Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at Community level of

Warfarin (ISO); 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-chromen-2-one

EC number: 201-377-6
CAS number: 81-81-2 [racemic mixture]

CLH-O-0000003175-78-11/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

14 March 2014

Proposal for Harmonised Classification and Labelling

Based on the CLP Regulation (EC) No 1272/2008,
Annex VI, Part 2

Substance Name: Warfarin

EC Number: 201-377-6

CAS Number: 81-81-2 [Racemic mixture]

Annex I Index Number: 607-056-00-0

Contact details for dossier submitter:

Pesticide Registration and Control Division

Department of Agriculture, Fisheries & Food

Backweston Laboratory Complex

Celbridge

Co. Kildare

Ireland

Email: biocides@agriculture.gov.ie, pcs@agriculture.gov.ie

Version number: Version 2 **Date: November 2012**

CONTENTS

1 BACKGROUND TO THE PROPOSAL

Warfarin has been reviewed as an existing active substance, by the Pesticide Registration & Control Division (PRCD) Department of Agriculture, Food & the Marine, Ireland, under both Council Directive 91/414/EEC concerning the placing on the market of Plant Protection Products (PPP) and Directive 98/8/EC concerning the placing on the market of biocidal products (BPD). These assessments were discussed and agreed by the respective technical committees under each review programme. Warfarin was added to Annex I of the PPP Directive in 2005 and to Annex I of the BPD Directive in September 2009. Warfarin is listed in Annex VI of Regulation (EC) No. 1272/2008.

1.1 History of the previous classification and labelling:

Directive on Dangerous Substances (Dir. 67/548/EEC):
The classification and labelling status for Warfarin was recorded in the Annex to Commission Directive 93/72/EEC (1 September 1993), Official Journal L 258, page 872 (reference A9/01). The classification of Warfarin was entered in the 19th ATP (Adaptation to Technical Progress) of Annex I to Directive 67/548 and was upgraded in the 25th ATP.

The current classification of Warfarin in Annex I of Directive 67/548/EEC is Repr. Cat. 1; R61, T; R48/25, R52-53.

Plant protection products (Dir. 91/414/EEC):
Warfarin, was re-evaluated upon the implementation of the first stage of the programme of work referred to in Article 8(2) of Council Directive 91/414/EEC (SANCO/10434/2004 final, 23.9.2005).

A review report for the active substance Warfarin was finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 23 September 2005 in view of the inclusion of Warfarin in Annex I of Directive 91/414/EEC. The draft assessment report, the peer review report and the comments and clarifications submitted after the peer review are considered as background documents to this review report.

The classification proposal from the Dir 91/414/EEC review was Repr. Cat.1; R61, T; R48/25, R52-53.

Biocides (Dir. 98/8/EC):
Warfarin (CAS no. 81-81-2, racemic mixture) was reviewed under the BPD and was notified as an existing active substance, by the "Warfarin Task Force". The assessment report evaluated Warfarin as product-type 14 (Rodenticides) and the assessment was carried out in the context of the work programme for the review of existing active substances provided for in Article 16(2) of Directive 98/8/EC concerning the placing of biocidal products on the market, with a view to the possible inclusion of this substance into Annex I or IA to the Directive.

On the basis of the final competent authority report, the Commission proposed the inclusion of Warfarin in Annex I to Directive 98/8/EC. In accordance with Article 15(4) of Regulation (EC) No 1451/2007, the assessment report was finalised and concluded at the Standing Committee on Biocidal Products during its meeting held on 17 September 2009.

The classification and labelling proposal from the Biocides review for Warfarin is Repr. Cat.1; R61, T^{+}, R26/27/28, R48/23/24/25, R52.

Technical Committee Classification & Labelling (TC C&L, European Chemicals Bureau (ECB)):

Ireland, as the RMS for Warfarin as a plant protection product, submitted a classification and labelling proposal in November 2006. The meeting agreed to add a classification for acute toxicity and also agreed that the classification of Warfarin Toxic: R48/25 for prolonged exposure (already in Annex 1 67/548) should be extended to the dermal and inhalation routes. This classification was based on a read-across from the oral sub-chronic exposure data. The TC C&L (May 2009) meeting agreed the final classification and labelling proposal as follows;

The C&L proposal was as follows; Repr. Cat.1; R61, T^+; R26/27/28-48/23/24/25-52-53.

Specific concentration limits for acute toxicity, prolonged exposure and developmental toxicity were proposed to the ECB technical committee on classification and labelling by the RMS. This proposal as presented below was not concluded.

C ≥ 7.0% T+; R61-26/27/28-48/23/24/25

1.0% ≤ C < 7.0% T; R61-23/24/25-48/20/24/25

0.1% ≤ C < 1.0% T; R61-20/21/22-48/24/25

0.01% ≤ C < 0.1% Xn; R48/21/22

1.2 Current Classification Proposal

Summary of the scientific justification for the harmonised proposal:

In accordance with Article 36(2) of Regulation (EC) No. 1272/2008, Warfarin should be considered for harmonised classification and labelling. This proposal is based on the data submitted for the assessment of Warfarin under Directive 91/414/EEC and Directive 98/8/EC. This proposal considers the classification currently in Annex I of Directive 67/548/EEC; the additional classification proposals following the Directive 91/414/EEC and Directive 98/8/EC reviews and recognises the classification already agreed by TC and L (May 2007).

NOTE: The developmental toxicity classification of Warfarin has been finalised and is not open for further discussion. Relevant background information on developmental toxicity data for Warfarin is included in this dossier to facilitate the discussion on read-across from this classification to the second generation rodenticides.

The current classification and labelling proposal for Warfarin based on the Directive 67/548 is:

Physical/chemical properties: None

Health Hazards: Repr. Cat.1; R61,

T^+; R26/27/28

T; 48/23/24/25

Environment: N; 52-53

Symbol: T+;

Risk phrases: 61-26/27/28-48/23/24/25-52-53

Safety phrases: (1/2)-28-36/37/39-45-60-61

The classification and labelling proposal for Warfarin based on Regulation EC No. 1272/2008 is:

Physical/chemical properties: None

Health Hazards: Acute Tox 1 – H330

Acute Tox 1 – H310

Acute Tox 2 – H300

STOT RE 1 H372

Repr. 1A H360D

Environment:

Env. Chronic Tox.2-H411

Signal word: Danger

Symbol: GHS06, GHS08, GHS09

Hazard statement codes: H300: Fatal if swallowed

H310: Fatal in contact with skin

H330: Fatal if inhaled

H372: Causes damage to organs through prolonged or repeated exposure.

H360D: May damage the unborn child

H411: Toxic to aquatic life with long lasting effects

This proposal for harmonised classification refers to the addition of acute toxicity, STOT-RE (dermal and inhalation) and specific concentration limits.

1.3 Proposed Specific Concentration Limits for Warfarin:

Previous proposal

Specific Concentration Limits (SCL) are required for Warfarin. There are three areas of classification concern because the endpoints that determine classification are numerically far removed from the general concentration limits normally used. These areas are; (1) acute lethal effects, (2) severe effects after repeated or prolonged exposure and in particular, (3) toxic effects for development. Applying general concentration limits to enable classification of products containing Warfarin would lead to an underestimation of the potential risk and communication of that risk to users of those products.

Specific Concentration Limits have been proposed for a number of anticoagulant rodenticides. The list of anticoagulant rodenticides includes Brodifacoum (IT), Bromadiolone (S), Difethialone (N), Coumatetralyl (DK), Flocoumafen (NL), Difenacoum (FIN) and Chlorophacinone (ES).

Ireland has previously proposed SCLs for Warfarin as part of the ECB TC C&L process and took into account acute toxicity, prolonged exposure and reproduction toxicity. This proposal was not finalised. The proposal at that time was as follows:

C ≥ 7.0% T+; R61-26/27/28-48/23/24/25

1.0% ≤ C < 7.0% T; R61-23/24/25-48/20/24/25

0.1% ≤ C < 1.0% T; R61-20/21/22-48/24/25

0.01% ≤ C < 0.1% Xn; R48/21/22

Current Proposal:

The anti-coagulant rodenticides are being considered/classified as a group on the basis of their similarity of biological action to Warfarin, SCLs therefore should also be considered in a similarly harmonised and consistent manner. The other rodenticides have no proposed SCL with regard to reproductive toxicity, instead their classification & labelling is determined by active substance concentrations in excess of the general concentration limit of 0.5% w/w (under Directive 67/548/EEC).

The available information on setting **Specific Concentration Limits (SCL) is sparse** and there appears to be no consensus in setting **(SCL)**, particularly with respect to reproductive toxicity. Recently however (2010), the ECHA working Group on Human Health Guidance for CLP has produced a draft document entitled "Guidance for Setting Specific Concentration Limits for Reproductive Toxicants within the CLP Regulation (EC/1272/2008)". This document has been referred to with regard to the setting of SCL for reproductive/developmental effects for Warfarin.

Specific Concentration Limits proposed for Warfarin based on the new CLP Regulation:

SCL for acute toxicity: Specific concentration limits are not applicable for acute toxicity classification.

SCL for repeat exposure (STOT-RE) classification (see 6.6 for detailed description):.

According to Regulation EC/1272/2008:

C ≥ 0.2% STOT RE 1

0.02% ≤ C < 0.2% STOT RE 2

SCL for reproductive toxicity classification (see 6.9 for detailed description):

C ≥ 0.0003% Repr. 1A

2 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Warfarin and its sodium salt are used in controlling rodents in domestic, agricultural, commercial and industrial sites. Warfarin is also a human medicine used in treatment of blood hyper-coagulation. These compounds used as rodenticides cause internal bleeding and haemorrhaging and death in rats and mice. They are applied as dry and liquid baits, and as a dust, which acts as a tracking powder.

Warfarin is currently in Annex VI of the CLP regulation with a classification as Cat 1 Repr; R61, R48/25 and N; R52/53. Ireland (via their competent authorities) is proposing the classification and labelling of Warfarin at EU level because additional hazard classes to those already finalised, have been proposed following the Plant Protection and Biocide Review programmes.

SCIENTIFIC EVALUATION OF THE RELEVANT DATA

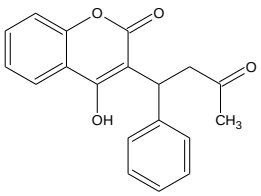
3 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

3.1 Name and other identifiers of the substance

Table 7: Substance identity

EC number:	201-377-6
EC name:	Warfarin
CAS number (EC inventory):	81-81-2
CAS number:	81-81-2 [racemic mixture]
CAS name:	2H-1-Benzopyran-2-one, 4-hydroxy-3-(3-oxo-1-phenylbutyl)-
IUPAC name:	4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one (CA)
Annex I index number	607-056-00-0
Molecular formula:	$C_{19}H_{16}O_4$
Molecular weight range:	308.25 g/mol

Structural formula:

Structural formula:	

3.2 Composition of the substance

Table 8: Constituents

The IUCLID dossier contains confidential information in relation to constituents. None of the constituents are considered relevant for classification purposes.

Table 9: Impurities

The IUCLID dossier contains confidential information in relation to impurities. None of the impurities are considered relevant for classification purposes.

Table 10: Additives

The IUCLID dossier contains confidential information in relation to additives. None of the additives are considered relevant for classification purposes.

3.3 Physico-chemical properties

Table 11: Summary of physico- chemical properties

REACH ref Annex,	Property	IUCLID section	Value	Comment/reference
VII, 7.1	Physical state at 20°C and 101.3 KPa	3.1	White, crystalline solid	Reference 1
VII, 7.2	Melting/	3.2	165°C (Purity: 100.4%)	Reference 1
VII, 7.3	Boiling point	3.3	494°C (Calculated)	Reference 2
VII, 7.4	Relative density	3.4 density	1.35 (Purity: 100.4%)	Reference 1
VII, 7.5	Vapour pressure	3.6	$p = \leq 3.47 \times 10^{-3}$ Pa (20°C)	Reference 1
VII, 7.6	Surface tension	3.10	72.8 mN/m (90% saturated soln., 20 C)	Reference 1
VII, 7.7	Water solubility	3.8	66.13 g/l (pH = 9.14) 267 mg/l (pH = 7.12) 4.9 mg/l (pH = 4.07) all at 20°C.	Reference 1
VII, 7.8	Partition coefficient n-octanol/water (log value)	3.7 partition coefficient	log P_{ow}= 2.9 at pH4 log P_{ow} = 0.7 at pH7 log P_{ow} = 0.6 at pH9 all at 30-35°C	Reference 1
VII, 7.9	Flash point	3.11	N.A.	Reference 2
VII, 7.10	Flammability	3.13	Non flammable	Reference 1
VII, 7.11	Explosive properties	3.14	Non explosive	Reference 1
VII, 7.12	Self-ignition temperature		N.A.	Reference 2
VII, 7.13	Oxidising properties	3.15	Non oxidising	Reference 1
VII, 7.14	Granulometry	3.5	No data	Reference 1
XI, 7.15	Stability in organic solvents and identity of relevant degradation products	3.17	N.A.	Reference 1
XI, 7.16	Dissociation constant	3.21	pK_a = 5.19 (20°C)	Reference 1
XI, 7.17	Viscosity	3.22	N.A.	Reference 1
	Auto flammability	3.12	Not auto-flammable	Reference 2
	Reactivity towards container material	3.18	Non reactive	Reference 2
	Thermal stability	3.19	Stable up to at least 290°C	Reference 1

4 MANUFACTURE AND USES

4.1 Manufacture

Not relevant for this dossier

Warfarin is manufactured and placed on the market, in this context, for application as a rodenticide pest control substance.

4.2 Identified uses

Warfarin is a first-generation single-dose anticoagulant rodenticide. It disrupts the normalblood clotting mechanisms resulting in increased bleeding tendency and, eventually, profusehemorrhage and death. Effectiveness of the active substance depends on exposure (i.e.consumption of the bait by the target organism). For effective and comprehensive control of rats and mice a bait concentration in wax blocks up to 0.079 % (m/m) ≡ 790 mg/kg in granular bait up to 0.079 % (m/m) ≡ 790 mg/kg is used.

Table 12: Description of identified uses

Identified use	Sector of Use (SoU)	Preparation Category (PC)	Process category (PROC)	Article category (AC)
Rodenticide - Pest control substance	Biocide (non-agricultural pesticide) Plant protection			

5 ENVIRONMENTAL FATE PROPERTIES

5.1 Degradation

Not relevant for this dossier. See Point 5.1.2 below.

5.1.1 Stability

Not relevant for this dossier.

5.1.2 Biodegradation

5.1.2.1 Warfarin can be classified as readily biodegradable. In the ready biodegradation study (Dengler, C., 2004-OECD 301D and EC C.4 (92/69/EEC), A7.1.1.2.1/01) the criteria of 60 % removal of ThOD within a 10 day window was exceeded. A degradation rate of 92.7 % (2mg Warfarin /l) was determined for a 28 day period. In this study, with a reliability of 1, no inhibitory effects of Warfarin were observed in the toxicity control (more than 25% degradation occurred within 14 days). In accordance with the 2nd ATP, Point 4.1.2.9.5, Warfarin is to be considered to be rapidly degradable.This is considered in the classification and labelling of Warfarin.Biodegradation estimation

Not relevant for this dossier.

5.1.2.2 Screening tests

Not relevant for this dossier.

5.1.2.3 Simulation tests

Not relevant for this dossier.

5.1.3 Summary and discussion of persistence

Not relevant for this dossier.

5.2 Environmental distribution

Not relevant for this dossier.

5.2.1 Adsorption/desorption

Not relevant for this dossier.

5.2.2 Volatilisation

Not relevant for this dossier.

5.2.3 Distribution modelling

Not relevant for this dossier.

5.3 Bioaccumulation

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

Method	Results	Remarks	Reference
N/A	See test below for results	Based on the log P_{ow}, as specified in the TGD: Experimentally determined log P_{ow} values are reported in reference A3.9/01. log P_{ow} (pH 4) = 2.9 log P_{ow} (pH 7) = 0.7 Calculated log P_{ow} value from BCFWIN v. 2.14, SRC Corporation. log P_{ow} = 2.23	Battersby RV (2003) Estimation of the bioconcentration factor (BCF) of Warfarin. EBRC Consulting GmbH, Hannover, Germany, Report dated September 10, 2003 (unpublished). A7.4.2/01

Based on experimentally determined partition coefficients (2.9 for pH = 4, 0.7 for pH = 7), the following bioconcentration factors were estimated:

BCF = 58 (pH 4)

BCF = 0.8 (pH 7).

Based on a calculated partition coefficient (2.23), the bioconcentration factor was estimated by QSAR at

BCF = 10.45.

Whether QSAR calculations (BCFWIN) or an extrapolation method based on an empirical relationship between log P_{ow} and BCF are used, the BCF is predicted to be well below 100 in both cases, indicating a low bioaccumulation potential of Warfarin. Since the estimation was performed using an officially recommended method, based on measured values determined by fully valid experimental procedures, this calculation is considered to be valid without restrictions. See Point 5.3.1.2 below for results of a full bioconcentration study in fish.

5.3.1.2 Measured bioaccumulation data

Method	Results	Remarks	Reference
OECD 305 E (1981)	NOEC 2.0 mg a.s./L	Measured concentration	Dommröse A-M (1990) Bioaccumulation (Flow-through Test) with the Test Substance Warfarin in Fish (Rainbow trout). NATEC GmbH, Hamburg, Germany, Report No: NA 88 9867/3.4, November 1989 (unpublished) A7.4.3.3.1/01

5.3.2 Terrestrial bioaccumulation

Method	Results	Remarks	Reference
N/A	BCF = 10.4	Based on the log P_{ow}, as specified in the TGD: Experimentally determined log P_{ow} values are reported in reference A3.9/01. log P_{ow} (pH 4) = 2.9 log P_{ow} (pH 7) = 0.7 Calculated log P_{ow} value from BCFWIN v. 2.14, SRC Corporation. log P_{ow} = 2.23	Bode, M. (2003) Estimation of the terrestrial bioconcentration factor (BCF) of Warfarin. EBRC Consulting GmbH, Hannover, Germany, November 19, 2003 (unpublished). A7.5.5.1/01

5.3.3 Summary and discussion of bioaccumulation

Warfarin is unlikely to bioaccumulate in aquatic organisms. The highest estimated BCF in the aquatic environment was only 58 at pH 4. In the study on Rainbow Trout the highest BCF determined was 21.6. In this study steady state was reached within 2 days. Similarly, in the terrestrial environment the highest estimated BCF was 10.4 at pH 4. These results are considered in the classification and labelling of Warfarin.

5.4 Secondary poisoning

Not relevant for this dossier.

6 HUMAN HEALTH HAZARD ASSESSMENT

RAC general comment
Warfarin belongs to a group of compounds known as the anticoagulant rodenticides, i.e. those with an anti-vitamin K (AVK) mode of action (MoA) which are used mainly as active substances in biocidal products for pest control of rats, mice and other rodents. Some of the substances had an existing harmonised classification. However, at the time of writing, only Warfarin is currently classified for toxicity to reproduction in category 1A. The current Annex VI entry of warfarin includes the racemic mixture of warfarin as well as the individual R- and S-enantiomers. The dossier submitter (IE) evaluated racemic warfarin (50:50 R:S) under the Biocides Directive (Dir. 98/8/EC) and the Plant Protection Product Directive (Dir. 91/414/EC). The dossier submitter used the Biocide and PPP EU evaluations as the basis for the CLH Report. The eight AVK rodenticides were previously discussed by the Technical Committee on Classification and Labelling of Dangerous Substances (TC C&L) of the European Chemicals Bureau (ECB) (2006 – 2008). However, the work was transferred to ECHA and to that end Member State Competent Authorities (MSCAs) were requested to prepare CLH proposals. CLH proposals for eight AVK rodenticides, Coumatetralyl (Denmark), Difenacoum (Finland), Warfarin (Ireland), Brodifacoum (Italy), Flocoumafen (The Netherlands), Difethialone (Norway) Chlorophacinone (Spain) and Bromodialone (Sweden), were submitted by eight different Dossier Submitters (DS). The dossiers were handled as a group but the Committee for Risk Assessment (RAC) proceeded to evaluate the proposals on a substance by substance basis comparing the human data available for Warfarin (and other AVKs) and relying on a weight-of–evidence approach as required by Regulation 1272/2008 (CLP).

6.1 Toxicokinetics (absorption, distribution, metabolism and elimination)

The following summary is prepared from the Warfarin (and Sodium Warfarin) assessment made under Directive 91/414/EEC (Plant Protection Products) and the Warfarin and Sodium Warfarin assessment made under Directive 98/8/EC (Biocides). The DAR and CAR documents are arttached/included in order to access greater details, where necessary.

The active substance Warfarin is marketed in rodenticide products either as the “free acid” or as its sodium salt. The active substance under physiologically and environmentally relevant conditions is the Warfarin molecule. Therefore, the plant protection and biocide dossier considers these entities as equivalentunder physiologically and environmentally relevant conditions. In light of this, the hazard assessment for sodium warfarin is identical to that carried out for Warfarin. It was the conclusion of the TC C & L review (2006/2007) to classify warfarin sodium in the same way as warfarin.

Based on studies in rats and the results of human clinical trials, the absorption of Warfarin and Sodium Warfarin following oral administration was effectively 100%, with maximal plasma concentrations rapidly achieved within 2 to 12 hours in humans. Absorption of Warfarin and Sodium Warfarin after oral intake may be seen as complete as unchanged Warfarin was not detected in faeces. Upon sustained skin contact, Warfarin may be dermally absorbed at a rate of approximately 15 % (13.3% and 14.4% were absorbed by rat skin after 6 h and 24 h exposure, respectively).

Warfarin is distributed quickly throughout the plasma. Only a small amount of circulating Warfarin is free in plasma (about 3% in rats, and 5% in humans), with most of the drug being bound to serum

albumin. Liver is the main target organ and has the greatest affinity of any tissue examined. After epicutaneous administration in rats, approximately 57% of the absorbed dose was distributed to tissues. Individual tissue amounts were small with the greatest recoveries from skin and the skin application site and minor amounts detected in the carcass, liver, GI tract contents and blood.

Warfarin, a racemic mixture of R and S enantiomers in a 50:50 ratio, is almost completely metabolised in man, predominantly by hydroxylation. No glucuronide or sulphated conjugates were identified. In terms of anticoagulant control, S-Warfarin is 3.8 times more potent than R-Warfarin. The metabolism of Warfarin is characterised in humans as follows: (R)-Warfarin is hydroxylated mainly to the (R, S)-alcohol, followed by minor amounts of 7-hydoxyWarfarin and (R, R)-alcohol, (S)-Warfarin mainly to 7-hydroxyWarfarin and to small amounts of both the (S, S) and (R, S)-alcohol. A small amount of parent Warfarin is also detected in urine. In liver, the Warfarin metabolites have either none or clearly decreased anticoagulant activity. The Warfarin alcohol stereoisomers have approximately 5–10% the anticoagulant activity of racemic Warfarin with the RS Warfarin alcohol stereoisomer being the most potent. Peak levels for the plasma metabolites occur between 30 and 60 hours after oral Warfarin administration. In rat, mainly 6- and 7-hydroxyWarfarin and an unidentified metabolite were identified. Additionally, 8-hydroxyWarfarin must also be present, as this compound is found in urine. The 6-, 7- and 8-hydroxy substitutions are without significant pharmacological activity in the rat. Peak levels for these metabolites occurred between 5 and 16 hours after oral administration.

Evidence of accumulation from a repeat dose human application study indicates a plasma half-life of ca. 40-163 hours after administration of 2, 5 and 10mg of Warfarin.

Unchanged Warfarin is excreted in small amounts in humans and animals (in rat approximately 7 % of the Warfarin metabolites). In faeces, the same metabolites are present but in different relative amounts than found in urine. About one-half to two-thirds of excreted Warfarin and its metabolites are found in urine. There is no indication that any metabolite of Warfarin is excreted via the exhalation route for example in rats, no $^{14}CO2$ was detected. In humans, 50% of orally or intravenously administered Warfarin is excreted within approximately 20 to 50 hours. There is a prolonged terminal phase of Warfarin elimination during which saturation binding of Warfarin to tissue enzymes probably occurs. In man, maximal reduction in Prothrombin complex activity was reached within 36 – 72 hours at a dose of 50 mg Warfarin. Of note, Vitamin K has an antidote action of the effect of Warfarin on Prothrombin complex activity.

