All-trans retinoic acid

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All-trans retinoic acid

Evaluation of the effects on reproduction, recommendation for classification

To: the State Secretary of Social Affairs and Employment
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Health Council of the Netherlands

contents

samenvatting

Op verzoek van de minister van Sociale Zaken en Werkgelegenheid (SZW) heeft de Gezondheidsraad beoordeeld of all-trans-retinoïnezuur invloed heeft op de voortplanting. Dit advies is opgesteld door de commissie Classificatie reproductietoxische stoffen, een subcommissie van de vaste commissie Gezondheid en beroepsmatige blootstelling aan stoffen (GBBS).

De Gezondheidsraad heeft een vaste rol bij de bescherming van werknemers tegen mogelijke schadelijke effecten van stoffen waar zij tijdens hun werk mee in aanraking kunnen komen. Op www.gezondheidsraad.nl staat meer informatie over die rol.

Gebruik van all-trans-retinoïnezuur

All-trans-retinoïnezuur komt van nature voor in het lichaam en speelt een cruciale rol tijdens de embryonale ontwikkeling. Het wordt in het lichaam gevormd uit vitamine A en kan cellen tot deling en differentiatie aanzetten. Daarom wordt deze stof – ook wel bekend als tretinoïne – veel gebruikt in therapeutische crèmes voor behandeling van huidaandoeningen zoals acne, alsmede in anti-rimpel crèmes. Ook wordt all-trans-retinoïnezuur therapeutisch ingezet bij de behandeling van een acute vorm van bloedkanker (promyelocytische leukemie) en van andere vormen van kanker (experimenteel). Mensen die werkzaam zijn in de farmaceutische industrie, in apotheken of in ziekenhuizen kunnen tijdens hun werk in aanraking komen met all-trans-retinoïnezuur.

Classificeren naar bewijskracht voor schadelijk effect

Bij de beoordeling van effecten op de voortplanting kijkt de commissie zowel naar de effecten op de fertiliteit (vruchtbaarheid) van mannen en vrouwen als naar de effecten op de ontwikkeling van het nageslacht. Daarnaast worden de effecten op de lactatie (hoeveelheid en kwaliteit van moedermelk) beoordeeld en de effecten via de moedermelk op de zuigeling.

De commissie beoordeelt of er aanwijzingen zijn dat de stof een schadelijk effect kan hebben. Als dergelijke aanwijzingen bestaan stelt ze voor om de stof in te delen in een bepaalde gevarencategorie, die aangeeft hoe sterk de bewijskracht is voor het schadelijke effect van de stof. Op basis van dat voorstel kan de minister van SZW besluiten om de stof al dan niet als reproductietoxische stof aan te merken. De indeling in gevarencategorieën is gebaseerd op EU-verordening (EG) 1272/2008.

Advies aan de minister

Op grond van de beschikbare wetenschappelijke gegevens stelt de commissie voor om all-trans-retinoïnezuur alleen voor effecten op de ontwikkeling van het nageslacht in te delen in

een gevarencategorie. Over de effecten op de vruchtbaarheid en op of via lactatie zijn onvoldoende geschikte gegevens beschikbaar.

Voorstel commissie voor indeling all-trans-retinoïnezuur:

- Voor effecten op de fertiliteit adviseert de commissie all-trans-retinoïnezuur niet in te delen in een gevarencategorie wegens onvoldoende geschikte gegevens.
- Voor effecten op de ontwikkeling adviseert de commissie all-trans-retinoïnezuur in te delen in categorie 1B (*stoffen waarvan verondersteld wordt dat zij toxisch zijn voor de menselijke voortplanting*) en te etiketteren met H360D (*kan het ongeboren kind schaden*).
- Voor effecten op of via lactatie adviseert de commissie om all-trans-retinoïnezuur niet te etiketteren wegens onvoldoende geschikte gegevens.

executive summary

At the request of the Minister of Social Affairs and Employment, the Health Council of the Netherlands evaluated the effects of all-trans retinoic acid on reproduction. This advisory report has been drafted by the Subcommittee on the Classification of Reproduction Toxic Substances of the Dutch Expert Committee on Occupational Safety (DECOS) of the Health Council. The Health Council has a permanent task in assessing the hazard of substances to which man can be exposed occupationally. More information about this task can be found at www.gezondheidsraad.nl.

Use of all-trans retinoic acid

All-trans retinoic acid occurs naturally in the body and plays a crucial role in cellular processes during embryogenesis. It is produced as a result of vitamin A metabolism. Due to its cell-activating properties, all-trans retinoic acid – also known as tretinoin – is widely used in therapeutic creams for skin disorders, as well as in 'anti-aging' creams. Other uses are the treatment of acute promyelocytic leukemia and other forms of cancer (experimental approaches). Workers can be occupationally exposed to all-trans retinoic acid in the pharmaceutical industry, in pharmacies or in hospitals.

Classification according to strength of evidence for toxic effect

For assessing the effects on reproduction, the Committee evaluates the effects on male and female fertility and on the development of the offspring. Moreover, the Committee considers the effects of a substance on lactation and on the offspring via lactation.
If data indicating hazardous properties are available, the Committee recommends classification in a category based on the strength of the evidence. Based on that proposal, the minister of Social Affairs and Employment can decide whether to classify the substance as toxic to reproduction.
The classification is performed according to EU-regulation (EC) 1272/2008.

Recommendations to the minister

Based on the available scientific data, the Committee recommends to classify all-trans retinoic acid for effects on offspring development only. There are insufficient data for classification with regard to effects on paternal and maternal fertility and effects on or via lactation.

The Committee's classification proposal for all-trans retinoic acid:

- For fertility, the Committee recommends not classifying all-trans retinoic acid due to a lack of appropriate data.
- For developmental toxicity, the Committee recommends classifying all-trans retinoic acid

in category 1B (*presumed human reproductive toxicant*) and to label it H360D (*may damage the unborn child*).

- For effects on or via lactation, the Committee recommends not labelling all-trans retinoic acid due to a lack of appropriate data.

01 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 regulation 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 1B and 2) or compound with effects on or via lactation.

1.2 Committee and procedure

This document contains the recommendations for classification of all-trans retinoic acid (all-trans RA) by the Health Council's Subcommittee on the Classification of Reproduction Toxic Substances, hereafter called the Committee. The members of the Committee are listed on the last page of this report. The classification is based on the evaluation of published human and animal studies concerning adverse effects with respect to fertility and offspring development as well as adverse effects on or via lactation.

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

Hazard statement codes

- H360F: May damage fertility
- H360D: May damage the unborn child
- H361f: Suspected of damaging fertility
- H361d: Suspected of damaging the unborn child
- H360FD: May damage fertility. May damage the unborn child
- H361fd: Suspected of damaging fertility. Suspected of damaging the unborn child
- H360Fd: May damage fertility. Suspected of damaging the unborn child

- H360Df: May damage the unborn child. Suspected of damaging fertility
- H362: May cause harm to breast-fed children

The classification and labelling of substances is performed according to the guidelines of the European Union (Regulation (EC) 1272/2008). The classification of compounds is the result of 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.

Additional considerations to Regulation (EC) 1272/2008

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 Regulation (EC) 1272/2008, 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[a] standard protocols) for the classification of compounds, but non-guideline studies are taken into consideration as well.

Regarding fertility, the Committee considers data on parameters related to fertility, such as seminal fluid volume and spermatozoa concentration, that are related to male fertility. The Committee excludes publications containing only data on sex hormone levels from the assessment, because the relationship between these hormone levels and functional fertility (ability to conceive children) is too uncertain.

In 2019, the President of the Health Council released a draft of the report for public review. The Committee has taken the comments received into account in deciding on the final version of the report. These comments, and the replies by the Committee, can be found on the website of the Health Council.

[a] Organisation for Economic Cooperation and Development

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 criteria define 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 on or via lactation is based on a 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 on or via 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 children, or if that level is unknown, the exposure limit for the general population, e.g. the acceptable daily intake (ADI).

1.4 Data

Literature searches were conducted in PubMed and Toxline up to and including October 2019. Literature was selected primarily on the basis of the abstracts. Publications cited in the selected articles, but not selected during the primary search, were reviewed if considered appropriate. Only original publications were included in the assessment, reviews were not.

The search criteria for PubMed were:

1. ((((((tretinoin) OR all tretinoin) OR all trans retinoic acid) OR retinoic acid) OR vitamin a acid)) AND (((((fertility effects[MeSH Terms]) OR fertility agents[MeSH Terms]) OR fertility[MeSH Terms])))
2. ((((((tretinoin) OR all tretinoin) OR all trans retinoic acid) OR retinoic acid) OR vitamin a acid)) AND ((((prenatal exposure delayed effects[MeSH Terms]) OR pregnancy outcomes[MeSH Terms]) OR pregnancy[MeSH Terms]) OR maternal exposures[MeSH Terms])
3. (((milk[MeSH Terms]) OR lactation[MeSH Terms])) AND ((((((tretinoin) OR all tretinoin) OR all trans retinoic acid) OR retinoic acid) OR vitamin a acid)

The search criteria for Toxline were:

4. (CAS-nr AND (fertility OR (developmental toxicity) OR teratogen* OR milk OR lactation).

The Committee evaluated both human and animal studies. The animal data are described in more detail in Annex A. For each study, the quality of its design (performed according to internationally acknowledged guidelines) and that of its documentation are considered.

In the assessment of the potential adverse effects of all-trans RA on reproduction, the Committee also used data on adverse effects related to its application as a therapeutic agent.

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:

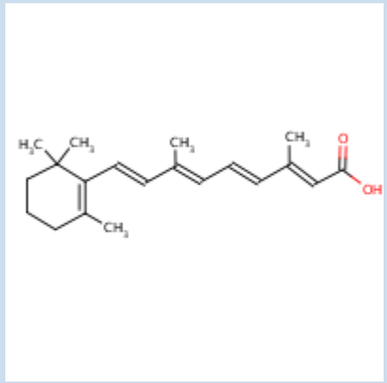
- Lack of appropriate data precludes assessment of the compound for reproductive toxicity.
- Sufficient data show that no classification for toxicity to reproduction is indicated.

1.6 Final remark

The classification of compounds is based on hazard evaluation (Niesink et al., 1995)[1] only, 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 characterisation, human exposure assessment and recommendations of other organisations.

02 all-trans retinoic acid

2.1 Properties

Name	All-trans retinoic acid
CAS name	Tretinoin
IUPAC name	(2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraenoic acid
CAS number	302-79-4
EINECS number	206-129-0
Synonyms	All-tretinoin, vitamin A acid
Colour and physical state	Yellow to light orange crystalline powder with floral odor
Molecular weight	300.44 g/mol
Molecular formula	$C_{20}H_{28}O_2$
Structural formula	
Melting point	180-182°C (experimental)
Vapour pressure	3.23 * 10^{-5} Pa at 25°C (estimated)
Log P (octanol-water)	6.3 (experimental)
Solubility	Practically insoluble in water and glycerin; Soluble in DMSO and ether; Slightly soluble in polyethylene glycol 400, octanol, ethanol and chloroform

Name	All-trans retinoic acid
Uses	All-trans retinoic acid (all-trans RA) is used for the treatment of acne.[2] It has also been shown to have beneficial effects in other dermatological disorders such as photo-induced epidermal dysplasia and solar keratosis. In addition, all-trans RA is employed in “anti-aging” creams, since it induces dermal and epidermal thickening with long-term application. Other uses include the treatment of senile lentigines (“liver spots”) and conditions characterized by hyperkeratotic disorders, the promotion of re-epithelialization and the treatment of keloid scars.[3] The substance is also applied systemically, in treatment of acute promyelocytic leukemia.[4] Its common name in medicines is tretinoin.
Occupational exposure	Can occur in the pharmaceutical industry, in pharmacies or in hospitals
General toxicity	In overdose, multiple organ systems can be affected and effects can be similar to symptoms of hypervitaminosis A.
Mechanism	Regulates cellular growth and differentiation
Metabolism	All-trans RA is produced naturally in the body of chordates during the breakdown of beta-carotene (provitamin A). This takes three oxidation steps: to retinol, retinal, and finally all-trans RA. The all-trans RA can isomerize to 13-cis retinoic acid (13-cis RA), which is a reversible reaction.[5,6]
Kinetics	The substance has been applied therapeutically either directly on the skin, or via the oral route. The pharmaco-kinetics of these application routes differ significantly. Topical application changes endogenous all-trans RA levels only minimally.[7] Oral administration, however, increases all-trans RA concentrations in the blood similar to those following intravenous administration, as demonstrated by the area under the concentration-time curve (AUC).[8-10]
Data from ECHA and HSDB, unless otherwise noted.[11,12]	

2.2 Context

Introduction

Vitamin A is an essential nutrient for humans. It consists of a group of substances possessing vitamin A activity (retinoids). Retinoids are vital for vision, reproduction, development, growth and immunity. A wealth of publications describes the adverse developmental effects of maternal vitamin A deficiency and the role of the different retinoids in development. The retinoid all-trans RA is found endogenously and is not only vital for the health of the mother, but also required for maintenance of the placenta and essential for the developing embryo, as recently reviewed by Spiegler and colleagues.[13] Maternal overdosing of vitamin A is also known to cause adverse developmental effects. All-trans RA may be the responsible metabolite. In this advisory report, its reproductive toxicity is assessed. In the human studies, the background intake of vitamin A, through food or supplements, may have had an effect adding to that of all-trans RA or 13-cis RA. However, due to inadequate information about the level of the vitamin A intake in these studies, this cannot be taken into account.

Isomerization

An important aspect in the evaluation is to what extent all-trans RA can isomerize to 13-cis RA, and vice versa. If isomerization occurs in the body, it cannot be excluded that the effects that seem to be caused by all-trans RA, are caused by 13-cis RA. This is relevant for the present report, because the medicine isotretinoin (13-cis RA) was already shown to be a human teratogen in 1985.[14] Oral isotretinoin medication during the first trimester of pregnancy was shown to be associated with a high relative risk (RR) of malformations in the offspring (RR=25.6; 95% confidence interval (CI) 11.4-57.5). The malformations were found to have a characteristic pattern involving craniofacial, cardiac, thymic and central nervous system structures. Furthermore, 17.4% of the pregnancies of the women taking isotretinoin orally around conception and during organogenesis ended in a miscarriage. The findings from this study and previous smaller studies led to worldwide consensus that women who are pregnant or want to become pregnant should not take isotretinoin.

