Committee for Risk Assessment

RAC

Annex 1

Background document

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

Melamine

EC Number: 203-615-4
CAS Number: 108-78-1

CLH-O-0000006932-69-01/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

10 December 2020

CLH report

Proposal for Harmonised Classification and Labelling

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

International Chemical Identification:

1,3,5-triazine-2,4,6-triamine; Melamine

EC Number: **203-615-4**

CAS Number: **108-78-1**

Index Number: **-**

Contact details for dossier submitter:

Federal Institute for Occupational Safety and Health (BAuA)
Federal Office for Chemicals
Friedrich-Henkel-Weg 1-25
D-44149 Dortmund, Germany
ChemG@baua.bund.de

Version number: 1.0 **Date: November 2019**

CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	1,3,5-triazine-2,4,6-triamine
Other names (usual name, trade name, abbreviation)	Melamine
ISO common name (if available and appropriate)	
EC number (if available and appropriate)	203-615-4
EC name (if available and appropriate)	1,3,5-Triazine-2,4,6-triamine
CAS number (if available)	108-78-1
Other identity code (if available)	
Molecular formula	$C_3H_6N_6$
Structural formula	
SMILES notation (if available)	Nc1nc(N)nc(N)n1
Molecular weight or molecular weight range	126.1199
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	
Description of the manufacturing process and identity of the source (for UVCB substances only)	
Degree of purity (%) (if relevant for the entry in Annex VI)	99.8-100 % w/w

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)
1,3,5-triazine-2,4,6-triamine	99.8-100 % w/w		

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
-				

Please refer to the confidential annex.

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
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2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 5:

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBA	1,3,5-triazine-2,4,6-triamine	203-615-4	108-78-1	Carc. 2 STOT RE 1	H351 H372 (urinary tract)	GHS08 Danger	H351 H372 (urinary tract)			
Resulting Annex VI entry if agreed by RAC and COM	TBA	1,3,5-triazine-2,4,6-triamine	203-615-4	108-78-1	Carc. 2 STOT RE 1	H351 H372 (urinary tract)	GHS08 Danger	H351 H372 (urinary tract)			

Table 6: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)	hazard class not assessed in this dossier	No
Oxidising gases	hazard class not assessed in this dossier	No
Gases under pressure	hazard class not assessed in this dossier	No
Flammable liquids	hazard class not assessed in this dossier	No
Flammable solids	hazard class not assessed in this dossier	No
Self-reactive substances	hazard class not assessed in this dossier	No
Pyrophoric liquids	hazard class not assessed in this dossier	No
Pyrophoric solids	hazard class not assessed in this dossier	No
Self-heating substances	hazard class not assessed in this dossier	No
Substances which in contact with water emit flammable gases	hazard class not assessed in this dossier	No
Oxidising liquids	hazard class not assessed in this dossier	No
Oxidising solids	hazard class not assessed in this dossier	No
Organic peroxides	hazard class not assessed in this dossier	No
Corrosive to metals	hazard class not assessed in this dossier	No
Acute toxicity via oral route	hazard class not assessed in this dossier	No
Acute toxicity via dermal route	hazard class not assessed in this dossier	No
Acute toxicity via inhalation route	hazard class not assessed in this dossier	No
Skin corrosion/irritation	hazard class not assessed in this dossier	No
Serious eye damage/eye irritation	hazard class not assessed in this dossier	No
Respiratory sensitisation	hazard class not assessed in this dossier	No
Skin sensitisation	hazard class not assessed in this dossier	No
Germ cell mutagenicity	**data conclusive but not sufficient for classification**	**Yes**
Carcinogenicity	**harmonised classification proposed**	**Yes**
Reproductive toxicity	hazard class not assessed in this dossier	No
Specific target organ toxicity-single exposure	hazard class not assessed in this dossier	No
Specific target organ toxicity-repeated exposure	**harmonised classification proposed**	**Yes**
Aspiration hazard	hazard class not assessed in this dossier	No
Hazardous to the aquatic environment	hazard class not assessed in this dossier	No
Hazardous to the ozone layer	hazard class not assessed in this dossier	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Melamine (1,3,5-triazine-2,4,6-triamine) is neither listed in the Annex VI of the CLP Regulation (EC) No 1272/2008 of the European Parliament and of the Council (latest consolidated version: 26.07.2017),

nor has a proposal for a Harmonised Classification and Labelling in Annex VI of the CLP previously been submitted for this substance. Melamine has been registered under REACH.

RAC general comment

Melamine has no existing entry to the CLP regulation. The proposal from the dossier submitter (DS) addressed the following endpoints: STOT RE, germ cell mutagenicity and carcinogenicity.

In the opinion, 'calculus' and 'stone' are used synonymously, likewise 'transitional cell epithelium' is used synonymously with 'urothelium'. Urolithiasis, nephrolithiasis and renal/kidney stones refer to the presence of calculi/stones in the urinary tract.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

None of the notifiers and/or registrants has self-classified melamine as STOT RE 1. The data assessed and discussed in the current CLH dossier, however, support classification in category STOT RE 1 (section 10.11). Thus, a justification that action is needed at community level is given due to disagreement of the dossier submitter with the current self-classification by the notifiers and/or registrants.

5 IDENTIFIED USES

This substance is used in articles, by professional workers (widespread uses), in formulation or re-packing, at industrial sites and in manufacturing.

6 DATA SOURCES

Sources: PUBMED, SCOPUS, WEB OF SCIENCE, ScienceDIRECT, Wiley, ECHA dissemination site, EMBASE, IUCLID (Reg data), OECD sids, IARC, Scifinder

7 PHYSICOCHEMICAL PROPERTIES

Table 7: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20 °C and 101,3 kPa	Solid white powder	Reuse P. and Halzschuh O.; 2009	Visual observation
Melting/freezing point	361°C	Reuse P. and Halzschuh O.; 2009	EU Method A.1
Boiling point	Decomposition and sublimation occur at temperatures close to and above the melting temperature.		Data waiving according to Annex VII 7.3
Relative density	1.57 at 20°C	Reuse P. and	EU Method A.3

Property	Value	Reference	Comment (e.g. measured or estimated)
		Halzschuh O.; 2009	
Vapour pressure	The study does not need to be conducted if the melting point is above 300 °C.		Data waiving according to Annex VII 7.5
Surface tension	Melamine has not both polar and non-polar groups, which are considered to be necessary for surfactant properties (from the guidance document). Surface activity is not a desired property of melamine.		Data waiving according to Annex VII 7.6
Water solubility	3.48 g/L at 20°C and pH 7.7	Reuse P. and Halzschuh O.; 2009	EU Method A.6
Partition coefficient n-octanol/water	Log Pow -1.22 at 22°C	Junghans M.; 2009	EU Method A.8
Flash point			
Flammability			
Explosive properties			
Self-ignition temperature			
Oxidising properties			
Granulometry	The various products under this joint submission are fine powders with a mass median diameter below 100 µm, except for the Cytec product (Rich 2010) with a mass median diameter of 120 µm.		Inter alia sieve analysis, dry powder laser diffraction,
Stability in organic solvents and identity of relevant degradation products	The stability of melamine is high and is not considered to be critical.		Data waiving according to Annex IX 7.15
Dissociation constant	pK_{b1} = 7.3 and pK_{b2} = 11.4.	Reuse P. and Halzschuh O.; 2009	OECD Guideline 112
Viscosity	Melamine is a solid. Therefore the determination of the viscosity is technically not feasible.		

8 EVALUATION OF PHYSICAL HAZARDS

Not assessed in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 8: Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
Toxicokinetic (AMDE) study, similar to OECD TG 417 Investigation of absorption (blood plasma), metabolism (analysis profile), distribution (investigated tissues: plasma, blood, liver, kidney, bladder), and excretion (urine, breath, faeces) Rats (Fisher 344) Oral (gavage) Male 3-4 animals/group Single oral dose: appr. 1.3 mg/kg bw); 0.025 mCi/rat (14C labelled melamine) Distribution study: rats were killed 0.5, 1, 4, 8, 24, 48 and 96 h after dosing. Vehicle: water	**Absorption:** • Rapid, maximum plasma level within less than 60 min **Metabolism:** • No metabolism indicated **Distribution:** • Distribution to liver, plasma, blood, kidney, and urinary bladder • Melamine levels in liver, and blood were similar to plasma • Melamine levels in the kidney were 2 – 3 times higher as compared to plasma (presumably due to renal concentration) • Melamine levels in the urinary bladder were 26.4 times higher as compared to plasma after 4 h (presumably back diffusion or contamination of the tissue by urine) **Excretion:** • Short plasma half-life: 2.7 h • Fast excretion via urine (93 %) by 96 h (about 90 % by 24 h) • Marginal excretion with breath (0.2 % by 96 h) and faeces (0.64 % by 96 h) • Urinary elimination half-life 3.0 h	**Considered reliable with restrictions** **Restrictions:** no GLP, only one dose level tested without justification, for distribution experiment only 3 animals used Key study **Test material:** melamine (14C labelled; 98.4 % radiochemically pure)	Mast et al. (1983)
Toxicokinetic study, no guideline followed Investigation of plasma related toxicokinetic parameters Pigs (Landrace-Yorkshire) Intravenous (ear vein) 5 animals (8 - 10 weeks) (no data on sex) Single dose: 6.13 mg/kg bw Sampling of plasma: 0, 1, 2, 4, 8, 12, 24, 36 and 48 h post-dosing Vehicle: not indicated	**Absorption:** • Data indicate rapid absorption **Distribution:** • Steady state volume of distribution: 0.61 L/kg • Steady state volume of distribution close to body water indicating that substance is not bound to blood components or tissues **Excretion** • Plasma t ½: 4.07 h • Data indicate fast plasma elimination	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, only plasma analysed Supporting study **Test material:** melamine (99 % pure)	Baynes et al. (2008)

Method	Results	Remarks	Reference
Toxicokinetic study, no guideline followed Investigation of plasma-related toxicokinetic parameters Rats (Sprague-Dawley) Intravenous and oral 3 male rats/group Single dose: 5 mg/kg bw (oral) 2 mg/kg bw (intravenous) Sampling of blood: 0, 0.5, 1, 2, 3, 6, 8, 24 post-dosing (oral) 0, 5, 15, 30 min and 1, 2, 4, 6 ,8 ,12 and 24 h post-dosing (intravenous) Vehicle: not indicated	**Absorption:** • Cmax was reached within 1 h, indicating rapid absorption after oral treatment **Distribution:** • Steady state volume of distribution: 102 ml/kg • Steady state volume of distribution less than rat total body water but larger than blood volume, indicating that melamine is predominantly restricted to blood and not extensively distributed to most organ tissues **Excretion:** • Plasma t ½: 4.9 h (intravenous), 3.9 h (oral) • After 24 h nearly all melamine eliminated from plasma (oral and intravenous)	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, only plasma analysed, only 3 animals/group, no data on substance purity Supporting study **Test material:** melamine (unknown purity; melamine reference standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China))	Yang et al. (2009)
Toxicokinetic study, no guideline followed Investigation of plasma-related toxicokinetic parameters, excretion in urine, metabolisation to cyanuric acid Monkey (Rhesus) Oral (intragastrically) 1 female and 2 males Single dose: 1.4 mg/kg bw Sampling: Blood: 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48 h post dosing Urine: every 24 h (0 - 24 h, 24 - 48 h, 48 – 72 h) Vehicle: glycerol solution Analysis: LC-MS/MS (melamine and cyanuric acid)	**Absorption:** • Tmax: 2.6 h • Data indicate rapid absorption **Metabolism:** • Elimination profiles of melamine and cyanuric acid did not correlate • Background cyanuric acid concentration was observed (about 1 µg) • Taken as indication that melamine is not metabolised to cyanuric acid **Elimination/excretion:** • Plasma t ½: 4.4 h • Fast elimination from plasma: after 36 h post–dosing, melamine concentration lower than quantification limit • Elimination from urine slower than from plasma but no exact conclusion on time-dependent profile presumably due to aggregated sampling (24 h intervals)	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, only plasma and urine analysed, only 3 animals/group, Supporting study **Test material:** melamine (99 % pure)	Liu et al. (2010a)

Method	Results	Remarks	Reference
Toxicokinetic study, no guideline followed Investigation of plasma-related toxicokinetic parameters Rat (Sprague-Dawley) Oral (gavage) 4 males, 4 females Single dose: 100 mg/kg bw Sampling of blood (vena orbitalis): 0, 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24 h post-dosing Vehicle: sodium carboxymethyl cellulose Analysis method: LC-MS/MS	**Absorption:** • Tmax: 1.21 h • Data indicate rapid absorption **Distribution:** • Volume of distribution: 1.3 L/kg) • Mainly distributed in body fluids with low volume of distribution **Elimination/Excretion:** • Plasma t ½: 2.51 h • Fast elimination from plasma; after about 12 h post-dosing, melamine concentration at about quantification limit in plasma	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, no other tissues than plasma Supporting study **Test material:** melamine (> 99 % pure)	Pang et al. (2013)
Toxicokinetic study, no guideline followed Concentration-time profile in heart, liver, spleen, lung, kidneys, urinary bladder, plasma, urine, feces Rat (Sprague-Dawley) Oral Females 4 animals per sampling time point Single dose: 1000 mg/kg bw Sampling of tissues, faeces and urine at 0, 12, 24, 48, 72, 96, 120, 144 and 168 h post-dosing Vehicle: not indicated Frozen tissues were pulverised under liquid nitrogen; formic acid added before analysis Analysis method: LC-MS/MS	**Absorption:** Plasma: • Increasing within 24 h after dosing, rapid declining up to 48 h (nearly 0 µg/ml), still detectable at 168 h at very low levels, Tmax approx. 24 h **Distribution:** • Fast distribution into all tissues Heart: • Increasing until 24 h after dosing, rapid declining up to 96 h (limit of detection at 96 h) Liver: • Increasing until 12 h after dosing, rapid declining up to 72 h (below limit of detection at 96 h) Spleen: • increasing until 24 h after dosing, rapid declining up to 72 h, (below limit of detection at 96 h) Lung: • Increasing until 12 h after dosing, rapid declining up to 72 h (below limit of detection at 96 h)	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, no information on toxic effects at the dose tested Supporting study **Test material:** melamine (> 99 % pure)	Wu et al. (2011)

Method	Results	Remarks	Reference
	Kidney: • Increasing until 24 h after dosing, rapid declining up to 72 h (below limit of detection at 96 h) Bladder: • Increasing until 24 h after dosing, rapid declining up to 72 h (below limit of detection at 120 h) **Excretion:** Faeces: increasing within 24 h after dosing, rapid declining up to 48 h (nearly 0 mg/g), still detectable at 168 h at very low levels; 61 % clearance at 24 h; high concentrations in faeces (10 mg/g) Urine: increasing within 24 h after dosing, rapid declining up to 48 h (nearly 0 µg/ml); still detectable at 168 h at very low levels; 25 % clearance at 24 h • 97 % of ingested melamine excreted at 24 h via urine and faeces		
Toxicokinetic study, no guideline followed Investigation of plasma-related toxicokinetic parameters such as Tmax, and t ½ Rat (Fisher 344) Oral (gavage) 6 males, 6 females Single dose: 1 mg/kg bw Sampling of blood (tail vein): 0, 0.5, 1, 2, 3, 4, 5, 6, 8 h post-dosing Vehicle: Carboxymethyl cellulose Analysis method: UPLC-MS/MS	**Absorption:** Males: Tmax: 1.0 h Females: Tmax: 0.75 h • Indicating fast absorption **Elimination:** Males: t ½ plasma: 1.62 h Females: t ½ plasma: 1.92 h • Indicating fast elimination	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, no other tissues than plasma, sampling no longer than 8h Supporting study **Test material:** melamine (> 99 % pure)	Jacob et al. (2012)

Method	Results	Remarks	Reference
Repeated dose toxicity study, no guideline followed, investigation of distribution into different organs Rat (Wistar) Oral (gavage) 3 males, 3 females Dose: 180 mg/kg bw/d for 28 days (daily doses) Collection of organs at 28 days after initiation of treatment and quantification of melamine Organs collected: kidney, liver, stomach, spleen, heart, uterus, ovaries, and testis Vehicle: not specified Analysis method: HPLC-MS	**Distribution:** • Distribution of melamine into kidney, liver, stomach, spleen, heart, uterus, ovaries, and testis was observed and steady state concentrations for melamine determined • The highest concentration compared to other organs analysed was found in the kidney (spleen: 4.1 ng/g, kidney: 11.83 ng/g, uterus: 9.3 ng/g, heart: 5.13 ng/g, testes: 4.57 ng/g, stomach: 4.1 ng/g, liver: 6.8 ng/g)	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, only distribution into some organs was investigated (no other toxicokinetic parameters), no data on purity Supporting study **Test material:** melamine (unknown purity; purchased from Sinopharm Chemical Reagent Co. (Shanghai, China))	Sun et al. (2016)
Distribution study, no guideline followed Rat (Sprague-Dawley) Intravenous (femoral vein) 5 males Single dose: 10 mg/kg bw Collection of organs 30 min after initiating of treatment and quantification of melamine, sampling of blood by cardiac puncture Organs collected: liver, kidney, spleen, urinary bladder, and brain Vehicle: sodium chloride solution Analysis method: HPLC-MS/MS	**Distribution:** Melamine levels in organs: • Plasma: 10.12 ± 1.6 µg/mL, • Liver: 4.18 ± 0.61 µg/g, (41.3 % of plasma) • Kidney: 19.48 ± 2.75 µg/g (192.5 % of plasma) • Spleen: 4.89 ± 0.78 µg/g (48.4 % of plasma) • Bladder: 1.74 ± 1.1 µg/g (17.2 % of plasma) • Brain tissues: 0.47 ± 0.20 to 1.18 ± 0.25 µg/g (4.6 to 11.7 % of plasma) The kidney had the highest melamine level	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, only distribution into some organs was investigated (no other toxicokinetic parameters), sampling only at one time point (30 min) after dosing Supporting study **Test material:** melamine (99 %)	Wu et al. (2009b)
Toxicokinetic study, no guideline followed Investigation of absorption, distribution, and excretion in melamine-treated sheep Sheep (Döhne-Merino) Oral (feed)	**Absorption:** • Significant melamine concentration in serum 2 d after beginning of treatment • Significant increase in concentrations from day 3 to 8 **Distribution:** • Melamine detected in muscle, liver, kidney, and	**Considered reliable with restrictions** **Restrictions:** no radiolabelling of melamine, only distribution into some organs was investigated, no data on purity	Cruywagen et al. (2011)

Method	Results	Remarks	Reference
6 males Daily dose: 11.6 mg/kg bw/d Treatment for 8 consecutive days • Collection of blood samples on day 1 (before treatment), 3, 6 and, 8 • Collection of urine and faeces quantitatively over 8-d period • All animals were slaughtered after 8 days, samples of muscle, liver, kidneys (left kidney) and, abdominal fat were taken and analysed Vehicle: corn gluten meal Analysis method: LC-MS/MS	abdominal fat • Content in muscle and kidney similar, about three times higher compared to liver, in fat lowest melamine level (about 200-fold less than the average of muscle and kidney) • No melamine detected in samples of control sheep **Excretion:** • 54.1 % of ingested melamine was excreted via urine • 23.7 % of ingested melamine was excreted via faeces	Supporting study **Test material:** melamine (unknown purity)	

Table 9: Summary table of human studies relevant for toxicokinetics

Method	Results	Remarks	Reference
Excretion study in exposed human volunteers Investigation of melamine excretion in urine from volunteers served hot noodle soup in melamine bowls 3 women and 3 men After 8h fasting time 500 ml hot noodle soup (initial temperature, 90 °C) in melamine bowl as a 30-minute breakfast Negative control: 3 women and 3 men served noodle soup in ceramic bowls Sampling of urine before soup consumption and in 2 h intervals after consumption until 12 h after start of experiment Analysis of melamine in urine: LC/MS	• Background melamine levels similar for ceramic and melamine bowls in urine samples prior to soup consumption • Total mean melamine excretion in urine for 12 h was 8.35 µg in melamine bowls and 1.31 µg in ceramic bowls (statistically significant difference) • Ceramic bowls: for 12 h melamine levels comparable to background level • Melamine bowls: increase in melamine concentration in urine until about 6 h after consumption, decline after that until end of experiment • Estimated half-life: 6 h	**Considered reliable with restrictions** **Limited evidence** **Restrictions:** test material unclear, no data on actual ingested dose levels, no data on melamine content in consumed noodle soup Supporting study **Test material:** unclear (melamine migrated from melamine resin plastic bowls)	Wu et al. (2013)

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

Toxicokinetic studies fully compliant with a standardised guideline such as OECD Test Guideline TG 417 and GLP were not available for melamine.

However, the toxicokinetic study by **Mast et al. (1983)** was performed similar to OECD TG 417 and is considered as key toxicokinetics study for melamine. Male Fisher 344 rats were orally treated with a single dose of 1.3 mg/kg bw ^{14}C-labelled melamine. Absorption, metabolism, distribution (in plasma, blood, liver, kidney, urinary bladder), and excretion of melamine were investigated. In addition, there exist several experimental *in vivo* toxicokinetic studies which were not performed in accordance with a standardised guideline but were considered as relevant and reliable supporting studies. These studies are documented in Table 8 and in the technical dossier. The results of the key and supporting studies are discussed below with respect to absorption, metabolism, distribution, and excretion.

Absorption

Melamine is a small (126.12 g/mol) polar substance (log P (octanol-water): -1.37) and a fast absorption can be expected. Indeed, a rapid absorption after oral administration was observed in rats in the key study by **Mast et al. (1983)**. Maximum plasma levels were found within less than 60 min after dosing. This is supported by findings in other toxicokinetic studies in rats (Jacob et al., 2012; Pang et al., 2013; Yang et al., 2011) where Tmax values of about 1 h were obtained and by a study in monkeys (Liu et al., 2010a) with a Tmax value of about 2.6 h.

Metabolism

There are only a few studies available where metabolism of melamine had been investigated. In the key study by **Mast et al. (1983)** in rats, no metabolism of melamine was indicated.

While detailed information concerning pharmacokinetics in humans is not available, melamine was, similar to observations in animals, detected unmetabolised in the urine of paediatric patients that had been exposed to melamine-tainted milk products (Cheng et al., 2009; Kong et al., 2011; Lam et al., 2009; Zhang et al., 2010b).

Distribution

In the key study by **Mast et al. (1983)**, distribution of radiolabelled melamine in rats to liver, kidney, and bladder at different time points after dosing (0.5 h to 96 h) was investigated. They found a fast distribution to these tissues with no enrichment. Highest concentrations were detected in the kidney and the urinary bladder. The authors concluded that the observed elevated kidney levels were probably due to renal concentration of the melamine prior to its urinary excretion and assumed that the elevated bladder content is caused by back diffusion or contamination of the tissue by urine. The results further indicate a fast melamine excretion from tissues. 96 h post-dosing, melamine was detectable in the liver and the kidney only at very low concentrations.

There are several studies which support the results obtained by **Mast et al. (1983)**. Distribution into different organs has been additionally observed by toxicokinetic studies (Wu et al. (2011), Wu et al. (2009b), Sun et al. (2016) and Cruywagen et al. (2011)). **Wu et al. (2011)** found a distribution into heart, liver, spleen, lungs, kidney, and urinary bladder with peak concentrations 12 or 24 h post-dosing after treatment of rats with a single high dose (1000 mg/kg bw) of melamine. **Wu et al. (2009b)** observed rapid distribution into liver, spleen, kidney, urinary bladder, and brain already within 30 min after dosing when rats were treated intravenously with a single melamine dose (10 mg/kg bw). **Sun et al. (2016)** found a distribution and steady-state concentrations of melamine in an oral 28 d repeated dose study in rats with a dose of 180 mg/kg bw/d in all organs investigated, namely kidney, liver, stomach, spleen, heart, uterus, ovaries, and testis. **Cruywagen et al. (2011)** detected melamine in muscle, liver, kidney, and abdominal fat in orally treated sheep. Hereby, except for **Wu et al. (2011)**, the results of the studies support that the kidney is one of the organs with the highest melamine levels. Results from several studies also indicate a fast elimination of melamine from tissues and that melamine enrichment in tissues is unlikely. **Wu et al. (2011)** found a fast decline of melamine concentration to the limit of detection (LOD) in many tissues mostly within 72 or 96 h post-

dosing. Here, melamine was longest detectable in the urinary bladder (until 120 h post-dosing) and plasma (168 h post-dosing) at very low concentrations. Low volume of distribution estimated in toxicokinetic studies by **Yang et al. (2009)** and **Pang et al. (2013)** in melamine-treated rats and by **Baynes et al. (2008)** in pigs provide additional information that melamine is not enriched in tissues.

In summary, the results regarding endogenous melamine distribution derived from key and supporting toxicokinetic studies indicate a fast distribution of melamine to most tissues (including kidney, liver, stomach, spleen, heart, uterus, ovaries, testis, brain, bladder, and lungs), an unlikely enrichment in tissues and that the kidney is one of the organs with the highest detected melamine concentrations.

Elimination

In the key study by **Mast et al. (1983)**, a fast clearance from plasma with a plasma half-time of 2.7 h and a fast excretion from the whole body were found. About 90 % of the dose was excreted 24 h post-dosing. The urinary excretion was found to be the sole route of elimination with a fast elimination half-life of 3.0 h.

At higher dose levels (Wu et al., 2011), excretion via faeces becomes predominant compared to excretion via urine (61 versus 25 % 24 h after dosing). In a study with sheep (Cruywagen et al., 2011) with a lower dose of 11.6 mg/kg bw/d for 8 consecutive days, the urine was the main excretion route supporting the results by **Mast et al. (1983)**. But excretion via faeces to a lower extent was observed as well.

Fast clearance from plasma with plasma half-times ranging from 1.62 h to 4.4 h and plasma elimination ranging from 12 to 36 h post-dosing were also observed in other supporting oral studies in rats, pigs, and monkeys (Baynes et al., 2008; Jacob et al., 2012; Liu et al., 2010a; Pang et al., 2013; Yang et al., 2009).

A fast excretion from the whole body, > 90 % at 24 h post-dosing, was observed in rats also after treatment with a high single dose of 1000 mg/kg bw (Wu et al., 2011). Nevertheless, very low levels of melamine were still detectable at 168 h post-dosing in plasma, urine, and faeces.

In a randomized crossover human study **Wu et al. (2013)** that investigated urinary melamine excretion subsequent to low-dose melamine exposure (migration from melamine resin plastic bowls), an estimated half-life of urinary melamine elimination was observed at approximately 6 h. Hence, as melamine undergoes rapid renal clearance in multiple mammalian species it appears likely that humans show a similar pharmacokinetics.

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

Hazard class not assessed in this dossier

10.2 Acute toxicity - dermal route

Hazard class not assessed in this dossier

10.3 Acute toxicity - inhalation route

Hazard class not assessed in this dossier

10.4 Serious eye damage/eye irritation

Hazard class not assessed in this dossier

10.5 Respiratory sensitisation

Hazard class not assessed in this dossier

10.6 Skin sensitisation

Hazard class not assessed in this dossier

10.7 Germ cell mutagenicity

Table 10: Summary table of mutagenicity/genotoxicity tests *in vitro*

Method, guideline, deviations if any	**Test substance**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
Bacterial gene mutation test Similar to OECD TG 471 GLP: yes Deviation: • Neither a *E.coli* WP2 strain nor the *Salmonella typhimrium* tester strain TA102 has been tested	**Melamine** Purity: no detailed information (melamine was provided by sponsor, purity information was given to test laboratory but not described in study report)	**Supporting study** **Reliable with restrictions** *Salmonella typhimurium* tester strains: TA100, TA98, TA1537, TA1535, TA1538 Test concentrations (with and without metabolic activation (S9 mix)): 0, 50, 100, 500, 1000, 2500, 5000 µg/plate Vehicle: DMSO Negative control: yes Positive control: yes	**Negative** (with and without metabolic activation) Cytotoxicity: no Precipitations: no Controls: valid negative (solvent control) and positive controls	Raltech Scientific Services (1981b)
Bacterial gene mutation test	**Melamine** Purity: no	**Supporting study**	**Negative** (with and without metabolic	NTP (1983) (cited also in

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Similar to OECD TG 471 GLP: no Deviations: • Neither a *E.coli* WP2 strain nor the *Salmonella typhimrium* strain TA102 has been tested • Justification for top dose (3333 µg/plate instead of 5000 µg/plate) in test without metabolic activation not considered sufficient (no information on higher concentrations)	detailed information (Reagent, purchased from Fisher)	**Reliable with restrictions** *Salmonella typhimurium* tester strains: TA100, TA98, TA1537, TA1535 Test concentrations (without metabolic activation): 0, 3.3, 10, 33, 100, 111, 333, 1000, 1111, 3333 µg/plate (justification for top dose without metabolic activation: melamine soluble and not cytotoxic) Test concentrations (with and without metabolic activation (S9 mix)): 0, 3.3, 10, 33, 100, 111, 333,1000, 1111, 3333, 5550 µg/plate Vehicle: DMSO Negative control: yes Positive controls: yes	activation) Cytotoxicity: no Precipitations: no Controls: valid negative (solvent control) and positive controls	Haworth et al. (1983)
Bacterial gene mutation test Similar to OECD TG 471 GLP: no Deviations: • Justification for top dose (500 µg /plate instead of 5000 µg/plate) considered to be insufficient (no information on higher concentrations)	**Melamine** Purity: no detailed information (melamine was provided by sponsor, purity information was given to test laboratory but not described in study report)	**Supporting study** **Reliable with restrictions** *Salmonella typhimurium* tester strains: TA100, TA98, TA1537, TA1535, TA 1538 *Escherichia coli* tester strain: WP2 uvrA Test concentrations (with and without metabolic activation (S9 mix)): 0, 0.1, 1.0, 10, 100, 500 µg/plate (justification for top dose: melamine soluble and not cytotoxic) Vehicle: DMSO Negative control: yes Positive controls: yes	**Negative** (with and without metabolic activation) Cytotoxicity: no Precipitations: no Controls: valid negative (solvent control) and positive controls	Litton Bionetics Inc. (1977)
Bacterial gene mutation test Similar to OECD	**Melamine** Purity: no detailed	**Supporting study** **(reliable with**	**Negative** (results with and without	Zhang et al. (2011)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
TG 471 GLP: no Deviations: • Recommended tester strain TA 1535 was not examined • No data on cytotoxicity • No data on vehicle	information (purchased from Sigma-Aldrich)	**restrictions)** *Salmonella typhimurium* tester strains: TA100, TA98, TA97, TA102 Test concentrations (with and without metabolic activation (S9 mix)): 0, 8.0, 40, 200, 1000, 5000 µg/plate Vehicle: no data Negative control: yes Positive controls: yes	metabolic activation) Cytotoxicity: no information Precipitations: no information Controls: valid negative and positive controls	
Bacterial gene mutation test Not similar to OECD TG 471 GLP: no Deviations: • Missing information on negative controls • Missing information on test concentrations • No details on results shown • Missing information on cytotoxicity	**Melamine** Purity: no information	**Disregarded study** **Not reliable** (Only overall information on negative result without any detailed information to allow a firm assessment of the study) *Salmonella typhimurium* tester strains: TA98 and TA100 Test concentrations (with and without metabolic activation (S9 mix)): no information Vehicle: no data Negative control: no data Positive controls: yes	**Negative** (no detailed results shown) Cytotoxicity: no information Precipitations: no information Controls: no detailed information * * about 255 substances were screened in the study, no individual results have been shown	Kubo et al. (2002)
Bacterial gene mutation test Not similar to OECD TG 471 GLP: no Deviations: • Missing information on negative and positive controls • Missing information on detailed test concentrations • No details on results shown	**Melamine** Purity: 99 %	**Disregarded study** **Not reliable** (Only overall information on negative result without any detailed information to allow a firm assessment of the study) *Salmonella typhimurium* tester strains: TA98, TA100, TA97, TA102 Test concentrations (with and without metabolic activation (S9 mix)): up to 5000 µg/plate	**Negative** (no detailed results shown) Cytotoxicity: no Precipitations: no information Controls: no data	Ishiwata et al. (1991)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
• Missing information on cytotoxicity		Vehicle: no data Negative control: no data Positive controls: no data		
Bacterial gene mutation test Not similar to OECD TG 471 GLP: no Deviations: • Missing information on negative and positive controls • Missing information on test design • No data on test concentrations • Missing information on cytotoxicity	**Melamine** Purity: no information	**Disregarded study** **Not reliable** (Only overall information on negative result without any detailed information to allow a firm assessment of the study) *Salmonella typhimurium* tester strains: TA1530, TA1531, TA1532, TA1534 No information if with or without metabolic activation Test concentration: no data Negative control: no data Positive controls: no data	**Negative** (no detailed results shown) Cytotoxicity: no information Precipitations: no information Controls: no data	Seiler (1973)
In vitro gene mutation study in mammalian cells (HPRT test) Similar to OECD TG 476 GLP: yes Deviation: • No justification why top dose tested was below 2 mg/ml as recommended in OECD TG 476 • No information on sampling time	**Melamine** Purity: no detailed information (melamine was provided by sponsor, purity information was given to laboratory but not described in study report)	**Supporting study** **Reliable with restrictions** CHO cells Test concentrations (with and without metabolic activation (S9 mix)): 5 concentrations between 0.6 and 1.0 mg/ml (exact concentrations confidential) Treatment time: 5 h Sampling time: no information Vehicle: DMSO Negative control: yes Positive controls: yes	**Negative** (with and without metabolic activation) **(≤ 1mg/ml, no information above)** Cytotoxicity: no Controls: valid negative (solvent control) and positive controls	Raltech Scientific Services (1981a) (cited in Mast et al. (1982))
In vitro gene mutation study in mammalian cells (MLA) Similar to OECD TG	**Melamine** Purity: no detailed information	**Supporting Study** **Reliable with restrictions** Mouse lymphoma L5178Y	**Negative** (with and without metabolic activation)	McGregor et al. (1988)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
490 GLP: no Deviation: • Colony size was not determined	(substance was supplied by National Toxicology Program Chemical Repository, Radian Corporation, Austin)	cells Test concentrations (without metabolic activation): 0,10, 20, 40, 80, 100, 120, 140, 160 µg/ml Test concentrations (with metabolic activation (S9 mix)): 0, 80, 100, 120, 140, 160 µg/ml (justification for top dose with and without S9 mix: poor solubility at higher concentrations in exposure medium) Treatment time: 4 h Sampling time: 48 h Vehicle: DMSO Negative control: yes Positive controls: yes	Cytotoxicity: no Controls: valid negative (solvent control) and positive controls	
In vitro mammalian chromosome aberration test Similar to guideline OECD TG 473 GLP: no information Deviations: • No short term exposure without metabolic activation • Short term exposure with metabolic activation too short (2 h instead of 3-6 h) • Only 100 instead of 300 metaphases scored	**Melamine** Purity: no detailed information (substance was supplied by National Toxicology Program Chemical Repository, Radian Corporation, Austin)	**Supporting study** **Reliable with restrictions** CHO cells Test concentrations (with and without metabolic activation (S9 mix)): 0, 240, 270, 300 µg/ml (Justification top dose: selected based on reduced growth by 50 %) Treatment time: • With S9:2 h • Without S9: continuously exposure Sampling time: 8-12 h after beginning of treatment (with/without S9) Vehicle: not specified (water, DMSO, ethanol or acetone) Negative control: yes Positive controls: yes	**Negative** (with and without metabolic activation) Cytotoxicity: no Controls: valid negative (solvent control) and positive controls	Galloway et al. (1987)

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
In vitro mammalian chromosome aberration test Not similar to guideline OECD TG 473 GLP: no Deviations: • Sampling after continuous exposure too early (4 h instead of 1.5 cell cycles) • No short term exposure (3-6 h) with and without metabolic activation • Only 100 instead of 300 metaphases scored • No justification why top dose tested was below 10 mM as recommended in OECD TG 473	**Melamine** Purity: no detailed information (purchased from Sigma-Aldrich)	**Disregarded study** **Not reliable** (It is not possible to conclude an overall negative outcome due to the lack of short term exposure with and without S9 mix and due to a too short sampling time after continuous exposure) CHO cells Test concentrations (with and without metabolic activation (S9 mix)): 0, 0.16, 0.8, 4 mM Treatment time: continuously for 24 and 48 h (with and without metabolic activation) Sampling times: 4 h after end of treatment Vehicle: no data Negative control: yes Positive controls: yes	**Negative** (with and without metabolic activation) Cytotoxicity: no Controls: valid negative (solvent control) and positive controls	Zhang et al. (2011)

Table 11: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells *in vivo*

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Mammalian erythrocyte micronucleus test Similar to OECD TG 474 GLP: yes Deviations: • Only 4 instead of 5 animals per group tested • Only one dose level included • No data on ratio of immature erythrocytes to total	**Melamine** Purity: no information	**Supporting study** **Reliable with restrictions** Species: CD1 mice; 4 males and 4 females per group Target organs: bone marrow Administration route: oral (gavage) Dose level: 0 and 1000 mg/kg bw/d	**Negative** • Negative results for all melamine treatment conditions Toxicity: • Single gavage (group I): no toxicity • Single gavage (group II): no toxicity in 7/8 mice, one female abnormal gait and ptosis • Second gavage (group III): abnormal	Pharmakon Research International (1981)

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
erythrocytes • Only 1000 instead of 4000 polychromatic erythrocytes screened		Justification for top dose: top dose selected was about the MTD chosen after preliminary test with doses 50, 166, 500, 1666.6, 5000 mg/kg bw/d (twice) Treatment: • Single gavage • Two consecutive daily gavages Sampling times: • 30 h after single gavage (group I) • 48 h after single gavage (group II) • 48 h after second gavage (group III) • 72 h after second gavage (group IV) Vehicle: distilled water Positive control: yes Negative control: yes	gait, decreased activity, ptosis, decreased body tone • Second gavage (group IV): abnormal gait, decreased activity, ptosis Cytotoxicity: no information Controls: valid negative (solvent control) and positive controls	
Mammalian erythrocyte micronucleus test Similar to OECD TG 474 GLP: not specified Deviations: • No information how many polychromatic erythrocytes were screened • No data on clinical signs • No data on cytotoxicity • No justification for intraperitoneal administration	**Melamine** Purity: no information	**Supporting study** **Reliable with restrictions** Species: B6C3F1 mice; 5 males/group Target organs: • bone marrow • peripheral blood Administration route: intraperitoneal injection (ip) Dose levels: 0, 500, 1000, 2000 mg/kg bw/d Treatment: one injection/day on 3 consecutive days Sampling time(s): 24 h after final treatment Vehicle: corn oil Positive control: yes Negative control: yes	**Negative** (negative results for bone marrow and peripheral blood) Toxicity: no information Cytotoxicity: no information Controls: valid negative (solvent control) and positive controls	NTP (1989b)

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Mammalian erythrocyte micronucleus test Not similar to OECD TG 474 GLP: no Deviations: • Sampling much earlier as recommended in the guideline (6 h instead of 18 to 24 h) • No justification for intraperitoneal administration • Number of screened polychromatic erythrocytes less than recommended (only 1000 polychromatic erythrocytes screened per animal) • Incomplete presentation of the results	**Melamine** Purity: no detailed information (purchased from Sigma-Aldrich)	**Disregarded study** **Not reliable** (Major deviations render the study not reliable; particularly due to an inappropriate sampling time) Species: NIH mice; 10 males/group Target organs: • bone marrow Administration route: intraperitoneal injection (ip) Dose levels: 0, 400, 800, 1600 mg/kg bw/d Treatment: two injections (24 h interval) Sampling time(s): 6 h after final treatment Vehicle: no data Positive control: yes Negative control: yes	**Negative** (negative results for bone marrow) Toxicity: no toxic manifestations observed Cytotoxicity: no Controls: valid negative and positive controls	Zhang et al. (2011)
Mammalian bone marrow chromosomal aberration test Similar to OECD TG 475 GLP: no Deviations: • No data on clinical signs and cytotoxicity • No justification for highest dose level • No justification for intraperitoneal substance administration • Only one sampling time (36 h) • No data on number of screened metaphases	**Melamine** Purity: no information	**Disregarded study** **Not reliable** (Major deviations render the study not reliable; particularly missing information regarding toxicity do not allow a firm assessment of the ambiguous result) Species: B6C3F1 mice; 8 males/group Administration route: intraperitoneal injection (ip) Dose levels: 0, 150, 300, 600 mg/kg bw Treatment: single ip injection Sampling time:	**Ambiguous** Negative: 150 and 600 mg/kg Positive: 300 mg/kg Toxicity: no data on cytotoxicity /clinical signs Controls: valid negative (solvent control) and positive controls	NTP (1989a)

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		36 h post ip Vehicle: Corn oil Positive control: yes Negative control: yes		
In vivo mammalian alkaline comet assay Not similar to OECD TG 489 Deviations: • No positive control included • 100 cells scored instead of 150 • Only two dose levels instead of three tested	**Melamine** Purity: no information (purchased by Wako)	**Disregarded study** **Not reliable** (Major deviations render the study not reliable; particularly due to missing positive controls the relevance of the negative result cannot be assessed) Species: rat (Sprague-Dawley); 5 animals/group Target organs: • Liver • Bladder Administration route: oral (gavage) Dose levels: 0, 1000, 2000 mg/kg bw Treatment: • doses given twice (21 h apart) Sampling time: • animals scarified 3 h after second administration Vehicle: 0.5 % methylcellulose Positive control: no positive controls Negative control: yes	**Negative** (Negative in liver cells and bladder cells) Toxicity (data for dose level 2000 mg/kg bw available only): • No clinical signs • Histopathological findings in the • Bladder cells (no other tissues investigated): haemorrhage, cell hyperplasia, cell mitosis, submucosal oedema, erosion of urothelium Cytotoxicity: • (at 2000 mg/kg bw): neutrophil infiltration • no data for 1000 mg/ bw Controls: negative controls valid	Wada et al. (2014)
In vivo mammalian alkaline comet assay Not similar to OECD TG 489 Deviations: • No data on positive control • No data on toxicity and on cytotoxicity • No justification for intraperitoneal	**Melamine** Purity: no detailed information (purchased from Sigma-Aldrich)	**Disregarded study** **Not reliable** **(**According to OECD TG 489, adopted 2016, the in vivo mammalian alkaline comet assay is not considered appropriate to measure DNA strand breaks in mature germ cells; particularly due to missing data on positive controls and toxicity the	**Positive** (positive in epididymides) • Dose-dependent increase in DNA content in the comet tail, in comet tail length and comet tail area Toxicity: no data	Zhang et al. (2011)

Method, guideline, deviations if any	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
administration • No validated protocol available for measure of DNA strand breaks in germ cells (OECD TG 489 not appropriate to measure DNA strand breaks in germ cells)		relevance of the positive result cannot be assessed) Species: NIH mice 10 males/group Target organs: - bilateral epididymises Administration route: intraperitoneal Dose levels: 0, 400, 800, 1600 mg/kg bw/d Treatment: • 5 consecutive days Sampling time: • 7 days after final treatment animals sacrificed and bilateral epididymises obtained Vehicle: no data Positive control: yes Negative control: yes	Cytotoxicity: no data Controls: negative controls valid, no data on positive controls shown	

10.7.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

Numerous *in vitro* and *in vivo* genotoxicity studies are available for melamine. Among those, studies performed using a preferred test system for the assessment of genotoxicity of chemicals (see: REACH guidance IR&CSA R.7a, tables R.7.7-2 to R. 7.7-4, 2017) are listed in Table 10 (*in vitro* tests) and Table 11 (*in vivo* tests). However, none of the studies were performed according to the respective standardised OECD test guidelines (TG) without deviations. Hence, a key study (OECD TG without deviations) that would provide data to conclusively assess the mutagenic potential of melamine (e.g. with confidence in the presence or absence of an effect) in a corresponding test system, was not identified. Deviations from the OECD TGs for the individual studies are documented in Table 10 and Table 11.

In vitro data

There is no evidence for melamine-induced genotoxic effects *in vitro* from the available data.

Four bacterial gene mutation tests **(Haworth et al., 1983; Litton Bionetics Inc., 1977; Raltech Scientific Services, 1981a; Zhang et al., 2011)** were performed similarly to OECD TG 471. All tests yielded negative results with and without metabolic activation. Despite deviations from OECD TG 471, (such as missing tested strains, insufficient justifications for the tested top dose, or missing data on cytotoxicity) the studies are considered reliable for the respective outcomes described. Accordingly, it can be concluded that, under the conditions of the tests, melamine does not induce gene mutations in bacteria with and without metabolic activation.

In addition, three negative bacterial gene mutation tests **(Ishiwata et al., 1991; Kubo et al., 2002; Seiler, 1973)** are considered as not assignable as the relevance of the results cannot be assessed due to missing information on controls and test concentrations.

There are two *in vitro* gene mutation studies in mammalian cell cultures available (McGregor et al., 1988; Raltech Scientific Services, 1981b). Both studies, a HPRT study by **Raltech Scientific Services (1981b)** performed similar to OECD TG 476 and a MLA test by **McGregor et al. (1988)**, performed similarly to OECD TG 490 yielded negative results with and without metabolic activation. The identified deviations such as missing justification of top dose or missing determination of colony size are not judged to impair with the reliability of the study results. Thus, it was shown that melamine did not induce gene mutations in mammalian cells cultures with and without metabolic activation.

Two *in vitro* chromosomal aberration tests in mammalian cell cultures by **Galloway et al. (1987)** and **Zhang et al. (2011)** are available. Both studies yielded negative results with and without metabolic activation. However, only the negative result obtained by the study of **Galloway et al. (1987)** without metabolic activation is considered reliable. Due to major deviations from the recommended exposure and/or sampling times as described in OECD TG 473, the results with metabolic activation found in the study by **Galloway et al. (1987)** and the results with and without metabolic activation obtained in the study by **Zhang et al. (2011)**, are not considered reliable and therefore disregarded. However, under the conditions of the tests, both studies did not indicate a potential for melamine to induce clastogenic effects with and without metabolic activation.

There are additional negative *in vitro* tests in mammalian cell cultures available which were not performed using a preferred test system according to the REACH guidance IR&CSA R.7a (2017; see: table R.7.7-2). These tests which are not included in are indicator tests such as unscheduled DNA synthesis (UDS) tests (Mirsalis and Butterworth, 1982; Naismith, 1982; Selden et al., 1994), sister chromatid exchange (SCE) assays (Galloway et al., 1987; Raltech Scientific Services, 1981b; Sorg, 1982), and a bioluminescence assay (Elmore and Fitzgerald, 1990). The only *in vitro* test in which a positive result was obtained is a microscreen assay (Rossman et al., 1991). This test, however, has not been validated as sufficient genotoxicity test system and the relevance of the results cannot be assessed.

In vivo data (soma cells)

There is no evidence for melamine-induced genotoxic effects *in vivo* (soma cells) from the available data.

There exist two *in vivo* mammalian micronucleus tests in mice (NTP, 1989b; Pharmakon Research International, 1981) which have been performed similar to OECD TG 474. The micronucleus test by **Pharmakon Research International (1981)** yielded negative results in bone marrow cells in mice following oral substance exposure of 1000 mg melamine/kg bw/d (either as single gavage administration or gavage administration for two consecutive daily). Deviations from OECD TG 474 (such as only one dose level tested and no data on cytotoxicity given), do not compromise the reliability of the negative test results. Negative results were also obtained in the micronucleus test by **NTP (1989b)** after intraperitoneal substance administration up to 2000 mg/kg bw/d in both, bone marrow and peripheral blood. Deviations from OECD TG 474 (such as the missing information on clinical signs and on cytotoxicity as well as the missing justification for intraperitoneal substance administration), do not compromise the reliability of the observed negative results.

Additionally, there are four *in vivo* genotoxicity studies available. These studies are, however, disregarded from the genotoxicity assessment of melamine as the results are considered not reliable due to experimental shortcomings or missing information compared to the respective OECD test guidelines as indicated in Table 11 (NTP, 1989a; Wada et al., 2014; Zhang et al., 2011).

In vivo data (germ cells)

There are two germ cell tests with *Drosophila melanogaster* (SLRL tests) available, described as negative (Lüers and Röhrborn, 1963) and ambiguous (Foureman et al., 1994) which are not included in Table 11. The respective OECD TG 477 has been considered not relevant for testing genetic toxicity and was, consequently, deleted in April 2014. (OECD, 2014)

Human data

No data available.

10.7.2 Comparison with the CLP criteria

Available *in vitro* and *in vivo* genotoxicity tests performed with melamine which are considered relevant and reliable (with restrictions) are consistently negative for the respective genotoxic test system. Hence, there is no evidence of induction of gene mutations, clastogenic effects or aneuploidy. Classification criteria for germ cell mutagens are not fulfilled for melamine.

10.7.3 Conclusion on classification and labelling for germ cell mutagenicity

Available reliable and relevant *in vitro* and *in vivo* genotoxicity studies with melamine are negative and do not indicate a mutagenic activity for that substance. Based on conclusive data, classification of melamine as mutagen is not warranted.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification for germ cell mutagenicity.

Melamine was negative in gene mutation tests in bacteria (Haworth *et al*., 1983; Litton Bionetics Inc., 1977; Raltech Scientific Services, 1981a and Zhang *et al*., 2011). All the tests, performed similarly to OECD TG with some limitations, were negative with and without metabolic activation. The DS considered the studies reliable for their respective outcome. In addition, three negative gene mutation assay in bacteria were available but disregarded due to missing information on controls and test concentrations.

Melamine did not induce *in vitro* gene mutations in mammalian cells with and without metabolic activation in two studies, performed similarly to OECD TG 476 or 490 (Raltech Scientific Services, 1981b, McGregor et al., 1988).

Based on the negative results observed in Galloway *et al*., 1987, melamine was not found to induce clastogenic effects with and without metabolic activation *in vitro* in mammalian cells. One additional negative *in vitro* chromosomal aberration tests in mammalian cells (Zhang *et al*., 2011) was not considered reliable by the DS due to major deviations from the recommended OECD TG 473 (sampling time after exposure too short).

Other negative *in vitro* studies (sister chromatide exchange, unscheduled DNA synthesis, bioluminescence assay) were considered of lower weight by the DS. The only positive study was a microscreen assay insufficiently validated (Rossman *et al*., 1991).

Based on two reliable *in vivo* mammalian micronucleus assays in mice (NTP, 1989b; Pharmakon Research international, 1981), the DS concluded that there is no evidence of genotoxic effects *in vivo* in somatic cells.

In addition, four *in vivo* genotoxicity studies were disregarded in the dossier: a negative mammalian Comet assay in liver and bladder cells (Wada *et al*., 2014), a positive mammalian Comet assay in epididymides (Zhang *et al*., 2011), a negative micronucleus test (Zhang *et al*., 2011) and an ambiguous mammalian bone marrow chromosomal aberration test (NTP, 1989a). These studies were disregarded by the DS due to experimental shortcomings or missing information compared to respective OECD TG.

Overall, the DS concluded that the classification criteria for germ cell mutagens were not fulfilled for melamine.

Comments received during consultation

Ten industry or trade association representatives agreed with the DS's proposal. One member state (MS) also agreed that no classification for germ cell mutagenicity was warranted for melamine.

