Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

VKM Report 2015: 20

Risk assessment of "other substances" - L-cysteine and L-cystine

Opinion of the Panel on Nutrition, Dietetic Products, Novel Food and Allergy of the Norwegian Scientific Committee for Food Safety

Report from the Norwegian Scientific Committee for Food Safety (VKM) 2015: 20
Risk assessment of "other substances" – L-cysteine and L-cystine

Opinion of the Panel on Nutrition, Dietetic Products, Novel Food and Allergy of the Norwegian Scientific Committee for Food Safety
20.11.2015

ISBN: 978-82-8259-175-1
Norwegian Scientific Committee for Food Safety (VKM)
Po 4404 Nydalen
N – 0403 Oslo
Norway

Phone: +47 21 62 28 00
Email: vkm@vkm.no

www.vkm.no
www.english.vkm.no

Cover photo: iStock Photo

Suggested citation: VKM. (2015). Risk assessment of "other substances" – L-cysteine and L-cystine. Opinion of the Panel on Nutrition, dietetic products, Novel Food an Allergy of the Norwegian Scientific Committee for Food Safety, ISBN: 978-82-8259-175-1, Oslo, Norway.

Risk assessment of "other substances" - L-cysteine and L-cystine

Author preparing the draft opinion

Tor A Strand

Assessed and approved

The opinion has been assessed by the Panel on Nutrition, Dietetic Products, Novel Food and Allergy of the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM). Per Ole Iversen (chair), Livar Frøyland, Margaretha Haugen, Kristin Holvik, Martinus Løvik, Tor A. Strand, Grethe S. Tell and Arild Vaktskjold.

(Panel members in alphabetical order after chair of the panel)

Acknowledgment

The Panel on Nutrition, Dietetic Products, Novel Food and Allergy has answered the request from the Norwegian Food Safety Authority. Project leader from the VKM secretariat has been Bente Mangschou. Tor A Strand is acknowledged for his valuable work on this opinion. Jan Alexander (the Scientific Steering Committee), Åshild Krogdahl (the Scientific Steering Committee) and Helle Margrete Meltzer (former member of the Panel on Nutrition, Dietetic Products, Novel Food and Allergy) constituted a reference group and are acknowledged for their valuable comments and suggestions on this opinion.

Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

Table of Contents

Summary

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has, at the request of the Norwegian Food Safety Authority (Mattilsynet; NFSA), assessed the risk of "other substances" in food supplements and energy drinks sold in Norway. VKM has assessed the risk of doses given by NFSA. These risk assessments will provide NFSA with the scientific basis while regulating the addition of "other substances" to food supplements.

"Other substances" are described in the food supplement directive 2002/46/EC as *substances other than vitamins or minerals that have a nutritional or physiological effect*. It is added mainly to food supplements, but also to energy drinks and other foods. VKM has not in this series of risk assessments of "other substances" evaluated any claimed beneficial effects from these substances, only possible adverse effects.

The present report is a risk assessment of L-cysteine and L-cystine, and is based on previous risk assessments of these amino acids and articles retrieved from a comprehensive literature search. In this report L-cysteine and L-cystine are often termed merely cysteine and cystine, respectively.

L-cysteine is a central compound in sulphur metabolism in the human body. L-cysteine is a conditionally essential sulphur-containing amino acid, obtained from L-methionine and from serine. Sulphur-containing amino acids are mainly found in cereal proteins and animal proteins, and less abundantly in pulses. Cysteine may occur in proteins either as cysteine itself or as cystine. Cystine is the disulphide dimer of cysteine, and is a more stable compound than cysteine.

According to information from the Norwegian Food Safety Authority (NFSA), cysteine and cystine are ingredients in food supplements purchased in Norway and NFSA has requested a risk assessment of the following doses of cysteine and cystine in food supplements: L-cysteine 10 mg/day and L-cystine 250, 500, 750 and 1000 mg/day. The mean usual daily intake of cysteine in the USA for all life stage- and gender groups is 1.0 g/day (NHANES II, USA).