The presence of all-trans RA and 13-cis RA and their isomerization in the human body are well-documented. Some findings from in vivo and in vitro studies are given below. The serum of healthy pregnant and non-pregnant women has been shown to contain approximately 20 percent more all-trans RA than 13-cis RA, demonstrating that both isomers can be present under normal circumstances.[5] The serum of patients taking all-trans RA orally for treatment of leukemia shows partial isomerization to 13-cis RA.[15,16] Some isomerization of all-trans RA to 13-cis RA was also seen in rats and rabbits exposed to all-trans RA orally.[17,18] Furthermore, the isomerization in this direction was compared directly to that in the opposite direction. The level of isomerization of all-trans RA to 13-cis RA observed in cultured human sebocytes and cultured human liver microsomes was lower than vice versa.[19,20] This was also demonstrated in cultured rat liver microsomes.

Thus, isomerization takes place in both directions, but all-trans RA generally seems to be present in higher concentrations than 13-cis RA. Together with the human teratogenicity of 13-cis RA, the above underpins that reproduction toxicity data on 13-cis RA should be taken into account when assessing the reproduction toxicity of all-trans RA, if the data on all-trans RA itself are insufficient.

Kinetics

All-trans RA and 13-cis RA have different kinetics.[6] All-trans RA has been shown to exhibit a higher tissue distribution than13-cis RA, which may be related to differences in binding to the cellular retinoid-binding proteins and receptors. On the other hand, all-trans RA may have limited access to the nucleus, whereas 13-cis RA may have extensive access. The half-life of all-trans RA (1 hour) is relatively short compared to that of 13-cis RA (16 hours).

Species differences in the kinetics of all-trans RA and 13-cis RA have been observed. For instance, the clearing rates of 13-cis RA are lower in target organs of primates than in those of rodents.

Foetal/maternal ratio

Maternal all-trans RA and maternal 13-cis RA can both be transferred to the foetus.[5] The all-trans RA concentration in the placental cord blood of newborns was shown to be lower than that in the blood of their mothers (1.0 µg/L vs 1.7 µg/L), amounting to around 60% of the concentration in maternal blood (range 44-100%). The 13-cis RA concentrations in newborn and maternal blood differed similarly (0.6 and 0.8 µg/L, respectively), the concentration in newborn blood being approximately 74% of that in maternal blood (range 54-95%).

Implications for this advisory report

This report focusses on the adverse effects on reproduction of high maternal exposure to all-trans RA. The Committee considers any publications concerning the human reproductive toxicity of 13-cis RA as supportive data. The reasons for doing so are the scarcity of human data concerning the reproductive toxicity of all-trans RA, the general consensus about 13-cis RA being a developmental toxicant and the isomerization between all-trans RA and 13-cis RA occurring in the body. It remains elusive, however, to what extent each of the isomers contributes to the reproductive effects observed in the human studies of the other isomer. The present assessment is restricted to all-trans RA and 13-cis RA exposures due to monotherapy. The substance names all-trans RA and 13-cis RA are used throughout the text, except in the description of the human studies, the primate study and a topical exposure study in rabbits. In these cases, the drug names tretinoin and isotretinoin are used, respectively.

2.3 Human studies

2.3.1 Fertility studies

Vogt and Ewers (1985) examined the sperm of men with acne conglobata treated with isotretinoin.[21] Twenty patients who were healthy otherwise, were voluntarily enrolled and received an oral dose of 1 mg/kg bw daily for twelve weeks. The sperm concentration of their ejaculate was analysed before and after treatment. After treatment, this concentration was increased ($p<0.05$). A subgroup of twelve men was available for follow-up, twelve weeks after the end of therapy. By then, the sperm concentration of their ejaculate had returned to before-treatment levels.

Török et al. (1987) examined the sperm of men with acne conglobata treated with isotretinoin.[22] Thirteen patients received systemic therapy, but the exposure route was not mentioned. The sperm was examined before treatment and after 16 weeks of treatment at a therapeutic dose (1 mg/kg). The following sperm parameters were investigated: ejaculate volume, sperm concentration, total sperm count, absolute and progressive motility, sperm morphology and fructose concentration. After isotretinoin treatment, absolute and progressive motility increased ($p<0.05$ and $p<0.01$, respectively), while the ejaculate volume, sperm concentation, total sperm count and fructose concentration were unchanged.

Çinar et al. (2016) investigated the effect of systemic isotretinoin treatment on male fertility.[23] Eighty-one adult male patients with severe or refractory acne vulgaris were included in the study. They were given a total dose of 120 mg/kg of isotretinoin orally over a period of six months. Before and after the study period, the spermiogram parameters of the patients were evaluated. All of the spermiogram parameters increased slightly ($p<0.05$), except for the percentage normal morphology, that decreased ($p<0.05$).

Çinar et al. (2017) also investigated the effect of systemic isotretinoin treatment on female fertility.[24] Seventy-nine women with acne were included in the study. They were given isotretinoin orally for six months and were examined before treatment, directly after treatment and twelve months later. The dose they received was gradually increased from 0.6-0.8 mg/kg to 120-150 mg/kg (reported in a previous paper concerning a shorter follow-up of the patients[25]). The patients' ovarian volume and antral follicle count were measured using ultrasound scanning. Treatment led to a decreased ovarian volume, as well as a decreased antral follicle count ($p<0.001$). These parameters had returned to pre-treatment levels twelve months later ($p<0.001$).

2.3.2 Developmental toxicity studies

Clinicians generally consider all-trans RA and 13-cis RA as teratogens.[26-30] The Committee assesses the strength of the evidence for the purpose of classification. With regard to the epidemiological data, this assessment is focused on data regarding monotherapy.

Oral exposure

Lammer et al. (1985) studied the pregnancy outcomes among women treated orally with isotretinoin for severe, recalcitrant cystic acne during the first trimester.[14] To this end, 154 human pregnancies with voluntarily reported foetal exposure to isotretinoin were investigated. A foetus was considered exposed, if the mother reported taking oral isotretinoin at any time between five days before and 70 days after her estimated date of conception. The duration of exposure ranged from seven to 124 days and daily dosages were within the range of 0.5 to 1.5 mg/kg bw. Pregnancies were classified as prospective (n=36) if they were reported before the foetal outcome was determined.These were all reported before the 12th week of gestation. All other pregnancies were classified as retrospective, including those for which diagnostic foetal ultrasound was performed before the exposure was reported. The investigators compared the rates of malformation in the exposed cohort with those in infants born in metropolitan Atlanta in 1982 and registered in the population-based Metropolitan Atlanta Congenital Defects Program. The outcomes of the 154 pregnancies were 95 elective first-trimester abortions, twelve spontaneous abortions, 26 infants without major malformations and 21 malformed infants. The outcomes in the prospective subcohort (n=36) were eight spontaneous abortions, 23 non-affected infants and five malformed infants. Exposure to isotretinoin was associated with an increased RR of malformations (RR=25.6; 95% CI 11.4-57.5) and with a higher risk of spontaneous abortion (no statistics reported).

Among the 21 malformed infants, the investigators found a characteristic pattern of malformations, involving craniofacial, cardiac, thymic, and central nervous system structures. The malformations included microtia/anotia (15 infants), micrognathia (6), cleft palate (3), conotruncal heart defects and aortic-arch abnormalities (8), thymic defects (7), retinal or optic-nerve abnormalities (4), and central nervous system malformations (18). In one case, the central nervous system malformation was a holoprosencephaly. None of the mothers reported the use of other known teratogenic agents, although information about alcohol use was not obtained. None of the mothers had diabetes, a seizure disorder, or another chronic illness that might increase the risk of bearing a malformed infant. No similar malformations were reported among the parents and siblings of the affected infants. Rosa et al (1986) reported some additional cases.[31]

In a conference abstract, Rosa et al.(1994) listed holoprosencephalies reported to the U.S. Food and Drug Administration.[32] From 1969 up to and including 1993, two holoprosencephalies were reported among infants born to women exposed to isotretinoin orally during the first trimester of pregnancy. Another two holoprosencephalies occurred after discontinued exposure before conception. Nineteen suspected holoprosencephalies were among 8700 non-retinoid exposure birth defect case reports to the FDA. The data concerning infants born to women exposed to tretinoin topically are described in the section *Topical exposure*.

Verma et al. (2016) reviewed the case studies of acute promyelocytic leukemia during pregnancy.[33] They obtained 43 publications with 71 patients. Several therapy regimens were involved, including treatment with tretinoin. Only sixteen patients received tretinoin monotherapy. Amongst other things, preterm delivery, intrauterine death and spontaneous/therapeutic abortion were noted. The foetal or neonatal complications in the subgroup receiving tretinoin monotherapy were not analysed separately.

Oral or topical exposure

De Wals et al. (1991) studied the association between holoprosencephaly and exposure to oral or topical retinoids in Europe using regional birth defect registries affiliated to the EUROCAT programme.[34] In the autumn of 1989, affiliated centres were asked to identify holoprosencephaly cases registered from 1986 to 1988 and to interview the mothers about the existence of any skin disease and the use of oral or topical retinoids at the time of conception and during the first trimester of pregnancy. Each centre was free to trace mothers using the method they found best, to obtain consent and to conduct the interviews either in person or by telephone. Information on 31 holoprosencephaly cases was received from ten centres covering 502,189 births. Holoprosencephaly cases with abonormal karyotype were excluded, as retinoids do not have any mutagenic activity. An interview specifically focusing on retinoids was conducted with the mothers of 16 cases: eight with normal and eight with unknown karyotype. No skin disease or use of oral or topical retinoids was identified in any instance. The authors mentioned that their study lacked power to identify an association.

Topical exposure

Loureiro et al. (2005) conducted a prospective epidemiological study to investigate the prevalence of congenital malformations associated with topical exposure of pregnant women to tretinoin between 1983 and 2003.[35] They compared the pregnancy outcomes and structural defects in infants of 106 women who had used topical tretinoin in their first trimester with those of 389 unexposed women. The two groups were similar with regard to age, primigravidity, previous spontaneous abortion, previous termination, socioeconomic status, caffeine use, alcohol and drug use, and week of gestation when entering the study. Women in the exposed group were more likely to be white and multiparous, and to have used tobacco in the index pregnancy than women in the control group, however. No differences were found between the groups in the proportion of pregnancies ending in spontaneous abortion (6.6% in exposed vs 8.5% in unexposed), or in infants with major structural defects (2.2% in exposed vs 1.2% in unexposed). In addition, the groups were similar in gestational length and birth weight, length, and head circumference. Infants from both groups (62 from exposed and 191 from unexposed mothers) were examined for minor malformations characteristic of retinoic acid embryopathy (as described [14]), e.g. flat nasal bridge, micrognathia, cleft uvula, et cetera), but no

differences were found (12% in exposed and 9.9% in unexposed). Information about the doses of topical tretinoin used on specific days in the first trimester was lacking, as was information on the serum levels of tretinoin in the pregnant women. Other limitations pointed out by the authors are that the women were not recruited early enough in gestation to examine the risk of early first trimester loss and that the limited number of participants precluded more appropriate time-dependent statistical analyses.

Shapiro et al. (1997) investigated the effects of gestational exposure to tretinoin within Motherisk, a service that collects and provides information about gestational exposure to drugs, chemicals, radiation and infectious diseases.[36] They compared the rates of malformation among infants born to women exposed and women not exposed to tretinoin. The exposed group consisted of women who contacted Motherisk voluntarity between 1988 and 1996 for information about the effects of gestational exposure to tretinoin. Controls were pregnant women who contacted Motherisk voluntarily to enquire about the effects of exposures not known or suspected to be teratogenic, or toxic to the foetus. No information was given about the time period of the exposure, the use of other medicines than tretinoin, or the number of women who contacted Motherisk, but were lost to follow-up. The 94 tretinoin-exposed women and the 133 controls were similar with respect to maternal age, pattern of smoking and alcohol use.[37] No differences were seen between the exposed women and the controls in the rates of live birth, miscarriage and elective termination of pregnancy. The prevalence of major malformations among live-born infants who had been exposed to topical tretinoin during the first trimester, did not differ from that among controls.

Jick et al.(1993) studied the effects of first trimester topical exposure to tretinoin on congential disorders.[38] They identified all women registered with Group Health Cooperative hospitals in Seattle, USA who obtained at least one prescription for topical tretinoin and were subsequently admitted to hospital for childbirth, abortion (spontaneous or induced), or foetal death between 1976 and 1991. For women who miscarried, delivered a stillborn foetus, or had an induced abortion, the timing of the exposure was calculated for gestational age at the termination of the pregnancy. Termination of the pregnancy for foetal anomaly was not mentioned. The researchers identified 215 women who delivered live or stillborn infants and were prescribed topical tretinoin in the four months before and/or the three months after the estimated date of conception (every prescription contained four months of medication). A comparison group consisted of 430 age-matched nonexposed women who delivered live or stillborn infants at the same hospitals. The prevalence of major malformations among live-born infants to the exposed women was 1.9% (4/212) and that among live-born infants to the nonexposed women 2.6% (11/427), leading to a RR of 0.7 (95% CI 0.2-2.3). Caron (1993) commented that the women considered as exposed did not seem to have been asked if they had used their prescriptions.[39] However, of the four mothers of infants with

a malformation, two obtained a second prescription of tretinoin (after conception). According to the Committee, this strongly suggests that they actually used their prescriptions.

Camera et al. (1992) reported a case of an infant born with a crumpled hypoplastic ear and atresia of the external auditory meatus on the right side.[40] The mother had used tretinoin cream (0.05%) during the month before her last menstruation and during the first 11 weeks of pregnancy.

Lipson et al. (1993) reported an infant born with supraumbilical exomphalos, an anterior diaphragmatic hernia, an inferior pericardial defect, dextroposition of the hart, and a right-sided upper limb reduction defect.[41] The mother had used a topical, alcohol-based liquid preparation of 0.05% tretinoin for severe facial acne before conception and during the first five weeks of gestation.