6.2 Acute toxicity

Method	Results	Remarks	Reference
Acute oral toxicity (No method stated but study conducted in a similar manner to OECD 401)	LD_{50}: 5.6 mg/kg b.w.	Warfarin technical, 0, 1, 2, 4, 8 and 12 mg/kg; single acute exposure. Rat (CbT Wistar), female	Bai, M.K., Krishnakumari, M.K., Majumder, S.K, Comp. Physiol. Ecol., Vol.17 (2), 75-82 (1992)
Acute oral toxicity (No method stated)	LD_{50}: 112-mg/kg b.w. (Male) LD_{50}: 10.4 mg/kg b.w. (Female)	Sodium Warfarin, 10, 20, 40, 80, 160 and 320 mg/kg b.w; single acute exposure. Rat (Sprague-Dawley), 6 males and 6 females per concentration.	Back, N., Steger, R., Glassman, J.M. Pharmacological Research Communications, Vol. 10 (5), 445-452 (1978)
Acute inhalation toxicity (EPA Guidelines for the testing of Pesticides, 1982)	LC_{50}: M: < 0.005 mg/l LC_{50}: F: < 0.005 mg/l	Warfarin technical, 0, 0.005, 0.021 and 0.044 mg/l (measured) 255 min. Rat (Sprague-Dawley), 5 males and 5 females per concentration.	Biesemeier, J.A., Unpublished report: FDRL study No.: 8359 (1985)
Acute dermal toxicity (OECD 402 and EC B.3 92/69/EEC)	LD_{50}: 20-80 mg/kg (Male) LD_{50}: 40 mg/kg (Female) Combined: 40 mg/kg	Warfarin technical, 5, 20, 80, 250, 400, 650 and 1000 mg/kg b.w. 24 h. Rat (Sprague-Dawley), 5 males and 5 females per concentration	Daamen, P.A.M., NOTOX project 110464; NOTOX substance 34794, (1994)

6.2.1 Acute toxicity: oral

The acute oral toxicity study (females only) submitted in the context of the Directive 91/414/EEC and Directive 98/8/EC reviews was not conducted according to GLP or recognised guidelines (Bai, M.K., Krishnakumari, M.K., Majumder, S.K., 1992). However, the study was well documented and meets basic scientific principles and was acceptable for acute oral toxicity classification. .

In this study, signs of toxicity were observed 2 – 3 days after administration of the test compound, and intensified within 4 – 5 days. Thereafter, signs of toxicity in surviving animals reversed gradually. Signs of intoxication were haemorrhages observed under the skin near the neck region, and around the nose, eyes and mouth. Food intake of all surviving animals was comparatively less than that of controls. Animals were found dead between days 4 – 6 after administration.

The relative weights of ovaries increased with increasing dose levels, while relative lung weights decreased. Histopathological examinations revealed mild to heavy haemorrhages in the kidneys, vasodilatation in ovaries and adrenals, mild bile duct proliferation, cellular infiltration, vasodilatation and haemorrhages in the livers, and atrophy of thyroid follicles. The body weight of surviving animals appeared lower than control (not statistical significant). The LD_{50} in female rats was calculated at 5.62 mg/kg bw (95 % confidence limit: 4.95 – 6.07 mg/kg bw).

The second acute oral toxicity study carried out with males and females (Back, N., et. al., 1978) was viewed as supportive only due to methodological shortcomings (LD_{50} 112 mg/kg bw ± 15.9 mg/kg bw for males, and 10.4 mg/kg bw ± 1.1 mg/kg bw for females. The two studies assessed indicate that the acute oral toxicity of Warfarin is dependent on strain and sex.

Apart from these studies presented above, further 'supportive" data on acute oral toxicity (expressed as LD_{50}) based on the results of a literature search were presented in the Biocides CAR. Since the reliability of the data and the level of documentation were considered to be questionable and did not warrant detailed presentation, these references are summarised briefly in

Table 6.2.1-1 1 below. In studies where male and female rats were tested, the LD_{50} values for females (range: 5–58 mg/kg b.w.) were lower than for males (range: 1.6–323 mg/kg b.w.). In contrast, the LD_{50} for other species were significantly higher, for example mice 374–675 mg/kg, rabbits ca. 800 mg/kg, dogs ca. 200–300 mg/kg. This data indicates the particular sensitivity of the rat species to Warfarin.

Table 6.2.1-1: Supportive data, acute oral toxicity of Warfarin technical

Animal species	**Sex**	**Vehicle**	**LD_{50} [mg/kg bw]**	**Reference***
Rat(R. argentiventer)	male, female	corn oil	315 (249 – 398) [b]	Ming LY (1979)
Rat(AW49,Fa. Wulf)			3.4 (2.9 – 4.3) [b]	Niedner R, (1973)
Rat (Osborne Mendel)	male	water	323 ± 70 [a]	Hagan EC, (1953)
Rat (Osborne Mendel)	female	water	58 ± 18 [a]	Hagan EC, (1953)
Rat (Sherman)	male	peanut oil	1.6 (1.4 – 1.9) [b]	Hayes WJ Jr. (1967)
Rat (Sherman)	male	peanut oil	3.0	Gaines TB (1969)
Mouse (NMRI)			640 (540 – 760) [b]	Niedner R, (1973)
Mouse	male, female	water	374 ± 84 [a]	Hagan EC, (1953)
Mouse			675	Popa L, (1980)
Guinea pig	male, female	water	182 ± 8 [a]	Hagan EC, (1953)
Rabbit	male, female	water	approx. 800	Hagan EC, (1953)
Rabbit		water	800	Hagan EC, (1953)

Dog	male, female	water	200 – 300	Hagan EC, (1953)

a) standard error; b) 95% confidence limit; CMC: 0.5% Carboxymethylcellulose

*References

Ming, L.Y, 1979. The toxicity of warfarin to the rice field rat, Rattus argentiventer (Robinson & Kloss) Malays. Agric. J. 52, 177-181.

Niedner, R.; Kayser, M.; Reuter, N.; Meyer, F.; Perkow, W., 1973. Toxicity of warfarin and its influence on blood coagulation in rats and mice. Arzneim.-Forsch. 23, 102.

Hagan, E.C.; Radomski, J.L. 1953 The toxicity of 3-(acetonylbenzyl)-4-hydroxycoumarin (Warfarin) to laboratory animals. J. Am. Pharm. Assoc. 42, 379-382

Hayes, W.J. Jr. 1967. The 90-dose LD50 and a chronicity factor as measures of toxicity. Tox. Appl. Pharm. 11, 327-335.

Gaines, T.B.,1969. Acute toxicity of pesticides. Tox. Appl. Pharm. 14, 515-534

Comparison to the criteria

DSD:

Classification was agreed in the TC and L meeting 2007 on the basis of the data from **the most reliable** study (CAR Ref A.6.1.1: Bai, M.K., *et. al.*, 1992) conducted on the most sensitive species, the rat. The LD_{50} from this study (5.62 mg/kg bw) falls within the criteria for R 28; Very toxic if swallowed ($LD_{50} \leq 25$ mg/kg bw).

Other data summarised are not sufficiently reliable for classification purposes. However, most of the LD_{50} values measured in rats (females range: 5–58 mg/kg b.w, males range: 1.6–323 mg/kg b.w.) fall within the criteria for classification for R28 also. In contrast, the LD_{50} for other species were significantly higher, for example mice 374–675 mg/kg, rabbits *ca*. 800 mg/kg, dogs *ca*. 200–300 mg/kg.

CLP:

Warfarin classifies according to the CLP regulation criteria as Acute Cat 2; H300 on the basis of the LD50 of 5.62 mg/kg bw from the most reliable study (Bai, M.K., *et. al.*, 1992). The criteria for classification are in Cat 2 are; $5 < LD_{50} \leq 25$ mg/kg bw.

6.2.2 Acute toxicity: inhalation

The acute inhalation toxicity of Warfarin (technical) was tested in Sprague-Dawley rats according to EPA guidelines for testing of pesticides (1982). The dose range was 0, 0.005, 0.021, 0.044 mg/l (actual concentration).

All animals appeared normal immediately following exposure until day 3 of the study. Significant incidences of decreased activity, increased respiration rate, bleeding at the ear notch, discoloration of ears and pale appearance were observed.

All males of the mid and high dose groups died by day 7. In the low dose group, deaths occurred between day 6 and 9. All females died between days 10 and 11.

Significant incidences of haemorrhages were observed in the axillary region, cranium, muscles, gonads, ears, abdominal and thoracic cavities.

Body weight gain of surviving males was decreased on day 4. Surviving males of the low dose group lost body weight until day 8. Surviving females maintained (low dose) or lost body weight until day 4.

The LD_{50} value for inhalation toxicity was therefore below the lowest aerosol concentration of 0.005 mg/l.

Comparison to the criteria

DSD:

Classification was agreed in the TC and L meeting 2007 on the basis of the acute inhalation study presented (CAR A6.1.3: Biesemeier JA (1985) conducted on the most sensitive species, the rat. The LD_{50} from this study (< 0.005 mg/l) falls within the criteria for R 26; Very toxic if swallowed ($LC_{50} \leq 0.05$ mg/l).

CLP:

Warfarin classifies according to the CLP regulation criteria as Acute Cat 1; H330 (Fatal if inhaled) on the basis of the LC_{50} of < 0.005 mg/l. The criteria for classification in Acute Cat 1 are; $0 < LC_{50} \leq 0.05$ mg/l.

6.2.3 Acute toxicity: dermal

The acute dermal toxicity of Warfarin was tested in Wistar rats according to EC method B.3 and OECD 402. 5/sex rats were exposed to dose levels of 0.5, 2, 8, 25, 40, 65 and 100 mg/mg in propylene glycol for 24 hours.

Animals showed clinical signs such as lethargy, hunched posture, ventro-lateral recumbency, laboured respiration, emaciation, swelling of the head, dark eyes, ptosis of both eyes, pale skin, piloerection, bleeding, scabs and dried blood in ears, dried blood on the head, chromodacryorrhea, adhesion of left eyelids. At the treated skin sites, erythema, scales and scabs were observed. Several animals showed haematomas (blue or green cutaneous areas) on back, shoulder, head, cheek and snout.

All deaths occurred between days 5 and 14. Several animals were killed in extremis

Macroscopic post mortem examination of animals that died or were killed in extremis revealed findings consistent with the administration of an anticoagulant. Haemorrhages, clotted blood, pale appearance and scabs were seen in various organs. Examination of surviving animals upon study termination revealed watery contents in uterus; nodule/nodules in the thymus, grown together with the heart and haemorrhages in the abdominal fat.

Loss of body weight, no or low body weight gain was observed in most of the surviving animals treated with 5, 20 or 80 mg/kg during the first week of the study. During the second week, improved body weight gain was observed in the majority of these animals

The dermal LD50 of the test compound in rats was 40 mg/kg for combined sexes and females alone. For males, the LD50 was estimated between 20 and 80 mg/kg.

Comparison to the criteria

DSD:

Classification was agreed in the TC and L meeting 2007 on the basis of the acute dermal study presented (CAR A6.1.2: Daamen, P., 1994) conducted on the most sensitive species, the rat. The LD_{50} from this study (40 mg/kg bw) falls within the criteria for R 27; Very toxic in contact with skin ($LD_{50} \leq$ 50mg/kg bw).

CLP:

Warfarin classifies according to the CLP regulation criteria as Acute Cat 1; H310 (Fatal in contact with skin) on the basis of the LC_{50} of < 0.005 mg/l. The criteria for classification in Acute Cat 1 are; $0 < LC_{50} \leq 0.05$ mg/l.

6.2.4 Acute toxicity: other routes

Not relevant for this dossier.

6.2.5 Summary and discussion of acute toxicity

Warfarin is classified as very toxic via the oral, dermal and inhalation routes based on the data from the animal studies.
In accordance with the provisions of Council Directive 67/548/EEC, Warfarin is assigned the symbol T+ and the indication of danger "very toxic". The following risk phrases R26-Very toxic by inhalation, R27-Very toxic in contact with skin and R28-Very toxic if swallowed are applied.

In accordance with the provisions of CLP Regulation (EC) No 1272/2008 Warfarin is assigned the Signal word "Danger" and the following hazard statements; Acute Tox 2 H300: Fatal if swallowed, Acute Tox 1-H310: Fatal in contact with skin and Acute Tox 1-H330: Fatal if inhaled.

RAC evaluation of acute toxicity
Summary of the Dossier submitter's proposal
Warfarin has no harmonised classification for acute toxicity. The dossier submitter (DS) proposed to add a classification for acute toxicity via oral, dermal and inhalation routes of exposure.
Acute oral toxicity
Two studies were presented by the Dossier Submitter (DS) in the CLH report. The first acute oral toxicity study (Bai *et al*. 1992) was not conducted according to GLP or recognised guidelines. In particular, only Wistar female rats were used. However, the study meets basic scientific principles and is considered suitable for acute oral toxicity classification. The LD_{50} in female rats was calculated as 5.62 mg/kg bw (95 % confidence limit: 4.95 – 6.07 mg/kg bw). In this study, signs of toxicity were observed 2 – 3 days after administration of the test compound, and intensified within 4 – 5 days. Thereafter, signs of toxicity in surviving animals reversed gradually. Signs of intoxication were haemorrhages observed under the skin near the neck region, and around the nose, eyes and mouth. Animals were found dead between days 4 – 6 after administration.
The second acute oral toxicity study carried out with male and female Sprague-Dawley rats (Back *et al*., 1978) was viewed as supportive only due to methodological shortcomings (LD_{50} of 112 mg/kg bw ± 15.9 mg/kg bw for males, and 10.4 mg/kg bw ±

1.1 mg/kg bw for females).

The two studies assessed indicate that the acute oral toxicity of Warfarin is dependent on strain and sex.

Further supporting data on acute oral toxicity (expressed as LD_{50}) based on the results of a literature search were presented in the Biocides CAR. The reliability of the data and the level of documentation were considered insufficient for a comparison with the CLP criteria.

In the studies where male and female rats were tested, the LD_{50} values for females (range: 5–58 mg/kg bw) were lower than for males (range: 1.6–323 mg/kg bw.). In contrast, the LD_{50} for other species were significantly higher, for example mice 374–675 mg/kg, rabbits ca. 800 mg/kg, dogs ca. 200–300 mg/kg. These data indicate the particular sensitivity of the rat to Warfarin.

Acute dermal toxicity

The acute dermal toxicity of Warfarin was tested in Wistar rats according to EC method B.3 and OECD Test Guideline (TG) 402. In that study rats (5/sex) per dose were exposed to dose levels of 0.5, 2, 8, 25, 40, 65 and 100 mg/kg in propylene glycol for 24 hours. Animals showed clinical signs such as lethargy, hunched posture, ventro-lateral recumbency, laboured respiration, emaciation, swelling of the head, dark eyes, ptosis of both eyes, pale skin, piloerection, bleeding, scabs and dried blood in ears, dried blood on the head, chromodacryorrhea and adhesion of left eyelids. At the treated skin sites, erythema, scales and scabs were observed. Several animals showed haematomas (blue or green cutaneous areas) on back, shoulder, head, cheek and snout. All deaths occurred between days 5 and 14. Several animals were killed *in extremis*.

The dermal LD_{50} of the test compound in rats was 40 mg/kg for combined sexes and females alone. For males, the estimated LD_{50} was between 20 and 80 mg/kg.

Acute inhalation toxicity

The acute inhalation toxicity of Warfarin (technical) was tested in Sprague-Dawley rats according to EPA guidelines for testing of pesticides (1982). The concentrations were 0, 0.005, 0.021, 0.044 mg/L (actual concentration). All animals appeared normal immediately following exposure (duration of exposure not stated in the CLH report) until day 3 of the study. Significant incidences of decreased activity, increased respiration rate, bleeding at the ear notch, discoloration of ears and pale appearance were observed. All males of the mid and high dose groups died by day 7. In the low dose group, deaths occurred between day 6 and 9. All females died between days 10 and 11.

Significant incidences of haemorrhages were observed in the axillary region, cranium, muscles, gonads, ears and abdominal and thoracic cavities.

The LC_{50} value for inhalation toxicity of Warfarin was the lowest aerosol concentration of 0.005 mg/L.

Classification proposed by the Dossier Submitter

Oral: Based on the oral LD_{50} for female rats (5.62 mg/kg bw), the DS proposed to classify Warfarin as Acute Tox. 2; H300 (criterion: oral, rat, $5 < LD_{50} \leq 25$ mg/kg bw).

Dermal: Based on the dermal LD_{50} for rats (40 mg/kg for combined sexes), the DS proposed to classify Warfarin as Acute Tox. 1; H310 (criterion: LD_{50}, dermal, rat or rabbit ≤ 50 mg/kg).

Inhalation: Based on the inhalatory LC_{50} value of 0.005 mg/Lfor the rat (both sexes combined), the DS proposed to classify Warfarin as Acute Tox. 1; H330 (criterion: LC_{50}, inhalation, rat, for dusts and mists ≤ 0.05 mg/L/4h).

The proposed classifications for acute toxicity for the oral and inhalation routes are a revision of the minimum classifications currently in Annex VI of CLP.

Comments received during public consultation

One Member State (MS) agreed with the classifications proposed by the DS for acute toxicity for Warfarin.

Assessment and comparison with the classification criteria

The RAC supported the proposal from DS to classify Warfarin according to CLP as follows:

- acute Tox. 2; H300 (Fatal if swallowed, criterion: oral, 5 < LD_{50} ≤ 25 mg/kg bw) based on the oralLD_{50} of 5.62 mg/kg bw from the most reliable study in female rats (Bai *et al.,* 1992);
- acute Tox. 1; H310 (Fatal in contact with skin, criterion: LD_{50}, dermal, rat or rabbit ≤ 50 mg/kg) based on the dermal LD_{50} for rats (40 mg/kg for combined sexes);
- acute Tox. 1; H330 (Fatal if inhaled, criterion: LC_{50}, inhalation, for dusts and mists ≤ 0.05 mg/l/4h) based on the inhalatory LC_{50} values of 0.005 mg/Lfor the rat (both sexes combined).

6.3 Irritation.

6.3.1 Skin

Species	Method	Average score 24, 48, 72 h		Reversibility Yes/No	Result	CAR Reference
		Erythema	Oedema			
New Zealand white rabbits	OECD 404, EC B.4 (92/69/EEC)	0	0	Not applicable	Non-irritating	A6.1.4/01

RAC evaluation of skin corrosion/irritation

Summary of the Dossier submitter's proposal

One skin irritation/corrosion study conducted in rabbit according to OECD TG 404 was considered by the DS. No classification is proposed by the DS as also agreed by the TC C&L in 2006/2007.

Comments received during public consultation

One MS supported the conclusion of non-classification of Warfarin as a skin irritant.

Assessment and comparison with the classification criteria

In the opinion of RAC the results of the study presented by the DS do not warrant classification of Warfarin for skin irritation/corrosion according to CLP criteria.

6.3.2 Eye

Species	Method	Average Score				Reversibility	Result	CAR Reference
		Cornea	Iris	Conjunctiva		Yes/No		
				Redness	Chemosis			
New Zealand white rabbits	OECD 405, EC B.5 (92/69/EEC)	0	0	0	0	Yes	Non-irritating	A6.1.4/02

RAC evaluation of eye corrosion/irritation

Summary of the Dossier submitter's proposal

One eye irritation/corrosion study conducted in rabbit according to OECD TG 405 was considered by the DS. No classification is proposed by the DS as also agreed by the TC C&L in 2006/2007.

Comments received during public consultation

One MS supported the conclusion of the DS on the non-classification of Warfarin as an eye irritant.

Assessment and comparison with the classification criteria

In the opinion of RAC the results of the study presented by the DS do not warrant classification of Warfarin for eye corrosion/irritation according to CLP criteria.

6.3.3 Respiratory tract

No information available.

6.3.4 Summary and discussion of irritation

Warfarin did not induce skin or eye irritation, and therefore does not require classification.

6.4 Corrosivity

Not relevant for thissubstance.

6.5 Sensitisation

6.5.1 Skin

Species	Method	Number of animals sensitized/ total number of animals	Result	CAR Reference

Albino Guinea pigs (Himalayan)	OECD 406, EC B.6 (92/69/EEC)	Test group: 0/20	Not sensitising	A6.1.5/01

RAC evaluation of skin sensitisation
Summary of the Dossier submitter's proposal One skin sensitisation study conducted in Guinea pigs according to OECD TG 406 (guinea pig maximisation test, GPMT) was considered by the DS. No classification is proposed by the DS as also agreed by the TC C&L in 2006/2007. **Comments received during public consultation** No comments were received. **Assessment and comparison with the classification criteria** In the opinion of RAC, the results of the GPMT do not warrant classification for skin sensitisation, because the observed effects do not meet the CLP classification criteria.

6.5.2 Respiratory system

No information available.

6.5.3 Summary and discussion of sensitisation

Experimental data indicate that Warfarin is not a skin sensitiser. No classification is required.

6.6 Repeated dose toxicity

Method	Results	Remarks	Reference
Repeat dose oral feeding study (40 days)	- Rattus norvegicus (5 males and 5 females) 12.5 ppm - 100% mortality 6.2 ppm - 95 % mortality 3.1 ppm - 95 % mortality 1.5 ppm - 50 % mortality - Rattus rattus (5 males and 5 females) 50 ppm - 100% mortality 25 ppm - 80 % mortality 12.5 ppm - 70 % mortality - Mus musculus (5 males and 5 females) 12.5 ppm - 100% mortality 6.2 ppm - 89 % mortality 3.1 ppm - 100 % mortality LO(A)EL or NO(A)EL not identified	Warfarin *1.5–800 ppm, daily. 12.5–800 ppm, daily 3.1–800 ppm, daily	Hayes, W.J. and Gaines, T.B. Public Health Reports 74: 2, 105-112. (1959)
Repeat dose oral feeding study (90 days)	Rat (Sherman), male. 90-day LD_{50} = 0.077 mg/kg b.w. /day LO(A)EL or NO(A)EL not identified	Warfarin 0.02-20 mg/kg b.w / day, daily	Hayes, W.J., Jr., Tox. Appl. Pharm. 11, 327-335, (1967)

*Purity not specified.

6.6.1 Repeated dose toxicity: oral

Detailed short/medium term repeat dose oral toxicity studies are not available due to the high susceptibility and sensitivity of animal model species to the anticoagulant effects of Warfarin. A few rat repeat dose Warfarin toxicity studies are available, however, some of these studies are not suitable for hazard evaluation. Rodents are the target species for Warfarin and in comparison to humans are particularly susceptible to the substance. In the available rat repeat dose Warfarin toxicity studies it is indicated that there is difficulty conducting the studies at low Warfarin levels. For example, the oral uptake of a dose of 0.077mg/kg b.w/day led to a mortality of 50% of test animals in a 90-day study notwithstanding the haemorrhaging that also occurred in rats at the levels tested. This dose given to a 60 or 70 kg person calculates to approximately 5 mg/day; by way of comparison the average therapeutic dose is 2-10 mg/person/day. It is also noted that the response to Warfarin is highly individual. In a therapeutic setting, this dose level should lead to a prothrombin time prolongation factor of between 1.5 and 2.5. The relevance of repeat dose oral toxicity studies conducted in animal models for extrapolation to humans, given the interspecies differences in pharmacological behaviour between the models species and humans, would be highly questionable even if testing was possible. A summary of the studies submitted is presented for completeness.

6.6.1a	(DAR Reference B.6.3.1.1)
Study title:	Laboratory Studies of Five Anticoagulant Rodenticides.
Author:	Hayes, W.J. and Gaines, T.B.
Date of report:	(1959)
Report identity:	Public Health Reports 74: 2, 105-112.
Testing facility:	Unknown
GLP:	No
Guidelines:	None

Materials and Methods:

Warfarin; Batch: no data; Purity: no data;
Animal species: Rattus norvegicus, Rattus rattus, Mus musculus;
No. of animals: at least 5 males and 5 females per group;
Dose levels: 1.5-800 ppm in the diet; Diet: a) unpoisoned, ground laboratory chow, b) yellow cornmeal containing warfarin;
Duration: 40 days;
Recordings: intake of poisoned bait and poison-free feed on a daily basis, body weight weekly, calculation of total dose of ingested rodenticide for each animal.

Findings:

The different susceptibilities of the three species of commensal rodents to warfarin were observed. A few new observations on pathological changes caused by warfarin supplement those reported previously.

Conclusions:

This study is of no relevance in evaluating warfarin for inclusion in Annex 1 of 91/414/EC.

6.6.1b	(DAR Reference B.6.3.2 Oral 90-day toxicity)
Study title:	The 90-day dose LD50 and a chronicity factor as measures of toxicity.
Author:	Hayes, W.J., Jr.
Date of report:	1967
Report identity:	Tox. Appl. Pharm. 11, 327-335
Testing facility:	Unknown

GLP: No
Guidelines: None
The purpose of this study was to use a 90-day dose LD50 and a chronicity factor (i.e. the ratio between 1-day dose and 90-day dose LD50 values for a compound, indicating the cumulative effect of the compound) to communicate the results of repeated doses. Warfarin was one compound which was mentioned in the paper.

Material and methods: Test material: Warfarin; Batch: no data; Purity: no data; Animal species: adult Sherman strain white rats; No. of animals: 110 male rats; Duration of treatment: 90 days; Recordings: The dose levels were calculated from measured food consumption.

Findings:
An inherent delay in the action of warfarin, even when given at high doses, was demonstrated. A gradual increase in the time necessary for intermediate doses to produce their effect, and the failure of sufficiently small dosages to produce an effect even when continued for the lifetime of the animal.

Conclusion:
The 90-day dose LD50 of warfarin was 0.077 mg/kg bw/day. Even with several limitations, the study is acceptable from the point of view of evaluating the classification of warfarin and this is supported by the human clinical exposures of warfarin as a therapeutic drug (2-10 mg/person/day).

6.6.2 Repeated dose toxicity: inhalation
Short and medium term repeat dose inhalation toxicity studies are not available due to the high susceptibility and sensitivity of animal model species to the anticoagulant effects of Warfarin.