Assessment and comparison with the classification criteria

In vitro results

Seven negative studies for gene mutation in the Ames test were provided on melamine. Three negative studies were disregarded due to missing information on methods and results (Seiler, 1983; Ishiwata *et al*., 1991; Kubo *et al*., 2002). Considering the overall database, all strains recommended in OECD TG 471 were tested up to maximum recommended concentration (5000 µg/plate), including strain TA 102 in studies similar to OECD TG 471. Both the preincubation methods or direct plate incorporation were used. Overall, RAC agrees with the DS that melamine did not induce gene mutation in bacteria in presence or absence of metabolic activation.

Two negative studies for gene mutation in mammalian cells, performed similarly to OECD TG, were available. RAC agrees with the DS that melamine did not induce gene mutation in mammalian cells in presence or absence of metabolic activation.

Melamine was negative in an in vitro mammalian chromosome aberration test (Galloway *et al*., 1987). RAC notes that in this test, time exposure was insufficient in presence of metabolic activation (only 2 hours). The study from Zhang *et al*., 2011 was disregarded by the DS as sampling after exposure was too early.

RAC agrees with the DS that other *in vitro* studies available in the dossier were of lower weight.

Overall, melamine did not induce gene mutation *in vitro* with and without metabolic activation. In addition, melamine was not clastogenic *in vitro* with and without metabolic activation. Nevertheless, RAC notes that the study investigating clastogenic effects with metabolic activation had limitations.

In vivo results

The available data are summarised in the table below:

Test method		Results	Reference
Micronucleus test CD1 mice (m, f)	2 consecutive days gavage study 0, 1000 mg/kg bw/day	Negative	Pharmakon Research, 1981
Bone marrow CA test B6C3F1 mice (m)	Single ip injection 0, 150, 300, 600 mg/kg	Negative : 150, 600 mg/kg Positive: 300 mg/kg	NTP, 1989a
Micronucleus test B6C3F1 mice (m)	Three ip injections 0, 500, 1000, 2000 mg/kg bw/day	Negative	NTP, 1989b
Micronucleus test	Two ip injection	Negative	Zhang *et*

NIH mice (m) *Limit: inappropriate sampling time (6h instead of 18-24h)*	0, 400, 800, 1600 mg/kg bw/day		*al*., 2011
Comet assay: epididymides NIH mice (m) *Limits: no positive controls, no data on cytotoxicity, non-validated method*	Five ip injections 0, 400, 800, 1600 mg/kg bw/day	Positive	
Comet assay: liver and kidney SD rats *Limits: non-standard positive controls*	Oral, single gavage 0, 1000, 2000 mg/kg	Negative	Wada *et al*., 2014

m: males, f: females

In vivo, melamine did not induce damage at chromosomal levels based on the negative results in the micronucleus assays up to the limit dose of 2000 mg/kg (Pharmakon, Research, 1981, NTP, 1989b, Zhang *et al*. 2011). No information on bone marrow exposure were provided in the dossier. The positive result observed at only the mid dose in the chromosomal aberration test (NTP, 1989a) is considered of low weight compare to the consistent negative results obtained in the micronucleus assays in mice.

The available Comet assay in liver and kidney was negative. Nevertheless, RAC notes that positive control used in the study were not in line the ones recommended in OECD TG. According to industry comments during the consultation, 3 out of 4 Ames-test positive substances were positive in this Comet assay. A positive result was observed in the Comet assay performed in epididymides following melamine ip exposure. Although this study may indicate an intrinsic potential of melamine to induce DNA damage in epididymides, the study had limitations as no information on cytotoxicity and general health of animals was provided and as positive control results were not published. Negative historical control range of the laboratory would also have been useful to assess the positive results as high background variability may have occurred. Moreover, RAC considers that the positive result in this Comet assay is of low weight as it was not supported by positive *in vitro* assays in mammalian cells.

Conclusion

Overall, based on negative results *in vitro* and mostly negative results *in vivo*, RAC agrees with the DS that **no classification for germ cell mutagenicity is warranted for melamine**.

10.8 Carcinogenicity

Non-human information

Oral administration

Table 12: Summary table of animal studies on carcinogenicity (oral administration)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Carcinogenicity study **Key study** Oral (feeding) F344/N **rats** Males/females (n = 50 / sex / group) Similar to OECD TG 451 (NTP standards) Deviation: only 2 concentrations tested No GLP	Melamine (> 95 % purity) ♂: 2250 and 4500 ppm (ca. **126 and 263** mg/kg bw/d) ♀: 4500 and 9000 ppm (ca. **262 and 542** mg/kg bw/d) Continuously administered **2 years** (103 weeks)	Neoplastic effects: **Male rats positive** (urinary bladder) **Female rats negative** In male rats: • A statistically significant trend ($P \leq 0.002$) for the occurrence of **transitional cell carcinomas** in the **urinary bladder** was observed (ctrl: 0/45, low-dose: 0/50, high-dose 8/49 (16 %)). The incidence in the high-dose group was significantly higher ($P \leq 0.016$). Transitional cell papillomas were observed in 1/49 (2 %) males of the high-dose group. The combined incidence of **transitional cell carcinomas and papillomas** showed a significant trend ($P < 0.001$) and the incidence in the high-dose group was significantly elevated ($P \leq 0.008$) (incidence table below) • Historical incidence of urinary bladder transitional-cell tumours in untreated male rats: papillomas (4/3551 (0.1 %)), carcinomas (0/3551) In female rats: • Neither transitional cell carcinomas nor papillomas were seen at a statistically higher incidence compared to controls (ctrl: 0/49, low-dose: 1/49 (2 %), high-dose: 1/47 (2 %); combined; incidence table below) • C-cell carcinomas in the thyroid of female rats were observed with a statistically significant positive trend (ctrl: 0/50, low-dose: 0/49, high-dose: 3/50 (6 %); $P \leq 0.038$). The pairwise comparison of the high-dose group with the control did not show statistical significance. When comparing the incidences of the high-dose group with the historical rate (98/3544 (2.8 %); overall historical range: high 5/50, low 0/50), no statistically significant difference was revealed. The tumours were therefore not considered treatment-related by the authors. Pre-/Non-neoplastic effects: • **Chronic inflammation of the kidney** (dose-	Melnick et al. (1984) and NTP (1983)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>related interstitial lymphoplasmacytic infiltration, and cortical fibrosis), distinguishable from the nephropathy observed in aging F344/ N rats, was detected dose-dependently in females with a significantly increased incidence (ctrl: 4/50 (8 %), low-dose: 17/50 (34 %)[#], high-dose: 41/50 (82 %)[#]; [#]P ≤ 0.01) and to a lesser, statistically insignificant, extent in males (ctrl: 2/49 (4 %), low-dose: 3/50 (6 %), high-dose: 6/49 (12 %))

Note: a later re-examination of the histopathologic changes revealed dose-dependent chronic lesions in the kidney
<table>
<tr><th rowspan="2">Melamine (mg/kg bw/d)</th><th colspan="2">Chronic renal lesions*</th></tr>
<tr><th>Males</th><th>Females</th></tr>
<tr><td>0</td><td>1/49 (2 %)</td><td>1/50 (2 %)</td></tr>
<tr><td>126</td><td>7/50 (14 %)</td><td>n.a.</td></tr>
<tr><td>263/262</td><td>19/49 (39 %)</td><td>20/50 (40 %)</td></tr>
<tr><td>542</td><td>n.a.</td><td>50/50 (100 %)</td></tr>
</table>
*fibrotic lesions (scars), stretching from superficial cortex into the medulla, associated with collecting duct dilatation and hyperplasia in the inner medulla, loss of tubule, tubule atrophy, and crowded glomeruli in the cortex; the observed renal changes were consistent with the features of human reflux nephropathy (chronic atrophic pyelonephritis) of early childhood and distinguishable from infarcts and foci of chronic progressive nephropathy (Hard et al., 2009)

• Calculi were seen in the urinary bladder of male but not female rats (low-dose: 1/50 (2 %), high-dose: 10/49 (20 %))

• A statistically significant association (P ≤ 0.001) was found between bladder calculi occurrence and transitional cell carcinomas (7/8 (87.5 %)) male rats with transitional cell carcinomas also displayed calculi)

• A significantly reduced survival rate was observed in the high-dose male group as compared to control animals (P = 0.03); a correlation between tumour incidence and low survival was not reported; 5/8 male rats with transitional cell carcinomas survived ≥ 98 weeks

• Weight gain depression was noted in all dosed rats (weight change relative to control after week 20: ♂ ca. - 4 % (low-dose), ca. - 9.2 % (high-dose); ♀ ca. - 4 % (low-dose), ca. - 8 % (high-dose))

<u>Tissue examined:</u>

• Macroscopic examination on major tissues or organs</td><td></td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
		• Histopathological examination on the following tissues: skin with mammary gland, mandibular lymph node, salivary gland, sternum with bone marrow, larynx or anterior trachea, esophagus, thyroid, parathyroid, lungs with mainstem bronchi, heart, stomach (glandular and nonglandular), duodenum, large intestine, liver, pancreas, spleen, kidneys, adrenal glands, urinary bladder, entire gonads, prostate or uterus, brain, and pituitary gland • examinations of the ureters and urethra were not performed • Tissue was preserved with 10% neutral buffered formalin embedded in paraffin	

Incidence table: Incidence of urinary bladder and kidney lesions in rats (NTP, 1983)

	Males			Females		
	Control	**Low-Dose (126 mg/kg bw/d)**	**High-Dose (263 mg/kg bw/d)**	**Control**	**Low-Dose (262 mg/kg bw/d)**	**High-Dose (542 mg/kg bw/d)**
Urinary Bladder						
➢ No. of animals with tissues examined microscopically	45	50	49	49	49	47
➢ Transitional cell carcinoma	0	0	8 (16 %)*	0	0	0
➢ Transitional cell papilloma	0	0	1 (2 %)	0	1 (2 %)	1 (2 %)
➢ Transitional cell hyperplasia	0	1 (2 %)	2 (4 %)	0	0	0
➢ Stones (calculi) ‡	0	1 (2 %)	10 (20 %)#	0	0	0
Kidney						
➢ No. of animals with tissues examined microscopically	49	50	49	50	50	50
➢ Chronic inflammation	2 (4 %)	3 (6 %)	6 (12 %)	4 (8 %)	17 (34 %)#	41 (82 %)#
➢ Nephropathy	32 (65 %)	36 (72 %)	30 (61 %)	19 (38 %)	23 (46 %)	28 (56 %)

* $P \leq 0.016$, relative to controls
# $P \leq 0.01$, relative to controls
‡ Observed at necropsy or by microscopic examination.

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Carcinogenicity study **Key study** Oral (feeding) B6C3F1 **mice** (hybrids) Males/females (n = 50 / sex / group) Similar to OECD TG 451 (NTP standards) Deviations: only 2 concentrations tested No GLP	Melamine (> 95 % purity) ♂/♀: 2250 and 4500 ppm (♂: ca. **327 and 688** mg/kg bw/d; ♀: **523 and 1065** mg/kg bw/d) Continuously administered **2 years** (103 weeks)	<u>Neoplastic effects:</u> **Male mice negative** **Female mice negative** <u>Pre-/Non-neoplastic effects:</u> • Dose-dependent **acute/chronic inflammation**, and mild epithelial (transitional cell) **hyperplasia** in the **urinary bladder** was found in male mice exposed to low- and high-dose melamine whereas in females comparable changes were only observed to a much lesser extent in the high-dose group (incidence table below) • **High incidence of calculi** in male mice and less frequently in females (incidence table below) • **Reduced survival** among male mice exposed to high-dose melamine (P = 0.013) as compared to control • The mean body **weights** of male mice in the high-dose group was slightly lower after week 50 <u>Tissue examined:</u> • Macroscopic examination on major tissues or organs • Histopathological examination on the following tissues: skin with mammary gland, mandibular lymph node, salivary gland, sternum with bone marrow, larynx or anterior trachea, esophagus, thyroid, parathyroid, lungs with mainstem bronchi, heart, stomach (glandular and nonglandular), duodenum, large intestine, liver, gallbladder, pancreas, spleen, kidneys, adrenal glands, urinary bladder, entire gonads, prostate or uterus, brain, and pituitary gland • Tissue was preserved with 10% neutral buffered formalin embedded in paraffin	Melnick et al. (1984) and NTP (1983)

<u>Incidence table: incidence of lesions in the urinary bladder in mice</u> (NTP, 1983)

	Males			Females		
	Control	**Low-Dose (327 mg/kg bw/d)**	**High-Dose (688 mg/kg bw/d)**	**Control**	**Low-Dose (523 mg/kg bw/d)**	**High-Dose (1065 mg/kg bw/d)**
➢ No. of animals with tissues examined microscopically	45	47	44	42	49	50
➢ Stones*	2 (4 %)	40 (85 %)	41 (93 %)	0	0	4 (8 %)
➢ Inflammation, acute	0	1 (2 %)	0	0	0	0

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results					Reference
➢ Inflammation, acute and chronic		0	25 (53 %)	24 (55 %)	0	0	4 (8 %)
➢ Inflammation, chronic		2 (4 %)	10 (21 %)	14 (32 %)	0	0	2 (4 %)
➢ Hyperplasia, epithelial		1 (2 %)	11 (23 %)	13 (30 %)	0	0	4 (8 %)
* Observed at necropsy							

Carcinogenicity study **Key study** Oral (feeding) F344 **rats** Males (n = 20 / group) Non-guideline study Deviations to OECD TG 451: reduced exposure time, only males, reduced number of animals, limited number of tissues examined (focused exclusively on urinary system), description of experimental procedures less detailed No GLP Study provides reliable information regarding the effects of melamine on the urinary system of male rats and is, hence, considered a key study The study does not provide information on melamine-related effects in other organs	Melamine (> 99 % purity) 3000, 10 000, and 30 000 ppm (ca. **100, 330, 1090** mg/kg bw/d*) Continuously administered **36 weeks** + 4 weeks recovery *Converted according to reported mean terminal body weight and food consumption...	<u>Neoplastic effects:</u> **Male rats positive** (urinary bladder and ureter) *Urinary bladder* • Dose-dependent **Carcinomas** (consisted of transitional cells) (ctrl: 0/20, low-dose: 0/20, mid-dose: 1/20 (5 %), high-dose: 15/19 (79 %, P < 0.01)) and dose-dependent **papillomas** (ctrl: 0/20, low-dose: 0/20, mid-dose 1/20 (5 %), high-dose: 12/19 (63 %, P < 0.01)) were seen in the urinary **bladder** at a significantly increased incidence in the high-dose group (incidence table below) • The incidence of **papillomatosis** (ctrl: 0/20, low-dose: 0/20, mid-dose: 5/20 (25 %, P < 0.05), high-dose: 17/19 (89 %, P < 0.01)) was dose-dependent and significantly elevated in the **urinary bladder** of the mid- and high-dose groups (incidence table below) (papillomatosis was distinguished from papillomas based on the presence of atrophic changes and apoptosis in the urinary epithelium) *Ureter* • **Papillomas** (3/19 (16 %)) and one **carcinoma** (1/19 (5 %)) in the **ureter** of the high-dose group were observed in the presence of calculi <u>Pre-/Non-neoplastic effects:</u> • Papillary or nodular **hyperplasia** of the (transitional cell) urothelium was observed in the urinary **bladder** (ctrl: 0/20; low-dose: 1/20 (5 %); mid-dose: 6/20 (30 %, P < 0.05); high-dose: 12/19 (63 %, P < 0.01)), the **ureter** (high-dose) and in the **renal pelvis** (mid- and high-dose) (incidences for the ureter and renal pelvis were not quantitatively specified) • **Calculus formation** was observed in the urinary bladder in a dose-dependent manner (ctrl: 0/20; low-dose: 4/20 (20 %); mid-dose: 9/20 (45 %, P < 0.05); high-dose: 8/19 (42 %, P < 0.01)) • A statistically significant correlation between calculus formation and tumour incidence was described	Okumura et al. (1992)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>

The ureter was slightly thickened in high-dose animals
Hematuria and polyuria was observed in the high-dose group
The terminal body weight was significantly decreased in the high-dose group
Spontaneous mortality was restricted to a single animal in the high-dose group

<u>Tissue examined:</u>

Animals were killed at week 40 and the tissues (urinary bladder, ureter, kidney) were histologically examined

</td><td></td></tr>
<tr><td colspan="4">Incidence table: Histological lesions and incidences of calculi in the urinary bladder of rats treated with melamine (Okumura et al., 1992)
<table>
<tr><th rowspan="2">Treatment (melamine mg/kg bw/d)</th><th rowspan="2">No. of rats</th><th colspan="5">No. (%) of rats with</th></tr>
<tr><th>PN hyperplasia</th><th>Papilloma</th><th>Carcinoma</th><th>Papillomatosis</th><th>Calculi</th></tr>
<tr><td>0</td><td>20</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>100</td><td>20</td><td>1 (5 %)</td><td>0</td><td>0</td><td>0</td><td>4 (20 %)</td></tr>
<tr><td>330</td><td>20</td><td>6 (30 %)*</td><td>1 (5 %)</td><td>1 (5 %)</td><td>5 (25 %)*</td><td>9 (45 %)*</td></tr>
<tr><td>1090</td><td>19</td><td>12 (63 %)#</td><td>12 (63 %)#</td><td>15 (79 %)#</td><td>17 (89 %)#</td><td>8 (42 %)#</td></tr>
</table>
#Significantly different from respective control group value at P ≤ 0.01
*Significantly different from respective control group value at P ≤ 0.05
PN = Papillary or nodulary hyperplasia</td></tr>
<tr><td>Carcinogenicity study

Key study

Oral (feeding)

F344/DuCrj rats

Males (n = 10 - 20 / group)

Non-guideline study

Deviations to OECD TG 451: reduced exposure time, only males, reduced number of animals, limited number of tissues examined (focused exclusively on</td><td>Melamine (99.9 % purity) in feed with and without NaCl supplementation (simultaneously administered)

Ctrl (n = 10);
Ctrl + 10 % NaCl (n = 10);
10 000 ppm (ca. 350 mg/kg bw/d*)
• w/o NaCl (n = 19),
• +5 % NaCl (n = 19),
• +10 % NaCl (n = 19);
30 000 ppm (ca. 1030 mg/kg bw/d*)</td><td><u>Neoplastic effects:</u>

Male rats positive (urinary bladder)

High incidence of transitional cell carcinomas (ctrl: 0/10, low-dose: 4/19 (21 %), high-dose: 18/20 (90 %)), papillomas (ctrl: 0/10, low-dose: 8/19 (42 %), high-dose: 10/20 (50 %)), and papillomatosis (ctrl: 0/10, low-dose: 9/19 (47 %), high-dose: 15/20 (75 %)) were found in the urinary bladder (incidence table below)
Simultaneous NaCl treatment reduced the incidences of these proliferative lesions (incidence table below)

<u>Pre-/Non-neoplastic effects:</u>

Transitional cell hyperplasia and ischemic changes (focal lesions demonstrating fibrosis, inflammation cell infiltration, and renal tubule regeneration) were observed in the papilla and cortex of the kidney respectively and attenuated in
</td><td>Ogasawara et al. (1995)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr>
<td>urinary system)

No GLP

Study provides reliable information regarding the effects of melamine on the urinary system of male rats and is, hence, considered a key study

The study does not provide information on melamine-related effects in other organs</td>
<td>• w/o NaCl (n = 20),
• +5 % NaCl (n = 20),
• +10 % NaCl (n = 20);

Continuously administered

36 weeks + 4 weeks recovery

*Converted according to reported mean terminal body weight and food consumption</td>
<td>the high-dose group and completely suppressed in the low-dose group by co-administration of NaCl (see table below)

<u>Histopathological findings in the kidney:</u>
<table>
<tr><th>Dose (mg/kg bw/d ♂/♀)</th><th>Papilla*</th><th>Cortex#</th></tr>
<tr><td>0</td><td>0/10</td><td>0/10</td></tr>
<tr><td>350</td><td>7/19 (37 %)</td><td>1/19 (5 %)</td></tr>
<tr><td>350 + 5 % NaCl</td><td>0/19</td><td>0/19</td></tr>
<tr><td>350 + 10 % NaCl</td><td>0/19</td><td>0/19</td></tr>
<tr><td>1030</td><td>20/20 (100 %)</td><td>20/20 (100 %)</td></tr>
<tr><td>1030 + 5 % NaCl</td><td>9/20 (45 %)</td><td>8/40 (40 %)</td></tr>
<tr><td>1030 + 10 % NaCl</td><td>1/20 (5 %)</td><td>2/20 (10 %)</td></tr>
</table>
*Transitional cell hyperplasia with angiectasis and thrombus formation
#Ischemic changes such as focal lesions demonstrating fibrosis, inflammation cell infiltration, and renal tubule regeneration

• The authors suggested epithelium stimulation secondary to microcalculus formation within the renal pelvis as a potential underlying cause

• Calculi were observed in the urinary bladder (ctrl: 0/10, low-dose: 7/19 (37 %), high-dose: 6/20 (30 %))

• A strong correlation between bladder tumours and calculus formation was noted

• Calculus formation in the 350 mg/kg bw/d melamine group was suppressed by NaCl in a dose-dependent fashion

• An elevated water intake was observed with increasing doses of NaCl and by high-dose melamine

• The urinary volume was increased in NaCl treated animals and in the high-dose melamine group

• The authors concluded that melamine-induced carcinogenesis is linked to calculi-induced irritation of the bladder epithelium and that NaCl-mediated polyuria as a consequence of elevated water intake prevents calculus formation and hence, bladder tumours

• Many microcrystals were observed in the urinary sediments in the high-dose group (1030 mg/kg bw/d) independent of NaCl supplementation

• Exfoliated epithelial cells were mainly found in the urine of high-dose rats; NaCl co-treatment attenuated the occurrence of those cells</td>
<td></td>
</tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
		• The **kidney weight** was reduced in the low- and high-dose melamine group • **Urinary occult blood** was seen in the high-dose melamine group and suppressed by concomitant NaCl (10 %) co-treatment • The **examination of the calculi** revealed that **melamine and uric acid** in equal molar ratios are the primary components of stones (total combined contents of melamine and uric acid in the stone was 61-81 %) • The final body **weight** of the high-dose group (1030 mg/kg bw/d melamine w/o NaCl) was considerably lower than that of the control • Spontaneous **mortality** was restricted to three animals in the 350 mg/kg bw/d group <u>Tissue examined:</u> • Histopathological examination was performed on the urinary bladder and kidney • Tissue fixation was done in formalin • No information on whether or not the ureter was examined	

Incidence table: Incidences of calculi and proliferative lesions in the urinary bladder (Ogasawara et al., 1995)

Treatment (melamine mg/kg bw/d)	No. of rats	Calculi (%)	Papillomatosis‡ (%)	Papilloma (%)	Carcinoma (%)
0	10	0	0	0	0
10 % NaCl	10	0	0	0	0
350	19	7 (37 %)	9 (47 %)	8 (42 %)	4 (21 %)
350 + 5 % NaCl	19	2 (11 %)	2 (11 %)*	0	0
350 + 10 % NaCl	19	1 (5 %)*	0	0	0
1030	20	6 (30 %)	15 (75 %)	10 (50 %)	18 (90 %)
1030 + 5 % NaCl	20	15 (75 %)	17 (85 %)	5 (25 %)	18 (90 %)
1030 + 10 % NaCl	20	6 (30 %)	2 (10 %)#	3 (15 %)#	0

‡Multiple papillomatous hyperplasias.
*Significantly different from the respective control group value at $P < 0.05$
#Significantly different from the respective control group value at $P < 0.001$

Carcinogenicity study **Key study** Oral (feeding) F344 **rats** Males/females (n = 65 / sex / group)	Melamine (no information on purity; test material was analysed) ♂: 100; 500; 1000 ppm (ca. **4, 20, 40** mg/kg bw/d*) ♀: 100; 1000; 2000 ppm (**5, 50,**	<u>Neoplastic effects:</u> **Male rats negative** **Female rats negative** • Four primary urinary bladder tumors were found (two transitional cell papillomas in control animals of both sexes, one transitional cell papilloma in the 1000 ppm male group, and one anaplastic transitional cell carcinoma in the 100 ppm female group)	Hazleton (1983)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Similar to OECD TG 451 Deviations: no information on purity GLP	**80** mg/kg bw/d*) Continuously administered **123 – 131 weeks** *Converted according to reported mean terminal body weight and food consumption	• Neoplastic lesions in other tissues were considered not related to melamine treatment <u>Pre-/Non-neoplastic effects:</u> • **Transitional epithelial hyperplasia** in the urinary bladder was observed (ctrl ♂: 2/39 (5 %), high-dose ♂: 6/37 (16 %)); however, the absolute incidences were insufficient to establish a treatment-related trend; hyperplasias were frequently identified in DOT (died on test) and moribund rats • Cystic calculi were found in three ♂ (one at 20 and two at 40 mg/kg bw/d) and two ♀ (at 80 mg/kg bw/d); one ♀ displayed both, calculi and transitional epithelial hyperplasia • Increased tubular pigments of unknown biological relevance in the kidney of ♀ rats of the high-dose group was seen with a statistically significant positive trend • Clinical pathology data did not reveal any treatment-related changes • A dose-dependent trend to develop dilated glands in glandular gastric mucosa and inflammation in non-glandular gastric mucosa was observed in female rats <u>Tissue examined:</u> • Complete necropsy on all animals • The following tissues were collected and preserved/fixed (alcohol-formalin-acetic acid) subsequent to gross necropsy: brain, spinal cord, lung, spleen, liver, kidneys, heart, aorta, eyes, pituitary, adrenals, bone marrow, sciatic nerve, thyroids (with parathyroids), urinary bladder, testes, prostate, seminal vesicle, ovaries, uterus, vagina, duodenum, jejunum, ileum, cecum, colon, pancreas, trachea, esophagus, stomach, salivary gland (submandibular), mesenteric lymph nodes, thymus, bone, tongue, skeletal muscle, skin, mammary gland • Urinary tract: the kidney and urinary bladder were examined grossly (necropsy) and microscopically • No information on whether or not the ureter was examined	
Carcinogenicity study Supporting study	Melamine (no information on purity)	<u>Neoplastic effects:</u> **Male rats positive** (gross papilloma and microscopic benign papilloma in the urinary bladder)	Hazleton (1953)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Oral (feeding) Albino **rats** Male/female (n = 10 / sex / group) Similar to OECD TG 451 Deviations: only 2 concentrations, inadequate number of animals, no statistics No GLP	1000 and 10 000 ppm (♂: ca. **30 and 350** mg/kg bw/d; ♀ ca. **40 and 470** mg/kg bw/d*) Continuously administered **2 years** *Converted according to reported mean terminal body weight and food consumption	**Female rats positive** (microscopic benign papilloma in the urinary bladder) • Gross papilloma (♂: ctrl: 0/7, low-dose: 0/8, high-dose: 4/7 (57 %); ♀: ctrl: 0/9, low-dose: 0/9, high-dose: 0/8) and microscopic benign papillomata (♂: ctrl: 0/3, low-dose: 0/8, high-dose: m: 4/7 (57 %); ♀: ctrl: 0/3, low-dose: 0/8, high-dose: 2/5 (40 %)) were observed • Microscopic lesions associated with gross findings were considered significant and related to melamine treatment by the authors <u>Pre-/Non-neoplastic effects:</u> 350/470 mg/kg bw/d • Microscopic epithelial (transitional cell) hyperplasia in the urinary bladder (♂: 6/7 (86 %); ♀: 5/5 (100 %)) • Urinary bladder calculi (♂: 5/7 (71 %); ♀: 2/8 (25 %)) • Small crystalline deposits in the kidney of 1 male and 2 females • No significant influence on weight or mortality 30/40 mg/kg bw/d • no treatment-related effects observed <u>Tissue examined:</u> • Histopathological examination was performed in addition to gross examinations on the following tissues: thyroid, parathyroid, lung, trachea, liver, kidney, bladder, adrenal, spleen, stomach, small and large intestine, testes, ovary, uterus, bone marrow • Urinary tract: the kidney and urinary bladder were examined grossly (necropsy) and microscopically • No information on whether or not the ureter was examined	
Carcinogenicity study Disregarded study Oral (feeding) WS/Shi **rats**	Melamine (> 95 % purity) 0.05 % N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)	Male rats were treated with 0.05 % of the initiating agent N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) for 4 weeks followed by a diet supplemented with 30 000 ppm melamine <u>Neoplastic effects:</u> **Male rats positive** (urinary bladder)	Mori et al. (2000)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>Males (ctrl: n = 20; mel (melamine): n = 19)

Non-guideline study

Deviations to OECD TG 451: pre-treatment with BBN, shorter treatment time, only one dose, only males, low number of animals, limited number of tissues examined

No GLP</td><td>30 000 ppm (ca. 1440 mg/kg bw/d*)

Continuously administered

4 weeks (BBN) + 32 weeks (melamine)

*converted with reported final body weight and daily food intake</td><td>Melamine promotes urinary bladder carcinogenesis induced by BBN in male rats

• Papillomas (Ctrl: 5/20 (25 %); mel: 14/19 (74 %), P < 0.05) and transitional cell carcinomas (Ctrl: 2/20 (10 %); mel: 8/19 (42 %), P < 0.05) were observed

<u>Pre-/Non-neoplastic effects:</u>

• Papillary of nodular hyperplasia (ctrl: 6/20 (30 %); mel 15/19 (78 %), P < 0.05)

• Urinary calculi (15/19 (79 %), P < 0.05)

• 14/15 rats that displayed calculi also developed tumours

<u>Tissue examined:</u>

• The urinary bladder was examined macroscopically and microscopically
• No information on whether or not the kidney and ureter was examined
• Tissue fixation was done in formalin</td><td></td></tr>
<tr><td>Long-term bioassay

Supporting study

Oral (feeding)

BALB/c mice

Males/females (ctrl: n = 21; mel: n = 27)

Non-guideline study

Deviations to OECD TG 451: short treatment time, only one dose, low number of animals, sex-specific effects not specified, limited number of tissues examined

No GLP</td><td>Melamine (obtained from sigma chemicals; no information on purity)

♂/♀: 12 000 ppm (1.2 %, ca. 1800 mg/kg bw/d*) in the presence or absence of different fatty acids

22 weeks

*Converted according to table 3.17, Guidance on the application of the CLP criteria (Version 5.0, July 2017)</td><td><u>Neoplastic effects:</u>

Male/female (not specified) mice positive (dysplasia/in situ carcinoma)

Neoplastic and preneoplastic proliferative lesions of the urinary tract:
<table>
<tr><th>Group</th><th>H[#]</th><th>D/CIS*</th></tr>
<tr><td>Renal pelvis</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>1/21(5 %)</td><td>1/21(5 %)</td></tr>
<tr><td>Melamine</td><td>2/27 (7 %)</td><td>4/27 (15 %)</td></tr>
<tr><td>Ureter</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/21</td><td>0/21</td></tr>
<tr><td>Melamine</td><td>3/27 (11 %)</td><td>7/27 (26 %)</td></tr>
<tr><td>Urinary bladder</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/21</td><td>0/21</td></tr>
<tr><td>Melamine</td><td>7/27 (26 %)</td><td>9/27 (36 %)</td></tr>
</table>
[#]transitional cell hyperplasia (H)
*transitional cell combined dysplasia/carcinoma in situ (D/CIS)

D/CIS: disorganization within hyperplastic layers of bladder urothelium, cells and nuclei not uniform in shape/size, mitotic figures are frequent

<u>Pre-/Non-neoplastic effects:</u></td><td>Cremonezzi et al. (2001)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>

Transitional cell hyperplasia in the urinary bladder, ureter, and renal pelvis (see table above)
Calculus formation in the bladder was observed but not described in detail (60 to 85 % of the animals in melamine treated groups and none in the control)
Calculus formation associated with an increased incidence of bladder transitional cell hyperplasia

Statistical significance between control and melamine group is not indicated

<u>Tissues examined:</u>

The urinary epithelia (urinary bladder, ureters, and renal pelves) was examined grossly and microscopically
Tissue fixation was done in formalin

</td><td></td></tr>
<tr><td>Long-term bioassay

Supporting study

Oral (feeding)

Wistar rats

Two sampling times (SP)

Males/females (SP 1 ctrl: n = 22, melamine n = 21; SP 2 ctrl: n = 36, melamine n = 20)

Non-guideline study

Deviations to OECD TG 451: short treatment time, only one dose, low number of animals, sex-specific effects not specified, limited number of tissues examined

No GLP</td><td>Melamine (obtained from Sigma chemicals; no information on purity)

♂/♀: 15 000 ppm (1.5 %, ca: 750 mg/kg bw/d) in the presence or absence of different fatty acids

Autopsies at 22-25 weeks (SP1) and 36-40 weeks (SP2)

*Converted according to table 3.17, Guidance on the application of the CLP criteria (Version 5.0, July 2017)</td><td>
<u>Neoplastic effects:</u>

Male/female (not specified) rats negative

<u>Pre-/Non-neoplastic effects:</u>

Proliferative lesions (metaplasia, hyperplasia, and dysplasia) were observed mainly at the proximal end of the urinary tract (papillae and renal pelvis)

Proliferative lesions of the urinary tract:
<table>
<tr><th>Group</th><th>SSM#</th><th>MSM*</th></tr>
<tr><td>Renal papillae (SP1)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/22</td><td>0/22</td></tr>
<tr><td>Melamine</td><td>9/21 (43 %)</td><td>1/21 (5 %)</td></tr>
<tr><td>Renal papillae (SP2)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/36</td><td>0/36</td></tr>
<tr><td>Melamine</td><td>6/20 (30 %)</td><td>0/20</td></tr>
</table>
#slight squamous metaplasia
*moderate squamous metaplasia
<table>
<tr><th>Group</th><th>H#</th><th>D*</th></tr>
<tr><td>Renal pelvis (SP1)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/22</td><td>0/22</td></tr>
<tr><td>Melamine</td><td>5/21 (24 %)</td><td>1/21 (5 %)</td></tr>
<tr><td>Ureter (SP1)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/22</td><td>n.a.</td></tr>
<tr><td>Melamine</td><td>3/21 (14 %)</td><td>n.a.</td></tr>
<tr><td>Urinary bladder (SP1)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/22</td><td>0/22</td></tr>
<tr><td>Melamine</td><td>1/21 (5 %)</td><td>0/21</td></tr>
<tr><td>Renal pelvis (SP2)</td><td></td><td></td></tr>
</table>
</td><td>Cremonezzi et al. (2004)</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
		Ctrl 0/36 0/36 Melamine 1/20 (5 %) 2/20 (10 %) *Ureter (SP2)* Ctrl 0/36 n.a. Melamine 2/20 (10 %) n.a. *Urinary bladder (SP2)* Ctrl 0/36 0/36 Melamine 1/20 (5 %) 0/20 #simple transitional cell hyperplasia without atypia *dysplasias Other non-specific kidney lesions including coarse retractile scarring, acute and chronic inflammation of renal parenchyma and dilatation with scattered eosinophilic casts in collecting tubules, were mainly observed at SP2 • No melamine treatment-related effects with statistical significance are reported • No evidence for urolithiasis • However, it was noted by the authors that *"Even though calculi or hydroureters were not observed during autopsy, the presence of minute areas of calcification in the papilla may suggest crystal depots which spontaneously dissolved thereafter"* and that *"precipitation can not be discarded"* <u>Tissue examined:</u> • The urinary epithelia (urinary bladder, ureters, renal pelves, and renal papillae) was examined grossly and microscopically • Tissue fixation was done in formalin	

Dermal application

Table 13: Summary table of animal studies on carcinogenicity (dermal administration)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Carcinogenicity study Disregarded study Dermal (topical application on dorsal skin) No OECD guideline, no GLP	Melamine (obtained from Pfaltz and Bauer; no information on purity) 12-0-tetradecanoylphorbol-13-acetate (TPA) Single application	• A single application of melamine followed by promotion with TPA (two application per week for a period of 31 weeks) had no tumour initiating activity • Melamine does not act as an initiator in this mouse skin model	Perrella and Boutwell (1983)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
CD-1 **mice** Females (n = 20)	(**1 µmol** in acetone) **31 weeks**		

Inhalative application

No relevant studies could be identified

Other forms of application

No relevant studies could be identified

Human information

No relevant studies could be identified

Additional information

Table 14: Summary table of other studies relevant for carcinogenicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Carcinogenicity study Oral (feeding) F344/N **rats** and C57BL/6 x C3H F1 **mice** Males/females (n = 50 / sex / group)	Uracil **Rats** 30 000 ppm 104 weeks **Mice** 30 000 ppm (from Wk 1 to Wk 6) and 25000 (from Wk 7 to Wk 96) 96 weeks	Uracil mediates its carcinogenic activity via an analogue mode of action (MoA) involving calculus formation and stimulation of the urothelium	**<u>Rats</u>** • **Transitional cell carcinomas** were observed in the **urinary bladder** of **males** (27/30 (90 %), P < 0.01) and **females** (5/27 (19 %), P < 0.05) • **Transitional cell papillomas** were observed in the **urinary bladder** of **males** (24/30 (80 %), P < 0.01) and **females** (8/27 (30 %)) • **Carcinomas** (7/30 (23 %), P < 0.05) and **papillomas** (4/30 (13 %), P < 0.05) were found in the **renal pelvis** in **male rats** • Carcinomas (3/27 (11 %)) were seen in the **renal pelvis** in **female rats** • Squamous cell carcinomas were seen in males (3/30 (10 %)) • Calculus formation was found in male and females (30 %) **<u>Mice</u>** • **Transitional cell carcinomas** were observed in the **urinary bladder** males (2/26 (8 %)) and females	Fukushima et al. (1992)

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
			(22/29 (76 %), P < 0.01)	

10.8.1 Short summary and overall relevance of the provided information on carcinogenicity

As summarized in Table 12 and in the technical dossier, several studies concerning the carcinogenic potential of melamine have been conducted in experimental animals. Evidence for melamine-related carcinogenic effects is mainly derived from multiple studies with rats. There is limited evidence from mice as only two studies address tumourigenesis in this species. For the purpose of classification, four key (Hazleton, 1983; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992) and three supporting studies were identified (Cremonezzi et al., 2004; Cremonezzi et al., 2001; Hazleton, 1953).

The National Toxicology Program (**NTP)** 2-years bioassay (1983) was performed in accordance with accepted scientific principles with minor deviations from OECD TG 451 and is considered a reliable source of information. The incidence of transitional cell carcinoma and the combined incidence of transitional cell carcinoma and papilloma were found to be significantly increased in the high-dose group (ca. 263 mg/kg bw/d) when compared to the control. A significant correlation between bladder calculi and the occurrence of transitional cell carcinomas was observed. The treatment-related tumour incidence markedly exceeded the historical control data from several laboratories including the test laboratory and can, therefore, considered to be related to melamine treatment and of biological significance. Tumour formation was neither observed in female rats nor in mice of either sex in a concurrent bioassay. However, preneoplastic lesions were seen in the kidneys (fibrotic lesions associated with collecting duct dilatation and hyperplasia in the inner medulla) of male and female rats and in the urinary bladder (hyperplasia of the transitional cell epithelium) of male and female mice (Hard et al., 2009; Melnick et al., 1984; NTP, 1983). Consistent with the results of the NTP study, two additional key studies in male rats reported high incidences of tumours in the urinary tract of male rats. Although both studies have not been conducted according to internationally recognised test guidelines and have some limitations in their study design, the provided information is considered reliable key information for the observed effects in the urinary tract system of male rates and hence, relevant for the purpose of classification. Accordingly, **Okumura et al. (1992)** reported transitional cell carcinomas and papillomas at a statistically significantly increased incidence in urinary bladders in the high-dose group (ca. 1090 mg/kg bw/d) following 36 weeks of melamine administration in addition to a four-week recovery period. The incidence of papillomatosis in the urinary bladder was statistically significantly elevated in the middle- and high-dose group. A correlation between tumour incidence and calculus formation was established with statistical significance. Papillomas and one carcinoma were also observed in the ureter. Preneoplastic lesions of the transitional cell epithelium such as hyperplasia in the ureter and renal pelvis were seen at a significantly increased incidence in the high-dose group (Okumura et al., 1992). In the study by **Ogasawara et al. (1995)**, male rats were treated with melamine in the presence and absence of different NaCl concentrations. High incidences of transitional cell carcinomas, papillomas, and papillomatosis in the urinary bladder were observed in rats that received a melamine dose of approximately 1030 mg/kg bw/d. A strong correlation between neoplastic lesions and the occurrence of uroliths was established. Preneoplastic lesions such as transitional cell hyperplasia in the renal papilla were observed in the kidney together with a reduced kidney weight (Ogasawara et al., 1995). Another reliable key carcinogenicity study (similar to OECD TG 451, GLP) by **Hazleton (1983)**, carried out to address melamine-related effects at lower doses (≤ ca. 40 mg/kg bw/d ♂, ≤ ca. 80 mg/kg bw/d ♀) subsequent to the NTP study, reported no treatment-related induction of cancerous lesions and only sporadic calculus formation (Hazleton, 1983). Preneoplastic transitional epithelial hyperplasias were observed with an increased incidence in high-dose males. A significant treatment-related trend, however, could not be established due to insufficient absolute incidence numbers. Increased incidences of tubular pigments in the kidney of female rats (high-dose) were found with a statistically significant positive trend. The biological relevance of this observation is, however, obscure as similar morphological pigments were also found in healthy control animals (Hazleton, 1983).

Altogether, a dose-response relationship with respect to urinary tumour formation can be established when combining the data from the four key studies (Table 15).

Table 15: Dose-response relationship regarding tumour formation in the urinary bladder of male rats

Dose (mg/kg bw/d)	**Incidence of hyperplasias** (# of animals examined)	**Incidence of papillomas** (# of animals examined)	**Incidence of carcinomas** (# of animals examined)	**Reference**
ca. 4 – 40	0 % (0/65)	0 % (0/65)	0 % (0/65)	Hazleton (1983)
ca. 100	5 % (1/20)	0 % (0/20)	0 % (0/20)	Okumura et al. (1992)
ca. 126	2 % (1/50)	0 % (0/50)	0 % (0/50)	Melnick et al. (1984) and NTP (1983)
ca. 263	4 % (2/49)	2 % (1/49)	16 % (8/49)	Melnick et al. (1984) and NTP (1983)
ca. 330	30 % (6/20)	5 % (1/20)	5 % (1/20)	Okumura et al. (1992)
ca. 350	47 % (9/19)*	42 % (8/19)	21 % (4/19)	Ogasawara et al. (1995)
ca. 1030	75 % (15/20)*	50 % (10/20)	90 % (18/20)	Ogasawara et al. (1995)
ca. 1090	63 % (12/19)	63 % (12/19)	79 % (15/19)	Okumura et al. (1992)

*multiple papillomatous hyperplasias

Three additional studies were identified as supporting studies with lower reliability and less relevance for the purpose of classification. A 2-year bioassay, with major deviations from OECD TG 451, found gross/microscopic papillomas in the urinary bladder of male rats and microscopic benign papillomas in the urinary bladder of female rats in the high-dose melamine group (♂/♀ 350/470 mg/kg bw/d) **(Hazleton, 1953)**. Two additional studies with non-guideline-conform study design reported proliferative lesions of the transitional cell epithelium of the urinary tract following melamine administration **(Cremonezzi et al., 2004; Cremonezzi et al., 2001)**. Accordingly, increased combined incidences of dysplasia/carcinoma *in situ* (D/CIS) in the bladder, the ureter, and the renal pelvis were seen in mice (ca. 1800 mg/kg bw/d) and proliferative lesions (metaplasia, hyperplasia, and dysplasia) were observed mainly in the renal papillae and renal pelvis of rats (ca. 750 mg/kg bw/d). For the reported incidence of D/CIS (combined) in the mice study, uncertainties regarding its defined cancerous potential exist. Dysplasias are generally described as intraurothelial neoplasias, composed of abnormal cells with precarcinogenic potential. They precede or accompany CIS and invasive tumours. The severity of dysplastic epithelial lesions ranges from mild to severe D/CIS with the latter having the highest potential of developing an invasive tumour (Spieler and Rössle, 2012). CIS alone appears as a flat non-invasive urothelial neoplasm composed of anaplastic cells (Oyasu, 1995). Progression to an invasive tumour occurs in a significant proportion of patients presenting with a CIS (Spieler and Rössle, 2012). As the authors do not specifically discriminate between dysplasia and CIS, the epithelial abnormalities are regarded as lesions of uncertain neoplastic potential. In addition, the authors reported having randomly distributed mice and rats of both sexes but do not specify their results according to sex. A sex-specific assessment of the results is, therefore, not possible.

Another study by **Mori et al. (2000)** investigated the effect of melamine following treatment with a tumour initiating agent. Although similar urinary bladder lesions were observed secondary to the formation of calculi, the results have to be interpreted in the context of the initial treatment with N-butyl-N-(4-hydroxybutyl)nitrosamine (a known inducer of bladder cancer) and are therefore not considered relevant within the scope of this classification (Mori et al., 2000). No additional evidence is derived from the dermal exposure study by **Perrella and Boutwell (1983)** (Perrella and Boutwell, 1983). In addition, data derived from a carcinogenicity study using uracil in rats and mice were considered relevant as the information provided strongly supports the sequence of key events related to the mode of action (MoA; described in detail in the following section) (Fukushima et al., 1992).

In summary, the results listed in Table 12 constitute convincing and sufficient evidence of carcinogenic activity evoked by dietary melamine exposure in experimental animals.

10.8.2 Comparison with the CLP criteria

"Carcinogen means a substance or a mixture of substances which induce cancer or increase its incidence (CLP Regulation 1272/2008, 3.6.1.1.). For the purpose of classification for carcinogenicity, substances are allocated to one of two categories based on strength of evidence and additional considerations (weight of evidence) (CLP Regulation 1272/2008, 3.6.2.1.)."

Hazard categories for carcinogens (CLP Regulation 1272/2008, Table 3.6.1)

Category 1:

"Known or presumed human carcinogens

A substance is classified in Category 1 for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as:

***Category 1A:** Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or*

***Category 1B:** Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence."*

Category 2:

"Suspected human carcinogens

The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies."

Strength of evidence (CLP Regulation 1272/2008, 3.6.2.2.3.):

"Strength of evidence involves the enumeration of tumours in human and animal studies and determination of their level of statistical significance.

*Evidence of carcinogenicity can be considered **sufficient** if a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites;*

*Evidence of carcinogenicity can be considered **limited** if the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs."*

Data concerning melamine-related carcinogenicity in humans are not available. Classification in category 1A is therefore not appropriate. However, data derived from long-term bioassays using experimental animals exist and provide evidence to assess the intrinsic property of melamine to induce neoplastic lesions. Accordingly, three independent and reliable studies in rats, conducted at different times and in different laboratories, established a causal relationship between melamine exposure and a significantly increased incidence of benign and malignant neoplasms in the urinary bladder of one species and one sex (male rats) (NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992). At high doses, tumour formation was reported at a high incidence (up to 90 % at the highest dose) and short latency (36 weeks of treatment) given the non-genotoxic MoA. The fourth key study by Hazleton (1983) indicates that carcinogenic effects require a certain threshold dose (Hazleton, 1983).

On the basis of the information provided, data from the key experimental animal studies are considered as sufficient evidence of carcinogenicity in animals as they show a causal relationship between melamine administration and an increased incidence of tumours which may potentially justify the classification in category 1B.

Classification in category 2 solely based on the key experimental animal studies may not be appropriate as the evidence cannot be considered limited for the following reasons:

(a) Carcinogenic effects have been observed in multiple experiments/studies.

(b) Three studies showing carcinogenic activity are considered sufficiently reliable and adequate in regard to design, conduct and interpretation.

(c) Melamine increases the incidence of both, malignant and benign neoplasms.

(d) The data provided by the key studies clearly demonstrate carcinogenic effects and not just promoting activity in a narrow range of tissues or organs.

Limited supportive evidence is additionally provided by two supplemental studies describing microscopic benign papillomata in the urinary bladder of male and female rats and melamine-related D/CIS of the urinary bladder, the ureter, and to a lesser extent in the renal pelvis in BALB/c mice at high-dose melamine exposure (1800 mg/kg/bw/d) which are regarded as lesions of uncertain neoplastic potential (Cremonezzi et al., 2001; Hazleton, 1953). No carcinogenic effect was seen in B6C3F1 mice at considerably lower concentrations (♂/♀ 327/688 and 523/1065 mg/kg/bw/d) (NTP, 1983).

Additional considerations / Weight of evidence (CLP Regulation 1272/2008, 3.6.2.2.4. - 3.6.2.2.6.):

"Beyond the determination of the strength of evidence for carcinogenicity, a number of other factors need to be considered that influence the overall likelihood that a substance poses a carcinogenic hazard in humans.

Some important factors which may be taken into consideration, when assessing the overall level of concern are (CLP Regulation 1272/2008, 3.6.2.2.6.):"

(a) tumour type and background incidence

Reliable experimental animal studies have demonstrated an increased incidence of papillomas and carcinomas arising from the transitional epithelium of the urinary tract (urothelium) secondary to the occurrence of calculi related to melamine administration (Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992). No incidence of transitional cell carcinomas (0/3551) and a very low incidence for papillomas (4/3551; 0.1 %) were reported in the historical background control data from several laboratories (NTP, 1983). Thus, the transitional cell tumour incidence exceeds the historical control data (HCD) and can, therefore, be considered treatment-related.

According to *ECHAs Guidance on the Application of the CLP Criteria (3.6.2.3.2.; section a) "By default, carcinogenic effects in experimental animals are considered relevant to humans and are considered for classification as carcinogens"*. However, certain types of tumours may not be considered for classification if sufficient evidence shows no relevance to humans. According to the recommendations of the *ECHA Guidance on the Application of the CLP Criteria* (3.6.2.3.2.; section k), based on an assessment by IARC (IARC, 1999b), urinary bladder tumours due to crystals in the bladder are considered not relevant to humans. However, the consensus section of the corresponding IARC report states:

"For chemicals producing bladder neoplasms in rats and mice as a result of calculus formation in the urinary bladder, the response cannot be considered to be species-specific; thus, the tumour response is relevant to an evaluation of carcinogenicity to humans. There are quantitative differences in response between species and sexes. Calculus formation is dependent on the attainment in the urine of critical concentrations of constituent chemicals which form the calculus; therefore, the biological effects are dependent on reaching threshold concentrations for calculus formation."(IARC, 1999b)

Accordingly, IARC did not exclude a carcinogenic response to chemical-mediated calculi in humans. It was rather discussed whether species have the ability to produce certain calculi based on specific chemical and physical conditions of the urine. Only the effect of sodium salts (e.g. saccharin or ascorbate) in terms of urinary precipitation followed by tumourigenic effects was considered a rat-specific phenomenon. Hereby, urinary precipitation is based on the presence of extraordinarily high urinary concentrations of alpha-2 (α2u) globulin and albumin. The interacting of these proteins with sodium salts deems necessary to form urinary precipitates in rats. Unlike rats, humans have a much lower urinary protein content (100-1000 times lower) and α2u-globulin or a similar protein does not occur (IARC, 1999b). It is worth noting that administration of saccharin leads to precipitation in rats but not in non-human primates, whereas melamine exposure causes calculi/crystal formation in rodents, non-human primates and humans (Early et al., 2013; IARC, 1999b; Lam et al., 2009; Takayama et al., 1998). In addition, several lines of evidence, explicitly discussed in section (k), suggest that melamine-related urinary stone formation may nevertheless pose a carcinogenic risk to humans. Concerning the classification of melamine, dismissing this tumour type may, therefore, not be justified.

Beside tumour formation in the urinary bladder of male rats, C-cell carcinomas in the thyroid of female rats were observed with a significant positive trend in the NTP (1983) study. Neither a pairwise comparison of the high-dose group with the concurrent control nor a comparison of the high-dose group with the HCD showed any statistical significance. These tumours were considered unrelated to melamine administration by the authors of the study (NTP, 1983).