In the conference abstract mentioned above, Rosa et al.(1994) listed holoprosencephalies reported to the U.S. Food and Drug Administration.[32] From 1969 up to and including 1993, five holoprosencephalies were reported among infants born to women exposed to tretinoin topically during the first trimester of pregnancy. These case reports were among a total of 25 birth defect case reports with maternal topical tretinoin exposure. Nineteen suspected holoprosencephalies were among 8700 non-retinoid exposure birth defect case reports to the FDA. The data concerning infants born to women taking isotretinoin orally have been described in the section *Oral exposure*.[34]

2.3.3 Lactation studies

There are no publications regarding the effects of all-trans RA on or via human lactation.

2.4 Animal studies

A number of animal studies has been performed with all-trans RA. Their designs and results are summarized in Annex A and described below. As oral and topical administration are the most relevant exposure routes for humans, the assessment in this report is limited to studies with these exposure routes. The presence or absence of maternal toxicity is relevant for the assessment, as it may influence the reproductive and developmental effects observed. Unless mentioned otherwise, the publications described provided no information as to whether maternal toxicity was investigated.

2.4.1 Fertility studies

Two publications on the effect of all-trans RA on the fertility of animals were found.

Ikemi et al. (2001) exposed five groups of five pregnant Sprague-Dawley rats to all-trans RA by gavage at 0, 1.25, 5, 20 or 80 mg/kg bw on

gestation day (GD) 14.[42] The rats were sacrificed on GD 20 and fertility and development were examined. Two fertility parameters were investigated: the mean numbers of corpora lutea and implantations. The mean maternal body weight and mean food consumption were similar among all groups (data not shown). No effects were seen on the mean numbers of corpora lutea and implantations. The developmental findings are described in 2.4.2.

The effect of all-trans RA exposure on implantation in mice was evaluated by Huang (2008).[43] Pregnant ICR albino mice were orally exposed to 0 or 50 mg/kg bw of all-trans RA in peanut oil (15 and 20 animals, respectively) on GD 2 and GD 3, a time point at which implantation of the fertilized eggs has not yet taken place. The mice were sacrificed on GD 8 and the numbers of implantation sites were recorded. The percentage of mice without implantation sites and the number of implantation sites per mouse did not differ between control animals and those treated with all-trans RA. The developmental findings are described in 2.4.2.

2.4.2 Developmental studies

Many research papers describe the effects of all-trans RA on the foetal development in several animal species. Below, the study outcomes relevant for classification are summarized. Only the aspects of the studies are reported that are relevant for the classification proposal for all-trans RA. Most studies were conducted in rats or mice. In some of the studies, all-trans RA was included as a positive control for other substances, because the investigators were convinced of the teratogenicity of all-trans RA.

Structural defects

Oral exposure

Primates

Hendrickx and Hummler (1992) tested the teratogenic potential of tretinoin in Cynomolgus monkeys (Macaca fascicularis).[44] Pregnant females were orally exposed to 5, 10, or 20 mg/kg bw (n=9, 6, or 1, respectively) once daily on GD 10-GD 20 and twice daily at approximately 8-hr intervals on GD 21-GD 24. Hysterotomies were performed on GD 100±2. Foetuses were weighed, sexed, measured and examined externally before skeletal investigation. A total of 77 vehicle control pregnancies from various teratology experiments was used for comparison (historical controls). Adverse clinical signs resembling hypervitaminosis A were observed: chapped mouth in one pregnant animal at 5 mg/kg bw, and chapped mouth, swollen eyelids, poor appetite and/or mild diarrhea in all pregnant animals at 10 mg/kg bw and in the pregnant animal treated with 20 mg/kg bw. The weight gain in the 5 mg/kg bw group was normal. Most of the pregnant animals treated with 10 or 20 mg/kg bw experienced weight loss during and/or after the period of treatment. A dose-related embryolethality

was observed: 2/9 (22%) at 5 mg/kg; 3/6 (50%) at 10 mg/kg and 1/1 (100%) at 20 mg/kg. Most of these losses occurred as early resorptions between GD 16 and GD 20. The incidence of these early losses was higher than in the controls. Two later losses occurred in the 10 mg/kg dose group, one due to resorption occurring between GD 26 and GD 30 and the other one due to spontaneous abortion with only tissue fragments on GD 48. Upon teratological examination of the remaining vital foetuses on GD 100±2, no malformations were found in the seven foetuses exposed to 5 mg/kg bw, but one foetus exhibited intrauterine growth retardation.
All three viable foetuses exposed to 10 mg/kg bw exhibited craniofacial defects, such as bilateral external ear defects and mandibular hypoplasia (foetus 1), temporal bone defects and unilateral auricular ear defects (foetus 2), and cleft palate and mandibular hypoplasia (foetus 3).
No malformations were observed in the historical controls. Furthermore, most viable foetuses in this study exhibited one or more skeletal variations, as revealed by internal examination. Five out of seven viable foetuses (71%) in the 5 mg/kg bw group showed extra ribs on the 7th cervical vertebrae (n=3) and variations in normal vertebrae count (n=3) or morphology (n=1). Two of the three viable foetuses (67%) in the 10 mg/kg group exhibited skeletal variations. One exhibited a hypoplastic 12th rib pair, the other one an absent rib pair, hypoplastic ribs and delayed ossification of the medial one-third of the scapulae. One or more variations in rib/vertebral number or morphology were observed in 25 of the 77 historical control foetuses (32%).

Rats

Nolen (1989) compared the teratogenic effects of single and multiple dosing of all-trans RA in rats.[45] Groups of twelve pregnant Sprague Dawley rats were either orally exposed to a single dose of 0, 5 or 10 mg/kg bw in corn oil on GD 9, or treated daily with 0, 4 or 8 mg/kg bw on GD 6-GD 15. Foetuses were collected on GD 20 and examined for skeletal and visceral anomalies. The body weight gains of dams exposed to single doses were not different from controls. Single exposure to 10 mg/kg bw increased the number of resorptions ($p \leq 0.05$), but did not affect the number of live foetuses. Foetal weight in this group was not reduced. A single dose of 5 mg/kg bw did not increase the incidence of external, soft-tissue or skeletal defects. After single exposure to 10 mg/kg bw increased numbers of foetuses with external defects ($p<0.01$; mainly exencephaly), soft-tissue defects ($p<0.01$; mainly cleft palate and exencephaly) and skeletal defects ($p<0.05$; mainly fused vertebrae and spina bifida) were recorded. The rats exposed on ten consecutive days showed no effects on dam body weight, number of resorptions, number of live foetuses, or mean foetal weight in either of the dose groups compared to controls. There were no increases in the number of foetuses with external defects. The number of foetuses with a soft tissue defect and the absolute number of soft tissue defects increased after ten-day exposure to 8 mg/kg bw ($p<0.01$; controls: 0/94 foetuses with defects; 4 mg/kg bw: 1/82; 8 mg/kg: 39/85). The recorded defects mainly consisted of protrusion of tongue, cleft palate, folded retina and hydronephrosis. At 4 mg/kg bw, the only increase observed was a rise

in the number of foetuses with the developmental variation of a rudimentary 14th rib ($p<0.01$). This was also observed at 8 mg/kg bw ($p<0.01$).

Ikemi et al. (2001) exposed groups of five pregnant Sprague-Dawley rats to all-trans RA at 0, 1.25, 5, 20 or 80 mg/kg bw by gavage on GD 14 and examined the palates on GD 20.[42] Rats were sacrificed on GD 20 and the mean numbers of early, late and total resorptions, as well as the mean numbers of live and dead foetuses were determined. Live foetuses were collected for inspection of palatal development. The mean maternal body weight and food consumption were similar among all groups (data not shown). No effects were seen on the mean numbers of late resorptions, or dead foetuses at any of the doses tested. The total number of resorptions was increased at 5 mg/kg bw only ($p<0.05$). According to the Committee, this is presumably due to an increase of early resorptions that was not treatment-related. No dose-dependent effects were seen in the number of viable foetuses, but the mean foetal body weight of both male and female foetuses was lower at 80 mg/kg bw ($p<0.05$). The frequencies of foetuses with variant patterns of palatal rugae were increased at 5 mg/kg bw and higher ($p<0.01$), and those of foetuses with anomalous patterns of palatal rugae at 1.25 mg/kg bw and higher ($p<0.05$). Both frequencies increased in a dose-dependent manner. Anomalous patterns of palatal rugae were rarely seen in the control group.

Astroff et al. (2002) exposed groups of twenty Sprague-Dawley rats to all-trans RA in corn oil at 15 or 25 mg/kg bw orally on GD 9 and GD 10.[46] Controls remained untreated. Foetuses were collected (from 17-19 litters) on GD 20 for microscopical examination of the head. Two tissue fixation techniques (chemical fixation and freezing) were compared, but gave similar findings. Incidences found were reported without statistics. Using chemical fixation, the percentages of foetuses with malformations were 10.5% at 15 mg/kg bw and 28.5% at 25 mg/kg bw. The main malformations found were cleft palate (1.6% and 5.4% of foetuses) and eye defects. The eye defects found were microphthalmia (2.4% and 3.2%), anophthalmia (3.2% and 7.6%), malposition (none and 1.2%) and folded retina in one foetus in each exposure group. No defects were found in the controls.

Yu et al. (2003) exposed pregnant Sprague-Dawley rats orally to 0 (n=3) or 125 mg/kg bw (n=5) of all-trans RA on GD 10.[47] Foetuses were collected on GD 21. Twenty-two out of 52 exposed foetuses and ten out of 27 unexposed foetuses were examined for external and internal malformations of the neural crest-derived organs. No effect of exposure on foetal body weight or heart weight expressed as percentage of body weight was observed. However, all exposed foetuses had craniofacial defects. Additionally, 94% had anorectal defects, 90% had limb defects, and 55% had neural tube defects. The thymus was absent or ectopic in 76%, the parathyroids were absent or single in 88%, and the thyroid was abnormal in 41% of treated foetuses. Neural crest type cardiovascular

malformations were observed in 90%, absent adrenals in 52% and renal malformations in 64% of treated foetuses. None of the controls showed any of these defects. An association was observed between absence of adrenal glands and the presence of neural tube defects ($p<0.05$).

Emmanouil-Nikoloussi et al. (2003) studied eye malformations associated with exencephaly in white rat embryos after exposure to all-trans RA during gestation.[48] The substance was suspended in corn oil and given to five pregnant rats by gastic intubation. Three of these animals received doses of 20 mg/kg bw/day from GD 7 to GD 11 with an additional dose on GD 14. The other two pregnant animals received doses of 20 or 30 mg/kg bw/day, according to the same scheme. These animals received a double dose on GD 11, or the paper contains typing errors. Control rats were treated with corn oil (n=4), or remained untreated (n=4). All animals were sacrificed on GD 21. The five treated animals had twelve embryos, whereas the control groups had 42 and 38 embryos, respectively. The treated animals showed three absorptions, eleven cases of compact embryonic masses, five cases of anophthalmia with exencephaly and one case of carioschisis. No abnormalities were found in the control groups.

Santos-Alvarez et al. (2003) investigated the presence of blastemic changes occurring during the embryonic period that may later cause foetal hindlimb deformity.[49] Pregnant Wistar rats received all-trans RA at 0 (n=10) or 120 mg/kg bw (n=10) as a single intragastric dose in mineral oil on day 10 of pregnancy. Embryos were examined for hindlimb abnormalities with various macro- and microscopical techniques on GD 11 and GD 15. The hindlimbs were examined for blastemic changes on GD 15 and the caudal somites on GD 11. Hypoplasia and misorientation of hindlimbs were present in 90% of the embryos in the all-trans RA group on GD 15. They were not present in any of the control embryos. The hindlimbs of the exposed embryos showed decreased mitotic activity ($p<0.05$). The caudal somites of the embryos in the all-trans RA group showed disruption on GD 11, including loss of normal morphology (percentage of embryos not mentioned). The morphology in the control group was normal. The cell activity in the exposed embryos was decreased at both time points ($p<0.05$).

Çolakoğlu and Kükner (2004) investigated the influence of exposure to all-trans RA on the developing cerebral cortex.[50] Groups of ten pregnant Wistar rats were exposed to 60 mg/kg bw all-trans RA dissolved in corn oil orally on GD 8 or GD 12 (before or after neurulation, respectively). A group of ten controls remained untreated. Three animals from each group were sacrificed on GD 15, 16 and 20, and foetuses were collected and examined. Administration at GD 8 resulted in exencephaly (48%), hydrocephaly (32%), gastroschisis (10%), omphalocele (15%), limb reduction defects (44%), exophtalmus (30%), abnormalities of osteogenesis (32%) and skin (14%), and mandibular-maxillar hypoplasia (28%). In the group that was exposed on GD 12, only omphalocele (8%) was found.

Most foetuses in this group appeared grossly normal. One rat in each group was allowed to give birth naturally. All pups born to the animal exposed on GD 8 were dead at birth. TGF-β expression in the foetal cerebral cortex was analysed with immunohistochemistry. TGF-β is a growth factor that plays a role in numerous developmental processes. In the group exposed on GD 8 differences in TGF-β expression compared to the control group were found. These differences were a decreased TGF-β expression on GD 15 and a more diffuse expression on GD 18. The TGF-β expression in the group exposed on GD 12 was comparable to that of the control group at both timepoints of analysis.

Danzer et al. (2005) exposed Sprague-Dawley rats (n=3-38) to a single dose of 20, 40, 50, 60 or 70 mg/kg bw all-trans RA by gavage on GD 10.[51] Foetuses were collected on GD 22 to investigate the occurrence of myelomeningocele-like defects of the central nervous system, such as spina bifida. The control group consisted of 265 vehicle-exposed and 63 untreated foetuses. The dams exposed to all-trans RA showed no adverse clinical signs, and their body weight gain, food consumption and survival rate were normal. They gave birth to fewer live foetuses than the controls (data not shown). Three hundred and seven of the 505 exposed foetuses (61%) were diagnosed with myelomeningocele-like defects, 31 of them also with exencephaly. One hundred and nineteen exposed foetuses were diagnosed with other malformations: curly tail, caudal regression syndrome, or sirenomelia. A clear dose-response relationship for the incidence of malformations was observed. The percentage of foetuses with defects increased from 78% at 50 mg/kg bw to 94% at 60 mg/kg bw and 100% at 70 mg/kg bw. In the control group and at 20 and 40 mg/kg bw, no defects were seen. Also, a correlation was seen between the dose of all-trans RA and the extent to which several features similar to human caudal regression syndrome developed: the higher the dose, the more severe the syndrome (data not shown). The offspring of the dams exposed to 60 mg/kg of all-trans RA, showed the highest incidence of isolated myelomeningoele-like defects (81.4%). Foetuses from this group were studied in more detail by magnetic resonance imaging and histopathological examination. According to the authors, the structural features of the malformations resembled those seen in humans.