6.6.3 Repeated dose toxicity: dermal
Short and medium term repeat dose dermal toxicity studies are not available due to the high susceptibility and sensitivity of animal model species to the anticoagulant effects of Warfarin.

6.6.4 Other relevant information
Warfarin is widely used in humans for both short-term (weeks-months) and long-term (years) oral anticoagulation therapy. It is also documented that Warfarin can have adverse effects in humans. The most frequent adverse effects noted were bleeding episodes, which can be regulated by monitoring prothrombin times. Only a small number of incidences of Warfarin-induced skin necrosis have been described. Other non-haemorrhagic skin conditions have also been described. Reduced bone mass in patients with long-term anticoagulant (not Warfarin) therapy has also been reported (see DAR for detail).
The administration of Warfarin to women during the first trimester of pregnancy is associated with about 5% incidence of foetal anomalies known as “foetal Warfarin syndrome” or “Warfarin embryopathy”. This is characterised by nasal hypoplasia, bone anomalies and bone-growth retardation, which might be related to interference with vitamin-K dependent bone proteins. Administration of Warfarin during the second or third trimester of pregnancy may lead to foetal loss and CNS lesions associated with haemorrhage. The reproductive toxicity will be dealt with in more detail in Section 6.9.

6.6.5 Summary and discussion of repeated dose toxicity
According to the DSD:

Warfarin is classified in Annex I to Directive 67/548/EEC as Toxic "T" and labelled with the risk phrase R48/25. This classification and labelling is derived from the repeat rat oral dose 90-day study where 0.02-20 mg/kg b.w/day of Warfarin was administered daily. The 90-day LD_{50} was calculated at 0.077 mg/kg b.w/day, approximately 1.4% of the acute LD_{50}. It is indicated in Annex VI to Directive 67/548, that classification is required when effects are observed at ≤ 50mg/kg (bodyweight)/day for oral intake. Substances are classified as Toxic when these effects are observed at levels one order of magnitude lower (i.e. 10-fold) than those set out for Harmful R48 (≤ 5mg/kg (bodyweight)/day for oral intake). The subchronic 90-day LD_{50} of 0.077 mg/kg b.w/day therefore triggers T; R48/25.

During the peer review of the Warfarin Biocides Competent Authority Report it was proposed to classify Warfarin as "Toxic" and label it with the risk phrase R48/25/24/23, therefore extrapolating the oral classification to the dermal and inhalation routes. This was agreed by the RMS and is based on the following:

Dermal absorption is calculated as 14% for Warfarin. When 14% of the oral subchronic cut-off value for classification as Toxic (≤ 5mg/kg b.w/day) is calculated a value of 0.7mg/kg bw/day is derived. Accordingly, 0.7mg/kg bw/day is greater than the oral 90-day LD_{50} of 0.077mg/kg bw/day and T R48/24 is applied.

Furthermore, extrapolation from acute inhalation is applied to subchronic inhalation for classification and labelling purposes. The acute LC_{50} value for Warfarin is <0.005mg/l. The inhalation subchronic cut-off for classification as Toxic is 0.025mg/l. As the acute LC_{50}, which has death as its endpoint, is less than < 0.005mg/l, T; R48/23 is applied.

According to CLP:
In accordance with the provisions of CLP Regulation (EC) No 1272/2008 Warfarin is assigned the Signal word "Danger" and the following hazard statement; H372: Causes damage to organs through prolonged or repeated exposure. It is proposed that Warfarin will be classified for Specific Target Organ Toxicity – Repeated Exposure (STOT-RE Category 1) on the basis of evidence from a limited number of studies in experimental animals, including the repeat rat oral dose 90-day. In this study, death occurred at low exposure concentrations. In addition, evidence from human cases in which significant toxicity occurred at low exposure concentrations, was taken into account.

SCL for repeat exposure (STOT-RE) classification: The formula set out in the Guidance to Regulation (EC) No 1272/2008 for setting of specific concentration limits for repeated exposure (STOT-RE) is as follows:

$$\text{SCL Cat. 1 for STOT-RE} = \frac{ED_{10}\text{ mg/kg body weight /day (oral) x 100\%}}{\text{10 mg/kg body weight /day (GV1)}}$$

and...

$$\text{SCL Cat. 2 for STOT-RE} = \frac{ED_{10}\text{ mg/kg body weight /day (oral) x 100\%}}{\text{100 mg/kg body weight /day (GV2)}}$$

A limited number of short and medium-term repeat dose oral toxicity studies are available for assessment of repeat dose toxicity. However, these studies do not allow for a LOAEL or NOAEL

setting or determination of an ED_{10} value for use in the above formulae due to the high susceptibility and sensitivity of rat model species to the anticoagulant effects of Warfarin.

In addition to the limitations of the oral repeat dose data, no data are available for assessment of repeat dose toxicity *via* the dermal and inhalation routes. The available rat oral studies show that a specific toxicity profile occurs in repeat dose rat studies at a dose / concentration between 2 and 3 orders of magnitude lower than the guidance values of 10 and 100mg/kg body weight /day.

On the other hand, robust evidence indicates that other species and human patients differ in sensitivity or susceptibility to the effect observed in the rat studies. The clinical dose range for humans reported in the submitted literature is from 2.5 to 20 mg/day. The dose prescribed depends on the prothrombin clotting times in individual patients and the dose is tailored specifically in each case.

An estimated (or surrogate) value for the ED_{10} can be derived for Warfarin in mg/kg bodyweight/day by dividing the lowest reported dose with clinical effect of 2.5 mg/day by 60kg (= 0.04 mg/kg bw/day). This value is used in setting the specific concentration limits for repeat exposure (STOT-RE) classification as follows:

$$\text{SCL Cat. 1 for STOT-RE} = \frac{0.04 \text{ mg/kg bw/day} \times 100\%}{10 \text{ mg/kg body weight /day (GV1)}} = 0.41\ \%$$

and...

$$\text{SCL Cat. 2 for STOT-RE} = \frac{0.04 \text{ mg/kg bw/day} \times 100\%}{100 \text{ mg/kg body weight /day (GV2)}} = 0.04\ \%$$

The resulting SCL values are rounded down (as described under 3.9.2.6 Guidance to Regulation (EC) No 1272/2008), resulting in an SCL for Cat. 1 of ≥ 0.2% w/w, and an SCL for Cat. 2 between 0.02% and < 0.2% w/w. Accordingly, if the Warfarin concentration in a product is above 0.2% then the classification STOT RE. 1 - H372 applies, and if the concentration of Warfarin is equal to or greater than 0.02% and less than 0.2% then classification with STOT RE. 2 - H373 applies.

Summary: According to Regulation EC/1272/2008:

$C \geq 0.2\%$ STOT RE 1

$0.02\% \leq C < 0.2\%$ STOT RE 2

RAC evaluation of specific target organ toxicity– repeated exposure (STOT RE)
Summary of the Dossier submitter's proposal
Detailed short/medium term repeat dose oral toxicity studies are not available due to the high susceptibility and sensitivity of experimental animals to the anticoagulant effects of Warfarin. A few repeat dose toxicity studies are available on Warfarin in rats and mice (Hayes and Gaines, 1959; Hayes, 1967). However, these studies are not suitable for hazard evaluation. Rodents are the target species for Warfarin and in comparison to humans are particularly susceptible to the substance. In the available rat repeat dose Warfarin toxicity studies it is indicated that there is difficulty conducting the studies at low Warfarin levels. For example, the oral uptake of a dose of 0.077

mg/kg bw/day led to a mortality of 50% of test animals in a 90-day study despite the haemorrhaging that also occurred in surviving rats at the levels tested (Hayes, 1967). This dose for a 60 or 70 kg person would correspond to approximately 5 mg/day; for comparison the average therapeutic dose is 2-10 mg/person/day. It is also noted that the response to Warfarin is subject to individual variation or susceptibility. In a therapeutic setting, this dose level should lead to a prothrombin time prolongation factor of between 1.5 and 2.5. The relevance of repeat dose oral toxicity studies conducted in animal models for extrapolation to humans, given the interspecies differences in pharmacological behaviour would be highly questionable even if testing was possible.

The DS did not report NOAEL or LOAEL based on animal studies of repeated toxicity testing.

Warfarin is widely used in humans for both short-term (weeks-months) and long-term (years) oral anticoagulation therapy. It is also documented that Warfarin can have adverse effects in humans. The most frequent adverse effects noted are bleeding episodes, which can be regulated based on monitoring prothrombin times. Only a small number of incidences of Warfarin-induced skin necrosis are described in the literature. Other non-haemorrhagic skin conditions have also been described. Reduced bone mass in patients with long-term anticoagulant therapy (not Warfarin) has also been reported.

Dossier Submitter's conclusion on classification

The DS proposed that Warfarin be classified for Specific Target Organ Toxicity – Repeated Exposure (STOT-RE Category 1) under CLP on the basis of evidence from a limited number of studies in experimental animals, including the repeat rat oral dose 90-day. In this study, death occurred at low doses (50% mortality at 0.077 mg/kg bw/day). In addition, evidence from human cases in which significant toxicity occurred at low doses was taken into account.

Specific concentration limits

The formula set out in the CLP Guidance for setting of specific concentration limits for repeated exposure (STOT-RE) is as follows:

$$\text{SCL Cat. 1 for STOT-RE} = \frac{ED_{10} \text{ mg/kg body weight /day (oral)} \times 100\%}{10 \text{ mg/kg body weight /day (GV1)}}$$

and

$$\text{SCL Cat. 2 for STOT-RE} = \frac{ED_{10} \text{ mg/kg body weight /day (oral)} \times 100\%}{100 \text{ mg/kg body weight /day (GV2)}}$$

A limited number of short and medium-term repeat dose oral toxicity studies are available for assessment of repeat dose toxicity. However, these studies do not enable setting a LOAEL or NOAEL or determination of an ED_{10} value for use in the above formulae due to the high susceptibility and sensitivity of rat model species to the anticoagulant effects of Warfarin.

In addition to the limitations of the oral repeat dose data, no data are available for assessment of repeat dose toxicity *via* the dermal and inhalation routes. The available rat oral studies show that a specific toxicity profile occurs in repeat dose rat studies at a dose / concentration between 2 and 3 orders of magnitude lower than the guidance values (GV) of 10 and 100mg/kg bw/day.

On the other hand, robust evidence indicates that other species and human patients differ in sensitivity or susceptibility to the effect observed in the rat studies. The clinical dose range for humans reported in the submitted literature is from 2.5 to 20 mg/day. The dose prescribed depends on the prothrombin clotting times in individual patients and the dose is tailored specifically in each case.

An estimated (or surrogate) value for the ED_{10} can be derived for Warfarin in mg/kg bw/day by dividing the lowest reported dose with clinical effect of 2.5 mg/day by 60kg (= 0.04 mg/kg

bw/day).

This value is used in setting the specific concentration limits for repeat exposure (STOT-RE) classification as follows:

$$\text{SCL Cat. 1 for STOT-RE} = \frac{0.04 \text{ mg/kg bw/day} \times 100\%}{10 \text{ mg/kg body weight /day (GV1)}} = 0.4\ \%$$

and

$$\text{SCL Cat. 2 for STOT-RE} = \frac{0.04 \text{ mg/kg bw/day} \times 100\%}{100 \text{ mg/kg body weight /day (GV2)}} = 0.04\ \%$$

The resulting SCL values are rounded down (as described in section 3.9.2.6 of the CLP Guidance), resulting in an SCL for Cat. 1 of ≥ 0.2% w/w, and an SCL for Cat. 2 between 0.02% and 0.2% w/w (0.02 % ≤ Cat. 2 < 0.2 %).

Proposed SCLs:

STOT RE 1; H372 above 0.2% and STOT RE 2; H373 between 0.02 and 0.2%.

Comments received during public consultation

One MS supported the classification of Warfarin as STOT RE 1 and the specific concentration limits for STOT RE proposed by the DS.

Assessment and comparison with the classification criteria

In humans Warfarin is used as oral anticoagulation drug at doses of 2 - 10 mg/person/day and this dose level is expected to lead to prolongation of prothrombin time by a factor between 1.5 and 2.5. The most frequent adverse effects in humans noted during repeated therapeutic exposure were bleeding episodes. There was also a low incidence of Warfarin-induced skin necrosis.

In the 90-day repeated oral toxicity study in rats with Warfarin, 50% mortality was observed at a very low dose (0.077 mg/kg bw/day).

The median lethal dose level for rats exposed orally to Warfarin during 90 days at 0.077mg/kg bw/day corresponds to a daily dose of 5.4 mg/person (70 kg person). In humans, this dose is used as a therapeutic dose to reduce prothrombin time by a factor between 1.5 and 2.5. This comparison suggests that rats are more sensitive to repeated exposure to Warfarin than humans.

In the opinion of RAC Warfarin warrants classification as STOT RE 1 with the hazard statement H372: Causes damage to organs through prolonged or repeated exposure. Classification is warranted because lethality is observed in animals at prolonged exposure well below the guidance value (≤ 10mg/kg bw/day, table 3.9.2 of the CLP Regulation).

RAC is of the opinion that Warfarin also fulfils the classification criteria by the dermal route. Indeed, taking into account the median lethal oral dose and the dermal absorption of Warfarin (14%) mortality would result as consequence of repeated dermal exposure at dose level of 0.55 mg/kg bw/day (0.077mg/kg bw/day x 100/14 = 0.55 mg/kg taking 100% for absorption of Warfarin by the oral route). This dose level is thus well below a GV of 10 mg/kg bw/day which fulfils the criteria for classification as STOT RE 1; H372.

Therefore, it is the opinion of RAC that Warfarin warrants classification as STOT RE 1 with hazard statement; H372: Causes damage to blood through prolonged or repeated exposure according to CLP criteria.

Specific Concentration Limits

In the opinion of RAC the specific concentration limit for STOT RE for Warfarin should be based

on the 90 - day oral study on rats with 90-day The median lethal dose level in rats = 0.077mg/kg/day.

An SCL for STOT RE 1 of 0.5% is proposed based on serious effects (death) seen at 0.077 mg/kg in the 90-day study in rats. Calculation: 0.077 mg/kg bw/day (adverse effect dose) / 10 mg/kg bw/day (GV for cat. 1) * 100% = 0.77% rounded down to 0.5% as required by the Guidance on the Application of the CLP Criteria.

An SCL for STOT RE 2 is proposed between 0.05% and 0.5% using the same data and method of calculation using guidance value of 100 mg/kg bw/day for Cat. 2. This calculation is performed according to the method described in the Guidance on the Application of the CLP Criteria.

6.7 Mutagenicity

No classification is included for this hazard class in Annex VI, Tables 3.1and 3.2, CLP Regulation and no classification is currently proposed as also agreed by TC C and L in 2006/2007.

6.7.1 *In vitro* data

6.7.2 NA*In vivo* data

6.7.3 NAHuman data
NA.

6.7.4 Other relevant information

6.7.5 NASummary and discussion of mutagenicity
NA.

6.8 Carcinogenicity

No classification is included for this hazard class in Annex VI, Tables 3.1and 3.2, CLP Regulation and no classification is currently proposed as also agreed by TC C and L in 2006/2007.

6.8.1 Carcinogenicity: oral

6.8.2 NACarcinogenicity: inhalation
NA.

6.8.3 Carcinogenicity: dermal

6.8.4 NACarcinogenicity: human data

6.8.5 NAOther relevant information

6.8.6 NASummary and discussion of carcinogenicity

6.9 NAToxicity for reproduction

6.9.1 Effects on fertility

Multi-generation studies: No multi-generation study data are available from published sources. However, the conduct of such studies is not considered feasible due to the particular sensitivity of the model species (rodents). For example, sodium warfarin, administered at a dose of 175µg/kg bw/day to Sprague-Dawley rats on gestational days 8-22 led to a mortality of 43% among dams (Feteih et al., 1990), which, for a 70 kg person would be calculated to equate to an exposure of 12 mg/day, which corresponds to a dosage within the usual therapeutic range (2.5 – 15 mg/day).

6.9.2 Developmental toxicity

Experimental animal data

Developmental toxicity studies: 5 experimental animal studies were submitted in support of the Pesticide review under EC 91/414. These are included here for completeness, although the current classification relies entirely on the human clinical evidence. The purity of the test substance has not been given in any of these studies/trials. An additional study is now available (BASF, 2010) which is a guideline study (OECD 414) and is evaluated below. The Pesticide Registration and Control Division (PRCD Ireland) reviewer of both the initial and current review is referred to as the 'Reviewer' in the following section.

6.9.2.1.a

Mirkova, E.; Antov, G. (1983): Experimental evaluation of the risk of prenatal pathology under effect of warfarin – coumarine rodenticide. Hig. Zdrav. 25, 476-482.

Guidelines: Not presented
GLP: No
Test material: Warfarin, Na-salt, technical pure;
Batch: No data;
Purity: No data;
No. of animals: A total of 260 pregnant Wistar rats;
Administration: Oral per gavage;
Vehicle: Water;
Controls: Included but not specified;
Sacrifice: Day 21;

I. single application: 8 mg/kg bw on days: 10, 11, 12, 13, 14 and 17;
II. repeated appl.: 0.32 mg/kg bw; Application period: days 1-7;
III. repeated appl.: 0.32 mg/kg bw; Application period: days 8-16;
IV: repeated appl.: 0.32, 0.16, 0.08, 0.04 mg/kg bw on days days 1-21.

Findings:
Following single applications of warfarin to groups of animals on days 10, 11, 12, 13, 14 and 17 of pregnancy, toxicological signs, abortion and massive vaginal haemorrhages (for an average of 3 days) were observed. Mortality rates of pregnant females were between 93.3 and 100%, except for the group dosed on day 10. In this group, mortality of dams was 48%. The overall and post-implantation loss was increased by 393.3 and 472%, and the mean foetal weights were decreased by 37%.

I. A significant increase of the incidences of depressed ossification of the skull bones (44.8%), and abnormal ossification of the sternum were observed in rat foetuses. A total absence of ossification was found in 31%, and lack of ossification of xiphoid in 41.4%.

II. Application of 0.32 mg/kg bw during the pre implantation stage from day 1-7 caused an increased incidence of haemorrhages (198%). No further signs of toxicity were described. Foetuses showed predominantly damage of cerebral vessels. Intracerebral haematoma were found in 42.8%, whereas the incidence of structural malformations of the rear limbs (pes varus) was only 5.4%.

III. Following application of 0.32 mg/kg bw during the period of organogenesis from days 8-16, increased incidences of post implantation loss (551,8%), and overall embryonic mortality (525%) were noted, and foetuses showed an increased incidence (182.7%) for the development of the haemorrhagic syndrome (haematoma and haemangioma).

Profound teratogenic effects such as increased incidences of structural malformations of the rear limbs (pes varus), internal hydrocephalus, intracerbral haematomas, massive haemorrhages into the abdominal cavity and delayed ossification of the parietal skull bones were found.

IV. Daily application of 0.32 and 0.16 mg/kg bw during the entire gestation period from day 1-21 resulted in a statistically significant increase of the total embryonic mortality (725.5 and 388.8%) in comparison to control. The post implantation loss was increased by 1074 and 501.8% for these dose levels, respectively. This dose-related effect was combined with the development of the haemorrhagic syndrome. Subcutaneous haematoma at different parts of the trunk and extremities, and haemangioma around the great vessels and the neck regions were observed. The incidence increased the rates of spontaneous occurrence and the control incidence by 193.8% for the 0.32 mg/kg bw dose, and 123.8% for the 0.16 mg/kg dose level.

In foetuses, significant increased incidences of structural malformations of the rear limbs (pes varus), internal hydrocephalus, intracerebral haematoma, and massive haemorrhages into the abdominal cavity were found. At 0.32 mg/kg and 0.16 mg/kg, the incidence of delayed ossification of the parietal skull bones was increased statistically significantly by 21.6 and 15.7%, respectively.

Following application of 0.32, 0.16 and 0.08 mg/kg bw, changes of biochemical parameters in livers of foetuses were observed. The activity of the cytochrome-oxidase ($P=0.818$, $p<0.01$) and succinate-dehydrogenase ($P=0.301$) was decreased. At 0.32 and 0.16 mg/kg bw, the activities of lactate-dehydrogenase ($P=0.956$, $p<0.05$) and glucose-6-phosphat-dehydrogenase ($P=0.980$, $p<0.02$) were statistically significantly increased. ATP and the amount of soluble proteins were significantly decreased in all dose level compared to control.

Conclusion:
The embrytoxic and teratogenic effects of the test compound warfarin resulted from the direct action of metabolites on embryonic cells following transplacental transfer. A certain role in the

pathogenesis is probably related to the circulatory changes and vasotoxic effects of warfarin that resulted in thrombosis of foetal blood vessels followed by tissue necrosis. The structural malformations of the limbs were explained by the formation of spot chondrodystrophy of the calcaneal epiphysis.

Reviewer's conclusion:
This submission is a translation of an (apparently) Russian language paper. There was very limited reporting of data in the original paper, none for most parameters. The test substance purity is unknown, other than that it is a technical pure a.s. The data can be considered as limited at best, but can be used to extract some general information.

-A single dose of 8 mg/kg/day on days 10, 11, 12, 13, 14, and 17 was highly toxic, causing up to 100% mortality, except on day 10 (48%). Foetal observations where mortality is so high are not meaningful.

-The incidence of intra-cerebral haematoma was increased in foetuses following administration of 0.32 mg/kg/day from days 1-7. The relevance of the data on malformations of the hind-limbs cannot be evaluated because of the lack of documentation of incidence data and the lack of background data.

-0.32 mg/kg/day administered from days 8-16 caused greatly increased embryonic loss, increased incidence of haemorrhage and greatly increased incidences of structural malformations. There was no information on maternal effects at this dose level. When the a.s. was administered throughout gestation, the same effects on the foetus were seen at ≥ 0.16 mg/kg/day. There was no information on maternal effects at this dose level. As the data were not reported, it is not possible to make any evaluation of the effects seen/not seen at doses lower than 0.16 mg/kg/day.

- 0.32 mg/kg/day from days 1-7, 8-16 or 10-21 of gestation have profound adverse effects on the foetus. 0.16 mg/kg/day caused similar effects when administered from days 1-21 of gestation. The maternal effects are not known at these dose levels.

6.9.2.1b
Howe, A.M.; Webster, W.S. (1992): The Warfarin embryopathy: A rat model showing maxillonasal hypoplasia and other skeletal disturbances. Teratology 46, 379-390.

ABSTRACT:
Sprague-Dawley rats were given daily subcutaneous doses of sodium warfarin (100 mgikg) and vitamin K1 (10 mgikg) for up to 12 weeks, starting on the day after birth. This dosing regimen creates an extrahepatic vitamin K deficiency while preserving the vitamin K-dependent processes of the liver. Control rats received either vitamin K1 only or were untreated. All rats survived without any signs of hemorrhage. The warfarin-treated rats developed a marked maxillonasal hypoplasia associated with a 11-13% reduction in the length of the nasal bones compared with controls. The length of the posterior part of the skull was not significantly different from controls. In the warfarin-treated rats, the septal cartilage of the nasal septum showed large areas of calcification, not present in controls, and abnormal calcium bridges in the epiphyseal cartilages of the vertebrae and long bones. The ectopic calcification in the septal cartilage may have been the cause of the reduced longitudinal growth of the nasal septum and the associated maxillonasal hypoplasia.
It is proposed that (1) the facial features of the human warfarin embryopathy are caused by reduced growth of the embryonic nasal septum, and the septal growth retardation occurs

because the warfarin-induced extrahepatic vitamin K deficiency prevents the normal formation of the vitamin K-dependent matrix gla protein in the embryo.

Guidelines: Non-guideline
GLP: No
Material and methods: This study was designed to investigate the developmental toxicity of warfarin, which in anticoagulant therapy in humans during the first trimester of pregnancy is known to cause various degrees of nasal hypoplasia and other anomalies known as Warfarin embryopathy. However, conventional studies in pregnant mice, rats or rabbits were not considered feasible since there appears to be a very narrow margin between the no-effect dose for the conceptus and the maternal lethal dose. Thus, in this investigation, the post-natal developmental toxicity of Warfarin was studied by dosing (new born) rats (sub-cutaneous injection) on post-natal day 1 with Warfarin in combination with Vitamin K. Thus, the extrahepatic Vitamin K deficiency induced by Warfarin is maintained, whereas Vitamin-K-dependant processes of the liver are not disturbed.
Test material: Warfarin, sodium-salt (Boots Company, North Rocks, Sydney); Batch: no data; Purity: no data; Species: Sprague-Dawley rats; No. of animals: a total of 13 litters (5 males and 5 females if possible); Administration: subcutaneous injection; Dose level: Group 1: 100 mg/kg Warfarin + 10 mg/kg Vitamin K1, Group 2: 10 mg/kg Vit. K1; Vehicle: distilled water; Controls: untreated litters; Duration of treatment: 12 weeks; Sacrifice: at various times throughout the study.

Group 1 (six litters) were given daily s.c. injections of Warfarin and Vit. K1, and the dams were also treated with Vit. K1 (10 mg/kg) to prevent haemorrhages from Warfarin ingestion by coprophagy. 11 males and 12 females from these litters were treated for 12 weeks until the final sacrifice.