(b) multi-site responses

Within the mammalian urinary tract system, the transitional cell epithelium covers the lining of the proximal urethra, the urinary bladder, the ureter, the renal pelvis and calyx as depicted in Illustration 1 (Apodaca, 2004; Hong et al., 2009; Oyasu, 1995). The ureter connects the urinary bladder with the renal pelvis which is the expanded funnel-shaped proximal end of the ureter. The extension of the renal pelvis is called calyx followed by the renal papilla, which is the apex of the pyramid where urine drains from the pyramid (Lote, 2012).

The urothelium (transitional cell epithelium) of parts of the urinary tract system is the sole site of melamine-related carcinogenicity (Cremonezzi et al., 2004; Cremonezzi et al., 2001; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992). The incidence of malignant neoplasms was significantly increased in the urinary bladder of male rats (Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992). Papillomas and a single tumour were found in the ureter of male rats (Okumura et al., 1992). D/CIS were reported in the urinary bladder, the ureter, and to a lesser extent in the renal pelvis of mice (Cremonezzi et al., 2001). Preneoplastic lesions such as hyperplasias and metaplasias were observed in the upper urinary tract (kidney, ureter) of rats and mice (Cremonezzi et al., 2004; Cremonezzi et al., 2001; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992).

(c) progression of lesions to malignancy

Dose-dependent benign and malignant epithelial neoplasms within urinary tract in rats have been described in three key studies (Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992). Other supporting studies have shown preneoplastic lesions (D/CIS) and benign epithelial neoplasms in mice and rats (Cremonezzi et al., 2001; Hazleton, 1953). Preneoplastic lesions such as hyperplasias or metaplasias were additionally seen in mice and rats (Cremonezzi et al., 2004; Cremonezzi et al., 2001; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992)

(d) reduced tumour latency

Following melamine administration, tumour formation in rats and D/CIS (combined) in mice have been observed already after 40 (36 weeks of treatment + 4 weeks recovery) and 22 weeks, respectively

(Cremonezzi et al., 2001; Ogasawara et al., 1995; Okumura et al., 1992). Hence, neoplastic effects were already induced and seen following sub-chronic exposure with durations substantially shorter as compared to the standard duration of the respective test guideline (24 months, OECD TG 451). Thus, melamine-mediated tumourigenesis does not necessarily require life-long exposures.

(e) whether responses are in single or both sexes

Reliable experimental animal studies (key studies) show increased tumour incidences with statistical significance exclusively in male rats (Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992).

Beyond that, other lesions related to carcinogenesis were reported in key and supporting studies such as microscopic benign papillomas in the urinary bladder of male and female rats and D/CIS (combined) in the urinary bladder, ureter and renal pelvis of mice of presumably both sexes (not specified in the study) (Cremonezzi et al., 2001; Hazleton, 1953). Preneoplastic lesions in the urinary bladder, ureter, and kidney were seen in mice and rats of both sexes (Cremonezzi et al., 2004; Cremonezzi et al., 2001; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992).

It is noteworthy that there is a species-independent male predisposition to melamine-mediated urolithiasis in rodents and humans. Greater urinary concentrations of protein, hormonal effects, and anatomical differences in the urethra have been discussed as underlying factors in rodents (Cohen and Lawson, 1995; De Sesso, 1995; Meek et al., 2003). In humans, a higher male-to-female ratio for general (paediatric) renal stone development and melamine-related urolithiasis (see section 10.11.) has been linked to different uric acid (higher in male) and hormone levels and to anatomical differences (Lu et al., 2011; Schulsinger, 2014; Sun et al., 2010c).

(f) whether responses are in a single species or several species

Reliable experimental animal studies (key studies) show increased tumour incidences with statistical significance exclusively in rats (Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992). In addition, melamine-mediated responses related to carcinogenesis were observed in key and supporting studies. Accordingly, preneoplastic lesions in the urinary tract rats have been observed (Cremonezzi et al., 2004; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992).

In mice, epithelial hyperplasia in the urinary bladder was reported at lower concentrations (♂/♀ 327/688 and 523/1065 mg/kg/bw/d) in the 2-years NTP mice study (mainly in males) (NTP, 1983). Increased incidences of hyperplasia and D/CIS (combined) in the urinary tract (bladder, ureter, and renal pelvis) of mice have been reported at high-dose melamine exposure (1800 mg/kg/bw/d). However, the latter lesions are regarded as lesions of uncertain neoplastic potential. As no tumour had been observed in any of the available studies, mice are considered less sensitive to melamine-mediated precipitation. For comparison, oral administration of uracil, a substance that promotes carcinogenesis via the same mode of action (MoA), increases the incidence of transitional cell carcinoma in both, rats and mice (Fukushima et al., 1992).

(g) structural similarity to a substance(s) for which there is good evidence of carcinogenicity

No data available.

(h) routes of exposure

Melamine was orally administered in long-term bioassays in experimental animals. This route of exposure also constitutes a relevant route in humans as melamine-related toxicity was observed following oral uptake (WHO / FAO, 2009). Consequently, in terms of the carcinogenic potential, the oral route is considered the most relevant route of exposure for both, rodents and humans.

One study using dermal application was identified. However, this study is considered not relevant (disregarded) as melamine was only tested for its property to initiate carcinogenesis (single application) followed by extended dermal application of the promoter TPA (31 weeks).

(i) comparison of absorption, distribution, metabolism and excretion between test animals and humans

Following a single oral administration of melamine in rats, the substance was rapidly absorbed with maximal plasma concentrations achieved after 1 h. 90 % of the administered melamine was excreted within 24 h via the urine (Mast et al., 1983). No significant biotransformation was observed (Mast et al., 1983). The urinary-excretion half-life was reported to be 3 h in rats and the plasma elimination half-life ranged from 2.7 h to 4.9 h in rats, 4 h in pigs, and 4.4 h in rhesus monkeys (Baynes et al., 2008; Liu et al., 2010a; Mast et al., 1983; Yang et al., 2009). While detailed information concerning pharmacokinetics in humans is not available, melamine was, similar to observations in animals, detected unmetabolised in the urine of paediatric patients that had been exposed to melamine-tainted milk products (Cheng et al., 2009; Kong et al., 2011; Lam et al., 2009; Zhang et al., 2010a). Hence, melamine undergoes rapid renal clearance in multiple mammalian species and it is likely that pharmacokinetics in humans is similar. Accordingly, in a randomized crossover human study that investigated urinary melamine excretion subsequent to low-dose melamine exposure (migration from melamine resin plastic bowls), an estimated half-life of approximately 6 hours was derived for urinary melamine elimination (Wu et al., 2013).

(j) the possibility of a confounding effect of excessive toxicity at test doses

Not identified. Tumour formation was seen at concentrations (330 - 350 mg/kg bw/d) that did not induce excessive toxicity (weight depression and/or survival) throughout the duration of the tests (Ogasawara et al., 1995; Okumura et al., 1992).

(k) mode of action and its relevance for humans

The following section aims to clarify whether the established MoA in experimental animals is relevant to humans.

MoA in animals - Experimental animals

As mentioned above, available animal data provide sufficient evidence on carcinogenic properties of melamine. As described in section 10.7 of this dossier, melamine is considered a non-genotoxic agent as it does not show any relevant genotoxic effects in the available test systems (IARC, 1999a; WHO / FAO, 2009) (see section 10.7). A dose-related formation of urinary bladder stones has been consistently observed in experimental animal studies and is considered the predominant adverse effect in terms of carcinogenicity. Based on a strong correlation between the occurrence of calculi in the bladder and neoplastic events at the same site, the established and commonly accepted MoA for melamine-associated carcinogenicity in rodents postulates transitional cell tumour formation in the bladder subsequent to melamine-induced urolithiasis (Cremonezzi et al., 2001; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992). Hence, the urinary bladder is considered as the primary target site related to carcinogenicity in rodents (Melnick et al., 1984; NTP, 1983).

Following oral ingestion, mainly unchanged melamine is rapidly excreted via the urine (see section 9) (Mast et al., 1983; Xie et al., 2010). Although the exact mechanisms, as of how melamine-mediated calculi form, has not been elucidated, it is thought that melamine interacts with uric acid to form melamine-uric acid salts that precipitate within the urine presumably as microcalculi within the renal pelvis, leading to transitional cell hyperplasia and ischemic changes in the kidney (papilla/pelvis), and the formation of larger calculi that are composed of equimolar amounts of melamine and uric acid (total combined content 61 - 81 %) (Ogasawara et al., 1995). Whether uric acid plays a major role in calculus formation in experimental animals is, however, a matter of debate as other studies failed to identify uric acid as a major stone component (Cong et al., 2014; Heck and Tyl, 1985; Shen et al., 2011a). In the urinary bladder, melamine-related stones have been strongly linked to local irritation and repeated physical damage to the transitional cell epithelium followed by urothelial proliferation, regenerative hyperplasia and subsequent progression to transitional cell papillomas and carcinomas (Bhat et al., 2010; Clayson et al., 1995; Cohen and Lawson, 1995; McGregor et al., 2010; Meek et al., 2003; NTP, 1983; Okumura et al., 1992). Simultaneous administration of melamine and high-doses of NaCl suppresses the formation of calculi and reduces the incidence of bladder tumours. This can be attributed to polyuria induced by NaCl supplementation which presumably facilitates the excretion of microcrystals, thus preventing the formation of larger calculi including their carcinogenic effects on the urothelium (Ogasawara et al., 1995). Administration of melamine concentrations below the level of

calculus formation does not increase the incidence of bladder tumours (Hazleton, 1983). A similar MoA has been described for other non-genotoxic calculus-forming chemicals such as uracil (Cohen et al., 2002). Male rats and mice are more susceptible to calculus formation as compared to females. Greater urinary concentrations of protein, hormonal effects, and anatomical differences in the urethra have been discussed as underlying factors (Cohen and Lawson, 1995; De Sesso, 1995; Meek et al., 2003). Mice are less sensitive than rats in regard to the progression of preneoplastic lesion to benign/malign tumours.

Melamine exposure was also associated with evidence of precarcinogenic effects in the kidney. Hyperplasia has been noted in the urinary bladder of mice (Cremonezzi et al., 2001; NTP, 1983). The incidence of chronic kidney inflammation was statistically significantly increased in female rats treated with high doses of melamine (ca. 542 mg/kg bw/d) as described in the NTP study. Male rats showed a slighter and statistically not significant increase at a lower dose (ca. 263 mg/kg bw/d). The renal inflammation was associated with lymphoplasmocytic infiltrates and fibrotic lesions(stretching from cortex into the medulla, associated with collecting duct dilatation and hyperplasia in the inner medulla, loss of tubule, tubule atrophy, and crowded glomeruli in the cortex) as revealed by a re-examination of the kidney histopathology (Hard et al., 2009). These observed renal effects in female rats were not associated with detectable stones (NTP, 1983). Chronic renal inflammation and proliferative lesions of the transitional epithelium (such as metaplasia, hyperplasia, and dysplasia mainly at the proximal end of the urinary tract (papillae and renal pelvis)) in the absence of urolithiasis were also observed in another study using rats (Cremonezzi et al., 2004). In the study conducted by Ogasawara et al. (1995), microcrystals/microcalculi, observed in the urinary sediment of male rats, were assumed to be formed in the renal pelvis and associated with lesions in papilla (transitional cell hyperplasia with angiectasis and thrombus formation) and lesions in the cortex (fibrosis, inflammation cell infiltration, renal tubule regeneration) after 36 weeks of high-dose melamine exposure (ca. 1030 mg/kg bw/d). As the observed preneoplastic renal lesions were attenuated in the high-dose group and completely suppressed in the low-dose group by concomitant administration of NaCl, it was concluded that the occurrence of microcrystals/microcalculi stimulates the epithelium giving rise to the reported kidney lesions (Ogasawara et al., 1995). Similar to the effects of larger calculi on the epithelium, microcrystalluria is associated with urothelial damages, regenerative hyperplasia, and tumour formation (Cohen and Lawson, 1995). Okumura et al. (1992) reported urothelial (transitional cell) hyperplasia not only in the urinary bladder but also at a high incidence in the ureter and in the renal pelvis of melamine-treated male rats that was accompanied by neoplastic lesions in the ureter (papillomas in 3/19 (16 %) and carcinoma in 1/19 (5 %)). Haematuria, often associated with renal injuries, was also observed in the high-dose group (ca. 1090 mg/kg bw/d) (Okumura et al., 1992).

A recent repeated dose toxicity study in rats revealed extensive crystal formation within the renal tubules (23/24 at 1000 mg melamine/kg bw/d, determined by wet mount analysis) and renal lesions such as tubular necrosis (see repeated dose toxicity section). Most notably, to establish a reliable method to evaluate tissue for crystals, the authors of the study employed different techniques (formalin fixation vs. wet mount). Unlike the in the study preferred wet mount analysis, tissue fixation with formalin (routinely done for histopathology) and exposure to ethanol dissolve melamine-related crystals, providing a potential explanation for the absence of renal precipitation in the NTP study and the study performed by Cremonezzi et al. (2004) (Stine et al., 2014). Accordingly, the authors of the latter study concluded that the observed renal effects may be linked to crystal casts that had been spontaneously dissolved. The histological examination in this study was done subsequent to formalin fixation (Cremonezzi et al., 2004). Another repeated dose toxicity study reported renal tubular cell debris, crystal deposition, and hyperactive regeneration of renal tubular epithelium in male and female rats subjected to 700 mg melamine/kg bw/day and crystalluria and nephrotoxicity (e.g. renal tubular degeneration/regeneration) in monkeys that had been treated with the same dose (Early et al., 2013). A 28-day study by Xu et al. (2010) reported increased mRNA expression of oncogenes in kidney tissue (c-myc, c-fos, and N-ras) of rats following melamine administration (due to a lack of available details, the results could not be assessed in full) (Xu et al., 2010). In addition, uracil exposure in rats, which is believed to exert toxicity via a similar MoA, significantly increases the incidence of papillomas and carcinomas in the renal pelvis (Fukushima et al., 1992). Thus, microcrystals/microcalculi appear to form in the kidney subsequent to oral melamine administration and are associated with preneoplastic renal effects that can be similar to those observed in the calculi-irritated bladder urothelium of rats such as hyperplasia of the transitional cell epithelium of the renal papillae and pelves. This suggests that microcrystals/microcalculi may induce proliferative lesions within the kidney by a

similar MoA, involving stimulation of the epithelium, epithelial damages, and hyperplasia. Microcrystals may also play an important role in renal inflammation and the formation of fibrotic lesions within the cortex and medulla. Although no tumours in the kidney have been observed in chronic bioassays with melamine, it appears conceivable that the observed renal lesions may have the potential to progress to papillomas or carcinomas in analogy to data obtained with uracil.

In summary, ample evidence from studies in rats, mice, and monkeys suggest that melamine-related precipitation originates in the kidneys and damages the epithelium along the urinary tract (including urinary bladder, ureter, and kidney), giving rise to transitional cell tumours in the bladder and precarcinogenic events (i.e. proliferative lesions of (a) the transitional cell epithelium (bladder, ureter, renal pelvis/papilla) and (b) renal tubular epithelium injuries and inflammation) that may be considered precursor lesions of neoplasms.

MoA in animals – Pets

In 2007, numerous cases of renal damage, kidney failure, and increased mortality had been reported in dogs and cats exposed to melamine-contaminated animal feed (Brown et al., 2007; WHO / FAO, 2009). Adverse effects observed in these animals were attributed to the presence of crystals in the kidney tubules and comprised renal tubular necrosis and inflammation, crystalluria, and haematuria (Cianciolo et al., 2008; Dobson et al., 2008). In contrast to the application of pure melamine in experimental animal studies, a mixture of several triazines (especially melamine and cyanuric acid but also ammeline and ammelide) was found in the animal feed (Puschner and Reimschuessel, 2011). The pattern of adverse renal effects seen in pets was consistent with results obtained from studies using a combination of melamine and cyanuric acid in experimental animals (Dalal and Goldfarb, 2011). Melamine-related toxicity is exacerbated in the presence of cyanuric acid (WHO / FAO, 2009). Crystals derived from a combined exposure to melamine and cyanuric acid are distinguishable from crystals that form upon exposure to melamine only (Stine et al., 2014).

Humans – Adulteration incident 2008 in China (the following section gives a short overview on the topic; relevant studies are listed in Table 20 *of the STOT-RE part of the current dossier)*

As described in experimental animal studies, the melamine-related MoA concerning carcinogenicity requires exposure sufficient to form precipitations in the urinary tract. Such high-level exposure was not anticipated to occur in humans (Meek et al., 2003). However, melamine-mediated urolithiasis in humans as a result of oral uptake was unfortunately seen in the wake of the melamine-tainted milk adulteration incident/scandal 2008 in China (WHO / FAO, 2009). Urinary tract calculi and renal toxicity were described in Chinese children who consumed melamine-containing infant formula (Chan et al., 2008; Guan et al., 2009; Lam et al., 2009; Zhang et al., 2009; Zhu et al., 2009). The prevalence of urolithiasis thereby correlated tightly with the estimated total consumption of contaminated formula (Table 16) (Shi et al., 2012). According to official numbers from the Chinese Ministry of Health, almost 300 000 children were affected, more than 50 000 underwent hospitalisation, and six confirmed deaths were related to the ingestion of melamine-contaminated infant formula (WHO / FAO, 2009). It is worth noting that melamine contamination was not limited to infant formula but has also been detected in other food products such as eggs or wheat gluten (Ingelfinger, 2008).

Table 16: Prevalence of urolithiasis according to estimated total Sanlu infant formula consumption among 344 children ≤ 3 years old who drank exclusively Sanlu infant formula (Shi et al., 2012)**.**

Estimated total consumption (g) of Sanlu infant formula	**No. Children**	**Urolithiasis**	**Prevalence (%)**
20+	19	0	0.0
400+	70	8	11.4
3200+	44	7	15.9
6400+	51	12	23.5
12800+	96	34	35.4
25600 - 76000	64	24	37.5
Total	344	85	24.7

Similar to what has been observed in studies with rodents, male human individuals were more susceptible to melamine-mediated urolithiasis when compared to females, suggesting a species-independent male predisposition (Liu et al., 2010b; Lu et al., 2011; Melnick et al., 1984; NTP, 1983). The reported melamine concentrations in the infant formula samples were in the range of 1212 mg/kg as the mean up to 4700 mg/kg as the maximum and the corresponding estimated intake was 10.4 to 28.4 mg/kg bw/d and 40.3 to 110.2 mg/kg bw/d dependent on the infant age, respectively (WHO / FAO, 2009). A tolerable daily intake (TDI) of 0.2 mg/kg bw/d was derived by WHO (WHO / FAO, 2009). However, it was reported that the risk of melamine-induced urolithiasis in children increases even at doses below the WHO TDI (Chen et al., 2009; Li et al., 2010). A considerably lower TDI of 0.0081 mg/kg bw/d was suggested by Hsieh et al. (2009). The European Food Safety Authority (EFSA), however, stated that human data were not sufficiently robust for the purpose of deriving an accurate TDI value, which is why the TDI had not been changed (EFSA, 2010). Uroliths in paediatric patients were mainly composed of melamine and uric acid and, thus, resembled the stone composition of experimental rats fed with pure melamine as reported by Ogasawara et al. (1995) (Chang et al., 2012; Grases et al., 2009; Ogasawara et al., 1995; Sun et al., 2010b; Sun et al., 2009; Sun et al., 2010c; Wang et al., 2011; WHO / FAO, 2009). However, as mentioned above, uric acid was not identified as a major stone component in other rodent studies (Cong et al., 2014; Heck and Tyl, 1985; Shen et al., 2011a). Based on the structural properties of melamine that allows for hydrogen bonding with uric acid, the formation of a crystalline lattice structure from melamine and uric acid was suggested (Dalal and Goldfarb, 2011; Grases et al., 2009; WHO / FAO, 2009). Uric acid was considered a main aetiological factor involved in the formation of melamine-mediated uroliths in humans (Chang et al., 2012). Other triazines were not found relevant for stone formation in humans (Grases et al., 2009; Lam et al., 2009; Sun et al., 2010b; Sun et al., 2010c). Importantly, as humans lack the enzyme urate oxidase (uricase) that, in most other mammals, converts uric acid to allantoin, uric acid levels are much higher in humans when compared to other mammals such as rats (e.g. 5-fold when comparing human infants to rats) (Alvarez-Lario and Macarron-Vicente, 2010; WHO / FAO, 2009). Higher uric acid levels may advance the formation of melamine-related kidney stones and thus, lower melamine levels might be sufficient for stone formation in humans (higher potency in humans possible) (WHO / FAO, 2009). A study in rats investigated the effects of a combined exposure to melamine and potassium oxonate (oxo); a nontoxic uricase inhibitor that induces hyperuricemia in rats. Interestingly, the results show that co-administration of oxo dramatically increases the toxicity of melamine leading to high mortality and severe renal damages (Zhang et al., 2015). Thus, there is evidence that humans may be more susceptible towards the development of urolithiasis attributed to melamine exposure as compared to rodent experimental animal models.

The predominant location of melamine-related calculi in exposed children was the kidney (mostly renal pelvis and calyx) whereas only a few stones were found in the ureter or the urinary bladder (Bhat et al., 2010; Ding, 2009; Guan et al., 2009; He et al., 2009; Lam et al., 2009; Shi et al., 2012; Sun et al., 2010a; Wang et al., 2009; Wang et al., 2011; Zhang et al., 2009; Zhu et al., 2009). For instance, He et al. (2009) observed almost all stones in the kidney (in one kidney: 431/562 children; in both kidneys: 131/562 children) and only a single stone in the bladder of one child (He et al., 2009). Shi et al. (2012) located 361/362 stones in the renal pelvis (Shi et al., 2012). Stones reached a size of 19-33 mm (Gao et al., 2011; Sun et al., 2010a; Zou et al., 2013).

Whether and how melamine forms microcrystals in the human renal tubular system is insufficiently elucidated (Guan et al., 2009). Ultrasonography, the most common diagnostic imaging technique, may not be sufficient to detect crystals, crystal aggregates, or smaller calculi (WHO / FAO, 2009). However, although not reported routinely, crystalluria was observed in some melamine-exposed paediatric patients (Lam et al., 2009; Ren et al., 2009) and intraluminal crystals and sand gravel-like material in the renal tubules were found in kidney biopsies (Jia et al., 2009a; Sun et al., 2010b). Crystalluria was also reported in a patient that had been treated with a melamine analogue triethylene melamine (Kravitz et al., 1951). Generally, lithogenic crystals are known to be cytotoxic and have been linked to renal inflammation as they trigger a general inflammatory response after being endocytosed by renal tubular cells (Boonla et al., 2007; Khan, 2004; Mulay et al., 2014). Accordingly, melamine has been shown to induce chronic kidney inflammation in experimental animal studies (Cremonezzi et al., 2004; Cremonezzi et al., 2001; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995), in murine macrophage cells and human embryonic kidney cells (Kuo et al., 2013), and in children exposed to contaminated infant formula (Lau and Tu, 2013; Sun et al., 2010b).

Inflammation is considered a significant risk factor for the development of urinary tract tumours (Burin et al., 1995).

While the majority of children with melamine-related nephrolithiasis did not show clinical signs and symptoms, nephropathy which in some cases progressed to obstructive acute renal failure was consistently reported (Dalal and Goldfarb, 2011; Lam et al., 2009; Sun et al., 2010c; Wang et al., 2013; Wu and Zhang, 2013). Nephrotoxicity including renal inflammation and renal injuries/lesions were seen in children secondary to melamine-induced renal precipitation (Gao et al., 2011; Guan et al., 2009; Lam et al., 2009; Lau and Tu, 2013; Zou et al., 2013). Lymphocytic infiltration in the glomeruli, sclerotic glomeruli, proliferation of fibrous tissue in the glomeruli and Bowman's capsule, swollen tubular cells, lymphocytic infiltration and fibrosis within the renal interstitium, and crystals within the lumen were observed in a kidney biopsy from a paediatric patient (Sun et al., 2010b). Markers for nephron impairment, tubular damage, and glomerular dysfunction were elevated in children with melamine-related stones and were still significantly different from healthy children, one year after the diagnosis (Shen et al., 2011b). Macroscopic and microscopic haematuria was described (Gao et al., 2011; Guan et al., 2009; Shang et al., 2012; Shen et al., 2011b; Sun et al., 2010a; Yang et al., 2010b; Zou et al., 2013) and may be a result of stone-related urothelial abrasion/irritation (Schulsinger, 2014; Yang et al., 2010b). The kidney was considered the predominant target site of melamine-related toxicity (Deng and Li, 2012; Hau et al., 2009; Wen et al., 2016; Wu and Zhang, 2013).

Information on the long-term effects of paediatric urolithiasis is limited. Whether there is a higher risk of urinary tract cancer (UTC) in children exposed to melamine is unclear (Ingelfinger, 2008). To date, no tumours in melamine-exposed individuals have been reported in follow-up studies up to 5 years (Chang et al., 2017; Wen et al., 2016). However, an increased risk of tumour formation in adulthood has been hypothesized for melamine-exposed children (Vara Messler et al., 2012; Wen et al., 2016). Persistent urolithiasis (up to 5 years) and chronic renal abnormalities have been reported and linked to potential irreversible damages as summarized in Table 20 (STOT-RE) and Table 24 (Annex II) (Gao et al., 2011; Liu et al., 2010b; Shen et al., 2011b; Wang et al., 2013; Zou et al., 2013). The most recent follow-up analysis reported that 91.4 % of the children (n = 198) expelled their stones after 5 years of their discharge and renal damages were not found. However, residual stones in the kidney were still observed in 17/198 (8.6 %) subjects (Chang et al., 2017). Another study demonstrated that the size of calculi increased in a small number of patients during a 12 month follow-up period (Dai et al., 2012). Long-term follow-up was suggested to detect early stage neoplastic events that may arise from melamine exposure in childhood (Puschner and Reimschuessel, 2011; Vara Messler et al., 2012; Wen et al., 2016).

Humans – Environmental low-dose exposure (relevant studies arey listed in Table 20: Summary table of human data on STOT RE *of the STOT-RE part of the current dossier)*

In addition to the numerous reports related to infant urolithiasis attributed to the consumption of melamine-tainted infant formula, exposure to lower melamine levels may be involved in the development of calcium urolithiasis in adults which is further elaborated in the STOT-RE part of the current dossier. Accordingly, a large-scale case-control study reported a strong association between urinary melamine concentrations, presumably derived from low-dose environmental exposure, and the risk of common calcium urolithiasis (urinary melamine level ≤ 3.11 ng/ml: adjusted odds ratio: 3.01; urinary melamine level ≥ 3.12 ng/ml: 7.64; trend test: $P < 0.0001$). Low-level melamine may, hence, be involved in the aethiology of calcium lithiasis. Melamine was also found as a component in all analysed stones from subjects with detectable urinary melamine concentrations (Liu et al., 2011).

Humans – Urinary calculi in general

The formation of urinary calculi in humans is generally related to supersaturation of the urine and crystallization of stone-forming salts in the kidney (Pak, 1998; Schissel and Johnson, 2011; Schulsinger, 2014). Stones vary in a wide range of size and are usually seen in the renal calyx and pelvis, the ureter, and urinary bladder (Evan, 2010). Most stones (approximately 80 %) are calcium oxalate stones. Depending on size, stones can either pass the ureter into the urinary bladder with a subsequent discharge through the urethra or obstruct the urinary tract, most commonly in the ureter. Whereas stones smaller than 4 mm have a 80 % chance of spontaneous passage, calculi above 10 mm are unlikely to pass spontaneously (Schulsinger, 2014). However, some renal stones have the ability to persistently accumulate in the human kidney where

they can grow to a large size. These stones, called staghorn calculi, are too large to move or obstruct the urinary tract and remain usually asymptomatic within the renal pelvis (Burin et al., 1995; Jongyotha and Sriphrapradang, 2015; Schulsinger, 2014).

Renal calcium stones, the most common type of nephrolithiasis in humans, have been linked to injured and necrotic renal epithelium associated with interstitial fibrosis, tubular epithelial hyperplasia, focal calcifications, and eroded papillary surface epithelium (Khan et al., 1984).

The lifetime risk for kidney stones amongst adults in industrial countries is approximately 10–12 % and both incidence and prevalence have risen worldwide, resuming a constant upward trend (Goldfarb, 2003; Moudi et al., 2017; Romero et al., 2010; Schulsinger, 2014). Kidney stones are uncommon in children, representing only 2 % to 3 % of the total population of patients with stones (Schwarz and Dwyer, 2006). The exact incidence of kidney stones amongst children is unknown (Moudi et al., 2017). In the United States, nephrolithiasis was reported to account for approximately 1/7600 to 1/685 hospital admission (Bush et al., 2010; Kokorowski et al., 2010). The prevalence of urolithiasis in China with regard to age was reported 0.27 % (< 20 years), 3.15 % (20–29 years), 5.96 % (30–39 years), 8.18 % (40–49 years), 9.14 % (50–59 years), and 9.68 % (> 60 years) (Wang et al., 2017). Similar data have been published for other countries (Romero et al., 2010).

Humans - urinary tract stones and urinary tract cancer

A considerably large body of evidence from epidemiological studies suggests a significant association between a history of urinary tract stones and various types of cancer in the kidney.

A meta-analysis published by Cheungpasitporn et al. (2015) assessed the association between a history of kidney stones and the incidence of the two common kidney cancers namely renal cell carcinoma (RCC) of the renal parenchyma and transitional cell carcinoma (TCC) of the upper urinary tract involving the renal pelvis (the predominant location of melamine-related stones in Chinese children). A statistically significantly increased risk of RCC and TCC in human beings with a history of kidney stones was demonstrated. In particular, nine observational studies were identified as relevant based on inclusion criteria in a comprehensive screening of the existing literature. Seven studies (six case-control studies and one retrospective cohort study) were considered relevant for analyzing the risk of RCC. A statistically significantly increased pooled relative risk (RR, 1.76 [95 % CI, 1.24–2.49]) was found. An elevated risk was reported in six out of seven studies. A subgroup analysis revealed an increased risk only in males (RR, 1.41 [95 % CI, 1.11–1.80]) but not in females (RR, 1.13 [95 % CI, 0.86–1.49]). 62 925 patients with kidney stones were included in the RCC analysis (Chow et al., 1997; Chung et al., 2013a; Maclure and Willett, 1990; McCredie and Stewart, 1992; McLaughlin et al., 1984; Schlehofer et al., 1996; Talamini et al., 1990). To assess the risk of TCC, five observational studies (four case-control studies and one retrospective cohort study) were taken into account and the analysis revealed a statistically significantly increased pooled relative risk of 2.14 (95 % CI, 1.35–3.40) in patients with a history of kidney stones. An elevated risk was reported in three out of five studies. 62 377 patients with kidney stones were included in the TCC analysis (Chow et al., 1997; Chung et al., 2013a; Liaw et al., 1997; McCredie and Stewart, 1992; Ross et al., 1989). Due to data limitation, a gender-related subgroup analysis of TCC risk was not performed. Although certain limitations are discussed, the included studies were of high quality as evaluated by Newcastle-Ottawa scale (Cheungpasitporn et al., 2015). The occurrence of TCC related to calculi in the renal pelvis has also been described in case reports and clinical studies (Inci et al., 2009; Katz et al., 2005; Kok et al., 1994).

An association between kidney stones and RCC and upper tract urothelial cancer (UTUC; in the ureter and renal pelvis) was also studied in the recently published Netherland Cohort Study, including 120 852 participants. As detailed information on risk factors commonly associated with kidney stones, RCC, and UTUT was available prior to tumour development, extensive adjustments for multiple confounders were possible. According to the outcome of the study, nephrolithiasis was associated with an increased risk of papillary RCC (HR: 3.08, 95% CI 1.55–6.11) and of UTUC (Hazard ratios: 1.66, 95% CI 1.03–2.68).

A nationwide population-based study using Taiwan's National Health Insurance Research Database revealed a statistically significantly increased cancer risk associated with urinary calculi (SIR, 1.75; 95 % CI: 1.68–1.83) with the highest risk observed for kidney cancer (SIR, 4.24; 95 % CI: 3.47–5.13) and bladder (SIR, 3.30; 95 % CI: 2.69–4.00). Most notably, an increased risk was still observed after individuals received urolithiasis treatment, suggesting that initial calculi-related damages may persist. Irritation of the epithelium,

chronic and systemic inflammation, and the presence of carcinogens was hypothesized as a potential underlying mechanism that may facilitate systemic tumourigenesis (Shih et al., 2014). A small-scale population-based cohort study including 27 cases reported a significantly increased risk of UTC in patients with urolithiasis (adjusted HR, 4.66; 95 % CI: 2.97-7.30) (Sun et al., 2013). Another cohort study conducted by Lin *et al.* (2016) found a 1.82-fold (95 % CI: 1.66–1.99, P < 0.001) increased risk of developing urinary tract tumours in individuals (n = 695) with previously diagnosed urolithiasis. With regard to the site of calculi-related cancer, the kidney was most commonly associated with malignancies followed by the ureter, the bladder, and the prostate gland (the adjusted hazard ratio (HR) for bladder, renal pelvis/ureter, renal, and prostate cancers were 1.94 (95 % CI: 1.62–2.33), 2.94 (95 % CI: 2.24–3.87), 2.94 (95 % CI: 2.29–3.77), and 1.45 (95 % CI: 1.27–1.65), respectively) (Lin et al., 2016).

Chronic nephrolithiasis (long-standing staghorn calculi) also predisposes for the development of squamous cell carcinoma (SCC), which is a rare malignancy of the renal pelvis (Deng et al., 2017; Li and Cheung, 1987; Nachiappan et al., 2016; Paonessa et al., 2011; Raghavendran et al., 2003). Long-standing staghorn calculi are present exclusively in renal pelvis or calyces where they persistently occupy most of the area without causing any obstructions. Hence, nephrolithiasis in this case usually does not cause pain and may be asymptomatic (Schulsinger, 2014). Chronic irritation, infection, and inflammation related to untreated chronic nephrolithiasis can lead to SCC (Jongyotha and Sriphrapradang, 2015). Urothelial proliferative lesions in the renal pelvis frequently associated with calculi-mediated irritation include metaplasia, dysplasia, squamous carcinoma *in situ*, and squamous cell carcinoma (Bhaijee, 2012; Kalayci et al., 2013; Kayaselcuk et al., 2003).

While several studies (Chow et al., 1997; Chung et al., 2013c; Lin et al., 2016; Shih et al., 2014) identified uroliths as significant predisposing factors for the development of bladder cancer, the overall evidence is not fully consistent. Whereas the aforementioned studies reported a statistically significantly increased risk, Burin et al. (1995) reviewed 7 studies regarding urolithiasis and bladder tumour formation and found a significant association in two case-controls out of five studies (Burin et al., 1995; Lin et al., 2016; Shih et al., 2014). Another recent meta-analysis including 13 studies (10 case-control studies, 3 cohort studies) with a total of 182418 participants revealed a significantly elevated risk of bladder cancer in individuals with a history of urinary calculi. The pooled odds ratio of bladder cancer was 1.87 (95% CI, 1.45–2.41) for patients with a prior episode of urolithiasis. The risk was increased for both, males and females (Yu et al., 2018).

The commonly suggested MoA that may explain the association between kidney stones and UTC located in the renal pelvis, ureter, or urinary bladder comprises stone-induced chronic irritation followed by inflammation, epithelial proliferation and ultimately the development of neoplastic changes (Cheungpasitporn et al., 2015; Chow et al., 1997; Chung et al., 2013a; Lin et al., 2016; van de Pol et al., 2019). Most notably, as shown by a population-based cohort study, neoplasms tended to occur at the same location within the urinary tract where the respective stone was found (Chow et al., 1997). Renal calcium stones, the most common type of nephrolithiasis in humans, have been linked to injured and necrotic renal epithelium associated with interstitial fibrosis, tubular epithelial hyperplasia, focal calcifications, and eroded papillary surface epithelium (Khan et al., 1984). Proliferative lesions of the renal pelvis urothelium, both preneoplastic (e.g. hyperplasia) and neoplastic (e.g. dysplasia, TCC) are associated with the presence of renal stones in urolithiasis patients (Inci et al., 2009). Concerning the elevated risk of papillary RCC associated with kidney stones, it has been hypothesised that stone-forming salts in the filtrate of the proximal tubules may affect the metabolism of the adjacent cells in such way that tumour formation is possible (van de Pol et al., 2019).

Especially for calculi-related induction of bladder cancer, urinary tract infection has been discussed as a potential confounding factor (Burin et al., 1995; McGregor et al., 2010; Meek et al., 2003). Since bacterial infection of the urinary tract is likewise associated with the formation of bladder cancer, identifying the potential cancer-inducing factor is difficult if calculi and infection are simultaneously present (Meek et al., 2003). However, a significantly increased risk of UTC was still observed in patients without a history of urinary tract infections (Chow et al., 1997; Kantor et al., 1984; Sun et al., 2013). Another study reported a statistically significantly increased risk after adjusting for urinary tract infections (Chung et al., 2013a).

A major inherent limitation of an observational epidemiological study, in general, is that it can only describe an association between a potential cause and a given outcome. Causation, however, cannot be established.

Several known or unknown confounders and biases may contribute to the outcome of the study. The authors of the two meta-analyses, for instance, discussed a possible surveillance bias, whereas urolithiasis patients may have undergone follow-up examinations that would increase the detection rate of urinary tumours (Cheungpasitporn et al., 2015; Yu et al., 2018). The issue was also discussed in the recent Netherland Cohort Study with the authors concluding that surveillance bias was an unlikely systematic error (van de Pol et al., 2019). In summary, epidemiological studies have established a link between a history of urolithiasis and an increased risk of urinary cancers. The association between kidney stones and kidney cancer, in particular, is considered strong.

Comparison of key events in experimental animal and humans

To assess whether the established MoA in experimental animals is relevant to humans, a comparative analysis of key events was performed. A summary of this analysis is provided in

Table 17 and a short justification is given in the following paragraphs.

Table 17: Comparative analysis of key events in animals and humans for melamine-induced calculi formation and tumour development (adapted from Meek et al. 2003)

Key events	Evidence in animals	Evidence in humans
Urinary concentration adequate for precipitation	Yes, at high dose exposures	Yes, at high dose exposures
Formation of calculi	Yes, at high dose exposures	Yes, at high dose exposures (maybe even at low doses)
Persistence of calculi	Yes	Yes
Urothelial* irritation/damage	Yes	Yes
Urothelial* proliferative lesions	Yes	Yes
Urothelial* tumour formation	Yes, occurs at high incidence	Plausible

*transitional cell epithelium

Urinary concentration adequate for precipitation and formation of calculi

Whether the described MoA that links melamine exposure to UTC in rodents is relevant to humans is a matter of debate. As described in experimental animal studies, the melamine-related MoA concerning carcinogenicity requires adequate exposure sufficient to form calculi in the urinary tract. The incidence of calculus formation was shown to correlate with the orally administered dose of melamine in experimental animal studies (Melnick et al., 1984; NTP, 1983; Research Triangle Institute, 1982).

Although high-dose exposure was not anticipated to occur in humans, melamine-mediated urolithiasis in humans was discovered in the wake of the melamine-tainted milk adulteration incident/scandal 2008 in China (Meek et al., 2003; WHO / FAO, 2009). The prevalence of paediatric urolithiasis thereby correlated with the estimated intake of infant formula (Shi et al., 2012) and a strong correlation between the size of calculi and the concentrations of melamine in the urine was reported (Lam et al., 2009). Thus, high-dose melamine exposure leads to sufficient urinary concentrations to allow for precipitation and calculus formation in animals and humans. Chronic low-dose exposure, on the other hand, may be a risk factor for the development of common calcium stones with a melamine fraction (Liu et al., 2011).

As aforementioned, the composition of calculi found in rodents that had been administered with melamine and in children that consumed contaminated infant formula consists of melamine and uric acid (WHO / FAO, 2009). Uric acid possesses imide groups which may interact with melamine to form melamine–urate complexes via hydrogen bond networks (WHO / FAO, 2009). Human infants are characterized by 5-fold higher levels of uric acid as compared to rats. Consequently, human infants may be more susceptible towards the development of melamine-uric acid kidney stones (WHO / FAO, 2009).

Altogether, a dose-response relationship between melamine exposure and urinary calculus formation can be established in animals and humans.

Persistence of calculi

Species-specific anatomical and physiological factors may play a role in the urolithiasis-mediated induction of neoplastic lesions and have been discussed in detail (Bhat et al., 2010; Burin et al., 1995; Cohen et al., 2002; Cohen and Lawson, 1995; De Sesso, 1995; Meek et al., 2003; WHO / FAO, 2009). The retention time of calculi and the concomitant potential to damage the urothelium, for instance, has been linked to the anatomy of the exposed species. Rodents are characterized by a horizontal body posture which may enable a long-lasting retention of calculi within the lumen of the bladder. Chronic irritation of the epithelium due to persistent urolithiasis is a key event in the established MoA in rodent animal models. However, ceasing melamine treatment in mice results in rapid calculi discharge, suggesting that the horizontal body posture may not prevent the passing of stones in rodents (Ren et al., 2012; Sun et al., 2014).

In contrast, the vertical deportment of humans presumably facilitates the elimination of urinary tract stones that spontaneous pass from the kidney to the bladder through the urethra (De Sesso, 1995). Depending on the size, calculi are either quickly voided or cause painful obstructions which are subject to surgical or lithotriptic removal. As a consequence of a shorter residence time of stones in the urinary tract, it has been hypothesised that humans are less susceptible to urolithiasis-mediated cancer and that an extrapolation of carcinogenicity seen in rodent bioassay to humans is uncertain (Burin et al., 1995; Cohen et al., 2002; Cohen and Lawson, 1995; Meek et al., 2003). However, in a significant number (ca. 9 %) of paediatric patients that had been exposed to melamine-tainted infant formula, asymptomatic intrarenal uroliths were found up to 5 years after the initial diagnosis and the cessation of melamine intake (Chang et al., 2017). As summarised in Table 20 (STOT-RE) and Table 24 (Annex II), follow-up studies have consistently revealed that kidney stones persist or even increase in size in approximately 8 - 10 % of the cases, indicating that long-standing chronic melamine-related urolithiasis can indeed be found in human urinary tract (Dai et al., 2012; Liu et al., 2010b; Shang et al., 2012; Wang et al., 2014; Wang et al., 2013; Yang et al., 2013; Zou et al., 2013). Thus, while anatomical species-specific differences may influence the residence time of calculi in the extrarenal urinary tract (urinary bladder), there is sufficient evidence on the existence of long-standing intrarenal stones in a certain number of melamine-exposed children that underwent follow-up examination.

Uroliths reported in melamine-exposed children ranged from smaller stones (≤ 10mm in diameter), representing the majority, to staghorn calculi (Hou et al., 2009; Ren et al., 2009; Wang et al., 2013). Melamine-related calculi up to 19-33 mm had been seen (Gao et al., 2011; Sun et al., 2010a; Zou et al., 2013). Staghorn calculi are known to accumulate in the human kidney in the absence of any clinical symptoms (Burin et al., 1995; Schulsinger, 2014). The size of the calculi was found to significantly impact the passing rate with larger stones being less frequently passed (Wang et al., 2011).

Hence, evidence for a long-standing presence of calculi in the urinary tract exists in rodent animals and humans.

Urothelial irritation (damages and proliferative lesions)

Within the mammalian urinary tract system, transitional cell epithelium covers the lining of the proximal urethra, the urinary bladder, the ureter, the renal pelvis and calyx as depicted in Illustration 1 below (Apodaca, 2004; Hong et al., 2009; Oyasu, 1995). The ureter connects the urinary bladder with the renal pelvis which is the expanded funnel-shaped proximal end of the ureter. The extension of the renal pelvis is called calyx followed by the renal papilla, which is the apex of the pyramid where urine drains from the pyramid (Lote, 2012).

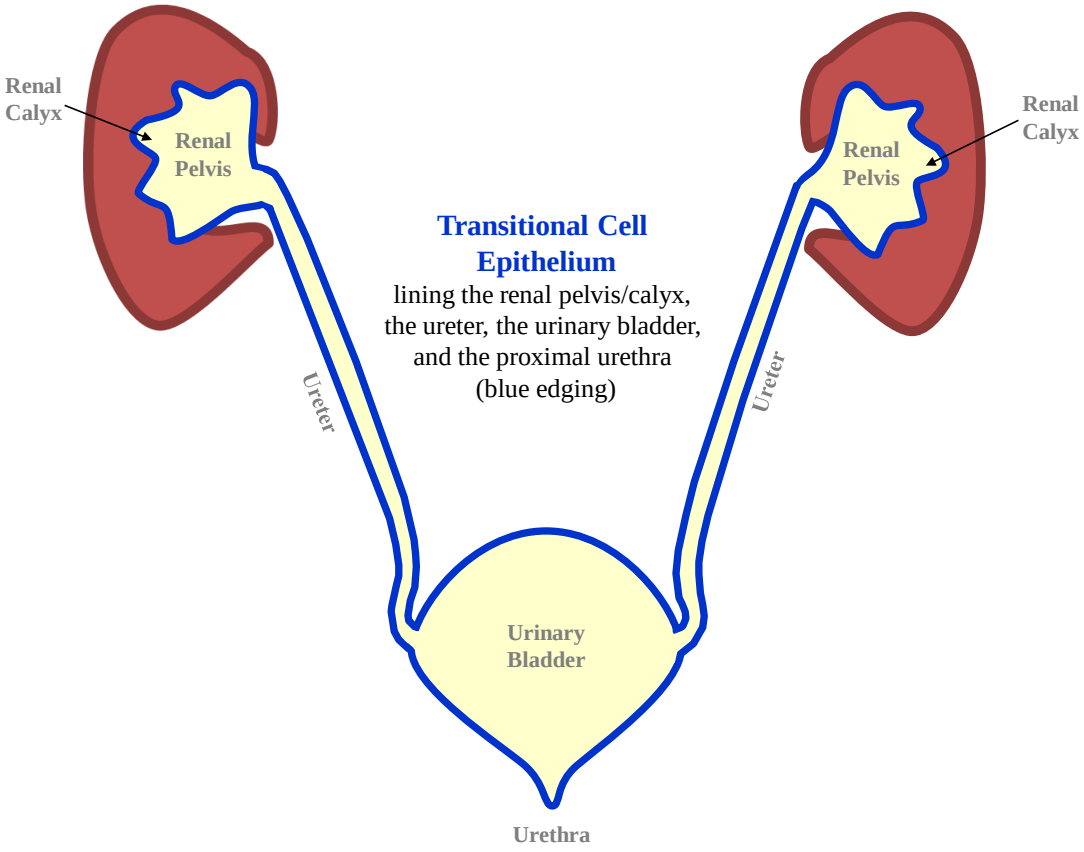

Illustration 1: Transitional cell epithelium lining the urinary tract system

Melamine-related carcinogenesis is considered based on precipitation-mediated injuries of the transitional cell epithelium within the urinary tract. Preneoplastic (e.g. hyperplasia) and neoplastic lesions of the transitional cell epithelium have been observed in the kidney (renal pelvis, renal papilla), the ureter, and the bladder of rats and mice (Cremonezzi et al., 2004; Cremonezzi et al., 2001; Hazleton, 1953; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992; Research Triangle Institute, 1982).

Consistent with these observations in experimental animal studies, melamine-exposed humans exhibit similar signs of epithelial injuries predominantly in the kidney (Lam et al., 2009; Lau and Tu, 2013; Sun et al., 2010b; Sun et al., 2009; Zou et al., 2013). In urolithiasis patients of non-melamine-mediated origin, the established association between nephrolithiasis and urinary cancer is assumed to be related to chronic irritation and proliferative epithelial changes (Chow et al., 1997). The presence of renal calcium stones is associated with kidney damages (e.g. eroded papillary epithelium, focal calcification) (Khan et al., 1984). Epithelial preneoplastic (e.g. hyperplasia) and neoplastic lesions (e.g. dysplasia, TCC) associated with the presence of renal stones were incidentally observed in the renal pelvis during percutaneous nephrolithotomy in urolithiasis patients (Inci et al., 2009). Other urothelial irritation (such as metaplasia, dysplasia, squamous carcinoma *in situ*, SCC) mediated by urinary stones have been described (Bhaijee, 2012; Kalayci et al., 2013; Kayaselcuk et al., 2003). Thus, while evidence for a calculi-related irritation-based MoA in animals is convincing, clinical observations from melamine-exposed children and common urolithiasis patients suggest a link between calculus formation in the kidney and irritation of the epithelium. Hence, evidence for calculi-mediated irritation of the urothelium exists in animal and humans.

Urothelial tumour formation

While the presence of melamine-related stones in experimental animals is clearly linked to tumour formation in the urinary bladder and in the ureter, preneoplastic lesions, that are considered carcinogenic precursor lesions in accordance with the MoA, are evident all along the urinary tract including the urinary bladder, the

ureter, and the kidney (Cremonezzi et al., 2004; Cremonezzi et al., 2001; Early et al., 2013; Hazleton, 1953; Melnick et al., 1984; NTP, 1983; Ogasawara et al., 1995; Okumura et al., 1992; Stine et al., 2014).

In humans, melamine-mediated stones were mostly found in the upper urinary tract of children (renal pelvis and calyx) where they induce pathophysiological changes related to urothelial toxicity. Whether these renal changes can progress to chronic damages and cancer formation is uncertain as the data derived from available follow-up studies are limited (i.e. insufficient duration of follow-up). However, the relationship of urolithiasis and UTC, in general, has been studied and evidence from epidemiological studies suggests an increased risk of UTC in individuals with a history of urolithiasis. The association between kidney stones and kidney cancer appears to be stronger as compared to urinary bladder cancer which is consistent with stones predominantly located in the kidney of patients (Burin et al., 1995; Cheungpasitporn et al., 2015). The proposed MoA that may link a history of urolithiasis to UTC formation comprises irritation of the urothelium, inflammation, and increased proliferation and thus, resembles the mechanisms that have been described in experimental animal studies (Cheungpasitporn et al., 2015; Chow et al., 1997). As calculi were mostly found in the renal pelvis of melamine-exposed children, injuries to the transitional cell epithelium may consequently occur at the same site of the urinary tract. These urothelial damages may, in analogy to the irritating effects of stones in rat bladders, give rise to the development of proliferative lesions (such as hyperplasia) eventually evolving into transitional cell tumour formation within the renal pelvis. It appears biologically plausible that transitional cell epithelium responds similarly to insults evoked by persistent urolithiasis and the corresponding MoA irrespectively of the actual location within the urinary tract. This assumption is strongly supported by the observation that tumours frequently arise at the same site where a respective stone is found and that TCCs have been seen in the renal pelvis of urolithiasis patients (Chow et al., 1997; Inci et al., 2009).

Summary:

A critical factor concerning the evaluation of melamine and its intrinsic property to induce neoplastic lesions in humans is the MoA that has been established in experimental animals and the question as to whether it is relevant to humans. A comprehensive assessment concerning the relevance of data derived from experimental animal studies concluded that besides possible quantitative differences in the carcinogenic response to calculi between species, the carcinogenic effect depends on reaching a threshold concentration of melamine in urine for calculi to form (IARC, 1999b). In the absence of epidemiological and toxicological data (before the tainted milk incidence in China), it had been concluded that due to a lack of substantial human exposure, melamine-mediated calculi are unlikely and that a carcinogenic risk by this MoA is not expected in humans (Cohen et al., 2002). Melamine was therefore classified by IARC as not classifiable as to its carcinogenicity to humans (group 3) (IARC, 1999a). Based on IARCs early assessment (IARC, 1999b), section 3.6.2.3.2. of the *ECHA Guidance on the Application of the CLP Criteria* states that the formation of urinary bladder tumours due to crystals in the bladder is a mechanism considered not relevant to humans.

