=10 litters) served as control group. Pregnant females were sacrificed on gestational day 18 and examined for malformations and the incidence of resorptions. The total number of implantations were 104 and 79 in treated and control animals, respectively. In the treated mice, the number of viable foetuses was decreased (39% vs. 94% in controls). Of the treated viable foetuses, 44% had median facial clefts with associated clefts of the secondary palate, 24% had isolated cleft palate, and 5% had absence of frontonasal nostrils and associated tissues. The incidence of these malformations in the control group was 0%. Ocular effects (anophthalmia, micropthalmia, open eye lid) were observed in 29% of the treated viable foetuses (vs. 4% in the controls). Whether the effects were seen in the presence or absence of maternal toxicity was not reported.[8]

Wilson *et al*. (1979) treated Wistar rats (n=10-17 litters/group) intravenously with doses of methotrexate of 0.3 mg/kg on gestational day 11 and rhesus monkeys (total of n=20) with doses of 3 mg/kg/day on gestational days 29-32. Autopsy was performed on gestational day 20 (rats) or 32 (monkeys). The embryos were examined externally, weighed, prepared for determination of methotrexate concentrations, and evaluated by the Wilson technique. Methotrexate induced embryotoxicity (20% resorptions vs. 5% in the control group) and teratogenicity (malformations in caudal vertebrae) in rats and embryotoxicity (resorptions, growth retardation), but no teratogenicity in monkeys. Methotrexate concentrations measured in rat embryos varied from 3.4-7.7 ng/g whereas the levels in monkeys were 108-209 ng/g. From the plasma levels measured it was concluded that the degree and type of embryotoxicity was not closely correlated to the level or duration of concentration in the embryos. Rat embryos seem more sensitive to methotrexate than monkey neonates. Whether the effects were seen in the presence or absence of maternal toxicity was not reported.[46]

DeSesso and Jordan (1977) studied the effects of methotrexate given single intravenous doses of 19.2 mg/kg bw (dissolved in sterile distilled water) to pregnant New Zealand White rabbits on gestational day 12 (n=10 litters/group). Untreated and saline control groups (n=12 and 13 litters, respectively) were included. Rabbits were killed two to 32 hours after treatment for histological analysis on embryos or at gestational day 29 for gross and skeletal examinations of the foetuses. The treated litters were reduced in size (25% resorptions vs. 13 and 10% in untreated and saline-treated groups, respectively) and had reduced foetal body weights ($p<0.005$) and higher malformation rates (94% vs. 4 and 6%, respectively; $p<0.005$). The malformations induced included cleft palate, micrognathia,

internal hydrocephalus, short tail, and limb defects. Forelimb defects mostly consisted of absence or fusion of bones of the paws and digits. Whether the effects were seen in the presence or absence of maternal toxicity was not reported.[10]

Jordan *et al*. (1977) studied the embryotoxicity of methotrexate in rats and rabbits. Wistar rats were treated with single intraperitoneal injections of doses of 0.3-2.5 mg/kg bw on various gestational days (4-12). The number of litters varied from 40 (control group) to five. New Zealand White rabbits were given single intraperitoneal doses of 0.3 mg/kg bw and single intravenous doses of 0.3-19.2 mg/kg bw on various gestational days (8-15). The number of pregnant animals varied from four to ten. Rats and rabbits were killed on gestational day 20 and 30, respectively, and litters were examined for intrauterine death and malformations. Jordan *et al*. concluded that the sensitivity varied during gestation in rats with day 10 as the most sensitive day for both embryotoxicity and teratogenicity (type of malformations was not mentioned). Rabbit embryos seemed much more resistent to embryolethal effects of low doses than rat embryos. Changing the route of administration from intraperitoneal to intravenous doubled the lethality and teratogenicity rate, but was still lower than in rats treated intraperitoneally. The frequency and type of malformations and the percentage embryo lethality varied during gestational days 8-15. Gestational days 10-12 seemed to be sensitive to various anomalies including hydrocephalus, microphthalmia, cleft lip and palate, micrognathia, dysplastic sacral and caudal vertebrae, and upper-limb defects, while treatment on day 15 produced only mild hind limb syndactyly. Whether the effects were seen in the presence or absence of maternal toxicity was not reported.[21]

Skalko and Gold (1974) treated ICR mice (n=7-15/group) with single intraperitoneal doses of methotrexate (in sodium hydrogen carbonate) of 0.3-50 mg/kg bw on gestational day 10. Untreated and vehicle control groups (n=10 and 9, respectively) were included. Females were killed on gestational day 17 and litters were observed for intrauterine death and malformations. Doses of 10 mg/kg and higher caused an increase in the resorption rate when compared to controls. Malformation rates were similar compared to the control groups at doses of 0.3-10 mg/kg bw, but increased to 27 and 92% at doses of 25 and 50 mg/kg, respectively (untreated and vehicle-treated controls: 1.5 and 0.9%, respectively). The malformations induced were ectrodactyly (reduction of the digits) and cleft palate. The increase in malformation rate occurred in parallel with an increase in intrauterine deaths. Whether the effects were seen in the presence or absence of maternal toxicity was not reported.[42]

Lactation

No animal data on lactation are available.