Because there are few intervention studies with cysteine or cystine, studies with N-acetylcysteine (or N-acetyl-L-cysteine, NAC), which is readily converted to cysteine, is included in this risk assessment. NAC is used as a pharmaceutical drug for various conditions, mainly as mucolytic agent, as paracetamol antidote, and has been included in numerous clinical trials.

Most of the cited studies have tested NAC in doses of about 600-1200 mg/day. The study groups have been various patient groups which included children, adolescents, adults and elderly, however relatively few studies have been conducted in children. In the randomised controlled trials there have been no differences in severe adverse events between the

placebo and NAC-groups. The adverse effects reported are generally limited to mild gastrointestinal symptoms.

The dose 1200 mg of NAC yields maximum 900 mg of L-cysteine or L-cystine. In adults, it is well documented that doses up to 900 mg per day for one year (corresponding to 13 mg/kg bw/day in a 70 kg adult) is without appreciable health risk. The data for doses above 900 mg/day are more scarce.

There are no data indicating that children and adolescent are more vulnerable than adults for L-cysteine or L-cystine. No tolerance level is set for cysteine or cystine specifically for children or adolescents, but an assumption is made that these age groups have similar tolerance per kg body weight as adults.

VKM concludes that:

- In adults (≥18 years), the specified doses 10 mg/day L-cysteine and 250, 500 and 750 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects, whereas the dose 1000 mg L-cystine per day may represent a risk of adverse health effects.
- In adolescents (14 to <18 years), the specified doses 10 mg/day L-cysteine and 250, 500 and 750 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects in adolescents, whereas the dose 1000 mg L-cystine per day may represent a risk of adverse health effects.
- In children (10 to <14 years), the specified doses 10 mg/day L-cysteine and 250 and 500 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects, whereas the doses 750 and 1000 mg L-cystine per day may represent a risk of adverse health effects.

Children below 10 years were not included in the terms of reference.

Short summary:

The Norwegian Scientific Committee for Food Safety (VKM) has, at the request of the Norwegian Food Safety Authority, assessed the risk of specified doses of L-cysteine and L-cystine in food supplements. VKM concludes that:

- In adolescents (14 to <18 years) and adults (≥18 years), the specified doses 10 mg/day L-cysteine and 250, 500 and 750 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects, whereas the dose 1000 mg L-cystine per day may represent a risk of adverse health effects.
- In children (10 to <14 years), the specified doses 10 mg/day L-cysteine and 250 and 500 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects, whereas the doses 750 and 1000 mg L-cystine per day may represent a risk of adverse health effects.

Key words: Adverse health effect, cysteine, cystine, food supplement, N-acetylcysteine, NAC, negative health effect, Norwegian Scientific Committee for Food Safety, other substances, risk assessment, VKM.

Sammendrag på norsk

På oppdrag for Mattilsynet har Vitenskapskomiteen for mattrygghet (VKM) vurdert risiko ved tilsetting av "andre stoffer" i kosttilskudd og energidrikk som selges i Norge. VKM har risikovurdert ulike bruksdoser oppgitt fra Mattilsynet. Disse risikovurderingene vil gi Mattilsynet vitenskapelige grunnlag for å regulere "andre stoffer" i kosttilskudd.

"Andre stoffer" er stoffer som har en ernæringsmessig eller fysiologisk effekt, og som ikke er vitaminer og mineraler. De tilsettes i hovedsak til kosttilskudd, men også til energidrikker og andre næringsmidler. I disse risikovurderingene har VKM ikke sett på potensielle gunstige helseeffekter, men kun vurdert mulige negative helseeffekter.