However, the 2008 food adulteration incidence in China has changed the weight of evidence assessment as it provided sufficient evidence for the formation of uroliths following melamine exposure in humans and urged a concern toward an increased risk of UTC in melamine-affected children (Li and Chow, 2017; Vara Messler et al., 2012; Wen et al., 2016). Subsequent follow-up studies show that melamine-mediated calculi and kidney abnormalities can persist and may cause irreversible damages (Wang et al., 2013; Zou et al., 2013). To date, the maximum duration of follow-up is 5 years which may be insufficient to detect long-term consequences of melamine exposure such as carcinogenic effects. Extensive long-term follow-up has been strongly warranted (Chang et al., 2017; Gao et al., 2011; Wang et al., 2013; Wen et al., 2016; Yang et al., 2013; Zou et al., 2013). Beyond a clear association between high-dose melamine exposure from infant formula and urinary tract stone occurrence, an elevated risk of urolithiasis in children and adults exposed to low levels of melamine has been reported (Chen et al., 2009; Lam et al., 2008; Li et al., 2010; Liu et al., 2011; Wu et al., 2010). Moreover, a history of urinary tract stones is associated with carcinomas in the urinary bladder and kidney (Burin et al., 1995; Cheungpasitporn et al., 2015; Chung et al., 2013b; Desai et al., 2016; La Vecchia and Airoldi, 1999; Shih et al., 2014; Sun et al., 2013; Wang et al., 2012). In summary, although anatomic and physiologic differences may influence the quantitative response to urolithiasis-mediated UTC development, a consistent MoA can be established in animals and humans. Thus, UTC as a consequence of melamine-mediated urolithiasis can be considered relevant to humans.

It has to be noted that IARC has recently revised its assessment of melamine with the conclusion to upgrade the classification from "not classifiable as to its carcinogenicity to humans" (group 3) to "possibly carcinogenic to humans" (group 2b) (IARC, 2019).

Conclusion on the Comparison with the CLP criteria

The results from several key studies in experimental animal models with oral exposure to pure melamine demonstrate strong evidence for neoplastic findings in the urinary bladder of male rats, thus providing sufficient evidence of melamine-mediated carcinogenicity in animals that may potentially justify the classification in category 1B. In addition to the clear effects in male rats, further supporting studies provide limited evidence of carcinogenic effects in female rats and mice.

Melamine-related tumourigenesis in rodents is based on a non-genotoxic mode of action secondary to the formation of calculi. Calculus formation occurs above a certain threshold at considerably high doses. According to the recommendations of the *ECHA Guidance on the Application of the CLP Criteria* (3.6.2.3.2., section k), *"the existence of a secondary mechanism of action with the implication of a practical threshold above a certain dose level…may lead to a downgrading of a Category 1 to Category 2 classification"*.

The *Guidance on the Application of the CLP Criteria* (3.6.2.3.2., section k) further states that "*Urinary bladder tumours due to crystals in the bladder"* is a mechanism that is not relevant for humans and that *"Where such a mechanism is identified then classification may not be appropriate"*. As particularized in the previous sections, a comprehensive analysis of the various key events related to melamine-mediated carcinogenesis was performed with the conclusion that although species-specific anatomical and physiological factors may play a role regarding the response to calculus formation, species-independent key events, common to both, rodents and humans, can be clearly identified. Thus, calculus formation as a consequence of melamine exposure poses a carcinogenic risk to humans.

Considering the overall evidence for melamine-mediated carcinogenesis, classification in category 2 rather than category 1B is considered most appropriate for the following reasons:

- Sufficient evidence of carcinogenicity (benign and malignant tumours) only in the urinary bladder of male rats (key studies in experimental animal studies)
- Supporting studies demonstrate the induction of only benign tumours and preneoplastic lesions
- Non-genotoxic mode of action
- Secondary mechanism of action with a threshold
- Sufficient evidence indicating relevance to human carcinogenicity

Category 2 according to CLP Regulation 1272/2008 (Table 3.6.1):

"Suspected human carcinogens

The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies."

10.8.3 Conclusion on classification and labelling for carcinogenicity

Based on experimental animal studies and human data, classification of melamine as Carc.2 (H351) is recommended.

Setting of specific concentration limit for carcinogenicity

Article 10.1 of the CLP regulation allows the use of specific concentration limits (SCL) based on the potency of carcinogens. The EU has adopted the T25 concept for carcinogenicity to assist in establishing SCLs for carcinogens.

The SCL considerations in this section are based on the respective EC guidance document "Guidelines for setting specific concentration limits for carcinogens" (European Commission, 1999) and the T25 concept.

T25 values for the key carcinogenic studies of melamine were calculated as described in **Dybing et al. (1997)** (Dybing et al., 1997). The key studies and the derived corresponding T25 values are shown in Table 18.

Table 18: T25 values derived from key carcinogenicity study

Study	**Methodological details**	**Calculation of T25**	**Resulting T25**
Carcinogenicity study in male and female rats by **NTP (1983)**	Duration: 103 weeks Observation period: no Start of treatment: at 6 weeks of age Oral: 7 days/week	Lowest dose with significantly increased tumour incidence (transitional cell carcinoma in ♂ rats): 263 mg/kg bw/d (incidence: 16 %) 97/104 (exposure) x 103/104 (observation) x 263 mg/kg bw/d = 242.9 mg/kg bw/d T25 after 24 month: 25/16 x 242.9 mg/kg bw/d= **379.5 mg/kg b/d**	**379.5 mg/kg bw/d** supporting low potency carcinogen (≥ 100 mg/kg bw/d)
Carcinogenicity study in male rats by **Okumura et al. (1992)**	Duration: 36 weeks Observation: 4 weeks Start of treatment: at 6 weeks of age Oral: 7 days/week	Lowest dose with significantly increased tumour incidence (transitional cell carcinoma in ♂ rats): 1090 mg/kg bw/d (incidence: 79 %) 30/104 (exposure) x 40/104 (observation) x 1090 mg/kg bw/d = 120.9 mg/kg bw/d T25 after 24 month: 25/79 x 120.9 mg/kg bw/d= **38.3 mg/kg b/d**	**38.3 mg/kg bw/d** supporting medium potency carcinogen (as 1 mg/kg bw/d < T25 ≤ 100 mg/kg bw/d)

Study	Methodological details	Calculation of T25	Resulting T25
Carcinogenicity study in male rats by **Ogasawara et al. (1995)**	Duration: 36 weeks Observation: 4 weeks Start of treatment: at 6 weeks of age Oral: 7 days/week	Lowest dose with significantly increased tumour incidence (transitional cell carcinoma in ♂ rats): 1030 mg/kg bw/d (incidence: 90 %) 30/104 (exposure) x 40/104 (observation) x 1030 mg/kg bw/d = 114.3 mg/kg bw/d T25 after 24 month: 25/90 x 38.8 mg/kg bw/d= **31.7 mg/kg bw/d**	**31.7 mg/kg bw/d** supporting medium potency carcinogen (as 1 mg/kg bw/d < T25 ≤ 100 mg/kg bw/d)

According to **Dybing et al. (1997)**, data for calculating the T25 should preferentially derive from lifetime oral or inhalation studies according to accepted guidelines. Thus, the 2-year carcinogenicity study by NTP (1983) was selected as the most relevant study for calculation of a T25. A T25 of 379.5 mg/kg bw/d was obtained using the T25 estimation procedure as described by **Dybing et al. (1997)** (see Table 18) for that study. According to the guidelines for setting SCLs for carcinogens (European Commission, 1999), section 3.4, a carcinogen with a T25 ≥ 100 mg/kg bw/d is considered a low potency carcinogen. Category 2 carcinogens (Carc. 2) showing low potency will normally be assigned an SCL of 1 - 5 % on a case by case basis (European Commission, 1999), section 5.3. There are two additional reliable carcinogenicity studies for melamine available in which a significant increase in tumour incidence was observed (Ogasawara et al., 1995; Okumura et al., 1992). In both cases, the T25 values (see Table 18) support a medium potency of melamine (as 1 mg/kg bw/d < T25 ≤ 100 mg/kg bw/d). Consequently, it is recommended not to deviate from the general Carc. 2 concentration limit of 1 %. The setting of an SCL for melamine is not proposed.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

For the purpose of classification, both experimental animal and human data were considered by the DS.

Four long-term studies in F344 rats were identified as key studies by the DS. Two oral feeding studies were performed similarly to OECD TG 451 (NTP, 1983; Hazleton, 1983). Two non-guideline long-term oral studies also investigated specifically the carcinogenicity potential of melamine on the urinary tract system (Okumura *et al.*, 1992; Ogasawara *et al.*, 1995). Although some deviations were noted in these two studies by the DS, they were considered relevant for classification purposes. In addition, two long-term oral studies (Cremonezzi *et al.*, 2004 and Hazleton, 1953) were identified as supporting studies. The positive tumour initiation study (Mori *et al.*, 2000) in rats was disregarded for classification purposes.

In mice, only one long-term study, similar to OECD TG 451 was identified as a key study (NTP, 1983). In addition, Cremonezzi *et al*., 2001 was used as a supporting study. The negative dermal initiation study in mice (Perrella and Boutwell, 1983) was conducted by applying one dose of melamine followed by treatment with a promotor. Due to this study design it was thus disregarded for classification purposes.

An NTP carcinogenicity study in rats and mice with uracil, acting through the same MoA, was also provided in the dossier as part of the discussion of the mode of action (MoA) of melamine in experimental animals.

Moreover, data were also derived from epidemiological studies in humans.

Based on the dose-related induction of urinary bladder tumours (transitional cell carcinoma and papilloma) in male rats in different studies conducted at different times and in different laboratories (NTP, 1983; Okumura *et al*., 1992; Ogasawara *et al.,* 1995), the DS considered that there was sufficient evidence of carcinogenicity in animals. Nevertheless, additional factors were considered to assess the overall level of concern and human relevance.

A critical factor concerning the evaluation of the carcinogenic potential of melamine is the MoA that has been established in experimental animals. Based on rats, mice and monkey data, the DS proposed the following MoA for experimental animals: *melamine-related precipitation originates in the kidneys and damages the epithelium along the urinary tract (including urinary bladder, ureter, and kidney), giving rise to transitional cell tumours in the bladder and precarcinogenic event (i.e. proliferative lesions of the transitional cell epithelium (bladder, ureter, renal pelvis/papilla) and renal tubular epithelium injuries and inflammation) that may be considered precursor lesions of neoplasms.*

ECHA guidance on the CLP criteria considers that tumours due to crystals in the urinary bladder are not relevant to humans, referring to IARC, 1999a. Based on the IARC, 1999b consensus report, the DS pointed out that, **only the formation of calcium phosphate-containing precipitates in the urine of rats were considered species-specific and not relevant to humans**. For substances inducing the formation of microcrystals, amorphous precipitates and/or calculi (e.g. melamine), IARC did not exclude a carcinogenic response to chemical-mediated calculi in humans. It is stated in the IARC, 1999b report that "F*or chemicals producing bladder neoplasms in rats and mice as a result of calculus formation in the urinary bladder, the response cannot be considered to be species-specific; thus, the tumour response is relevant to an evaluation of carcinogenicity to humans. There are quantitative differences in response between species and sexes. Calculus formation is dependent on the attainment in the urine of critical concentrations of constituent chemicals which form the calculus; therefore, the biological effects are dependent on reaching threshold concentrations for calculus formation."*

The evidence of an association between melamine exposure and the formation of calculi and renal damage in humans came mainly from adverse health effects reported in children following the accidental consumption of melamine-tainted infant formula (China voluntary adulteration scandal).
The DS acknowledged that there are uncertainties as to whether exposure to melamine at low-doses may promote urolithiasis. Some reports related to infant urolythiasis suggested that the risk of calculi may even be increased at low dose (Chen *et al*., 2009; Lam *et al*., 2008, Li *et al*., 2010). Nevertheless, there are uncertainties regarding the actual level of accidental exposure to melamine in children. In some studies investigating potential kidney effects in human exposed to environmentally chronic low-doses of melamine, it was suggested that melamine

may promote the development of calcium-related urinary stones including the formation of a nidus that subsequently promotes the growth of calcium uroliths, renal tubular injuries, or enhanced precipitation of calcium oxalate. Nevertheless, major study methodology deficiencies were identified in these studies. Thus, the DS concluded that whether the calculi could be induced at low dose levels remain to be elucidated.

Another important factor to evaluate is whether melamine-induced calculi could persist in humans. Persistence in humans is an important factor to assess if melamine can produce chronic irritation followed by inflammation and proliferation and the development of neoplastic changes. The DS stressed that some human follow-up studies suggested that melamine-mediated calculi and kidney abnormalities could persist (Wang *et al*., 2014; Zou *et al*., 2013, Dai *et al*. 2012, Yang *et al*., 2013). A more recent 5-year follow-up study showed that although most of the children expelled their stones, asymptomatic intrarenal uroliths in kidney were seen in around 9% of children (Chang *et al*., 2017).
The DS considered that there are insufficient data and long-term follow-up studies on melamine exposure to conclude on a higher incidence of urinary tract cancer in humans. To date, no follow-up studies reported an increase cancer risk. Nevertheless, the DS pointed out that the history of urinary tract stones in humans is associated with carcinomas in urinary bladder and kidney.

Potential quantitative differences in response to calculi between experimental animals species, sexes and humans have been extensively discussed by the DS for each key event of the proposed MoA (e.g. horizontal body posture in rodents compared to humans, differences in localisation of stones between rodents and humans, higher sensitivity of human to uric acid).

Overall, the DS concluded that a consistent MoA can be established in experimental animals and humans. The data provides sufficient evidence that the MoA observed in experimental animals may be relevant to humans.

In conclusion, the DS proposed to classify melamine as Carc. 2, H351 on the following basis:

- Sufficient evidence of carcinogenicity only in urinary bladder of male rats;
- Non-genotoxic mode of action;
- Secondary mechanism of action with a threshold;
- Sufficient evidence indicating relevance to human.

Based on T25 calculation from transitional cell carcinoma observed in male rats in the available life-time exposure (NTP, 1983) study, the substance would fall into the low potency group. Nevertheless, based on the two additional key 36-week exposure studies (Ogasawara *et al*., 1995; Okumura *et al*, 1992) a T25 values supporting a medium potency of melamine was calculated. Therefore, the DS did not recommend SCL.

Comments received during consultation

Three MS agreed that a classification of melamine as a carcinogen is warranted. One MS agreed with Carc. Cat. 2, while another MS was in favour of at least a Cat. 2 classification and one expressed the view that both Cat. 2 and Cat. 1B could be justified. Two of these MSs agreed to use the general concentration limit but provided comments on the methodology used for specific concentration limit (SCL) derivation.

One non-governmental organisation (NGO) considered that classification as Carc. Cat. 1B is more appropriate than Cat. 2 due to extensive evidence in experimental animals supported by human data. They considered speculative to assume species differences in the urinary tract and indicated that it is too early to understand the long-term effects of melamine exposure in humans.

Thirty-six comments were received from industrial organisations or individuals, which were all in favour of no classification.

Industry questioned the selection of studies, the reliability and the weight of the studies used for classification purposes. In particular, they highlighted some recent relevant review papers that were dismissed in the dossier (Swaen *et al*., 2019, Cohen *et al*., 2018a and b). Moreover, an in-depth analysis of the quality of the carcinogenicity studies in mice and rats was provided.

As stated in the ECHA CLP guidance document, industry supported that urinary bladder tumours due to crystals observed in male rats are not relevant to humans and noted that the guidance document was not limited to specific type of crystals. Extensive commentary was provided on each of the key events of the proposed MoA of melamine-induced tumours.

The precipitation of uroliths in human urinary tract is the first step of carcinogenesis. Uroliths will only be formed if a threshold exposure level is exceeded. There are evidence that melamine exposure is associated with uroliths at high dose exposure in humans (deliberate adulteration scandal in China) but industry pointed out that they are no evidence that uroliths could be formed at low exposure. They highlighted the low reliability and the limitations of the studies, quoted by the DS, suggesting effects even at low exposure (Li, 2010; Liu, 2011, Wu, 2010, Lam, 2008 and Chen, 2009). Indeed, one of the major limitation is that the non-reversible stones observed in the follow-up studies of children accidentally exposed to melamine may be due to confounding factors such as the presence of non-reversible non-melamine stones not distinguished from melamine stones.

The first step is followed by chronic irritation and cytotoxicity through prolonged exposure. The uroliths must be persistent over a long period to become a decisive step in carcinogenesis. Industry strongly disagree that bladder stone may persist in humans**:**

- Early lesions such as bladder stones and hyperplasia are reversible through cessation of exposure;
- Humans will seek medical assistance in case a stone has formed (medical treatment, stone removal);
- Life-long conditions of criminal use of melamine is not plausible;
- There are variety of differences in anatomy between rats and humans that could explain that humans is not susceptible to the carcinogenic effects of urinary tract solids.

Other practical consideration on species-specificity were discussed by industry. Differences in locations of urinary tract stones in rats and humans did not allow a direct extrapolation between species. Indeed, based on the NTP study, proliferative lesions were only seen in the urinary bladder and not in the kidney. In addition, tumours did not developed in male mice in this study. There is differences in susceptibility to carcinogenesis from urinary solids that has not been completely explain for melamine. The rat is the most susceptible species.

Moreover, industry commented on other factors that need to be taken into account for classification. Tumours were only seen in presence of excessive primary toxicity (e.g. necrosis), associated with hyperplasia that lead to tumours as a secondary consequence. Therefore, according to the ECHA guidance, melamine tumours in rats should be considered as a secondary consequence of a very high dose that cause excessive local toxicity, pointing to a

doubtful potential for carcinogenicity in humans. On this basis, melamine should not be classify.

Industry also commented the T25 approach used by the DS. They considered that the T25 should be derived from the NTP study as the presence of bladder stone is driven by melamine concentration to reach the solubility limit and not exposure duration. On this basis a high specific concentration limit of 5 % should be applied to melamine.

Specific comments received during the consultation have been considered by RAC and discussed below.

Assessment and comparison with the classification criteria

Assessment of key data

Animal data

Seven oral long-term studies were included in the CLH dossier to evaluate carcinogenicity in rats. In addition, a tumour initiation study was available in rat. Two oral carcinogenicity studies were available in mice. Initiating activity by skin application was also tested in one study in mice.

In the **NTP, 1983** study, performed similarly to OECD TG 451 in rats and mice of both sexes, a dose-related increase in transitional cell carcinoma was found with a statistically significant trend. The increase was statistically significant compare to control at 263 mg/kg bw/day in rats and was considered treatment-related and biologically relevant. Transitional cell carcinoma were not seen in the available historical control data (0/3351 through the bioassay program). Seven of the eight rats had bladder stones and an association was found between bladder stones and bladder tumours in rats. A transitional cell papilloma was seen in an additional high-dose male rat. The historical control data for papilloma were 4/3351 (0.1%). A correlation between bladder calculi and the occurrence of bladder transitional cell carcinoma was noted. Pre-neoplastic lesions, consisting of transitional cell hyperplasia, were also increased at 263 mg/kg bw/day. In female rats, an increase in chronic inflammation (different from chronic progressive nephropathy) was seen but no calculi or bladder tumours were induced except one transitional cell papilloma at each dose level. On the three male rats having bladder stones without carcinoma, one had papilloma and the two other had epithelial hyperplasia in the bladder. In the male rat having carcinoma without the presence of stone, the authors suggest that the stone may have passed before the post-mortem examination.

Survival was decreased in male rats at the top dose (38%) but 60% survived 92-week of treatment. Mean body weight was decreased after 10 weeks in low and high dose groups (5-10%). No excessive general toxicity was noted.

	Males			**Females**		
Dose (mg/kg bw/day)	**Control**	**126**	**263**	**Control**	**262**	**542**
Urinary bladder						
Transitional cell carcinoma	0/45	0/50	8/49* (16%)	0/49	0/49	0/49
Transitional cell papilloma	0/45	0/50	1/49** (2%)	0/49	1/49 (2%)	1/47 (2%)
Transitional cell hyperplasia	0/45	1/50 (2%)	2/49 (4%)	0/49	0/49	0/49
Stones (calculi)	0/45	1/50	10/49**	0/49	0/49	0/49

		(2%)	(20%)			
Kidney						
Chronic inflammation	2 /49 (4%)	3/50 (6%)	6 /49 (12%)	4/50 (8%)	17/50** (34%)	41/50** (82%)

*p≤ 0.05, **≤0.001;

In male mice, an increase in stones (4%, 85% and 93% in control, low and high dose, respectively), chronic inflammation and pre-neoplastic lesions (hyperplasia) were also observed in urinary bladder but no tumours were induced. Similar lesions were noted in female mice but only at the top dose of 1090 mg/kg bw/day and showing low incidences.

In **Okumura *et al*., 1992,** male rats were treated during 36 weeks through diet followed by a 4-week recovery period. Only kidney lesions were investigated. In line with the above results, the authors reported a dose-related increase in transitional cell carcinoma and papilloma in the urinary bladder of male rats. The increase was statistically significant at 1090 mg/kg bw/day and significantly correlated with calculi formation. In addition, a statistically significant increase in transitional cell hyperplasia was noted in the ureters and renal pelvis at the top dose group (only in the renal pelvis at the mid dose group). A carcinoma and 3 papilloma in the ureters were also reported at the top dose. A dose-related increase in urinary bladder weight was noted at mid and high dose groups. Haematuria and polyuria was noted in the top dose group.

With the exception of one male rats, the authors reported that all rats survived until the end of the study. Body weight gain was markedly affected at the top dose (no detailed data available). Terminal body weight was significantly less than control at the top dose following the 4-week recovery period (323 g vs 450g in controls). Body weight was not significantly affected at the low and mid dose groups.

Although a low number of animals per group was used and only kidney findings were analysed in male rats, RAC agrees with the DS that the published results of the study are sufficiently reliable and relevant to assess the carcinogenic potential of melamine. Based on body weight changes, RAC notes that the MTD may have been exceeded at the top dose. Although not statistically significant, an increase in carcinoma (5%) was already noted in absence of general toxicity at 330 mg/kg bw/day

Dose (mg/kg bw/day)		**Control**	**100**	**330**	**1090**
Urinary bladder					
Transitional cell carcinoma		0/20	0/20	1/20 (5%)	15/19* (79%)
Transitional cell papilloma		0/20	0/20	1/20 (5%)	12/19** (63%)
papillomatosis		0/20	0/20	5/20* (25%)	17/19** (89%)
Transitional cell hyperplasia		0/20	1/20	6/20* (30%)	12/19 ** (63%)
Calculi		0/20	4/20 (20%)	9/20* (45%)	8/19 ** (42%)

*p≤ 0.05, **≤0.001;

In a similar study design (36-week exposure followed by 4-week recovery), a statistically significant increase in transitional cell carcinoma and papilloma in the urinary bladder were observed in male rats at ≥ 350 mg/kg bw/day in **Ogasawara *et al*., 1995**. In this study a correlation with uroliths and preneoplastic lesions (e.g. hyperplasia) was also found at this dose. An increase in transitional cell hyperplasia was also noted at 350 and 1030 mg/kg bw/day in kidney papilla. No effect on survival was noted in the study. The final body weight in the groups treated with melamine at 1030 mg/kg bw/day (top dose) was reported to be particularly very low. Food consumption was also decreased in this group. Urinary blood was observed in most of the rats at this dose. RAC notes that 1030 mg/kg bw/day may have been above MTD. Similarly to Okumura *et al*., 1992, the study was not performed according to OECD TG but for similar reason was considered acceptable for classification purposes. RAC

acknowledges that the limitations raised by industry during the consultation on the inconsistency between the urinary volume and the water consumption and the low urine volumes in control raised doubt on the reporting in the study. Nevertheless, the study still provide useful information on the implication of bladder stones in the induction of tumours and the chemical composition of melamine-induced stones in rats. Indeed, in this study, male rats were also treated with melamine in the presence of different NaCl concentrations. Urinary bladder tumours were prevented in the presence of NaCl presumably through the facilitation of excretion of microcrystals. An analysis of four stones reported that the composition of the calculi was melamine and uric acid in an equimolar ratio.

Treatment (dose of NaPT expressed as mg/kg bw/day)	**No.**	**Calculi (%)**	**Papillomatous hyperplasia (%)**	**Papilloma (%)**	**Carcinoma (%)**
0	10	0	0	0	0
10% NaCl	10	0	0	0	0
350	19	37	9	42	21
350 + 5% NaCl	19	11	11	0	0
350 + 10% NaCl	19	5	0	0	0
1030	20	30	75	50	90
1030 + 5% NaCl	20	75	75	25	90
1030 + 10% NaCl	20	30	10**	15*	0

*p≤ 0.05, **≤0.001

In **Hazleton, 1983,** performed in rat similarly to OECD 451, no treatment-related neoplastic lesions in the urinary bladder were noted using lower doses (≤ 40 mg/kg bw/day in males and 80 mg/kg bw/day in females). Transitional cell hyperplasia in the urinary bladder was only noted at the top dose in males (6/37 vs 2/39 in controls). Calculi were seen in 1 male at 20 mg/kg bw/day and 2 males at 80 mg/kg bw/day in the urinary bladder.

RAC agrees with the DS that with respect to transitional cell carcinoma in the urinary bladder, a dose-response relationship can be established based on the four above studies (table 15 of the CLH report).

Two additional rat studies and one additional mice study were identified by the DS as supportive due to lower reliability. Major limitations (e.g. exposure time, number of animals, number of dose levels) were identified in these studies. In Hazleton, 1953, urinary bladder calculi, epithelial hyperplasia and benign papilloma were noted in the urinary bladder of rats at the top dose (350 mg/kg bw/day in males and 470 mg/kg bw/day in females). In this study small deposit of crystalline deposits in the kidney were also noted in 1 male and 2 females. Cremonezzi *et al*., 2004 reported an increase in proliferative lesions (metaplasia, hyperplasia and dysplasia) in the renal papilla and renal pelvis of rats (male or female not specified) at around 750 mg/kg bw/day. In mice, Cremonezzi *et al.,* 2001 published an increase combined incidence of dysplasia and/carcinoma in situ in the bladder, the ureter and the renal pelvis in mice (sex not specified) at around 1800 mg/kg bw/day. According to the DS there are uncertainties on the cancerous potential of this type of lesion. In this study, increase transitional cell hyperplasia and calculus formation was also noted.

In addition, an oral tumour initiation study was available in WS/Shi rat (Mori *et al*., 2000). An increase in urinary bladder lesions and formation of calculi was observed in the studies following treatment with a known inducer of bladder cancer. In this study, 14/15 rats that displayed calculi developed tumours. In contrast, in tumours dermal initiation study in female

mice (Perrella and Boutwell 1983), no tumours were observed. Nevertheless, very few details on study methods were available to RAC to assess the reliability of the study.

Human data

As described in detail in the STOT RE section of the CLH dossier (Table 20), in children accidentally exposed to melamine, stones were mostly found in the kidney and a few were also found in the ureters and in the bladder. They were mainly composed of acid uric and melamine. In most of the reported cases, the stones were successfully treated but some larger stones required surgical treatment. Persistent urolithiasis and kidney abnormalities (urinalysis, hydronephrosis) were also reported by several authors in children in longitudinal or follow-up studies as described in the STOT RE section of the opinion. There is no data showing an association between melamine exposure in humans and kidney tumours. Nevertheless, follow-up in melamine-exposure studies may have been to date insufficient (short follow-up).

Mode of action

It is commonly accepted that melamine-induced carcinogenicity acts through the formation of calculi. The postulated MoA is that the urinary tumours in rats may be due to the formation of urinary crystals or calculi producing persistent irritation/inflammation and consequent transitional cell epithelium proliferation and urinary tract tumours.

Based on the available data, RAC considered that melamine is not genotoxic.

MoA in male rats

The following key events were described by the DS for tumour induction in the urinary bladder of male rats:

- Urinary concentration above the solubility limit adequate for precipitation and formation of calculi;
- Transitional cell epithelium irritation due to the persistence of calculi;
- Transitional cell epithelium proliferation;
- Transitional cell tumour formation.

Based on the four carcinogenicity key studies identified by the DS, all the events were found in male rats (presence of calculi, transitional cell hyperplasia and tumour formation). RAC agrees with the DS that there is supporting evidence that the presence of melamine-related stones in experimental male rats is linked to pre-neoplastic lesions along the urinary tract and tumour formation in the urinary bladder. RAC notes that the tumours observed at the highest dose in the NTP study in male rats were not seen in the presence of excessive general toxicity.

MoA in female rats and other species

In male and female mice and in female rats, formation of calculi and transitional cell hyperplasia was seen in the carcinogenicity studies (NTP, 1983). Nevertheless, no urinary tract tumours were seen up to 542 mg/kg bw/day in female rats and up to 688 and 1065 mg/kg bw/day in male and female mice, respectively. In the study of Cremonezzi *et al*., 2001, an increase in lesions in the urinary tract of unclear neoplastic potential were seen only at very high dose (c.a. 1800 mg/kg bw/day) in mice. Although the proposed MoA is plausible in female rats and mice, RAC notes a clear difference between species and sex susceptibility to urinary calculi induced by melamine. This is further supported by the data available on uracil, acting via a similar MoA, for which transitional cell carcinoma were seen in both rats and mice in both sexes. As commented by industry during the consultation, species-specificity for tumour induction observed with melamine in the NTP study may disappear at higher dose levels

maybe exceeding MTD.

In monkeys, following a 13-week exposure, the kidney was also identified as the primary target organ (Early *et al*., 2013). In the urinalysis, urine crystals were noted in weeks 9 and 13. Renal tubular degeneration/regeneration, mononuclear cell infiltration, tubular dilation and single cell necrosis were noted in the kidneys at 700 mg/kg bw/day. Due to the use of formalin in the study, the melamine induced crystals may have been underestimated. Kidney findings in monkeys were consistent with the findings observed in rats in 90-day studies (nephropathy) but no findings in the urinary bladder were found in monkeys. No longer-term studies were available in monkeys to investigate the potential carcinogenic potential of the observed proliferative kidney lesions.

In domestic pets (cats and dogs), crystals and kidney lesions (renal tubular necrosis and inflammation, crystalluria, haematuria), were also noted following accidental exposure though contaminated diet. Nevertheless, in these studies, animals were exposed to a mixture of triazine, notably cyanuric acid, which is a known synergist of melamine-induced renal damages.

MoA in humans

The relevance of the proposed MoA to humans needs to be carefully evaluated.

- *First step : urinary concentration adequate for precipitation and formation of calculi*

The first step of the presumed MoA may be relevant to humans if a threshold above a certain exposure is exceeded to form precipitation in the urinary tract.
Human infants were exposed to melamine following an adulteration incident in China in 2008. Kidney damage caused by stones in the urinary tract was found in exposed children. The predominant location of melamine induced calculi were the kidney, although fewer stones were found in the urinary bladder or the ureters.

RAC agrees with the DS that whether melamine induced calculi at low exposure remains to be elucidated (e.g. below the WHO Tolerable Daily Intake of 0.2 mg/kg bw) due to the limitations in the available human epidemiological studies suggesting an increased risk (See table below).

(Reference), study method	**Source of exposure**	**Prevalence of urolithiasis**	**Main limitations**
(Lam *et al*., 2008) Cross-sectional	Melamine-tainted formula	1/3170 urolithiasis + 7 suspected at 0.01-0.21 mg/kg bw/day	High uncertainties on exposure, study methodology
(Li *et al*., 2010) observational	Melamine-tainted formula	OR: 1.7, 95% CI: 1.3-2.4 at < 0.2 mg/kg bw/day	High uncertainties on exposure, enrolment bias
(Chen *et al*., 2009) observational	Melamine-tainted formula	63/3976 at 0.01-62.67 mg/kg bw/day (one kidney stone at 0.04 mg/kg bw/day) vs one case in the control group	High uncertainties on exposure

(Liu *et al.*, 2011) Case-control	Unknown	Increased risk of calcium urolithiasis: RR= 7.64 (95% CI: 1.98-29.51) at > 3.11 mg/ml melamine in urine	Study methodology, potential confounders
(Wu *et al.*, 2010) Cross-sectional	Environmental	Increased risk of human calcium and uric acid urolithiasis	High uncertainties on melamine measurements in urine, no analysis of melamine content in stones

During the consultation, industry also highlighted that background levels of non-melamine related stones may bias the results. Although the background incidence of stones in infants is low compare to adults, it was reported by Swaen *et al.*, that in China, at the time of the accidental exposure, the prevalence rates were in the range of 2.5-3.61% in some part of the China. Lower prevalence was reported in Hong-Kong (0.03-0.6%). RAC notes that melamine can be distinguished from other stones such as calcium stones in humans. Nevertheless, RAC acknowledges that it contributes to the uncertainties on melamine effects at low exposure levels.

Overall, RAC considered the studies of low reliability and weight, due to the inherent limitation of the study design (case-control study do not allow to assess causality) and potential confounding factors (e.g. considerableuncertainties on exposure).

The DS also notes that humans may be more susceptible to stone formation than rats. When analysed, melamine-mediated uroliths in humans were mainly composed of melamine and uric acid naturally present in urine. It is thought that melamine interact with uric acid to form melamine-uric acid salts that precipitate to form microcalculi within the renal pelvis. Humans lack enzyme uricase compared to other mammals and higher level of uric acid could make them more susceptible. Moreover, uric acid concentration in neonates is higher compared to older children and adults that could also make them even more susceptible (WHO, 2009). Several studies investigated the composition of calculi induced by melamine in rats. Ogasawara *et al.*, 1995 reported that the analysed stones induced by melamine were constituted of melamine and uric acid as seen in humans. Quantitative differences were noted as higher concentration of uric acid were reported in humans than in rats (2:1 ratio in humans vs 1:1 ratio in rats). Nevertheless, the presence of uric acid in melamine-induced calculi in rodents was not consistently found in the studies (e.g. Research triangle institute, 1982).

Overall, RAC considered the first step of the MoA is plausible in humans. The precise threshold of exposure in humans leading to precipitation of melamine is not possible to derive based on the available data. Humans may be more sensitive than rats to the formation of uric-acid calculi. Thus, there are no strong evidence that low exposure to melamine could not form uroliths in humans.

- *Second step: transitional cell epithelium irritation due to the persistence of calculi*

In order to induce prolonged irritation of the urinary tract, uroliths should persist in humans. Several studies suggested potential persistence of uroliths and kidney abnormalities following children exposure to melamine tainted formula.

(Reference), study method	Results	Main limitations
(Chang *et al*., 2017) 5-year follow up study N=207 children	No renal damage observed in any children. Asymptomatic residual stones (< 4mm) in 17/198, proteinuria (10/198) and hematuria (6/198). No need to treat these residual stones clinically but further follow-up was suggested by the authors	Selection bias, limited examination, no controls. Stone analysis on 12 stones in the retrospective study (not in the follow-up study)
(Wang *et al*., 2014) 18-month follow-up N=73	5/73 (15%) persistent calculi	Selection bias
(Wang *et al*., 2013) Meta-analysis N= 26 studies	Persistent kidney abnormalities at 12-month follow-up: 7.7%	Possible selection bias, lack of sub-group analysis
(Yang *et al*., 2013) 48-month follow-up N=45	6/45 stones dissolved partially, 4/45 did not change and 1/ 45 increase in size	Selection bias
(Zou *et al*., 2013) 2-year longitudinal study N=240	At 24-month, 8.85% had persistent urolithiasis, obstruction features (hydronephrosis, hydroureter) were observed in 1.3% of patients, haematuria and leucocyturia were also still observed in a few cases	Selection bias, only 5 stones from melamine-exposed children were analysed, no measurement of melamine level in patients
(Dai *et al*., 2012) 12-month follow-up N=36	9/36: residual stones (6 with decreasing size and three stone with increasing size)	Selection bias
(Liu *et al*., 2010b) Population-based screening study	Remaining abnormalities (nephrolithiasis or hydronephrosis) in 5/48 (12%) patients 6 months after cessation of exposure	Selection bias, uncertainties on exposure in controls, lack of information on maternal feeding behavior
(Shang *et al*., 2012) 18-month follow-up N=38	5/38 (13%) showed residual renal stones	Selection bias

During the consultation, industry highlighted that uroliths of non-melamine origin may be a confounding factor in the available epidemiological studies. Thus, non-melamine stones may have been erroneously diagnosed as irreversible melamine stones. Moreover, industry pointed out that cyanuric acid may be present at low level in food and may play a role in infant kidney lithiasis. The DS was in the view that the background incidence of stones in children is low and that following accidental exposure of children, melamine induced stones were analysed and distinguishable from calcium stones. Moreover, cyanuric acid was only present at trace levels and was not identified to play a role in the aetiology of urolithiasis in Chinese children (WHO, 2009). According to the DS, there is no evidence that the stones were formed independently of melamine exposure.

Industry also commented that in case of stones in humans, depending on the size, calculi will

be either spontaneously voided or lead to painful obstruction and be subject to surgical removal. Thus, stones will not persist beyond removal. In addition, early lesions that might occur before treatment will be reversible. This was supported by data in mice, as following cessation of exposure, rapid dissolution and discharge of stones were seen in mice exposed to melamine (Sun *et al*., 2014 and Ren *et al*., 2012). According to the DS, persistent stones were seen in numerous follow-up studies. These stones (usually < 4mm) remained in the urinary tract system and did not obstruct the urinary tract as these would have cause severe symptoms. RAC agrees that in humans, higher size stones will lead to medical assistance and potentially stone removal. Nevertheless, it is plausible based on the available data that asymptomatic stones may persist. Indeed, kidney stones may not always cause symptoms. According to the systematic review and meta-analysis of Wang *et al*., 2013, 76.2% of the patients were asymptomatic.

In order to assess the relevance of this key events in humans, potential differences in the anatomical and physiological aspects of the bladders in rodents and humans need also to be carefully evaluated.
Species-specific anatomical and physiological factors in the urolithiasis-mediated induction of neoplastic lesions have also been discussed in the dossier and during the consultation. The retention time of calculi has been linked to the anatomy of the rodents. Calculi in rodents are sustained as they are normally horizontally positioned favouring the remaining of the calculi within the lumen of the urinary bladder, with less chance of elimination. The vertical deportment of humans may facilitate the elimination of stones. It has thus been a hypothesis that humans will be less susceptible to urolithiasis-mediated cancer compared to rodents. Nevertheless, as highlighted by the DS, following cessation of treatment in mice, rapid calculi discharge was found in mice (Ren *et al*., 2012, Sun *et al*., 2014) and thus elimination of stones was also possible in rodents.

Overall, RAC agrees that humans may be less susceptible to the persistence of stones due to anatomical and physiological aspects. Although the available studies have limitations, it is plausible that asymptomatic residual stones may persist in humans. Longer follow-up studies would be needed to exclude persistence of residual stones in patient with melamine-induced urolithiasis.

Therefore, although quantitative differences in humans and uncertainties have been identified (higher susceptibility of rats, limitation in the available studies), the data do not indicate that the persistence of stones in humans can be disregarded.

- Last step: transitional cell epithelium proliferation and tumour development

The last key events are the inflammation and proliferation of urothelium and lastly tumour induction. In humans, nephrotoxicity including renal inflammation and renal injuries/lesions have been seen. In a case report in paediatric patient having acute renal failure following accidental exposure to melamine, lymphocytic infiltration in the glomeruli, sclerotic glomeruli, proliferation of fibrous tissue in the glomeruli and Bowman's capsule, swollen tubular cells, lymphocytic infiltration and fibrosis within the renal interstitium, and crystals within the lumen were observed in a kidney biopsy (Sun *et al*., 2010b). In this case report, at follow-up, renal damage were fully resolved.

Differences in localisation of calculi in rats and humans have been noted. Indeed, rat

carcinoma were seen in the urinary bladder only whereas humans developed stones mostly in the upper urinary tract (kidney). Differences were also noted in the 90-day studies in monkey and rats. Whereas rats showed kidney and urinary bladder toxicity, toxicity was limited to kidney in monkeys. According to the DS, preneoplastic lesions in rats and mice were not limited to the urinary bladder and were also seen in kidney and ureters. It has been suggested that renal cancer in humans could occur at the same site as the site of calculi. RAC notes that as shown by several studies, stones in the ureters and bladder were also noted in few children. It may also be noted that with uracil, acting with a similar MoA as melamine, carcinoma were also noted in the renal pelvis in male and female rats.

RAC notes that preneoplastic lesions seen in the ureters and bladder in rats and mice were only seen at very high dose (possibly exceeding MTD) compared to urinary bladder preneoplastic changes and considered that chronic inflammation and regenerative lesions did not always lead to cancer as seen in mice and female rats at the tested dose levels. Nevertheless, also potential differences in location were seen between rats and humans, renal inflammation and proliferative lesions were observed.

There is no data showing an association between melamine exposure in humans and kidney tumours. Nevertheless, follow-up in melamine-exposure studies may have been to date insufficient (small size cohorts, short follow-up).

More in general, according to IARC, 2019, there is epidemiological evidence that cancer of the urinary tract in humans is associated with a history of calculi in the bladder. Nevertheless, RAC notes that according to IARC, 1999, there are uncertainties on the association between micro-crystalluria (associated with irritation and cell proliferation) and bladder carcinomas.

Overall, although there are potential quantitative differences between rats and humans (e.g. localisation of tumours), the proposed MoA in humans cannot be disregarded.

<u>IARC assessment and CLP guidance document</u>

In the CLP guidance document (Version 5.0, 2017), urinary bladder tumours due to crystals in the bladder were considered not relevant to humans. The ECHA guidance document referred to the IARC, 1999 document.

IARC, 1999 published a consensus report on kidney tumours. Regards to urinary bladder neoplasms, melamine is quoted as a non-genotoxic chemical that have been shown to induce formation of microcrystals, amorphous precipitate and/or calculi in the urine of mice and rats. The report quoted also uric acid, calcium oxalate, uracil, thymine acting with a similar MoA. Inert materials such as glass beads and paraffin were also considered as potentially leading to calculus formation (following surgical implantation).

With regards to human relevance, IARC considered that *the risk in humans may not be as great as that in rodents because the calculi are usually voided spontaneously or removed by surgical procedures. Thus through quantitative differences in the carcinogenic response to calculi between species, the effect is not species-specific. However, calculus formation is dependent of the attainment in the urine of critically high concentrations of the constituent chemicals which form calculus. The carcinogenic effects are also dependent on reaching a threshold concentration for calculus formation.*

In contrast, regarding urinary bladder carcinogenesis produced by chemicals such as sodium salts (e.g. saccharine or ascorbate) causing calcium phosphate-containing precipitates in the urine of rats was considered as a species- and dose specific phenomenon that does not occur in humans. Urinary precipitation is based on the presence of high urinary concentrations of alpha-2u globulin and albumin. The interaction with these proteins with sodium salts is necessary to form precipitates.

As also pointed by the DS during the consultation, unlike substances such as sodium saccharin or sodium ascorbate, melamine induces urolithiasis in both experimental animals and humans. This is also one of the reasons why the DS considered the established MoA in animals relevant to humans.

RAC acknowledges that quantitative differences between species may exist but considered that the proposed MoA cannot be disregarded as potentially relevant in humans.

Conclusion on human relevance of the proposed MoA

Overall, RAC considered the evidence on urolithiasis in humans is sufficient to consider the proposed MoA plausible in humans. Nevertheless, the Committee notes that there are potential quantitative differences between rats and humans and some uncertainties in the assessment (low dose exposure, persistence of stones in humans, limitations in the available studies).

Comparison of the evidence for carcinogenicity with the classification criteria

In humans, a positive association has been observed between exposure to melamine and urolith formation which is the first step of the proposed MoA in experimental animals. Nevertheless, there is no evidence of carcinogenicity reported in the available epidemiological studies. Therefore, RAC agrees with the DS that classification in category 1A is not appropriate. However, the negative epidemiology data do not overrule the animal data.

In experimental animals, a statistically significant increase in transitional cell carcinoma was noted in male rats in three key studies (NTP, 1983; Ogasawara *et al*., 1995; Okumura *et al*., 1992). In the NTP study, tumours were seen in the absence of general toxicity. Moreover, RAC notes that the increase in malignant tumours was already observed following only 40-week exposure (36 week exposure followed by 4-week recovery) in male rats. On this basis, RAC agrees with the DS that there is sufficient evidence for carcinogenicity in experimental animals.

According to the CLP regulation (Annex I: 3.6.2.2.4), additional considerations like human relevance have to be taken into account for a classification for carcinogenicity. These are assessed in the following table:

Factor	Evidence with melamine	Conclusion
Tumour type and background incidence	- Transitional cell papilloma and carcinoma in male F344 rats - Rare tumours, above HCD - MoA may be relevant to humans (IARC, 1999)	Sufficient evidence in animals: category 1B
Multi-site responses	No. Tumours were only seen in the urinary tract system	Decreased

		concern
Progression of lesions to malignancy	Yes, transitional cell carcinoma are malignant lesions.	Increased concern
Reduced tumour latency	Yes. Reduced tumour latency was noted as following 36 weeks treatment in rats, tumours were already observed (Okumura *et al*., 1992, Ogasawara *et al*., 1995).	Increased concern
Whether responses are in single sex or both	Responses were only seen in male rats. Male predisposition to calculus was also seen in humans. Potential differences in anatomy, hormone and uric acid levels in humans has been suggested in the dossier.	-
Whether responses are in a single species or several	Single species	Decreased concern
Structural similarity to a substance(s) for which there is good evidence of carcinogenicity	Other substances such as uracil, acting via a similar MoA, provide evidence of urinary tract carcinogenicity in both sexes and species in rodents	-
Routes of exposure	The oral route of exposure used in the long-term carcinogenicity studies is considered a relevant route in humans.	-
Comparison of ADME between test animals and humanss	No species specific differences identified in the available toxicokinetics studies.	-
The possibility of a confounding effect of excessive toxicity at test doses	No excessive general toxicity was not found in rats in the NTP, 1983 study at the high dose.	-
Mode of action and its relevance for humanss	Melamine is not genotoxic. Precipitation of melamine within the urine is responsible of calculi and subsequent tumour formation. The MoA is considered potentially relevant to humans also some unresolved question on potential quantitative differences have been noted. RAC notes the existence of a secondary mode of action, with the implication of a practical threshold above a certain dose level for calculi formation and chronic stimulation of cell proliferation.	downgrade to category 2

- … no influence on the concern (neither increase nor decrease)

During the consultation, industry noted that the bladder tumours are formed as a results of the physical presence of bladder stones and that such particle effects should be exempt from classification. The MoA is not considered by industry to be related to specific intrinsic properties of melamine, as only the dose leading to uroliths results in cancer. Moreover, they considered that calculi by themselves are not carcinogenic to the human urinary tract.
RAC considers nonetheless that the precipitation of crystals in the urinary tract is most likely to be responsible for tumour formation. The CLP regulation does not exclude a carcinogenicity classification due to the physico-chemical properties of a chemical.

In conclusion, RAC considers that in principle there is sufficient evidence of carcinogenicity in experimental animals (urinary bladder tumours in male rats) to justify classification in category 1B.

Regarding the relevance of the proposed MoA to humans, calculi have been associated with high melamine exposure. As calculi formation represents the first step of the proposed MoA of carcinogenesis, RAC agrees with the DS that there is sufficient evidence indicating that the MoA is of relevance to human carcinogenicity. Nevertheless, RAC notes potential quantitative differences in response to calculi between species. Notably, the following differences and uncertainties were noted:

- Humans may be more sensitive than rodents to calculi formation due to higher level of uric acid;
- Although calculi were seen in male and female mice, no tumours were induced below MTD
- humans may be less sensitive than rat to persistence of calculi in the urinary tract system due to anatomical differences;
- Differences in localisation of uroliths in animals (bladder) and humans (mostly kidneys) have been noticed. Nevertheless, RAC considers the MoA relevant within the urinary tract system and that stones were also noted in the ureters and bladder in few children.
-

Overall, also some quantitative differences were noted, the MoA cannot be disregarded as potentially relevant in humans.

The proposed mode of action is non-genotoxic and secondary to the formation of calculi that will only occur above a practical threshold. On this basis, according to the CLP guidance document, a downgrading of a category 1 classification to category 2 may be considered (Guidance to CLP version 5.9, 2017 3.6.2.3.2 (k); *In addition, the existence of a secondary mechanism of action with the implication of a practical threshold above a certain dose level (e.g., hormonal effects on target organs or on mechanisms of physiological regulation, chronic stimulation of cell proliferation) may lead to a downgrading of a Category 1 to Category 2 classification.*

Thus, the critical issue is the ability of melamine to reach a threshold concentration in human urine in order for calculi to form. Such a threshold cannot be established based on the available human data as there are too many uncertainties on actual exposure levels in the available studies. RAC notes that, to date, there is no strong evidence that calculi could occur following low exposure of melamine. Due to the uncertainties on potential effect of melamine at low dose exposure, **RAC agrees with the DS to classify melamine as Carc. 2 (H351).**

Specific concentration limit

In line with the EC (1999) guidance RAC, calculated the following T25 values based on the combined transitional cell carcinoma or papilloma in the urinary bladder of male rats observed following life-time dietary exposure (NTP, 1983). Start of treatment was 6 week of age and the duration of the study was 103 weeks. The lowest effective dose in male rats was 263 mg/kg bw/day (top dose in this study). At this dose, 9/49 male rats showed urinary tract tumours (18.4%). No background correction is needed as no tumours were seen in controls. The T25 is equal to 354 mg/kg bw/day (T25 = 103/104 x 25/18.4x 263 mg melamine/kg bw/day).

According to defaults situation, a T25 ≥ 100 mg/kg bw/day is considered a low potency carcinogen and an SCL of 1-5% could be assigned according to the CLP guidance document.

Nevertheless, other considerations should be considered for assigning a potency class:

Dose-response relationship: there is no data indicating a supralinear dose-response that would justify to move the substance into a high potency group.

Site/species/strain/gender activity: melamine induced tumours in rats in a single specific tissue in a single gender of a single species. This would be in favor of a low potency carcinogen.

Mechanism including genotoxicity: melamine is not considered as a genotoxicant. Melamine is a threshold carcinogen which is probably determined by the concentration at which melamine will form calculi.

As melamine is threshold-based, one of the MSs proposed to use the NOAEL of 126 mg/kg bw/day instead of the LOAEL in the NTP study to derive the T25 as recommended in EC, 1999. A T25 slightly above 100 mg/kg bw/day was obtained by the DS. As melamine might be more sensitive than rats due to higher uric acid levels relevant for the formation of melamine-uric acid calculi, the MS preferred not to derive an SCL.

RAC agrees that the use of a NOAEL instead of the LOAEL could be appropriate in the case of melamine. RAC notes that a T25 of 170 mg/kg bw/day would be obtained (T25=103/104 x 25/18.4 x 126) that would still support a low potency class.

Mechanistic relevance to humans

Mechanistic relevance to humans may also need to be taken into account. RAC considers that there is no reason to change the potency class from the starting assumption of low potency. Quantitative differences in rats and humans have been identified. Potential differences in susceptibility have been identified in both directions (high sensitivity of human formation of calculi but lower sensitivity of humans for persistence of calculi and differences in localisation).