2.4 Conclusion

Fertility

Data on women previously treated with methotrexate for ectopic pregnancy indicated that methotrexate treatment does not compromise ovarian reserve[36] or the ability to become pregnant[4]. In addition, no effect on the ability to become pregnant was observed in women treated with methotrexate for gestational trophoblastic disease.[39,47] Case reports on male psoriatic patients showed that treatment with methotrexate may temporarily cause decreases in sperm counts, sperm motility, and the percentage of normal sperm cells. The decreases vary from less than 10% to more than 50%, but were still within normal limits in many cases.[16,17,40,43,45]

In laboratory animals, intravenous administration of methotrexate induced reduced sperm head counts in rats at single doses of 100 and 300 mg/kg bw[20] and statistically significantly decreased tubular fertility indices and mean spermatogonia numbers per tubule, accompanied by cytoplasmic swelling and vacuolization of spermatogonia in rabbits at 14 weekly doses of 6 mg/kg bw[23].

The Committee is of the opinion that the human data indicate that methotrexate is not likely to impair female fertility; data on male fertility stem from case reports only and are considered insufficient to draw conclusions.

There are only two animal studies, both addressing male fertility following intravenous administration.[20,23] Although the Committee considers this route less relevant, methotrexate has been shown to be distributed widely into body tissues and extracellular fluids following intravenous injection. Because the Committee assumes that occupational exposure leads to relevant internal exposure, these intravenous studies are taken into consideration. They showed that methotrexate may induce such histological changes in the testes that functional defects are very likely to occur. However, functional tests were not available.

Therefore, the Committee proposes to classify methotrexate for fertility in category 2 (*suspected human reproductive toxicant*) and to label with H361f.

Developmental toxicity

The Committee is of the opinion that the use of methotrexate as an abortifacient agent justifies to propose classifying methotrexate for development in category 1A (*known human reproductive toxicant*). Classification into category 1A is supported by human case reports[9,11,14,25,29,34] and studies in mice[8,42], rats[21,46], and rabbits[10,21]. These reports and studies showed that methotrexate administered during pregnancy induced malformations involving the central nervous system, skull, face, and limbs as well as developmental delay and intellectual impairment.

Lactation

Methotrexate is excreted in human breast milk in an amount of 0.26 µg/100 mL (Johns *et al*. (1972)[29]. This value is based on one single case study only. Since there is no information about a safe/acceptable daily intake of methotrexate it was not possible to calculate a safe level for methotrexate in human breast milk.

Therefore, the Committee proposes not labelling methotrexate for effects during lactation due to a lack of appropriate data.

Proposed classification for fertility

Category 2; H361f

Proposed classification for developmental toxicity

Category 1A; H360D

Proposed labelling for effects during lactation

Lack of appropriate data precludes the assessment of methotrexate for labelling for effects during lactation.

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A	The Committee
B	Regulation (EC) 1272/2008 of the European Community
C	Additional considerations to Regulation (EC) 1272/2008
D	Comments on the public draft
E	Fertility and developmental toxicity studies

Annexes

Annex **A**

The Committee

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The first draft of the present document was prepared by MM Tegelenbosch-Schouten (TNO Triskelion BV, Zeist. the Netherlands) by contract with the Ministry of Social Affairs and Employment.

The Health Council and interests

Members of Health Council Committees are appointed in a personal capacity because of their special expertise in the matters to be addressed. Nonetheless, it is precisely because of this expertise that they may also have interests. This in itself does not necessarily present an obstacle for membership of a Health Council Committee. Transparency regarding possible conflicts of interest is nonetheless important, both for the President and members of a Committee and for the President of the Health Council. On being invited to join a Committee, members are asked to submit a form detailing the functions they hold and any other material and immaterial interests which could be relevant for the Committee's work. It is the responsibility of the President of the Health Council to assess whether the interests indicated constitute grounds for non-appointment. An advisorship will then sometimes make it possible to exploit the expertise of the specialist involved. During the inaugural meeting the declarations issued are discussed, so that all members of the Committee are aware of each other's possible interests.