Danzer et al. (2011) administered a single dose of 0 or 50 mg/kg bw of all-trans RA in olive oil to Sprague-Dawley rats orally at GD 10 and investigated the incidence and development of myelomeningocele lesions in their offspring at different gestational ages.[52] All-trans RA-exposed foetuses with myelomeningocele, all-trans RA-exposed foetuses without myelomeningocele and control foetuses were harvested at GD 14, 16, 18, 20 and 22. The overall incidence of myelomeningocele lesions after exposure to all-trans RA was 66%. These lesions were all confined to the lumbosacral area. The mean cranio-caudal and transverse diameter of the myelomeningocele lesion appeared to increase as pregnancy progressed. The mean foetal body weight of exposed foetuses with myelomeningocele

was lower than that of unexposed foetuses and that of exposed foetuses without myelomeningocele at GD 20 and GD 22 ($p<0.05$ and $p<0.01$, respectively). At the end of term, by GD 22, 57% of the exposed foetuses with myelomeningocele also had club foot deformity, and 87% a curly tail. All foetuses with myelomeningocele showed a normal pain response after forepaw pinching. Only 58% and 13% of these foetuses showed a normal response to pain after hindpaw and tail pinching, respectively. None of the myelomeningocele foetuses with large defects and clubfoot deformity had a normal pain response in these tests.

Cai et al. (2007) orally exposed groups of 24 pregnant Wistar rats to all-trans RA at 0 or 135 mg/kg bw in mineral oil on GD 10.[53] A total of 231 embryos was harvested from rats treated with all-trans RA on GDs 13.5 to 20.5. About 11% of the embryos was dead or resorbed and spina bifida was externally visible in the lumbosacral region in 48%. Some older foetuses of the treated group had anorectal malformations, short or absent tail, curly tail, omphalocele, cranioschisis, palatoschisis, or eye abnormalities (not specified). No visible malformations were observed in the foetuses of the control group.

Liu et al. (2010) investigated skeletal and hindlimb development of Sprague-Dawley rats exposed to all-trans RA *in utero*.[54] Pregnant dams (n=6) were exposed to 0, 120, 130 or 140 mg/kg in paraffin oil by intragastric injection on GD 10. The animals were sacrificed and foetuses were collected on GD 21. Body weight, body length, as well as ossification and malformation of the hindlimbs (abnormal talus-calcaneus angle) were assessed. The foetuses exposed to all-trans RA showed a smaller mean body weight and a smaller mean body length compared with controls ($p<0.01$), effects that were dose-dependent. They also showed a higher weight/length ratio compared with controls ($p<0.01$) that was dose-dependent. Furthermore, they showed less skeletal ossification of the hindlimbs compared with controls ($p<0.05$), an effect that was dose-dependent and a smaller talus-calcaneus angle of the hindlimbs compared with controls ($p<0.05$), an effect that was not dose-dependent.

The effects of 0, 25 or 50 mg/kg bw of all-trans RA given by single gavage on GD 10 in rats (n=5 per group) were examined by Wise et al. (2010).[55] In another experiment, the effects of 0, 2.5, 5 or 10 mg/kg bw were examined. Foetuses were collected on GD 21, inspected for external malformations and for skeletal changes using micro-computed tomography. All-trans RA-related effects were determined using descriptive statistics, including absolute or relative reference to the control group. No unscheduled deaths, or treatment-related clinical signs were observed in the pregnant animals treated with all-trans RA. There were no changes in their body weight (data not shown). No effect was seen on foetal survival at doses up to 50 mg/kg bw, as shown by percent postimplantion loss. Mean live foetal weights were 2-7% lower at doses ≥ 5 mg/kg bw. External malformations were observed in 80% of foetuses in the 50 mg/kg bw group. They primarily

involved the eyes (exophtalmia), ears (anotia, microtia, detached, malpositioned pinna), and cleft palates (data not shown). Similar external malformations were only seen in 6 out of 57 and 2 out of 64 foetuses at 10 and 25 mg/kg bw, respectively. Micro-computed tomographic evaluation of the foetuses revealed a dose-dependent increase of skeletal malformations: the incidence was 6% at 0 and 2.5 mg/kg bw, 45% at 5 mg/kg bw, 83% at 10 mg/kg bw. In the other experiment the incidence was 0% at 0 mg/kg bw, 97% at 25 mg/kg bw and 100% at 50 mg/kg bw. The main malformations were an increased incidence of extra vertebrae (≥ 2.5 mg/kg bw) and skull bone malformations (≥ 10 mg/kg bw). Variations included an increased incidence of supernumerary ribs (≥ 2.5 mg/kg bw) and a decreased incidence of ossified sacrocaudal vertebra at ≥5 mg/kg bw.

Using a toxicogenomic approach, Robinson et al. (2012) compared the effects of all-trans RA in vivo with the responses in rat Whole Embryo Culture (WEC), a potential alternative for in vivo studies.[56] Wistar rats (n=3) were exposed to 0 or 50 mg/kg bw of *all-trans* RA in corn oil by gavage on GD 10. Embryos were collected 48 hours after exposure. The dose administered in vivo did not cause embryonic death, nor signs of maternal toxicity. For WEC, nonexposed embryos were cultured with 0 or 0.5 µg/ml of all-trans RA in culture medium for 2-48 hours. All-trans RA in vivo induced alterations in the development of the forebrain and caudal regions of the neural tube, branchial bars, mandible, maxillary, forelimb, and hindlimb ($p \leq 0.05$). Similar effects on morphological development were recorded in WEC ($p \leq 0.05$). Gene expression of embryos exposed in vivo and in WEC was compared, which lead to identification of several gene clusters affected by all-trans RA exposure. Furthermore, the embryo's exposed in vivo and ex vivo showed great similarity at the gene (directionality and significance) and functionality (e.g. embryonic development, cell differentiation) level, although they differed in timing of all-trans RA gene induction.

Wei et al. (2012) treated pregnant Wistar rats with 0 or 140 mg/kg bw of all-trans RA in olive oil by gavage on GD 10 and performed gross morphological examination of the embryos on GD 11, 12 or 13.[57] A total of 135 live embryos was harvested from the 15 all-trans RA-treated rats. The incidence of dead or absorbed embryos was about 11%. The embryos showed a spectrum of anomalies such as a stunted tail (100%) and spina bifida aperta (49%). The other anomalies were not mentioned. The 132 embryos harvested from the 12 control animals did not show any anomalies.

Turner et al. (2013) examined the presence of neural tube defects in foetuses from pregnant Sprague-Dawley rats exposed to 60 mg/kg all-trans RA in olive oil (n=42) through gavage on GD 10.[58] Controls (n=20) remained untreated. Foetuses were examined between GD 19 and GD 21. None of the control foetuses (n=267) had a structural abnormality, whereas at least one neural tube defect was present in 52% (217/418)

of the foetuses exposed to all-trans RA. The defects observed were spina bifida, exencephaly, or a combination of the two.

Wei et al. (2013) treated pregnant Wistar rats with all-trans RA and examined the occurrence of foetal spina bifida aperta.[59] Pregnant Wistar rats were given a single oral dose of 0 or 140 mg/kg bw of all-trans RA (n=16 and n=12, respectively) by intragatric injection in olive oil on GD 10. Foetuses were obtained at GD 12, 13, 15, or 18. The results were presented in a pooled fashion. The frequency of dead or absorbed foetuses was approximately 11%. From the treatment group, 127 foetuses were obtained. Spina bifida aperta was present in 48% (61/127). From the control group, 121 normal live foetuses were obtained. The number of dead or absorbed foetuses in this group was not mentioned.

Jiang et al. (2014) exposed pregnant Sprague-Dawley rats (n=10) to 0 or 120 mg/kg bw of all-trans RA in mineral oil by gavage on GD 10.[60] Foetuses were examined for external malformations on GD 20. The body weight of all-trans RA-exposed and control foetuses did not differ. The fraction of foetuses with malformations in the all-trans RA group was increased compared to the controls (63/122 vs 0/131; p<0.001). With regard to the malformations found, only those of the clubfoot-type were mentioned.

Agarwal et al. (2015) treated pregnant Sprague-Dawley rats with 0 (n=7) or 60 (n=9) mg/kg bw of all-trans RA dissolved in olive oil by gavage on GD 10.[61] Foetuses were collected and examined for myelomeningocele (MMC) on GD 15, 17, 19, or 21. Of the 82 all-trans RA-exposed foetuses, 65 (79%) developed MMC, with no other gross anatomical defects. None of the 54 foetuses in the control group demonstrated an MMC-like defect.

Mice

In the following studies, mice were exposed early in embryogenesis to specifically address effects on development like resorptions, retarded growth and abnormalities. In all but one study (Huang (2008)[43]) the animals received a single dose.

Creech Kraft et al. (1987) investigated the effect of a single oral dose of 0, 60 or 100 mg/kg bw of all-trans RA in soybean oil given to ICR mice (n=5-12) on GD 9 or GD 11.[62] Exposure to 60 mg/kg bw on GD 9 resulted in developmental defects in 90% of live foetuses (vs 2% in controls, p<0.05). These defects were skeletal defects (90% vs 2% in controls, p<0.05), or a cleft palate (43% vs none in controls, p<0.05). Exposure to 100 mg/kg bw on GD 9 or GD 11 resulted in a similarly high teratogenic activity.

In a similar setting, Creech Kraft et al. (1989) treated NMRI mice with a single dose of 0 (n=21), or 10 mg/kg bw (n=8) of all-trans RA in soybean oil by gavage on GD 11.[63] Mice were sacrificed on GD 18. Exposure had

no effect on the number of implantations per litter, percentage of resorbed implantations, number of foetuses per litter, or mean foetal weight. However, 63% of exposed foetuses showed anomalies of the forelimbs, 60% showed anomalies of the hind limbs and 18% of the exposed foetuses showed skull anomalies (vs 0.8%, 2% and 3% in unexposed foetuses, respectively). In this study, pharmacokinetics were also assessed. Maternal plasma levels were maximal at one hour after administration (4.5 µg/ml), and were undetectable after eight hours. In embryos, the concentration of all-trans RA was highest at two hours after application and almost as high as in maternal plasma at this point in time (±2.5 µg/g embryo). The maternal concentration at four and eight hours after application was exceeded by the embryonal concentration.

The effects of 0, 40 and 60 mg/kg bw all-trans RA were examined in ICR mice after oral exposure on GD 8 by Yasuda et al. (1986).[64] Foetuses were examined on GD 18. Neither 40 mg/kg bw, nor 60 mg/kg bw had affected the number of implantation sites, or the foetal body weight. The percentage of dead foetuses was increased in the group exposed to 60 mg/kg bw (36.5% vs 10.7% in the control group, $p<0.001$). The number of malformations was increased in both groups (84.8% and 82.5% at 40 and 60 mg/kg bw, respectively, vs 3.4% in the control group, both $p<0.001$). At 40 mg/kg bw, mainly the incidences of cleft palate and imperforate anus were increased ($p<0.001$). At 60 mg/kg bw, the incidences of a greater variety of malformations were increased, including exencephaly, exophthalmos, cleft palate, spina bifida, no tail, agnathia and club foot ($p<0.01$, or $p<0.001$).

Nugent et al. (2002) tested the teratogenic effect of all-trans RA in TGF-β2 knockout and wildtype mice.[65] TGF-β2 is an important growth factor for prenatal growth and development. Heterozygous TGF-β2 mice (+/-) were cross-bred to get three foetal genotypes (+/+ (wildtype), +/- and -/-). Dams (n=9) were exposed to 100 mg/kg bw of all-trans RA by gavage on GD 11 and sacrificed on GD 15. Control dams either received vehicle (n=2), or remained untreated (n=3). One out of nineteen all-trans RA-exposed wildtype foetuses (5%) was dead at GD 15, whereas none of the fifteen control wildtype foetuses were dead. Fourteen out of the nineteen all-trans RA-exposed foetuses (74%) showed a cleft palate. All fifteen foetuses in the control groups had a normal, fused palate.

Rengasamy and Padmanabhan (2004) treated TO mice with single doses of 0, 100, 150 or 200 mg/kg bw of all-trans RA by gavage at GD 7, 8, 9, 10, 11 or 12 and investigated the presence of supernumerary ribs and other skeletal deformations at GD 18.[66] Six to thirteen litters were examined. Maternal food and water consumption and body weight gain were similar in all-trans RA-exposed and control groups. Treatment on GD 7 resulted in total embryonic resorption (all doses, including a dose of 50 mg/kg bw). A high rate of resorption was also seen at 200 mg/kg bw administered on GD 8. Treatment on different days resulted in different patterns

of deformation, that were not always dose-dependent, but deformations were present at all tested doses. At every dose and dosing day, multiple types of deformation were increased ($p<0.05$) GD 8-12 were susceptible for induction of extra lumbar ribs and GD 9-12 for induction of extra cervical ribs. The increased incidence of extra cervical ribs peaked in the GD 10 and GD 11 groups and that of the extra lumbar ribs in the GD 8 and GD 11 groups. Although the incidence of supernumerary ribs generally increased with increasing all-trans RA dose, a dose-response relationship was lacking. All-trans RA reduced the presacral vertebral number on GD 8 and GD 9. It caused sternal anomalies on GD 9-12. The incidence of sternal anomalies generally increased with increasing dose and advancing developmental stage at which all-trans RA exposure occurred.

CD-1 mice (10-25 animals per group) were exposed to a single oral dose of 0, 2.5, 10, 30, 60 or 100 mg/kg bw of all-trans RA on GD 11 by Campbell et al. (2004).[67] They were sacrificed on GD 18 and the foetuses obtained were examined for the presence of cleft palate and forelimb malformations. There were no maternal deaths in any group. Exposure did not have any effect on the average number of foetuses per dam, nor on average foetal weight. The number of foetuses with cleft palate and/or forelimb malformations was higher in all-trans RA-exposed foetuses than in controls. An increase was observed at doses of 10 mg/kg bw and higher ($p<0.05$) and this effect was dose-dependent. In the group exposed to 100 mg/kg bw 95% of foetuses was affected, in the control group only 2%.