I denne rapporten har VKM vurdert risiko ved L-cystein og L-cystin. Risikovurderingen er basert på tidligere risikovurderinger av disse aminosyrene og artikler som er funnet ved et omfattende litteratursøk. Videre i denne rapporten omtales L-cystein og L-cystin ofte kun som henholdsvis cystein og cystin.

L-cystein er en sentral forbindelse i kroppens metabolisme av svovel. L-cystein er en semi-essensiell svovelholdig aminosyre som kan dannes fra L-metionin og serin. I kosten finner vi de svovelholdige aminosyrene hovedsakelig i kornprodukter og animalsk protein, og i mindre grad i belgvekster som bønner og linser. Cystein kan forekomme i proteiner enten som cystein eller som cystin. Cystin består av to cysteinmolekyler, og er en mer stabil forbindelse enn cystein.

Ifølge informasjon fra Mattilsynet er cystein og cystin ingredienser i kosttilskudd som selges i Norge. Oppdraget fra Mattilsynet var å risikovurdere følgende doser i kosttilskudd: L-cystein 10 mg/dag og L-cystin 250, 500, 750 og 1000 mg/dag. Gjennomsnittlig inntak av cystein fra kosten i USA i alle aldersgrupper, inkludert både menn og kvinner, er 1,0 g/dag (NHANES II, USA).

Ettersom det finnes få intervensjonsstudier med cystein eller cystin, er studier med N-acetylcystein (eller N-acetyl-L-cystein, NAC) som lett konverteres til cystein, inkludert i denne risikovurderingen. NAC er brukt som legemiddel for en rekke tilstander, hovedsakelig som slimløsende middel, og det er en viktig antidot ved paracetamolforgifting, og inngår i mange kliniske studier.

I de fleste studiene er NAC undersøkt i doser på omlag 600-1200 mg/dag, men med et dosespenn fra ca. 600 til 2400 mg/dag. Gruppene som er undersøkt er for det meste varierende pasientgrupper i ulike aldre; barn, unge, voksne og eldre, men det er relativt få studier med barn. I de randomiserte kliniske studiene finner man ingen signifikant forskjell i alvorlige negative helseeffekter mellom placebo- og NAC-gruppene. Negative helseeffekter som er rapport i studiene er stort sett begrenset til milde gastrointestinale symptomer.

En dose på 1200 mg NAC gir maksimum 900 mg L-cystein eller L-cystin. Det er godt dokumentert at 900 mg/dag oralt administrert tilskudd av cystein eller cystin i opptil ett år

hos voksne (tilsvarer 13 mg/kg kroppsvekt/dag i en voksen person som veier 70 kg) sannsynligvis ikke vil forårsake negative helseeffekter. Data for doser over 900 mg/dag er mer usikkert.

Det er ikke framkommet data som indikerer at barn og ungdom er mer sårbare enn voksne for L-cystein eller L-cystin. Det er ikke fastsatt et eget toleransenivå for barn og unge, men forutsatt at disse aldersgruppene har samme toleranse per kg kroppsvekt som voksne.

Vitenskapskomiteen for mattrygghet (VKM) konkluderer med at:

- For voksne (≥18 år) er det usannsynlig at dosene 10 mg/dag L-cystein og 250, 500 og 750 mg/dag L-cystin i kosttilskudd vil forårsake negative helseeffekter, mens en dose på 1000 mg L-cystin per dag vil kunne representere en risiko for negative helseeffekter.
- For ungdom (14 til <18 år) er det usannsynlig at dosene 10 mg/dag L-cystein og 250, 500 og 750 mg/dag L-cystin i kosttilskudd vil forårsake negative helseeffekter, mens en dose på 1000 mg L-cystin per dag vil kunne representere en risiko for negative helseeffekter.
- For barn (10 til <14 år) er det usannsynlig at dosene 10 mg/dag L-cystein og 250 og 500 mg/dag L-cystin i kosttilskudd vil forårsake negative helseeffekter, mens doser på 750 og 1000 mg L-cystin per dag vil kunne representere en risiko for negative helseeffekter.