Annex **B**

Regulation (EC) 1272/2008 of the European Community

3.7 Reproductive toxicity

3.7.1 Definitions and general considerations

3.7.1.1 Reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring. The definitions presented below are adapted from those agreed as working definitions in IPCS/EHC Document No 225, Principles for Evaluating Health Risks to Reproduction Associated with Exposure to Chemicals. For classification purposes, the known induction of genetically based heritable effects in the offspring is addressed in Germ Cell Mutagenicity (section 3.5), since in the present classification system it is considered more appropriate to address such effects under the separate hazard class of germ cell mutagenicity.

In this classification system, reproductive toxicity is subdivided under two main headings:

(a) adverse effects on sexual function and fertility;

(b) adverse effects on development of the offspring.

Some reproductive toxic effects cannot be clearly assigned to either impairment of sexual function and fertility or to developmental toxicity. Nonetheless, substances with these effects, or mixtures containing them, shall be classified as reproductive toxicants.

3.7.1.2 For the purpose of classification the hazard class Reproductive Toxicity is differentiated into:

- adverse effects
  - on sexual function and fertility, or
  - on development;
- effects on or via lactation.

3.7.1.3 Adverse effects on sexual function and fertility

Any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems.

3.7.1.4 Adverse effects on development of the offspring

Developmental toxicity includes, in its widest sense, any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation. However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women, and for men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means adverse effects induced during pregnancy, or as a result of parental exposure. These effects can be manifested at any point in the life span of the organism. The major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency.

3.7.1.5 Adverse effects on or via lactation are also included in reproductive toxicity, but for classification purposes, such effects are treated separately (see Table 3.7.1 (b)). This is because it is desirable to be able to classify substances specifically for an adverse effect on lactation so that a specific hazard warning about this effect can be provided for lactating mothers.

3.7.2 Classification criteria for substances

3.7.2.1 Hazard categories

3.7.2.1.1 For the purpose of classification for reproductive toxicity, substances are allocated to one of two categories. Within each category, effects on sexual function and fertility, and on development, are considered separately. In addition, effects on lactation are allocated to a separate hazard category.

Table 3.7.1(a) Hazard categories for reproductive toxicants.

Categories		Criteria
CATEGORY 1		Known or presumed human reproductive toxicant Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility, or on development in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A) or from animal data (Category 1B).
	Category 1A	Known human reproductive toxicant The classification of a substance in Category 1A is largely based on evidence from humans.
	Category 1B	Presumed human reproductive toxicant The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.
CATEGORY 2		Suspected human reproductive toxicant Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

Table 3.7.1(b) Hazard category for lactation effects.

EFFECTS ON OR VIA LACTATION
Effects on or via lactation are allocated to a separate single category. It is recognised that for many substances there is no information on the potential to cause adverse effects on the offspring via lactation. However, substances which are absorbed by women and have been shown to interfere with lactation, or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child, shall be classified and labelled to indicate this property hazardous to breastfed babies. This classification can be assigned on the: (a) human evidence indicating a hazard to babies during the lactation period; and/or (b) results of one or two generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk; and/or (c) absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk.

3.7.2.2 Basis of classification

3.7.2.2.1 Classification is made on the basis of the appropriate criteria, outlined above, and an assessment of the total weight of evidence (see 1.1.1). Classification as a reproductive toxicant is intended to be used for substances which have an intrinsic, specific property to produce an adverse effect on reproduction and substances shall not be so classified if such an effect is produced solely as a non-specific secondary consequence of other toxic effects.

The classification of a substance is derived from the hazard categories in the following order of precedence: Category 1A, Category 1B, Category 2 and the additional Category for effects on or via lactation. If a substance meets the criteria for classification into both of the main categories (for example Category 1B for effects on sexual function and fertility and also Category 2 for development) then both hazard differentiations shall be communicated by the respective hazard statements. Classification in the additional category for effects on or via lactation will be considered irrespective of a classification into Category 1A, Category 1B or Category 2.

3.7.2.2.2 In the evaluation of toxic effects on the developing offspring, it is important to consider the possible influence of maternal toxicity (see section 3.7.2.4).

3.7.2.2.3 For human evidence to provide the primary basis for a Category 1A classification there must be reliable evidence of an adverse effect on reproduction in humans. Evidence used for classification shall ideally be from well conducted epidemiological studies which include the use of appropriate controls, balanced assessment, and due consideration of bias or confounding factors. Less rigorous data from studies in humans shall be supplemented with adequate data from studies in experimental animals and classification in Category 1B shall be considered.