Huang (2008) evaluated the effect of pre-implantation exposure to all-trans RA on resorptions in mice.[43] Pregnant ICR albino mice were orally exposed to 0 or 50 mg/kg bw all-trans RA in peanut oil (15 and 20 animals, respectively) on GD 2 and GD 3, a time point at which implantation of the fertilized eggs has not yet taken place. The mice were sacrificed on GD 8 and the numbers of resorptions (early and late) in the uterine horns were recorded. The number of mice with visible implantation sites without developing decidua (early resorptions) was higher in the treated animals ($p<0.05$).

Qin et al. (2014) treated pregnant Kumming mice (4 or 5 animals per group) with 0, 30, 70, or 100 mg/kg bw of all-trans RA in sesame oil by gavage on GD 11.[68] They examined the foetuses obtained on GD 17 for the presence of cleft palate. One hundred and fifty-six foetuses were obtained from the all-trans RA-exposed dams and 55 from the controls. The incidence of cleft palate was 100% at both 70 and 100 mg/kg bw. It was 40% at 30 mg/kg bw. None of the control foetuses showed cleft palate.

Billington et al. (2015) determined the impact of all-trans RA on midfacial shape variation and manifestation of holoprosencephaly in mice.[69] Pregnant female C57Bl/6 mice were treated by gavage with all-trans RA in corn oil at doses of 3.75 (n=67) or 7.5 (n=50) mg/kg bw on GD 7. Controls (n=20) remained untreated. Embryos were isolated at GD 9 or

GD 10, and assessed for external phenotypes. For geometric morphometric shape analysis, embryos were collected at GD 11. Treatment with 7.5 mg/kg bw led to 94% of embryos showing defects with about two thirds of the embryos showing holoprosencephaly and one third with a neural tube defect. Treatment with 3.75 mg/kg bw led to 7% of embryos being affected, with a vast majority of the affected embryos showing holoprosencephaly. None of the controls had holoprosencephaly or neural tube defects.

Yan et al. (2015) determined the frequency of limb malformation in ICR mice exposed to all-trans RA prenatally.[70] Pregnant dams (n=56) received 0 or 80 mg/kg bw of all-trans RA in soybean oil by gavage on GD 11. Eight dams from each group were sacrificed daily from GD 12 to GD 18 and their foetuses were examined. The morphological comparison of the foetal limbs from all-trans RA-exposed and control foetuses was only reported for GD 18. Both the forelimbs and the hindlimbs of the foetuses of the treatment group were shorter than those of the foetuses in the control group. The mean lengths of the left and right forelimbs of the control group were 11.0±0.5 mm and 10.7±0.5 mm, respectively, whereas those of the treatment group were 8.0±03 mm and 8.0±0.3 mm ($p<0.05$). The mean lengths of the left and right hindlimbs of the control group were 9.6±0.3 mm and 9.5±0.3 mm, respectively, whereas those of the treatment group were 6.0±0.2 mm and 6.0±0.2 mm ($p<0.05$).

Wang et al. (2016) analysed the incidence of cleft palate in pregnant C57BL/6J mice gavaged with 0 or 80 mg/kg bw of all-trans RA in corn oil on GD 11.[71] On GD 18, 18 animals per group were sacrificed and foetuses examined. The weight gain of the all-trans RA-exposed animals did not differ from that of the unexposed animals. The frequencies of cleft palate were 74 and 3 percent in all-trans RA-exposed and unexposed foetuses, respectively ($p<0.001$).

Wang et al. (2017) analysed the prevalence of craniofacial malformations in the offspring of pregnant C57BL/6N mice gavaged with 0 or 100 mg/kg bw of all-trans RA in corn oil on GD 10 or GD 13 (group size not mentioned).[72] Foetuses were collected and examined on GD 19, offspring was examined at postnatal day (PND) 35. No noticable differences in maternal food consumption and body weight were observed between the all-trans RA-treated and control groups. There were no maternal deaths in either group. Pups exposed at GD 10 died right after birth, wheras six pups exposed at GD 13 and a nonspecified control group survived until PND 35. In the group exposed at GD 10, all-trans RA induced cleft palate in 82 percent of the foetuses at GD 19, whereas exposure at GD 13 did not induce cleft palate, but caused craniofacial asymmetry in 44% (10/23) at GD 19 and 50% (3/6) at PND 35.

Gao et al. (2017) investigated the induction of cleft palate and tail abnormalities by all-trans RA in mice.[73] Pregnant C57BL/6 mice (n=12) received 0 or 100 mg/kg bw of all-trans RA in corn oil orally on GD 10. Foetal morphology was analysed on GD 12, 13, 14, 15, 16 and 17.
All foetuses exposed to all-trans RA showed a cleft palate. The frequency of cleft palate in the controls was not reported. Foetuses exposed to all-trans RA demonstrated a shorter tail than controls at all timepoints except GD 12 ($p<0.05$).

Zhang et al. (2017a) determined the frequency of neural tube defects after prenatal exposure to all-trans RA.[74] Pregnant Kumming mice (n=12) received 0 or 30 mg/kg bw in olive oil by gavage at GD 7. Six animals were sacrificed on GD 9 or GD 10 and their embryos examined. In the all-trans RA-exposed group, 56/98 embryos demonstrated neural tube defects (57%), in the control group 0/107 ($p<0.05$). The numbers of dead or absorbed embryos in these groups were 7 and 2, respectively ($p<0.05$).

Zhang et al. (2017b) analysed the occurrence of cleft palate in mice exposed to all-trans RA prenatally.[75] Pregnant C57BL/6 mice (n=30) received 0 or 100 mg/kg in corn oil by gavage at GD 10. Foetuses were examined at GD 12, 13, 14 or 16. Treatment with all-trans RA induced cleft palate in 99% of the foetuses (99/100), whereas none of the foetuses in the control group exhibited cleft palate (0/100).

Rabbits

The effects of 0, 6.25, 12.5 or 25 mg/kg bw of all-trans RA in corn oil given by single gavage on GD 9 in rabbits (n=4 per group) was examined by Wise et al. (2010).[55] Foetuses were collected on GD 28, inspected for external malformations and inspected for skeletal changes using micro-computed tomography. All-trans RA-related effects were determined with a variety of nonstatistical techniques, including absolute or relative reference to the control group. No unscheduled deaths, or treatment-related clinical signs were observed in the pregnant animals treated with all-trans RA. There were no changes in their body weight (data not shown). No effects on percent postimplatation loss and mean live foetal weight were observed. External malformations were observed only at 25 mg/kg bw as small ears (43%) and absent or small tails (75%). Skeletal evaluation revealed an increased incidence of malformations of the caudal vertebrae and a decrease in the number of ossified sacrocaudal vertebrae. The skeletal malformations were only observed at 25 mg/kg. They occurred in 16/23 foetuses (69%).

Hamsters

Shenefelt (1972) examined the teratogenicity of all-trans RA in golden hamsters by giving pregnant females a single dose of all-trans RA (as sodium salt in buffer) in the range of 7-116 mg/kg bw by gavage in the period of GD 5–12, with ¼-day intervals around GD 8 (n=5-31 per dose group; controls n=328).[76] Foetuses were collected on GD 14 and

examined for malformations. The foetal LD_{50} and the incidences of internal malformations at this dose were determined. The number of live foetuses was reduced at a dose of about 23 mg/kg bw and higher when given around GD 7. The LD_{50} was found to be lowest when given at GD 7 (ca 17 mg/kg bw) and highest when given at GD 12 (ca 110 mg/kg bw). Malformations observed include exencephaly, malformations of the pituitary, thyroid, thymus and umbilical artery, malpositioned hindlimbs, rib fusions, omphalocele, malformed uterus, absent kidney, absent gall bladder, malformed external ear, hypoplastic mandible, spina bifida. The incidence of any malformation was 100%, when the LD_{50} was given in the period GD 7-GD 12. An intraperitoneal dose of 34 mg/kg given at GD 7 and intravenous doses of 34 mg/kg given at GD 7 or GD 8 led to a similar pattern of mortality and malformations, when compared to about the same dose given orally, indicating that all-trans RA is well absorbed.

Topical administration

Rabbits

Christian et al. (1997) treated pregnant New Zealand white rabbits with tretinoin topically and investigated the incidence of abortions and resorptions and the incidence of malformations and variations in the offspring.[77] Twenty rabbits received 10 or 100 times the clinical dose (0.005 mg/kg bw, according to the authors) on GD 7 through GD 19. Controls received no treatment (n=20), or vehicle (n=20). Five does in the lowest-dose group and three does in the highest-dose group died during the experiment, whereas one of the vehicle-treated animals died and none of the untreated ones. On GD 29, all remaining rabbits were killed and weighed, and uteri and foetuses were examined. Maternal body weight and gravid uterine weight were reduced in the highest-dose group ($p<0.01$ and $p<0.05$, respectively). The highest dose reduced foetal weight ($p<0.01$). The treatment did not reduce the number of implantations, the number of does with resorptions, the total number of resorptions, the number of early resorptions, or the number of live foetuses per litter. The incidences of two kinds of malformation, open eyelids and cleft palate, were increased in the lowest-dose group ($p<0.01$). Some variations were increased in the lowest-dose group, others in the highest-dose group.

Functional and cognitive effects

Oral exposure

Rats

Coluccia et al. (2008a) treated Sprague-Dawley rats (n=9) on GD 11, 12 and 13 with 0 or 2.5 mg/kg bw of all-trans RA in sesame oil by gavage and examined long-term developmental toxicity.[78] Dams were allowed to give birth and offspring was weighed until weaning and behavioural tests were performed until PND 90. No effect of treatment on dam weight gain, percentage of dams giving birth, pregnancy length or litter size at birth

was observed. However, postnatal mortality of pups (at birth) was found to be affected (40/108 (37%) in the exposed group vs 3/139 (2%) in the control group, p<0.0001). Body weight gain in both male and female offspring was lower in exposed animals from PND 12 on (for both sexes p<0.001 at PND 90). From PND 2 to 18, one male and one female from different litters were examined for somatic development and reflexive behaviour. In total, eight males and seven females from exposed and control groups each were examined. A delay in somatic development was observed in exposed animals compared to controls (eye opening (male: p<0.01; female: p<0.05) and hair growth (male and female: p<0.05); no effect on ear unfolding or auditory conduit opening). A delay in the onset of reflexive behaviour was also observed (maturation of righting reflex, cliff aversion and pole grasping: all p<0.05 for both sexes). Other behavioural tests were directed at locomotor activity and locomotor coordination and learning ability. All these tests were performed with eight or nine males and six or nine females from the control group and nine animals per sex from the exposed group. The tests were carried out on days 21, 40 and 90 after birth. Ambulatory activity was impaired in exposed rats compared to controls. On PND 90, a reduced distance travelled in a cage was observed in exposed rats compared to control rats (male and female: p<0.001). Exposed rats stayed longer in the centre of the cage than control rats (p<0.05). However, the number of rearings in an open field test did not differ between exposed and control groups. Motor learning performance was impaired in exposed rats compared to controls. This was demonstrated with an accelerating rotarod test at 40 and 90 days after birth in four sessions per time point. The difference in latency to fall between exposed and control animals increased with every session. A grip strength test showed that muscular strength was not affected. Microscopical analyses of the cerebellum of one animal per sex per litter were performed at PNDs 1, 3, 8 and 40. It showed that the cerebellar size was reduced by 10.7% compared to controls (no p-value mentioned). The cerebellar surface was reduced by 9.4% compared to controls (p<0.05). In 3-day old rats, the cerebellar size was reduced by 21.5% compared to controls (p<0.01). Cerebellar foliation was impaired at PNDs 1 and 3. The morphological effects disappeared in the next developmental steps examined (PNDs 8 and 40).

Coluccia et al. (2008b) treated pregnant Sprague-Dawley rats on GDs 8, 9 and 10 with 0 (n=10) or 2.5 mg/kg bw (n=9) of all-trans RA in sesame oil by gavage and examined long-term developmental toxicity.[79] Dams were allowed to give birth and offspring was weighed until weaning and behavioural tests were performed until PND 90. On the day of birth, all pups were weighted, checked for any external malformations (outcome not reported), sexed and then randomly culled to eight pups per litter. One male rat from each litter was used for behavioural studies at 90 days of age. No effect of treatment on dam weight gain, percentage of dams giving birth, pregnancy length or litter size at birth was observed. Postnatal mortality of pups (at birth) was increased as compared to control animals

(p<0.05). Their body weight gain did not differ from that of the controls (data not shown). Similar to the previously investigated treatment at GDs 11-13, the treatment at GDs 8-10 impaired the postnatal onset of reflexive behaviour, locomotion, motor coordination and motor learning. The postnatal somatic development and muscular strength were not affected.

In a third paper, Coluccia et al. (2009) again addressed the long-term effects of prenatal exposure to all-trans RA, but examined the consequences of exposure at a later embryonic stage, i.e. GD 14, 15 and 16.[80] All-trans RA was given to Sprague-Dawley rats (control: n=10; all-trans RA: n=10) at 0 or 2.5 mg/kg bw in sesame oil by gavage at GD 14, 15 and 16. Postnatal somatic development, onset of reflexive behaviour and locomotor activity and ability were examined in pups (males only, control: n=10; all-trans RA: n=10) until they were 90 days old. No effects were found on dam weight gain, percentage of dams giving birth, pregnancy length, litter size at birth, postnatal mortality of pups (at birth), or pup weight gain throughout lactation. Also, no differences in physical feature maturation between exposed and control groups were detected (ear unfolding, auditory conduit opening, eye opening and hair growth). However, when the exposed pups were tested between days 2 and 18 after birth, they showed a delay in maturation of the righting reflex, cliff aversion and negative geotaxis (for all parameters: p<0.05). Furthermore, treatment with all-trans RA decreased the distance travelled in a cage when measured at 90 days after birth (p<0.01). Treatment did not affect the time spent in the central part of the arena. Motor coordination however, assessed by measuring the latency to fall in single sessions in the rotarod performance test at constant speed mode, was reduced in 40- and 90-day old exposed rats when compared to age-matched unexposed rats (p<0.05 and p<0.01, respectively). Motor learning ability, assessed by repeatedly measuring the latency to fall in accelerating rotation speed mode, was also negatively affected in the exposed animals at PND 40 and 90 (p<0.05, <0.01, or <0.001, depending on the session). The all-trans RA exposure did not affect muscular strength, as measured by a grip strength test.