Toxicokinetics

There is no data suggesting that the toxicokinetic behavior will be different in animals and humans.

Other elements

The short latency period observed in the studies increase the concern. Indeed, transitional cell tumours were already observed following 36-week exposure to melamine (Ogasawara *et al*., 1995; Okumura *et al*., 1992). Based on these studies, a T25 < 100 mg/kg bw/day is obtained as calculated by the DS. These data support a medium potency of melamine. Although it is recommended to use a 2-year study over the 36-week study in the EC guidance, the short latency period strongly increases the concern. RAC notes that due to the specific melamine MoA, the 36-week studies may be relevant for deriving a T25 value.

Overall, RAC considers that **the generic concentration limit is appropriate for melamine**.

10.9 Reproductive toxicity

Hazard class not assessed in this dossier.

It should be noted that a testing proposal for the conduction of an extended one-generation reproductive toxicity study (EU B.56./OECD TG 443) was submitted by a registrant (Submission number: WS600383-16).

10.10 Specific target organ toxicity-single exposure

Hazard class not assessed in this dossier.

10.11 Specific target organ toxicity-repeated exposure

Table 19: Summary table of animal studies on STOT RE

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr>
<td>Sub-acute, non-guideline repeated dose toxicity study

Supporting study

Hyperuricemic model

Oral (gavage/intraperitoneal injection)

Wistar rats

Males (n = 6 / group)

No international accepted test guideline followed

No GLP</td>
<td>Melamine (> 99 % purity)

Vehicle: 1 % carboxymethyl cellulose (CMC)

Co-treatment (intraperitoneal injection): Potassium oxonate (oxo; > 97 %)

Dosing groups:
<table>
<tr><th colspan="2">Group</th><th>mg/kg bw/d</th></tr>
<tr><td>1</td><td>ctrl</td><td>-</td></tr>
<tr><td>2</td><td>o</td><td>600</td></tr>
<tr><td>3</td><td>m</td><td>200</td></tr>
<tr><td>4</td><td>m/o</td><td>200+ 200</td></tr>
<tr><td>5</td><td>m/o</td><td>200+ 400</td></tr>
<tr><td>6</td><td>m/o</td><td>200+ 600</td></tr>
<tr><td>7</td><td>m/o</td><td>400+ 400</td></tr>
<tr><td>8</td><td>m/o</td><td>400+ 600</td></tr>
</table>
Ctrl (CMC), o (oxo), m (melamine), m/o (co-treatment)

Melamine was administrated immediately after Oxo injection in the co-treatment group

3 days

Continuously administered</td>
<td>Effective dose (ED): 200 mg/kg bw/d melamine in combination with ≥ 200 mg/kg bw/d potassium oxonate ♂ (renal pathologies)

Hyperuricemia and renal effects induced due to oxo treatment

• The administration of the nontoxic uricase inhibitor potassium oxonate (oxo) led to an rapid increase in serum uric acid levels followed by a reduction (hyperuricemia lasted for ca. 3 h)
• Renal toxicity was minimal even at the highest dose of 600 mg/kg bw/d (no histological changes and mild effects on renal function (increased serum blood urea nitrogen (BUN) and serum creatinine (SCr), and reduced urine osmolality (Uosm); no changes in urine urea nitrogen (UUN) and urine creatinine (UCr)

Combined effects of melamine and oxo

• Mortality (supporting table below) was high in the high-dose melamine + oxo co-treatment groups (group 6: 4/6; group 7 and 8: 12/12)
• Severe renal toxicity (supporting table below): (a) histological changes of the kidney were observed in surviving rats in all co-treatment groups but not in the control (brownish color, enlarged size, and uneven surfaces with needle-like brown-colored spots; expanding gradually with increasing concentrations of oxo) (b) Microscopically, obstructed tubule pathologies were observed (including dilated renal tubules with visible hyperemia, interstitial vascular dilation, and hydropic degeneration in proximal tubule epithelial cells) (c) Crystals (brownish, needle-like) were seen as radial aggregates in tubular lumina in group 6

Freeze-dried kidneys were analysed for their melamine and uric acid content (supporting table below)
• melamine concentrations were dose-dependent and substantially higher as compared to the control
• Uric acid concentrations were oxo-dose-dependent and undetectable in control animals

A saturated solution of melamine mixed with oxo did not precipitate in an in vitro experiment (pH 5.5)

No effects were noted when melamine was administered alone</td>
<td>Zhang et al. (2015)</td>
</tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		Tissue examined: • The kidney was histologically examined (fixed in formalin) • The kidney composition was analysis after freeze-drying with ESI-TOF-MS	

Supporting table: Pathological changes and uric acid and melamine content in kidneys 3 days after administering melamine plus oxo; adapted according to Zhang *et al.* (2015)

Group	Melamine (mg/kg bw/d)	Oxo (mg/kg bw/d)	Number of survivals	Gross Morphological Changes Scores*	Histological Changes[#]	Uric Acid (mg/g Dried Kidney)[a]	Melamine (mg/g Dried Kidney)[a]
8	400	600	0/6	+++	+++	NA	NA
7	400	400	0/6	+++	+++	NA	NA
6	200	600	2/6	+++	+++	12.58±0.57	1.68±0.04
5	200	400	6/6	++	+	8.04±0.86	1.58±0.19
4	200	200	6/6	+	+	5.35±0.05	1.36±0.00
1	0	0	6/6	-	-	0	0.03±0.00

*****Gross morphological changes include changes in colour and size. (+++ was defined as golden-brown colour of the whole kidney surface and longitudinal section; ++ was defined as brown colour of the whole kidney, and brown colour was confined within only medulla from longitudinal sections; + was defined as scarce brown colour of the kidney surface, brown colour was confined within only longitudinal section). [#]Histological changes includes hyperaemia, interstitial vascular dilation, and hydropic degeneration (+++ was defined as obvious tubular dilation, hyperaemia, and interstitial vascular dilation; ++ was defined as medium tubular dilation, hyperaemia, and interstitial vascular dilation; + was defined as scare tubular dilation, hyperaemia, and interstitial vascular dilation). [a]The uric acid and melamine contents were measured only in the survived rats. The uric acid and melamine were measured for two kidney samples per group. NA not analysed

Sub-acute, non-guideline repeated dose toxicity study Supporting study Oral (gavage) Sprague-Dawley **rats** Males (Ctrl n = 6, low-dose n = 6, high-dose n = 8) No international accepted test guideline followed No GLP	Melamine (> 99 % purity) Vehicle: 0.5 % hydroxypropyl methylcellulose **0, 350,** and **1050** mg/kg bw/d **5 days** Continuously administered	**ED:** 350 mg/kg bw/d ♂ (mild renal injuries) Low-dose: • No obvious adverse effects was observed • 3/6 (50 %) had altered urine (cloudy/discolored) • Kidney histopathology: multifocal crystal (0/6), multifocal dilation of distal nephron tubules (1/6, (17 %)), multifocal necrosis/degeneration/regeneration of distal nephron tubular epithelium (1/6, (17 %)) High-dose: • Red discoloration in the urine, red material around the mouth and nose, pale skin • Decreased body weight and food consumption • Clinical pathologies: increased neutrophils, monocytes, total protein and globulin, decreased lymphocytes and albumin/globulin ratio, increased creatinine and serum urea nitrogen, decreases in total bilirubin and chloride (changes were considered indicative of renal tissue injuries) • The adrenal gland and kidney weight were increased while the thymus weight was decreased • **Kidney pathologies** were found (degenerative and necrotic changes) in 3/8 (37,5 %) animals that died on day 4 and 5	Early et al. (2013)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		• **Kidney histopathology**: multifocal crystal (8/8), multifocal dilation of distal nephron tubules (8/8), multifocal necrosis/degeneration/regeneration of distal nephron tubular epithelium (8/8) • Lymphoid depletion of the thymus (8/8) • A correlation between the kidney injuries and a dysregulation of genomic biomarkers was found <u>Tissue examined:</u> • Tissues collected at necropsy included the adrenal glands (bilateral), abdominal aorta, bone (femur/sternum), bone marrow (sternum), brain, epididymis (bilateral), esophagus, eye (with optic nerve) (bilateral), heart, duodenum, jejunum, ileum (Peyer's patches), appendix, colon, rectum, kidney (bilateral), liver, lung, lymph nodes (submandibular lymph nodes and mesenteric lymph nodes), breast sciatic nerve, ovaries (bilateral), fallopian tubes (bilateral), pancreas, pituitary, prostate, salivary glands (submandibular gland, sublingual gland), seminal vesicles, skeletal muscle (biceps femoris), skin (groin), spinal cord (neck, chest, and waist), spleen, stomach, testes (bilateral), thyroid (with parathyroid) (bilateral), trachea, bladder, uterus, and thymus. • Tissue was examined crossly and microscopically • No specific information on whether or not the urinary bladder, the ureters, and the renal pelvis were examined (text stats only kidney and bladder)	
Sub-acute, non-guideline repeated dose toxicity study Supporting study Oral (feeding) F344 **rats** Males/females (n = 6 / sex / group) No international accepted test guideline followed No GLP	Melamine (99 % purity) **123.7** mg/kg bw/d **7 days** Continuously administered (cyanuric acid alone and a combination of melamine and cyanuric acid was additionally tested)	**ED:** 123.7 mg/kg bw/d ♂/♀ (renal crystals) • few **crystals with scattered distribution** were observed in the **renal tubules** of 3/6 (50 %) males and 2/6 (33 %) females (combined 5/12 (42 %) rats) (examined by wet mount procedure which prevents the dissolving of crystals) • Reduced food consumption was noted <u>Tissue examined:</u> • Urinary bladder was crossly examined • The kidneys were examined macroscopically and histopathologically (wet-mount preparation)	Jacob et al. (2011)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Sub-acute, non-guideline repeated dose toxicity study Supporting study Oral (gavage) Caesarean-derived CD IGS VAF/+ **rats** Pregnant **females** (n = 22) Non-pregnant **females** (n = 24) No international accepted test guideline followed No GLP	Melamine (99 % purity) Vehicle: 1 % carboxymethyl-cellulose *4 dose groups* (1) non-pregnant ctrl (n = 11); (2) non-pregnant **1000** mg/kg bw/d (n =11); (3) time-pregnant ctrl (n =11); (4) time-pregnant **1000** mg/kg bw/d (n =13) **10 days** Continuously administered (cyanuric acid was additionally tested)	**ED:** 1000 mg/kg bw/d ♂ (compromised renal function and renal injuries associated with renal crystals) *pregnant and non-pregnant female rats:* • The mean weight gain was significantly lower in non-pregnant and pregnant cohorts • The kidney weight was significantly higher • **Renal crystals** (elongated rectangular box shaped) were observed within the renal tubules in 23/24 (96 %) non-pregnant rats using wet-mount analysis as compare to no renal crystals in the control • **Lager uroliths** in 4/24 (17 %) were found adjacent to the papilla • It was noted that formalin fixation dissolves crystals in the kidney which made them disappear in the histopathologic section • Histopathological examination of the kidney revealed **tubular necrosis** (24/24 (100 %)) and **tubular dilation** (22/24 (92 %)) **The presence of renal crystals correlated with increased renal size, weight, and histologic lesions.** • No significant findings were observed in the urinary bladder (epithelial (mild) necrosis in 3/24 (13 %)) • Serum chemistry revealed elevated levels of blood urea nitrogen and creatinine (2-4 times over control) • Serum melamine levels were increased (due to reduced kidney function as a result of extensive crystal formation followed by intratubular obstruction) *Pregnant rats:* • The mean heart weight was significantly elevated • Early death were significantly higher • The litter size, average fetal body weight, and average crown rump length was significantly reduced <u>Tissue examined:</u> • The kidney and the urinary bladder was examined histopathologically (both by formalin-based fixation and wet-mount preparation)	Stine et al. (2014)
Sub-acute, non-guideline repeated dose toxicity study Supporting study Oral (feeding)	Melamine (> 95 % purity) ♂ ppm: / mg/kg bw/d*: 5000 / **400** 10 000 / **790** 15 000 / **1260**	**ED:** 790 mg/kg bw/d ♂; 3030 mg/kg bw/d ♀ (bladder uroliths) • Only necropsies performed • No effect on survival in either group • Weight loss was observed in the two high-dose group in male and female rats *Males:*	NTP (1983)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
F344/N **rats** Male/female (n = 5 / sex / group) Non-guideline study NTP standards No GLP	20 000 **1980** 30 000 **3430** ♀ ppm: mg/kg bw/d*: 5000 **660** 10 000 **1220** 15 000 **2010** 20 000 **3030** 30 000 **4650** Continuously administered **14 days** *Converted according to table 3.17, Guidance on the application of the CLP criteria (Version 5.0, July 2017)	• ≥ 790 mg/kg bw/d 'hard crystalline solids' were observed in the urinary bladder (4/5 (80 %) to 5/5 (100 %)) • At 3430 mg/kg bw/d, 2/5 (40 %) rats presented kidneys described as "pale and pitted" at necropsy *Females* • ≥ 3030 mg/kg bw/d 'hard crystalline solids' were observed in the urinary bladder (4/5 (80 %)) <u>Tissue examined:</u> • Only necropsy on all animals was performed • No histopathology	
Sub-acute repeated dose toxicity study **Key study** Oral (gavage) Sprague-Dawley rats Males/females (n = 6 / sex / group; recovery animals: 2 / sex / control group and high-dose group) Similar to OECD TG 407 Deviations: shorter duration, wider dose spacing GLP	Melamine (> 99 % purity) Vehicle: 0.5 % hydroxypropyl methylcellulose ♂/♀ **140, 700, and 1400** mg/kg bw/day (lowered to **1000** mg/kg bw/day due to mortality) **14 days** + 8 days recovery Continuously administered	**ED:** 140 mg/kg bw/d ♀ (renal crystals); 700 mg/kg bw/d ♂/♀ (renal injuries) **BMD_{10}*:** 292.04 mg/kg bw/d ♂/♀ (renal injuries) • The kidney was identified as the primary target organ *Low dose:* • histopathological findings revealed slight crystal deposition in the papillary area of the kidney in 2/6 (33 %) female rats (incidence table below) • No other observations related to melamine administration were noted *Mid-dose/high-dose:* • **Reduced kidney function** (increased serum urea nitrogen and creatinine at ≥7 00 mg/kg bw/d) • red urine and decreased body weights • 1400/1000 mg/kg bw/d: high incidence of mortality (4/10 (40 %) ♂, 6/10 (60 %) ♀), decreased activity, hunched posture, thin, red materials around the mouth (porphyrin staining), ocular discharges, dehydration, decreased body weight and body weight gain, and reduction in food consumption • ≥ 700 mg/kg bw/d: **treatment-related gross pathologies in the kidney** (enlarged, sometimes with a yellowish cut surface), spleen, and thymus • Spleen/thymus had a reduced size (high-dose group) • Histopathological findings (incidence table below): 1. Kidney	Early et al. (2013)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		- 700 mg/kg bw/d: dilation of distal nephron tubule, degeneration and necrosis of tubular epithelium, and regeneration of the tubular epithelium, crystals in the distal tubular lumen (especially in the outer medulla and papillary area; 12/12 ♂/♀), regeneration of the tubular epithelium in all animals (12/12 ♂/♀), - 1400/1000 mg/kg bw/d: dilation of distal nephron tubules, necrosis and degeneration of tubular epithelial cell, and desquamated epithelial cell with luminal crystals (most severe in the dead or moribund rats) 2. Heart - 1400/1000 mg/kg bw/d: multifocal myocardial cell necrosis with hemorrhage and neutrophil infiltration 3. Immune system - 1400/1000 mg/kg bw/d: lymphoid depletion in lymph node, spleen, and thymus <u>Tissue examined:</u> • Tissues collected at necropsy included the adrenal glands (bilateral), abdominal aorta, bone (femur/sternum), bone marrow (sternum), brain, epididymis (bilateral), esophagus, eye (with optic nerve) (bilateral), heart, duodenum, jejunum, ileum (Peyer's patches), appendix, colon, rectum, kidney (bilateral), liver, lung, lymph nodes (submandibular lymph nodes and mesenteric lymph nodes), breast sciatic nerve, ovaries (bilateral), fallopian tubes (bilateral), pancreas, pituitary, prostate, salivary glands (submandibular gland, sublingual gland), seminal vesicles, skeletal muscle (biceps femoris), skin (groin), spinal cord (neck, chest, and waist), spleen, stomach, testes (bilateral), thyroid (with parathyroid) (bilateral), trachea, bladder, uterus, and thymus. • Tissue was examined crossly and microscopically • No specific information on whether or not the urinary bladder, the ureters, and the renal pelvis were examined (text stats only kidney and bladder) *Benchmark doses were calculated using the US EPA Benchmark Dose Response Software (version 2.7) employing different models for dichotomous data and further explained in the summary section below	

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td colspan="4">Incidence table: Histopathologic findings in the 14-day study in rats (Early et al., 2013)
<table>
<tr><th>Group</th><th colspan="2">C1</th><th colspan="2">T1</th><th colspan="2">T2</th><th colspan="2">T3</th></tr>
<tr><th>Dosage (mg/kg bw/d)</th><th colspan="2">0</th><th colspan="2">140</th><th colspan="2">700</th><th colspan="2">1400 → 1000</th></tr>
<tr><th>Sex</th><th>M</th><th>F</th><th>M</th><th>F</th><th>M</th><th>F</th><th>M</th><th>F</th></tr>
<tr><td>Kidneys
Eosinophilic granules in papillary tubular epithelium
Multifocal crystal*
Multifocal dilation of distal nephron tubules
Multifocal necrosis/degeneration/regeneration of distal nephron tubular epithelium</td><td>
0/6
0/6
0/6
0/6</td><td>
0/6
0/6
0/6
0/6</td><td>
0/6
0/6
0/6
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0/6
7/7
6/7
6/7</td><td>
2/10
9/10
8/10
9/10</td></tr>
<tr><td>Heart
Multifocal myocardial necrosis and hemorrhage with neutrophil infiltration</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>3/7</td><td>5/10</td></tr>
<tr><td>Lymph node (mandibular, mesenteric)
Lymphoid depletion</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>4/7</td><td>6/10</td></tr>
<tr><td>Peyer's patch
Lymphoid depletion</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>3/7</td><td>6/10</td></tr>
<tr><td>Spleen
Lymphoid depletion</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>4/7</td><td>2/10</td></tr>
<tr><td>Thymus
Lymphoid depletion</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td><td>4/6</td><td>8/10</td></tr>
</table>
*w/o degenerated cell deposited distal nephron tubular lumen</td></tr>
<tr><td>Sub-acute, non-guideline repeated dose toxicity study

Supporting study

Oral (gavage)

Wistar rats

Males (n = 7 / group)

No international accepted test guideline followed

No GLP</td><td>Melamine (obtained from Sigma-Aldrich chemicals; purity not specified)

Vehicle: 1 % sodium carboxymethylcellulose

100, 300, 600 mg/kg bw/d

15 days

Continuously administered

(cyanuric acid alone and a combination of melamine and cyanuric acid was additionally tested)</td><td>ED: 100 mg/kg bw/d ♂ (renal crystals); 300 mg/kg bw/d ♂ (mild haemorrhage)

• Crystal depositions were observed near the papilla of the renal tubule in all treatment groups
• A lower level of uric acid was found in the serum of the middle- and high-dose group with statistical significance
• Extensive tubular dilatation was observed in the distal tubules of the high-dose group
• Haemorrhage was found in the high-dose (severe) and middle-dose (mild)
• Inflammation of the renal interstitium was noted in the high-dose group

<u>Tissue examined:</u>

• Histopathological examination of the kidney (formalin fixation)</td><td>Xie et al. (2010)</td></tr>
<tr><td>Sub-acute repeated dose toxicity study

Supporting</td><td>Melamine (≥ 99.5 % purity)

ppm: mg/kg bw/d*:</td><td>ED: 240 mg/kg bw/d ♂ (cyrstalluria); 832 mg/kg bw/d ♂ (significantly increases incidence of urolithiasis)

(BMD_{10}*: 609.08 mg/kg bw/d ♂ (significantly increases incidence of urolithiasis))</td><td>Research Triangle Institute (1982)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr>
<td>study

Oral (feeding)

F344 rats

Males (n = 40 / group; except for 2000 ppm n = 19)

Similar to OECD TG 407
Deviations: examinations restricted to the urinary system, renal, only males, renal histopathology was only done in ctrl and animals of the highest dose

No GLP</td>
<td>2000 240
4000 475
7000 832
10 000 1184
13 000 1524
16 000 1865
19 000 2140

28 days

Continuously administered

*mean of the reported values</td>
<td>The study focused on the effects of melamine-related urinary bladder stones

Urinary bladder calculi:
• Dose-dependent increased incidence of urinary bladder calculi (≥ 475 mg/kg bw/d) that correlated with the incidence of urinary bladder transitional cell hyperplasia (≥ 832 mg/kg bw/d)

Incidence of macroscopic urinary bladder calculi and transitional cell hyperplasia:
<table>
<tr><th>Dose*</th><th>0</th><th>240</th><th>475</th><th>832</th></tr>
<tr><td>Calculi</td><td>0/39</td><td>0/19</td><td>3/40 (7.5 %)</td><td>8/40# (20 %)</td></tr>
<tr><td>Hyperplasia</td><td>0/20</td><td>0/20</td><td>0/20</td><td>2/20 (10 %)</td></tr>
<tr><th>Dose*</th><th>1184</th><th>1524</th><th>1865</th><th>2140</th></tr>
<tr><td>Calculi</td><td>29/40## (72.5 %)</td><td>32/40## (80 %)</td><td>36/40## † (90 %)</td><td>34/40## † (85 %)</td></tr>
<tr><td>Hyperplasia</td><td>4/20 (20 %)</td><td>12/20## (60 %)</td><td>16/20## (80 %)</td><td>16/20## (80 %)</td></tr>
</table>
*mg/kg bw/d #P < 0.01 ##P < 0.001
†At very high doses ≥ 1865 mg/kg bw/d, calculi were also found in the ureter and kidney (1865 mg/kg bw/d: 1/40 (2.9 %) ureter; 2140 mg/kg bw/d: 2/40 (5.9 %) ureter, 2/40 (5.9 %) kidney)

Kidney
• Notable clinical signs observed at necropsy with a dose-related incidence: white flecks or streaks in the kidney (1184*: 1/40 (2.5 %), 1524*: 7/40 (17.5 %), 1865*: 26/40 (65 %), 2140*: 30/40 (75 %);*mg/kg bw/d)
• Histopathology of the kidney revealed focal nephropathy in all animals of the 2140 mg/kg bw/d group (5/5) vs. 0/5 in the control (other concentrations were not examined)

Others
• The incidence of crystalluria was dose-dependent and significantly increased in all treated animals:
<table>
<tr><th>Dose*</th><th>0</th><th>240</th><th>475</th><th>832</th></tr>
<tr><td>Calculi</td><td>15/36 (41.7 %)</td><td>13/18# (72.2 %)</td><td>27/40# (67.5 %)</td><td>32/40## (80 %)</td></tr>
<tr><th>Dose*</th><th>1184</th><th>1524</th><th>1865</th><th>2140</th></tr>
<tr><td>Calculi</td><td>37/40## (92.5 %)</td><td>37/40## (92.5 %)</td><td>38/39## (97.4 %)</td><td>34/35## (97.1 %)</td></tr>
</table>
*mg/kg bw/d #P < 0.05 ##P < 0.001

• A shift to aciduria was observed
• Elevated water consumption in all treatment groups
• Significant inhibition of body weight gain in the groups ≥ 1524 mg/kg bw/d
• The composition of two calculi was analysed (by</td>
<td></td>
</tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		electron probe x-ray and fourier transform infrared spectroscopy), melamine was considered the principal component <u>Tissue examined:</u> • Macroscopic examination of the urethra, urinary bladder, ureter, and kidney • The urinary system was fixed with formalin • Histopathological examination was only done for the urinary bladder *Benchmark doses were calculated using the US EPA Benchmark Dose Response Software (version 2.7) employing different models for dichotomous data and further explained in the summary section below	
Sub-acute, non-guideline repeated dose toxicity study Supporting study Oral (gavage) Wistar **rats** Males/females (n = 3 / sex / group) No international accepted test guideline followed No GLP	Melamine (obtained from Sinopharm Chemical Reagent Co.; purity not specified) Vehicle: 0.9 % sodium chloride Males/females: **180** mg/kg bw/d **28 days** (cyanuric acid alone and the combination were additionally tested)	• A significant reduction of chief cells in the stomach was observed • No pathological changes were observed in the kidney, testes and ovaries • Melamine was detected in various organs (with statistical significance in the spleen, kidney, uterus, testes, stomach, and liver) • No crystals were observed which was discussed as a result of formaldehyde fixation <u>Tissue examined:</u> • Histopathological examination was done for the kidney, liver, stomach, spleen, heart, uterus, ovaries and testis (paraformaldehyde fixation)	Sun et al. (2016)
Sub-acute, non-guideline repeated dose toxicity study Supporting study Oral (feeding) Albino Wistar **rats** Males: number of animals per	Melamine (99 % purity) 30 000 ppm (ca. **2430** mg/kg bw/d*) **28 days** Continuously administered *converted with reported body weight and daily	**ED:** 2430 mg/kg bw/d ♂ (severe pathologies in multiple organs with the kidney being most affected) Morphological/anatomical changes: rats treated with melamine turned yellow and swollen with hypertrophic and congested organs (especially ureter, kidney, and liver) Creatinine, uric acid, and urea levels were significantly elevated in the serum <u>Histopathology:</u> the majority of the examined organs was severely injured (especially the kidney, liver, and testes) • Kidney: severely renal damages accompanied by 'salt particles' and crystals in renal tissue (uriniferous and collecting tubules and glomeruli) and ureter	El Rabey et al. (2014)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
group not specified (20 animals in total and two groups) No international accepted test guideline followed No GLP	food intake	• Urinary bladder: severe epithelial injuries and crystal accumulation • Liver: necrotic changes, broad infiltration of lymphocyte infiltration, accumulation of hepatic granules, and melamine crystals • Testis: reduced number of spermatogonia and primary spermatocytes • Spleen: vascular obstruction, hemorrhage, and accumulation of melamine crystals • Heart: muscle degeneration (hyalinization of muscle fibers, focal cell infiltration or necrosis) Bilirubin, creatinine, uric acid, and urea were significantly increased in the serum Serum melamine concentration was 33.17 ± 10.63 mg/ml ($P < 0.001$) Melamine treated rats showed a statistically significant reduced body weight gain (-66 %) Terminal body weight was significantly lower in the melamine group as compared to control (-20 %) Food intake following melamine treatment was significantly elevated in week 2, significantly lower in week 4, and not different in week 1 and 3 <u>Tissue examined:</u> • Necropsy on all animals was performed • Histopathological examination was done for the kidney, urinary bladder, heart, liver, spleen, and testis (formalin fixation)	
Sub-chronic repeated dose toxicity study (1st NTP 13-week study) **Key study** Oral (feeding) F344/N **rats** Males/females: n = 12/12 Similar to OECD TG 408 (NTP standards)	Melamine (> 95 % purity) Dosing groups: ♂ ppm: / mg/kg bw/d: 6000 / **560** 9000 / **850** 12 000 / **1100** 15 000 / **1400** 18 000 / **1700** ♀ ppm: / mg/kg bw/d: 6000 / **560** 9000 / **880** 12 000 / **1200** 15 000 / **1400**	**ED:** 560 mg/kg bw/d ♂ (urolithiasis, renal abnormalities); 1400 mg/kg bw/d ♀ (urolithiasis) **1st Study:** *Histopathologic evaluations:* <u>Low-dose</u> (560 mg/kg bw/d, ♂/♀ n = 10) • Focal epithelial (transitional cell) **hyperplasia of the urinary bladde**r was observed in only 1/10 (10 %) males and in 0/10 females <u>High-dose</u> (1700/1600 mg/kg bw/d; ♂/♀ n = 10) • Diffuse epithelial (transitional cell) **hyperplasia of the urinary bladder** was found in 8/10 (80 %) males and 2/10 (20 %) females	Melnick et al. (1984) and NTP (1983)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr>
<td>Deviation: tissue examination restricted

No GLP</td>
<td>18 000 1600

Continuously administered

13 weeks</td>
<td>
Incidence of urolithiasis:
<table>
<tr><th rowspan="2">Dose (mg/kg bw/d ♂/♀)</th><th colspan="2">Number of rats with urinary bladder stones</th></tr>
<tr><th>males</th><th>females</th></tr>
<tr><td>0</td><td>0/12</td><td>0/12</td></tr>
<tr><td>560</td><td>6/12 (50 %)</td><td>0/12</td></tr>
<tr><td>850/880</td><td>8/12 (67 %)</td><td>0/12</td></tr>
<tr><td>1100/1200</td><td>12/12 (100 %)</td><td>0/12</td></tr>
<tr><td>1400</td><td>10/12 (83 %)</td><td>3/12 (25 %)</td></tr>
<tr><td>1700/1600</td><td>12/12 (100 %)</td><td>5/12 (42 %)</td></tr>
</table>
No other compound-related effects were seen

<u>Re-evaluated renal histopathological findings</u> (Hard et al., 2009):

• The observed cortical and medullary tubular acute and chronic changes in the kidney of rats were considered similar to features of human reflux nephropathy (chronic atrophic pyelonephritis) of early childhood
• Melamine-mediated lesions required discrimination from spontaneous chronic progressive nephropathy
• No crystals were observed in renal tissue
• In some rats, solitary concretions were noted in the upper fornix of the renal pelvis

Renal lesions were seen in the cortex and inner medulla associated with retrograde nephropathy

Incidence of reflux nephropathy:
<table>
<tr><th>Dose (mg/kg bw/d ♂/♀)</th><th>males</th><th>females</th></tr>
<tr><td>0</td><td>0/10</td><td>0/10</td></tr>
<tr><td>560</td><td>1/9 (11 %)</td><td>0/10</td></tr>
<tr><td>1700/1600</td><td>10/10 (100 %)</td><td>8/10 (80 %)</td></tr>
</table>
In the high-dose males and females, cortical lesions included:
• Irregular areas of tubule basophilia involving the proximal and distal convoluted tubules of the cortex
• Conspicuous tubule dilatation (mainly distal tubules and collecting tubules in the cortex)
• Proximal tubules in varying stages of compression
• Modest mononuclear cell infiltration
• Dilated collecting ducts in the inner medulla
• Simple hyperplasia (basophilic inner medulla characterized by a crowding of the lining epithelial cells)

<u>Tissue examined:</u>
• Necropsies performed on all animals
• A variety of tissues were examined histologically on
</td>
<td></td>
</tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>10 animals only in control and high-dose (1700/1600 mg/kg bw/d ♂/♀) animals (gross lesions, tissue masses, abnormal lymph nodes, skin, mandibular lymph nodes, mammary gland, salivary gland, thigh muscle, sciatic nerve, bone marrow, costochondral junction (rib), thymus, larynx, trachea, lungs and bronchi, heart, thyroid, parathyroid, esophagus, stomach, duodenum, jejunum, ileum, colon, mesenteric lymph nodes, liver, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles/ prostate/ testes or ovaries/ uterus, nasal cavity, brain, and pituitary gland)
• In the lowest dose group (560 mg/kg bw/d) only the kidney and the urinary bladder was microscopically examined</td><td></td></tr>
<tr><td>Sub-chronic repeated dose toxicity study (2nd NTP 13-week study)

Key study

Oral (feeding)

F344/N rats

Males/females: n = 10/10

Similar to OECD TG 408 (NTP standards)
Deviation: tissue examination restricted

No GLP</td><td>Melamine
(> 95 % purity)

Dosing groups:
♂ ppm: / mg/kg bw/d:
750 / 72
1500 / 150
3000 / 300
6000 / 590
12 000 / 1300
♀ ppm: / mg/kg bw/d:
750 / 84
1500 / 150
3000 / 300
6000 / 600
12 000 / 1300

Continuously administered

13 weeks</td><td>ED: 72 mg/kg bw/d ♂ (urolithiasis); 84 mg/kg bw/d ♀ (calcareous deposits)

2nd Study:

<u>Incidence of urolithiasis:</u>
<table><tr><th rowspan="2">Dose (mg/kg bw/d ♂/♀)</th><th colspan="2">Number of rats with urinary bladder stones</th></tr><tr><th>males</th><th>females</th></tr><tr><td>0</td><td>1/10 (10 %)</td><td>0/10</td></tr><tr><td>72/84</td><td>2/10 (20 %)</td><td>0/10</td></tr><tr><td>150</td><td>5/10 (50 %)</td><td>0/10</td></tr><tr><td>300</td><td>7/10 (70 %)</td><td>0/10</td></tr><tr><td>590/600</td><td>9/10 (90 %)</td><td>0/10</td></tr><tr><td>1300</td><td>9/9 (100 %)</td><td>0/10</td></tr></table>
Males:
• Hyperplasia of the transitional epithelium of the bladder was observed in males (300: 1/10 (10 %), 590: 3/10 (30 %), 1300: 9/9 (100 %); conc. in mg/kg bw/d) but not in females
• Hyperplastic changes in males coincides with calculi in all cases and were accompanied by prominent capillaries and occasional edema and scattered mast cell in the submucosa
Females:
• Neither calculi nor hyperplasia was found
• Dose-related calcareous deposits were observed in the straight segments of the proximal tubules in the kidney of female rats (0: 2/10 (20 %), 84: 3/10 (30 %), 150: 4/10 (40 %), 300: 10/10 (100 %), 600: 8/10 (80 %), 1300: 10/10 (100 %); conc. in mg/kg bw/d)*

<u>Re-evaluated renal histopathological findings</u> (Hard et al., 2009):

• The observed cortical and medullary tubular acute and chronic changes in the kidney of rats were considered</td><td>Melnick et al. (1984)
and
NTP (1983)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>similar to features of human reflux nephropathy (chronic atrophic pyelonephritis) of early childhood
• Melamine-mediated lesions required discrimination from spontaneous chronic progressive nephropathy
• No crystals were observed in renal tissue
• In some rats, solitary concretions were noted in the upper fornix of the renal pelvis

Incidence of reflux nephropathy:
<table><tr><th>Dose (mg/kg bw/d ♂/♀)</th><th>males:</th><th>females:</th></tr><tr><td>0</td><td>0/10</td><td>0/9</td></tr><tr><td>590/600</td><td>0/10</td><td>0/10</td></tr><tr><td>1300</td><td>6/9 (67 %)</td><td>2/10 (20 %)</td></tr></table>
<u>Tissue examined:</u>
• Necropsies performed on all animals
• Microscopically examination was performed for the kidney and the urinary bladder of all animals</td><td></td></tr>
<tr><td>Sub-chronic repeated dose toxicity study (3rd NTP 13-week study)

Supporting study

Oral (feeding)

F344/N rats

Males/females: n = 10/10

NTP standards

No GLP</td><td>Melamine (> 95 % purity)

Dosing groups: 18 000 ppm (1700/1600 mg/kg bw/d ♂/♀ +/-1 % ammonium chloride in the drinking water)

Continuously administered

13 weeks</td><td>ED: 1700 mg/kg bw/d ♂ (urolithiasis); 1600 mg/kg bw/d ♀ (urolithiasis)

3rd Study:

Incidence of urolithiasis:
<table><tr><th rowspan="2">Dose (mg/kg bw/d ♂/♀)</th><th colspan="2">Number of rats with urinary bladder stones</th></tr><tr><th>males</th><th>females</th></tr><tr><td>0</td><td>1/10 (10 %)</td><td>0/10</td></tr><tr><td>0 + NH_4Cl</td><td>0/10</td><td>0/10</td></tr><tr><td>1700/1600</td><td>10/10 (100 %)</td><td>3/10 (30 %)</td></tr><tr><td>1700/1600 + NH_4Cl</td><td>8/8 (100 %)</td><td>3/9 (33 %)</td></tr></table>• The addition of 1 % NH_4Cl (ammonium chloride) did not influence the occurrence of bladder calculi
• No other treatment-related effect was seen

<u>Tissue examined:</u>
• Only necropsy was performed</td><td>NTP (1983)</td></tr>
<tr><td>Sub-chronic, non-guideline repeated dose toxicity study

Supporting study

Oral (drinking water)

Sprague-Dawley</td><td>Melamine (obtained from Sigma-Aldrich; purity not specified)

Males:
0, 60, 300, and 600 mg/kg bw/d

Females for mating:</td><td>ED: 60 mg/kg bw/d (Inflammatory changes in the kidney)

Males (3 months exposure)

• No change in body weight
• The endothelial function of the renal arteries showed impairment in a dose-dependent manner (reduced acetylcholine-induced relaxation endothelium-dependent (EDR), increased ACh-induced endothelium-dependent contractions (EDCs))
• A dose-dependent reduction of renal blood flow was</td><td>Tian et al. (2016)</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
rats Males/females: number of animals per group not specified (4-8 rats were used for the experiments) No international accepted test guideline followed No GLP	**600 mg/kg bw/d** (treatment started two weeks before mating and was continued during gestation) *F1 offspring from exposed females:* Group 1: on-going treatment with **600** mg/kg bw/d (male pups for another 3 months) Group 2: control pubs with no further exposure Continuously administered **3 months**	observed (maximal relative enhancement of the renal cortex/aorta; P< 0.05 for the mid- and high-dose group) • Markers for **inflammation** (fibronectin, TGF-β, BMP4 and COX-2 expression↑; fibronectin protein levels↑) and **fibrotic changes** were found in renal arteries and kidneys • The authors were unable to detect renal stones due to poor resolution of the applied method (computerized tomography) **Statistically significant effects in the low-dose group:** • Inflammatory changes in the kidney (elevated fibronectin protein level in the kidney, elevated TGF-β, BMP4 and COX-2 expression) and some in the renal arteries (elevated BMP4 expression in the renal arteries) *F1 offspring* • Melamine concentrations in kidney, plasma and urine were strongly elevated in pups that underwent on-going treatment (group 1) but also in untreated pubs (group 2) from exposed mothers with statistical significance • The authors concluded that *'melamine is able to retain in the offspring after 3 months'* • Renovascular dysfunction (impaired EDRs, increased EDCs) was observed in the untreated offspring of melamine-exposed female rats (group 1 and 2) • Increased chronic inflammation marker were found in group 1 <u>Tissue examined:</u> • Histopathological examination of the kidney (paraformaldehyde fixation)	
Sub-chronic, non-guideline repeated dose toxicity Supporting study Oral (feeding) Wistar **rats** Two sampling times (SP)	Melamine (obtained from Sigma Chemical Co.; no information on purity) M/F: 15 000 ppm (1.5 %, ca: **750 mg/kg bw/d***) in the presence or absence of different fatty acids	**ED:** 750 mg/kg bw/d ♂/♀ (kidney lesions) • No melamine treatment-related effects with statistical significance are reported • No evidence for urolithiasis • However, it was noted that *"Even though calculi or hydroureters were not observed during autopsy, the presence of minute areas of calcification in the papilla may suggest crystal depots which spontaneously dissolved thereafter."* • Proliferative lesions (metaplasia, hyperplasia, and dysplasia) were observed mainly at the proximal end	Cremonezzi et al. (2004)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>Male/female (SP 1 ctrl: n = 22, melamine n = 21; SP 2 ctrl: n = 36, melamine n = 20)

No international accepted test guideline followed

No GLP</td><td>Continuously administered

Autopsies at 22-25 weeks (SP1) and 36-40 weeks (SP2)

*Converted according to table 3.17, Guidance on the application of the CLP criteria (Version 5.0, July 2017)</td><td>of the urinary tract (papillae and renal pelvis)

Proliferative lesions of the urinary tract:
<table>
<tr><th>Group</th><th>SSM#</th><th>MSM*</th></tr>
<tr><td>Renal papillae (SP1)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/22</td><td>0/22</td></tr>
<tr><td>Melamine</td><td>9/21 (43 %)</td><td>1/21 (5 %)</td></tr>
<tr><td>Renal papillae (SP2)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/36</td><td>0/36</td></tr>
<tr><td>Melamine</td><td>6/20 (30 %)</td><td>0/20</td></tr>
</table>
#slight squamous metaplasia
*moderate squamous metaplasia
<table>
<tr><th>Group</th><th>H#</th><th>D*</th></tr>
<tr><td>Renal pelvis (SP1)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/22</td><td>0/22</td></tr>
<tr><td>Melamine</td><td>5/21 (24 %)</td><td>1/21 (5 %)</td></tr>
<tr><td>Ureter (SP1)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/22</td><td>n.a.</td></tr>
<tr><td>Melamine</td><td>3/21 (14 %)</td><td>n.a.</td></tr>
<tr><td>Urinary bladder (SP1)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/22</td><td>0/22</td></tr>
<tr><td>Melamine</td><td>1/21 (5 %)</td><td>0/21</td></tr>
<tr><td>Renal pelvis (SP2)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/36</td><td>0/36</td></tr>
<tr><td>Melamine</td><td>1/20 (5 %)</td><td>2/20 (10 %)</td></tr>
<tr><td>Ureter (SP2)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/36</td><td>n.a.</td></tr>
<tr><td>Melamine</td><td>2/20 (10 %)</td><td>n.a.</td></tr>
<tr><td>Urinary bladder (SP2)</td><td></td><td></td></tr>
<tr><td>Ctrl</td><td>0/36</td><td>0/36</td></tr>
<tr><td>Melamine</td><td>1/20 (5 %)</td><td>0/20</td></tr>
</table>
#simple transitional cell hyperplasia without atypia
*dysplasias

• Other non-specific kidney lesions including coarse retractile scarring, acute and chronic inflammation of renal parenchyma and dilatation with scattered eosinophilic casts in collecting tubules, were mainly observed at SP2

<u>Tissue examined:</u>

• The urinary epithelia (urinary bladder, ureters, renal pelves, and renal papillae) was examined grossly and microscopically
• Tissue fixation was done in formalin</td><td></td></tr>
<tr><td>Sub-chronic, non-guideline</td><td>Melamine (> 99 % purity)</td><td>ED: 100 mg/kg bw/d ♂ (urinary bladder calculi, urinary bladder hyperplasia)</td><td>Okumura et al. (1992)</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
repeated dose toxicity/carcinogenicity study Supporting study Oral (feeding) F344 **rats** Males (n = 20 / group) No GLP No international accepted test guideline followed	3000, 10 000, and 30 000 ppm (ca. **100, 330, 1090** mg/kg bw/d*) Continuously administered 36 weeks + 4 weeks recovery *Converted according to reported mean terminal body weight and food consumption	• **Calculus formation** was observed in the urinary bladder in a dose-dependent manner (ctrl: 0/20; low-dose: 4/20 (20 %); mid-dose: 9/20 (45 %, P<0.05); high-dose: 8/19 (42 %, P<0.01)) • A statistically significant correlation between calculus formation and tumour incidence was described • Papillary or nodular **hyperplasia** of the urothelium was observed in the urinary **bladder** (ctrl: 0/20; low-dose: 1/20 (5 %); mid-dose: 6/20 (30 %, P<0.05); high-dose: 12/19 (63 %, P<0.01)), the **ureter** (high-dose) and in the **renal pelvis** (mid- and high-dose) • The Ureter was slightly thickened • **Hematuria** and **polyuria** was observed in the high-dose group <u>Tissue examined:</u> • Animals were killed at week 40 and the tissues (urinary bladder, ureter, kidney) were histological examined	
Sub-chronic, non-guideline repeated dose toxicity/carcinogenicity study Supporting study Oral (feeding) F344/DuCrj **rats** Males (n = 10 – 20 / group) No international accepted test guideline followed No GLP	Melamine (99.9 % purity) in feed with and without NaCl supplementation Ctrl (n =10); Ctrl + 10 % NaCl (n =10); 10 000 ppm (ca. **350 mg/kg bw/d***) • w/o NaCl (n =19), • +5 % NaCl (n =19), • +10 % NaCl (n = 19); 30 000 ppm (ca. **1030 mg/kg bw/d***) • w/o NaCl (n = 20), • +5 % NaCl (n = 20), • +10 % NaCl (n = 20);	**ED:** 350 mg/kg bw/d ♂ (urinary bladder calculi, proliferative kidney lesions and renal damages) • **Calculi** were observed in the urinary bladder (ctrl: 0/10, low-dose: 7/19 (37 %), high-dose: 6/20 (30 %)) • A strong correlation between bladder tumours and **calculus formation** noted • Calculus formation in the 350 mg/kg bw/d melamine group was suppressed by NaCl in a dose-dependent fashion • **Microcrystals** were observed in the urinary sediments in the high-dose group (1030 mg/kg bw/d) independent of NaCl supplementation • **Transitional cell hyperplasia** (with angiectasis and thrombus formation in some cases) and **ischemic changes** (focal lesions demonstrating fibrosis, inflammation cell infiltration, and renal tubule regeneration) were observed in the **kidney** and attenuated in the high-dose group and completely suppressed in the low-dose group by co-administration of NaCl (see table below). The authors suggested epithelium stimulation secondary to micro calculus formation within the renal pelvis as a potential underlying cause	Ogasawara et al. (1995)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td>Continuously administered

36 weeks + 4 weeks recovery

*Converted according to reported mean terminal body weight and food consumption</td><td><u>Histopathological findings in the kidney:</u>
<table><tr><th>Dose (mg/kg bw/d ♂/♀)</th><th>Papilla*</th><th>Cortex#</th></tr><tr><td>0</td><td>0/10</td><td>0/10</td></tr><tr><td>350</td><td>7/19 (37 %)</td><td>1/19 (5 %)</td></tr><tr><td>350 + 5 % NaCl</td><td>0/19</td><td>0/19</td></tr><tr><td>350 + 10 % NaCl</td><td>0/19</td><td>0/19</td></tr><tr><td>1030</td><td>20/20 (100 %)</td><td>20/20 (100 %)</td></tr><tr><td>1030 + 5 % NaCl</td><td>9/20 (45 %)</td><td>8/40 (40 %)</td></tr><tr><td>1030 + 10 % NaCl</td><td>1/20 (5 %)</td><td>2/20 (10 %)</td></tr></table>*Transitional cell hyperplasia with angiectasis and thrombus formation
#Ischemic changes

• Urinary occult blood was seen in the high-dose melamine group and suppressed by concomitant NaCl (10 %) co-treatment

• Examination of the calculi revealed that melamine and uric acid in equal molar ratios are the primary components of stones (total combined contents of melamine and uric acid in the stone was 61-81 %)

<u>Tissue examined:</u>

• Histopathological examination was performed on the urinary bladder and kidney
• Tissue fixation was done in formalin
• No information on whether or not the ureter was examined</td><td></td></tr>
<tr><td>Chronic repeated dose toxicity/carcinogenicity study

Supporting study

Oral (feeding)

F344/N rats

Males/females (n = 50 / sex / group)

Similar to OECD TG 451 (NTP standards)
Deviations: only 2 concentrations tested</td><td>Melamine (> 95 % purity)

♂: 2250 and 4500 ppm (ca. 126 and 263 mg/kg bw/d)

♀: 4500 and 9000 ppm (ca. 262 and 542 mg/kg bw/d)

Continuously administered

2 years (103 weeks)</td><td>ED: 126 mg/kg bw/d ♂ (urinary bladder calculi and reflux nephropathy); 262 mg/kg bw/d ♀ (chronic inflammation of the kidney and reflux nephropathy)

• Calculi were seen in the urinary bladder of male rats (ctrl: 0/45; low-dose: 1/50 (2 %); high-dose: 10/49 (20 %); positive trend: P≤0.002) but not in females

• Chronic inflammation of the kidney (dose-related interstitial lymphoplasmacytic infiltration, and cortical fibrosis), distinguishable from the nephropathy observed in aging F344/ N rats, was detected dose-dependently in female with a significantly increased incidence (ctrl: 4/50 (8 %); low-dose: 17/50 (34 %)#; high-dose: 41/50 (82 %)#; #P≤0.01) and to a lesser, statistically insignificant, extent in males (ctrl: 2/49 (4 %), low-dose: 3/50 (6 %), high-dose: 6/49 (12 %))

<u>Re-evaluated renal histopathological findings</u> (Hard et al., 2009)</td><td>Melnick et al. (1984)
and
NTP (1983)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>No GLP</td><td></td><td>

The observed cortical and medullary tubular acute and chronic changes in the kidney of rats were considered similar to features of human reflux nephropathy (chronic atrophic pyelonephritis) of early childhood
Melamine-mediated lesions required discrimination from spontaneous chronic progressive nephropathy
No crystals were observed in renal tissue
In some rats, solitary concretions were noted in the upper fornix of the renal pelvis

Incidence of reflux nephropathy*:
<table>
<tr><th>Dose (mg/kg bw/d ♂/♀)</th><th>males:</th><th>females:</th></tr>
<tr><td>0</td><td>1/49 (2 %)</td><td>1/50 (2 %)</td></tr>
<tr><td>126/262</td><td>7/50 (14 %)</td><td>20/50 (40 %)</td></tr>
<tr><td>263/542</td><td>19/49 (39 %)</td><td>50/50 (100 %)</td></tr>
</table>
*fibrotic lesions (scars) associated with collecting duct dilatation and hyperplasia in the inner medulla, loss of tubule, tubule atrophy, and crowded glomeruli in the cortex;

<u>Tissue examined:</u>

Macroscopic examination on major tissues or organs
Histopathological examination on the following tissues: skin with mammary gland, mandibular lymph node, salivary gland, sternum with bone marrow, larynx or anterior trachea, esophagus, thyroid, parathyroid, lungs with mainstem bronchi, heart, stomach (glandular and nonglandular), duodenum, large intestine, liver, pancreas, spleen, kidneys, adrenal glands, urinary bladder, entire gonads, prostate or uterus, brain, and pituitary gland
examinations of the ureters and urethra were not performed
Tissue was preserved 10% neutral buffered formalin embedded in paraffin

</td><td></td></tr>
<tr><td>Chronic repeated dose toxicity/carcinogenicity study

Supporting study

Oral (feeding)

F344 rats

Males/females (n = 65 / sex / group)

Similar to</td><td>Melamine (no information on purity; test material was analysed)

♂: 100; 500; 1000 ppm (ca. 4, 20, 40 mg/kg bw/d*)
♀: 100; 1000; 2000 ppm (ca. 5, 50, 80 mg/kg bw/d*)

Continuously administered</td><td>

No urinary bladder calculi were found
Clinical data did not reveal any treatment-related changes
A Dose-dependent trend to develop dilated glands in glandular gastric mucosa and inflammation in non-glandular gastric mucosa was observed in female rats

<u>Tissue examined:</u>

Complete necropsy on all animals
The following tissue was collected and preserved/fixed (alcohol-formalin-acetic acid) subsequent to gross necropsy: brain, spinal cord, lung, spleen, liver, kidneys, heart, aorta, eyes, pituitary, adrenals, bone

</td><td>Hazleton (1983)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>OECD TG 451
Deviations: purity was not specified

GLP</td><td>123 – 131 weeks

*Converted according to reported mean terminal body weight and food consumption</td><td>marrow, sciatic nerve, thyroids (with parathyroids), urinary bladder, testes, prostate, seminal vesicle, ovaries, uterus, vagina, duodenum, jejunum, ileum, cecum, colon, pancreas, trachea, esophagus, stomach, salivary gland (submandibular), mesenteric lymph nodes, thymus, bone, tongue, skeletal muscle, skin, mammary gland
• Urinary tract: the kidney and urinary bladder were examined grossly (necropsy) and microscopically
• No information on whether or not the ureter was examined</td><td></td></tr>
<tr><td>Sub-acute, non-guideline repeated dose toxicity study

Supporting study

Oral (feeding)

Balb/c mice (n = 5 / group / sex) and C57BL/6 mice (n = 6 / group)

Males/females

No international accepted test guideline followed

No GLP</td><td>Melamine (99.5 % purity)

9373 ppm

<u>Balb/c mice:</u>
2810 mg/kg bw/d*

<u>C57BL/6 mice:</u>
1940 mg/kg bw/d*

14 days
Continuously administered

*converted using the median reported weights and daily consumption according to table 3.17, Guidance on the application of the CLP criteria (Version 5.0, July 2017)</td><td>ED: 2810 (Balb/c mice) and 1940 (C57BL/6 mice) mg/kg bw/d

<u>Histopathological results:</u>

• Active mucosal inflammation (moderate/ severe cystitis) and hyperplasia of the transitional cell epithelium was seen in the urinary bladder exclusively in all animals with stones (incidence table)

• No melamine-related precipitations (crystals/stones) and/or lesions were observed in the ureter and kidney

Incidence table: Incidence of calculi in the urinary bladder and associated hyperplasia of the transitional epithelium
<table><tr><th rowspan="2">Group</th><th colspan="2">Incidence of bladder stones</th><th colspan="2">Incidence of bladder epithelial hyperplasia</th></tr><tr><th>males</th><th>females</th><th>males</th><th>females</th></tr><tr><td>Balb/c mice</td><td></td><td></td><td></td><td></td></tr><tr><td>Ctrl</td><td>0/5</td><td>0/5</td><td>0/5</td><td>0/5</td></tr><tr><td>Melamine</td><td>5/5</td><td>5/5</td><td>5/5</td><td>5/5</td></tr><tr><td>C57BL/6 mice</td><td></td><td></td><td></td><td></td></tr><tr><td>Ctrl</td><td>0/6</td><td>0/6</td><td>0/6</td><td>0/6</td></tr><tr><td>Melamine</td><td>6/6</td><td>6/6</td><td>6/6</td><td>6/6</td></tr></table>
<u>Tissue examined:</u>

• The urinary bladder was macroscopically examined
• The urinary bladder, the ureters, and the kidney were fixed in formalin for histopathological examination</td><td>Xu et al. (2011)</td></tr>
<tr><td>Sub-acute, non-guideline repeated dose toxicity study

Supporting study

Oral (feeding)

Balb/c mice</td><td>Melamine (99.5 % purity)

2810 mg/kg bw/d* (9373 ppm)

3 groups + 4 subgroups (see table)</td><td>ED: 2810 mg/kg bw/d

• The observed urothelial hyperplasia was characterised by many mitotic figures, 4–7 rows of nuclei, and well-defined umbrella/intermediate cells

• Urinary bladder calculi rapidly disappeared after melamine withdrawal and were absent starting from day 4 of

• The calculi mediated urothelial hyperplasia regressed</td><td>Sun et al. (2014)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>Males/females (no. of animals as indicated in the table)

No international accepted test guideline followed

No GLP</td><td>14 days / 56 days

Continuously administered

*converted using the median reported weights and daily consumption of 300 g/kg bw as reported in the study</td><td>dependent on the duration after melamine withdrawal (incidence table)

Incidence table: Incidence of calculi in the urinary bladder and associated hyperplasia of the transitional epithelium and its regression
<table>
<tr><th>Group</th><th>No. of mice</th><th>ED/ ER* (days)</th><th>Inc. of BC#</th><th>Inc. of BEH†</th><th>No. of mice with RT/R/ ST/CR††</th></tr>
<tr><td>Untreated</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>1</td><td>10</td><td>14/0</td><td>0/10</td><td>0/10</td><td></td></tr>
<tr><td>2</td><td>10</td><td>14/0</td><td>0/10</td><td>0/10</td><td></td></tr>
<tr><td>3</td><td>10</td><td>14/0</td><td>0/10</td><td>0/10</td><td></td></tr>
<tr><td>4</td><td>8</td><td>56/0</td><td>0/8</td><td>0/8</td><td></td></tr>
<tr><td>Melamine (2810 mg/kg bw/d)</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>1</td><td>10</td><td>14/0</td><td>10/10</td><td>10/10</td><td></td></tr>
<tr><td>2</td><td>10</td><td>14/0</td><td>10/10</td><td>10/10</td><td></td></tr>
<tr><td>3</td><td>10</td><td>14/0</td><td>10/10</td><td>10/10</td><td></td></tr>
<tr><td>4</td><td>8</td><td>56/0</td><td>8/8</td><td>8/8</td><td></td></tr>
<tr><td>Recovery</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>1</td><td>10</td><td>14/4</td><td>0/10</td><td></td><td>6/4/0/0</td></tr>
<tr><td>2</td><td>10</td><td>14/8</td><td>0/10</td><td></td><td>0/6/4/0</td></tr>
<tr><td>3</td><td>10</td><td>14/42</td><td>0/10</td><td></td><td>0/0/6/4</td></tr>
<tr><td>4</td><td>16</td><td>56/42</td><td>0/16</td><td></td><td>0/0/10/6</td></tr>
</table>
Inc: incidence; *ED/RD: experiment-days/recovery-days (i.e., days after melamine withdrawal); #BC: bladder calculus; †BEH: bladder epithelial hyperplasia; ††RT/R/SR/CR: regressive tendency/regression/significant regression/complete regression phenotypes of BEH.