3.7.2.3 Weight of evidence

3.7.2.3.1 Classification as a reproductive toxicant is made on the basis of an assessment of the total weight of evidence, see section 1.1.1. This means that all available information that bears on the determination of reproductive toxicity is considered together, such as epidemiological studies and case reports in humans and specific reproduction studies along with sub-chronic, chronic and special study results in animals that provide relevant information regarding toxicity to reproductive and related endocrine organs. Evaluation of substances chemically related to the substance under study may also be included, particularly when information on the substance is scarce. The weight given to the available evidence will be influenced by factors such as the quality of the studies, consistency of results, nature and severity of effects, the presence of maternal toxicity in experimental animal studies, level of statistical significance for inter-group differences, number of endpoints affected, relevance of route of administration to humans and freedom from bias. Both positive and negative results are assembled together into a weight of evidence determination. A single, positive study performed according to good scientific principles and with statistically or biologically significant positive results may justify classification (see also 3.7.2.2.3).

3.7.2.3.2 Toxicokinetic studies in animals and humans, site of action and mechanism or mode of action study results may provide relevant information which reduces or increases concerns about the hazard to human health. If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expressed in humans then a substance which produces an adverse effect on reproduction in experimental animals should not be classified.

3.7.2.3.3 If, in some reproductive toxicity studies in experimental animals the only effects recorded are considered to be of low or minimal toxicological significance, classification may not necessarily be the outcome. These effects include small changes in semen parameters or in the incidence of spontaneous defects in the foetus, small changes in the proportions of common foetal variants such as are observed in skeletal examinations, or in foetal weights, or small differences in postnatal developmental assessments.

3.7.2.3.4 Data from animal studies ideally shall provide clear evidence of specific reproductive toxicity in the absence of other systemic toxic effects. However, if developmental toxicity occurs together with other toxic effects in the dam, the potential influence of the generalised adverse effects shall be assessed to the extent possible. The preferred approach is to consider adverse effects in the embryo/foetus first, and then evaluate maternal toxicity, along with any other factors which are likely to have influenced these effects, as part of the weight of evidence. In general, developmental effects that are observed at maternally toxic doses shall not be automatically discounted. Discounting devel-

opmental effects that are observed at maternally toxic doses can only be done on a case-by-case basis when a causal relationship is established or refuted.

3.7.2.3.5 If appropriate information is available it is important to try to determine whether developmental toxicity is due to a specific maternally mediated mechanism or to a non-specific secondary mechanism, like maternal stress and the disruption of homeostasis. Generally, the presence of maternal toxicity shall not be used to negate findings of embryo/foetal effects, unless it can be clearly demonstrated that the effects are secondary non-specific effects. This is especially the case when the effects in the offspring are significant, e.g. irreversible effects such as structural malformations. In some situations it can be assumed that reproductive toxicity is due to a secondary consequence of maternal toxicity and discount the effects, if the substance is so toxic that dams fail to thrive and there is severe inanition, they are incapable of nursing pups; or they are prostrate or dying.

3.7.2.4 Maternal toxicity

3.7.2.4.1 Development of the offspring throughout gestation and during the early postnatal stages can be influenced by toxic effects in the mother either through non-specific mechanisms related to stress and the disruption of maternal homeostasis, or by specific maternally-mediated mechanisms. In the interpretation of the developmental outcome to decide classification for developmental effects it is important to consider the possible influence of maternal toxicity. This is a complex issue because of uncertainties surrounding the relationship between maternal toxicity and developmental outcome. Expert judgement and a weight of evidence approach, using all available studies, shall be used to determine the degree of influence that shall be attributed to maternal toxicity when interpreting the criteria for classification for developmental effects. The adverse effects in the embryo/foetus shall be first considered, and then maternal toxicity, along with any other factors which are likely to have influenced these effects, as weight of evidence, to help reach a conclusion about classification.

3.7.2.4.2 Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species. However, the limited number of studies which have investigated the relationship between developmental effects and general maternal toxicity have failed to demonstrate a consistent, reproducible relationship across species. Developmental effects which occur even in the presence of maternal toxicity are considered to be evidence of developmental toxicity, unless it can be unequivocally demonstrated on a case-by-case basis that the developmental effects are secondary to maternal toxicity. Moreover, classification shall be considered where there is a significant toxic effect in the offspring, e.g. irreversible effects such as structural malformations, embryo/foetal lethality, significant post-natal functional deficiencies.

3.7.2.4.3 Classification shall not automatically be discounted for substances that produce developmental toxicity only in association with maternal toxicity, even if a specific maternally-mediated mechanism has been demonstrated. In such a case, classification in Category 2 may be considered more appropriate than Category 1. However, when a substance is so toxic that maternal death or severe inanition results, or the dams are prostrate and incapable of nursing the pups, it is reasonable to assume that developmental toxicity is produced solely as a secondary consequence of maternal toxicity and discount the developmental effects. Classification is not necessarily the outcome in the case of minor developmental changes, when there is only a small reduction in foetal/pup body weight or retardation of ossification when seen in association with maternal toxicity.