Group 2 (three litters) were treated only with Vit. K1, and 11 males and 10 females were subjected to the final sacrifice.

Group 3 (four litters) served as untreated control. 13 males and 14 females were sacrificed upon study termination.
Findings:
All rats survived without any signs of haemorrhage. For Warfarin treated males and females, there was a statistically significant reduction in tail length (12-17%), nasal length (7-13%), overall length (6-12%) and weight (7-13%) upon study termination (week 12). The snout of these animals was shorter and broader, and the pinnae of the ears were reduced in size. These features were particularly evident after 3 weeks of treatment. The growth parameters upon study termination are presented in Table 7.9.2.1.2b-1.

Table 6.9.2.1b-1: Growth measurements in male and female rats upon study termination (week 12)

Group	Sex	No. of litters	No. of rats	Weight [g]	Body length [mm]	Tail length [mm]	Nasal length [mm]
Warfarin	male	6	11	311.1 ± 20.2[1,2]	380 ± 11.8[2,3]	168.5 ± 8.1[2,3]	21.4 ± 1.2[2,3]
Vit. K1	male	3	11	334.6 ± 22.8	406.8 ± 9.0[2]	190.9 ± 8.6[2]	23.8 ± 0.6
Control	male	4	13	344.8± 34.2	424.0 ± 18.3	201.8 ± 9.6	24.5 ± 0.8

Warfarin	female	6	12	207.3 ± 18.1[3]	338.8 ± 14.8[3,2]	152.8 ± 9.4[3,2]	20.8 ± 1.0[3,2]
Vit. K1	female	3	10	237.4 ± 37.2[4]	380.5 ± 9.0	184.5 ± 7.6	22.7 ± 0.5
Control	female	4	14	224.2 ± 23.0	384.3 ± 13.9	186.0 ± 8.2	22.4 ± 0.6

1) significantly different from Vit. K group ($p<0.05$)
2) significantly different from untreated group ($p<0.01$)
3) significantly different from Vit. K group ($p<0.01$)
4) significantly different from untreated group ($p<0.05$)

Warfarin treatment had a differential effect on the growth of various skull bones (skull length reduced by 5-6% in male and 5% in females). The results of measurements of alizarin-stained skulls after 12 weeks of treatment are presented in Table 7.9.2.1.2b-2, the anterior third of the skull was most affected. The results of forelimb bone length in Table 7.9.2.1.2b -3. The bones from treated rats were slightly shorter (4-5% reduction in both sexes) than controls.

Table 6.9.2.1b-2 : Skull measurements [mm] upon study termination (week12)

	Males			**Females**		
	Warfarin	**Vit. K1**	**Control**	**Warfarin**	**Vit. K1**	**Control**
No of rats	11	11	13	12	10	14
Skull length	42.7 ± 1.0[1,2]	45.2 ± 0.9	45.5 ± 1.8	41.3 ± 1.2[1,2]	43.6 ± 1.4	43.3 ± 1.6
Nasal bone length	15.2 ± 1.1[1,2]	17.5 ± 0.6	17.3 ± 0.6	14.6 ± 0.7[1,2]	17.0 ± 0.7	17.0 ± 0.6
Frontal bone length	13.2 ± 0.7[3]	13.8 ± 0.6	13.5 ± 0.6	12.4 ± 0.5[1,4]	13.1 ± 0.4	12.8 ± 0.5
Parietal bone length	7.8 ± 0.8	7.6 ± 0.6	7.7 ± 0.7	7.5 ± 0.3	7.1 ± 0.7	7.4 ± 0.4
Interparietal bone length	6.3 ± 0.6[4]	6.7 ± 0.4	6.8 ± 0.5	6.3 ± 0.3	6.4 ± 0.6	6.6 ± 0.6
Premaxilla length	10.7 ± 0.5	11.5 ± 0.4	11.5 ± 1.6	10.4 ± 0.6[3]	11.1 ± 0.5	10.9 ± 1.0
Maxilla length	15.9 ± 1.2[1,4]	17.7 ± 0.7	17.2 ± 1.7	14.9 ± 1.5[1,4]	17.0 ± 0.7[4]	15.9 ± 0.7
Mandibular length	24.0 ± 0.9	24.5 ± 1.1	24.8 ± 1.3	24.1 ± 1.0[4]	23.5 ± 0.9	23.7 ± 1.4
Bizygamatic width	23.1 ± 0.8	23.3 ± 0.5	23.5 ± 0.7	22.0 ± 0.8	22.6 ± 0.8	22.3 ± 0.6
Width of snout (max.)	8.5 ± 1.0[2,3]	9.3 ± 0.8	9.7 ± 0.4	8.4 ± 0.4[2,3]	8.9 ± 0.4	9.1 ± 0.6
Transfrontal width (min.)	6.5 ± 0.2[1,2]	6.9 ± 0.3	6.9 ± 0.3	6.4 ± 0.3[4]	6.6 ± 0.3	6.7 ± 0.3
Facial height	13.4 ± 0.4[1,2]	14.0 ± 0.5	14.1 ± 0.3	12.8 ±	13.4 ± 0.3	13.1 ± 0.4

				0.3[1,4]		
Max. nasal height	8.1 ± 0.4[4]	8.5 ± 0.5	8.7 ± 0.7	7.9 ± 0.3[3]	8.3 ± 0.4	8.0 ± 0.5

1) significantly different from Vit. K group ($p<0.01$)
2) significantly different from untreated group ($p<0.01$)
3) significantly different from Vit. K group ($p<0.05$)
4) significantly different from untreated group ($p<0.05$)

Table 6.9.2.1.b-3: Forelimb bone length [mm] upon study termination (week 12)

Group	**No.**	Sex	**Scapula**	**Humerus**	**Ulna**	**Meta-carpal**	**Prox. phalanx**	**Middle phalanx**	**Distal phalanx**
Warfarin	11	male	25.3 ± 1.0[1]	25.8 ±0.6[1,2]	30.2 ±0.6[3,4]	8.2 ± 0.7[3,4]	5.2 ± 0.2[1,3]	2.8 ± 0.1[3]	2.7 ± 0.1
Vit. K1	11	male	25.8 ± 1.2	27.0 ± 0.5	31.6 ± 0.6	8.5 ± 0.2	5.6 ± 0.2	3.0 ± 0.1[4]	2.7 ± 0.2
Control	13	male	26.8 ± 1.5	26.9 ± 1.5	31.3 ± 0.9	8.5 ± 0.3	5.5 ± 0.3	2.9 ± 0.2	2.7 ± 0.1
Warfarin	12	female	23.4 ± 1.2	24.0 ±0.8[3,4]	28.2 ±1.7[3,4]	7.9 ± 0.2[3,1]	5.2 ± 0.3	2.6 ± 0.1[3,4]	2.5 ± 0.1[2]
Vit. K1	10	female	24.4 ± 0.9	25.2 ± 0.4[3]	30.6 ± 0.4	8.2 ± 0.2	5.3 ± 0.3	2.8 ± 0.1	2.6 ± 0.1
Control	14	female	24.3 ± 1.3	25.0 ± 0.7	30.5 ± 0.8	8.1 ± 0.3	5.2 ± 0.2	2.8 ± 0.1	2.5 ± 0.2

1) significantly different from untreated group ($p<0.05$)
2) significantly different from Vit. K group ($p<0.05$)
3) significantly different from Vit. K group ($p<0.01$)
4) significantly different from untreated group ($p<0.01$)

The alizarin-stained nasal septa from Vit. K1 and control rats did not show evidence of calcification in the septal cartilage. In contrast, all septal cartilages from Warfarin-treated rats showed extensive areas of calcification. The calcification appeared 2 weeks after the start of Warfarin administration, and increased progressively during the following weeks. This calcification remained visible up to 15 months after cessation of treatment. There were no abnormal calcifications in the limbs or axial skeleton that might correspond to the "stipplings" described in the human Warfarin embryopathy. The growth plates from the femur and tail vertebrae showed many calcium bridges which transverse the growth plate from postnatal day 10 onwards. Similar structures were not seen in the controls. The primary and secondary ossification centres appeared to be normal.

Study Conclusion:
Under the conditions of this test, post-natal Warfarin treatment (100 mg/kg bw) in combination with Vit. K1 (10 mg/kg bw) induced extrahepatic Vit. K deficiency in the neonatal rat which caused differential growth retardation of the developing skull resulting in maxillonasal hypoplasia, calcium deposits in the cartilage of the nasal septum, and calcium bridges in the epiphyseal cartilage of vertebrae and long bones. These findings indicate a generalised disturbance in the maintenance of uncalcified cartilage. The authors concluded that it was unclear whether calcification of the nasal septum is the cause of maxillonasal hypoplasia, by reducing the longitudinal growth of the septum, or if they were the result of another more fundamental disturbance of the chondrocytes.

Reviewer's conclusion:
The study appeared to be carried out to a good standard and the data well reported. It is considered to be acceptable. The test substance purity was not reported (not known). This was a hypothesis testing study in which the apparent lack of teratogenicity of warfarin (nasal hypoplasia and bone stippling) in animal models was explored. It was proposed that the reason for the difference in response may relate to the critical periods for nasal and skeletal development, which are prenatal (during the first trimester) in man and occur during late foetal and early postnatal life in the rat. The study clearly demonstrated a post-natal warfarin induced effect on nasal cartilage and facial/skull bone growth, not dissimilar to the human embryopathy but significantly less marked. This effect may be linked to the vitamin K-dependent bone protein-matrix gla (γ-carboxyglutamic acid) protein (MGP). This protein is synthesised in the growth plate cartilage and has a role in prevention of calcification. In the presence of warfarin, the MGP remains decarboxylated and therefore unable to prevent calcification of the cartilage. The study provided evidence that abnormal calcification of the nasal septum may be the underlying cause of the warfarin embryopathy.

6.9.2.1.c
Feteih, R. et al. (1990): Effect of Sodium Warfarin on Vitamin K-dependent proteins and skeletal development in the rat foetus, Journal of Bone and Mineral Research 5 (8), 885-894.

Guidelines: Not presented
GLP: No

Abstract
Sodium warfarin was administered daily to Sprague-Dawley rats from gestational day 8 to day 22 to examine the effects of this compound on the developing fetal skeleton and on the vitamin K-dependent bone and cartilage proteins. At a dose of 175 µkg of sodium warfarin there was a 43% mortality rate among the dams. Maternal prothrombin times and serum osteocalcin levels were slightly elevated but not significantly. In the surviving litters, fetal bone osteocalcin and y-carboxyglutamic acid were significantly reduced (50 and 57%, respectively, on gestational day 22) when compared to age- and/or weight-matched control pups. The high correlation of osteocalcin content in long bone (R = 0.64) and calvariae (R = 0.77) to fetal body weight observed in control fetuses was not seen in the warfarin-exposed pups. Examination of alizarinstained warfarin-exposed fetal skeletons for ossification centers showed no difference from controls. However, analysis of the tibia1 growth showed several changes compared to control that included (1) widened hypertrophic zones, (2) increased calcification of the hypertrophic zones, and (3) disorganization of the hypertrophic cells. These results suggest that the growth plate abnormalities seen with prenatal warfarin exposure relate to the inhibition of the vitamin K-dependent proteins of the skeletal system.

Note: Two vitamin K-dependent proteins have been characterized in the skeleton. Osteocalcin (bone Gla protein) is a 3 Gla residues per 5700 MW protein associated with hydroxyapatite crystals in the extracellular matrix and is present in bone in proportion to the mineral. In newborn rat pups, osteocalcin is about 0.2% of bone dry weight and increases to 2% in the adult rat skeleton,") accounting for one of the major noncollagenous proteins. A second vitamin K- dependent protein, the matrix Gla protein, is a 5 Gla residues per 11 ,000 MW protein that predominates in embryonic bone and cartilage extracellular matrix but is also synthesized by lung, heart, and kidney. Its presence in bone is not correlated to mineral composition but remains at a constant level (- 0.4 mg/g bone) in adult bone.

Material and methods: The purpose of this study was to investigate the effects of Warfarin (sodium warfarin, EndoLabs, Wilmington, DE) administered to rats on gestational days 8-22 on the developing foetal skeleton and on vitamin-K-dependant bone and cartilage proteins. Pregnant Sprague-Dawley rats received daily subcutaneous doses of 175 μg/kg bw of Sodium Warfarin on days 8 -to 22 of gestation. Control animals received physiological saline. Litters were examined on days 20, 21 and 22, the number of foetuses and resorptions was recorded, and each foetus was examined for gross abnormalities. Individual maternal and foetal blood samples were assayed for prothrombin time and osteocalcin levels. Foetuses were further subjected to whole skeletal, biochemical or histological evaluation at random.

Findings

Sodium Warfarin administration at a dose of 175 g/μkg bw/day to Sprague-Dawley rats on gestational days 8-22 led to a mortality of 43% among dams, whereby maternal prothrombin times were only slightly (but not significantly) elevated.

Mean litter size and fetal weights, although reduced for warfarin-exposed animals, were not significantly different from controls. The mean numbers of resorptions were also not significantly different from control litters. There was also no significant difference in the numbers of ossification centers examined between controls and warfarin- exposed foetuses. First analysis of craniofacial dimensions showed significant decreases in measures of mandibular length and depth and maxillary length, but when these proportions were adjusted for foetal body weight no significant differences were found.

Analysis of tibial growth of warfarin treated-foetuses showed changes such as widened hypertrophic zones, increased calcification of these zones and disorganisation of the hypertrophic chondrocytes, suggesting that the growth plate abnormalities seen with prenatal warfarin exposure relate to the inhibition of vitamin-K-dependant proteins of the skeletal system.

The morphologic defects in the development of bone were associated with biochemical effects of warfarin on the skeleton as seen by analysis of the bones for y-carboxyglutamic acid (Gla) and osteocalcin in both calvariae and long bones. On gestational day (GD) 21, Gla was decreased from 46 to 53% based on the number of residues per 10^3 glutamic acid. When Gla concentration was normalised to bone dry weight (Table 4) the decrease was even greater (65-67% of control). In contrast to serum osteocalcin values, which were not statistically different in the two groups , osteocalcin levels in calvariae of warfarin-exposed pups were decreased 23-43% by day 22. In long bones of these foetuses, osteocalcin was decreased from 25 to 50% (Table 5). The osteocalcin concentration in both long bone and calvariae was highly correlated to the foetal body weight of controls only (Table 3).

Table 1. Summary of levels of Gla in long bone and Calvaria (mean ± SE *(n)*)

Day	*Long bone*		*Calvaria*	
	*Gla residues/1000glu**	*Gla NM/mg*	*Gla residues/1000glu*	*Gla NM/mg*
20 Control	1.80 ± 0.22 (7)	0.34 ± 0.08 (7)	1.05 ± 0.13 (7)	0.28 ± 0.07
21 Control Warfarin-treated *% reduction*	2.21 ± 0.21 (16) 1.27 ± 0.09 (26) *43.9*	0.35 ± 0.03 (16) 0.12 ± 0.04 (25) *65*	1.34 ± 0.06 (16) 0.62 ± 0.01 (15) *53.8*	0.31 ± 0.01 (16) 0.10 ± 0.02 (14) *67*
22 Control	2.40 ± 0.09 (17)	0.28 ± 0.05 (17)	1.50 ± 0.08 (12)	0.38 ± 0.04 (12)

Warfarin-treated *% reduction*	1.29 ± 0.14 (7) *46.1*	0.18 ± 0.03 (7) *52*	0.81 ± 0.11 (8) *46*	0.16 ± 0.04 (8) 57

*gla residues per 1000 glutamic acid residues

Table 2 Summary of levels of osteocalcin in long bones and calvaria

Day	*Long bones* [a]	*Calvaria* [b]
20 control	2.84 ±0.5	6.70± 0.76 (9)
21 control Warfarin	6.08 ±0.41 (16) 4.20 ±0.91 (24)	13 ± 0.80 (16) 10 ± 0.71 (15)
% reduction of control	31.0	23.1
22 Control Warfarin	20.32 ± 1.43 (17) 9.97 ±2.12 (8)[a]	40.13± 1.95 (17) 22.83 ± 2.71 (9)[a]
% reduction of control	50.0	43.1

[a] $p < 0.001$ compared to controls

[b] ng/mg bone dry weight (LS mean SE) (*n*)

Table 3 Correlation of body weight and level of osteocalcin in bone for control (C) and warfarin exposed (W) foetuses

Group	Age	Weight (gm)	Osteocalcin (long bone) ng/mg dry weight	Coerrelation coefficient	Osteocalcin (calvaria) ng/mg dry wt	Correlation coefficient
C	GD 20	2.54±0.1	2.84 ±0.5	0.99	6.70 ±0.76	0.77
C	GD 22	5.46±0.4	20.32± 1.4	0.64	40.13± 1.96	0.64
W	GD 22	5.22±0.2	9.97 ±2.1	0.19	22.8 ± 2.7	0.19

Since foetal bones contain two known vitamin K-dependent proteins, a calculation was carried out (Table 4) to determine quantitatively how much Gla in foetal bone is accounted for by the presence of immunoreactive osteocalcin. Table 4 shows that, at most, 3% of the Gla content in foetal long bone and 6% of the Gla content of foetal calvariae can be accounted for by the presence of osteocalcin. A large portion of the remainder of Gla-containing protein is likely to be the matrix Gla protein, although as yet unidentified proteins may also be present. Since matrix Gla protein is present in much greater quantities than osteocalcin in embryonic bone and cartilage extracellular matrix, the large reduction in Gla content of the warfarin-exposed bones suggests that this protein was inhibited by the prenatal warfarin exposure.

Table 4 Calculated estimation of % Gla on osteocalcin in long bones and calvaria of the control rat foetus

Total Gla*[a] *Nmoles/mg bone* *X ± SD		***Theoretical amount*[b] *of Gla in osteocalcin* *nmoles***	
Long bones			
GD 20	**0.34 ± 0.08**	**0.0015**	**0.44**
GD 21	**0.35 ± 0.03**	**0.0032**	**0.91**
GD 22	**0.38 ± 0.05**	**0.01.7**	**2.81**

Calvaria			
GD 20	**0.28 ± 0.07**	**0.0035**	**1.26**
GD 21	**0.31 ± 0.01**	**0.0068**	**2.21**
GD 22	**0.38± 0.04**	**0.0211**	**5.56**

[a]Total Gla measured after alkaline hydrolysis

[b]nmoles Gla = ostelcalcin ng/5700 x 3 (Gla res/molecule). Osteocalcin was measured by RIA (Table 5)

Reviewer's conclusion:
The pharmacological action of warfarin involves inhibition of vitamin K-dependent synthesis of γ-carboxyglutamic (Gla) residues in proteins of the liver, bone and other tissues. Two vitamin K-dependent proteins have been characterised in the skeleton, i.e., osteocalcin, which is associated with hydroxyapatite crystals in the extracellular matrix, and matrix Gla protein that predominates in embryonic bone and cartilage extracellular matrix. In the present study, the effects of prenatal treatment with warfarin on bone histology and morphology, and associated biochemical effects (levels of osteocalcin and Gla protein) were investigated in the rat.

Mean litter size and foetal weights were reduced compared to controls (not statistically significant). The mean number of resorptions was not significantly increased. There was no significant difference in ossification centres in warfarin-treated foetuses. Apparent differences in facial dimensions (mandibular length and depth, maxillary length) were not significant when adjusted for foetal body weight. The results clearly demonstrate and adverse effect on the hypertrophic region of the long bones, with marked disruption of the columnar arrangement of the hypertrophic chondrocytes. These morphological defects were correlated with biochemical effects on the skeleton with marked reductions in matrix Gla protein in calvariae and long bones (by up to 53%). This data suggests that decreased synthesis of this protein in response to warfarin exposure may account for bone abnormalities.

The dose causing effects on the foetus in this study was 175 µg/kg b.w. At this dose, there was 43% maternal mortality. While the cause of death is not actually stated, it must be assumed that haemorrhage was involved. Maternal prothrombin times were not affected at this dose, except for a single dam whose prothrombin times were increased by 1.6 times normal. The litter from this dam were most profoundly affected by adverse bone change.

6.9.2.1.d
Kronick, J. et al. (1974): Effects of Sodium Warfarin administered during pregnancy in mice, Am. J. Obstet. Gynecol. 118 (6), 819-823.

Guidelines: Not presented
GLP: No
Material and methods: Test design: The purpose of this study was to investigate teratogenic and foetotoxic effects in mice. Virgin mice (F1 generation derived from crossing C3H and A/J strains) were caged with males overnight. Upon detection of vaginal plugs, Warfarin sodium (salt)(Coumadin drug) was administered i.p. at doses of 1, 2, 3 and 4 mg/kg bw/day at various stages of pregnancy. Control animals received physiological saline or distilled. water. Mice were sacrificed at various intervals following treatment, and uterine contents were preserved for later inspection, plus withdrawal of blood samples (prothrombin assay).
Findings:
In groups treated from days 3-11 of gestation with 2 and 4 mg/kg bw/day, there was a very high incidence of haemorrhaged placentae and foetal deaths (including both dead and resorbed foetuses).

These doses of Warfarin prolonged the prothrombin time by 3.5-5 times the control at 24 hours after the final injection. In contrast, there was no evidence of haemorrhaged placentae and no significant increase in either prothrombin time or foetal deaths in animals treated with 1 mg/kg bw/day. In addition, none of the doses employed in this study lead to an increase of the frequency of malformations. However, the authors conclude that this may also be due to the high incidence of severely haemorrhaged placentae which subsequently could have resulted in early foetal death, and so obscured any embryotoxic effect of Warfarin. For this reason, the effects of single doses of Warfarin were investigated.

Table 6.9.2.1de-1 Effects of sodium warfarin administered to pregnant mice from days 3-11 of gestation (taken from Kronick et. al., 1974)

Treatment group							
days	Dose mg/kg/day	No. pregnant	No. implantations	Haemorrhaged placentas	Foetal deaths (%)	Mat. deaths (%)	Mean prothrombin time
3-11	4	14	92	92.7	92.7	14.3	48.9
3-11	2	8	66	100	71.2	12.5	33.6
3-11	1	11	88	0	13.6	0	10.4
3-11	0	10	88	0	4.5	0	10.1

Single injections of 4 mg/kg from days 5-14 of gestation showed a significant increase of foetal deaths for an administration on days 10 and 11 compared to control. No foetal or placental haemorrhages were observed in this series, and there was only a low incidence of gross foetal malformations (all of which were cleft lip and/or cleft palate). Mean prothrombin times 24 hours after Warfarin administration were elevated by a factor of 2.0 – 3.3 compared to control.

Single doses of 1, 2, 3 and 4 mg/kg were administered on either one of the gestation days 8 through 11 (period of organogenesis), and all animals were sacrificed on day 18. No incidence of foetal or placental haemorrhage was observed. Statistical analysis revealed that there was significant linear regression of over-all foetal deaths on log dose, suggesting a dose-dependant increase of foetal mortality irrespective of the day of gestation.

Treatment group						
days	Dose mg/kg/day	No. litters	No. implantations	Foetal deaths (%)	Live and ext. malformed (%)	Mean prothrombin time
5	4	7	62	4.8	3.2	25.1
6	mg/kg/day	7	56	10.8	0	15.6
7	‘	7	56	1.8	0	20
8	‘	7	60	1.6	0	25.2
9	‘	7	56	9.1	0	24.4
10	‘	14	118	29.6*	2.5	32.6
11	‘	14	112	17.0**	2.7	25.6
12	‘	7	57	7.1	0	27.4
13	‘	7	62	11.3	0	23.8
14	‘	7	59	10.2	1.7	20.2
11	control	8	62	3.2	0	9.4

* $p < 0.001$, ** $p < 0.01$ compared to controls.

Day 10 was found to be the most sensitive day to warfarin induced embryotoxicity. Analysis of malformation data showed a significant difference between the Warfarin-treated and the control

groups. However, the low incidence of malformations is only suggestive of a teratogenic effect, and the majority of malformations were described as very minor (open eyelid, skeletal and ossification abnormalities.

Treatment; Day 10 (pooled groups)	No. of litters	Implantations	Dead/resorbed foetuses	Foetal deaths (%)
Warfarin, 4 mg/kg/day	31	278	70	25.2
Warfarin, 4 mg/kg/day + Vit K1	11	93	11	11.8
Control	9	84	7	8.3

Co-administration of 8 mg/kg Vitamin K together with 4 mg/kg Warfarin on day 10 of gestation prevented Warfarin-induced foetal death.

Conclusion.
It was concluded by the authors that the developmental effects of warfarin could be classified into three categories; foetal death associated with haemorrhaged placentas, foetal death not associated with haemorrhage and foetal malformation. The haemorrhaged placentaes were associated with high mortality rates and increased prothrombin times of 3.5 to 5 times controls.

6.9.2.1.e

In order to address the Specialised Experts' doubts (Specialised Experts, September 2006 Commission Doc ECBI/121/06) regarding the capability of OECD 414 compliant teratogenicity protocols to detect adverse effects on embryos and foetuses due to maternal exposure to Warfarin, an experimental study employing this test protocol was commissioned by the CEFICC Rodenticide Data Development Group ((RDDG) (Kubaszky, 2009). This study is reported below.