<u>Tissue examined:</u>

• Only the urinary bladder was examined macroscopically and microscopically
• The urinary bladder was fixed in formalin for histopathological examination</td><td></td></tr>
<tr><td>Sub-acute repeated dose toxicity study

Supporting study

Oral (feeding)

B6C3F1 mice

Male/female (n = 5 / sex / group)

Non-guideline study</td><td>Melamine (> 95 % purity)

♂ ppm: mg/kg bw/d*:
5000 710
7500 980
10 000 1200
12 500 1440
15 000 1880
30 000 3330
♀ ppm: mg/kg bw/d*:
5000 790
7500 1250
10 000 1670
12 500 1790</td><td>ED: 3330 mg/kg bw/d ♂; 4740 mg/kg bw/d ♀ (hard, crystalline solid)

• Stones (“hard, crystalline solid”) were found in the urinary bladder of all (5/5) male mice and in 40 % (2/5) of the female mice in the highest dose group
• No other effect related to melamine treatment was noted

<u>Tissue examined:</u>

• Only necropsy on all animals was performed
• No histopathology</td><td>NTP (1983)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>NTP standards

No GLP</td><td>15 000 2370
30 000 4740

Continuously administered

14 days

*Converted according to table 3.17, Guidance on the application of the CLP criteria (Version 5.0, July 2017)</td><td></td><td></td></tr>
<tr><td>Sub-chronic repeated dose toxicity study

Key study

Oral (feeding)

B6C3F1 mice

Males/females (n = 10 / sex / group)

Similar to OECD TG 408 (NTP standards)
Deviation: tissue examination restricted

No GLP</td><td>Melamine (>95 % purity)

♂ ppm: / mg/kg bw/d:
6000 1400
9000 2000
12 000 2800
15 000 3900
18 000 4700
♀ ppm: / mg/kg bw/d:
6000 1800
9000 2700
12 000 3500
15 000 4800
18 000 5900

Continuously administered

13 weeks</td><td>ED: 2800 mg/kg bw/d ♂; 3500 mg/kg bw/d ♀ (urinary bladder stones)

• Mean body weight gain was reduced in all treatment groups (≥ 9 %)

Urinary bladder

• Dose-dependent incidence of ulceration of the urothelium (urinary bladder)
• Ulcers observed in the bladder were multifocal and associated with inflammation
• Epithelial hyperplasia was seen in 2/10 males in the highest dose group
• Dose-dependent incidence of calculi in the urinary bladder with males being more sensitive

Incidence of urolithiasis:
<table><tr><th rowspan="2">Dose (mg/kg bw/d ♂/♀)</th><th colspan="2">Number of rats with urinary bladder stones</th></tr><tr><th>males</th><th>females</th></tr><tr><td>0</td><td>0/10</td><td>0/10</td></tr><tr><td>1400/1800</td><td>0/10</td><td>0/10</td></tr><tr><td>2000/2700</td><td>0/10</td><td>0/10</td></tr><tr><td>2800/3500</td><td>6/10 (60 %)</td><td>1/10 (10 %)</td></tr><tr><td>3900/4800</td><td>9/10 (90 %)</td><td>3/10 (30 %)</td></tr><tr><td>4700/5900</td><td>7/10 (70 %)</td><td>7/10 (70 %)</td></tr></table>
kidney

• According to the re-evaluation of the kidney histopathology, renal lesions indicative of a retrograde nephropathy were observed with lower incidence and severity in mice too (not reported) (Hard et al., 2009)</td><td>Melnick et al. (1984)
and
NTP (1983)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td><u>Tissue examined:</u>
Necropsies performed on all animals
A variety of tissues were examined histologically only in control and high-dose animals (gross lesions, tissue masses, abnormal lymph nodes, skin, mandibular lymph nodes, mammary gland, salivary gland, thigh muscle, sciatic nerve, bone marrow, costochondral junction (rib), thymus, larynx, trachea, lungs and bronchi, heart, thyroid, parathyroid, esophagus, stomach, duodenum, jejunum, ileum, colon, mesenteric lymph nodes, liver, gallbladder, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles/ prostate/ testes or ovaries/ uterus, nasal cavity, brain, and pituitary gland)
In the lowest dose group only the kidney and the urinary bladder was microscopically examined</td><td></td></tr>
<tr><td>Sub-chronic, non-guideline repeated dose toxicity study

Supporting study

Oral (feeding)

BALB/c mice

Males/females (ctrl: n = 21; mel: n = 27)

No international accepted test guideline followed

No GLP</td><td>Melamine (obtained from Sigma Chemicals; purity not specified)

M/F: 12000 ppm (1.2 %, ca. 1800 mg/kg bw/d*) in the presence or absence of different fatty acids

22 weeks

*Converted according to table 3.17, Guidance on the application of the CLP criteria (Version 5.0, July 2017)</td><td>ED: 1800 mg/kg bw/d ♂/♀ (hyperplasia and dysplasias/in situ carcinomas in the urinary tract)
Calculus formation in the bladder was observed but not described in detail (60 to 85 % of the animals in melamine treated groups and none in the control)
Calculus formation was associated with an increased incidence of bladder hyperplasia (see incidence table below)
Proliferative lesions of the urothelial tract:<table><tr><th>Group</th><th>H#</th><th>D/CIS*</th></tr><tr><td>Renal pelvis</td><td></td><td></td></tr><tr><td>Ctrl</td><td>1/21(5 %)</td><td>1/21(5 %)</td></tr><tr><td>Melamine</td><td>2/27 (7 %)</td><td>4/27 (15 %)</td></tr><tr><td>Ureter</td><td></td><td></td></tr><tr><td>Ctrl</td><td>0/21</td><td>0/21</td></tr><tr><td>Melamine</td><td>3/27 (11 %)</td><td>7/27 (26 %)</td></tr><tr><td>Urinary bladder</td><td></td><td></td></tr><tr><td>Ctrl</td><td>0/21</td><td>0/21</td></tr><tr><td>Melamine</td><td>7/27 (26 %)</td><td>9/27 (36 %)</td></tr></table>#transitional cell hyperplasia
*transitional cell dysplasias/in situ carcinomas (combined)

Statistical significance between control and melamine group is not indicated

<u>Tissue examined:</u>
The urinary epithelia (urinary bladder, ureters, and renal pelves) was examined grossly and</td><td>Cremonezzi et al. (2001)</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		microscopically • Tissue fixation was done in formalin	
Chronic repeated dose toxicity/carcinogenicity study Supporting study Oral (feeding) B6C3F1 **mice** (hybrids) Males/females (n = 50 / sex / group) Similar to OECD TG 451 (NTP standards) Deviation: only 2 concentrations tested No GLP	Melamine (> 95 % purity) ♂/♀: 2250 and 4500 ppm (♂: ca. **327 and 688** mg/kg bw/d; ♀: **523 and 1065** mg/kg bw/d) Continuously administered **2 years** (103 weeks)	**ED:** 327 mg/kg bw/d ♂ (urinary bladder calculi and acute/chronic inflammation, and mild epithelial hyperplasia); 1065 mg/kg bw/d ♀ (urinary bladder calculi and acute/chronic inflammation, and mild epithelial hyperplasia) • **High incidence of urinary bladder calculi** (males: ctrl 2/45(4 %), low-dose 40/47 (85 %), high-dose 41/44 (93 %); females: high-dose 4/50 (8 %)), **acute/chronic inflammation**, and **mild epithelial hyperplasia** in the urinary bladder was found in male mice exposed to low- and high-dose melamine whereas similar chances were observed only in the high-dose group of female mice to a much lesser extent • **Reduced survival** among male mice exposed to high-dose melamine <u>Tissue examined:</u> • Macroscopic examination on major tissues or organs • Histopathological examination on the following tissues: skin with mammary gland, mandibular lymph node, salivary gland, sternum with bone marrow, larynx or anterior trachea, esophagus, thyroid, parathyroid, lungs with mainstem bronchi, heart, stomach (glandular and nonglandular), duodenum, large intestine, liver, gallbladder, pancreas, spleen, kidneys, adrenal glands, urinary bladder, entire gonads, prostate or uterus, brain, and pituitary gland • Tissue was preserved 10% neutral buffered formalin embedded in paraffin	Melnick et al. (1984) and NTP (1983)
Sub-chronic repeated dose toxicity study **Key study** Oral (nasal-gastric gavage) **Cynomolgus monkey** (Macaca fascicularis) Age: 3 – 4 years (both sexes) Male/female (n = 3 / sex / group; recovery	Melamine (> 99 % purity) Vehicle: 0.5 % hydroxypropyl methylcellulose ♂/♀ **60, 200,** and **700** mg/kg bw/day Continuously administered **91 days** + 28 days recovery (without dosing)	**ED:** 200 mg/kg bw/d ♀ (histopathological changes in the kidney) • Overall, the kidney was identified as primary target organ • Estimated NOAEL: 60 mg/kg bw/d <u>Low-dose</u> • No histopathological findings <u>Mid-dose</u> • Nephrotoxicity (histopathological changes in the kidney: minimal to mild tubular nuclear pyknosis with interstitial mononuclear cell infiltration, and cortical lymphoid nodules) was observed in 2/3 ♀ monkeys <u>High-dose</u> • Turbid and whitish urine in ♂/♀, starting from day 25 and 20, respectively • Urinary crystals • Elevated alanine aminotransferase, indicative of hepatocellular injuries, was found	Early et al. (2013)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
animals: 2 / sex / only control group and high-dose group) Similar to OECD TG 409 Deviations: lower number of animals, primates are not recommended, GLP		• Red blood cell changes were observed • Increased kidney weights in ♂/♀ Histopathological observation: *Kidney* • Minimal to moderate nephrotoxicity-related changes (e.g. renal tubular degeneration/regeneration with or without concurrent interstitial mononuclear infiltration, cortical lymphoid nodules, tubular dilation occasionally containing granular or hyaline casts, tubular nuclear pyknosis, tubular single-cell necrosis, and/or thickening of the glomerular capsule) were found in 2/3 ♂ and 3/3 ♀ • Changes were generally not resolved within 4 weeks of recovery *Heart* • Minimal pericarditis (secondary to uremia) in 1/3♀ *Bone marrow* • Minimal to mild Increased hematopoiesis in 1/3♂ and 3/3 ♀ *Spleen* • Minimal to moderate depletion of lymphoid in 2/3 ♂ and 1/3 ♀ *Thymus* • Minimal depletion of lymphoid in 1/3 ♂ *Liver* • Minimal extramedullary hematopoiesis in 1/3 ♀ *Adrenals* • Minimal extramedullary hematopoiesis in 1/3 ♀ In addition: diffuse cardiomyocytic vacuolation, cholecystis with cholangitis, and thyroiditis were seen in 2/3 ♂ and 1/3 ♀ <u>Tissue examined:</u> • Tissues collected at necropsy included the adrenal glands (bilateral), abdominal aorta, bone (femur/sternum), bone marrow (sternum), brain, epididymis (bilateral), esophagus, eye (with optic nerve) (bilateral), heart, duodenum, jejunum, ileum (Peyer's patches), appendix, colon, rectum, kidney (bilateral), liver, lung, lymph nodes (submandibular lymph nodes and mesenteric lymph nodes), breast sciatic nerve, ovaries (bilateral), fallopian tubes (bilateral), pancreas, pituitary, prostate, salivary glands (submandibular gland, sublingual gland), seminal vesicles, skeletal muscle (biceps femoris), skin (groin), spinal cord (neck, chest, and waist), spleen, stomach, testes (bilateral), thyroid (with parathyroid) (bilateral), trachea, bladder, uterus, and thymus. • Tissue was examined crossly and microscopically	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		• No specific information on whether or not the urinary bladder, the ureters, and the renal pelvis were examined (text stats only kidney and bladder)	

Table 20: Summary table of human data on STOT RE

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference

Observational study
 	
Cross-sectional study
	Melamine (intentional adulteration of milk products)	Investigation of the renal outcomes in children that were exposed to **low-dose melamine** in Hong Kong
		
		Participants: 3170 children (1748 ♂ and1422 ♀; age ≤ 12 years)
		
		Exposure: oral, mildly contaminated milk products for ≥ one month (from twice a week to daily)
		
		Estimated daily intake: 0.01 to 0.21 mg/kg mg/day (for the eight children with stones/deposits)
		
		No reliable biomarker of melamine exposure
		
		The level of exposure in the Hong Kong area was considerably lower as compared to mainland China (Wen et al., 2016)
		
		Examinations: screening of renal effects using ultrasonography and urinalysis:
			Renal deposits in 8/3170 (0.25 %)
			
			Renal calculus (1/3170 (0.03 %)); suspected renal deposits* (7/3170 (0.22 %)); other renal abnormalities (17/3170 (0.54 %); haematuria (208/3170 (6.56 %)); proteinuria (59/3170 (1.86 %)); leucocytes (108/3170 (3.40 %)); other abnormalities on urinalysis (5/3170 (0.16 %))
			
			*small hyperechoic renal foci (< 4 mm) at or close to the renal papillae
			
			No severe adverse effects were observed in Children exposed to low-dose melamine in the Hong Kong area
			
			<u>Reported uncertainties/limitations:</u>
			• Absence of a reliable biomarker of melamine exposure
			• An accurate calculation of the exposure was not possible (misclassification bias)
			  ○ Documentation of history of consumption not systematically
			  ○ Melamine levels in formula may vary between batches
			  ○ Possible overestimation by parents
				Lam et al. (2008)

Observational study	Melamine (intentional adulteration of milk products)	Ultrasonographic screening of melamine-exposed children in China plus a reinvestigation of a subset of patients **Participants:** 3976 infants with and 358 without a history of melamine-contaminated milk product consumption (defined as	**Initial screening:** Renal stones and/or hydronephrosis were detected in 63 cases (63/3976) in the initial screening vs. one case (1/358) in the control group (no consumption of melamine-tainted formula)	Chen et al. (2009)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		formula known to be contaminated) **49 paediatric patients were reinvestigated** (32 ♂ and 17 ♀; mean age: 24 months) **Exposure:** oral, melamine-contaminated milk product consumption Estimated daily intake: 0.01 to 62.67 mg/kg bw/day, geometric mean: 1.28 mg/kg bw/day (done for the 49 reinvestigated patients using reported melamine levels (AQSIQ*) in formula products and an estimated formula intake values) Estimated length of exposure: 17 months (geometric mean) **Note:** • The daily intake estimation was done using melamine concentrations provided by AQSIQ (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ) (2008) Results of Nationwide Melamine Special Inspection on Powered Infant Formula. AQSIQ, Beijing.) • A few formula samples were also analysed as part of this study whereas melamine levels were considerably lower (no specified) than those reported by AQSIQ and no cyanuric acid was found in any sample • Overestimation of exposure based on AQSIQ data seems likely **Examinations:** ultrasonographic screening, assessments of clinical signs	**Reinvestigation:** • Nephrolithiasis: 36/49 (74 %) • Hydronephrosis: 16/49 (33 %) (hydronephrosis was assumed to be caused by nephroliths) • Haematuria: 4/49 (8 %) The authors conclude that low-dose melamine exposure (below the recommended WHO TDI) may be a risk factor of renal stones (one kidney stone case with an estimated intake of 0.04 mg/kg bw/d)	
Observational study Cross-sectional	Melamine (intentional adulteration of milk products)	Screening of renal effects in Chinese children shortly after the announcement of formula contamination	**Prevalence of renal stones:** Overall prevalence 50/589 (8.5 %) With respect to estimated	Guan et al. (2009)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
study		**Participants:** 589 children (341 ♂ and 248 ♀; age: 0 - 36 months) were screened, 421 were exposed to melamine **Exposure:** oral, assessment based on information on the history of exposure to contaminated formula, including the brand, melamine content, duration of exposure, use of formula alone or a combination of breast milk and formula **Melamine content** in powdered-milk infant formulas was classified as: presumably no melamine (0 ppm), moderate (< 150 ppm), and high (> 500 ppm) Estimated daily intake: no data Estimated length of exposure: ≥ 30 days **Examinations:** ultrasonography of kidney and laboratory analysis (serum and urinalysis), questionnaire	melamine content: • No melamine: 8/168 (4.8 %) • Moderate: 19/300 (6.3 %) • High: 23/121 (19 %) **Prevalence of suspected renal stones:** Overall 112/589 (19 %) With respect to estimated melamine content: • No melamine: 24/168 (14.3 %) • Moderate: 58/300 (19.3 %) • High: 30/121 (24.8 %) **Stone characteristics:** Stones were grainy and gobbet-shaped (irregular and nubby) and **mostly localised to the renal pelvis** **Laboratory analysis** (incidence table below) **Evidence for glomerular dysfunction** (elevated urinary levels of microalbumin, and transferrin) was significantly increased in patients with suspected stones (P = 0.01) Children exposed to high-melamine levels had a significantly elevated risk to have stones <u>Reported uncertainties/limitations:</u> • Uncomplete questionnaires in some cases • Possible enrolment bias • Urine sample collection was untimed • Lack of data on renal pathological characteristics (e.g. no renal biopsies)	

Incidence table: Laboratory results in the children studied, according to the presence or absence of urinary tract stones (Guan et al., 2009)

Group	Haematuria	Leukocyturia	Proteinuria	Glomerular Dysfunction[#]	Renal Tubular Dysfunction
Children with stones	2/34 (5.9)	1/34 (2.9)	0/34	4/41 (9.8)	0/41
Children with suspected stones	0/76	1/76 (1.3)	1/76 (1.3)	12/88 (13.6)	4/88 (4.5)
Children without stones	4/262 (1.5)	4/262 (1.5)	2/262 (0.8)	15/269 (5.6)	8/269 (3.0)
All children	6/372 (1.6)	6/372 (1.6)	3/372 (0.8)	31/398 (7.8)	12/398 (3.0)
P value*	0.10	0.63	0.65	**0.04**	0.42

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
#Evidence for glomerular dysfunction included elevated urinary levels of microalbumin and/or increased transferrin levels *P values were calculated for the comparisons among the three subgroups of children with the use of Fisher's exact test, except in the case of glomerular dysfunction, for which Pearson's chi-square test was used.				
Observational study	Melamine (intentional adulteration of milk products)	Ultrasonographic examination of Chinese children exposed to melamine **Participants:** 15 577 children (7988 ♂ and 7589 ♀; mean age: 22 months (ranging from 1 month - 15 years) with melamine exposure (minimal exposure duration was set to 30 days) **Exposure:** oral, melamine content of the formula estimated according to official numbers; 22 brands with melamine content ranging from 0.09 to 2563 ppm **Examinations:** ultrasonography of kidney, questionnaire (demographic characteristics, history of exposure, symptoms) **For further analysis** as published in Zhang *et al.* (2009): 846 children (417 ♀ and 429 ♂ mean age: 18 months (ranging from 1 month - 5 years)	**Urolithiasis** was seen in 562/15577 (3.6 %) children **Calculi** were mostly found in the kidney (431 in a single kidney, 131 in both kidneys) and a few in the ureter (7), bladder (1), urethra (1), and gallbladder (1) Children with stones were mainly (88.6 %) ≤ 36 months The highest incidence rate (155/2496 (6.21 %)) was seen in the age group 6 - 12 months **Calculi characteristics:** • Large calculi (≥ 10mm ø) in n = 9 in the **renal pelvis** and ureter • Medium sized calculi (4-9 mm ø) in n = 108 • Small calculi (< 4mm ø) in n = 371 • Sand-like calculi in n = 64 Detected calculi were less dense, more sand-like and different from calcium-oxalate calculi (ultrasonographically distinguishable) 15 children presented signs of urinary tract obstruction **Liver:** 3 children exposed to high-dose melamine for 6 months presented with biliary calculi **Further results from Zhang et al. (2009):** The incidence of renal calculi was closely related to the melamine content of the formula (up to **15.7 %** for Sanlu (2563 mg/kg)) Reduced weight and head circumference SDSs amongst patients with stones One child, fed with highly contaminated formula (Sanlu) for 42 days developed bilateral multiple calculi (> 0.5 cm diameter)	He et al. (2009) and Zhang et al. (2009) (further analysis)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			Liver abnormalities (hepatomegaly, elevated aspartate aminotransferasemia, and gallstone) were observed in 5 nephrolithiasis patients Reported uncertainties/limitations: • Possible enrolment bias	
Intake assessment	Melamine (intentional adulteration of milk products)	Estimation of dietary melamine exposure originated from tainted infant formula in Chinese children Four age groups were selected (3, 6, 12, and 24 months) The mean body weight was estimated **Exposure:** consumption of infant formula was estimated according to the recommended usage level in the package insert Melamine concentrations were derived from official numbers (AQSIQ, SAC (Standardization Administration of China) (2008). Determination of melamine in raw milk and dairy products. GB/T 22388-2008; in Chinese) The intake of melamine was calculated by the actual measured melamine concentration (mg/kg) by the daily maximum amount of infant formula consumption (kg/d), and then divided by the mean body weight (bw in kg).	**Estimated melamine intake:** Using mean conc. (1212mg/kg) Age (months) / Mel. intake (mg/kg bw/d)* 3 / 28.4 6 / 26.0 12 / 18.2 24 / 10.4 Using max conc. (4700mg/kg) Age (months) / Mel. intake (mg/kg bw/d)* 3 / 110.2 6 / 100.7 12 / 70.5 24 / 40.3 *based on the mean and maximum melamine concentration that was found as mean value in Sanlu infant formula Reported uncertainties/limitations: • Uncertainty regarding intake estimation: ○ Formula samples are not necessarily representative ○ Variation in melamine concentrations in the adulterated Sanlu infant formula ○ Consumption of other infant formula brands with unknown concentrations	Jia et al. (2009b)
Observational study Case-control study	Melamine (intentional adulteration of milk products)	Chinese children with melamine-related urolithiasis were analysed in a clinicopathological study **Participants:** 35 children (**15 cases** with a confirmed history of melamine consumption and diagnosed urolithiasis vs. **20 controls** (asymptomatic, no calculi, with detectable melamine	**Stones** (2.5-18 mm ø) were mostly located in the **renal pelvis** **Kidney pathologies:** Acute renal failure (1/15), hydronephrosis (4/15), dysuria (2/15), haematuria (1/15), proteinuria (3/15), β-2 microglobulin (indicator of tubular damages) in the urine (2/15), microalbuminuria (indicator of	Lam et al. (2009)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		in urine); age < 3 years) **Exposure:** oral, confirmed history of melamine consumption Estimated length of exposure: 3-24 months (mean ca. 8 months calculated from available data) Clinical data are reported but were not generated in the scope of this publication **Examinations:** analysis of blood and urine Melamine and cyanuric acid concentrations were measured in the urine using triple–quadruple tandem mass spectrometry and gas-chromatography mass-spectrometry, respectively	glomeruli injuries in 2/15), crystalluria (1/15) (crystals appeared golden-brown with globular to flattened shape with fine linear radiations) **Melamine concentrations in the urine:** *Ctrl group:* 0.08 to 37 (median, 6.6) µg/mmol Cr *Urolithiasis group:* 0.87 to 2002 (median: 21) µg/mmol Cr (P = 0.008) A statistically significant correlation between urinary melamine level and the size of calculi was found (r = 0.86, P = 0.0007) which, according to the authors, strongly suggests that melamine exposure in humans is related to nephrolithiasis **Cyanuric acid concentrations in the urine:** *Ctrl group:* 6.4 to 86 (median, 15) µg/mmol Cr *Urolithiasis group:* 4.2 to 50 (median, 19) µg/mmol Cr (P = 0.59) No correlation between urinary cyanuric acid level and the size of calculi was found (r = 0.081, P = NS). No correlation between urinary melamine and urinary cyanuric acid was established The authors concluded that cyanuric acid may not be important for stone formation in humans Predisposing urinary lithogenic factors: • The pH of the urine was significantly lower in cases as compared to control • Urinary urate level (mean ctrl: 0.73 mmol/mmol Cr, mean cases: 1.36 mmol/mmol;	

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			P = 0.033) (one patient with microscopic crystalluria had a high urate-to creatinine ratio (1.8 mmol/mmol Cr)) The authors noted that urine pH < 5.8 promotes uric acid precipitation	
Observational study Case-control study	Melamine (intentional adulteration of milk products)	Evaluation of associations between nephrolithiasis-related clinical findings, exposure patterns, biomarkers and potential melamine exposure in children **Participants:** 1222 children (14 cases and 1208 controls; age from 0 - 16 years) exposed to melamine Cases were defined as diagnosed urolithiasis **Exposure:** oral, estimated exposure based on duration and quantity of contaminated formula per day and classified as: control (< 0.05 ppm), low (0.05-2.5 ppm), and high exposure (> 2.5 ppm) Estimated length of exposure: 20 days up to 4 years **Examinations:** blood pressure, urinalysis, urine calcium and creatinine, renal function tests and renal ultrasonography, questionnaires (age, gender, birth history, history of having resided in China, past history of urinary tract infection (UTI) or vesico-urethra reflux, family history of nephrolithiasis and clinical symptoms such as abdominal pain, flank pain, dysuria, urinary frequency, granule in urine, decrease urine output)	The presence of renal calculi in paediatric patients was significantly associated with: • Longer exposure duration (consumption of contaminated products) • Higher exposure level Ctrl (< 0.05 ppm): 2/504 (0.4 %) Low (0.05-2.5 ppm): 3/672 (0.5 %) High (> 2.5 ppm): 9/46 (19.6 %) • The nephrolithiasis risk clearly increases with exposure level 2/10 cases presented melamine in their urine vs. 0/20 in the control at the time of assessment Stones were mostly located over the **renal calyx** (2.1- 7.5 mm ø) <u>Reported uncertainties/limitations:</u> • Possible enrolment bias • Recall bias from parents about the amount and duration of dairy products consumed by children • misclassification bias (calculation of the exact exposure dose)	Wang et al. (2009)
Observational study	Melamine (intentional adulteration of milk products)	Diagnostic screening and management of Chinese children with melamine-mediated renal stones **Participants:** 1091 children (age < 4 years) with suspected melamine exposure **Exposure:** suspected melamine exposure	**Renal calculi** were observed in 12/1091 (1.1 %) children Stones were mostly located in the renal pelvis and calyx 11/12 (91.7 %) had consumed Sanlu brand infant formula (ca. 955 – 2563 ppm) 1/12 (8.3 %) had consumed milk products with low-level melamine content (6.2 – 17 ppm)	Zhu et al. (2009)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		Estimated length of exposure: 1-24 months (mean ca. 12 months calculated from available data) **Examinations:** Stones detected by B-ultrasonography, renal function was analysed by urinalysis and renal function tests	Amongst the nephrolithiasis cases: • 6/12 had dysuria • 12/12 had normal renal function (4/12 proteinuria, 1/12 hematuria)	
Observational study	Melamine (intentional adulteration of milk products)	Retrospective evaluation of continuous renal replacement therapy (CRRT) in Chinese children with acute kidney injury attributed to melamine-related urolithiasis **Participants:** 8 children (6 ♂ and 2 ♀; age: 9 - 33 months, median: 18 months) of 562 children diagnosed with urolithiasis in a previous screening involving 15 577 children (He et al., 2009; Zhang et al., 2009) **Exposure:** oral, high-content melamine tainted formula for a considerably long duration Formula brand: Sanlu (Shijiazhuang, Hebei Province, China; melamine levels reported as exceeding 2.5 ppm*). Mean duration of consumption: 11.1 months (range: 5-18 months) **Examinations/therapy:** laboratory blood test (blood urea nitrogen (BUN), creatinine (Cr)), abdominal X-ray and ultrasonographic, CRRT (PRISMA machine), urinalysis after CRRT, * List of milk products that are confirmed to be positive for melamine. Geneva: International Food Safety Authorities Network, 2008.	**Conditions on admission to the paediatric intensive care unit** All children presented with **renal stones** (kidney and ureter) and met the criteria for **acute kidney injury** (abrupt reduction of kidney function, increase of serum creatinine (Cr), oliguria) Calculi appeared sand-like and less dense than calcium oxalate stones • **Hydronephrosis** in 4/8 patients • **Diffused pathological changes** in bilateral kidneys in 2/8 patients • Unilateral or bilateral **dilated upper ureters** in 4/8 patients • **Oliguria or anuria** in 8/8 patients • **Haematuria** in 1/8 patients (assumed to be caused by stone-related injuries) • **Hypertension** in 6/8 patients • **Reduced renal function** (elevated BUN (13.11 - 35.6 mmol/L) and Cr (238.8 - 773.7 μmol/L)) **After CRRT:** Renal function recovered (normal renal function in all children at 6-months follow-up) Blood pressure returned to normal Urinalysis normal 4/8 patients passed their stones (residual stones were passed or	Yang et al. (2010b)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			removed within 2 months) <u>Reported uncertainties/limitations:</u> • no analysis of melamine in urine, blood, and stones	

<table>
<tr><th>Type of data/report</th><th>Test substance</th><th>Route of exposure
Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr>
<td>Observational study</td>
<td>Melamine (intentional adulteration of milk products)</td>
<td>Clinical evaluation of urolithiasis attributed to melamine consumption in Chinese children

Participants: 2235 children (1242 ♂ and 993 ♀; age: 4 - 72 months, median: 15 months) with a presumed history of melamine exposure participated in a screening

182 children with detailed data were enrolled in two groups:

(1) n = 79 with calculi (cases)
(2) n = 103 without calculi (control)

Exposure: oral, daily intake was calculated based on reported melamine concentrations in milk products*

Mean estimated daily intake:
Group (1): 5.17 ± 4.53
Group (2): 2.38 ± 3.39
(mg/kg bw/day)

Mean duration of consumption:
Group (1): 12.53 ± 8.47
Group (2): 8.65 ± 3.40
(months)

Examinations: questionnaire (history of exposure to contaminated formula, including the brand, duration of exposure and daily intake of milk powder), B-ultrasound, laboratory data (serum blood urea nitrogen (BUN) and serum creatinine (sCr), urinalysis, and treatment (conservative and surgical)

*The list of names of melamine contaminated milk products. General administration of quality supervision, inspection and quarantine of P.R.C. (2009)</td>
<td>Prevalence of urolithiasis: 3.53% (79/2235)

Children with stones (1) had significantly elevated daily melamine intake (5.17 ± 4.53 vs. 2.38 ± 3.39 mg/kg bw/day, P < 0.001) and duration of formula consumption (12.53 ± 8.47 vs. 8.65 ± 3.40 months, P < 0.001) as compared to children without stones (2)

Melamine exposure in group (1) was 25.85-fold higher than the TDI derived by WHO (0.2 mg/kg bw/d)

There was no significant difference between group (1) and (2) with regard to age and sex

Clinical symptoms:
<table>
<tr><th rowspan="2">Clinical symptoms</th><th colspan="2">Groups</th></tr>
<tr><th>(1)
n = 79</th><th>(2)
n = 103</th></tr>
<tr><td>Microscopic haematuria</td><td>15*
(19%)</td><td>0</td></tr>
<tr><td>Pyuria</td><td>12*
(15%)</td><td>1
(1%)</td></tr>
<tr><td>Passing of gravel[1]</td><td>4
(5%)</td><td>0</td></tr>
<tr><td>Dysuria</td><td>9#
(11%)</td><td>0</td></tr>
<tr><td>Impaired renal function</td><td>5†
(6%)</td><td>0</td></tr>
</table>
*P < 0.001,#P < 0.01, †P < 0.05
[1]gravel in the urine

haematuria was assumed to be related to the movement of the stone

The pH was lower in group (1) as compare to group (2) (5.47 ± 0.63 vs. 6.08 ± 0.52, P < 0.05)

65.82 % of children with stones were asymptomatic

Stones were located in the kidney of all children (79/79), 8/79 had stones in both kidneys and ureter, 2/79 had additional stones in the bladder

Mean stone size: 6.17 mm (range 3–19)</td>
<td>Sun et al. (2010a)</td>
</tr>
</table>

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			Smaller stones (< 10 mm) were successfully treated in the majority of group 1 cases (n = 75) Children with larger stones (> 10 mm) required surgical treatment	
Observational study	Melamine (intentional adulteration of milk products)	Investigation of the relationship between the daily intake of melamine-tainted formula and nephrolithiasis in Chinese children **Participants:** 7181 (683 cases and 6498 controls; age: < 3years) Cases were defined as being diagnosed with nephrolithiasis by ultrasonography Controls were defined as not being diagnosed with nephrolithiasis by ultrasonography **Exposure:** oral, information on current and past formula consumption was collected, a daily intake was calculated (based on a questionnaire and reported melamine concentrations in the formula), and 12 exposure groups were defined Table 22 **Data collection:** information was gathered **(**questionnaire, interview) from a subset of children that had been subjected to ultrasonographic examination as part of the Survey of Children's Health and Feeding Status in Beijing	Increasing daily intake and a prolonged duration of exposure were associated with an elevated risk of nephrolithiasis • No exposure: 115/3062 (3.8 %) • High-dose exposure: **50/139 (36 %)** (> 102.4 mg/kg bw/d) (see Table 22) Even low-dose exposure below the TDI recommended by the WHO (< 0.2 mg/kg bw/d) increased the nephrolithiasis risk by 1.7 times (OR 1.7; 95 % CI: 1.3-2.4; P = 0.001) At the two highest dose (≥ 51.2 mg/kg bw/d) the nephrolithiasis risk increased by 11.3 times (OR 11.3; 95 % CI: 5.9-21.8; P < 0.001) <u>Reported uncertainties/limitations:</u> • melamine exposure only retrospectively estimated • possible enrolment bias • no information on overall fluid intake and non-formula dietary constituents • no laboratory investigations	Li et al. (2010)
Observational study and follow-up Population-based screening	Melamine (intentional adulteration of milk products)	Population-based screening study based on ultrasonographic examination of Chinese children **Participants:** 7933 children (4321 ♂ and 3612 ♀; age: < 3 years) with suspected melamine consumption **Exposure:** oral, information on consumption collected (mean estimated exposure dose: 116 mg/d (range 36 - 220) for children with urinary tract abnormalities) Children with evidence of nephrolithiasis or hydronephrosis were monitored after 1, 3, and 6	Urinary tract abnormalities (including nephrolithiasis (24/48) or hydronephrosis without nephrolithiasis (24/48)) were observed in **48/7933 (0.61 %)** children (30 ♂ and 18 ♀) All cases had consumed Sanlu products (estimated consumption in the 48 cases was 116 mg/d) 3/48 patients had urinary abnormalities (haematuria (1/3), leukocyturia (1/3), proteinuria (1/3)) 43/48 children were asymptomatic	Liu et al. (2010b)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		months **Examinations:** population-based screening (ultrasonography conducted by trained sonographers, who were blinded to exposure history), urinary tract abnormalities were confirmed by a second team, interview of mothers of children with stones (dairy consumption behaviour), urinalysis	5/48 children presented with symptoms (oliguria and crying when urinating) Boys were 3.1 times more likely to exhibit abnormalities Abnormalities disappeared in most children during the 6 months follow-up **Remaining abnormalities** were observed in 5/48 **(12 %)** patients 6 months after cessation of melamine consumption (at the 6 months follow-up) <u>Reported uncertainties/limitations:</u> • Not all children of the area were screened • No information on exposure for children with negative diagnosis • Exposure assessment relied on information provided by maternal recall • No information on breast feeding behaviour	
Observational study Case report	Melamine (intentional adulteration of milk products)	Histopathological results from a percutaneous **kidney biopsy** from a 8-months-old male infant subsequent to melamine-mediated acute renal failure **Exposure:** oral Estimated length of exposure: ca. 8 months with Sanlu brand milk powder (from the first week of age until 8 months) Follow-up (repeated biopsy) was performed 13 months after discharge **Examinations:** abdominal ultrasonography, peritoneal dialysis, renal-function tests, percutaneous renal biopsy, HPLC (calculi composition)	Bilateral renal stones causing acute obstructive renal failure Renal function tests did not show abnormalities at the time of examination **Histopathological findings from the biopsy:** Lymphocytic infiltration in the glomeruli, sclerotic glomeruli, proliferation of fibrous tissue in the glomeruli and Bowman's capsule, swollen tubular cells, crystals within the lumen of the tubular cells, lymphocytic infiltration and fibrosis within the renal interstitium, swollen tubular capillary endothelial cells, dilatation, abnormal structure of organelles within some renal tubular epithelial cells, and pyknotic nuclei Calculi were found to be composed of melamine (29.2 %),	Sun et al. (2010b)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			uric acid (52.2 %) and other unidentifiable material (18.6 %) Cyanuric acid was not present in calculi At follow-up (repeated biopsy): resolved renal damages (no calculi or crystals, no degeneration of the renal tissue)	
Observational study Cross-sectional	Melamine (unknown source of exposure)	Preliminary study to investigate a potential association between urinary melamine concentrations arising from low-dose environmental exposure and common types of urolithiasis in Taiwanese adults **Participants:** 11 patients with uric acid urolithiasis, 22 patients with calcium urolithiasis, 22 sex- and age-matched controls (participants of a regular health check-up in the same hospital) **Exposure:** environmental exposure **Examinations:** health examination and urinary melamine measurements by LC-MS/MS	Patients presenting with either uric acid or calcium urolithiasis had significantly higher urinary melamine levels (P = 0.019) **Melamine level (µg/mmol Cr):** Ctrl: 0.06 (0.02–0.20) Calcium urolithiasis: 0.14 (0.07–0.93) Uric acid urolithiasis: 0.50 (0.07–1.18) Reported uncertainties/limitations: • Low sample size • Only one measurement of urinary melamine levels (one-spot overnight urine samples) • No measurement of melamine content in stones	Wu et al. (2010)
Observational study and follow-up	Melamine (intentional adulteration of milk products)	Diagnosis, treatment, and follow-up of melamine exposed Chinese paediatric patients with kidney stones and acute obstructive renal failure **Participants:** 25 patients (17 ♂ and 8 ♀; age: 6 – 36 months) **Exposure:** oral Estimated length of exposure: 9.5 months (median) **Examinations**: clinical signs, estimated consumption of contaminated formula, urinalysis, ultrasonography of the urinary tract, routine serum chemistry and haematology parameters (including serum urea nitrogen, and creatinine), stone composition was	All patients had stones in the kidney and ureters The appearances of calculi were either sand-like crystals or larger size, clump-like stones The function of the kidney was significantly impaired as indicated by increased serum values of blood urea nitrogen (BUN), creatinine and uric acid All patients had oliguria, anuria or dysuria Haematuria was observed in two patients **Stone composition:** Uric acid and melamine (molar ratio 1.2:1 to 2.1:1) without cyanuric acid or	Sun et al. (2010c)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		analysed (using HPLC-MS, GC-MS, and FTIR (fourier transform infrared spectrometry)), treatment was performed, follow-up at 12 to 17 months	other melamine analogues The authors concluded that cyanuric acid does not contribute to the calculus formation Treatment: liquid plus alkalisation of urine and surgery Follow-up: renal function returned to normal	
Observational study	Melamine (intentional adulteration of milk products)	Screening study based on ultrasonographic examination and urinalysis of Chinese children **Participants:** 14 256 children (7802 ♂ and 6438 ♀; age: 10 days to 17 years; 2283 had a history of melamine (mel) consumption) Children with obvious symptoms were not enrolled **Exposure:** oral, history of melamine consumption (formula brand was not recorded) **Investigations:** ultrasonography of the kidneys and urinary tract and urinalysis	**Abnormalities of the urinary system** (congenital anomalies of kidney and urinary tract, urinary stones and/or hydronephrosis, leucocyturia and haematuria and/or proteinuria) were observed in **869/14 256 (6.10 %)** children using either ultrasound or urinalysis **Prevalence of kidney stones:** No exposure: 0.3 % (38/11 973) Mel exposure: 1.6 % (37/2283)* * P < 0.001 The majority of stones was small- and medium-sized (ø < 10 mm) **Urinalysis:** 572/14 256 (4%) with abnormalities The risk of nephrolithiasis was 5.17 times higher amongst children with dietary melamine exposure	Yang et al. (2010a)
Observational study Follow-up	Melamine (intentional adulteration of milk products)	Investigation of the relation between urolithiasis and secondary renal injuries in Chinese children **Participants:** 8335 children (♂ 3473 and ♀ 4862 ; age: ≤ 6 years; with a history of melamine consumption) **Exposure:** oral, history of melamine-contaminated milk powder consumption Melamine concentrations in formula products were set to high (Sanlu milk powder: 162 – 2563 mg/kg*), middle (combination of Sanlu and other brands), and low (other milk powder brands: 0.09 –	**Renal calculi** were observed in 105/8335 (1.3 %; 68 ♂ and 37 ♀) children (77 of whom were asymptomatic) Detection rate dependent on estimated melamine consumption (see Table 22): Low: 26/5443 (0.5 %) Middle: 15/617 (2.4 %) High: 64/2284 (2.8 %) The size of stones (ø) ranged from 1.1 mm to 19.3 mm Duration of exposure significantly correlated with the size of stones (r = 0.262; P < 0.010) Melamine was considered the only	Gao et al. (2011)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		150 mg/kg*) Estimated length of exposure: 1 - 36 months **Examinations:** urinalysis and urinary system ultrasonography **Follow-up** after 6 months of the initial diagnosis *General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ) (2008)	underlying cause of urolithiasis (other causes such as hypercalcinuria, cystine metabolic abnormality or congenital abnormalities of the urinary tract were not found) The concentration of melamine, gender (higher incidence in males), and age (infants more prone than 1-6 years old children) were statistically significant risk factors **At follow-up (n = 96):** 67/96 (69.8 %) children passed their stones at 6 months follow-up 29/96 (30.2 %) children presented with persistent stones after 6 months **Urinalysis (n = 85):** *Proteinuria:* 6/85 (7.1 %) (persistent stones: 5/6; passed stones: 1/6; P = 0.009) *Microscopic haematuria:* 13/85 (15.3 %) (persistent stones: 8/13; passed stones: 5/13; P = 0.018) *Leukocyturia:* 14/85 (16.5 %) (persistent stones: 6/14; passed stones: 8/14; P = 0.344) Macroscopic haematuria in one case **urinary microprotein profiles (n = 76)** (microalbumin (ALB), immunoglobulin G (IgG), and N-acetyl-β-D-glucosidase (NAG) as marker for detecting glomerular and tubular injury, respectively**):** 32/76 (42.1 %) children presented abnormalities (persistent stones: 52.4 %; passed stones: 38.2 %) Marker for detecting glomerular injuries: • 12/32 elevated ALB/Cr • 3/32 elevated IgG/Cr • 10/32 elevated ALB/Cr and IgG/Cr Marker for detecting tubular injuries:	

<table>
<tr><th>Type of data/report</th><th>Test substance</th><th>Route of exposure
Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td></td><td>• 16/32 elevated NAG

<u>Reported uncertainties/limitations:</u>
• Unable to control for potential bias in patient selection
• No exclusion of confounding bias caused by non-melamine-related renal stones possible
• Urinary microprotein profile data from the initial screening not available</td><td></td></tr>
<tr><td>Observational study

Case-control study (cross-sectional design)</td><td>Melamine (unknown source of exposure)</td><td>Examination of a possible link between low-level environmental melamine exposure and urolithiasis in Taiwanese adult patients

Participants: 211 patients diagnosed with calcium urolithiasis and 211 age- and gender-matched controls

Exposure: environmental exposure

Examinations: questionnaire, blood analysis, urinalysis (triple-quadrupole liquid chromatography tandem mass spectrometry), stone composition analysed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)</td><td>Elevated urinary melamine concentrations were associated with an increased risk of calcium urolithiasis:

<table><tr><th>urinary melamine levels (ng/ml)</th><th>risk of calcium urolithiasis (adjusted odds ratio#)</th></tr><tr><td>MDL* – 3.11</td><td>3.01 (95 % CI: 0.76–11.89)</td></tr><tr><td>≥ 3.12</td><td>7.64 (95 % CI: 1.98–29.51)
Trend test: P< 0.0001)</td></tr></table>(*method detection limit; #adjusting for educational level, fluid intake, cigarette smoking, betel quid chewing, alcohol drinking, urinary uric acid, urinary calcium, urinary creatinine, and estimated creatinine clearance rate)

Population attributable risk (PAR %) of calcium urolithiasis with urinary melamine: 47.9–52.9 %

Melamine was detected in all analysed stones (9/9) from patients with urinary melamine levels above the MDL

A correlation between the risk of calcium urolithiasis and melamine exposure was found

<u>Reported uncertainties/limitations:</u>
• Controls were chosen from the same hospital (no control that represents the general population)
• Potential confounders (e.g. consumption of animal proteins) due to missing</td><td>Liu et al. (2011)</td></tr>
</table>

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			information • Only one measurement of urinary melamine levels (one-spot overnight urine samples)	
Observational study	Melamine (intentional adulteration of milk products)	The impact of gender, age, and urinary pH was evaluated in a retrospective analysis of Chinese children that had been exposed to melamine **Participants:** 208 (136 ♂ and 72 ♀; age: < 3 years; all had a history of melamine consumption) **Exposure:** oral, 3 months consumption Sanlu milk powder Estimated length of exposure: 3 months **Examinations**: retrospective review of data acquired during a clinical screening in 2008/2009 (ultrasonographic examination and urinalysis)	**Kidney stones** were found in **83/208 (39.9 %)** children that had a history of Sanlu milk powder consumption Of the 83 cases 62 were boys and 21 were girls (boys/girls ratio: 3/1; control: 74♂/51♀) **Gender** was significantly associated with nephrolithiasis risk (OR: 2.03; 95 % CI: 1.11 - 3.74, P = 0.02) **Acidic urine** was found to be another significant risk factor (OR: 1.78; 95 % CI: 1.01-3.11; P = 0.04) The age did not have a significant influence on the nephrolithiasis risk <u>Reported uncertainties/limitations:</u> • prevalence of kidney stone may be affected by geographical and environmental factors • individual risk factors (e.g. weight index and exposure dose of melamine, and stone analysis) were not analysed due to the lack of relevant information	Lu et al. (2011)
Observational study Follow-up	Melamine (intentional adulteration of milk products)	Follow-up investigation of renal effects associated with melamine-mediated kidney stones over the course of 12 months **Participants:** 32 530 children subjected to an urinary tract screening were initially enrolled (Inclusion criteria: age 0-36 months, history of melamine consumption (≥ 2 weeks), with (A) or without (B) melamine-related urolithiasis, without obstruction and need not to be hospitalized, consent formed signed) 462 were selected according to these criteria (274 ♂ and 188 ♀;	**Stone discharging rate of 265 urolithiasis patients:** Months after diagnosis / Calculi discharging rates 1 — 139/265 (52.5 %) 3 — 178/265 (67.2 %) 6 — 234/265 (88.3 %) 12 — 253/265 (95.5 %) **Liver and renal function:** Transient increase of liver AST levels and no permanent liver	Shen et al. (2011b)