3.7.2.4.4 Some of the end points used to assess maternal effects are provided below. Data on these end points, if available, need to be evaluated in light of their statistical or biological significance and dose response relationship.

Maternal mortality:

an increased incidence of mortality among the treated dams over the controls shall be considered evidence of maternal toxicity if the increase occurs in a dose-related manner and can be attributed to the systemic toxicity of the test material. Maternal mortality greater than 10 % is considered excessive and the data for that dose level shall not normally be considered for further evaluation.

Mating index

(no. animals with seminal plugs or sperm/no. mated × 100) (*)

Fertility index

(no. animals with implants/no. of matings × 100)

Gestation length

(if allowed to deliver)

Body weight and body weight change:

Consideration of the maternal body weight change and/or adjusted (corrected) maternal body weight shall be included in the evaluation of maternal toxicity whenever such data are available. The calcula-

* () It is recognised that the Mating index and the Fertility index can also be affected by the male.

tion of an adjusted (corrected) mean maternal body weight change, which is the difference between the initial and terminal body weight minus the gravid uterine weight (or alternatively, the sum of the weights of the foetuses), may indicate whether the effect is maternal or intrauterine. In rabbits, the body weight gain may not be useful indicators of maternal toxicity because of normal fluctuations in body weight during pregnancy.

Food and water consumption (if relevant):

The observation of a significant decrease in the average food or water consumption in treated dams compared to the control group is useful in evaluating maternal toxicity, particularly when the test material is administered in the diet or drinking water. Changes in food or water consumption need to be evaluated in conjunction with maternal body weights when determining if the effects noted are reflective of maternal toxicity or more simply, unpalatability of the test material in feed or water.

Clinical evaluations (including clinical signs, markers, haematology and clinical chemistry studies):

The observation of increased incidence of significant clinical signs of toxicity in treated dams relative to the control group is useful in evaluating maternal toxicity. If this is to be used as the basis for the assessment of maternal toxicity, the types, incidence, degree and duration of clinical signs shall be reported in the study. Clinical signs of maternal intoxication include: coma, prostration, hyperactivity, loss of righting reflex, ataxia, or laboured breathing.

Post-mortem data:

Increased incidence and/or severity of post-mortem findings may be indicative of maternal toxicity. This can include gross or microscopic pathological findings or organ weight data, including absolute organ weight, organ-to-body weight ratio, or organ-to-brain weight ratio. When supported by findings of adverse histopathological effects in the affected organ(s), the observation of a significant change in the average weight of suspected target organ(s) of treated dams, compared to those in the control group, may be considered evidence of maternal toxicity.

3.7.2.5 Animal and experimental data

3.7.2.5.1 A number of internationally accepted test methods are available; these include methods for developmental toxicity testing (e.g. OECD Test Guideline 414), and methods for one or two-generation toxicity testing (e.g. OECD Test Guidelines 415, 416).

3.7.2.5.2 Results obtained from Screening Tests (e.g. OECD Guidelines 421 — Reproduction/ Developmental Toxicity Screening Test, and 422 — Combined Repeated Dose Toxicity Study with

Reproduction/Development Toxicity Screening Test) can also be used to justify classification, although it is recognised that the quality of this evidence is less reliable than that obtained through full studies.

3.7.2.5.3 Adverse effects or changes, seen in short- or long-term repeated dose toxicity studies, which are judged likely to impair reproductive function and which occur in the absence of significant generalised toxicity, may be used as a basis for classification, e.g. histopathological changes in the gonads.

3.7.2.5.4 Evidence from in vitro assays, or non-mammalian tests, and from analogous substances using structure-activity relationship (SAR), can contribute to the procedure for classification. In all cases of this nature, expert judgement must be used to assess the adequacy of the data. Inadequate data shall not be used as a primary support for classification.

3.7.2.5.5 It is preferable that animal studies are conducted using appropriate routes of administration which relate to the potential route of human exposure. However, in practice, reproductive toxicity studies are commonly conducted using the oral route, and such studies will normally be suitable for evaluating the hazardous properties of the substance with respect to reproductive toxicity. However, if it can be conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expressed in humans then a substance which produces an adverse effect on reproduction in experimental animals shall not be classified.

3.7.2.5.6 Studies involving routes of administration such as intravenous or intraperitoneal injection, which result in exposure of the reproductive organs to unrealistically high levels of the test substance, or elicit local damage to the reproductive organs, including irritation, must be interpreted with extreme caution and on their own are not normally the basis for classification.