Barn under 10 år inngår ikke i oppdraget.

Kort sammendrag:
Vitenskapskomiteen for mattrygghet (VKM) har på oppdrag for Mattilsynet har vurdert risiko ved inntak spesifikke doser av L-cystein og L-cystin i kosttilskudd. VKM konkluderer med at:

- For ungdom (14 til <18 år) og voksne (≥18 år) er det usannsynlig at dosene 10 mg/dag L-cystein og 250, 500 og 750 mg/dag L-cystin i kosttilskudd vil forårsake negative helseeffekter, mens en dose på 1000 mg L-cystin per dag vil kunne representere en risiko for negative helseeffekter.
- For barn (10 til <14 år) er det usannsynlig at dosene 10 mg/dag L-cystein og 250 og 500 mg/dag L-cystin i kosttilskudd vil forårsake negative helseeffekter, mens doser på 750 og 1000 mg L-cystin per dag vil kunne representere en risiko for negative helseeffekter.

Abbreviations and glossary

Abbreviations

AESAN - Spanish Agency for Food Safety and Nutrition
ANSES - French Agency for Food, Environmental and Occupational Health and Safety
AUC - area under the curve
bw - body weight
COPD - chronic obstructive pulmonary disease
CRP - C-reactive protein
EFSA - European Food Safety Authority
FDA - Food and Drug Administration, USA
GI - gastrointestinal
HDL - high density lipoprotein
IOM - Institute of Medicine, USA
LD_{50} - lethal dose for 50% of the animals
LDL - low density lipoprotein
NAC - N-acetylcysteine
NADH - reduced nicotinamide adenine dinucleotide
NFSA - Norwegian Food Safety Authority [*Norw.*: Mattilsynet]
NHANES - National Health and Nutrition Examination Survey
NOAEL - no observed adverse effect level
UL - tolerable upper intake level
VKM - Norwegian Scientific Committee for Food Safety [*Norw.*: Vitenskapskomiteen for Mattrygghet]
VLDL - very low density lipoprotein
WHO - World Health Organization

Glossary

"Other substances": a substance other than a vitamin or mineral that has a nutritional or physiological effect (European Regulation (EC) No. 1925/2006, Chapter I, Article 2; https://www.anses.fr/sites/default/files/documents/NUT2007sa0314EN.pdf).

"Negative health effect" and "adverse health effect" are broad terms and World Helath Organization (WHO) has established the following definition for "adverse effect": a change in morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences (WHO, 1994).

An adverse event is considered serious if it:

- results in death
- is life-threatening
- requires or prolongs hospitalisation
- is a congenital anomaly or birth defect
- is a persistent or significant disability/incapacity
- is another serious or important medical event

Background as provided by the Norwegian Food Safety Authority

"Other substances" are substances other than vitamins and minerals, with a nutritional and/or physiological effect on the body. "Other substances" are mainly added to food supplements, but these may also be added to other foods and beverages, such as sports products and energy drinks. Ingestion of these substances in high amounts presents a potential risk for consumers.

In Norway, a former practice of classification of medicines had constituted an effective barrier against the sale of potentially harmful "other substances". Ever since this practice was changed in 2009, it has become challenging to regulate and supervise foods with added "other substances". Meanwhile, in the recent years, the Norwegian market has witnessed a marked growth in the sales of products containing "other substances". In 2011, food supplements containing "other substances" constituted more than 50% of the market share.

While at the EU level, these substances fall under the scope of the European Regulation (EC) No. 1925/2006 on the addition of vitamins, minerals and certain other substances to foods and the European Regulation (EC) No 258/97 concerning novel foods and novel food ingredients, "other substances" remain largely unregulated. In order to ensure safe use of "other substances" many countries have regulated their use at a national level. For example, Denmark regulates these substances in a positive list i.e. a list of substances with maximal daily doses, permitted for use in food supplements and other foods (https://www.retsinformation.dk/Forms/R0710.aspx?id=163394).