Study: **Teratogenicity Study of test item Warfarin sodium with Rats.**
Author: Kubaszky, R.
Date of Report: 21st May, 2009
Report identity: Report No.07/396-105P; BASF Id; 2009/1122956.
Testing facility: LAB Research Ltd., Szabadságpuszta, Hungary.
Test substance: Warfarin sodium technical, 98% (Batch GC922).
GLP: Yes.
Guidelines: OECD 414
Deviations: Additional high dose groups were added to ascertain maternal toxicity.
Acceptable: Yes.

Materials and Methods:

Teratogenic effects of Warfarin were investigated by oral administration (gavage) of Warfarin sodium (vehicle: carboxymethylcellulose) to groups of twenty-five pregnant female Wistar rats at dose levels of 0, 0.125, 0.150 or 0.200 mg/kg bw/day from either gestation day 6 to 15 (TP1, groups 1–4) or from g.d. 6 to 19 ((TP2, groups 5–8). Thus, dosing regimens following both the most recent and earlier test guideline versions are covered. Two further groups of twelve pregnant female Wistar rats, respectively, were dosed 0.250 mg/kg bw/day (g.d. 6–15, TP1, group 9, and g.d. 6–19, TP2, group 10). The extra groups at 0.250 mg/kg bw/day were added after the start of the study to demonstrate clear maternal toxicity following treatment with Warfarin sodium.

All surviving animals were terminated on gestation day 20. Records of body weight, food consumption and clinical signs were maintained in life and at termination the maternal rats were subject to Caesarean section and examined macroscopically. Terminal investigations also included examination of ovaries to determine the number of corpora lutea and of gravid uterine weight. Each uterine horn was examined for live foetuses and stillbirths; implantations and foetal resorptions. Foetuses were weighed and examined for external malformations. Foetuses were preserved (ca. 50 %% for skeletal and ca. 50 % for visceral examination), sexed and examined for malformations, minor abnormalities and skeletal changes. Subcutaneous haemorrhages recorded during macroscopic examination of the dams or foetuses were photographed.

Results and discussion:

Maternal observations:

Mortalities (0, 0, 2, 2, 5) and clinical signs of toxicity occurred in the 0.150, 0.200 and 0.250 mg/kg TP 1 treatment regimen. 8 dams died or were sacrificed at 0.250 mg/kg TP 2 treatment regimen. The majority of mortalities (and sacrifices) occurred between gestation days 14 and 17 (one dam was sacrificed on g.d. 19). Clinical signs (pilo-erection, pallor, reduced activity, vaginal bleeding and an open vaginal orifice), death and morbidity were considered to be treatment-related and consistent with the pharmacological action of the substance.

Body weight and body weight gain for surviving dams, gravid uterine weight, or the corrected body-weight, or corrected bodyweight gain, were not affected by treatment at any dose level and treatment regimen.

Necropsy revealed gross pathological changes including blood filled uterus (all dams found dead or sacrificed, 9 in TP1 and 9 in TP2, also in 4 and one surviving dams in TP1 and TP2, respectively), blood stains around the vaginal orifice (8 females in each of TP1 and TP2, including one control female which showed several postembryonic deaths), intestinal bleeding (2 females TP1) and pale organs (5 females in TP1 and 2 in TP2).

Table 1. Maternal pregnancy data and mortalities (including sacrifices in extremis)

Dose groups	Control		0.125 Mg/kg		0.150 mg/kg		0.200 mg/kg		0.250 mg/kg	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
TP1										
No. sperm positive females	**25**		**25**		**25**		**25**		**12**	
No. dams with viable foetuses	18	**72**	22	**88**	21	**84**	18	**72**	6	**50**
No. of pregnant dams (including with no implantations but with corpora lutea)	21	**84**	22	**88**	23	**92**	23	**92**	11	**92**
No. of non-pregnant females	4	**16**	3	**12**	2	**8**	1	**4**	1	**8**
No. of dams with 5 or less implantations	0	**0**	1	**4**	0	**0**	0	**0**	0	**0**
Clinical signs **-piloerection** **-paleness** **-reduced activity** **-vaginal bleeding** **-open vaginal oriface**					 3 2 2 1 1		 4 3 4 4		 5* 5* 5* 6*	

Mortality due to toxicity	0	**0**	0	**0**	2	**8**	2	**8**	5	**42**
Euthanized due to toxicity	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
No. dams evaluated	20	**80**	20	**80**	21	**84**	21	**84**	11	**92**
TP2										
No. sperm positive females	25		25		25		25		12	
No. dams with viable foetuses	20	**80**	23	**92**	20	**80**	22	**88**	4	**33**
No. of pregnant dams (including with no implantations but with corpora lutea)	20	**80**	23	**92**	21	**84**	23	**92**	12	**100**
No. of non-pregnant females	5	**20**	2	**8**	4	**16**	2	**8**	0	**0**
No. of dams with 5 or less implantations	0	**0**	1	**4**	0	**0**	1	**4**	0	**0**
Clinical signs **-piloerection** **-paleness** **-reduced activity** **-vaginal bleeding** **-open vaginal oriface**					 1 1 1 1		 1 2 1		 8* 8* 8* 8*	
Mortality due to toxicity	0	**0**	0	**0**	0	**0**	0	**0**	5	**42**
Euthanized due to toxicity	0	**0**	0	**0**	1	**4**	0	**0**	3	**25**
No. dams evaluated	20	**80**	22	**88**	21	**84**	21	**84**	12	**100**

*statistically significant

There were no significant differences in the number of corpora lutea, pre- or post-implantation losses, number of implantations or the number of viable foetuses at any of the dose levels or treatment regimens. Mean foetal weights were similar in all groups and unaffected by Warfarin treatment.

Foetal investigations:

External examination

Placental changes attributable to Warfarin treatment were noted, namely statistically significantly increased incidences of greenish discoloured placenta at 0.150, 00.200 and 00.250 mg/kg bw/d in TP 1 and also increased at 0.150, 0.200 mg/kg with statistical significance at 0.250 mg/kg bw/d in TP2.

The incidence of foetal haemorrhages, external or visceral, was increased in all treatment groups in comparison with the controls. Since Warfarin is an anticoagulant, this effect was expected and consistent with the known characteristics of the test material.

Yellowish discolouration in the lens was recorded in one foetus at 0.200 mg//kg bw/d (TP1), one foetus of the 0.125 mg/kg TP2 group, two foetuses (1 litter) at 0.150 mg//kg bw/day and four foetuses (2 litters) in the 0.200 mg/kg bw/d TP2 groups. Samples of affected eyes were examined histologically and central cataract diagnosed in all eyes except the single 0.125 mg/kg TP2 foetus. This malformation is reported as very rare for Wister rats (not seen in >5000 foetuses in this test house and >17000 foetuses of Charles River data base) and so was attributed to an effect of treatment with Warfarin sodium by the study director. It is noted, however, that the statistical analysis was not performed on a per litter basis, which is considered more relevant, particularly in the case of a rare malformation. The litter incidence was included in the tabulated summary by the reviewer for information.

Any other variations and malformations were considered spontaneous and not related to treatment.

Note: A single litter (TP1, 0.150 mg/kg bw/d), which had been excluded from the statistical analysis due to uncertainty about the exact day 0 of gestation) had with four foetuses showing abnormally high bodyweights and facial skeletal malformations. 2/7 foetuses had malformed skulls with wide nasal and/or frontal bone/cartilage. One had unossified nasal bone, one had malformed vertebra and both had malformed sternum. The malformations were considered attributable to treatment by the study director. However, it is noted that this was a single mid dose litter with a number of malformed foetuses. There is no evidence of such effects in other litters at any dose levels. It must be concluded that relationship to treatment is equivocal, at best.

Table 2 Summary of foetal findings[1]

TP1					
Parameter	**0 mg/kg**	**0.125 mg/kg**	**0.150 mg/kg**	**0.200 mg/kg**	**0.250 mg/kg**
Litter data					
Number of litters examined	18	20	19	17	6
Number of foetuses examined	251	292	267	221	84
Preimplantation loss (%)	9	7	4	5	16
Postimplantation loss (%)	7	5	10	18*	3
-early embryonic death (%)	5	4	7	6	3
-late embryonic death (%)	3	1	3	11*	0
-total intrauterine death (%)	15	13	13	22	19
External examinations:					
No. of malformations (No. litters affected)	0 (0)	1 (1)	0(0)	0(0)	0 (0)
No. of variations (no. litters affected)					
Placental abnormalities:					
-greenish discolouration	3(1)	2(1)	12(4)*↑	37(6)**↑	5(1)*↑
-foetal haemorrhage (≥2, pinhead-sized) [2]	0(0)	7(7)*	9(6)**↑	6(5)**↑	2(1)*↑
Visceral examinations:					
Number of foetuses examined	108	129	117	99	37
-yellow discolouration of lens [3]	0 (0)	0 (0)	0 (0)	1 (1)	0(0)
Skeletal examinations:					
Number of foetuses examined (litters)	107(18)	124(18)	112(19)	89(16)	35(6)
No. of variations (foetuses affected)	28	38	38	25	7
No. malformations (foetuses affected)	2	5	6	3	1
TP2					
Litter data					
Number of litters examined	20	22	20	21	4
Number of foetuses examined	296	306	281	300	60
Preimplantation loss (%)	5	9	5	4	0
Postimplantation loss (%)	7	6	8	8	5

TP1					
External examinations:					
No. of malformations (No. litters affected)	1(1)	0(0)	0(0)	0(0)	0(0)
No. of variations (no. litters affected)	6(6)	7(5)	6(5)	6(5)	2(1)
Placental abnormalities:					
-greenish discolouration	11(4)	13(6)	16(5)	27(9)**↑	0(0)
-fibrinoid degenerated	20(4)	9(7)*↓	4(3)*↓	6(2)**↓	2(2)
-foetal haemorrhage (pinhead-sized) [2]					
-1	1(1)	12(8)**↑	8(5)*↑	4(4)	3(3)**↑
≥2	1(1)	3(3)	6(4)*↑	6(7)	2(2)*↑
Visceral examinations:					
Number of foetuses examined	129	134	124	132	26
-Great arteries (brachiocephalic trunk short or extremely short)[3]	2(2)	3(3)	2(1)	8(6)**↑	0(0)
-yellow discolouration of lens[3]	0(0)	1(1)	2(1)	4(3)*↑	0(0)
Skeletal examinations:					
Number of foetuses examined	126(20)	127(22)	116(20)	126(21)	7
No. of variations (foetuses affected)	45	27	21**↓	25**↓	13
No. malformations (foetuses affected)	8	4	1	6	8

[1] Data taken from the study data summary tables; Some parameters were analysed as mean foetal incidences without analysis of the litter incidence.
[2]quantitation of foetal haemorrhages is unclear *e.g.*, TP2/ 0.200 mg/kg /placental abnormalities/foetal haemorrhage/≥2 = 6(7) i.e., 6 foetuses from 7 litters.
[3] The litter numbers were inserted by the reviewer, statistical analysis was conducted on the foetal incidence data only.

There were no statistically significant or otherwise relevant effects on the skeletal development. The statistically significant reduction in skeletal variations seen at 0.150 and 0.200 mg/kg bw/day in the TP2 phase was not likely to be related to treatment. In the TP2, 0.250 mg/kg bw/d group, there were several cases of reduced skull bone ossification that were considered to be treatment-related by the notifier. It was suggested by the study director that a possible trend may have been present in differences in skull ossification (Table 3).

Table 3. Summary of skull ossification data

Group (mg/kg)	No. Foetuses	Normal	Whole skull	1 skull bone	2 skull bones	≥3 skull bones	Zyg.Inc.[1]	≥3 skull bones marked
Control TP1	**107**	**49 (46%)**	**13 (12%)**	**12 (11%)**	**14 (13%)**	**32(30%)**	**20 (19%)**	**11 (10%)**
200 TP1	**89**	**38 (43%)**	**9 (9%)**	**8(9%)**	**10(11%)**	**33(37%)**	**14(16%)**	**8 (9%)**
250 TP1	**35**	**26 (74%)**	**3 (9%)**	**1 (3%)**	**1(3%)**	**7(20%)**	**3(9%)**	**0**
Control TP2	**126**	**38 (30%)**	**34 (27%)**	**22 (17%)**	**15(12%)**	**51(40%)**	**33(26%)**	**11 (9%)**
200 TP2	**126**	**66 (52%)**	**11 (9%)**	**18 (14%)**	**17(13%)**	**25(20%)**	**13(10%)**	**3 (2%)**
250 TP2	**26**	**5 (19%)**	**7 (27%)**	**3 (12%)**	**2(8%)**	**16(62%)**	**7(27%)**	**5 (19%)**

[1] **incomplete ossification of *os zygomaticum.***

Incomplete ossification of the whole skull was statistically significantly ($p<0.01$) *increased* in the 0.125 and 0.150 mg/kg TP1 groups, when foetal data were analysed. Incomplete ossification of the whole skull was statistically significantly ($p<0.01$) *reduced* in the 0.150 and 0.200 mg/kg TP1 groups, when foetal data were analysed. The incidence of marked incomplete ossification of one bone of the skull was significantly higher ($p<0.01$) in TP2 at 0.250 mg/kg. Marked incomplete

ossification of more than one bone of the skull was higher (not statistically significant) in TP2 at 0.250 mg/kg, but significantly lower in the TP1/0.250 mg/kg group ($p<0.05$). The possibility that a trend may exist for lower percentage incidence of normal skull ossification and higher incidence of 3 or more skull bones with incomplete ossification in the TP2 groups was suggested by the study director.

However, there is no clear relationship between dose and effect when the TP1 and TP2 groups are examined. It should also be noted that the 0.250 mg/kg dose levels had 12 dams not 25 (of which 5 and 7 died/were euthanized in TP1 and TP2, respectively) therefore litter numbers are greatly reduced in both 0.250 mg/kg groups making comparison to other groups questionable.

Conclusions

Notifiers conclusion:

The present study shows that Warfarin induces a definite increase in the incidence of subcutaneous and internal foetal haemorrhage, and foetal ocular effects (central cataract, also a foetal effect due to Warfarin medication in humans). There are also some indications of disturbed ossification in skull bone at higher dose levels.

There was one litter of 7 foetuses from a TP1 dam at 150 mg/kg bw/d, where a combination of typical-sized (3 × ~3g) and abnormally large (44 × ~6g) foetuses were recorded. Pups are normally around or less than 6 g at birth (GG22). It is impossible to know what happened in this case: Interpretations include the female mating twice and having two ovulations or a single ovulation where not all eggs were fertilised at the same time. Another interpretation is that mating was normal and the large foetuses showed abnormal growth.

In conclusion, the recent teratogenicity study on Warfarin according to OECD guideline 414 produced treatment-related adverse foetal effects that are consistent with symptoms observed in infants born to Warfarin-treated women. It is therefore concluded that standard teratogenicity studies are capable off identifying the teratogenic potential of Warfarin.

Reviewers conclusion.

There were a number of substance-related deaths/ sacrifices *in extremis* in both TP1 (from 0.150 mg/kg) and TP2 (0.259 mg/kg). Other than significant clinical signs associated with the pharmacological/toxicological mechanism of action, there were no additional treatment-related effects on dams. Pregnancy-related parameters (number of corpora lutea, pre- or post-implantation losses, number of implantations or the number of viable foetuses) were not affected at any of the dose levels or treatment regimens in surviving dams. Mean litter weights were similar in all groups.

A number of placental changes related to Warfarin treatment were noted, namely statistically significantly increased incidences of greenish discoloured placenta at 0.150, 00.200 and 00.250 mg/kg bw/d in TP 1 and also increased at 0.150, 0.200 mg/kg with statistical significance at 0.250 mg/kg bw/d in TP2. Toxicity to the foetuses was seen in the form of haemorrhages of different sizes and numbers detected on external examination and seen at all dose levels.

Yellowish discolouration in the lens was recorded at 0.200 mg//kg bw/d (TP1) and from 0.125 mg/kg in the TP2 group. This was confirmed as central cataract on histological examination (except the single 0.125 mg/kg TP2 foetus). This malformation is reported as very rare for Wister rats (not seen in >5000 foetuses in this test house and >17000 foetuses of Charles River data base) and so was attributed to an effect of treatment with Warfarin sodium by the study director.

The possibility that a trend may exist for lower percentage incidence of normal skull ossification and higher incidence of 3 or more skull bones with incomplete ossification in the TP2 groups was

suggested by the study director. The evidence is equivocal, at best. There was no other clear treatment-related increase in external, visceral or skeletal variations and malformations in either group. All findings were considered spontaneous and not related to treatment.

Therefore, there was clear maternal toxicity from 0.0150 mg/kg. This was demonstrated as treatment-related clinical signs and mortalities. 0.125 mg/kg was an NOEL for maternal toxicity.

Treatment-related external haemorrhage was increased in foetuses at all dose levels. A treatment related increase in central cataract was seen from 0.125 mg/kg (TP1) and 0.200 mg/kg (TP2). The lack of this finding at the highest dose group may reflect the fact that a significant number of litters were lost through maternal deaths (5/12 and 8/12, respectively). There was no NOEL for teratogenicity (central cataract)

RAC evaluation of reproductive toxicity

The DS did not propose any change to the existing Annex VI classification of toxicity to reproduction of warfarin, noting as follows (p4 of CLP report). *"The developmental toxicity classification of Warfarin has been finalised and is not open for further discussion. Relevant background information on developmental toxicity data for Warfarin is included in this dossier to facilitate the discussion on read-across from this classification to the second generation rodenticides."* They did however propose Specific Concentration Limits (SCL). The proposed SCL is dealt with below, followed by an evaluation of the available data on the reproductive toxicity of warfarin in support of the aforementioned evaluations of 7 other anticoagulant rodenticides evaluated by RAC for toxicity to reproduction at the same time.

Specific concentration limits

Summary of the Dossier submitter's proposal

The DS proposed setting an SCL for reproductive toxicity according to the then Draft Guidance on the setting of concentration for reproductive toxicants with the CLP Regulation (Draft 2, Feb 2010).

Their argumentation was that Warfarin at doses of 2.5 mg/day (0.04 mg/kg bw/day, female bodyweight of 60kg) have been reported to result in nasal hypoplasia and vertebral stippling. Higher doses have resulted in a high percentage of embryofoetal mortality (A NOAEL cannot be set and the value of 0.04 mg/kg bw/day represents a LOAEL which in turn approximates to an ED_{10} value.

Based on the Guidance for Setting Specific Concentration Limits for Reproductive Toxicants within the CLP Regulation (EC/1272/2008), substances with an ED_{10} value less than or equal to 4 mg/kg bw/day in animal studies are considered as high potency substances. Warfarin is considered to have very high potency in terms of developmental toxicity simply because its LOAEL is approximately 2 orders of magnitude below the upper limit value for high potency classification. The general concentration limit (GCL, 0.3% w/w) is applied to all medium potency reproductive toxicants. For high potency substances, the ECHA Guidance for CLP has proposed an SCL of 0.03% w/w. Furthermore, extremely potent developmental toxicants with ED_{10} values deviating 10-fold or greater, below the upper limit value of 4 mg/kg bw/day must lead to a further revision of the SCL value. The high potency SCL must be reduced by a factor of 10 for each 10-fold disparity between the ED10 and the upper limit value of 4 mg/kg bw/day.

Warfarin has a LOAEL of 0.04 mg/kg bw/day which approximates to the ED_{10}. This value is about 2 orders of magnitude below the upper limit value for high potency classification. Based

on the draft guidance, a value of 0.03/100 = 0.0003% w/w is calculated for Warfarin. Any preparation containing Warfarin equal to or in excess of 0.0003% w/w shall be classified with respect to reproductive toxicity, Repr. 1A – H360D, i.e.

C ≥ 0.0003% Repr. 1A

Comments received during public consultation

Comments from industry disagreed with the proposed SCL (comments with detailed justification were submitted as a confidential attachment). Two MS agreed that the current classification of Warfarin as Repr. 1A (developmental toxicity) was appropriate

RAC Assessment and comparison with the classification criteria

Based on human data, doses of 2.5-5 mg/person/day (equivalent to 0.04-0.08 mg/kg/day) may cause developmental toxicity and could be regarded as an ED_{10} level. This human ED_{10} value would, if using the guidance for setting SCLs based on animal data, belong to the high potency group (< 4 mg/kg/day). The guidance states that for an ED_{10} < 4 mg/kg/day, the SCL is 0.03%, and for ED_{10} below 0.4 mg/kg/day the SCL becomes 0.003%. Also if starting from an ED_{10} value obtained from animal studies (0.125 mg/kg/day; Kubaszky et al. 2009), it would qualify Warfarin for the high potency group and this would result in a SCL of 0.003%. Thus, the RAC has concluded on a SCL on 0.003% for the developmental toxicity of Warfarin.

Data on reproduction toxicity in support of other anticoagulant rodenticide classifications

Summary of information supplied by the Dossier Submitter

Fertility

No multi-generation study data are available from published literature. However, the conduct of such studies is not considered feasible due to the particular sensitivity of the model species (rodents). For example, sodium Warfarin, administered at a dose of 175 µg/kg bw/day to Sprague-Dawley rats on gestational days 8-22 led to a mortality of 43% among dams (Feteih *et al*., 1990), which, for a 70 kg person would be calculated to equate to an exposure of 12 mg/day, which corresponds to a dosage within the usual therapeutic range (2.5 – 15 mg/day).

Warfarin did not show any effect on fertility after many years of human use or in a two generation reproduction study in rats with Vitamin-K supplementation. Therefore no classification has been proposed for fertility.

Developmental toxicity

NOTE: The classification for developmental toxicity of Warfarin is already harmonised and the substance has an entry in Annex VI of the CLP Regulation as toxic to reproduction, Repr. 1A; H360D. Harmonised classification for developmental toxicity of Warfarin was neither subject of the current DS proposal, nor of the RAC discussion. Relevant background information on developmental toxicity data for Warfarin was included in the CLH report in order to facilitate the weight of evidence assessment and the discussion on harmonised classification for developmental toxicity of other anticoagulant rodenticides and to allow for setting of specific concentration limits.

Developmental toxicity: Human evidence

Warfarin, which is also used as an oral anticoagulant drug in order to prevent formation of clots in the blood of people with mechanical heart valves or with deep vein thrombosis, has been found to cause death of embryos or foetuses and malformations, mainly nasal hypoplasia in humans. Since deformation of the naso-maxial part of the face is very specific, it is also referred

to as "Warfarin embryopathy".

The evidence that Warfarin has the intrinsic property to induce alterations of development in humans doesn't come from cohort or case-control studies, but mostly from clinical reports each describing one or a few cases with various effects on the health of neonates, abortions or stillbirths where pregnant women were treated with Warfarin as an anticoagulant drug.

The DS presented summaries of over 10 case reports of pregnancy outcomes in women treated with Warfarin as an oral anticoagulant as well as two reviews (Schardein, 1985; Hall et al., 1980) of case reports, in which the administration of Warfarin during pregnancy induced birth defects. The daily dose of Warfarin was usually between 5-10 mg/day.

Such exposure during the first trimester may cause a well-defined complex of malformations, with hypoplastic nose being the most characteristic feature. Bone abnormalities of the axial and appendicular skeleton (radiological stippling of the vertebral column) often also occur. Punctate calcification of other bone sites may also be present. Kyphoscoliosis, abnormal skull development, and brachydactyly have been observed as associated skeletal effects.

The risk of malformation to the foetus of a mother treated with Warfarin is not known with certainty. Schardein (1985) assessed the risk of malformation due to exposure to Warfarin during pregnancy as in the order of 1:5, but details of how this estimate was derived are not available. More recently, a review of the maternal and foetal risks (Chan, 2000) associated with oral anticoagulants indicated that the use of oral anticoagulants throughout pregnancy was associated with embryopathy in 6.4% (95% confidence interval [CI], 4.9% - 8.9%) of live births. Substitution with heparin at or prior to 6 weeks and up to 12 weeks was reported to remove this risk (Chan, 2000). In the more recent literature survey provided by industry (BASF, 2010) in support of the Flocoumafen CLH dossier, which has been summarised by the DS in the respective CLH report, the risk of embryopathy due to Warfarin treatment in sensitive periods of gestation is 4.3 %, relative to the number of pregnancies. This is in agreement with other authors, estimating the malformation risk to be "probably below 5 %" (De Swiet, 1987), or otherwise frequently in the range of 4–7 %, with some studies even reporting 0 % (Chan, Anand & Ginsberg, 2000; Hung & Rahimtoola, 2003; van Driel *et al*., 2002; Oakley, 1955; Hall, Pauli, & Wilson, 1980; Schaefer *et al*., 2006).

In addition to skeletal malformation there are other hazards caused by the toxic properties of Warfarin:

- spontaneous abortion (14 - 47 %, aggregated figure based on Blickstein && Blickstein, 2002; Chan, Anand & Ginsberg, 2000; Oakley & Doherty, 1976; Arnaout et al., 1998; Khamooshi et al., 2007; Shannon et al., 2008),
- neonatal death (1.4 – 4.5 %; Blickstein & Blickstein, 2002; Oakley & Doherty, 1976; Arnaout et al., 1998; Khamoshi et al., 2007), CNS defect (4.33 %; Hall, Pauli & Wilson, 1980; Oakley & Doherty, 1976),
- premature delivery (4.6 – 13.9 %; Blickstein & Blickstein, 2002; Hall, Pauli, & Wilson, 1980),
- ocular atrophy (Greaves, 1933; Hall, Pauli, & Wilson, 1980).