3.7.2.5.7 There is general agreement about the concept of a limit dose, above which the production of an adverse effect is considered to be outside the criteria which lead to classification, but not regarding the inclusion within the criteria of a specific dose as a limit dose. However, some guidelines for test methods, specify a limit dose, others qualify the limit dose with a statement that higher doses may be necessary if anticipated human exposure is sufficiently high that an adequate margin of exposure is not achieved. Also, due to species differences in toxicokinetics, establishing a specific limit dose may not be adequate for situations where humans are more sensitive than the animal model.

3.7.2.5.8 In principle, adverse effects on reproduction seen only at very high dose levels in animal studies (for example doses that induce prostration, severe inappetence, excessive mortality) would

not normally lead to classification, unless other information is available, e.g. toxicokinetics information indicating that humans may be more susceptible than animals, to suggest that classification is appropriate. Please also refer to the section on maternal toxicity (3.7.2.4) for further guidance in this area.

3.7.2.5.9 However, specification of the actual 'limit dose' will depend upon the test method that has been employed to provide the test results, e.g. in the OECD Test Guideline for repeated dose toxicity studies by the oral route, an upper dose of 1 000 mg/kg has been recommended as a limit dose, unless expected human response indicates the need for a higher dose level.

3.7.3 Classification criteria for mixtures

3.7.3.1 Classification of mixtures when data are available for all ingredients or only for some ingredients of the mixture

3.7.3.1.1 The mixture shall be classified as a reproductive toxicant when at least one ingredient has been classified as a Category 1A, Category 1B or Category 2 reproductive toxicant and is present at or above the appropriate generic concentration limit as shown in Table 3.7.2 for Category 1A, Category 1B and Category 2 respectively.

3.7.3.1.2 The mixture shall be classified for effects on or via lactation when at least one ingredient has been classified for effects on or via lactation and is present at or above the appropriate generic concentration limit as shown in Table 3.7.2 for the additional category for effects on or via lactation.

Table 3.7.2 Generic concentration limits of ingredients of a mixture classified as reproduction toxicants or foreffects on or via lactation that trigger classification of the mixture.

Ingredient classified as:	Generic concentration limits triggering classification of a mixture as:			
	Category 1A reproductive toxicant	Category 1B reproductive toxicant	Category 2 reproductive toxicant	Additional category for effects on or via l actation
Category 1A reproductive toxicant	≥ 0,3 % [Note 1]			
Category 1B reproductive toxicant		≥ 0,3 % [Note 1]		
Category 2 reproductive toxicant			≥ 3,0 % [Note 1]	
Additional category for effects on or via lactation				≥ 0,3 % [Note 1]

Note The concentration limits in the table above apply to solids and liquids (w/w units) as well as gases (v/v units).
Note 1 If a Category 1 or Category 2 reproductive toxicant or a substance classified for effects on or via lactation is present in the mixture as an ingredient at a concentration above 0,1 %, a SDS shall be available for the mixture upon request.

3.7.3.2 Classification of mixtures when data are available for the complete mixture

3.7.3.2.1 Classification of mixtures will be based on the available test data for the individual ingredients of the mixture using concentration limits for the ingredients of the mixture. On a case-by-case basis, test data on mixtures may be used for classification when demonstrating effects that have not been established from the evaluation based on the individual components. In such cases, the test results for the mixture as a whole must be shown to be conclusive taking into account dose and other factors such as duration, observations, sensitivity and statistical analysis of reproduction test systems. Adequate documentation supporting the classification shall be retained and made available for review upon request.

3.7.3.3 Classification of mixtures when data are not available for the complete mixture: bridging principles

3.7.3.3.1 Subject to paragraph 3.7.3.2.1, where the mixture itself has not been tested to determine its reproductive toxicity, but there are sufficient data on the individual ingredients and similar tested mixtures to adequately characterise the hazards of the mixture, these data shall be used in accordance with the applicable bridging rules set out in section 1.1.3.

3.7.4 Hazard Communication

3.7.4.1 Label elements shall be used for substances or mixtures meeting the criteria for classification in this hazard class in accordance with Table 3.7.3

Table 3.7.3 Label elements for reproductive toxicity.

Classification	Category 1A or Category 1B	Category 2	Additional category foreffects on or via lactation
GHS Pictograms			No pictogram
Signal Word	Danger	Warning	No signal word
Hazard Statement	H360: May damage fertility or the unborn child (state specific effect if known)(state route of exposure if it is conclusively proven that no other routes of exposure cause the hazard)	H361: Suspected of damaging fertility or the unborn child (state specific effect if known) (state route of exposure if it is conclusively proven that no other routes of exposure cause the hazard)	H362: May cause harm to breast-fed children.
Precautionary Statement Prevention	P201 P202 P281	P201 P202 P281	P201 P260 P263 P264 P270
Precautionary Statement Response	P308 + P313	P308 + P313	P308 + P313
Precautionary Statement Storage	P405	P405	
Precautionary Statement Disposal	P501	P501	