<table>
<tr><th>Type of data/report</th><th>Test substance</th><th>Route of exposure
Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td>age: 2 – 36 months)

Two groups were formed: (A) children with diagnosed melamine-related urolithiasis (n = 265), (B) no urolithiasis (n = 197)

A control group (C) (n = 500; 267 ♂ and 233 ♀; age: 4 – 36 months) consisted of healthy children without melamine consumption* and urolithiasis

Exposure: oral, history of melamine consumption ≥ 2 weeks

Examinations: urinary tract screening (ultrasonography), quantification of early tubular and glomerular damage markers including urinary albumin (Alb), transferrin (TRF), α1-microglobulin (α1MG), immunoglobulin G (IgG), β2-microglobulin (β2MG), and N-acetyl-β -D-glucosaminidase (NAG) after 1, 3, 6, and 12 months of diagnosis, biochemical testing (parameters related to liver and renal function such as serum Alb, blood urea nitrogen (BUN), creatinine (Cr), uric acid, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) at the end of 3 and 12 months

*non-melamine-contaminated milk powder was consumed for 1 – 36 months in the control group</td><td>damage was observed

At 3 months: mild increase in AST levels (group A: 24/265 (9.1 %); group B: 13/197 (6.1 %); group C: 15/500 (3 %))

At 12 months: mild increase in AST levels (group A: 6/265 (2.2 %); group B: 1/197 (0.6 %))

ALT values, serum Alb, uric acid and renal functions (BUN and Cr) were normal in both exposure groups

Urinalysis:

At 3 months: abnormalities* observed (group A: 18/265 (6.8 %); group B: 12/197 (6.1 %))

At 12 months: abnormalities* observed (group A: 13/265 (4.9 %); group B: 9/197 (4.6 %))

*Microscopic haematuria, leucocyturia, and proteinuria was mostly found in children with kidney stones

Haematuria or leucocyturia was also found in children who had a history of melamine consumption but did not develop urolithiasis

After 12 months, 13 children remain to show abnormalities

Early renal injury markers (compared to rages in group C):

<table>
<tr><th>Months</th><th colspan="2">Abnormality rate of glomerular filtrating membrane (%) vs. 5.4 % in group (C)</th></tr>
<tr><th></th><th>(A)</th><th>(B)</th></tr>
<tr><td>1</td><td>43†</td><td>14.2†</td></tr>
<tr><td>3</td><td>22.6</td><td>n.a.</td></tr>
<tr><td>6</td><td>16.2</td><td>n.a</td></tr>
<tr><td>12</td><td>10.2#</td><td>6.6</td></tr>
</table>
†P < 0.001
#P = 0.049

The authors concluded that early renal injury markers are more sensitive as compared to urinalysis</td><td></td></tr>
</table>

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
Observational study Short-term follow-up	Melamine (intentional adulteration of milk products)	Investigation of health effects and possible risk factors in Chinese children with melamine-related kidney stones **Participants:** 7328 children (4077 ♂ and 3249 ♀) with a concern of melamine-mediated urinary stones **Exposure:** oral, feeding history was analysed (brands of formula, duration of feeding, breast feeding), levels of melamine concentrations in the corresponding brands was obtained from AQSIQ* **Examinations:** renal ultrasound, urinalysis, analysis of calculi composition (LC- MSMS, ICP-MS, FTIR, XRD, SEM), short-term follow-up after 15.3 ± 8.9 days (n = 51) *General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ) (2008)	**Urinary stones** 189/7328 (2.58 %; 101 ♂ and 88 ♀; ratio: 1.1:1; mean age: 27.4 ± 25.5 months) 186/189 stones were located in the kidney , 8/189 in the ureter, and 4/189 in the bladder The location of kidney stones was as followed: renal calyx (70 right, 67 left), pelvis (62 right, 58 left), parenchyma (6 right, 5 left), and renal hilus (1 right, 3 left) 55/62 (88.7 %) children who consumed high-level melamine-tainted formula (Sanlu formula only with estimated melamine concentration of > 5500 mg/kg) (see Table 22) 29/3133 (0.9 %) children who consumed low-level melamine-tainted formula (estimated < 200 mg/kg) (see Table 22) The age of the child, melamine consumption, and the age of the father were identified as significant risk factors 2 children presented with acute obstructive renal failure (peak creatinine: 482 and 596 mmol/L) Proteinuria and haematuria was observed Case example (5-months-old girl): both kidneys enlarged, parenchymal damage, bilateral renal and ureteric stones and hydronephrosis Urinary calculi contained melamine and uric acid (cyanuric acid was not found) **Short-term follow-up (n = 51):** 33/51 (65 %) children passed their stones The size of the stone had an significant impact on the passing rate (larger stones were more less frequently passed than small calculi)	Wang et al. (2011)

<table>
<tr><th>Type of data/report</th><th>Test substance</th><th>Route of exposure
Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td>Observational study</td><td>Melamine (intentional adulteration of milk products)</td><td>The prevalence of urolithiasis amongst Chinese children in relation to the consumed quantity of melamine-tainted infant formula was studied in a population-based survey

Participants: 2186 children (1329 ♂ and 857 ♀; ≤ 3 years)

Exposure: oral, melamine-contaminated infant formula (information on the melamine concentration, feeding frequency and duration to estimate an accumulated consumption)

Examinations: population-based survey to determine the prevalence of urolithiasis (ultrasonographic screening), urinalyses, questionnaire (feeding history, socio-demographic information, observations about urinary tract signs and symptoms), quantification of melamine and cyanuric acid in infant formula collected form affected families using by GC-MS</td><td>Urolithiasis was diagnosed in 362/2186 (16.6 %; 222 ♂ and 140 ♀) children

Stones were mainly located in the renal pelvis (361/362)

The prevalence was higher amongst children (n = 350) who were exposed exclusively to highly contaminated Sanlu infant formula (24.6 % vs. 17.8 % for Sanlu + others, 17.3 % for any infant formula, 9.3 % for any infant formula excluding Sanlu, 8.5 % for other milk products, and 0 % for exclusively breast-feeding)

The prevalence of urolithiasis correlated with the estimated total amount of consumed Sanlu infant formula (P < 0.001):
<table><tr><th>Estimated total consumption (g) of Sanlu infant formula*</th><th>Prevalence of urolithiasis (%)</th></tr><tr><td>20+</td><td>0.0</td></tr><tr><td>400+</td><td>11.4</td></tr><tr><td>3200+</td><td>15.9</td></tr><tr><td>6400+</td><td>23.5</td></tr><tr><td>12800+</td><td>35.4</td></tr><tr><td>25600-76000</td><td>37.5</td></tr><tr><td>Total</td><td>24.7</td></tr></table>*for 344 children (≤ 3 years), caregiver reported the number of consumed infant formula bags

No calculi observed in children that nourished exclusively by breastfeeding

Hydronephrosis: 104/2168 (4.8%)

Urinalysis:

Haematuria (26/336, 7.7 %), proteinuria (13/306, 4.2 %), or leucocyturia (2/306, 0.7 %) was detected in < 8 % of the urolithiasis cases</td><td>Shi et al. (2012)</td></tr>
</table>

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			Formula content: *Sanlu infant formula:* 44/48 (91.7 %) contained melamine (45 - 4700 ppm; median: 1800 ppm); 32/48 (67 %) contained cyanuric acid (0.4 - 6.3 ppm; median: 1.2 ppm) *Non-Sanlu infant formula:* 8/36 (22 %) of non-Sanlu infant formula contained melamine (4 - 50 ppm; median: 27.5 ppm); 23/36 (64 %) contained cyanuric acid (0.3 - 5.0 ppm; median: 1.0 ppm) <u>Reported uncertainties/limitations:</u> • Prevalence was estimated from a single screening • Other milk products were not tested for melamine • Recall bias regarding feeding history	
Observational study Case-control study Follow-up	Melamine (intentional adulteration of milk products)	Comparison of the clinical features and the renal ultrasound findings between heavily exposed (Sichuan region) and minimal exposed Chinese children (Hong Kong) **Participants:** 66 children (44 cases and 22 controls (age from 0 - 16 years)) Cases were defined as heavily exposed Sichuan children with suspected melamine-related stones 22 children with minimal exposure form the Hong Kong area suspected to have melamine-related stones were enrolled as controls **Exposure:** oral, Sichuan region characterized by the availability of heavily contaminated brands (e.g. Sanlu) as compared to the Hong Kong area were minimal contaminated brands were available (concentrations were derived from AQSIQ* **Examinations:** renal ultrasonography and urinary analysis of interleukin 8 (IL-8) and monocyte chemotactic	The concentration of melamine in the formula, the number of stones in affected patient (median: 4 (Sichuan) vs. 1 (Hong Kong)), and the largest stone size (mean: 6.3 mm (Sichuan) vs. 3.8 mm (Hong Kong)) were significantly higher in Sichuan children **Follow-up:** At 12 months, 28 % of Sichuan children and 48 % of Hong Kong children still presented with renal stones (P = 0.1302) At 9 months, the ratio of urinary IL-8/creatinine was significantly elevated in Sichuan children with stones as compared to Sichuan children that had passed theirs stones and as compared to Hong Kong children with stone (indicative of renal interstitial inflammation as suggested by the authors) IL-8/creatinine in Sichuan children with stones declined over the course of the follow-up <u>Reported uncertainties/limitations:</u> • possible enrolment bias	Lau andTu (2013)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		protein-1 (MCP-1) *General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ) (2008)	• incomplete follow-up	
Observational study Descriptive longitudinal study over 2 years	Melamine (intentional adulteration of milk products)	Study aims to characterise the adverse urinary tract effects in Chinese children that had been exposed to melamine **Participants:** 240 children with diagnosed melamine-related urolithiasis (145 ♂ and 95 ♀; age 1 – 82 months) **Exposure:** oral Estimated length of exposure: 14.48 ± 11.28 months (mean value) **Examinations:** ultrasonography, blood tests, and urinalysis (analysed in 128 patients), analysis of stone morphology, microstructure and crystal structure (scanning electron microscopy, XRD; n = 5 derived from melamine-exposed children as compared to n = 2 form non-exposed adults)	**Calculi** were observed at single or multiple sides (146/240, 60.8 %): kidney 240/240 (100 %), ureter 16/240 (6.7 %), bladder 6/240 (2.5 %), urethra 3/240 (1.3 %) **Obstruction features** (hydronephrosis, hydroureter) were seen in 40/240 (16.7 %) **Urinalysis** showed haematuria in 26/128 (20.3 %), leukocyturia in 27/128 (21.1 %), and proteinuria in 8/128 (6.3 %) **Evidence of renal lesions** (glomerulus and tubule): • Markers of renal tubule injuries such as urinary microalbumin, α 1- and β 2-macroglobulin, n-acetyl-β-d-glucosaminidase, and retinol-binding protein were elevated • Markers for renal glomerulus such as serum creatinine, β 2-macroglobulin, and cystatin C were increased **Calculi passing rate**: 1 month: 59.6 % (130/218) 6 months: 85.4 % (193/226) 24 months: 91.2 % (206/226) 8.85 % had persistent urolithiasis after 24 months Urinary α 1- and β 2-macroglobulin level did not recover until 6 months after diagnosis At 24 months, obstruction features were seen in 3/226 (1.3 %), haematuria in 2/79 (2.5 %), and leukocyturia in 1/79 (1.3 %) **Stone composition** Uroliths were composed of loose	Zou et al. (2013)

<table>
<tr><th>Type of data/report</th><th>Test substance</th><th>Route of exposure
Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td></td><td>crystals that could be easily crushed

The XRD pattern of calculi from exposed children was significantly different from non-exposed adults

<u>Reported uncertainties/limitations:</u>
• no random recruitment
• incomplete clinical data in some cases
• no measurement of melamine level in patients
• only 5 stones were analysed</td><td></td></tr>
<tr><td>Systematic review and meta-analysis</td><td>Melamine (intentional adulteration of milk products)</td><td>Systematic review and meta-analysis of the available literature regarding clinical profile and recovery status of Chinese children that had been exposed to melamine

26 studies were identified according to the selection criteria (reported recovery rate) including 2164 children with kidney abnormalities

Quality assessment was performed including the following parameters: sampling methods, the description of melamine exposure and kidney abnormalities, the description of demographic characteristics, the description of therapeutic measures, and the rate of loss to follow-up

22 were assigned as high-quality and 4 as low-quality</td><td>Clinical Characteristics

• 2044/2164 (94.5 %) of the patients presented with urinary calculi
• 103/2164 (4.8 %) of the patients had hydronephrosis
• 17/2164 (0.7 %) of the patients showed urinary obstructions

• 95.5 % of the calculi had a diameter of ≤ 10mm (based on 13 studies)
• 76.2 % of the patients were asymptomatic (based on 16 studies)
• The pooled ratio of male to female was 1.49:1

Pooled recovery rates:

<table><tr><th>Follow-up at (months)</th><th>Pooled recovery rate (%)</th><th># of studies</th></tr><tr><td>1</td><td>67.1</td><td>15</td></tr><tr><td>3</td><td>76.3</td><td>10</td></tr><tr><td>6</td><td>85.4</td><td>7</td></tr><tr><td>12</td><td>92.3</td><td>5</td></tr></table>
• In 7.7 % of the children, kidney abnormalities persisted at 12 months after diagnosis and treatment initiation (the authors estimated that a total of > 20 000 affected children failed to recover)

• Recovery rates for 18 and 24 months were 82.4 % and 99.5 %, respectively, and</td><td>Wang et al. (2013)</td></tr>
</table>

<table>
<tr><th>Type of data/report</th><th>Test substance</th><th>Route of exposure
Relevant information about the study (as applicable)</th><th>Observations</th><th>Reference</th></tr>
<tr><td></td><td></td><td></td><td>based on a single study

<u>Reported uncertainties/limitations:</u>
no subgroup analyses by gender, diameter of calculus, consumption of milk powder, and disease severity possible due to the lack of detailed information
possible selection bias (based on the inclusion criteria)</td><td></td></tr>
<tr><td>Observational study</td><td>Melamine (ambient exposure from tableware manufacturing)</td><td>Examination of the relationship between urinary melamine levels and renal injuries in professional users (melamine tableware manufacturing factories) in Taiwan

Participants: 44 melamine exposed workers (from two melamine tableware manufacturing factories in Taiwan) compared to 105 non-exposed workers (control group, no history of melamine exposure or exposure to other chemicals known to cause renal injury)

Exposed workers (n = 44): manufacturers (n = 16), grinders (n = 8), packers (n = 10), administrators (n = 10)

Exposure: ambient exposure (tableware manufacturing workplaces are characterised by many coarse and fine melamine containing particles)

Length of exposure: exposed group had a work history of ≥ 1 year

Examinations: quantification of melamine levels in the air (area air samples and personal breathing-zone), urine, and serum (using LC-ESI-MS/MS), analysis of biomarkers for early renal injuries (urinary N-acetyl β-D-glucosaminidase (NAG), microalbumin, β2-microglobulin (β2-MG))</td><td>Melamine concentrations:

Manufacturers had the highest exposure (area and personal melamine) and the highest urinary melamine concentrations
Administrators had the lowest exposure and urinary melamine concentrations
Grinders and packers were in between
<table><tr><th>Group</th><th>Melamine concentration</th></tr><tr><td colspan="2">Urine</td></tr><tr><td>non-exposed workers (ctrl)</td><td>0.7 ± 0.9 µg/mmol Cr</td></tr><tr><td>exposed workers* (highest level)</td><td>84.4 ± 47.4 µg/mmol Cr</td></tr><tr><td colspan="2">Serum</td></tr><tr><td>non-exposed workers (ctrl)</td><td>below the MDL</td></tr><tr><td>exposed workers* (highest level)</td><td>7.2 ± 4.6 ng/ml</td></tr></table>*manufacturers

Melamine exposure (as determined by measuring particular-phase melamine within the personal breathing-zone and area air samples) was significantly associated with higher melamine levels in urine and serum (P < 0.001)
Urinary melamine concentrations were associated with the working schedule (increasing on Monday, remaining high throughout the workweek, decreasing over the weekend)</td><td>Wu et al. (2015)</td></tr>
</table>

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			• Urinary melamine was highly correlated to serum melamine levels (r = 0.808; P = 0.001) **Preclinical renal injury markers:** • A positive correlation between urinary melamine levels and NAG levels was observed (ctrl: 0.4±0.2, melamine expo: 1.8±3.5 IU/mmol Cr; r = 0.339, P = 0.002) • No correlation between urinary melamine levels and microalbumin was found • The rate of detectable β2-MG was significantly elevated in highly exposed workers (P = 0.007) • Clinical parameters related to renal function (serum blood urea nitrogen (BUN), creatinine, uric acid, estimated glomerular filtration rate and estimated creatinine clearance rate) were found not to be abnormal <u>Reported uncertainties/limitations:</u> • No adjustment for the impact of formaldehyde • No investigation on other occupational nephrotoxins (e.g. lead, cadmium) • Small sample size	
Observational study Cross-sectional study	Melamine (unknown source of exposure)	Examination of adverse renal effects associated with calcium urolithiasis that had been linked to low-level environmental melamine exposure and urolithiasis in Taiwanese adult patients **Participants:** 309 (226 ♂ and 83 ♀; mean age of 54.7 ± 12.8 years) patients who had been diagnosed with calcium urolithiasis compared to normal healthy controls (n = 105) from a previous study (Wu et al., 2015) Exclusion criteria: a history of chronic urinary tract infection, renal failure, chronic diarrhoea, gout, renal tubular acidosis, primary and secondary	Patients with calcium urinary tract calculi had statistically significantly elevated urinary levels of NAG (calcium urolithiasis patients: 1.35 ± 1.51 µg/mmol Cr vs. normal healthy control: 0.4 ± 0.2 µg/mmol Cr) and microalbumin (calcium urolithiasis patients: 16.61 ± 52.62 µg/mmol Cr vs. normal healthy control: 1.9 ± 6.8 µg/mmol Cr) Urinary melamine levels in calcium urolithiasis patients (3.24 ± 6.66 µg/mmol Cr) were significantly higher (P < 0.001) as compared to normal healthy controls from a previous study (0.7 ± 0.9 µg/mmol Cr)	Liu et al. (2017)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		hyperparathyroidism, or cancer **Exposure:** environmental exposure **Examinations**: interviews with a questionnaire (detailed demographic data, medical history, history of substance use, total number of stone episodes), urinalysis (to measure melamine levels and markers of early renal damages such as N-acetyl β-D-glucosaminidase (NAG), β2-microglobulin (β2-MG), and microalbumin) Melamine was measured using a isotopic liquid chromatography/ tandem mass spectrometry method (LC-MS/MS) Urinary microalbumin, NAG, and β2-MG were measured by enzyme-linked immunosorbent assay	**Urinary melamine concentrations were found to significantly correlate with NAG** (spearman correlation coefficient, r = 0.157, P = 0.006, n = 309) No association was observe between urinary melamine concentration and urinary microalbumin levels β2-MG was only detectable in 16 patients and hence could not be analysed NAG is a particular marker for early renal tubular injuries <u>Reported uncertainties/limitations:</u> • Only one measurement of urinary melamine levels (one-spot overnight urine samples)	
Observational study Follow-up	Melamine (intentional adulteration of milk products)	Retrospective characterisation of urolithiasis treatment and 5-year follow up in Chinese children that had been exposed to melamine **Participants:** 207 (♂125 and 82♀; mean age: 13.6 months) with melamine-related urolithiasis (follow-up in 198/207) **Exposure:** oral, melamine contaminated milk product **Examinations:** retrospective analysis of clinical data regarding urolithiasis treatment, stone composition and morphology (n = 12, using a combination of infrared spectrum, SEM, XRD, and HPLC as already published by Chang et al. (2012)), and follow-up (5 years including ultrasonography, renal function tests, and urinalysis)	**Retrospective analysis of urolithiasis treatment** • Comparison between patients that received conservative treatment (CTr) vs. surgical intervention (SIn) • There were significant differences between the two groups in terms of age of onset (higher in CTr), clinical presentations (lower incidence in CTr), size (smaller in CTr) and location of stones (higher incidence of multiple stones in SIn), renal function (less severe in CTr; e.g. incidence of hydronephrosis, serum BUN, CR, and UR), and mean time of hospitalisation (shorter in CTr) **Follow-up** • **Residual stones** were still present in 17/198 (**8.6 %**; 11/149 (7.4 %) in the CTr and 6/49 (12.2) in the SIn group) • The renal function was normal in the followed-up children	Chang et al. (2017)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
			(10/198 (5 %) proteinuria, 6/198 (3 %) microscopic hematuria) **Stone composition:** The main stone component was urate (UA dehydrate and ammonium UA) <u>Reported uncertainties/limitations:</u> • No control samples from healthy patients • 412/619 patients were unavailable for follow-up • Limited examinations at follow-up	
Observational study Cross-sectional study	Melamine (unknown source of exposure) Possible interaction with di-(2-ethylhexyl) phthalate (DEHP)	Examination of a possible association between urinary melamine levels and markers of early renal damages in Taiwanese children Examination of the relationship between melamine and di-(2-ethylhexyl) phthalate (DEHP) related to renal effects was additionally investigated **Participants:** 224 children (132 ♂ and 92 ♀; ≤ 12 years (mean age 5.5 years)) presumably exposed to DEHP due to consumption of phthalate-tainted foodstuffs (intentional addition of phthalates to a variety of foodstuffs 2011 in Taiwan) **Exposure:** environmental exposure **Examinations:** urinalysis (to measure current melamine and urinary oxidative DEHP metabolites levels and biomarkers of early renal injury such as microalbumin, N-acetyl β-D-glucosaminidase (NAG), and β2-microglobulin (β2-MG); all values were normalised to urinary creatinine levels), interviews with a questionnaire to estimate past DEHP exposure Melamine was measured using a isotopic liquid chromatography/ tandem mass spectrometry method (LC-MS/MS; one-spot overnight)	Melamine levels were significantly positive correlated with urinary NAC and urinary microalbumin levels (both Spearman correlation coefficient $r = 0.24$, $p < 0.001$) Median urinary melamine level were 1.5 – 1.6 µg/mmol creatinine A significant dose-response relationship was noted between urinary melamine, past DEHP exposure, and urinary microalbumin Study shows environmental background exposure to melamine in Taiwanese children <u>Reported uncertainties/limitations:</u> • No external comparison group without exposure (reference group) • Only one measurement of urinary melamine levels (one-spot overnight urine samples)	(Wu et al., 2018)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		as described (Liu et al., 2011; Wu et al., 2010) All measured values were above the method of detection limit Urinary microalbumin, NAG, and β2-MG were measured by enzyme-linked immunosorbent assay		
Observational study Prospective cohort study	Melamine (unknown source of exposure)	Examination of a possible association between baseline urinary melamine levels and adverse renal effect in Taiwanese patients with early-stage CKD to determine whether low-dose environmental melamine exposure plays a role in kidney injury/declining kidney function **Participants:** 293 children (159 ♂ and 134 ♀; mean age 57 ± 14 years) with an estimated glomerular filtration rate (eGFR) ≥ 30 ml/min/1.73m^2 in 2006–2010 were enrolled and urinary melamine concentrations were determined at enrolment (median urinary corrected melamine level was 0.97 (0.43–2.08) µg/mmol creatinine) Participants were followed until the end of observation in December 2016, or until the last contact happened, or the occurrence of targeted kidney outcomes*, cancer, or death **Exposure:** environmental exposure **Examinations:** urinary melamine was measured using a isotopic liquid chromatography/ tandem mass spectrometry method (LC-MS/MS; one-spot overnight) Follow-up (median follow-up period of 7 years): clinical status and kidney function (serum creatinine levels and eGFR) were monitored at 3-month intervals Adjustment for well known risk factors was done *Primary kidney outcome: doubling of serum creatinine levels	**At baseline:** Significant negative correlation between corrected urinary melamine levels and eGFR, serum albumin/ haemoglobin Significant positive correlation between corrected urinary melamine levels and urinary protein/creatinine ratio (UPCR) and serum uric acid **During follow-up:** Urinary melamine levels at the time of enrolment correlated with the deterioration of CKD progression during a 7 years follow-up period (median) in patients with early-stage CKD (significant positive correlation between baseline urinary corrected melamine levels and the doubling of serum creatinine levels and rapid deterioration of renal function (eGFR decline > 3 ml/min /1.73m^2 per year and 30% decline in eGFR in the first 2 years)) <u>Reported limitations:</u> • No information on the source of melamine exposure • Only one measurement of urinary melamine levels (at enrolment), i.e. not representative/conclusive for long-term/cumulative exposure • Low number of participants	(Tsai et al., 2019)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
		and end-stage kidney disease during follow-up Secondary kidney outcome: eGFR slope during entire follow-up or a 30% decline in eGFR in the first 2 years of follow-up		

10.11.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

A substantial body of evidence concerning melamine-related toxicity following repeated oral exposure exists. Relevant data are derived from both experimental animal studies and observational studies in humans.

Animal data

Significant adverse effects in experimental animals derived from sub-acute, sub-chronic, and chronic studies were mainly and consistently observed in the urinary tract system, comprising urolithiasis and signs of nephrotoxicity such as chronic inflammation and renal injuries. Consequently, the urinary tract system, specifically the kidney and the urinary bladder, was identified as the main target organ system (Bhat et al., 2010; Dalal and Goldfarb, 2011; Deng and Li, 2012; Early et al., 2013; Hau et al., 2009; NTP, 1983; WHO / FAO, 2009). Effective dose levels (lowest dose inducing significant/severe target organ toxicity) were derived and compared with the guidance values provided by *ECHAs Guidance on the Application of the CLP Criteria* (Annex I: 3.9.2.9.6., table 3.9.2 and Annex I: 3.9.2.9.7., table 3.9.3.).

In the current dossier, studies provided by **NTP (1983)** and **Early et al. (2013)** have been identified as key information source as they are similar to internationally accepted guideline studies and/or performed according to GLP. In addition, a substantial number of non-guideline studies from the literature exist which have been regarded as supplemental information in experimental animals that may provide insufficient/inadequate information on its own but nevertheless contribute to the overall weight of evidence and thus, were considered relevant for classification.

Animal data – rats

Multiple studies addressing melamine-related toxicity in a sub-acute repeated exposure setting in rats have been identified. Key information was provided by **Early et al. (2013)**. Accordingly, the effects of repeat oral (gavage) melamine administration (140, 700, 1400/1000 mg/kg bw/d) for 14 consecutive days in rats were investigated (Early et al., 2013). The study was conducted according to GLP and can be assigned as similar to OECD guideline TG 407 with some deviations mostly in terms of the test duration. The study identified the kidney as the main target organ related to melamine-mediated repeated dose toxicity. At the lowest melamine dose (140 mg/kg bw/d), slight crystal depositions in the papillary renal area were observed in 2/6 (33 %) of the female rats. No other treatment-related effect was observed in the low-dose group. At ≥ 700 mg/kg bw/d, severe renal pathologies including dilation of distal nephron tubule, degeneration and necrosis of tubular epithelium, and regeneration of the tubular epithelium were observed in addition to a reduced renal function (increased blood serum urea and creatinine) and crystal depositions. Renal injuries were associated with the presence of renal crystals in the distal tubular lumen which were seen in all animals of either sex. In addition to the kidney injuries, pathophysiological effects in the heart and immune system were described in animals of the high-dose group (1400/1000 mg/kg bw/d) which were accompanied by a high incidence of mortality (which prompted the authors to reduce the highest dose from 1400 to 1000 mg/kg bw/d). The authors were, however, unable to clarify whether the high mortality was due to kidney or heart toxicity. A NOAEL of 140 mg/kg bw/d was derived by the authors of the study. However, a 33 % increase in the renal crystal incidence in females may be considered adverse as crystals are regarded to be nephrotoxic and a clear threshold concentration as to when crystals become toxic has yet not been established. Beyond that, renal crystal formation is regarded as initial key event in the MoA culminating in severe epithelial damages and cancer. Thus, from a conservative perspective, 140 mg/kg bw/d was set as the first effective dose level (Table 21: Extrapolation of equivalent effective dose for toxicity studies of greater or lesser

duration than 90 days (only key and supporting studies that report effects at doses relevant for classification are listed)). Renal damages (necrosis/degeneration/hyperactive regeneration of distal nephron tubular epithelium of ♂/♀ rats, reduced renal function, crystal depositions) were observed in animals subjected to ≥ 700 mg/kg bw/d, which, when extrapolated to a 90-day study design, would give an effective dose level of 108.9 mg/kg bw/d. However, at this dose level, the incidence of renal injuries is 100 % and it is biologically plausible that such effects at lower incidences would occur at lower doses. Thus, due to widely spaced dose selection (2- to 4-fold recommended by OECD TG 407/408 vs. 5-fold in the current study), uncertainty exists as to whether exposure levels below the mid-dose are lacking effects. Benchmark modelling using the US EPA Benchmark Dose Response Software (version 2.7) and the implemented Cochran-Armitage trend test was, therefore, employed to better reflect the pattern of this dose-response relationship (Table 23 Annex II). The trend test was highly significant ($P < 0.0001$) and the benchmark modelling revealed a BMD_{10} of 292.036 mg/kg bw/d which, when extrapolated to a 90-day study design, would give an effective dose of 45.4 mg/kg bw/d. Hence, although the actual observed effective dose for renal damages is slightly above the guidance value of 100 mg/kg bw/d (108.9 mg/kg bw/d), renal damages are still considered relevant for classification as stated in Table 21. As stated in ECHAs Guidance on the Application of the CLP Criteria (Annex I: 3.9.2.9.8.), guidance values "are intended only for guidance purposes, i.e., to be used as part of the weight of evidence approach, and to assist with decisions about classification" and *"are not intended as strict demarcation values"*.

In addition, numerous non-guideline studies have been conducted to further investigate the adverse renal effects mediated by melamine in rats. They provide additional and supporting information on melamine-related adverse renal effects that is largely consistent with the results of the aforementioned key studies and consequently relevant for risk characterisation. Supporting information in the context of sub-acute repeated exposure test settings is provided by the following studies. A hyperuricemic model was established in the study conducted by **Zhang et al. (2015)**, providing evidence that higher uric acid levels in rats remarkably exacerbate the renal toxicity mediated by melamine (Zhang et al., 2015). Combined treatment with low-dose melamine (200 mg/kg bw/d; oral gavage) and potassium oxonate (nontoxic uricase inhibitor that does not co-precipitate with melamine in a saturated solution; intraperitoneal injection) for 3 consecutive days induced adverse renal abnormalities while no effects were noted when rats were exposed to melamine alone. Potassium oxonate treatment in the absence of melamine showed only mild renal effects only at the highest tested dose. As humans lack the enzyme uricase and consequently have higher uric acid levels (which is considered the main aetiological factor in melamine-related urolith formation), a hyperuricemic rat model may be more suitable to describe the conditions in humans. In addition, **Early et al. (2013)** conducted a second 5-day study in rats to evaluate genomic markers in kidney tissue (Early et al., 2013). Certain genes (Kim-1, Clu, Spp1, A2m, Lcn2, Tcfrsf12a, Gpnmb, CD44, Tff3) were found to be differentially regulated upon oral (gavage) melamine exposure and thus suggested as diagnostic markers for kidney injuries. Besides one animal that exhibited mild kidney pathologies, discoloration of the urine was the most prominent finding in the low-dose (350 mg/kg bw/d). The high-dose group (1050 mg/kg bw/d) was characterized by pathophysiological effects (renal tubular dilation with mild to marked degeneration and necrosis, and mild to marked cell debris with crystal deposition) in the kidney similar to what had been found in the first 14-day study by Early et al. (2013). In the study by **Jacob et al. (2011)**, rats were orally exposed to 123.7 mg/kg bw/d melamine for only 7 days with renal crystal formation (♂ 3/6 (50 %); ♀ 2/6 (33 %)) as the main adverse effect reported (Jacob et al., 2011). In a study by **Stine et al. (2014)**, extensive renal crystal formation (23/24 (96 %)) and even larger stones (4/24 (17 %)) were seen in the kidney of female rats that had been orally exposed (gavage) to 1000 mg/kg bw/d melamine for 10 consecutive days (Stine et al., 2014). The histopathology of the kidney revealed tubular necrosis (24/24 (100 %)) and tubular dilatation (22/24 (92 %); consisted with features of crystal nephropathy) in almost all animals which correlated well with the presence of renal crystals. Hence, the authors concluded that crystal formation contributes to the reduced renal function. The kidney weight was significantly increased and markers of renal function such as plasma urea nitrogen and creatinine were strongly elevated. Another important observation was that the use of formalin during tissue fixation/preservation (a commonly used standard procedure) effectively dissolves melamine crystals giving rise to false negative results in histopathological examinations. This critical aspect was first reported by a study in 2008, later acknowledged by WHO in its 2010 assessment report, and may account for the absence of renal crystals in numerous studies such as NTP (1983), Ogasawara et al. (1995), Cremonezzi et al. (2004), and Sun et al. (2016) (Reimschuessel et al., 2008; Stine et al., 2014; WHO / FAO,

2009). Hence, the ability of melamine to induce renal crystal formation as evident by histopathological examination of the kidney may, in general, be underestimated. In the oral 14-day study in rats provided by **NTP (1983)**, 'hard crystalline solids' were observed in the urinary bladder of male (≥ ca. 834 mg/kg bw/d) and female rats (≥ ca. 1668 mg/kg bw/d) at high doses. At the highest dose (ca. 2500 mg/kg bw/d), 40 % of the male rats had "pale and pitted" kidneys (NTP, 1983). **Xie et al. (2010)** found crystals near the papilla of the renal tubule in all treatment groups (≥ 100 mg/kg bw/d) following oral (gavage) melamine administration for 15 days (Xie et al., 2010). Tubular dilatation in distal tubules (600 mg/kg bw/d), hemorrhage (mild at 300 and severe at 600 mg/kg bw/d), and inflammation became obvious mostly in the high-dose group (600 mg/kg bw/d). The sub-acute 28-day study by **Research Triangle Institute (1982)** was designed and conducted to investigate the formation of urinary stones following oral exposure to melamine in a wide range of doses in male rats (seven dose levels starting from ca. 240 mg/kg bw/d) (Research Triangle Institute, 1982). The main finding was dose-dependent urinary bladder calculus formation (≥ ca. 475 mg/kg bw/d), dose-dependent hyperplasia of the urinary bladder epithelium (≥ ca. 1184 mg/kg bw/d; correlated with the occurrence of stones), and dose-dependent crystalluria (≥ ca. 240 mg/kg bw/d). In the kidney, clinical signs such as white flecks or streaks were noted in a dose-dependent fashion (≥ ca. 1184 mg/kg bw/d). Renal histopathology was only done at the highest dose level and revealed focal nephropathy in all animals. It is, therefore, not possible to correlate the incidence of crystalluria with histopathological changes within the kidney. The study is not suitable to assess melamine-related effects in the kidney. **Sun et al. (2016)**, found no renal pathologies following oral (gavage) repeated melamine treatment (180 mg/kg bw/d) for 28 days (Sun et al., 2016). The authors noted, however, that lack of visible renal crystals may have been due to formaldehyde fixation. A significant reduction of chief cells in the stomach was observed. No other adverse effect was detected. In another study conducted by **El Rabey et al. (2014)**, rats were exposed to very high oral doses (ca. 2430 mg/kg bw/d) of melamine and developed severe pathologies within the urinary system. In addition, toxic effects were shown in other tissues such as liver, testis, spleen, and heart (El Rabey et al., 2014). Thus, at very high levels of exposure, the toxicity of melamine goes beyond the urinary system.

Beyond that, a number of studies addressing melamine-related toxicity in a sub-chronic repeated exposure setting have been identified. Key information was provided by a comprehensive and reliable dataset from the United States NTP collected according to accepted scientific principles **(NTP, 1983)**. The data set concerning sub-chronic repeated dose toxicity consists of three 13-week (91-day) studies with repeated oral melamine administration in rats. The studies were aimed at identifying the cumulative toxic effects of melamine, the primary target organ, and the appropriate concentration for a subsequent carcinogenicity study (described below). In the first 13-week study, urinary bladder stones were observed at all doses in male rats (≥ ca. 560 mg/kg bw/d) and at the two highest doses in female rats (≥ ca. 1400 mg/kg bw/d). Hyperplasia of the urinary bladder epithelium was mostly observed in the high-dose (ca. 1700 mg/kg bw/d) male group. The effective dose level can be set at 560 and 1400 mg/kg bw/d for male and female rats, respectively. In the second 13-week study, urinary bladder stones were seen at all doses exclusively in males (≥ ca. 72 mg/kg bw/d; dose-dependent incidence). Hyperplasia of the transitional epithelium of the bladder was observed in males at ≥ ca. 300 mg/kg bw/d with a dose-related incidence that was only seen in males with concurrent urolithiasis. Neither calculi nor hyperplasia was found in the urinary bladder of females. However, females were characterised by calcareous deposits in the straight segments of the proximal renal tubules that occurred in a dose-dependent manner (0*: 2/10 (20 %), 84*: 3/10 (30 %), 150*: 4/10 (40 %), 300*: 10/10 (100 %), 600*: 8/10 (80 %), 1300*: 10/10 (100 %); *mg/kg bw/d). The effective dose level may be set at 72 and 84 mg/kg bw/d for male (urolithiasis) and female (calcareous renal deposits) rats, respectively. As analysed using a Cochran-Armitage trend test that is implemented in the US EPA Benchmark Dose Response Software (version 2.7), urinary bladder calculi in males and renal calcareous deposits in females occurred with a statistically significant positive trend ($P < 0.0001$). Benchmark modelling revealed BMD_{10} values of 41.7 mg/kg bw/d and 28.8 mg/kg bw/d for males and females, respectively (Table 23 Annex II). The outcome of the third 13-week study was that the addition of ammonium chloride (which inhibited stone formation in mice fed with 4-ethylsulfonylnaphthalene-1-sulfonamid) does not influence the occurrence of calculi in the urinary bladder of male and female rats exposed to high-dose melamine. A more recent re-assessment of the kidney-related histopathology performed in the sub-chronic 1983 NTP studies by **Hard et al. (2009)** revealed melamine-mediated pathophysiologic effects in the kidney. Cortical and medullary tubular changes indicative of retrograde nephropathy were found at a low incidence in the low-dose group in males (♂ 1/9 (11 %) at 560 mg/kg bw/d), and at high incidences in the high-dose group in male and female

rats of the first- (♂ 10/10 at 1700 and ♀ 8/10 at 1600 mg/kg bw/d) and second 13-week study (♂ 6/9 (67 %) and ♀ 2/10 (20 %) at 1300 mg/kg bw/d) (Hard et al., 2009).

Supporting information in the context of sub-chronic repeated exposure test settings is provided by the following studies. Accordingly, a recent study by **Tian et al. (2016)**, observed impairment of the endothelial function of the renal arteries, reduced renal blood flow, fibrotic changes in the kidney, and increased expression of inflammatory markers in male orally rats exposed to three different melamine doses for three months. In addition, female rats were treated with melamine for two weeks and F1 male pubs were studied after additional three months of treatment (Tian et al., 2016). A sub-chronic study related to melamine-mediated carcinogenic effects (also described in the carcinogenicity section of the current dossier) by **Cremonezzi et al. (2004)**, reported renal effects (squamous metaplasia in the renal papillae, hyperplasia and dysplasia mainly in the renal pelvis) largely in the proximal end of the urinary tract (papillae and renal pelvis) in rats that had been orally exposed to 750 mg melamine/kg bw/d for 22 to 40 weeks (Cremonezzi et al., 2004). Two additional sub-chronic studies by **Okumura et al. (1992)** and **Ogasawara et al. (1995)** were conducted to investigate the carcinogenic potential of melamine specifically in regard to urolith formation in the urinary tract system following sub-chronic repeated oral exposure (Ogasawara et al., 1995; Okumura et al., 1992). Both studies are non-guideline studies and described in detail in the carcinogenicity section of the current dossier. The main findings comprise urinary tract stones in the bladder associated with an increased incidence of tumour formation in the urinary bladder and nephrotoxicity. According to **Okumura et al. (1992),** an increased incidence of hyperplasia of the urothelium was found in the renal pelvis (≥ 330 mg/kg bw/d), ureters (1090 mg/kg bw/d), and urinary bladder (≥ 100 mg/kg bw/d) in a dose-dependent manner. Urolithiasis was observed in all melamine treatment groups with a dose-dependent incidence. In the study by **Ogasawara et al. (1995)**, adverse renal effects were observed at doses ≥ 350 mg/kg bw/d and manifested in hyperplasia of the transitional cell epithelium in the renal papillae and ischemic lesions including fibrosis, inflammation, and renal tubules regeneration in the renal cortex. Urolithiasis and urinary tumours were seen in animals treated with ≥ 350 mg/kg bw/d.

Supporting information in the context of chronic repeated exposure test settings is provided by the following studies. A carcinogenicity study was conducted by **NTP (1983)** with low-dose (♂ 126 and ♀ 262 mg/kg bw/d) and high-dose (♂ 263 and ♀ 542 mg/kg bw/d) oral melamine administration (Melnick et al., 1984; NTP, 1983). The results are described in detail in the carcinogenicity section of the current dossier. Urinary bladder calculi were observed in males only (ctrl: 0/45; low-dose: 1/50 (2 %); high-dose: 10/49 (20 %)) and associated with the occurrence and transitional cell carcinomas. Chronic inflammation of the kidney, distinguishable from the nephropathy observed in aging F344/ N rats, was detected dose-dependently in female with a significantly increased incidence (ctrl: 4/50 (8 %), low-dose: 17/50 (34 %)$^{\#}$, high-dose: 41/50 (82 %)$^{\#}$; $^{\#}P \leq 0.01$) and to a lesser, statistically insignificant, extent in males (ctrl: 2/49 (4 %), low-dose: 3/50 (6 %), high-dose: 6/49 (12 %)). A re-evaluation of the renal histopathology by **Hard et al. (2009)** revealed a dose-dependent incidence of reflux nephropathy (fibrotic lesions (scars) associated with collecting duct dilatation and hyperplasia in the inner medulla, loss of tubule, tubule atrophy, and crowded glomeruli in the cortex;) in both sexes, whereas female rats were more affected (Hard et al., 2009). Another chronic study (carcinogenicity) by **Hazleton (1983)**, conducted similar to OECD TG 451 and GLP, in male and female rats subjected to low-dose melamine (4 - 40/5 - 80 mg/kg bw/d ♂/♀) failed to show clear/significant adverse effects to the urinary system. Preneoplastic transitional epithelial hyperplasias were observed with an increased incidence in high-dose males. A significant treatment-related trend, however, could not be established due to insufficient absolute incidence numbers. Increased incidences of tubular pigments in the kidney of female rats (high-dose) were found with a statistically significant positive trend. The biological relevance of this observation is, however, obscure as similar morphological pigments were also found in healthy control animals. Calculus formation was only sporadic (Hazleton, 1983).

Animal data – mice

Key information from studies in mice is provided by an oral sub-chronic 13-week (91-day) **NTP** study that had been conducted according to accepted scientific principles in 1983 (NTP, 1983). The main adverse effect in the study was dose-dependent calculus formation in the bladder which, as in rats, was more severe in male mice. Ulceration of the urinary bladder epithelium was also noted in a dose-dependent fashion (♂ ≥ 2800, ♀ 1/10 at 2700 and ≥ 4800 mg/kg bw/d). Hyperplasia was noted in 2/10 males of the highest dose group (4700 mg/kg bw/d). An effective dose level based on urinary bladder stones can be set at 2800 and 3500 mg/kg

bw/d for male and female mice, respectively. Renal lesions were not reported in the NTP study but mentioned in the publication by Hard et al. (2009). Accordingly, retrograde nephropathy was also seen in the kidney of mice with a low incidence and severity (Hard et al., 2009).

Supporting information is provided by the following studies. A 14-day sub-acute study by **NTP (1983)** reported urinary bladder stones in male and female mice of the highest dose group (♂ 5/5 at 3330 and ♀ 2/5 at 4740 mg/kg bw/d) (NTP, 1983). According to the authors, there was no other melamine-related effect at necropsy. Two additional sub-acute studies by **Xu et al. (2011)** and **Sun et al. (2014)** in mice observed urinary bladder calculi associated with hyperplasia of the transitional cell epithelium, whereas the latter study also analysed the retention time of calculi and the regression of the hyperplasia following withdrawal of melamine from the feed (Sun et al., 2014; Xu et al., 2011). In addition, a sub-chronic study related to melamine-mediated carcinogenic effects (also described in the carcinogenicity section of the current dossier) by **Cremonezzi et al. (2001)**, investigated the effects of 1800 mg melamine/kg bw/d orally administered for 22 weeks (Cremonezzi et al., 2001). The authors found proliferative lesions (hyperplasia, dysplasia/in situ carcinoma) with increasing incidence in the renal pelvis, ureter, and urinary bladder. The observed lesions were associated with the occurrence of stones in the bladder. A chronic study (carcinogenicity) by **NTP (1983)**, conducted analogously to the aforementioned chronic study in rats, reported a high incidence of urolithiasis in the low- and high-dose group in males (40/47 at 327 and 41/44 at 688 mg/kg bw/d) and in the high-dose group in females (4/50 at 1065 mg/kg bw/d) (NTP, 1983). The occurrence of calculi was associated with acute/chronic inflammation and mild epithelial hyperplasia in the urinary bladder mostly in low- and high-dose males and to a lesser extent in females.

Animal data – other experimental animals

Key information from other experimental animals is provided by **Early et al. (2013)**. In a sub-chronic study that had been conducted to GLP and can be assigned as similar to OECD guideline TG 409, Cynomolgus monkeys were orally (nasal-gastric gavage) treated with three melamine doses (60, 200, and 700 mg/kg bw/d) for 91 days followed by 28 days recovery. No histopathological finds were seen in the low-dose group. Nephrotoxicity was observed in animals subjected to melamine at ≥ 200 mg/kg bw/d (2/3 ♀) which may represent the effective dose level. While the effects in the kidney were more pronounced in the high-dose group, some effects were also noted in other organs such as the heart, bone marrow, spleen, thymus, liver, and adrenal glands. The authors identified the kidney as the primary target related to melamine-mediated health hazards (Early et al., 2013).

Summarizing the findings in experimental animals, it is apparent that melamine exerts toxicity to the urinary system, mainly the kidney and urinary bladder, in a variety of species including rats, mice, and monkeys. As explicitly addressed in the MoA section of the carcinogenicity part of the current dossier, crystal formation related to melamine exposure can be considered as the initial adverse effect that appears to be causally involved in the development of nephrotoxicity and subsequent stone formation in the urinary tract. Hereby, the toxic effects of melamine seem to be tightly dose-dependent whereas crystal formation starts at a low concentration level followed by severe nephrotoxicity at higher doses. It is worth noting that due to improper experimental procedures, the occurrence of melamine-mediated crystals may be underestimated.

Table 21: Extrapolation of equivalent effective dose for toxicity studies of greater or lesser duration than 90 days (only key and supporting studies that report effects at doses relevant for classification are listed)

Study reference	Effective dose (mg/kg bw/d)	Length of exposure	Effective dose when extrapolated to 90-day exposure	Classification supported by the study
Effective dose levels derived from key studies that support classifications				
NTP (1983) 2nd 90-day study (rats)	**Effective dose: 72** (calculi in the urinary bladder in ♂ rats) (BMD_{10}: 41.7)[#] **Effective dose: 84** (dose-related incidence of calcareous deposits in the	90 days		category 2 (urinary bladder) category 2 (kidney)

Study reference	Effective dose (mg/kg bw/d)	Length of exposure	Effective dose when extrapolated to 90-day exposure	Classification supported by the study
	straight segments of the proximal tubules in ♀ rats) (BMD_{10}: 28.8) #			
Early et al. (2013) (rats)	**Effective dose: 140** (renal crystals in ♀ rats) (BMD_{10}: 21.08) # **Effective dose: 700** (renal injuries in ♂/♀ rats) **BMD_{10}: 292.04**# (renal damages in ♂/♀ rats) BMD modelling was used as uncertainty regarding the dose-response relationship is high (widely spaced dose selection and a 100 % incidence at the effective dose)	14 days	**21.8** mg/kg bw/d (renal crystals in ♀ rats) **108.9** mg/kg bw/d (renal injuries in ♂/♀ rats) **45.4** mg/kg bw/d# (based on BMD_{10} in support of the inclusion of the actual ED in category 2)	category 2 (kidney)
Effective dose levels derived from supporting studies that support classifications derived from key studies				
Zhang et al. (2015) (rats)	**Effective dose: 200** (renal injuries in ♂ rats) in combination with oxo	3 days	**20** mg/kg bw/d[1]	in support of category 2 (kidney)
Early et al. (2013) (rats)	**Effective dose: 350** (mild kidney pathologies in ♂ rats) **Effective dose: 1050** (severe kidney pathologies in ♂ rats)	5 days	**35** mg/kg bw/d[1] (mild kidney pathologies in ♂ rats) **105** mg/kg bw/d[1] (severe kidney pathologies in ♂ rats)	in support of category 2 (kidney)
Jacob et al. (2011) (rats)	**Effective dose: 123.7** (crystals in the renal tubules in ♂/♀ rats)	7 days	**12.37** mg/kg bw/d[1]	in support of category 2 (kidney)
Stine et al. (2014) (rats)	**Effective dose: 1000** (renal crystals, tubular necrosis, tubular dilation in ♀ rats)	10 days	**111** mg/kg bw/d	in support of category 2 (kidney)
Xie et al. (2010) (rats)	**Effective dose: 100** (renal crystals in ♂ rats) **300** (mild haemorrhage)	15 days	**16.7** mg/kg bw/d (renal crystals in ♂ rats) **50** mg/kg bw/d (mild haemorrhage)	in support of category 2 (kidney)
Research Triangle Institute (1982) (rats)	**Effective dose: 240** (dose-related incidence of crystalluria in ♂ rats) (BMD_{10}: 77.1)#	28 days	80 mg/kg bw/d	in support of category 2 (kidney)
Tian et al. (2016) (rats)	**Effective dose: 60** (inflammatory	84 days (3 months)		in support of category 2

Study reference	Effective dose (mg/kg bw/d)	Length of exposure	Effective dose when extrapolated to 90-day exposure	Classification supported by the study
	changes in the kidney of in ♂/♀ rats)			(kidney)
NTP (1983) 1st and 2nd 90-day study combined (rats) according to WHO*	**Effective dose: 44.6*** (calculi in the urinary bladder in ♂ rats)	90 days		in support of category 2 (kidney)

[#] for details see Table 23 Annex II

*according to WHO assessment report (WHO / FAO, 2009)

[1]according to ECHAs Guidance on the Application of the CLP Criteria (section 3.9.2.2. and table 3.16), for studies with exposure durations shorter than 9 days, guidance values should be no greater than 10 times the default guidance value

Human data

Information regarding specific target organ toxicity following repeated exposure in humans is mainly provided by extensive literature concerning adverse health effects in children following consumption of melamine-tainted infant formula which was seen in the wake of the deliberate adulteration scandal in China. For the purpose of classification, the epidemiological evidence provided by these observational studies was considered in a weight of evidence approach. Additional, albeit currently inconclusive, information is provided by studies describing a link between low-dose environmental and occupational melamine exposure and adverse renal effects.