Borracci and Carratù (2014) also investigated the long-term cognitive deficits in the offspring of all-trans RA-treated pregnant rats, but continued behavioural testing until PND 180.[81] Pregnant Sprague-Dawley rats received 0 (n=18), or 2.5 (n=19) mg/kg bw of all-trans RA in sesame oil by gavage once daily on GD 11, 12 and 13. Body weight gain of pregnant control rats and pregnant rats treated with all-trans RA was monitored every day. No difference in final (GD 20) body weight gain was found between pregnant control rats and pregnant rats treated with all-trans RA. On the day of birth, all pups were weighted, checked for any external malformations (outcome not reported), sexed and then randomly culled to eight pups per litter. One male rat from each litter was used for behavioural studies (passive and active avoidance tasks) at 180 days of age.

Treatment with all-trans RA did not affect pregnancy length, litter size at birth and number of dams giving birth, whereas it caused an increase in pup mortality at birth. The body weight of the all-trans RA-treated animals was not different from that of the controls at the time of behavioural testing. Treatment with all-trans RA compromised the ability of offspring to learn an active avoidance task, shown by a decreased improvement in performance over blocks of training, compared to controls. Their memory ability, assessed with a passive avoidance test, was not affected.

2.4.3 Lactation studies

No studies were found regarding the effects of all-trans RA on or via lactation in animals.

2.5 Conclusions

2.5.1 Fertility

Fertility effects in humans

No data are available on the effects of all-trans RA or 13-cis RA on functional fertility (the ability to have children). There is only some information about effects on parameters related to male or female fertility.[21-24] The data regarding male fertility concern the effects of 13-cis RA on sperm characteristics, such as concentration and motility.[21-23] These sperm characteristics showed increases as well as decreases, for instance a reversible increase of the sperm concentration in the ejaculate and a decrease of the fraction of sperm with normal morphology. The data regarding female fertility concern the effects of 13-cis RA on ovary characteristics.[24] Reversible reductions of ovarian volume and antral follicle count were demonstrated.
Together, these human data are not sufficient for classifying all-trans RA for effects on fertility.

Fertility effects in animals

Only two studies have been carried out on the effect of all-trans RA on the fertility of rodents, both concerning females.[42,43] In these studies, no adverse effects on reproductive parameters, such as the number of corpora lutea and the number of implantations, were observed. Consequently, the animal data do not provide sufficient evidence for classification.

Overall conclusion on fertility

Overall, the human and animal data are insufficient for classifying all-trans RA for effects on fertility. Therefore, the Committee proposes not to classify all-trans RA for fertility due to a lack of appropriate data.

2.5.2 Development

Developmental effects in humans

The Committee has assessed the strength of the epidemiological evidence concerning developmental effects of all-trans RA, including those of 13-cis RA. The data have been obtained with various study designs. The studies include a prospective cohort study (Loureiro et al. (2005)[35]), surveillance system studies (De Wals et al. (1991)[34], Jick et al. (1993)[38]), follow-up studies of pharmacovigilance centre data (Lammer et al. (1985)[14], Shapiro et al. (1997)[36]), a review of case reports (Verma et al. (2016)[33]) and two additional case reports (Camera et al. (1992)[40], Lipson et al. (1993)[41]. The available data mainly concern oral and topical exposure. The data all relate to the occurrence of structural abnormalities; information on functional and cognitive development is lacking.

Among the studies having a control group, the follow-up study of pharmacovigilance centre data by Lammer et al. (1985)[14] is the only study demonstrating developmental toxicity. This study shows that 13-cis RA taken orally during pregnancy can lead to spontaneous abortion and to children born with major and minor structural defects. A characteristic pattern of external and internal malformations, involving craniofacial, cardiac, thymic and central nervous system structures was observed. The malformations included microtia/anotia, micrognathia, cleft palate, conotruncal heart defects and aortic-arch abnormalities, thymic defects, retinal or optic-nerve abnormalities and central nervous system malformations. Lammer et al.[14] mentioned that the mothers in their study did not take any medicines with known teratogenic properties, but did not record alcohol use. Neither the surveillance system study of oral or topical retinoids (De Wals et al. (1991)[34]), nor three studies on topical exposure to all-trans RA (Jick et al. (1993)[38], Loureiro et al. (2005)[35], Shapiro et al. (1997)[36]) showed a difference between the exposed and the unexposed groups. On the other hand, the anomalies found in the case reports on topical all-trans RA exposure[33,40,41] and the pharmacovigilance study of Rosa et al. (2004)[32] on oral 13-cis RA and topical all-trans RA exposure are similar to those found by Lammer et al., who studied 13-cis RA. Thus, oral exposure to 13-cis RA has been shown to cause developmental toxicity in humans. Despite the corresponding clinical features described in some case reports, topical exposure to all-trans RA has not been demonstrated to cause this effect. The different outcomes could be related to the substance, or to the dose and route of administration. The oral therapeutic dose of 13-cis RA is 1 mg/kg bw[4,22], whereas the topical therapeutic dose of all-trans RA is 0.005 mg/kg bw[2,77]. Therefore, the oral administration may have led to a higher systemic concentration than the topical treatment. As a consequence, it remains elusive whether the different substances are responsible for the different outcomes of the epidemiological studies.

Although isomerization of all-trans RA to 13-cis RA and vice versa occurs, the kinetics data do not allow assessment of the contributions of the two isomers to the developmental toxicity observed.

Consequently, the Committee considers the epidemiological evidence insufficient to conclude that all-trans RA is a human developmental toxicant.

Developmental effects in animals

A large number of animal studies has been performed on the effects of prenatal exposure to all-trans RA. The Committee has only taken into account data on oral and topical exposure, the two relevant human exposure routes.

Increased incidences of foetal resorptions or pup mortality were found by Nolen et al. (1989) and Rengasami and and Padmanabhan (2004), but not by Wise et al. (2010).[45,55,66]

Increased incidences of major and minor external and internal malformations were observed in oral studies in rats (Nolen et al. (1989), Ikemi et al. (2001), Danzer et al. (2005), Wise et al. (2010) and Robinson et al. (2012)[42,45,51,55,56]), mice (Rengasamy and Padmanabhan (2004), Wang et al. (2016) and Wang et al. (2017)[66,71,72]) and rabbits (Wise et al. (2010)[55]) in the absence of maternal toxicity. The types of malformation observed include cleft palate, exencephaly, spina bifida and skeletal changes.

In the remaining animal studies, including those in monkeys and hamsters, maternal toxicity was observed, or not excluded. Those studies generally showed similar abnormalities, and the findings from these studies are supportive evidence for developmental toxicity of all-trans RA.

This also holds true for the only study with primates, by Hendrickx and Hummler (1992).[44]

All four animal studies on the functional and cognitive effects of prenatal all-trans RA exposure, carried out by Carratù and colleagues, demonstrate functional and cognitive effects in the absence of maternal toxicity.[78-81]

Prenatal all-trans RA exposure was shown to retard maturation of reflexes. Furthermore, it impaired locomotor activity, motor coordination and motor learning ability. The motor deficits persisted into adulthood. Prenatal exposure to all-trans RA did not affect somatic characteristics, or muscle strength.

Thus, the animal experiments show that prenatal exposure to all-trans RA can cause major and minor structural malformations, as well as functional and cognitive deficits lasting into adulthood.

Overall conclusion on development

Together, the human and animal studies provide sufficient evidence for developmental toxicity caused by prenatal exposure to all-trans RA. The structural abnormalities caused in animals correspond to those seen in humans. In addition, the animal studies show that prenatal exposure to all-trans RA can induce various functional and cognitive adverse effects. Therefore, classification for developmental toxicity in category 1B is warranted.

2.5.3 Lactation

Effects of all-trans RA on or via lactation have not been examined in humans or animals. Indirectly, however, the behavioural rat studies shed some light on the presence or absence of effects on lactation. [78-81] In these studies, the pups were followed-up until they were 90 or 180 days of age. In three out of these four studies, the pups exhibited normal growth until the end of this period, whereas in the fourth study, all-trans RA reduced body weight gain compared to controls during lactation.[78] This study suggests that all-trans RA-treated mothers may produce subnormal amounts of breast milk. The cause of the difference in results between this study and the other ones is elusive. Anyhow, only one study, in mice, indirectly suggests that maternal all-trans RA exposure may have an adverse effect on milk production.

Breast milk of humans or animals has not been analysed as to the presence of all-trans RA. Therefore, whether effects can occur via lactation cannot be assessed.

In conclusion, no labelling of all-trans RA for effects on or via lactation is proposed due to lack of appropriate data.

Proposed classification for fertility

Lack of appropriate data precludes the assessment of all-trans RA for effects on fertility.

Proposed classification for developmental toxicity

For developmental toxicity, the Committee recommends classifying all-trans RAin category 1B (*presumed human reproductive toxicant*) and to label it H360D (*may damage the unborn child*).

Proposed labelling for effects on or via lactation

Lack of appropriate data precludes the assessment of all-trans RA for effects on or via lactation.

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annex

A Fertility and developmental studies in animals

Abbrevations used:

GD = gestation day

PND = postnatal day

Table 1. Fertility studies in animals

species	experimental period/ design	dose/route	general toxicity	effects on reproduction	ref
Sprague-Dawley rats (n=5 per dose), F	Exposed on GD 14. Rats sacrificed on GD 20 and reproductive parameters were investigated.	0, 1.25, 5, 20 or 80 mg/kg bw all-trans RA; gavage	Mean maternal body weight and food consumption similar among all groups (data not shown)	No effects on mean numbers of corpora lutea and implantations at any of the doses tested.	Ikemi et al., 2001
ICR albino mice (n=20; controls: n=15), F	Mice exposed to all-trans RA on days 2 and 3 of gestation. On day 8, mice sacrificed and number of implantation sites in each uterine horn recorded.	0 or 50 mg/kg bw; gavage	Not described	No effects on percentage of mice without implantation sites and number of implantation sites per mouse.	Huang, 2008

Table 2a. Developmental toxicity studies in animals, structural defects

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
Cynomolgus monkey (Macaca fascicularis; n=9, 6, or 1, respectively; F)	Exposure once daily on GD 10-20 and twice daily at approximately 8-hr intervals on GD 21-24; Hysterotomy on GD 100 ± 2; Foetuses were weighed, sexed, measured and examined externally before skeletal investigation. A total of 77 vehicle control pregnancies from various teratology experiments was used for comparison	5, 10 or 20 mg/ kg bw; gavage	Weight loss at 10 and 20 mg/kg bw during and/or after treatment. Chapped mouth in 1/9 at 5 mg/kg bw. Chapped mouth, swollen eyelids, poor appetite and/or diarrhea at 10 and 20 mg/kg bw	Dose-related embryolethality: 2/9 (22%) at 5 mg/kg; 3/6 (50%) at 10 mg/kg and 1/1 (100%) at 20 mg/kg. Most losses were early resorptions between GD 16 and 20. Incidence of these losses higher than in historical controls. Two later losses at 10 mg/kg, one of them due to resorption between GD 26 and GD 30 and the other due to spontaneous abortion with only tissue fragments on GD 48. On GD 100±2, no malformations in seven foetuses exposed to 5 mg/kg bw, but one foetus with intrauterine growth retardation. All three viable foetuses exposed to 10 mg/kg bw exhibited craniofacial defects: bilateral external ear defects and mandibular hypoplasia (foetus 1), temporal bone defects, unilateral auricular ear defects (foetus 2), cleft palate and mandibular hypoplasia (foetus 3). No malformations in historical controls. 5/7 viable foetuses (71%) in the 5 mg/kg bw group showed extra ribs on the 7th cervical vertebrae (n=3) and variations in normal vertebrae count (n=3) or morphology (n=1). 2/3 viable foetuses (67%) in the 10 mg/kg group exhibited skeletal variations: one a hypoplastic 12th rib pair, the other one an absent rib pair, hypoplastic ribs and delayed ossification of the medial one-third of the scapulae. One or more variations in rib/vertebral number or morphology in 25/77 historical control foetuses (32%). Dose-dependent increase in embryolethality (2/9; 3/6; 1/1). At 5 mg/kg bw no malformations, apart from one growth-retarded foetus. At 10 mg/kg bw, craniofacial malformations (external ear defects, mandibular hypoplasia, cleft palate and temporal bone abnormalities). Skeletal variations in all exposed foetuses (77%, historical controls: 32%).	Hendrickx and Hummler, 1992
Sprague Dawley rats (n=12; F)	1) Single exposure on GD 9; 2) Daily exposures GD 6-15. Foetuses collected on GD 20 and examined for skeletal and visceral anomalies	1) 0, 5 or 10 mg/kg bw (acute); 2) 0, 4 or 8 mg/kg bw (chronic); oral	No effect on body weight gain	1) increased number of resorptions at 10 mg/kg bw ($p \leq 0.05$). Number of live foetuses and foetal weight not reduced in this group. Incidence of external, soft-tissue and skeletal defects unaffected by 5 mg/kg bw. Ten mg/kg bw increased numbers of foetuses with external defects ($p<0.01$; mainly exencephaly), soft-tissue defects ($p<0.01$; mainly cleft palate and exencephaly) and skeletal defects ($p<0.05$; mainly fused vertebrae and spina bifida). 2) no effects on number of resorptions, number of live foetuses, or mean foetal weight in either of the dose groups compared to controls. No increases in the number of animals with external defects. Increased number of foetuses with a soft tissue defect and increased absolute number of soft tissue defects after exposure to 8 mg/kg bw ($p<0.01$; controls: 0/94 foetuses with defects; 4mg/kg bw: 1/82; 8 mg/kg: 39/85). Recorded defects mainly protrusion of tongue, cleft palate, folded retina and hydronephrosis. No increased numbers of malformations at 4 mg/kg bw, except for a rise in number of foetuses with developmental variation of rudimentary 14th rib ($p<0.01$). This was also observed at 8 mg/kg bw ($p<0.01$)	Nolen, 1989