Annex **C**

Additional considerations to Regulation (EC) 1272/2008

The classification and labelling of substances is performed according to the guidelines of the European Union (Regulation (EC)1272/2008) presented in Annex B. The classification of compounds is ultimately dependent on an integrated assessment of the nature of all parental and developmental effects observed, their specificity and adversity, and the dosages at which the various effects occur. The guideline necessarily leaves room for interpretation, dependent on the specific data set under consideration. In the process of using the regulation, the committee has agreed upon a number of additional considerations:

- If there is sufficient evidence to establish a causal relationship between human exposure to the substance and impaired fertility or subsequent developmental toxic effects in the offspring, the compound will be classified in category 1A, irrespective of the general toxic effects (see Annex B, 3.7.2.2.1.).
- Adverse effects in a reproductive study, reported without information on the parental or maternal toxicity, may lead to a classification other than category 1B, when the effects occur at dose levels which cause severe toxicity in *general* toxicity studies.
- Clear adverse reproductive effects will not be disregarded on the basis of reversibility per se.

- The committee does not only use guideline studies (studies performed according to OECD* standard protocols) for the classification of compounds, but non-guideline studies are taken into consideration as well.

* Organisation for Economic Cooperation and Development

Annex **D**

Comments on the public draft

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- J. Zawierko, the Netherlands

Annex **E**

Fertility and developmental toxicity studies

Table 1 Summary of studies the potential effects of methotrexate in the treatment of rheumatoid arthritis on pregnancy outcome (adapted from Martínez Lopéz *et al*.[28]).

	Chakravarty[5]	Donnenfeld[12]	Kozlowski[24]	Lewden[27]	Østensen[37]	Østensen[38]	
	survey among rheumatologists n=65 100% with RA	survey among teratology centres n=3 67% with RA	exposed case series n=8 mean age: 28.4 y; 88% with RA	exposed case series n=28 mean age: 32.9 y; 100% with any rheumatic disease	exposed case series n=4 mean age: 26.8 y; 100% with any rheumatic disease	survey among patients n=34 100% with any rheumatic disease	survey among rheumatologists n=19 100% with any rheumatic disease
pregnancies	38[a]	3	10	28	4	3	5[b]
weeks of exposure (range)	1.5-15	2-5	2-15	2-9[c]	3-6	-[d]	-[d]
elective abortions (%)	8 (21)[e]	0	2 (20)[f]	5 (18)[g]	0	2 (67)[g]	0
miscarriages[h] (%[i])	7 (23)	0	3 (30)	4 (17)	1 (25)	1 (100)	1 (20)
live births[j] (%[i])	23 (74)	3 (100)	5 (50)	19 (83)	3 (75)	0	1 (20)
congenital malformations (%[i])	3 (10)[k]	0	0	1 (4)	0	0	0

RA: rheumatoid arthritis
[a] one woman still pregnant at the time of the survey excluded
[b] outcome of three pregnancies unknown
[c] one patient was exposed from week 6 to 11
[d] exposed at conception or during first trimester
[e] four were counselled by their rheumatologist to consider termination, because of taking methotrexate during pregnancy and concern about its teratogenicity
[f] these patients accepted the offer for termination of a pregnancy while bearing a potentially damaged foetus
[g] no information on reasons
[h] percentage of miscarriages before 20 weeks in the general population: approximately 12-15%[28] [note of the committee: this percentage depends on the level of ascertainment, which may vary]
[i] percentage of outcome from all pregnancies except those in which abortion was induced electively
[j] percentage of birth defects in the general population: 3-5%[28] [note of the committee: this percentage depends on the level of ascertainment, which may vary]
[k] one case of malformations concerned a spontaneous abortion

Table 2 Fertility studies in laboratory animals with methotrexate: intravenous administration.

authors	species	experimental period/ design	dose	general toxicity	effects on reproductive organs/ effects on reproduction
Johnson (1994)	Sprague Dawley rats (n=8/ group)	one single injection; necropsy on day 56; testicular toxicity evaluated qualitatively and quantitatively	100, 300, 500, 700 mg/ kg bw	mortality in half the number of rats treated with 300 mg/kg bw and all rats treated with 500 and 700 mg/kg bw; all other rats healthy	reduction in sperm head count (measured in testis) at 100 and 300 mg/kg bw; no effect on histology, repopulation index, epididymal index
Koehler (1988)	New Zealand White rabbits (n=20)	one injection once/week for 14 weeks. In the 15th week an 5-hour infusion;. measured: FSH, LH, testosterone, androstendione levels in plasma; methotrexate levels in testes; tubular fertility index (TFI)[a] and mean spermatogonia count per tubule in 100 cross sections	6 mg/kg bw; infusion: 57.5 mg/kg bw	not reported	TFI significantly lower in the treated group as compared to the controls; reduced number of spermatogonia and increased cell size and swelling of cytoplasm of spermatogonia; no changes at the level of cellular junctions. methotrexate concentration in testes (17±2 µg/L) significantly higher than in serum (6±2 µg/L) increased plasma FSH and androstendione levels; decreased testosterone levels; LH unaltered

bw=body weight; FSH=follicle stimulating hormone; LH=luteinizing hormone; n=number
[a] tubular fertiltiy index: percentage of seminiferous tubules containing identificable spermatogonia (per 100 cross sections of tubules)