In summary, it is noted that oral administration of Warfarin leads to various developmental toxicity effects in humans, spontaneous abortion and stillbirth being the most frequent ones (ca. 27% of pregnancies), and naso-maxial hypoplasia being the most frequent malformation among alive births (ca. 5% of pregnancies). Substitution of Warfarin by Heparin during first trimester of pregnancy removes the risk of naso-maxial hypoplasia.

When compared with the classification criteria for developmental toxicity, the existing evidence in humans is sufficient for category Repr. 1A with hazard statement H360D: 'May damage the unborn child'.

Developmental toxicity: Animal evidence

The DS used 6 experimental studies to evaluate the developmental toxicity in animals: Mirkova & Antov, 1983; Howe & Webster, 1992; Feteih *et a*l. 1990 (containing 2 studies); Kronick *et al*., 1974 and Kubaszky, 2009).

The study of Kubaszky (2009) was carried out in accordance with the OECD TG 414 in order to address the doubts of Specialised Experts (September 2006 Commission Doc ECBI/121/06) regarding the capability of OECD 414 compliant teratogenicity protocols to detect adverse effects on embryos and foetuses due to maternal exposure to Warfarin.

Four other studies (Mirkova & Antov, 1983, Feteih *et al*., 1990 and Kronick *et al*., 1974) followed the general design of OECD 414, because the animals were exposed during various periods of pregnancy, but with considerable deviations in methodology.

A sixth study (Howe & Webster, 1992) did not follow the OECD 414 design, since treatment of mothers after parturition of offspring was applied in order to deliberately produce malformations of facial skull (mostly maxillonasal hypoplasia) resembling those being induced by Warfarin in humans.

Kubaszky, 2009

In the study of Kubaszky (2009), teratogenicity of Warfarin was investigated by oral administration (gavage) of Warfarin sodium (vehicle: carboxymethylcellulose) to groups of twenty-five pregnant female Wistar rats at dose levels of 0, 0.125, 0.150 or 0.200 mg/kg bw/day according to two test protocols (TP):

TP1, groups 1–4 - from gestation day (GD) 6 to 15

TP2, groups 5–8 - from GD 6 to 19

Two other groups of twelve pregnant female Wistar rats were dosed with 0.250 mg/kg bw/day (GD 6–15, TP1, group 9, and GD 6–19, TP2, group 10).

Thus, dosing regimens following both the most recent and earlier test guideline versions were covered.

Maternal mortality (0, 0, 2/25, 2/25, 5/12) and clinical signs of toxicity occurred in the 0.150, 0.200 and 0.250 mg/kg bw/day TP 1 treatment. In total, 8 dams died or were sacrificed at 0.250 mg/kg bw/day in the TP 2 treatment. The majority of mortalities (and sacrifices) occurred between gestation days 14 and 17 (one dam was sacrificed on GD 19). Clinical signs (piloerection, pallor, reduced activity, vaginal bleeding and an open vaginal orifice), death and morbidity were considered to be treatment-related and consistent with the pharmacological action of the substance. In the opinion of the DS, there was clear maternal toxicity from 0.0150 mg/kg bw/day (LOAEL). This was demonstrated as treatment-related clinical signs and mortalities. 0.125 mg/kg bw/day was an NOEL for maternal toxicity.

In the groups exposed from day 6 to day 21of gestation the mortality of dams was observed only at dose of 0.250 mg/kg bw/day but not at lower doses. Thus, severe maternal toxicity was observed at a dose of 0.250 mg/kg bw/day, mild or moderate maternal toxicity was observed at doses 150 and 0.200 mg/kg bw/day and no maternal toxicity was seen at dose of 0.0125 mg/kg bw/day.

A single litter (TP1, 0.150 mg/kg bw/day), which had been excluded from the statistical analysis due to uncertainty about the exact day 0 of gestation) had four foetuses showing abnormally high body weights and facial skeletal malformations. In total, 2/7 foetuses had malformed skulls with wide nasal and/or frontal bone/cartilage. One foetus had unossified nasal bone, while another one had malformed vertebra and both of them had malformed sternum. The malformations were considered attributable to treatment by the study director. However, it is noted that these foetuses were part of a single mid dose litter. There is no evidence of such effects in other litters at any dose levels. It must be concluded that a relationship with treatment is equivocal, at best.

Warfarin at a dose of 0.2 mg/kg bw/day given by gavage to female rats from day 6 to day 15 of

pregnancy caused a significant increase in late embryonic death (11% vs 3% in control females), increased number of dead foetuses (5 foetuses vs 0 in control group), increased intrauterine mortality (23% vs 16% in control group), increased percentage of post-implantation loss (19% vs 7% in the control group) and number of runts (6% at 0.2 mg/kg bw/day, 5% at 0.15mg/kg bw/day vs 2% in the control group. Warfarin given at 0.2 mg/kg bw/day by gavage to female rats from day 6 to day 19 of pregnancy (TP 2) caused an increase in bloody infiltration in viscera (21% vs 5% in control group). In addition, at doses of 0.125; 0.125 and 0.200 mg/kg bw/day Warfarin caused cataracts in 1 up to 4 animals per a dose group of ca. 200 foetuses. This malformation is reported as very rare for Wistar rats (not seen in >5000 foetuses in this test laboratory and >17 000 foetuses of Charles River data base) and so was attributed to an effect of treatment with Warfarin sodium by the study director.

Mean foetal weights were similar in all groups and were thus unaffected by Warfarin treatment.

There was an increase in incidence of foetal external haemorrhages in all treatment groups in comparison with the controls, but a clear steep dose-response relationship was not observed due to low dose increment. When dosed at 0.2 mg/kg bw/day Warfarin given by gavage to female rats from day 6 to day 19 of pregnancy (TP 2) caused an increase in bloody infiltration in viscera (21% vs 5% in the control group).

Mirkova & Antov, 1983

In the study of Mirkova & Antov (1983), the application of Warfarin at a dose of 0.32 mg/kg bw/day during the period of organogenesis from days 8-16, caused increased incidences of post implantation loss (551,8%), overall embryonic mortality (525%) and an increased incidence in foetuses (182.7%) of haematoma and haemangioma (foetal haemorrhagic syndrome). In addition, increased incidences of structural malformations of the rear limbs (*pes varus*), internal hydrocephalus, intracerebral haematomas, massive haemorrhages into the abdominal cavity and delayed ossification of the parietal skull bones were observed.

In the other experimental groups in the study of Mirkova and Antov (1983), a daily application of Warfarin at doses of 0.32 and 0.16 mg/kg bw during the entire gestation period from day 1-21 resulted in a statistically significant increase of the total embryonic mortality (725.5 and 388.8%, respectively) in comparison to the control. The post implantation loss was increased by 1074 and 501.8% for these dose levels, respectively. In foetuses, significant increased incidences of structural malformations of the rear limbs (*pes varus*), internal hydrocephalus, intracerebral haematoma, and massive haemorrhages into the abdominal cavity were found. At 0.32 mg/kg and 0.16 mg/kg, the incidences of delayed ossification of the parietal skull bones were increased statistically significantly by 21.6 and 15.7%, respectively.

There was also no information on maternal toxicity at the doses at which developmental effects were observed.

Kronick et al., (1974)

In the Kronick, *et al*. study (1974)_Warfarin sodium (salt) (Coumadin drug) was administered intraperitoneally (i.p.) at doses of 1, 2, 3 and 4 mg/kg bw/day at various stages of pregnancy in mice. Control animals received physiological saline or distilled water.

In female mice treated from days 3-11 of gestation with 2 and 4 mg/kg bw/day, there was a very high incidence of haemorrhaged placentae and foetal deaths (including both dead and resorbed foetuses). These doses of Warfarin prolonged the prothrombin time by 3.5-5 fold compared to controls at 24 hours after the final injection.

In contrast, there was no evidence of haemorrhaged placentae and no significant increase in either prothrombin time or foetal deaths in animals treated with 1 mg/kg bw/day. None of the doses administered in this study from 3 to 11 day of pregnancy led to an increase of the frequency of malformations, probably due to high foetal death rates.

In female mice treated with single daily i.p. injection of Warfarin at 4 mg/kg on day 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 of pregnancy there was an increase of foetal death rate in mice treated

on days 10 and 11 of pregnancy.

Co-administration of 8 mg/kg Vitamin K together with 4 mg/kg Warfarin on day 10 of gestation prevented Warfarin-induced foetal death. No foetal or placental haemorrhages were observed in this series, and there was only a low incidence of gross foetal malformations (all of which were cleft lip and/or cleft palate). Mean prothrombin times 24 hours after Warfarin administration were elevated by a factor of 2.0 – 3.3 compared to control.

In female mice given a single daily i.p. injection of Warfarin at a dose of 1, 2, 3 or 4 mg/kg on gestation day 8, or 9, or 10 or 11 (period of organogenesis) no increased incidence of foetal or placental haemorrhage was observed. Statistical analysis revealed that there was a significant linear regression of over-all foetal deaths on log dose, suggesting a dose-dependent increase of foetal mortality irrespective of the day of gestation. Analysis of malformation data showed a significant difference between the Warfarin-treated mice and the controls. However, low incidence of malformations is only suggestive of a teratogenic effect, and the majority of malformations were described as very minor (open eyelid, skeletal and ossification abnormalities).

Feteih et al., 1990

In the first study of Feteih, et al. (1990), sodium Warfarin was administered subcutaneously at a daily dose of 0.175 mg/kg bw to Sprague-Dawley rats from gestational day 8 to day 22 to examine the effects of this compound on the developing foetal skeleton and on the Vitamin K-dependent bone and cartilage proteins. At a dose of 0.175 g/mg bw/day, Warfarin induced significant maternal toxicity leading to a mortality of 43% among dams, whereby maternal prothrombin times were only slightly (but not significantly) elevated. Mean litter size and foetal weights, although reduced for Warfarin-exposed animals, were not significantly different from controls. The mean numbers of resorptions were also not significantly different from control litters.

There was also no significant difference in the numbers of ossification centers examined between controls and Warfarin-exposed foetuses. First analysis of craniofacial dimensions showed significant decreases in measures of mandibular length and depth and maxillary length, but when these proportions were adjusted for foetal body weight no significant differences were found. The morphologic defects in the development of bone were associated with biochemical effects of Warfarin on the skeleton as seen by analysis of the bones for gamma-carboxyglutamic acid (Gla) and osteocalcin (bone Gla protein).

As noted by the DS, two vitamin K-dependent proteins have been characterised in the skeleton, i.e., osteocalcin, which is associated with hydroxyapatite crystals in the extracellular matrix and matrix Gla protein (MPG) that predominates in embryonic bone and cartilage extracellular matrix. Osteocalcin, also known as bone Gla-containing protein (BGLAP), is a non-collagenous protein found in bone and dentin. Matrix Gla protein (MGP) is a protein found in numerous body tissues that requires Vitamin K for its optimum function. It is present in bone (together with the related vitamin K-dependent protein osteocalcin). Osteocalcin and MPG are both calcium-binding proteins that may participate in the organisation of bone tissue. Both have glutamate residues that are post-translationally carboxylated by the enzyme gamma-glutamyl carboxylase in a reaction that requires Vitamin K hydroquinone. This process also occurs with a number of proteins involved in coagulation: prothrombin, factor VII, factor IX and factor X, protein C, protein S and protein Z.

In the second study of Feteih *et al*. (1990), the effects of prenatal treatment with Warfarin were investigated on bone histology and morphology, and associated biochemical effects (levels of osteocalcin and Gla protein) in the rat.

On GD 21, Gla was decreased from 46 to 53% of controls based on the number of residues per 10^3 moities of glutamic acid. When Gla concentration was normalised to bone dry weight the decrease was even greater (65-67% of control). In contrast to serum osteocalcin values, which were not statistically different between the two groups, osteocalcin levels in the calvariae of Warfarin-exposed pups were decreased by 23-43% of control values by day 22. In the long

bones of these foetuses, osteocalcin was decreased from 25 to 50%. The osteocalcin concentration in both long bone and calvariae was highly correlated to the foetal body weight of controls only.

Since foetal bones contain two known vitamin K-dependent proteins, osteocalcin and matrix Gla protein (MPG), a calculation was carried out to determine quantitatively how much Gla in foetal bone is accounted for by the presence of immunoreactive osteocalcin. The results indicated that, at most, 3% of the Gla content in foetal long bone and 6% of the Gla content of foetal calvariae can be accounted for by the presence of osteocalcin. A large portion of the remainder of Gla-containing protein is likely to be the matrix Gla protein, although as yet unidentified proteins may also be present. Since matrix Gla protein is present in much greater quantities than osteocalcin in embryonic bone and cartilage extracellular matrix, the large reduction in Gla content of the Warfarin-exposed bones suggests that this protein was inhibited by the prenatal Warfarin exposure.

In spite of the reduction of level of osteocalcin and Gla in foetal bones the ossification centres revealed by staining with alizarin were not different in Warfarin treated animals from the control animals. However, analysis of the tibial growth showed several changes compared to control that included (1) widened hypertrophic zones, (2) increased calcification of the hypertrophic zones, and (3) disorganization of the hypertrophic cells. These results suggest that the growth plate abnormalities seen in foetal rats prenatally exposed to Warfarin are related to the inhibition of the vitamin K-dependent proteins of the skeletal system.

Howe & Webster, 1992

The study of Howe & Webster (1992) was not performed in accordance with OECD TG 414 but was designed to investigate the developmental toxicity of Warfarin, which is known to cause various degrees of nasal hypoplasia and other anomalies known as Warfarin embryopathy. However, conventional studies in pregnant mice, rats or rabbits were not considered feasible since there appears to be a very narrow margin between the no-effect dose for the conceptus and the maternal lethal dose. Thus, in this investigation, the post-natal developmental toxicity of Warfarin was studied in new born rats given sub-cutaneous injection of Warfarin in combination with Vitamin K starting on post-natal day 1. Thus, the extra-hepatic vitamin K deficiency induced by Warfarin is maintained, whereas vitamin-K-dependant processes of the liver are not disturbed.

The following groups were created:

- Warfarin group: six litters were given daily s.c. injections of Warfarin and Vit. K1 and the dams were also treated with Vit. K1 (10 mg/kg) to prevent haemorrhages from Warfarin ingestion by coprophagy. 11 males and 12 females from these litters were treated for 12 weeks until the final sacrifice;
- Vit. K1 group: three litters were treated only with Vit. K1, and 11 males and 10 females were subjected to the final sacrifice;
- Control group: four litters served as untreated control. 13 males and 14 females were sacrificed upon study termination.

In Warfarin-treated male and female offspring, there was a statistically significant reduction in tail length (12-17%), nasal length (7-13%), overall length (6-12%) and weight (7-13%) upon study termination (week 12). The snout of these animals was shorter and broader, and the pinnae of the ears were reduced in size. The measurements of alizarin-stained skulls after 12 weeks of treatment also revealed a small but statistically significant reduction in length of nasal bone length, frontal bone length, maxilla length and reduction of transfrontal width, width of snout and facial height. The forelimb bones length of Warfarin-treated rats were slightly shorter (4-5% reduction in both sexes) than Vit. K1 and/or untreated control animals.

The alizarin-stained nasal septa from Vit. K1 and control rats did not show evidence of calcification in the septal cartilage, while all nasal septal cartilages from Warfarin-treated rats showed extensive areas of calcification. The calcification appeared 2 weeks after the start of

Warfarin administration, and increased progressively during the following weeks. This calcification remained visible up to 15 months after cessation of treatment. There were no abnormal calcifications in the limbs or axial skeleton that might correspond to the "stipplings" described in the human Warfarin embryopathy. The growth plates from the femur and tail vertebrae showed many calcium bridges which transverse the growth plate from postnatal day 10 onwards. Similar structures were not seen in the controls.

RAC assessment of the toxicity to reproduction of warfarin in support of other anticoagulant rodenticide classifications

Although the classification of developmental toxicity of Warfarin is based on humans studies it is of great importance to note that in the studies of developmental toxicity in rats carried out with methodology compliant with OECD TG 414 (Kubaszky, 2009) or with a design resembling that methodology (Mirkova & Antov, 1983) and also in mice (Kronick *et al*. 1974) Warfarin induced clear developmental toxicity at doses within the same order of magnitude as therapeutic doses used in humans.

In the opinion of RAC Warfarin has caused developmental toxicity (intrauterine death of foetuses , increased postimplantation loss, internal and subcutaneous haemorrhages, intracerebral haematomas, cataract of lens) resembling the effects of developmental toxicity of Warfarin in humans except the naso-maxial hypoplasia which is observed only in humans due to difference in time of skull ossification between humans and rats. In the Kronick, et al. study (1974) Warfarin induced a very high incidence of haemorrhaged placentae and foetal deaths (including both dead and resorbed foetuses) when female mice were treated i.p. from days 3-11 of gestation with 2 and 4 mg/kg bw/day. These effects warrant classification of Warfarin as a developmental toxicant according to CLP criteria, although these effects were not systematically seen in all studies.

The developmental toxicity in humans has been observed at dose levels of 2-18 mg/person (equivalent to 2/60 = 0.033mg/kg/day – 18/60= 0.3 mg/kg/day), which in rats is probably lethal (the median lethal dose in a 90-day rat study is 0.077 mg/kg bw/day).

The developmental toxicity in humans and animals is linked with relatively effective transplacental transport of Warfarin to the foetus. The concentration of radioactive Warfarin residues in the liver of foetal rats are ca. 5 times lower than in maternal liver, in foetal blood cells and plasma, less than 2 times lower than in maternal blood, and in the foetal carcass slightly lower than in foetal plasma but higher than in blood cell fraction.

It is noted that the developmental toxicity of Warfarin has been demonstrated in humans at the dose levels of 2- 18 mg/person at which this substance exerts clear anticoagulation effects leading to substantial prolongation of prothrombin time by a factor of 1.5 to 2.5.

There are no data on developmental toxicity of Warfarin in humans at lower doses or data on dose response-relationship between developmental effects and level of exposure since the dose given to individual patients is adjusted to avoid the development of complications. The level of exposure is controlled to maintain level of anticoagulation required by the therapy.

Therefore, taking into account available evidence, it is concluded that Warfarin has to affect the coagulation system leading to moderate prolongation of prothrombin time through alteration of the Vitamin K cycle in order to induce developmental toxicity. In the opinion of RAC the existing evidence from case studies on humans is sufficient to establish a causal relationship between human exposure to Warfarin and subsequent developmental toxic effects in the progeny.

6.9.3 Data from human clinical use.

The following extract from Pesticide DAR summarises the human clinical data ,and reviews of clinical data, which are key to the current classification and labelling of Warfarin as Toxic for reproduction Category 1, T; R 61 May cause harm to the unborn child/

Schardein, J.L. (Ed.) 1985 Anticoagulants: Chemically Induced Birth Defects, Marcel Dekker, 89-106. Not GLP, published

Hall, J.G.; Pauli, R.M.; Wilson, K.M., 1980 . Maternal and foetal sequelae of anticoagulation during pregnancy. Am. J. Med. 68, 122-140. Not GLP, published

In two reviews (Schardein, 1985; Hall et al., 1980), retrospective summaries of case reports in which the administration of Warfarin during pregnancy induced birth defects were presented, together with a description of the encountered malformations or other effects, and the dosage of Warfarin involved. The duration of exposure in most of the 22 cases reviewed in detail by Hall et al. (1980) extends far beyond the first trimester (> week 30 of gestation). The daily dose of Warfarin was usually between 5-10 mg/day, only in one case at 2.5-5 mg/day. The following case reports were submitted and represent a selection from the published literature of warfarin-associated adverse developmental outcomes.

Reference	**Patient Treatment**	**Time of treatment**	**Outcome**
Kerber, I. J. et al. (1968)	*Warfarin (7.5 mg/day)* Digitalis Penicillin	Preconception to 31 weeks	Nasal hypoplasia Mental retardation Brachydactyly Scoliosis and other skeletal abnormalities
Bloomfield, D. K.; Rubinstein, L.I. (1969)	*Warfarin sodium (av. 6.25 mg/day)* Penicillin Digoxin	Preconception to 36 weeks	Normal female.

Reference	Patient Treatment	Time of treatment	Outcome
Becker, M. H. et al. (1975)	1. *Warfarin (-)* Digoxin Sulfisoxazole Erythromycin 2. *Warfarin (7.5 mg/day)* Digoxin	Preconception to 26 weeks. Throughout pregnancy	Nasal hypoplasia Optic atrophy Mental retardation Kyphoscoliosis Shortened proximal extremities Nasal hypoplasia Opacification of optic lens. Poorly developed ears Punctate calcification of vertebra and epiphyseal regions.
Shaul, W. L. et al. (1975)	*Warfarin sodium (2.5-5 mg/day)* Diazapam (briefly) Furosemide (2 wks at 26 weeks)		Nasal hypoplasia Vertebral stippling
Fourie, D. T. ; Hay, I. T. (1975)	*Warfarin sodium (5 mg/day)* Digoxin Furosemide Pottassium Isoptin	Preconception to week 36.	Nasal hypoplasia Choanal stenosis Short fingers, dysplastic nails Chondrodysplasia punctata
Barr, M.; Burd, A. R (1976)	*Warfarin sodium (7.5 mg/day*) Propanolol	Preconception to 17 weeks (elective abortion)	Nasal hypoplasia Large protuberant eyes Short fingers Hypertelorism
Carson, M.; Reid, M. (1976)	Warfarin (20 mg –3 mg – 4.5 mg/day)	Wk 12.5 to wk 36	Microcephaly Bifrontal narrowing Mental retardation Spastic

Reference	Patient Treatment	Time of treatment	Outcome
Holzgreve, W. et al. (1976)	*Warfarin (-)*	6mths preconception to wk 12 of gestation.	No abnormalities apparent at birth Retarded psycomotor development at 5 mths.
Abbott, A. et al. (1977)	*Warfarin* (6-7 mg/day)	Preconception to 24 wks.	Nasal hypoplasia Epiphyseal stippling Chonrodysplasia punctata.
Smith, M. F.; Cameron, M. D. (1979)	*Warfarin (-)*	Throughout pregnancy	Nasal hypoplasia Hypertelorism Tachycardia Hepatomegaly Generalised oedema
Stevenson, R. E. et al. (1980)	*Warfarin (5 mg/day)*	Throughout	Nasal hypoplasia Optic atrophy Developmental retardation

The administration of Warfarin to women during pregnancy has been shown to cause a well-defined complex of malformations in some of the offspring. This occurs as a result of exposure during the first trimester. This syndrome has been designated as "warfarin embryopathy" or "foetal warfarin syndrome' (FWS). The risk of malformation to the foetus of a mother treated with warfarin is not known with certainty. Schardein (1985) assessed the risk of malformation due to exposure to Warfarin during pregnancy as in the order of 1:5. More recently, a review of the maternal and foetal risks associated with oral anticoagulants (OA) indicated that the use of OA throughout pregnancy was associated with embryopathy in 6.4% (95% confidence interval [CI], 4.9% - 8.9%) of live births. Substitution with heparin at or prior to 6 weeks and up to 12 weeks was reported to remove this risk (Chan, 2000). Such malformations are still being reported in the literature, due to the necessity of treatment of patients (with e.g., mechanical heart valves), with warfarin, even after pregnancy has been detected (Howe, et. al., 1997, Chan and Ginsberg, 2002, Gohlke-Barwolf, 2001 , Ginsberg and Hirsh, 2001 ,).

The most consistent feature of FWS is a hypoplastic nose, caused by underdeveloped nasal cartilage. The degree of severity is varied from mild abnormality to severe breathing and feeding difficulties. Bone abnormalities of the axial and appendicular skeleton (radiological stippling of the vertebral column) often also occur. Punctate calcification of other bone sites may also be present. Kyphoscoliosis, abnormal skull development, and brachydactyly have been observed as associated skeletal effects. It is believed that avoidance of exposure to OA during weeks 6-12 of gestation should avoid warfarin embryopathy. It should be noted that exposure to coumarins during the first trimester was associated with a high rate of spontaneous abortions, in addition to the incidences of specific embryopathy. Likewise, exposure during the first and second trimester was also associated with a high rate of spontaneous abortion, stillbirths and warfarin-related complications (developmental abnormality) (Hall et. al.,1980).

Exposure after this time interval (first trimester) is associated with an apparently separate series of warfarin-related adverse effects, not related to warfarin embryopathy, *per se.* Adverse effects on the central nervous system predominate and include hydrocephaly or microcephaly, microphthalmia, various eye abnormalities, Dandy-Walker malformation and other CNS malformations often associated with degrees of mental retardation (Kaplan, 1985, Pati and Helmbrecht, 1993).

References

Chan, WS, Anand, S, and Ginsberg, J.S., 2000. Anticoagulation of pregnant women with mechanical heart valves: a systemic review of the literature. Arch. Intern. Med., 120: 191-6.

Howe, A.M., Lipson, A.H., de Silva, M., Ouvrier, R., Webster, W.S., 1997. Severe cervical dysplasia and nasal cartilage calcification following prenatal warfarin exposure. Am. J. Med. Genet., 71: 391-6.