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
Sprague-Dawley rats (Crj:CD (SD)) (n=5; F)	Exposure on GD 14; foetuses collected on GD 20. Examination of uteri for number of live and dead foetuses, and resorptions. Live foetuses weighed and palates examined	0, 1.25, 5, 20 or 80 mg/kg bw; gavage	Mean maternal body weight and food consumption similar among all groups (data not shown)	No effects on the mean numbers of late resorptions, or dead foetuses at any of the doses tested. Total number of resorptions increased at 5 mg/kg bw only ($p<0.05$). No dose-dependent effects in the number of viable foetuses, but mean foetal body weight of both male and female foetuses lower at 80 mg/kg bw ($p<0.05$). Increased frequency of foetuses with variant patterns of palatal rugae at 5 mg/kg bw and higher ($p<0.01$). Increased frequency of foetuses with anomalous patterns of palatal rugae at 1.25 mg/kg bw and higher ($p<0.05$). Both frequencies increased in a dose-dependent manner. Anomalous patterns of palatal rugae rare in control group.	Ikemi et al, 2001
Sprague-Dawley rats (CRL:CD (SD) IGS BR; n=20; F)	Exposure on GD 9 and 10. Foetuses collected from 17-19 litters on GD 20, heads examined microscopically. Two tissue fixation techniques (chemical fixation and freezing) were compared	Untreated, 15 or 25 mg/kg bw; gavage	Not described	Tissue fixation techniques gave similar findings. Incidences reported without statistics. Results obtained with chemical fixation are the following. Percentages of foetuses with malformations: 10.5% at 15 mg/kg bw and 28.5% at 25 mg/kg bw. Main malformations found: cleft palate (1.6% and 5.4% of foetuses) and eye defects. Eye defects found: microphthalmia (2.4% and 3.2%), anophthalmia (3.2% and 7.6%), malposition (none and 1.2%) and one foetus with a folded retina in each exposure group. No defects found in the controls.	Astroff et al, 2002
Sprague-Dawley rats (all-trans RA: n=5; controls: n=3; F)	Exposure on GD 10; foetuses collected on GD 21 and examined; 22/52 exposed foetuses and 10/27 unexposed foetuses examined for external and internal malformations of the neural crest-derived organs	0 or 125 mg/kg; intragastric dosing	Not described	No effect on foetal body weight, or heart weight expressed as percentage of body weight. All exposed foetuses had craniofacial defects, 94% anorectal defects, 90% limb defects, and 55% neural tube defects. Thymus absent or ectopic in 76%, parathyroids absent or single in 88%, thyroid abnormal in 41% of treated foetuses. Neural crest type cardiovascular malformations in 90%, absent adrenals in 52% and renal malformations in 64% of treated foetuses. None of the controls showed any of these defects. Association between absence of adrenal glands and presence of neural tube defects ($p<0.05$)	Yu et al., 2003
White rats (n=2-4; F)	Exposure to 20 mg/kg bw on GD 7.5, 8.5, 9.5, 10.5, 11.5 and 14.5 (n=3), or exposure to 30 mg/kg bw on GD 7.5, 8.5, 9.5, 11.5 and to 20 mg/kg bw on GD 10.5 and 11.5 (n=2). The latter animals received a double dose on GD 11.5, or the paper contains typing errors. Foetuses were collected on GD21 and examined for malformations of the head.	Untreated, 0, 20 or 30 mg/kg bw; gavage	Not described	The five treated animals had twelve embryos at GD 21, the control groups 42 and 38 embryos. The treated animals showed three absorptions, eleven cases of compact embryonic masses, five cases of anophthalmia with exencephaly and one case of carioschisis. No abnormalities were found in the control groups.	Emmanouil-Nikoloussi et al., 2003
Wistar rats (n=10; F)	Exposure on GD10; embryos collected on GD11 or GD15 and examined for hindlimb abnormalities with various macro- and microscopical techniques. Hindlimbs examined on GD15 and caudal somites on GD11.	0 or 120 mg/kg bw; intragastric administration	Not described	GD15: hypoplasia and misorientation of hindlimbs in 90% of embryos in the all-trans RA group, but in none of the control embryos. Decreased mitotic activity in exposed animals ($p<0.05$). GD11: disruption, including loss of normal morphology, in the caudal somites of embryos in the all-trans RA group (percentage of embryos not mentioned); normal morphology in the control group. Decreased cell activity at both time points in exposed embryos ($p<0.05$)	Santos-Alvarez et al., 2003

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
Wistar rats (n= 10; F)	Exposure on GD8 or GD12 (before or after neurulation, respectively). Foetuses collected on GD 15, 18, 20 (n=3 each) and examined macroscopically. Heads examined by immunohistochemistry (TGF-β expression in the foetal cerebral cortex). One rat per exposure group gave birth naturally.	Untreated, or 60 mg/kg bw; gavage	Not described	Exposure on GD8: exencephaly (48%), hydrocephaly (32%), gastroschisis (10%), omphalocele (15%), limb reduction defects (44%), exophtalmus (30%), abnormalities of osteogenesis (32%) and skin (14%), and mandibular-maxillar hypoplasia (28%). Exposure on GD12: only omphalocele (8%). Most foetuses in this group appeared grossly normal. All pups born to the animal exposed on GD 8 were dead at birth. Exposure on GD8: decreased TGF-β expression on GD15 and more diffuse expression on GD18. Exposure on GD12: no effect on TGF-β expression on GD15 or GD18.	Çolakoğlu and Kükner (2004)
Sprague-Dawley rats (n=3, 4, 7 or 38; F)	Exposure on GD10; Foetuses collected on GD22 and examined for myelomeningocele (MMC)-like defects of the central nervous system, such as spina bifida.	20, 40, 50, 60 or 70 mg/kg; gavage; control group part vehicle-treated, part untreated	No effects on maternal food intake, weight gain or survival rate; no adverse clinical signs	Fewer live foetuses (data not shown). MMC-like defects: 307/505 exposed foetuses (61%), 31 of them also showed exencephaly. Other malformations: 119 (curly tail, caudal regression syndrome, or sirenomelia). Clear dose-response relationship for all defects together: 78% of foetuses at 50 mg/kg bw, 94% at 60 mg/kg bw and 100% at 70 mg/kg bw. No defects in the control group, nor at 20 and 40 mg/kg bw. The higher the dose, the more severe the features similar to human caudal regression syndrome (data not shown). The group exposed to 60 mg/kg bw showed the highest incidence of isolated MMC-like defects (81.4%). Foetuses from this group were studied in more detail by magnetic resonance imaging and histopathological examination. The structural features of the malformations in the rats resembled those seen in humans.	Danzer et al, 2005
Sprague-Dawley rats (n= not described; F)	Exposure on GD10. Foetuses harvested on GD 14, 16, 18, 20, or 22. All-trans RA-exposed myelomeningocele foetuses, all-trans RA-exposed foetuses without myelomeningocele and control foetuses were compared as to neurofunction development. Sensorimotor reflex of forepaws, hindpaws and tails tested.	0, 50 mg/kg bw; gavage	Not described	Overall incidence of myelomeningocele lesions after all-trans RA 66%; lesions all confined to the lumbosacral area. Mean cranio-caudal and transverse diameter of the myelomeningocele lesion increased as pregnancy progressed. Mean foetal body weight of exposed foetuses with myelomeningocele lower than that of unexposed foetuses and that of exposed foetuses without myelomeningocele at GD20 and 22 ($p<0.05$ and $p<0.01$, respectively). By GD22, 57% of exposed foetuses with myelomeningocele also had club foot deformity, and 87% a curly tail. All foetuses with myelomeningocele showed a normal pain response after forepaw pinching at GD22. Only 58% and 13% of these foetuses showed a normal pain response after hindpaw and tail pinching, respectively. These pain responses were absent in myelomeningocele foetuses with large defects and clubfoot deformity.	Danzer et al, 2011
Wistar rats (n=24; F)	Exposure on GD10; embryos collected on GD 13.5-20.5 and examined for malformations.	0 or 135 mg/kg; intragastric administration	Not described	11% dead or resorbed foetuses. Several malformations (e.g. 48% with externally visible spina bifida in the lumbosacral region).	Cai et al, 2007
Sprague-Dawley rats (n=6; F)	Exposure on GD10. Foetuses collected on GD21 and examined for weight, length and skeletal ossification and malformation of hindlimbs (abnormal talus-calcaneus angle).	0, 120, 130 or 140 mg/kg; intragastric injection	Not described	Smaller increase of body weight and length and higher weight/length ratio (all $p<0.01$ and dose-dependent). Less skeletal ossification of hindlimbs ($p<0.05$; dose-dependent). Smaller talus-calcaneus angle of hindlimbs ($p<0.05$, no dose-dependence).	Liu et al, 2010

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
Sprague-Dawley Crl:CD(SD) rats (n=5 per dose; F)	Exposure on GD10; Foetuses collected on GD 21, examined externally. Skeletal morphology was examined using micro-computed tomography	1) 0, 25 or 50 mg/kg; gavage 2) 0, 2.5, 5, 10 mg/kg; gavage	No deaths, no treatment-related clinical signs, no effect on body weight	No effects on foetal survival. Mean live foetal weights 2-7% lower at doses ≥ 5 mg/kg bw. External malformations in 80% of foetuses at 50 mg/kg bw (primarily involving eye (exophtalmia), ears (anotia, microtia, detached, malpositioned pinna), and cleft palates (data not shown)). Similar external malformations in 6/57 and 2/64 foetuses at 10 and 25 mg/kg bw, respectively. Dose-dependent increase of skeletal malformations: 6% at 0 and 2.5 mg/kg bw, 45% at 5 mg/kg bw, 83% at 10 mg/kg bw; in the other experiment 0% at 0 mg/kg bw, 97% at 25 mg/kg bw and 100% at 50 mg/kg bw. Main malformations: increased incidence of extra vertebrae (≥ 2.5 mg/kg bw) and skull bone malformations (≥ 10 mg/kg bw). Variations included increased incidence of supernumerary ribs (≥ 2.5 mg/kg bw) and decreased incidence of ossified sacrocaudal vertebra at ≥5 mg/kg bw.	Wise et al 2010
Wistar (HsdCpd:WU) rats (n =3; F))	Exposure on GD10, embryos collected after 48 hours and examined morphologically. Part of nonexposed embryos cultured with 0 or 0.5 µg/ml for 2-48 hours.	0, 50 mg/kg bw; gavage	No signs of toxicity	No statistically significant embryonic death. Alterations in the development of the forebrain and caudal regions of the neural tube, branchial bars, mandible, maxillary, forelimb, and hindlimb ($p \leq 0.05$). Similar effects on morphological development recorded in culture ($p \leq 0.05$).	Robinson et al, 2012

Wistar rats (n=15, F; controls n=12, F)	Exposure on GD10, embryos collected on GD 11, 12 or 13.	0 or 140 mg/kg bw; gavage	Not described	- 11% dead or absorbed embryos
				- 135 live embryos:
				  - 100% with stunted tail
				  - 49% with spina bifida aperta
				- controls: 132 embryo's without anomalies
					Wei et al., 2012

Sprague-Dawley rats (n=42; controls n=20)	Exposure on GD10, foetuses collected between GD 19 and GD 21.	0 or 60 mg/kg bw; gavage	Not described	Spina bifida, exencephaly or both present in 217/418 (52%) exposed foetuses and in 0/267 (0%) control foetuses	Turner et al., 2013
Wistar rats (n=16; controls n=12)	Exposure on GD10, embryos collected on GD 12, 13, 15 or 18.		Not described	Results were pooled. All-trans RA: 11% dead or absorbed foetuses; Spina bifida aperta in 61/127 (48%) of foetuses. Controls: 121 normal live foetuses; number of dead or absorbed foetuses not reported	Wei et al., 2013
Sprague-Dawley rats (n=10)	Exposure on GD10; animals sacrificed on GD 20 and foetuses examined externally	0 or 120 mg/kg bw: gavage	Not described	Foetal body weight not affected. Percentage of foetuses with malformations (club foot) increased (63/122 vs 0/131 in controls ; $p<0.001$)	Jiang et al., 2014
Sprague-Dawley rats (n=9, F; controls n=7, F)	Exposure on GD10; animals sacrificed on GD 15, 17, 19 or 21 and foetuses examined externally	0 or 60 mg/kg bw; gavage	Not described	65/82 all-trans RA-exposed foetuses (79%) developed MMC, with no other gross anatomical defects. 0/54 control foetuses demonstrated MMC.	Agarwal et al., 2015