Table 3.1 Developmental toxicity studies in laboratory animals with methotrexate: gavage.

authors	species	experimental period/ design	dose	general toxicity	developmental toxicity
Khera (1976)	short-haired European and Persian cats (n=17/20 group)	gd 11-14, 14-17, or 17-20; Caesarean section at gd 44; foetuses observed for abnormalities.	0.5 mg/kg/d (in gelatin capsules)	in all treated groups maternal toxicity: vomiting, leukopenia, decreased neutrophile/lymphocyte ratio, progressive bw loss, mortality	number of live foetuses and foetal weight not affected; increased malformation rate in all treated groups; anomalies: umbilical hernia, retarded calvarial ossification, hydrocephalus, spina bifida, malformed limbs, cleft palate, talipes varus, bent tail, subcutaneous oedema.

Table 3.2 Developmental toxicity studies in laboratory animals with methotrexate: parenteral administration

authors	species	experimental period/ design	dose/route	general toxicity	developmental toxicity
Darab (1987)	C57BL/6J mice (n=10/15 group)	single dose on gd 9; sacrifice on gd 18	20 mg/kg bw; ip	not reported	in the viable treated foetuses: 44% with median facial clefts; 25% with isolated cleft palate; 5% with absent frontonasal nostrils and associated tissues; 29% with ocular effects (anophthalmia, micropthalmia, open eye lid); percentage of resorptions in the treated group 61% vs. 6% in control group.
Wilson (1979)	rhesus monkeys (n=20); Wistar rats (n=10-17 litters/group)	rats: single dose on gd 11; monkeys gd 29-32; autopsy on gd 20 (rats), gd 32 (monkeys); embryos examined externally, weighed, prepared for methotrexate assay and Wilson technique	rats: 0.3 mg/kg bw; iv monkeys: 3 mg/kg bw/d; iv	not reported.	rats: embryotoxicity and teratogenicity (malformations in caudal vertebrae); methotrexate levels in embryos: 3.4-7.7 ng/g monkeys: embryotoxicity; methotrexate levels in embryos: 108-209 ng/g.
DeSesso/Jordan (1977)	New Zealand white rabbits (n=10/12 litters /group)	single dose on gd 12; sacrifice 2-32 hours after treatment (embryo analysis) or on gd 29 (gross malformations and skeleton)	19.2 mg/kg bw; iv	not reported.	resorptions: 25% vs. 13% in controls; reduced foetal bw; increased malformation rate (94% vs. 4% in control); malformations: cleft palate, micrognathia, internal hydrocephalus, short tail, limb defects

Jordan (1977)	Wistar rats (5-20/group); New Zealand white rabbits (n=4-10)	rats: single dose on various gd (4-12); sacrifice on gd 20 rabbits: single dose on various gd (8-15); sacrifice on gd 30; litters observed for intrauterine death, malformations.	rats: 0.3-2.5 mg/kg bw/d; ip rabbits: 0.3 mg/kg bw/d; ip and 0.3-19.2 mg/kg bw/d; iv	not reported.	rats: gd 10 most sensitive day for both embryotoxicity and teratogenicity (type of malformations not mentioned); rabbits: frequency and type of malformations, and the percentage embryolethality varied during gd 8-15. during gd 10-12: sensitive to various anomalies including hydrocephalus, microphthalmia, cleft lip and palate, micrognathia, dysplastic sacral and caudal vertebrae, upper-limb defects; on gd 15: only mild hind limb syndactyly.
Skalko/Gold (1974)	ICR mice (n=7-15/ group)	single dose on gd 10; sacrifice: gd 17; litters observed for intrauterine death and malformations	0.3-50 mg/kg bw; ip	not reported.	increased resorption rate; increased malformation rate at doses of 25 and 50 mg/kg to 27 and 92% respectively (vs. 0.9% in vehicle controls); malformations induced: ectrodactyly (reduction of the digits), cleft palate; increase in malformation rate in parallel with increase in intrauterine mortality.

bw = body weight; d=day(s); gd=gestational day(s); ip=intraperitoneal; iv=intravenous; n=number