Gohlke-Barwolf, C., 2001. Anticoagulation in pregnancy and post-partum in heart valve diseases, thrombosis or atrial fibrillation: fetal risk versus maternal thromboembolism. Z. Kardiol., 90 (Suppl. 4): 49-56.

Ginsberg, J.S., Greer, I., and Hirsh, J., 2001. Use of anticoagulants in pregnancy. Chest, 119: 122S-131S.

Kaplan, L.C.1985. Congenital dandy walker malformation associated with first trimester warfarin: a case report and literature review. Teratology 32, 333-337.

Pati, S.; Helmbrecht, G.D. 1994Congenital schizencephaly associated with *in utero* warfarin exposure. Reprod. Tox. 8, 115-120

Pauli, R.M., Lian, J.B., Mosher, Suttie, J.W., 1987. Association of congenital deficiency of multiple vitamin K-dependent coagulation factors and the phenotype of the warfarin embryopathy: clues to the mechanism of teratognicity of coumarin derivatives. Am. J. Hum. Genet., 41: 566-583.

Menon, R.K., Gill, D.S., Thomas, M., Kernoff, P.B.A., Danona, P., 1987. Impaired carboxylation of osteocalin in warfarin treated patients. J. Clin. Endocrinol. Metab., 64: 59-61.

Franco, B., Meroni, G., Parenti, G., Leviers, J., Bernard, L., Gebbia, M., Cox, L., Maroteaux, P., Sheffield, L., Rappold, G.A., 1995. A cluster of sulfatase genes on Xp22.3: mutations on chondrodysplasia punctata (CDPX) and implications ofr warfarin embryopathy. Cell, 81: 15-25.

Saxena, S.P., Fan, T., Li, M., Isreals, E.D., and Israels, L.G., 1999. A novel role for vitamin K1 in tyrosine phosphorylation cascade during chick embryogenesis. J. Clin. Invest., 99: 602-607.

Saxena, S.P., Israels, E.D., Israels, L.G., 2001. Novel vitamin K-dependent pathways regulating cell survival. Apoptosis, 6: 57-68.

Van Driel, D., Wesseling, J., Sauer, P.J., van Der Veer, E., Touwen, B.C., Smrkovsky, M., 2001. *In utero* exposure to coumarins and cognition at 8-14 years old. Paediatrics 107: 123-9.

Van Driel, D., Wesseling, J., Rosendaal, F.R., Odink, R.J., Van der Veer, E., Gerver, W.J., Geven-Boere, L.M., and Sauer, P.J., 2000. Growth until puberty after *in utero* exposure to coumarins. Am. J. Med. Genet., 95: 438-443.

Wesseling, J., Van Driel, D., Smrkovsky, M., van Der Veer, E., Geven-Boere, L.M., Sauer, P.J., Touwen, B.C., 2001. Neurological outcome in school-age children after *in utero* exposure to coumarins. Early Hum. Dev., 63: 83-95.

A more recent literature survey has been provided by industry (BASF, 2010) in support of the floucoumafen CLH dossier, which includes literature published since that submitted for

the Warfarin Plant Protection DAR and the Biocide CAR (up to 1994) and also the some of older literature. An extract of industry summary of this survey is included below:

The risk of adverse foetal effects due to Warfarin treatment in humans is difficult to estimate, due to the inhomogeneous data base: Some review articles evaluate complication rates in relation to pregnancies, others to live births, and this cannot always be resolved, due to incomplete information given in some articles. Nevertheless, since the number of pregnancies is predominantly referred to, this approach is adopted for the current overall evaluation. In case of significant overlap between review articles only the most comprehensive and reliable one was considered for deriving an overall foetal complication rate based on most recent data, resulting in the selection presented in Table 1. Furthermore, the data base has been restricted to Warfarin exposures only (ignoring other anticoagulants, e.g. Acenocoumarol) where possible. For details also see discussion of individual articles above.

Accordingly, based on the available data the risk for embryopathy due to Warfarin treatment in sensitive periods of gestation is 4.3 %%, relative to the number of pregnancies. This is in agreement with other authors, estimating the malformation risk to be "probably below 5 %" (De Swiet, 1987), or otherwise frequently in the range of 4–7 %, with some studies even reporting 0 % (Chan, Anand & Ginsberg, 2000; Hung & Rahimtoola, 2003; van Driel et al., 2002; Oakley, 1955; Hall, Pauli, & Wilson, 1980; Schaefer et al., 2006).

Other significant risks to the foetus or the newborn are associated with Warfarin treatment: Spontaneous abortion (27.3 %%, aggregated figure based on Blickstein && Blickstein, 2002; Chan, Anand & Ginsberg, 2000; Oakley & Doherty, 1976; Arnaout et al., 1998; Khamooshi et al., 2007; Shannon et al., 2008), stillbirth (27..1 %, based on the same articles except Oakley,, 1976), neonatal death (3.1 %; Blickstein & Blickstein, 2002; Oakley & Doherty, 1976; Arnaout et al., 1998; Khamoshi et al., 2007), CNS defect (4.33 %; Hall, Pauli & Wilson, 1980; Oakley & Doherty, 1976), premature delivery (66.2 %; Blickstein & Blickstein, 2002; Hall, Pauli, & Wilson, 1980), haemorrhage (2.2 %; Hall, Pauli, & Wilson, 1980), and ocular atrophy (Greaves, 1933; Hall, Pauli, & Wilson, 1980).

Table 1. Compilation and analysis of literature on Warfarin embryopathy in humans (a number of papers cited were not considered, either due to extensive overlap with the selected articles, or since merely citing and reiterating conclusions from other evaluations, Bates et al. (2008)), Srivastava et al. (20007), Ginsberg et al. (2003), van Driel et al. (2002), Oakley (1995), and Pauli (1988)).

Reference	No.	Embryopathy	
		No.	%
Hung and Rahimtoola, 2003 -pregnancies -live births	637 472 84	28 44 2	4.4 9.3 2.4
Blinckstein & Blickstein, 2002 -pregnancies -live births	792 549 224	35 35 16	4.4 6.4 7.1
Hall, Pauli & Wilson, 1980	224	16	7.1
Schafer, et al., 2006 -warfarin -All AVKs (live births)	66 356	0 2	0 0.6
Cotrufo et al., 2002 -pregnancies	71	4	5.6
Oakley & Doherty, 1976 -pregnancies	11	1	9.1
Arnaout et al., 1998	18	0	0.0
Srivastava et al., 2002	30	3	10
Geelane et al., 2005	150	0	0
Khamooshi et al., 2007	142	7	4.9
Akhtar et al., 2007	43	0	0
Shannon et al., 2008	11	1	9.1
Total, relative to number of pregnancies	**2279**	**97**	**4.3**

6.9.4 Other relevant information

Two separate mechanisms have been proposed for the specific embryopathy identified following first trimester exposure and the adverse CNS effects seen with second/third trimester exposure.

Two vitamin K-dependent proteins have been characterised in the skeleton i.e. osteocalcin, (bone gla [γ-carboxyglutamic acid]), which is associated with hydroxyapatite crystals in the extracellular matrix, and matrix gla protein (MGP) that predominates in embryonic bone and cartilage extracellular matrix. It has been proposed that in the presence of Warfarin, γ-carboxylation of glutamate residues in osteocalcin is inhibited by preventing the reduction of vitamin K epoxide, resulting in poor calcium binding and the observed anomalies in bone formation. In normally developing cartilage MGP, which is synthesised in the growth plate cartilage, remains

decarboxylated; this prevents the calcification of cartilage. In the presence of Warfarin, inappropriate calcification of cartilage occurs. Evidence has been provided to show that abnormal calcification of the nasal septum may be the underlying cause of this particular symptom of Warfarin embryopathy (Howe and Webster, 1992).

Inhibition of carboxylation of vitamin K-dependent clotting factors leading to intracranial haemorrhage is considered responsible for the CNS effects seen following exposure during the second and third trimesters (Pati and Holmbrecht, 1994). No specific pattern of CNS abnormalities has been identified, and there is no correlation between time of exposure and CNS effects (Hall et al., 1980). Coumarins, during the first trimester, were associated with a high rate of spontaneous abortions, in addition to the incidences of specific embryopathy. Likewise, exposure during the first and second trimesters was also associated with a high rate of spontaneous abortion, stillbirths and related complications (developmental abnormality) (Hall et al., 1980).

6.9.5 Summary and discussion of reproductive toxicity

It has been demonstrated clearly that Warfarin is both teratogenic and causes developmental toxicity when administered to pregnant women. The foetal outcome is considered to be dependent on the timing and duration of exposure; exposure during the first trimester is associated with Foetal Warfarin Syndrome and exposure throughout pregnancy or during the second and third trimester is associated with adverse effects on CNS development. Two separate mechanisms are proposed for the specific embryopathy identified following first trimester exposure and the adverse CNS effects seen with second/third trimester exposure.

The dose range reported in the submitted literature is from 2.5 to 20 mg/day, with 5.0-7.5 mg/day the most frequently used dose level. The dose prescribed relates to the prothrombin clotting times in individual patients and cannot to related to mg/kg/day dose level. The dose levels were not reported in some papers submitted. It is noted that the exact nature of the prescribed drug, e.g., chemical identity and purity, is not reported in all cases.

Classification: DSD

The information presented above is the basis for the classification of Warfarin as;

Toxic for Reproduction Repr. Cat. 1, R 61).

In accordance with the provisions of CLP Regulation (EC) No 1272/2008 Warfarin is assigned the Signal word “Danger” and the following hazard statement; H360: May damage the unborn child.

Proposal for a SCL for reproductive toxicity:

A proposal has been for the setting of a specific concentration limit for reproductive toxicity, according to the Draft Guidance on the setting of concentration for reproductive toxicants with the CLP Regulation (Draft 2, Feb 2010).

The Warfarin dose range for humans reported in the submitted literature is from 2.5 to 20 mg/day. Doses of 2.5 mg/day (0.04 mg/kg bw/day, female bodyweight of 60kg) have been reported to result in nasal hypoplasia and vertebral stippling. Higher doses have resulted in a high percentage of embryofoetal mortality (A NOAEL cannot be set and the value of 0.04 mg/kg bw/day represents a LOAEL which in turn approximates to an ED_{10} value.

Based on the Draft document entitled "Guidance for Setting Specific Concentration Limits for Reproductive Toxicants within the CLP Regulation (EC/1272/2008)", substances with an ED_{10} value less than or equal to 5 mg/kg bw/day are considered as Class 1 or high potency substances. Warfarin is considered to have very high potency in terms of developmental toxicity simply because its LOAEL is approximately 2 orders of magnitude below the upper limit value for high potency classification. The general concentration limit (GCL, 0.3% w/w) is applied to all medium or class 2 potency reproductive toxicants. For high potency substances, the ECHA working Group on Human Health Guidance for CLP has proposed an SCL of 0.03% w/w. Furthermore, extremely potent developmental toxicants with ED_{10} values deviating 10-fold or greater, below the upper limit value of 5 mg/kg bw/day must lead to a further revision of the SCL value. The high potency SCL must be reduced by a factor of 10 for each 10-fold disparity between the ED10 and the upper limit value of 5 mg/kg bw/day.

Warfarin has a LOAEL of 0.04 mg/kg bw/day which approximates to the ED_{10}. This value is about 2 orders of magnitude below the upper limit value for high potency classification. Based on the draft guidance, a value of 0.03/100 = 0.0003% w/w is calculated for Warfarin. Any preparation containing Warfarin equal to or in excess of 0.0003% w/w shall be classified with respect to reproductive toxicity, Repr. 1A – H360D, i.e.

$C \geq 0.0003\%$ Repr. 1A

6.10 Other effects
Not relevant for this dossier.

6.11 Delayed neurotoxicity
Not relevant for this dossier.

6.12 Medical data
Not relevant for this dossier.

7 HUMAN HEALTH HAZARD ASSESSMENT OF PHYSICO-CHEMICAL PROPERTIES

7.1 Explosivity
Not relevant for this dossier.

7.2 Flammability
Not relevant for this dossier.

7.3 Oxidising potential
Not relevant for this dossier.

8 ENVIRONMENTAL HAZARD ASSESSMENT

8.1 Aquatic compartment (including sediment)

8.1.1 Toxicity test results

8.1.1.1 Fish

Acute Toxicity to Fish:

Method	Results	Remarks	Reference
Merkblatt 33 der Biologischen Bundesanstalt vom September 1975 (2. Auflage)	LC_{50} = 65 mg a.s./L	Nominal concentration. High concentration of acetone used due to solubility problem Static 96 hrs **Key study**	Günther (1984) Fish Toxicity, rainbow trouts. Ökolimna GmbH, Burgwedel, Germany, Report No: (no report no. allocated), May 10, 1984 (unpublished)
EPA-660/3-75-009 (1975)	LC_{50} = 88 mg a.s./L	Nominal concentration. White precipitate formed due to solubility problem Static 96 hrs **Supportive study**	McAllister WA, Cohle P (1984) Acute toxicity of Warfarin technical to bluegill sunfish (Lepomis macrochirus). Analytical Bio-Chemistry Laboratories, Columbia, Missouri, Report No: 32460, December 28, 1984 (unpublished) A7.4.1.1/02
Merkblatt 33 der Biologischen Bundesanstalt vom September 1975 (2. Auflage)	LC_{50} = 66 mg a.s./L	Nominal concentration. White precipitate formed due to solubility problem Static 96 hrs **Supportive study**	Günther (1984) Fish Toxicity, Orfe. Ökolimna GmbH, Burgwedel, Germany, Report No:(no report no. allocated), May 10, 1984 (unpublished) A7.4.1.1/03

Acute Toxicity to Fish:

In the key study, rainbow trout exhibited signs of toxicity such as a high level of agitation, disequilibrium and/or fish on the bottom of the vessel, even at sub-lethal concentrations, immediately after the initiation of exposure until 8 hours later. Many of the surviving fish recuperated after about 48 hours and conveyed a completely normal impression upon termination of the test. It should be noted that acetone concentrations of 70 and 100 mg/L were used as a vehicle in this study and that the concentration of acetone used as a vehicle was above permissible limits in some cases. Mortality in the 100-mg/L solvent control was at 40 % throughout the study. The Warfarin levels tested were from 0 to 110 mg/L.

In the supportive studies (A7.4.1.1/02 and A7.4.1.1/03), Bluegill Sunfish and Golden Orfe exhibited signs of similar toxicity. In study A7.4.1.1/02 260 mg/L acetone was used as a vehicle. While signs of toxicity (surfacing, quiescence, dark discoloration and/or fish on the bottom of the vessel) were reported in this study there was no mortality in the solvent control. Nominal test concentrations of Warfarin in this study were from 0 to 1000 mg/L. In study A7.4.1.1/03 940 mg/L acetone was used as a vehicle with fish exhibiting toxicity symptoms (agitation, balance impairment and/or fish on the bottom of the vessel) but with no mortality in the solvent controls. Most of the surviving fish recuperated after 48 hours. Nominal test concentrations of Warfarin were from 0 to 120 mg/L.

The LC_{50} (96 hour) values in each of the three studies were 65 mg/L, 88 mg/L and 66 mg/L, a finding that was fairly consistent for each of the three species. There is a question as to whether the high levels of acetone used in the studies were responsible for the toxic response observed in the fish. According to the OECD guideline 203 (1992) 'the concentration of organic solvents, emulsifiers, or dispersants should not exceed 100 mg/L'. This concentration was clearly exceeded in both the supportive studies. However, in these studies there were no mortalities seen in the solvent controls. Furthermore there is conflicting evidence in the open literature as to the toxicity of acetone to fish. In general, fish appear to have a high degree of tolerance to acetone. One document cites LD_{50} values of 6070 mg/L (Brook trout) and 15,000 mg/L (Fathead minnow) (www.inchem.org/documents/sids/sids/67641.pdf). Normally acetone is miscible in water. The observations of the white precipitate in the supportive studies have not been accounted for. It is likely that Warfarin was the cause of the toxicity symptoms in the fish. However, the concentrations used in each of the studies exceed the PECs estimated for surface water (with correct use) by several orders of magnitude. The risk of Warfarin contamination in the hydrosphere is considered to be negligible.

Short-term toxicity to fish: Not relevant for this dossier.

Long-term toxicity to fish:

Method	**Results**	**Remarks**	**Reference**
OECD 204 (1984)	NOEC 2.0 mg a.s./L	Measured concentration. High concentration of acetone used due to solubility problem Flow-through 21 days **Key study**	Dommröse A-M (1989) Investigation of the test substance Warfarin in a prolonged toxicity test on fish (Rainbow Trout). NATEC GmbH, Hamburg, Germany, Report No. NA 88 9867/3.3, August 1989 (unpublished). A7.4.3.1/01

In this study the concentrations were actually 29-73 % below nominal. This was thought to be due to the low solubility of the test substance. Solvent concentration was higher than in Guideline i.e. 100 mg/L. However, the results are reported as measured concentrations. At concentrations above 2.0 mg a.s./L mortalities of the Rainbow Trout occurred. Abnormal effects such as reduced food consumption, reduced sensitivity and fish swimming at the bottom of the test vessel were also observed at concentrations above 2.0 mg a.s./L.

8.1.1.2 Aquatic invertebrates

Short-term toxicity to aquatic invertebrates:

Method	Results	Remarks	Reference
OECD 202 and EC method C.2 (92/69/EEC)	EC_{50} >105	Nominal concentration. Limit test Static 48 hrs **Key study**	Hertl J (2001) Acute toxicity of Warfarin techn. to *Daphnia magna* in a 48-hour immobilization test. IBACON GmbH, Rossdorf, Germany, Report No: 12001220, October 2, 2001 (unpublished). A7.4.1.2/01
EPA, Ecol. Res. 660/3-75009 (1975)	LC_{50} = 130 mg./L (48-hour)	Nominal concentration. Static 48 hrs **Supportive study**	Forbis, A.D. Georgie L, Burgess D (1984) Acute toxicity of Warfarin technical to *Daphnia magna*. Analytical Bio-Chemistry Laboratories, Columbia, Missouri, Report No: 32462, December 18, 1984 (unpublished) A7.4.1.2/02
Test method I 5.3-97125-2/6 issued by the German Federal Ministry of the Environment on June 2, 1981	LC_{50} = 180 mg L (24-hour)	Nominal concentration. White precipitate formed due to solubility problem Static 24 hrs **Supportive study**	Günther (1984) Daphnia toxicity. Ökolimna GmbH, Burgwedel, Germany, Report No: (no report no. allocated), June 20, 1984 (unpublished) A7.4.1.2/03

There was no effect on mobility or mortality of Daphnids at the highest rate tested in the key study. In this study no solvent was used. In the supportive studies (A7.4.1.1.111202 and A7.4.1.1/03), exhibited signs had similar toxicity profiles with LC50 values of 160 and 180 mg a.s./L respectively. In Study A7.4.1.1/03 there were no adverse effects noted in the acetone control treatments.

Long-term toxicity to aquatic invertebrates:

Method	Results	Remarks	Reference
OECD 202, Part 2	NOEC=0.059 mg/L	Measured concentration.	Dommröse A-M (1990) Investigation of

(OECD 211 was adopted in 1998, and therefore after conduct of the study)		Immobilisation and reproduction were assessed Flow-through 21 days **Key study**	the test substance Warfarin in a prolonged immobilisation and reproduction test on *Daphnia magna*. NATEC, Hamburg, Germany, Report No: NA 88 9867/3.2, April 23, 1990 (unpublished) A7.4.3.4

The results of the immobilisation study were comparable to the second test which is not reported whereby high rates of immobilisation occurred at the 3.67mg/l concentration level and significantly lower rates at the highest concentration level [25.7mg/l]. The study authors do not present any reasoned explanation to explain this outcome, however the analytical determinations of the test concentrations do confirm the validity range of the exposure concentrations offered. The first offspring (F_1 generation) were observed on day 7, and at the lower concentration levels the findings appeared to be dose responsive. The reproduction rate was increased at a test concentration of 0.525 g/l, however this coincided with high mortality [92.5%] in this group.

In the highest dose group, there was an evident lack of reproduction until day 14, and thereafter a very low reproduction rate as compared to the control. The NOEC for aquatic invertebrates was determined to be 0.059 mg a.s./L/.

8.1.1.3 Algae and aquatic plants

Method	Results	Remarks	Reference
OECD 201 and EC method C.3 (92/69/EEC)	E_rC_{50} and E_bC_{50} >83.2 mg/L	Measured concentration. Continuous stirring for 72 h hrs E_rC_{50} and E_bC_{50} could not be determined they were above the highest treatment rate **Key study**	Hertl J (2001) Toxicity of Warfarin techn. to *Scenedesmus subspicatus* in an Algae Growth Inhibition Test. IBACON GmbH, Rossdorf, Germany, Report No: 12002210, October 2, 2001 (unpublished) A7.4.1.3/01
OECD 201 (1984)	E_bC_{50} > 8.5 mg/L	Nominal concentration. Static 72 hrs E_bC_{50} could not be determined as they were above the highest treatment rate **Supportive study**	Dommröse A-M (1989) Growth inhibition Test on Algae, Test Substance: Warfarin NATEC, Hamburg, Germany, Report No: NA 88 9867/3.1, February 17, 1989 (unpublished) A7.4.1.3/02

Warfarin is of low toxicity to algae and for this reason no definitive E_rC_{50} and E_bC_{50} could be determined from the key study. The justification for non-submission of data on aquatic plant toxicity has been accepted.

8.1.1.4 Sediment organisms
Not relevant for this dossier.

8.1.1.5 Other aquatic organisms
Not relevant for this dossier.

8.2 Terrestrial compartment

8.2.1 Toxicity test results
Not relevant for this dossier.

8.2.1.1 Toxicity to soil macro organisms
Not relevant for this dossier.

8.2.1.2 Toxicity to terrestrial plants
Not relevant for this dossier.

8.2.1.3 Toxicity to soil micro-organisms
Not relevant for this dossier.

8.2.1.4 Toxicity to other terrestrial organisms
Toxicity to birds: Not relevant for this dossier.
Toxicity to other above ground organisms: Not relevant for this dossier.

8.3 Atmospheric compartment
Not relevant for this dossier.

8.4 Microbiological activity in sewage treatment systems
Not relevant for this dossier.

8.4.1 Toxicity to aquatic micro-organism
Not relevant for this dossier.

8.5 Conclusion on the environmental classification and labelling

In some of the aquatic toxicity tests, considerable difficulty was experienced in obtaining homogenized samples even with using high concentrations of the solvent acetone. However, more recent tests were performed using filtrate obtained from supersaturated solutions. Overall, the resulting toxicity profile for Warfarin was generally consistent, and is considered of sufficient quality to characterize the parent compound as regards its hazard classification.

The acute toxicity of Warfarin was investigated in fish, daphnia and algae. The critical endpoint for acute toxicity was from the study on Rainbow Trout (Günther (1984)). The LC_{50} from this study

was 65 mg a.s./L. The critical endpoint for chronic toxicity was from the study on Daphnids (Dommröse A-M (1990). The NOEC from this study was 0.059 mg a.s./L. These endpoints are used for the classification and labelling of Warfarin. As Warfarin is readily biodegradable and has a very low BCF the classification is as follows:

In accordance with the provisions of the 2nd ATP to CLP Regulation (EC) No 1272/2008, Warfarin is assigned the following hazard statement Env. Chronic Tox.2-H411: Toxic to aquatic life with long lasting effects. There is no M factor associated with the endpoints used in the classification (as per Table 4.1.3 of the 2nd ATP to CLP). The Signal Word 'Warning' and the environmental hazard pictogram are required. The Precautionary Statements are: P273, P391, P501.

RAC evaluation of environmental hazards

Summary of Dossier submitter's proposal

The current harmonised environmental classification of Warfarin is Aquatic Chronic 3 (H412) according to CLP. The DS proposed to harmonise the classification for Warfarin as Aquatic Chronic 2 (H411) according to CLP.

Degradation
Degradation was studied in a ready biodegradability test (OECD TG 301D), and based on this', Warfarin can be classified as readily biodegradable. The criterion of 60 % removal of ThOD within a 10 day window was exceeded. A degradation of 92.7% was determined for a 28 day period.

No other degradation tests were submitted.

Based on the available data rapid degradation was proposed for Warfarin.

Bioaccumulation
The experimental log K_{ow} of Warfarin is 0.7 at pH 7 and the calculated log K_{ow} is 2.23. These values are lower than the cut-off values of log $K_{ow} \geq 4$. Furthermore, the experimental bioconcentration factor (BCF) was obtained following OECD TG 305E and the resulting BCF was 21.6 L/kg, which is lower than the cut-off value of 500 L/kg. This value was not indicated in the CLH report, but the DS reported it after public consultation.

In conclusion, since the low log K_{ow} as well as the low experimental BCF indicated low potential for bioaccumulation, the DS concluded that warfarin has no potential for bioaccumulation.

Aquatic toxicity
Three acute toxicity studies in fish (*Oncorhynchus mykiss*), three in invertebrates (*Daphnia magna*) and two in algae (*Desmodesmus subspicatus*) were reported by the DS. There are also chronic tests available for the same species of fish and invertebrates following OECD TG204 and 202 part II, respectively. However, chronic endpoints for algae have not been included in the CLH report.