Human data – adulteration scandal

According to official numbers from the Chinese Ministry of Health, almost 300 000 children were affected, more than 50 000 with urinary problems underwent hospitalisation, and six confirmed deaths were related to the ingestion of melamine-contaminated infant formula (WHO / FAO, 2009). Based on investigations by the General Administration of Quality Supervision, Inspection and Quarantine of China (AQSIQ), 69 batches from 22 infant formula manufacturers were found to be contaminated with melamine levels ranging from 0.09 mg/kg to 2563 mg/kg (WHO / FAO, 2009). A second investigation by the Institute of Nutrition and Food Safety, Chinese Center for Disease Control and Prevention revealed melamine levels in formula produced by Sanlu, a major manufacturer, ranging from < 0.05 to 4700 mg/kg (mean: 1212 mg/kg) (WHO / FAO, 2009). Similar levels were found in other surveys (Shi et al., 2012; Wu et al., 2009a). Melamine concentrations in tainted infant formula were generally high in mainland China, whereas products from other regions such as the Hong Kong area were less affected (Wen et al., 2016). The estimated daily intake depending on the age of the children was 10.4 to 28.4 mg/kg bw/d considering the reported mean melamine concentration (1212 mg/kg) and 40.3 to 110.2 mg/kg bw/d considering the maximum melamine concentration (4700 mg/kg) (WHO / FAO, 2009). A TDI of 0.2 mg/kg bw/d was derived by WHO (WHO / FAO, 2009). However, it was noted that the risk of melamine-induced urolithiasis in children increases even at low-dose exposure below the WHO TDI (Chen et al., 2009; Li et al., 2010). A considerably lower TDI of 0.0081 mg/kg bw/day was suggested by Hsieh *et al.* (2009) (Hsieh et al., 2009). According to an EFSA assessment, however, human data are not sufficiently robust for the purpose of deriving a TDI which is why the TDI had not been changed (EFSA, 2010). According to a WHO assessment report, affected children were exposed to melamine with a considerably high purity and only insignificant traces of other triazine compounds (Bhalla et al., 2009; WHO / FAO, 2009). Uncertainty regarding the exposure of individual subjects is given as the level of dietary melamine intake was based on estimations (e.g. the amount of infant formula that had been consumed per day and total duration of consumption). However, a clear and consistent relationship between repeated melamine exposure and significant adverse health effects in humans can be established.

The main adverse effect consistently described in numerous observational studies was the occurrence of melamine-caused stones in the urinary tract system in affected children (Dalal and Goldfarb, 2011; Wen et

al., 2016; WHO / FAO, 2009). The predominant location of calculi was the kidney (mostly renal pelvis and calyx) whereas only a few stones were found in the ureter or the bladder (Bhat et al., 2010; Ding, 2009; Guan et al., 2009; He et al., 2009; Lam et al., 2009; Shi et al., 2012; Sun et al., 2010a; Wang et al., 2009; Wang et al., 2011; Zhang et al., 2009; Zhu et al., 2009). There was a strong correlation between melamine levels found in urine and the size of the kidney stone in the corresponding patient (Lam et al., 2009). The prevalence of urolithiasis was closely related to the level of exposure (Table 22). Given the uncertainties related to the exposure assessment (i.e. usually retrospective estimates based on parenteral reporting and melamine concentrations derived from official numbers or concentration measurements within the respective study), the exact prevalence according to a specific quantity of melamine intake cannot be derived. However, the available data conclusively show that the prevalence correlates with the level of melamine exposure. Additional risk factors such as duration of consumption of contaminated products, prematurity (higher risk for infants), and male gender were consistently identified (Gao et al., 2011; Li et al., 2010; Liu et al., 2010b; Lu et al., 2011; Wang et al., 2009; Wang et al., 2011). Calculi attributed to melamine consumption were different and distinguishable from common calcium-oxalate calculi. At hospital admission following the announcement of the outbreak, paediatric patients presented with melamine-related calculi that were described as radiographical and ultrasonographically distinguishable from common calcium stones (when compared to calcium stones, melamine-related calculi are: (1) radiolucent on conventional radiographs; (2) lesions less echogenic, more "sandy" appearance, structurally less dense and associated with a feeble or absent acoustic shadow when examined by ultrasonography) (Dalal and Goldfarb, 2011; He et al., 2009; Yang et al., 2010b). Melamine and uric acid were commonly identified as major stone components and considered the main aetiological factors involved in the formation of melamine-mediated nephroliths (Chang et al., 2012; Grases et al., 2009; Sun et al., 2010b; Sun et al., 2009; Sun et al., 2010c; Wang et al., 2011). Based on the structural properties of melamine that allows for hydrogen bonding with uric acid, the formation of a crystalline lattice structure from melamine and uric acid was suggested (Dalal and Goldfarb, 2011; Grases et al., 2009; WHO / FAO, 2009). Other triazines were not found relevant for stone formation (Grases et al., 2009; Lam et al., 2009; Sun et al., 2010b; Sun et al., 2010c). Importantly, the WHO noted in its 2009 assessment that humans, especially infants, may be more sensitive to renal calculus formation attributed to melamine and uric-acid interaction when compared to rats. Accordingly, as humans lack the enzyme urate oxidase (uricase) that, in most other mammals, converts uric acid to allantoin, uric acid level are much higher in humans as compared to other mammals such as rats (e.g. 5-fold when compared human infants to rats) (Alvarez-Lario and Macarron-Vicente, 2010; WHO / FAO, 2009). Higher uric acid levels may advance the formation of melamine-related kidney stones and melamine levels sufficient to allow for stone formation may be lower in human subjects (higher potency in humans possible) (WHO / FAO, 2009). The study by Zhang et al. (2015) demonstrated that the serum uric acid levels of rats can be elevated by treating them with potassium oxonate (oxo) which is a nontoxic uricase inhibitor that induces hyperuricemia. Most notably, in a subsequent experiment, the authors reported that a combined administration of oxo and melamine greatly exacerbated the renal toxicity, leading to high mortality and severe renal damages (Zhang et al., 2015). It was suggested that the results derived from this hyperuricemia model closely resemble clinical findings in paediatric patients.

Nephrotoxic effects in exposed children including renal injuries/lesions and renal inflammation were seen secondary to melamine-mediated renal precipitation (Gao et al., 2011; Guan et al., 2009; Lam et al., 2009; Lau and Tu, 2013; Yang et al., 2010b; Zou et al., 2013). Lymphocytic infiltration in the glomeruli, sclerotic glomeruli, proliferation of fibrous tissue in the glomeruli and Bowman’s capsule, swollen tubular cells, lymphocytic infiltration and fibrosis within the renal interstitium, and crystals within the lumen were observed in a kidney biopsy from a paediatric patient (Sun et al., 2010b). Markers for nephron impairment, tubular damage, and glomerular dysfunction were elevated in children with melamine-related stones which were still significantly different as compared to healthy children one year after the diagnosis (Shen et al., 2011b). Macroscopic and microscopic haematuria was described (Gao et al., 2011; Guan et al., 2009; Shang et al., 2012; Shen et al., 2011b; Sun et al., 2010a; Yang et al., 2010b; Zou et al., 2013) and may be a result of stone-related urothelial abrasion/irritation (Schulsinger, 2014; Yang et al., 2010b). Melamine-related renal pathologies progressed to acute obstructive renal failure and death in some cases (Hau et al., 2009; Sun et al., 2010a; Sun et al., 2010c). Several follow-up studies revealed that urolithiasis and renal abnormalities persisted in approximately 8 – 10 % of the cases (Chang et al., 2017; Gao et al., 2011; Shen et al., 2011b; Wang et al., 2013; Zou et al., 2013). Additional follow-up studies, not included in Table 20 and not included

in the meta-analysis by Wang et al. (2013), are summarised in Table 24 of Annex II and support consistently that although the majority of paediatric patients passed their stones and recovered, melamine-related nephrolithiasis persisted in a certain percentage of subjects. In some cases, it was even reported that the stone size had been increased during a 12 months follow-up in 8 % of the study participants (Dai et al., 2012). Hence, there is a particular concern regarding long-term effects originating from melamine-related renal injuries at an early age. Paediatric patients with acute renal failure (also described as acute kidney injury (AKI) (Shang et al., 2012)), for instance, may have an elevated risk to develop cardiovascular events and an increased mortality risk (Coca et al., 2009). A current meta-analysis revealed that a history of kidney stones is associated with an increased risk of chronic kidney disease (CKD) (Shang et al., 2017). Beyond that, nephrolithiasis is associated with an increased risk of urinary tract carcinogenesis (see carcinogenicity section).

Taken together, it can be concluded that the urinary tract system is the main target system in humans which is consistent with what has been observed in experimental animals.

Table 22: Prevalence of urolithiasis in children exposed to melamine-tainted formula

Reference	Estimated exposure	No. of children	No. of urolithiasis cases	Prevalence (%)
Lam et al. (2008)	very low (0.01 – 0.21 mg/kg bw/d)	3170	1 (7 suspected)	0.03 % (0.3 %)
Guan et al. (2009)	Presumably no exposure	168	8	4.8 %
	Moderate (< 150 ppm)	300	19	6.3 %
	High (> 500 ppm)	121	23	19.0 %
He et al. (2009)	n.a.	15 577	562	3.6 %
Wang et al. (2009)	Ctrl group (< 0.05 ppm)	504	2	0.4 %
	Low (0.05 – 2.5 ppm)	672	3	0.5 %
	High (> 2.5 ppm)	46	9	19.6 %
Zhu et al. (2009)	n.a.	1091	12	1.1 %
Sun et al. (2010a)	n.a. for all children screened	2235	79	3.53 %
Li et al. (2010) ***using birth weight**	0 mg/kg bw*/d	3062	115	3.8 %
	0 – 0.2 mg/kg bw*/d	575	38	6.6 %
	0.2 – 0.4 mg/kg bw*/d	475	41	8.6 %
	0.4 – 0.8 mg/kg bw*/d	456	37	8.1 %
	0.8 – 1.6 mg/kg bw*/d	567	67	11.8 %
	1.6 – 3.2 mg/kg bw*/d	539	70	13 %
	3.2 – 6.4 mg/kg bw*/d	288	51	17.7 %
	6.4 – 12.8 mg/kg bw*/d	200	36	18 %
	12.8 – 25.6 mg/kg bw*/d	202	22	10.9 %
	25.6 – 51.2 mg/kg bw*/d	346	72	20.8 %
	51.2 – 102.4 mg/kg bw*/d	332	84	25.3 %
	> 102.4 mg/kg bw*/d	139	50	36 %
Yang et al. (2010a)	Presumably no melamine	11 973	38	0.3 %
	Exposed to melamine	2283	37	1.6 %
Gao et al. (2011)	Low (0.09 ppm to 150 ppm)	5443	26	0.5 %
	Mid (combined high and low)	617	15	2.4 %
	High (162 ppm to 2563 ppm)	2284	64	2.8 %
Lu et al. (2011)	History of feeding highly contaminated Sanlu mild formula	208	83	39.9 %
Wang et al. (2011)	Low-dose formula (estimated < 200 ppm)	3133	29	0.9 %
	High-dose formula (estimated > 5500 ppm)	62	55	88.7 %
Shi et al. (2012)	Exclusively breast-feeding	64	0	0 %
	Any infant formula	2063	357	17.3 %
	Exclusively high-dose formula (ca. 1800 ppm)	350	86	24.6 %

Human data – Environmental chronic low-dose exposure and occupational ambient exposure

In addition to the numerous reports related to infant urolithiasis attributed to melamine exposure presumably at relatively high doses (estimated dietary exposure ca. 40 – 120 times the TDI established by the WHO (WHO / FAO, 2009)), uncertainty as to whether exposure to low-dose melamine may promote the formation of uroliths exists. Four studies, conducted by the same group, have described a possible role of low-level melamine exposure in the development of common urinary stones such as calcium urolithiasis (the most common type) and early markers of impaired renal function in Taiwanese adults. In the first preliminary study, Wu et al. (2010) showed that patients with uric acid urolithiasis and calcium urolithiasis have significantly higher melamine concentrations in their urine (Wu et al., 2010). Based on these initial results and to further investigate the effects of low-dose melamine exposure, a large-scale case-control study was conducted, enrolling a higher number of calcium urolithiasis cases (n = 211) and matched controls (n = 211). Consistent with the first study, a strong association between urinary melamine concentrations, supposedly caused by low-dose environmental exposure, and the risk of calcium urolithiasis was reported (urinary melamine level ≤ 3.11 ng/ml: adjusted odds ratio: 3.01; urinary melamine level ≥ 3.12 ng/ml: 7.64; trend test: $P < 0.0001$). In addition, melamine was found as a component in all analysed stones from subjects with detectable urinary melamine concentrations (Liu et al., 2011). In a third study, the authors investigated whether a link between low-level melamine exposure and early renal damages can be established. A significant association between urinary melamine concentrations and the levels of some early renal tubular injury markers (NAG) but not others (microalbumin) was found (Liu et al., 2017). In the fourth study, a positive significant correlation between urinary melamine levels and the expression of markers of early renal damage (e.g. urinary NAG and urinary microalbumin/creatinine ratio) was again reproduced (Wu et al., 2018). While melamine exposure in Chinese children was clearly attributed to intentionally tainted milk formula, the source of exposure in the three low-dose studies was obscure and discussed in the context of ubiquitous occurrence as a result of the widespread use. Accordingly, multiple sources of exposure beyond intentional adulteration have been identified (WHO / FAO, 2009). Contamination of food following migration from melamine resin tableware products, for instance, has been frequently demonstrated (WHO / FAO, 2009). Significantly elevated melamine concentrations in the urine were, hence, detected following consumption of hot soup form melamine resin plastic bowls (Wu et al., 2013). Low-level melamine was also seen in the blank urine of control patients in another independent study (Zhang et al., 2010a). Low-level environmental exposure has also been shown in the US population (Panuwet et al., 2012). Low concentrations of melamine have been detected in indoor dust, human breast milk, and meat/dairy (Manav et al., 2019; Zhu and Kannan, 2018; Zhu et al., 2019). Chronic low-dose melamine exposure has been considered a significant risk factor for urolithiasis in humans by others (Dalal and Goldfarb, 2011). The results of the four studies by Liu et al. (2017); Liu et al. (2011) and Wu et al. (2018); Wu et al. (2010) show that the Taiwanese population is exposed to low concentrations of melamine. They suggest that low-dose melamine exposure might as well contribute to urolithiasis and kidney injuries in humans. However, certain limitations of the studies have to be taken into account to assess a potential risk derived from low-level melamine exposure. For instance, all four low-dose studies were conducted as a cross-sectional design which generally does not imply causation. In particular, with regard to the study by Wu et al. (2010) and Liu et al. (2011), urinary melamine levels have been measured at the time where a stone episode was already diagnosed. Information on the actual urinary melamine concentration at the time the stone formed is unavailable. Hence, the authors' conclusion that low-dose urinary melamine levels measured in the study, i.e. low-dose exposure, have been contributed to the formation of calcium stones may not be justified. Other limitations are discussed in the corresponding publications and elsewhere (Lopez and Quereda, 2011).

Several mechanisms have been postulated to explain how low-dose melamine exposure may promote the development of calcium-related urinary stones including the formation of a nidus that subsequently promotes the growth of calcium uroliths, renal tubular injuries, or enhanced precipitation of calcium oxalate (Liu et al., 2011; Wu et al., 2010; Wu et al., 2014). There are several additional studies suggest an interaction of melamine with other lithogenic substances such as calcium oxalate or calcium phosphate. Several *in vitro* studies have shown that melamine promotes the formation of calcium crystals (Gombedza et al., 2019; Poon et al., 2012; Thanasekaran et al., 2012). Moreover, it has been suggested that melamine-related calculi, found in Chinese paediatric patients, may change their chemical characteristics. Accordingly, the authors of the study by Sun *et al.* (2010a) hypothesized that large conservative therapy-resistant melamine-related calculi may undergo calcification. In a study by Wen *et al.* (2011), it was uncovered that residual melamine-related

calculi, while remaining in the same location, changed their radiographic features from being radiolucent at the time of hospital discharge to radio-opaque at follow-up. According to the authors of the study, the analysis of the residual melamine-related calculi that became radio-opaque revealed melamine as the major component of the core enclosed within a calcium/calcium oxalate dihydrate containing shell, resembling common calcium stones. However, the authors failed to provide sufficient analytical data to substantiate this claim. In addition, some studies have found that melamine-related calculi contain a certain level of calcium oxalate which inversely correlated with the effectiveness of conservative treatment (Li et al., 2011; Li et al., 2012). It had also been suggested that predisposing lithogenic factors may determine the development, the composition, and the persistence of stones. The commonly observed elevated male-to-female ratio in the exposed paediatric population, for instance, may be explained by hormonal differences which can have an impact on urinary saturation of calcium oxalate (Heller et al., 2002; Lu et al., 2011). Hence, there is a particular concern regarding the interaction of melamine with other lithogenic salts such as calcium oxalate. The available data point towards a possible promoting role of melamine in the formation of calcium crystals and the development of chemically mixed stones.

In addition, ambient melamine exposure and its impact on renal function (as assessed by measuring markers of early renal injuries) in an occupational setting was examined by **Wu et al. (2015)**. Accordingly, ambient melamine exposure is significantly associated with elevated melamine levels in urine and serum as well as with increased levels of urinary N-acetyl b-D-glucosaminidase and detectable b2-microglobulin, suggesting possible damages to renal tubular cells (Wu et al., 2015). However, the relevance of these urinary biomarkers regarding their validity as indicators of effects that can progress to significant disturbance of the kidney function is not fully elucidated.

A recently published prospective cohort study (same group as Liu et al. (2017); Liu et al. (2011); Wu et al. (2018); Wu et al. (2010); Wu et al. (2015)) including 293 participants studied the role of environmental melamine in the progressive decline of kidney function in patients with renal abnormalities (subjects with early-stage chronic kidney disease (CKD)). Urinary levels of melamine were determined at the time of enrolment and correlated with kidney function at baseline and progression of CKD during a 7 years follow-up period. The results of the study show a correlation between urinary melamine and compromised kidney function at baseline and deterioration of CKD progression during follow-up. The results suggest that chronic low-dose melamine exposure may have adverse effects especially in vulnerable sub-populations such as individuals with early-stage CKD (Tsai et al., 2019)

In summary, information on low-dose human exposure, provide mostly by one Taiwanese research group, is available. The data show a correlation between urinary melamine and adverse effects within the urinary tract (contribution to common calcium urolithiasis, impaired renal function), suggesting adversity even at low doses. However, as there are significant uncertainties concerning the relevance and validity of these data, a final conclusion cannot be reached at this point. More data have to be generated to convincingly support a potentially harmful effect of low-dose environmental melamine exposure.

10.11.2 Comparison with the CLP criteria

"Target organ toxicity (repeated exposure) means specific, target organ toxicity arising from a repeated exposure to a substance or mixture (CLP Regulation 1272/2008, 3.9.1.1.). According to CLP Regulation 1272/2008, 3.9.2., substances are placed in one of two categories, depending upon the nature and severity of the effect(s) observed.

CLP Regulation 1272/2008, 3.9.2., Table 3.9.1:

***Category 1:** Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of:*

- *reliable and good quality evidence from human cases or epidemiological studies; or*
- *observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations.*

Guidance dose/ concentration values are provided below (see 3.9.2.9), to be used as part of a weight-of- evidence evaluation.

Category 2: *Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure. Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided below (see 3.9.2.9) in order to help in classification. In exceptional cases human evidence can also be used to place a substance in Category 2 (see 3.9.2.6).''*

Significant toxicity in humans has been observed as a consequence of repeated dietary melamine consumption. Experimental epidemiological studies related to melamine-induced toxicity in humans are non-existent. However, findings from numerous observational epidemiological studies related to the consumption of melamine-tainted milk products in China, consistently show melamine-induced urinary precipitation and nephrotoxicity as the main adverse health effects seen in exposed humans. While these studies are characterized by limitations and uncertainties mostly related to the quantification of melamine exposure, the selection of the study population, and the level of detail to which kidney abnormalities were examined, they clearly establish a causal association between melamine exposure and adverse renal abnormalities. The specific nature of adverse health effects consistently observed in exposed children reached from asymptomatic/symptomatic urolithiasis, compromised renal function, renal injuries and inflammation, to acute obstructive renal failure and death. The consistency with findings in experimental animal studies provides good biological understanding and plausibility of the observed effects. Thus, the sum of information that has emerged following the melamine-adulteration incident provides sufficient evidence for renal toxicity attributed to oral melamine consumption in humans. On the basis of these observations and in line with the findings in experimental animals, the urinary tract system is considered primary target organ system.

As stated in the CLP Regulation 1272/2008, 3.9.1.1. (Table 3.9.1), reliable and good quality evidence from human cases or epidemiological studies justify classification in category 1. The large body of data derived from the melamine adulteration incident as a whole can be considered reliable and good quality evidence for the following reasons: (a) high level of consistency regarding the reported outcomes (calculi mostly in the renal pelvis/calyx, nephrotoxicity, melamine stones composed of melamine and uric acid clearly distinguishable from common calcium-oxalate calculi), (b) the existence of a dose-response relationship, albeit not quantitative (prevalence of urolithiasis depending on exposure level), (c) conformity with experimental animal data, (d) the specificity of the nature of adverse health effects (mode of action; see 10.8.2, section (k)), (d) the biological plausibility based on observations in experimental animals, (e) no significant confounding factor could be identified.

Hence, according to the weight of evidence derived from epidemiological studies in humans, classification of melamine in category 1 (toxicity to the urinary system following repeated oral exposure) is considered justified. Species-specific differences in uric acid metabolism (higher uric acid levels attributed to the lack of the enzyme urate oxidase) may increase the potency of melamine in humans as compared to other mammals such as rats.

In line with observations in humans, significant adverse effects on the urinary system have been documented following repeated oral exposure to melamine in experimental animals. Based on information derived from key studies, the spectrum of toxic effects considered relevant for classification includes calculus formation in the urinary bladder of male rats (NTP, 1983), dose-related calcareous deposits in the straight segments of the proximal renal tubules in female rats (NTP, 1983), renal crystals in female rats (Early et al., 2013), and renal damages in male and female rats (Early et al., 2013). Information derived from supporting studies is consistent with the effects described in the key studies and largely supports the classification as part of the weight of evidence approach. Significant adverse effects on the urinary system were consistently reported at doses close to or below the guidance value of 100 mg/kg bw/d and above 10 mg/kg bw/d (Table 39) which would potentially justify a classification in category 2 (CLP Regulation 1272/2008, 3.9.1.1.; Table 3.9.1). However, since significant melamine-induced urinary precipitation and nephrotoxicity was observed in numerous observational human studies, classification in category 1 is considered justified.

10.11.3 Conclusion on classification and labelling for STOT RE

Based on significant adverse health effects in the urinary tract system of humans orally exposed to melamine, classification of melamine as STOT-RE 1 for the urinary tract system as primary target organ system is recommended (H372 (urinary tract system)).

Setting of specific concentration limit (SCL):

An SCL is not proposed as melamine did not induce target organ toxicity at a dose level clearly below the guidance values according to CLP Annex I, Table 3.9.2.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The DS identified the urinary tract system as the main target organ in rats, mice, monkeys and humans.

In rats, mice and monkeys, adverse toxic effects at dose levels relevant for classification STOT RE 2 (> 10 mg/kg and ≤ 100 mg/kg) were identified by the DS in two key reliable GLP studies:

- 90-day rat study (NTP, 1983): Calculus formation in the urinary bladder in males and calcareous deposits in the straight segments of the proximal tubules in females;
- 14-day rat study (Early *et al*., 2013): Renal crystals in female rats and renal injuries in male and female rats in a 14-day study.

In a weight-of-evidence assessment, the DS considered that other identified supportive studies showed consistent findings and justified classification as STOT RE 2. When exposure duration was different from 90-day, Haber's rule was used by the DS to derive an extrapolated effective dose.

The DS highlighted that due to improper experimental procedure, the occurrence of melamine crystals in experimental studies may have been underestimated (the use of formalin during tissue fixation could dissolved melamine crystals).

According to the DS, human urinary tract findings were in line with rats, mice and monkeys. Extensive literature was available in the dossier on the adverse health effects in children following consumption of melamine-tainted infant formula (deliberate adulteration scandal in China). The main limitation noted by the DS is the uncertainty related to individual exposure. Indeed, melamine exposure duration and daily intake was based on retrospective estimation of batch concentration analysis of infant formula performed in the study or, based on official numbers from the Chinese Ministry of Health. Variability in the concentration of melamine in infant formula has been observed. For example, higher concentrations were found in mainland china than in the Hong-Kong area. Nevertheless, a causal relationship between melamine exposure and significant adverse effects in humans has been established. The main effect was the occurrence of melamine-caused stones in the urinary tract of the affected children.

The DS summarised several published observational studies and noted that:

- Stones were most commonly found in the kidney, but to a minor extent also in the urinary bladder and ureter;
- A correlation between the prevalence of urolithiasis and exposure to the substance were observed. Nevertheless, the exact prevalence according to a specific quantity of melamine intake cannot be derived due to uncertainties in exposure assessment (a summary of the prevalence is provided in Table 22 of the CLH report);
- Several risk factors have been identified: male gender, prematurity, duration of consumption of contaminated products;
- Calculi from melamine exposure were distinguished from common calcium-oxalate calculi. The induced stones were mainly composed of uric acid and melamine. Other triazines (e.g. cyanuric acid) were not found relevant for stone formation;
- Humans and most particularly children may be more sensitive to melamine-induced calculi as uricase is not present in humans compared to other mammals (e.g. rats);
- Also, some studies suggested adversity at low dose (impaired renal function, contribution to common calcium urolithiasis), but effect in humans at low-dose exposure remain to be elucidated as major limitations were noted in these studies (e.g. study methodology).

Nephrotoxic effects observed in children accidentally exposed to melamine include renal injuries/lesions and renal inflammation. Macroscopic haematuria was described and may be the result of stone-related urothelial irritation/abrasion. Progression to acute obstructive renal failure and death was seen in some cases. Only in a few subjects was persistent nephrolithiasis reported in follow-up studies. According to the DS, paediatric patients with acute renal failure, may have an elevated risk to develop cardiovascular events and an increased mortality risk. The DS also highlighted that in a current meta-analysis, history of kidney stones is associated with an increased risk of chronic kidney disease and urinary tract carcinogenicity.

Based on adverse renal abnormalities observed in reliable and good quality human observational studies, a classification of melamine as STOT RE 1 was proposed by the DS.

No SCL was proposed.

Comments received during consultation

One member state (MS) agreed to classify melamine as STOT RE 1 for urinary tract system. Nevertheless, the they questioned if calculus formation could be considered as a significant/severe adverse effect.

One NGO supported the proposal to classify melamine as category 1.

Ten comments were received from mainly industrial organisations. All were in favour of no classification. Some comments provided on the mode of action (MoA) of melamine urinary tract toxicity were similar to the comments provided for the carcinogenicity hazard class and are addressed in the corresponding section of the opinion.
The main comments provided by industry were:

- The Early *et al*., (2013) study is of low reliability, with excessive dosage, no statistical analysis and too short an exposure duration;

- Other relevant 90-day key studies were available in rats and monkeys and did not support STOT RE classification (e.g. NTP, 1983).
- Melamine deposits will only occur if the solubility limit of melamine is exceeded in urine. The use of time extrapolation (Haber's rule) is questionable in this context as the threshold-based MoA is mainly dependant to concentration and mostly independent from exposure duration.
- The NOAEL/LOAEL set by the DS using the NTP 13-week study in rats for calculi formation is not supported in the absence of statistical significance at the LOAEL.
- Criminal adulteration is not relevant for classification as it is not a reasonably expected use.
- There is a major bias in the human data as the stones formed from melamine exposure and those of other origin (e.g. calcium stones) were not differentiated in the published human studies. Industry representatives considered that it is necessary to consider the potential confounding influence of stones not formed from melamine exposure when interpreting the data, notably the persistence of stones and effects at low levels of melamine.

An industry representative pointed out that the proposal for STOT RE classification is based on the primary effects of the MoA of tumour formation in male rats (formation of urinary bladder stones and its sequels). Consequently, the proposed STOT RE classification would serve as a double classification.

Assessment and comparison with the classification criteria

RAC considered the same studies as the DS for STOT RE hazard assessment. In addition, results of a recent EOGRTS study were also taken into account (Study report, 2020, results as summarised on the ECHA dissemination website).

In experimental animals, the urinary tract system was identified as the target organ in all the repeated-dose toxicity studies (including carcinogenicity and reproductive toxicity) in rats, mice and monkeys.

Urinary tract findings induced by melamine in mice and monkeys were observed only above the guidance value (GV) which would potentially justify classification in category 2.

In rats, at dose levels below the GV that may trigger STOT RE 2 classification, the following findings were noted (See also in-depth analysis by RAC of the studies in the BD):

Urinary tract	Findings	Study duration (days)	Effective dose (mg/kg)*
Urinary bladder	Stone formation	90	LOAEL: 72 BMD10: 41.7
Kidney	Crystal deposits	14	LOAEL: 140 BMD10: 21.1
	Nephropathy: Tubular dilatation, tubular basophilia, infiltration of mononuclear inflammatory cells, degeneration/necrosis/ regeneration of the tubular epithelium, fibrosis	14	NOAEL: 700 BMD10: 292

* As calculated by the DS

With regards to the formation of **calculi in the urinary bladder**, as described in more detail in the carcinogenicity section of this opinion, the MoA of transitional cell epithelium changes induced by melamine is commonly accepted to be related to the precipitation of melamine above a certain threshold in urine, the formation of calculi and subsequent inflammation, and epithelium hyperplasia/proliferation leading to potential carcinogenesis. The first key-event of the MoA is threshold-based and involves stone formation. This is considered as an early event of urinary tract toxicity. Subsequent hyperplasia seen in the urothelium is considered to be an adaptive effect with no adverse consequences on its own upon cessation of exposure unless neoplasia develops, which is addressed under the carcinogenicity hazard class. Moreover, RAC notes that Haber's rule should be used with care in the case of stone formation as the first key events of the MoA is mainly concentration-related. Exposure duration may also be involved in stone formation as seen in several studies in humans (e.g. Wang *et al*., 2009, Gao *et al*., 2011), but might be more correlated to the size of stones.

With regards to **kidney nephropathy** (distinguishable from age-related chronic progressive nephropathy), Hard *et al*., 2009 considered that melamine precipitation in the lower urinary tract could create pressure effects through transient obstruction leading to the observed renal changes (termed retrograde nephropathy by the authors). It may also be hypothesised that crystals deposits in the tubular lumen of the kidney may produce irritation and inflammation, resulting in tubule degeneration and/or necrosis and subsequent acute to chronic inflammation or obstructive nephropathy. In monkeys nephrotoxicity seen following 90-day exposure (Early *et al*., 2013), at 700 mg/kg was not fully reversible. RAC considered retrograde nephropathy relevant for classification of melamine as STOT RE. With regards to dose levels, classification for STOT RE 2 based on nephropathy would only be triggered by one 14-day key study in rats, supported by some short-term studies identified by the DS with study durations of 14 days or less (Early *et al*., 2013 five-day study, Stine *et al*., 2014). In the available 28-day supporting study, also renal crystals were seen in rats but no nephrotoxicity was noted up to 240 mg/kg (Research triangle institute). Moreover, nephrotoxicity observed in 90-day short-term studies or EOGRTS study (77-day exposure in males) was only observed at dose above GV for classification. For example, according to the reanalysis of Hard *et al*., 2009, nephrotoxicity was not seen below 100 mg/kg bw/day and only 1/9 males had nephropathy at around 500 mg/kg bw/day (NTP, 1983). Therefore, due to uncertainties on time extrapolation with Haber's rule and as nephrotoxicity was seen above the GV of 100 mg/kg in studies of more than 15-day duration and only following Haber's rule extrapolation in studies ≤ 14- day duration, the criteria for STOT RE 2 classification, based on animal data (rats, mice monkeys), are not fulfilled.

In humans, in line with the available animal data, the primary effect of consuming high doses of melamine was **calculi formation**. Numerous studies have established that the consumption of melamine at high dose results in urolithiasis in children. According to the available human observational studies (table 20 of the CLH report), stones were reported to be mainly located in kidney renal pelvis or calyx. In some studies, stones were also reported to be located, in few children, in ureter and to a lesser extent in bladder. Abnormalities at urinalysis (Haematuria, proteinuria and leukocyturia) were consistently reported in some children exposed to melamine. Obstruction features such as

hydronephrosis were also noted (hydroureter was reported only in one study). Based on urinary microprotein profile (ex: microalbumine, acetyl-beta-D-glycosidase), tubular injuries and renal glomerulus injuries were also described. According to the 2-year longitudinal study from Zou *et al*., 2013, renal tubular and glomerular damage were resolved by 6-month after diagnosis. Obstruction features, haematuria and leukocyturia were still noted in few patients at 24-month follow-up. Persistent urolithiasis was noted in 8.85% of the patients at 24-month.

No renal function impairment (creatinine, blood urea nitrogen) was reported except in case of **acute obstructive renal failure** in children. In these patients, stones were found either in kidneys or ureters (Sun *et al.,* 2010c). Acute renal failure was reported in several studies (Lam *et al*., 2009; Yang *et al*., 2010b and Sun *et al*., 2010b and c, Wang *et al*., 2011). In a case report, Sun *et al*., 2010b reported the following histopathological findings from a biopsy of a paediatric patient with acute renal failure: lymphocytic infiltration in the glomeruli, sclerotic glomeruli, proliferation of fibrous tissue in the glomeruli and Bowman's capsule, swollen tubular cells, lymphocytic infiltration and fibrosis within the renal interstitium, and crystals within the lumen. According to the available follow-up studies (Sun *et al*., 2010b, 2010c; Yang *et al*., 2010b), following treatment, the renal function was fully recovered in patients with acute renal failure. During the consultation, industry highlighted that background levels of non-melamine stones may bias these published results. RAC acknowledges that it could be a potential bias in the human data and contributes to the uncertainties on melamine effects at low exposure levels. Nevertheless, it does not affect the conclusion that at high exposure, kidney injuries were seen following melamine human exposure.

Although an association between stone formation and melamine exposure levels was found, the estimated daily exposures in the studies were uncertain (high variability from low to very high concentration in the analysed melamine-tainted infant formula batches).

Calculi formation is a key event for renal acute or chronic diseases in human. Other acute renal findings observed in humans, abnormalities at urinalysis (e.g. haematuria), hydronephrosis, glomerular and tubular injuries may be assumed to be cause by uroliths. Although these effects are expected to be reversible following stone treatment or removal, they may be considered as relevant for classification. In all the available studies, kidney renal function (creatinine, Serum blood urea nitrogen levels) was normal except in patients with acute obstruction renal failure. According to official numbers, in 2008, over the 22,384,000 infants examined following suspected melamine exposure, 294 000 infants had been diagnosed with urinary tract abnormalities, 51,900 infants were hospitalised, and 6 deaths were confirmed. Sun *et al*., 2010 also reported that 25 cases were diagnosed with complications of acute obstructive renal failure. According to WHO, the infants consumed the infant formula for 15 days to 13 months (median 8 months). The estimated daily intake depending on the age of children was around 10 to 110 mg/kg considering mean/maximum melamine levels in some batch of infant formula (WHO, 2009). RAC considers acute renal obstruction failure induced by melamine to be a severe adverse complication of concern and relevant for classification. Death has also been seen in 6 patients. Nevertheless, no diagnostic investigation was performed in these six children but the reason is thought to be the lack of, or delay of treatment (WHO, 2009). RAC notes that, in the case of melamine-tainted products, the deficiency in protein in the children's diet may additionally favour kidney failure. Moreover, the very low number of exposed subjects with complications decrease the concern. RAC also notes

uncertainties on exposure-effect levels and particularly at low dose exposure (uncertainties on individual daily intake, background incidence of urolithiasis).

With regards to potential chronic renal disease, following calculi or crystal formation, lesions as observed in rats (retrograde nephropathy) and monkeys are expected to occur in humans. Supporting evidence are available from the human biopsy showing that fibrosis, tubular diseases and inflammation may occur. Moreover, chronic nephropathy seen in monkey following 90-day exposure that was fully reversible, increasing the concern.

Overall, based on its potential to cause human acute and chronic renal diseases, classification as STOT RE 1 would be required according to the CLP criteria. Nevertheless, RAC considers that a downgrading to category 2 is appropriate, mainly in view of the uncertainties on the effects of melamine at low doses. RAC also notes that animal data, showing similar effect as in humans, are supportive of the classification under STOT RE.

RAC considers a **classification of melamine as STOT RE 2 (urinary tract system) warranted**.

Supplemental information - In depth analyses by RAC

The table below summarised in selected key findings in kidney after repeated exposure to melamine in rats, monkeys and humans for STOT RE classification:

	Crystals/calculi, organ weight, clinical analysis	**Histopathological findings**
RATS		
2-year study (similar to OECD 451) 0, 126/262, 263/542 mg/kg bw/day in m/f, (NTP, 1983) (STOT RE 2 ≤ 12.5 mg/kg bw/day)	Urinary bladder ↑ Stones formation in males starting at 126 mg/kg bw/day in 1/50 and 10/40 at 262 mg/kg bw/day	Kidney ↑ nephropathy in f (not stat. significant) ↑ chronic inflammation at ≥ 263 mg/kg bw/day (stat. significant in females, increase non stat. in males) Presence of residual fibrotic scars. (Re-analysis, Hard *et al*., 2009, retrograde nephropathy in 7/50 m at 126 mg/kg bw/day graded minimal to moderate and 20/50 f at 262 mg/kg bw/day graded minimal to mild) Urinary bladder 262 mg/kg bw/day: ↑Transitional cell hyperplasia, combined carcinoma and papilloma in males
2-year study (similar to OECD 451) 4/5, 20/50, 40/80 mg/kg bw/day in m/f (Hazleton, 1983)	Urinary bladder Stone in 1 male at 20 mg/kg bw/day and 2 males at 40 mg/kg bw/day 2 f at 80 mg/kg bw/day	Urinary bladder Increase transitional cell hyperplasia (unknown biological relevance)

(STOT RE 2 ≤ 12.5 mg/kg)		
36-week + 4 week recovery study 0, 350, 1030 mg/kg bw/day +/- 5 or 10% NaCl in males **(Ogasawara et al., 1995)** (STOT RE 2 ⩽ 32 mg/kg bw/day)	Crystals in the urinary sediments at 1030 mg/kg independent of NaCl supplementation Reduced kidney weight, urinary occult blood <u>Urinary bladder</u> ↑ Stones formation in males at 350 mg/kg bw/day (7/19 vs zero in control), suppressed by NaCl treatment	<u>Kidney</u> Transitional cell hyperplasia and ischemic changes (focal lesions demonstrating fibrosis, inflammation cell infiltration, renal tubule regeneration) in papilla and cortex at ≥ 350 mg/kg bw/day. Attenuated following NaCl treatment <u>Urinary bladder</u> Increased papillomatous hyperplasia at ≥ 350 mg/kg
36-week + 4 week recovery study 0, 100, 330, 1090 mg/kg in males (Okumura *et al*., 1992) STOT RE 2 ⩽ 32 mg/kg bw/day)	Hematuria, polyuria at 1090 mg/kg <u>Urinary bladder</u> ↑ Stones formation starting at ≥ 100 mg/kg bw/day (1/20 males)	<u>Urinary bladder</u> Increased transitional cell hyperplasia at 330 mg/kg bw/day
EOGRTS (according to OECD 443, GLP) Rats 0, 65/87, 268/355, 883/1124 mg/kg bw/day in m/f, mean of the means of all periods in F0 (STOT RE 2 ≤ ~ 117 mg/kg bw/day for 11 weeks treatment in m) (Study report, 2020)	833 mg/kg bw/day: ↑ urea levels, in males and females ↑red blood cell in the urine of males <u>Kidney</u> ↑ relative kidney weight in males	<u>Kidney</u> F0: Retrograde nephropathy at 268 mg/kg bw/day in kidney in m, f (massive in males, moderate in females) <u>Urinary bladder</u> F0: Diffuse hyperplasia of the urinary bladder urothelium in males at 833 mg/kg (not seen in F1), and at ≥ 355 mg/kg bw/day in females
13-week studies (similar to OECD 408) 1st study 0, 560, 850/880, 1100/1200, 1400, 1700/1600 mg/kg bw/day in m/f 2nd study 0, 72/84, 150, 300, 590/600, 1300 mg/kg bw/day in m/f 3rd study: 0, 1700/1600, 1700/1600 mg/kg bw/day + NH4CL (STOT RE 2 ≤ 100	<u>Urinary bladder:</u> Stone formation (starting at 560 mg/kg bw/day in 6/12 m in the first study, 2/10 males in the second study at 72 mg/kg; BMD10: 41.7 mg/kg bw/day; ≥1400 mg/kg bw/day in f) Addition of 1% ammonium chloride did not influence the occurrence of calculi <u>Kidney</u> Solitary concretions in the upper fornix of the renal pelvis in a few rats (Hard *et al*., 2009) Calcareous deposit in renal tubules	Only low and high dose investigated in the 1st study <u>Urinary bladder</u> Dose-related increase in diffuse epithelial hyperplasia in m, f (incidence higher in m) starting at 300 mg/kg bw/day in males (1/10) and 1700 mg/kg bw/day in females (2/10). <u>Kidney</u> (Re-analysis, Hard *et al*., 2009, low and high dose only) Dose-related ↑ retrograde nephropathy at ≥ 1300 mg/kg bw/day in m, f (more severe in males)

mg/kg bw/day) (NTP, 1983)	in f≥ 84 mg/kg bw/day (Dose-related increased in tubular mineralization in the distal outer stripe of outer medulla described by Hard *et al*., 2009 in females in the 1st study)	Not observed in control and 72/84, mg/kg bw/day, 1/9 m and 0/10 f at 560 mg/kg bw/day
14-day GLP study + 7-day recovery 140, 700, 1400/1000 mg/kg bw/day (STOT RE 2 ≤ 600 mg/kg bw/day) (Early *et al*., 2013) Few animals/groups	↑ blood serum urea and creatinine at 700 mg/kg bw/day <u>Kidney</u> Crystal deposition in the renal outer medulla and papilla area (2/6 females ≥ 140 mg/kg bw/day; BMD10: 21.08 mg/kg bw/day)	<u>Kidney</u> Nephropathy ≥ 700 mg/kg bw/day in m, f (6/6m and 6/6f) (BMD10: 292 mg/kg bw/day as calculated by the DS)
MICE		
2-year study (similar to OECD 451) (0, 327/523, 688/1065 mg/kg bw/day) (NTP, 1983) (STOT RE 2 ≤ 12.5 mg/kg bw/day)	<u>Urinary bladder</u> Stones increased in males at both doses and in females at 1065 mg/kg bw/day	<u>Urinary bladder</u> Inflammation, epithelial hyperplasia in males at ≥ 327 mg/kg bw/day and in females at 1065 mg/kg bw/day
13-week study (similar to OECD 407) 0, 710/790, 980/1250, 1200/1670, 1440/1790, 1880/2370, 3330/4740 in m/f (STOT RE 2 ≤ 100 mg/kg bw/day) (NTP, 1983)	<u>Urinary bladder</u> Stones starting at 2800 mg/kg bw/day in m and f (6/10 in m and 1/10 in f)	<u>Urinary bladder</u> Ulceration, inflammation of the epithelium, hyperplasia of epithelium in 2/10 m at 3330 mg/kg bw/day <u>Kidney (</u>reanalysis by Hard *et al*., 2009) Retrograde nephropathy were observed with lower incidence in mice than rats (no further information)
MONKEYS		
13-week + 4-week recovery 0, 60, 200, 700 mg/kg bw/day (Early *et al*., 2013)	Urine crystal formation in the urinalysis ≥ 700 mg/kg bw/day <u>kidney</u> ↑ kidney weight in m, f (not reversible following recovery)	<u>Kidney</u> Nephrotoxicity: Inflammation, single-cell necrosis in 2/3 females ≥ 200 mg/kg bw/day (graded minimal to mild) Minimal to moderated renal tubular degeneration/regeneration with or without interstitial mononuclear infiltration, tubular nuclear pyknosis, tubular single-cell necrosis, thickening of the glomerular capsule in 2/3 males and 3/3 females at 700 mg/kg bw/day (graded minimal to

		moderate). Not observed at 60 mg/kg. Not resolve in all animals at 700 mg/kg

10.12 Aspiration hazard

Hazard class not assessed in this dossier

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Not assessed in this dossier.

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13 ANNEXES

13.1 List of abbreviations

ALB	Microalbumin
Alb	Albumin
ALT	Alanine aminotransferase
AMDE	Absorption, metabolism, distribution, and elimination
AQSIQ	General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China
AST	Aminotransferase
BUN	Serum blood urea nitrogen
CIS	Carcinoma in situ
CMC	Carboxymethyl cellulose
Cr	Creatinine
CRRT	Continuous renal replacement therapy
CTr	Conservative treatment
D/CIS	Dysplasia/carcinoma in situ (combiend)
D/CIS	Dysplasia
ED	Effective dose
ESI-TOF-MS	Electrospray ionisation time-of-flight mass spectrometry
FTIR	Fourier transform infrared spectrometry
GC-MS	Gas chromatography coupled with mass spectrometry
GLP	Good laboratory practice
H	Hyperplasia
HCD	Historical control data
HPLC-MS	High-performance liquid chromatography coupled with mass spectrometry
HPLC-MS/MS	High-performance liquid chromatography coupled with tandem mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IgG	Immunoglobulin
IL-8	Interleukin 8
LC-ESI-MS/MS	Liquid chromatography electrospray ionisation coupled with tandem mass spectrometry
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MCP-1	Monocyte chemotactic protein-1
MDL	Method detection limit
MoA	Mode of action
MSM	Moderate squamous metaplasia
NaCl	Sodium chloride
NAG	N-acetyl-β-D-glucosidase
NTP	National Toxicology Program
oxo	Potassium oxonate
RCC	Renal cell carcinoma
SAC	Standardization Administration of China
SCC	Squamous cell carcinoma
SCL	Specific concentration limits
SCr	Serum creatinine
SEM	Scanning electron microscopy
SIn	Surgical intervention
SSM	Slight squamous metaplasia
TCC	Transitional cell carcinoma
TPA	12-0-tetradecanoylphorbol-13-acetate
TRF	Transferrin

UA	Uric acid
Ucr	Urine creatinine
Uosm	Urine osmolality
UPLC-MS/MS	Ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry
UTC	Urinary tract cancer
UTI	Urinary tract infection
UUN	Urine urea nitrogen
XRD	X-ray diffractometry
α1MG	α1-microglobulin
β2MG	β2-microglobulin

13.2 Benchmark dose modelling

The LOAEL was used to derive the effective dose where applicable. However, limitations of the NOAEL/LOAEL approach have been broadly discussed (EPA, 2012). For instance, it does not account for variability and uncertainty in the experimental results that may arise from limitations in the study design such as a low number of dose groups, low sample size, and wide spacing (EPA, 2012). Thus, in case limitations in study design hindered a full range capture of the dose-response characteristics regarding melamine-induced toxicity, benchmark dose (BMD) modelling was applied as an alternative approach to better describe the pattern of a given dose-response relationship. The response level BMD_{10} for dichotomous data refers to a 10 % increase in response compared with the background response (Hardy et al., 2017). The $BMDL_{10}$, defined as the lower 95 % confidence dose of the BMD_{10}, may be used to reflect the uncertainties and statistical errors. As stated in ECHAs Guidance on the Application of the CLP Criteria (3.7.2.6.1.), the BMD methodology may be applicable if scientific justification is provided. BMD modelling was performed using the US EPA Benchmark Dose Response Software (version 2.7). Table 1 summarises BMDs derived using different models for dichotomous data for several experimental animal studies.

Table 23: Result of benchmark modelling* from various studies

Reference	Endpoint	Model	Goodness-of-fit (P value)	AIC	BMD_{10}	$BMDL_{10}$
NTP, 1983 1st 90-day study (rats)	urolithiasis (bladder) ♂ rats	Weibull Hill Multistage	0.3117 0.3147 0.3094	52.8866 52.9035 52.9228	**158.03** 276.95 116.684	50.83 79.81 50.71
NTP, 1983 2nd 90-day study (rats)	urolithiasis (bladder) ♂ rats	Weibull Hill Multistage	0.9391 0.9371 0.9254	55.5206 55.6809 55.5865	**41.73** 61.95 34.29	19.66 18.92 19.57
NTP, 1983 2nd 90-day study (rats)	calcareous deposits in the kidney	Weibull Hill Multistage	*0.0975* 0.1807	*60.3624* 58.3863	*32.74* **28.82**	*18.95* 18.92
Research Triangle Institute, 1982 (rats)	urolithiasis (bladder) ♂ rats	Weibull Hill Multistage	*0.0231* 0.3121 *0.0489*	*226.577* 219.98 *224.881*	*461.03* **609.08** *427.82*	*356.60* 500.72 *352.66*
Research Triangle Institute, 1982 (rats)	crystalluria ♂ rats	Weibull Hill Multistage	0.8003 0.6199 0.8044	230.078 231.306 230.051	73.37 157.62 **77.05**	57.81 36.94 57.87
Early, 2013 (rats)	renal crystals ♀ rats	Weibull Hill Multistage	0.7051 0.4237 0.7051	18.1264 21.1195 18.1264	**34.78** 122.69 **34.78**	21.08 8.36 21.08
Early, 2013 (rats)	necrosis/degeneration/regeneration of distal nephron tubular epithelium ♂/♀ rats	Weibull Hill Multistage	*0.0460* 0.4685 *0.0934*	*25.1297* 18.5554 23.1629	*168.146* **292.036** *182.307*	*62.17* 138.40 *61.93*

*Benchmark doses were calculated using the US EPA Benchmark Dose Response Software (version 2.7) employing different models for dichotomous data; BMD_{10} is defined as 10 % extra risk; goodness-of-fit values below 0.1 have been disregarded; AIC = Akaike's Information Criterion, the model with the lowest AIC was selected

13.3 Additional follow-up studies

Table 24: Additional follow-up studies

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, route of exposure, dose levels, duration of exposure**	**Results**	**Reference**
Follow-up study	Melamine (intentional adulteration of milk products)	Follow-up duration: **12 months** Participants: n = 36 Reported recovery rates: • 27/36 (75 %) stones disappeared completely • **9/36 (25 %)** had residual stones • 6/36 (17 %) stone size decreased during follow-up • 3/36 (8 %) stone size increased during follow-up	(Dai et al., 2012)
Follow-up study	Melamine (intentional adulteration of milk products)	Follow-up duration: 18 months Participants: 38 Reported recovery rates: • 5/38 (13.16 %) showed residual renal stones	(Shang et al., 2012)
Follow-up study	Melamine (intentional adulteration of milk products)	Follow-up duration: 48 months Participants: 45 Reported recovery rates: • 34/45 stones disappeared completely • 6/45 dissolved partially • 4/45 did not change • 1/45 increased in size	(Yang et al., 2013)
Follow-up study	Melamine (intentional adulteration of milk products)	Follow-up duration: 18 months Participants: 73 Reported recovery rates: • 5/73 intrarenal calculi still present (6.85 %) • 1/73 suffered from hydronephrosis	(Wang et al., 2014)