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
ICR mice (1. n=10; 2. n=6)	1. Exposure on GD 11; 2. Exposure on GD 9; Animals sacrified on GD 17; external/visceral/ skeletal examination	1. 0, 100 mg/kg bw; 2. 0, 60 or 100 mg/kg bw; gavage	Not described	No effect on incidence of resorptions. Live foetuses: - 60 mg/kg bw on GD 9 gave developmental defects in 90% (vs 2% in controls, p<0.05). These defects were skeletal defects (90% vs 2% in controls, p<0.05), or cleft palate (43% vs none in controls, p<0.05). - 100 mg/kg bw on GD 9 gave similar teratogenic effects, 100 mg/kg bw on GD 11 as well. Embryonic substance concentration 58% of maternal plasma concentration; metabolite concentrations about 40%.	Creech Kraft et al., 1986
NMRI mice (n=8, F; controls n=21, F)	Exposure on GD 11; foetuses collected on GD 18 and skeletal anomalies (forelimbs, hindlimbs and skull) assessed. Levels of substance and metabolites measured in foetal and maternal plasma at 0.5-8 hrs after administration.	0 or 10 mg/kg; gavage	Not described	No effects on the number of implantations per litter, percentage of resorbed implantations, number of foetuses per litter, or mean foetal weight. Anomalies of the forelimbs 63%, hindlimbs 60% and skull 18% of treated foetuses, resp. (vs 0.8%; 2% and 3% in untreated foetuses, respectively). Most frequent anomalies were bent radius (23%), short humerus (11%), bent tibia (26%), short fibula (18%) and cleft palate (13%). Maternal plasma levels maximal 1 hr after administration (4.5 µg/ml), undetectable after 8 hrs. In embryos, the all-trans RA concentration was highest at 2 hrs after application and almost as high as in maternal plasma (±2.5 µg/g embryo), and higher than in maternal plasma at 4 and 8 hrs.	Creech Kraft et al, 1989
ICR mice (n=13, F)	Exposure on GD8; collection of foetuses on GD18, external/visceral/ skeletal examination	0, 40 or 60 mg/kg bw; gavage	Not described	No effect on number of implantation sites or foetal weight. Percentage of dead foetuses increased in group exposed to 60 mg/kg bw (36.5% vs 10.7% in controls, p<0.001). Number of malformations increased in both groups (84.8% and 82.5% at 40 and 60 mg/kg bw, respectively, vs 3.4% in controls, both p<0.001). At 40 mg/kg bw, mainly increased incidences of cleft palate and imperforate anus (p<0.001). At 60 mg/kg bw, increased incidences of variety of malformations, including exencephaly, exophthalmos, cleft palate, spina bifida, no tail, agnathia and club foot (p<0.01, or p<0.001).	Yasuda et al, 1986
TGF-β2 KO mice (heterozygous); n=9	TGF-β2 KO mice (+/-) were mated to obtain three foetal genotypes (+/+, +/- and -/-). Exposure on GD11; foetuses collected on GD15, genotyped and examined for malformations	Untreated, 0 or 100 mg/kg bw; gavage	Not described	1/19 all-trans RA-exposed +/+ foetuses (5%) was dead at GD 15, whereas 0/15 control +/+ foetuses was dead. 14/19 +/+ all-trans RA-exposed foetuses (74%) showed a cleft palate. All 15 foetuses in the control +/+ groups had a normal, fused palate. 6/40 (15%) all-trans RA-exposed +/- foetuses were dead at GD 15, whereas 0/16 control +/- foetuses was dead. In 31/40 (77%) +/- all-trans RA-exposed foetuses cleft palate was recorded. All 16 foetuses in the control +/- groups had a normal, fused palate.	Nugent et al, 2002
TO mice	Exposure on GD 7, 8, 9, 10, 11 or 12; 6-13 litters sacrificed and examined for skeletal deformations on GD 18	0, 100, 150 or 200 mg/kg bw; gavage	Food and water consumption and body weight gain were similar in all-trans RA-exposed and control groups.	Treatment on GD 7 resulted in total embryonic resorption (all doses, including 50 mg/kg bw). High rate of resorption at 200 mg/kg bw administered on GD 8. Treatment on different days resulted in different patterns of deformation (not always dose-dependent, but deformations present at all tested doses). At every dose and dosing day, multiple types of deformation increased (p<0.05). GD 8-12 were susceptible for induction of extra lumbar ribs and GD 9-12 for induction of extra cervical ribs. The increased incidence of extra cervical ribs peaked in GD 10 and 11 groups and that of extra lumbar ribs in GD 8 and 11 groups. Although the incidence of supernumerary ribs generally increased with increasing dose, a strict dose-response relationship was lacking. Reduced presacral vertebral number on GD 8 and 9. Sternal anomalies on GD 9-12. Incidence of sternal anomalies generally increased with increasing dose and advancing developmental stage at which exposure occurred.	Rengasamy and Padmanabhan, 2004

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
CD-1 mice (10-25 animals per group)	Exposure on GD 11; sacrificed on GD 18 and examined for malformations	0, 2.5, 10, 30, 60 or 100 mg/kg bw; orally	No deaths in any group	No effect on average number of foetuses per dam, or on average foetal weight. Increased number of foetuses with cleft palate and/or forelimb malformations at doses of 10 mg/kg bw and higher ($p<0.05$). This effect was dose-dependent. At 100 mg/kg bw, 95% of foetuses were affected (control group: 2%).	Campbell et al., 2004
ICR albino mice (n=20; controls n=15)	Exposure on GD2 and GD3; sacrificed on GD8 and numbers of resorptions (early and late) in uterine horns recorded	0 or 50 mg/kg bw; orally	Not described	Increased number of mice with visible implantation sites without developing decidua (early resorptions) ($p<0.05$).	Huang, 2008
Kumming mice (n=4 or 5)	Exposure on GD11, foetuses collected on GD17 and examined for cleft palate	0, 30, 70, or 100 mg/kg bw; gavage	Not described	In total, 156 all-trans RA-exposed foetuses and 55 control foetuses were obtained. Incidence of cleft palate 100% at 70 and 100 mg/kg bw, 40% at 30 mg/kg bw.	Qin et al., 2014
C57Bl/6 mice (n=67 or 50; controls n=20)	Exposure on GD7, foetuses collected on GD 9, 10 or 11, and examined externally	0, 3.75 or 7.5 mg/kg bw; gavage	Not described	7.5 mg/kg bw: 94% showed defects, about two thirds holoprosencephaly and one third a neural tube defect 3.75 mg/kg bw: 7% showed defects, the vast majority being holoprosencephaly Controls: 0% showed defects	Billington et al., 2015
ICR mice (n=56)	Exposure on GD11, foetuses collected on GD 12-18 and examined externally	0 or 80 mg/kg bw; gavage	Not described	At GD18 decreased left and right fore limbs and decreased left and right hind limbs ($p<0.05$).	Yan et al., 2015
C57Bl/6J mice (n=18)	Exposure on GD11, foetuses collected on GD 18 and examined for cleft palate	0 or 80 mg/kg bw; gavage	Weight gain not affected	74% cleft palate in all-trans RA-exposed foetuses and 3% in controls.	Wang et al., 2016
C57BL/6N mice (group size not mentioned)	Exposure on GD10 or GD13, foetuses collected on GD 19 and examined; offspring examined on PND35	0 or 100 mg/kg bw; gavage	food consumption and body weight not affected; no deaths	- GD10-group: cleft palate in 82% at GD19, pups died right after birth. - GD13-group: no cleft palate; 6 pups survived; craniofacial asymmetry in 10/23 (44%) at GD19 and 3/6 (50%) at PND35	Wang et al., 2017
C57BL/6 mice (n=12; F)	Exposure on GD10, fetuses collected on GD 12, 13. 14, 15, 16 and 17, and examined morphologically	0 or 100 mg/kg bw in corn oil orally	not described	- All foetuses exposed to all-trans RA showed a cleft palate. The frequency of cleft palate in the controls was not reported. - Foetuses exposed to trenitoin demonstrated a shorter tail than controls at all timepoints except GD 12 ($p<0.05$).	Gao et al., 2017
Kumming mice (n=12)	Exposure on GD7, fetuses collected on GD 9 or 10, and examined morphologically	0 or 30 mg/kg bw; gavage	Not described	56/98 foetuses showed neural tube defects (57%) vs 0/107 in controls ($p<0.05$); 7 dead or absorbed embryos vs 2 controls ($p<0.05$).	Zhang et al., 2017a
C57BL/6 mice (n=30)	Exposure on GD10, fetuses collected on GD 12, 13. 14 or 16, and examined morphologically	0 or 100 mg/kg; gavage	Not described	99/100 foetuses showed cleft palate vs 0/100 controls	Zhang et al., 2017b

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
Dutch Belted rabbits (n=4; F)	Exposure on GD9; Foetuses collected on GD 28, inspected for external malformations,and examined for skeletal changes using micro-computed tomography	0, 6.25, 12.5 or 25 mg/kg; gavage	No unscheduled deaths, no treatment-related physical signs, no effect on body weight	No effect on percent postimplantation loss, no effect on mean live foetal weight. External malformations (small ears, absent or small tails), skeletal malformations (caudal vertebrae) and decreased mean number of ossified sacrocaudal vertebra at 25 mg/kg	Wise et al, 2010
Golden hamsters (n=5-31; controls n=328)	Single exposure at a timepoint in the period GD 5 – GD 12¾, with ¼-day intervals around GD 8; foetuses collected on GD 14.5; foetal LD_{50} and incidences of internal malformations at this dose determined.	0, 7-116 mg/kg bw all-trans RA sodium salt; gavage	Not described	Number of live foetuses reduced at about 23 mg/kg bw and higher around gestation day 7.5. LD_{50} lowest at GD 7¼ (ca 17 mg/kg bw) and highest at GD 12½ (ca 110 mg/kg bw). Malformations observed include exencephaly, malformations of the pituitary, thyroid, thymus and umbilical artery, malpositioned hindlimbs, rib fusions, omphalocele, malformed uterus, absent kidney, absent gall bladder, malformed external ear, hypoplastic mandible, spina bifida. Incidence of any malformation 100% when LD_{50} given in period GD7-GD12. An intraperitoneal dose of 34 mg/kg at GD 7¾ and intravenous doses of 34 mg/kg at GD 7¾ or GD 8¾ led to a similar pattern of mortality and malformations, when compared to about the same dose given orally.	Shenefelt 1972
New Zealand white rabbits (n=20)	Exposure to tretinoin on GD7 through 19, foetuses collected on GD 29 and examined morphologically	10 or 100 times the clinical dose; topically	5/20 lowest dose group died, 3/20 in highest dose group, 1/20 vehicle controls, 0/20 untreated controls; body weight and gravid uterine weight reduced ($p<0.01$ and $p<0.05$, respectively) in highest-dose group	Highest-dose group showed reduced foetal weight ($p<0.05$); No effects on the number of implantations, the number of does with resorptions, the total number of resorptions, the number of early resorptions, or the number of live foetuses per litter. Increased incidences of open eyelids and cleft palate in lowest–dose group. Some variations increased in one dose group, some in the other.	Christian et al., 1997

Table 2b. Developmental toxicity studies in animals, functional and cognitive effects

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
Sprague-Dawley rats (n=9; F)	Exposure on GD11, 12 and 13. Behavioural tests conducted in 1/sex/litter on PND 21, 40, 90. Morphological analysis of brain of 1/sex of 2 control and 2 treated litters on PND 1, 3, 8, 40	0, 2.5 mg/kg bw; gavage	No effect on weight gain, percentage giving birth, or pregnancy length	No effect on litter size at birth. Increased postnatal mortality (at birth) (40/108 (37%) in the exposed group vs 3/139 (2%) in the control group, p<0.0001). Decreased pup weight gain from PND 12 on (for both sexes p<0.001 at PND 90). Delay in postnatal somatic development (eye opening (M: p<0.01; F: p<0.05) and hair growth (M and F: p<0.05); no effect on ear unfolding or auditory conduit opening). Delay in onset of reflexive behaviour (maturation of righting reflex, cliff aversion and pole grasping: all p<0.05 for both sexes). Affected locomotor activity: reduced distance travelled in a cage (M and F: p<0.001) at PND90; longer stay in the centre of the cage (p<0.05); no effect on number of rearings in open field test. Impaired motor learning performance, as demonstrated with an accelerating rotarod test at PND 40 and 90 in four sessions per time point. The effect on latency to fall increased with every session (PND 40, M: p<0.05; p<0.005; p< 0.01; p<0.001, F: p<0.05; p<0.001; p< 0.001; p<0.0001 for 1st, 2nd, 3rd and 4th session, respectively. PND 90, M: p<0.005; p<0.0001; (3rd and 4th session), F: p<0.05; p<0.005; p< 0.005; p<0.0001 (1st, 2nd, 3rd and 4th session, respectively)). Muscular strength (grip strength test) not affected on PND 21, 40 or 90. Reduction of cerebellar size by 10.7% on PND 1. Reduction of cerebellar surface by 9.4% (p<0.05). Reduction of cerebellar size by 21.5% on PND 3 (p<0.01). Impaired cerebellar foliation at PND 1 and 3. Morphological effects disappeared at PND 8 and 40.	Coluccia et al., 2008a
Sprague-Dawley rats (n=10; controls n=9; F)	Exposure on GD8, 9 and 10. Behavioural tests conducted in 1 male per litter on PND 21, 40, 90.	0, 2.5 mg/kg bw; gavage	No effect on weight gain, percentage giving birth, or pregnancy length	No effect on litter size at birth. Postnatal mortality (at birth) increased. No effect on body weight gain (data not shown). Impaired postnatal onset of reflexive behaviour, locomotion, motor coordination and motor learning. No effect on postnatal somatic development and muscular strength.	Coluccia et al., 2008b
Sprague-Dawley rats (n=10; F)	Exposure on GD14, 15 and 16. Behavioural tests conducted in 1 male/litter. Postnatal somatic development, onset of reflexive behaviour and locomotor activity and ability were examined	0, 2.5 mg/kg bw; gavage	No effect on weight gain, percentage giving birth, or pregnancy length	No effect on litter size at birth, postnatal mortality (at birth), or pup weight gain throughout lactation. No effect on physical feature maturation (ear unfolding, auditory conduit opening, eyes opening and hair growth). Delay in maturation of righting reflex, cliff aversion and negative geotaxis on PND 2-18 (for all parameters: p<0.05). Decreased distance travelled in a cage on PND 90 (p<0.01). No effect on time spent in the central part of the arena. Reduced motor coordination, as assessed by measuring the latency to fall in single sessions in the rotarod performance test at constant speed mode, on PND 40 and 90 (p<0.05 and p<0.01, respectively). Reduced motor learning ability, assessed by repeatedly measuring latency to fall in accelerating rotation speed mode, on PND 40 and 90 (on PND 40: 1st session: p<0.001, 2nd: p<0.05, 3rd: p<0.01, 4th: p< 0.001; PND 90: 1st session: p<0.05, 2nd: p<0.05, 3rd: p<0.05, 4th: p< 0.001). Muscular strength not affected on PND 21, 40 or 90, as measured by a grip strength test.	Coluccia et al 2009

species	experimental period/design	dose/route	general toxicity	developmental toxicity	ref
Sprague-Dawley rats (n=19; controls n=18, F)	once daily on gestation days 11, 12 and 13; On the day of birth, pups were weighted, checked for external malformations (outcome not described), sexed and then randomly culled to eight pups per litter; one male rat used for behavioural studies (passive and active avoidance tasks) at 180 days of age	0 or 2.5 mg/kg bw in sesame oil; gavage.	No difference in final (GD 20) body weight gain between control and all-trans RA-treated rats.	- Body weight at the time of behavioural testing not affected. - pregnancy length, litter size at birth and number of dams giving birth not affected - pup mortality at birth increased ($p<0.0001$) - ability to learn an active avoidance task affected, shown by decreased improvement in performance over blocks of training ($p<0.05$) - memory ability, assessed with a passive avoidance test, not affected.	Borracci and Carratù, 2014

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