All the acute endpoints ($L(E)C_{50}$) reported in the CLH dossier for the three trophic levels are higher than 1 mg/L: fish (*Oncorhynchus mykiss,* LC_{50}(96h) = 65-88 mg/L); invertebrate (*Daphnia* magna, one study for which an EC_{50}(48h) > 105 mg/L and two other studies for which LC_{50}(48h) = 130 mg/L and LC_{50}(24h) = 180 mg/L were reported) and algae (*Desmodesmus subspicatus,* E_rC_{50}(72h) > 83.2 mg/L). Fish and invertebrate toxicities ($L(E)C_{50}$) were based on nominal concentrations because the concentrations were not measured during the tests, while algae toxicity was based on measured concentrations. *Oncorhynchus mykiss* is the most sensitive species in the acute studies with an LC_{50} value of 65 mg/L from the key study. This report of test was dated 1984 following guideline EPA-660/3-75-009. Acetone was

used as the vehicle due to the low solubility of warfarin. However, the concentration of acetone as a vehicle was well above the recommended 100 mg/L (OECD TG 203). In addition, the mortality in the 100 mg/L solvent (acetone) control was 40%.

Regarding the chronic toxicity, *Daphnia magna* is the most sensitive species with a NOEC value of 0.059 mg/L while fish showed lower toxicity with a NOEC value of 2 mg/L. The toxicity of both tests was based on mean measured concentrations and the both studies were considered valid by the DS.

An LC_{50} of 65 mg/L (*Oncorhynchus mykiss*) and a NOEC of 0.059 mg/L (*Daphnia magna*) were used to establish the classification by the DS.

Comments received during public consultation

Four Member States made comments during the public consultation. The main concern raised in the comments was the reliability of the reported acute toxicity studies. For example, the provided studies for fish were old (1984) and high concentrations of solvent were used, resulting in 40% of death in the solvent control in the key study, and furthermore several signs of toxicity were observed in the solvent controls of the supportive studies. Additionally, in the supportive studies, unidentified white precipitates were observed. Moreover, the substance is readily biodegradable, its solubility is low and the endpoints are based on nominal concentrations. It was also pointed out that the known acute toxicity of acetone in fish is well above the applied concentrations but the reported fish studies showed some toxic effects in the solvent controls.

One MS requested to include a missing NOEC value (algae) from the biocidal dossier to the CLH report as well.

In their response to comments received during public consultation the DS agreed that the acute studies are unreliable due to the physical and chemical properties of Warfarin.

Regarding the use of these acute studies to classify Warfarin, the DS noted that it should be discussed by the ECHA experts.

The DS included the following additional information about the NOEC values from the algae tests:
Hertl J. (2001) NOErC = 21.3 mg/L

Dommröse A.-M. (1989-supportive study) NOEC = 8.5 mg/L

Additional key elements

Additional information is taken from the biocides CAR concerning hydrolysis and photolysis.

Hydrolysis as a function of pH and identification of breakdown products (OECD TG 111)

Less than 4% of the tested Warfarin hydrolysed in the preliminary test during five days at 50 ± 0.5 °C and any pH value. Hence, k_H and DT_{50} or DT_{90} values could not be established. Therefore, Warfarin is considered to be hydrolytically stable. The half-lives at pH 4, 7 and 9 are expected to exceed one year at 25 °C.

Warfarin is expected to be hydrolytically stable under environmentally relevant conditions. No major degradation products were or could be identified in view of the negligible hydrolysis rate.

Phototransformation in water including the identity of the products of transformation (UBA Test Guideline "Phototransformation of chemicals in water, part A, Direct Phototransformation")

Under exclusion of light, i.e. in the dark control, Warfarin was stable in water for a period of 14 days at 20–25 C (ambient temperature). Under irradiation, there was no significant decrease of the Warfarin concentration in the test solutions during the entire (330 hours) test. Consequently, only an "upper limit of quantum efficiency" was estimated for Warfarin in water at $\leq$ 0.0004. From this, a lower limit of the environmental half-life in surface water was calculated to be $\geq$ 54 days (light path 1 cm, average spectral photon irradiance corresponding to conditions during November in Central Europe).

RAC assessment and comparison with criteria

Degradation
Information on hydrolysis and photolysis from the biocides CAR has been included in the additional elements section.

According to this information, Warfarin is hydrolytically stable under environmentally relevant conditions with a DT_{50} possibly higher than 1 year. Direct photolysis in water is not expected to contribute significantly to abiotic degradation in aqueous systems under normal environmental conditions.

Regarding the data which appears in the CLH report, RAC agreed that Warfarin is readily biodegradable under test conditions (OECD TG 301D), with a level of degradation of 92.7% after 28 days also meeting the criterion of 60% removal within a 10 day window. Therefore, based on these data and the decision scheme in the CLP Guidance, RAC agreed with the DS that warfarin should be considered **rapidly degradable** according to CLP.

Bioaccumulation
The experimental log K_{ow} for warfarin is 0.7 (pH 7) and the experimental BCF (OECD TG 305) is 21.6 L/kg, both values are below the cut-off values of log $K_{ow} \geq 4$ (CLP) and BCF $\geq$ 500 (CLP). RAC agreed with the DS that warfarin has **low potential for bioaccumulation**.

Aquatic toxicity
The acute toxicity category should be based on the lowest valid $L(E)C_{50}$. In this case, all the acute endpoints ($L(E)C_{50}$) reported in the CLH dossier for the three trophic levels are higher than 1 mg/L. The reported three fish studies were based on nominal concentrations and taking into account that the substance is readily biodegradable and shows low solubility, the reported toxicity values could be underestimates of acute toxicity in fish. Furthermore, the three acute fish studies were performed in 1984, and acetone was used as a vehicle in order to dissolve warfarin, but the acetone concentrations used exceeded in some cases the maximum concentration for solvents suggested in the current guideline (100 mg/L). Although there are data which showed that the fish had a high degree of tolerance to acetone, the mortality in the 100 mg/L solvent control of the key study was 40% throughout the test. Although the LC_{50} values of the three studies were fairly consistent (65 mg/L for the key study, 66 and 88 mg/L for the supportive studies), all these tests showed the same deficiencies. In addition in the studies used as supportive tests a white precipitate was formed due to solubility problems, so the reliability of these tests is questionable.

In the CLH report a prolonged toxicity test in fish (*Oncorhynchus mykiss,* OECD TG 204), is reported as a chronic test. This test, as well as the acute tests, used acetone as vehicle to dissolve Warfarin, and for some tested concentrations at a level higher than that allowed in the guidelines (100 mg/L). However, for the NOEC concentration of 3.8 mg/L (nominal concentration), the concentration of acetone was 45.6 mg/L, therefore this value is in agreement with the guideline and may be considered as reliable. Measured concentrations were actually 29-73% below nominal and a 21 day NOEC (mortality, 1.8 mg/L if the geometric mean is considered) of 2.0 mg/L based on mean measured concentrations were reported. This value cannot be used as a chronic value but it strengthens the evidence that the aquatic acute toxicity for fish is higher than 1 mg/L (see "In *depth analysis by RAC*" section).

Regarding the *Daphnia* test used as a key study, although the results are based on nominal concentrations, the concentration of Warfarin was determined in duplicate at 0 and 48 h. The measured concentrations were within ± 20% of the nominal or measured initial concentration, thus the results can be based on nominal concentrations, i.e. the EC_{50} value > 105 mg/L (nominal) is acceptable. Two more acute *Daphnia* tests were also submitted in the CLH report and their results, based on nominal concentration are in agreement with this value, although it is not possible to conclude based on the provided information if the measured concentrations were within the 20% of the nominal concentration throughout the whole tests.

Two algae tests were summarised in the CLH report. Warfarin showed low toxicity to algae with E_rC_{50} and E_bC_{50} > 83.2 and >8.5 mg/L, respectively. After public consultation NOEC values of 21.3 mg/L (NOE_rC) and 8.5 mg/L (NOEC), respectively were submitted by the DS for both tests. The first test was used as a key study for this trophic level and the values are based on measured concentrations.

In conclusion, the acute tests for fish cannot be considered reliable and valid for classification, taking into account the prolonged toxicity test, the reported information suggests that the LC_{50} for fish is higher than 1 mg/L. Reliable acute toxicity tests for algae and daphnia are available and according to the results, no classification for aquatic acute toxicity is justified.

Regarding chronic toxicity, no adequate chronic data is available for all three trophic levels. Chronic information was included only for daphnia in the CLH report and for algae after public consultation. Taking into account the lowest reliable chronic toxicity value (*Daphnia*, NOEC = 0.059 mg/L) and that the substance is rapidly degradable, a classification as Aquatic Chronic 2 (H411) would be applicable for Warfarin.

Due to the lack of chronic data for fish, the surrogate approach must to be applied and compared to the classification based on chronic toxicity. However, the observed low acute toxicity in fish would result in a less stringent classification and therefore, the chronic classification should be based on the chronic toxicity in *Daphnia*.

In conclusion, RAC agreed with the DS's proposal and concluded that the provided data did not justify classification for aquatic acute toxicity butwhile classification for long-term aquatic hazards is warranted as follows: **Aquatic Chronic 2 (H411)**.

It is recommended to revise the classification if reliable acute and chronic data for fish become available.

In depth analyses by the RAC

Prolonged toxicity to an appropriate species of fish.

Concentrations of acetone.

Nominal Concentration [mg/L]	**Concentrations of acetone [mg/L]**
2.4	**29.2**
3.8	**46.2**

6.2	**75.4**
9.9	**120.5***
15.9	**193.5***
25.4	**309.1***
40.6	**494.1***
65.0	**791.0***

*Concentrations higher than the level allowed for the guidelines (100 mg/L).

The concentrations were calculated with a density for acetone of 791 mg/mL and a final volume of 18 L and considering that the preparation of test solution was carried out dissolving 6 g of Warfarin in 100 mL of acetone

Table A7.4.3.1-1: Analytical verification of Warfarin

	t=0 d	t=11 d	t=21 d	Geometric mean [mg/L]	Recoveries [%]
3.8	0.91	2.42	2.67	1.80	47.4
6.2	1.41	1.96	2.54	1.91	30.8
15.9	8.52	4.61	-	6.27	39.4

Table A7.4.3.1-2: Cumulative mortality rates in Oncorhynchus mykiss during the 21-day exposure with Warfarin

Nominal conc. [mg/l]	**Mortality [%] at day**									
	1	**3**	**5**	**7**	**9**	**11**	**14**	**16**	**18**	**21**
Control	0	0	0	0	0	0	0	0	0	0
Solvent control	0	0	0	10	10	20	20	20	30	30
2.4	0	0	0	0	0	0	0	0	10	10

3.8	0	0	0	0	0	0	0	0	0	0
6.2	0	0	0	0	0	0	40	40	40	50*****
9.9	0	0	0	0	0	0	10	10	20	30
15.9	0	0	0	0	0	0	20	20	30	30
25.4	0	0	0	0	0	0	10	10	30	30
40.6	0	0	0	0	0	10	20	30	30	30
65.0	0	0	0	0	0	20	50	50	60	60

***** high mortality caused by social stress

OTHER INFORMATION

The draft version of the guidance on the setting of specific concentration limits for reproductive toxicity (which is currently being reviewed for incorporation into the Guidance to Regulation (EC) No 1272/2008 on CLP of substances and mixtures) was consulted in the setting specific concentration limits for reproductive toxicity. New studies were conducted in support of the floucuomafen CLH Report and were circulated by the sponsor. Information considered pertinent to Warfarin were summarised for this report, i.e.,

Kubaszky, R. (2009). Teratology study of the test item Warfarin sodium with rats. LAB Research Ltd, Veszpém, Hungary.

Literature survey on outcomes of clinical use of Warfarin in pregnant women, abstracted from;

BASF (2010). Flocoumafen: Applicants statement on the pending classification proposal for developmental toxicity by read-across from Warfarin. Confidential report BASF Doc ID 2010/1018983. Hannover, 4th Feb 2010.
The individual publications relied on are listed in Annex 1 to this report.

The data sources (e.g. registration dossiers, other published sources) used for the generation of the dossier are indicated in the Reference list.

REFERENCES

Reference 1: Warfarin (Pt 14) Biocide Competent Authority Report including Document I, Document II A, B & C, and Document III A & B.

Reference 2: Warfarin (PT 14) Pesticide Draft Assessment Report Volume 1-6 (2009).

Reference 3: Review report for the active substance Warfarin finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 23 September 2005 in view of the inclusion of Warfarin in Annex I of Directive 91/414/EEC. SANCO/10434/2004 final (23.9.2005)

Reference 4: ECBI/54/06 PCS, DAFF, Ireland report on developmental studies used for analysis of Warfarin.

Reference 5: ECBI/54/06 Addendum 1. RE: RMS Classification proposal for Warfarin (CAS no. 81-81-2).

Reference 6: ECBI/54/06 Addendum 2. Comments for FU II following on from the Meeting on Health Effects of Plant Protection Products and Biocides, Arona, 15 – 16 May, 2007.

Reference 7: ECBI/54/06 Addendum 2. Comments from FU IV following on from the Meeting on Health Effects of Plant Protection Products and Biocides, Arona, 15 – 16 May, 2007.

ANNEX 1

References from BASF 2010 literature survey.

Akhtar, R. P., Abidd, A. R., Zafar, H., Cheema, M. A, & Khan, J. S.(2007). Anticoagulation in pregnancy with mechanical heart Valves: 10-year experience. *Asian Cardiovascular & Thoracic Annals , 15* (6), S . 497-501.

Al-Lawati, A. A., Venkitraman, M., Al-Delaime, T., & Valliathu, J.(2002). Pregnancy and mechanical heart valves replacement; dilemma of anticoagulation. *European Journal of Cardio-thoracic Surger, 22*, S. 223–227.

Arnaout, M., Kazma, H., Khalil, A., Shasha, N., Nasrallah, A., Karam, K., et al. (1988). Is there a safe anticoagulation protocol for pregnant women with prosthetic valves? *Clinical & Experimental Obstetrics & Gynecology, 25* (3), S. 101-104.

Bates, S. M., Greer, I. A., Pabinger, I., Sofaer, S., & Hirsh, J. (2008). Venous thromboembolism, thrombophilia, antithrombotic therapy, and pregnancy. *Chest , 133,* S. 844S–886S.

Ben Ismail, M., Abid, F., Trabelsi, S., Taktak, M., & Fekih, M. (1986). Cardiac valve prostheses, anticoagulation, and pregancy. *British Heart Journal, 55*, S. 101-105.

Blickstein, D., & BBlickstein, I . (2002). Thhe risk of feetal loss assoociated withh Warfarin anticoagulation.. *Internationnal Journal of Gynecollogy and Obbstetrics , 78*, S. 221-2225.

Born, D., Martinez, E. E., Almeida, P. A., Santos, D. V., Carvalho, A. C., Moron, A. FF., et al. (1922). Pregnancy in patients with prosthetic heart valves: the effects off anticoagulation on mother, foetus, and neonate. *American Heart Journal , 124* (2), S. 413-417.

Caruso, A., De Carolis, S., Ferrazzani, S. Paradisi, GG., Pomini, F., & Pompei, A. (19994). Pregnancy outcome in women with cardiac valve prosthesis. *European Journal of Obstetrics and Gynecolgyy and Reproductive Biology , 54*, S. 7-11.

Chan, WW. S., Anand, S., & Ginsberg, J. S. (2000). Anticoagulation of pregnant women with mechanical heart valves. *Archives of Internal Medicine , 160* S. 191-196.

Chen, WW. W., Chan, C. S., Lee, P. K., Wang, R. Y., & Wong, VV. C. (1982). Pregnancy in patients with prosthetic heart valves: An experience with 45 pregnancies. *Quarterly Journal of Medicine , 203*, S. 358-365.

Chong, M., Harvey, D., & De Swiet, M. ((1984). Follow-up study of children whose mothers were treated witth warfarin during pregnancy. *British Journal of Obstetrics and Gynaecology , 91*, S. 1070--1073.

Cotrufo, M., De Feo, M., De Santo, L. S., Romano, GG., Della Corte, A., Renzulli, A., et al. (2002). Risk of warfarin during pregnancy with mechanical valve protheses. *Obstetrics & Gynecology , 99*, S. 35-40.

Cotrufo, M., de Luca, T., Calabrò, R., Mastrogiovanni, G., & Lama, D. (1991). Coumarin anticoagulation during pregnancy in patients with mechanical valve prostheses. *European Journal of Cardio-thoracic surgery , 5*, S. 300-305.

De Swiet, M. (1987). Anticoagulants. *British Mediaal Journal, 294*, S. 428-430.

Duhl, A. J., Paidas, M. J., Ural, S. H., Branch, W., Casele, H., Cox-Gill, J., et al. (2007).

Antithrombotic therapy andd pregnancy: consensus report and recommendations for prevention and treatment off venous thromboembolism and adverse pregnancy outcomes. *American Journal of Obstetrics and Gynecology , 197*, S. 457.e1-457.e21.

Gelani, M. A., Singh, S., Verma, A., Nagesh, A., Betigeri, V., & Nigam, MM. (2005). Anticoagulation in patients with mechanical valves during pregnancy. *Asian Cardiovascular & Thoracic Annals , 113* (1), S. 300-33.

Ginsberg, J. S., Chan, W. S., Bates, S. M.., & Katz, S. (2003). Anticoagulation of pregnant women with mechanical heart valves. *Archives of Internal Medicine , 163*, S. 694-698.

Ginsberg, J., Hirsh , J., Turner,, D., Levine, M., & Burrows, R. (1989). Risks to the fetus of anticoagulant therapy during pregnancy. *Thrombos. Haemostas. , 61*, S. 197-203.

Greaves, M. (1993). Anticoagulants in pregnancy. *Pharmacology & Therapeutics , 59*, S. 311-327.

Hall, J. G. (1965). Embryopathy associated with oral anticoagulant therapy. *Birth Defects , 12*, SS. 133-140.

Hall, J. G., Pauli, R. M., & Wilson, K. M. (1980). Maternal and fetal sequelae of anticoagulation during pregnancy. *The American Journal of Medicine , 68*, S. 122-140.

Hanania, G., Thomas, D., Michel, P., Garbarz, E., Age, C., Millaire, A., et al. (1994). Grosseses chez les porteuses de prothèses valvulaires. Étude coopérative rétrospective française (15 cas). *Arch. Mal. Coeur, 87*, S. 429-437.

Hung, L., & Rahimtoola, S. H. (2003). Prosthetic heart valves and pregnancy. *Circulation , 107*, S. 1240-1246.

Iturbe-Alessio, I., del Carmen Fonseca, M., Mutchinik, O., Santos, M. A., Zajarias, A., & Salazar, E. (1986). Risks of anticoagulant therapy in pregnant women with artificial heart valves. *The New England Journal of Medicine , 315* (22), S. 1390-1393..

Khamooshi, A. J., Kashfi, F., Hoseini, S., Tabatabaei, M. B., Javadpour, H., & Noohi, F. (2007). Anticoagulation for prosthetic heart valves in pregnancy. Is there an answer? *Asian Cardiovascular & Thoracic Annals, 115* (6), S. 493-496.

Kort, H., & Cassel,, G. (1981).. An appraisal of warfarin therapy during pregnancy. *South African medical Journal, 60*, S. 578-579.

Larea, J. L., Núñez, L., Reque, J. A., Aguado, M. GG, Mataros,, R., & Minguez, J. A. (1983). Pregnancy and mechanical valve prostheses: a high-risk situation for the mother and the foetus. *Ann. Thoracic Surgery , 36*, S. 4459-463.

Lécuru, F., Desnos, M., & Taurelle, R. (1 996). Anticoagulant therapy in pregnancy. Report of 54 cases. *Acta Obstetricia et Gynecooogica Scandinavica , 75*, S. 217-221.

Lee, C.-N., Wu, C.-C., Lin, P.-Y., Hsieh, F.-J.,& Chen, H.-Y. (1994). Pregnancy following cardiac prosthetic valve replacement. *Obstetrics && Gynecology , 83* (3), S. 353-3566.

Meschengieser, S., Fondevila,, C., Santarelli, M., & Lazzari, M. (1999). Anticoagulation in pregnant women with mechanical heart valve prostheses. *Heart, 82*, S.23-26.

Nybo Andersen, A.-M., Wohlfahrt, J., Christens, P., Olsen, J., & Melbye, M. (2000). Maternal age and fetal loss : population based register linkage study. *BM , 320* (7251), S. 1708-1712.

Oakley, C. M. (1995). Anticoagulants in pregnancy. *British Heart Journal , 74*, S. 107-111.

Oakley, C., & Doherty, P. (1976). Pregnancy in patients after valve replacement. *British Heart*

Journal , 38, S. 1140-1148.

Pauli, R. M. (1988)). Mechanism of bone and cartilage maldevelopment in the Warfarin embryopathy. *Pathology & Immunopathology Research , 7*, S. 107-112.

Pavankumar, P., Venugopal, P., Kaul, U., Iyer, K., Das, B., Sampathkumar, A., et al. ((1988). Pregnancy in patients with prosthetic cardiac valve. *Scandinavian Journal of Thoracic & Cardiovascular Surgery, 22*, S. 19-22.

Peters, P. W. (2006). Vitamin K antagonists in pregnancy: An overestimated risk? *Thrombosis and Haemostasis , 95*, S. 922-923.

Sadler, L., McCowan, L., White, H., Stewart, A., Bracken, M., & North, R. (2000). Pregnancy outcomes and cardiac complications in women with mechanical, bioprosthetic and homograft valves. *BJOG , 107* (2), S. 245-253.

Salazar, E., Izaguirre, R., Verdejo, J., & Mutchinick,, O. (1996). Failure of adjusted doses of subcutaneous heparin to prevent thromboembolic Phenomena in pregnant patients with mechanical cardiac valve prostheses. *JACC , 27*, S. 1698-1703.

Sareli, P., England, M. J., Berk, M. R., Marcus, R. H., Epstein, M., Driscoll, J., et al. (1989). Maternal and fetal sequelae of anticoagulation during pregnancy in patients with mechanical heart valve prostheses. *The American Journal of Cardiology, 63*, S. 1462-1465.

Sbarouni, E., & Oakley, C. (1994). Outcome of pregnancy in women with valve prostheses. *British Heart Journal, 27*, S. 196-201.

Schaefer, C., Hanneman, D., Meister, R., Eléfant, E., Paulus, W., Vial, T., et al. (2006). Vitamin K antagonists and pregancy outcome. *Thrombosis and Haemostasis , 95*, S. 949 957.

Shannon, M. S., Edwards, M.-B., Long, F., Taylor, K. M., Bagger, J. P., & De Swiet, M. (2008). Anticoagulant management of pregnancy following heart valve replacement in the United Kingdom, 1986-2002. *Journal of Heart Valve Disease , 17* (5), S. 526-532.

Siguret, V., Pautas, E. & Gouin-Thibault,, I. (2008). Warfarin therapy: Influence of pharmacogenetic and environmental factors on the anticoagulant response to Warfarin. In: Litwack, G., Vitamin K, Academic Press, ISBN: 978-0-12-374113-4.

Srivastava, A. K., Gupta, A. K., Singh, A. V. & Husain, T. (2002). Effect of oral anticoagulant during pregnancy with prosthetic heart valve. *Asian Cardiovascular && Thoracic Annals , 10* (4), S . 306-309.

Srivastava, A. R., Modi, P., Sahi, S., Niwariya, Y., Singh, H., & Banerje, A. (2007). Anticoagulation for pregnant patients with mechanical heart valves. *Annals of Cardiac Anaesthesia , 100*, S. 95-107.

van Driel, D., Wesseling, J., Sauer, P. J., Touwen, B. C., van der Veer, E., & Heymans, H. S. (2002). Teratogen update: Fetal effects after in utero exposure to coumarins. Overview of cases, follow-up findings, and pathogenesis. *Teratology , 66*, S. 127-140.

van Driel, D., Wesseling, J., Sauer, P. J., van der Veer, E., Touwen, B. C., & Smrkovsky, M. (2001). In utero exposure to coumarins and cognition at 8 to 14 years old. *Pediatrics , 107* (1), S. 123-129.

Vitale, N., De Feo,, M., De Santo, L. S., Pollice, A., Tedesco, N., & Cotrufoo, M. (1999). Dose-dependent fetal complications of warfarin in pregnant women with mechanical heart valves. *Journal of the Amerrican College of Cardiology , 33* (6), S. 1637-1641.

Vitali, E., Donatelli, M., Quaini, E., Groppelli, G., & Pellegrini,, A. (1986). Pregnancy in patients with mechanical prosthetic heart valves. *J Cardiovasc Surg , 27*, S. 221-227.

Wellesley, D., Moore, I., Heard, M., & Keeton, B. (1998). Two cases of warfarin embryopathy: a re-emergence of this condition? *British Journal of Obstetrics and Gynaecology , 105*, S. 805--806.

Wong, V., Cheng, C., & Chan, K. (1993). Fetal and neonatal outcome of exposure to anticoagulants during pregnancy. *American Journal of Medical Genetics , 45*, S. 17-21.

Zurawski, J. M., & Kelly, E. A. (1997). Pregnancy outcome after maternal poisoning with Brodifacou, a long-acting Warfarin-like rodenticide. *Obstetrics & Gynecology , 90* (4), S. 672-674.