NFSA is working on the establishment of a regulation on the addition of "other substances" to foods at a national level. The regulation will include a list of substances with permitted maximal doses, based on the substances and doses found in products on the Norwegian market. NFSA has therefore requested the Norwegian Scientific Committee for Food Safety (VKM) to assess the safety of "other substances" found on the Norwegian market. NFSA, in consultation with the industry, has compiled a list of "other substances" found in products marketed in Norway. Only substances with a purity of minimum 50% or concentrated 40 times or more have been included in the list. Substances regulated by other legislations like those for novel foods, food additives, aromas, foods for special medical purposes, etc. have been excluded from the list.

Terms of reference as provided by the Norwegian Food Safety Authority

The Norwegian Food Safety Authority (NFSA) requested the Norwegian Scientific Committee for Food Safety (VKM) to assess the safety of L-cysteine and L-cystine in food supplements at the following doses:
L-cysteine: 10 mg/day
L-cystine: 250, 500, 750 and 1000 mg/day

NFSA requested VKM to assess the safety of "other substances" (in accordance to the guidance document developed in Phase 2) at the doses specified (Phase 3).

Safety assessments for "other substances" present in food supplements shall be carried out for a general population, ages 10 years and above.

Assessment

1 Introduction

"Other substances" are described in the food supplement directive 2002/46/EC as *substances other than vitamins or minerals that have a nutritional and/or physiological effect*, and may be added to food supplements or e.g. energy drinks.

This risk assessment regards the substances L-cysteine and L-cystine per se, and no specific products. In this report L-cysteine and L-cystine are often termed merely cysteine and cystine, respectively.

VKM has in this series of risk assessments of "other substances" not evaluated any claimed beneficial effects from these substances, but merely possible adverse effects at specified doses used in Norway.

According to information from the Norwegian Food Safety Authority (NFSA), cysteine and cystine are ingredients in food supplements purchased in Norway and NFSA has requested a risk assessment of the following doses of cysteine and cystine in food supplements: L-cysteine 10 mg/day and L-cystine 250, 500, 750 and 1000 mg/day.

L-cysteine is a central compound in sulphur metabolism in the human body. L-cysteine is a conditionally essential sulphur-containing amino acid, obtained from L-methionine and from serine. In normal physiological conditions the body is able to obtain L-cysteine in sufficient quantity. However premature babies cannot synthesise it and must obtain it through diet. The thiol groups (-SH) of two molecules of cysteine are readily oxidised to yield the disulphide group of cystine. Cystine plays a special role in the structure of proteins. Cysteine and cystine are interconvertable and are therefore considered together in this report.

NFSA has requested assessment of the L-isomers of the two amino acids. There are very few published studies investigating the effects of L-cysteine or L-cystine. However, N-acetylcysteine (or N-acetyl-L-cysteine, NAC) is used as a pharmaceutical drug for various conditions, mainly as mucolytic agent, as a paracetamol antidote, and is included in numerous clinical trials. The acetyl-group in NAC acts merely as a molecule stabiliser, and is not believed to change the physiological properties, and studies with NAC are therefore included in this risk assessment.

The common mean daily intake of cysteine in the USA for all life stage and gender groups is about 1.0 g/day (NHANES II, USA) and daily turnover is about 7 g/day in a 70 kg person (Fukagawa et al., 1998).

2 Hazard identification and characterisation

2.1 Literature

In this risk assessment we have evaluated previous risk assessments of cysteine and articles retrieved from a comprehensive literature search.

2.1.1 Previous risk assessments

Risks related to L-cysteine has previously been evaluated by the Institute of Medicine (IOM) in USA in 2005, the European Food Safety Authority (EFSA) as flavouring substance and in another EFSA opinion as food additive for technical purposes in foods intended for infants and young children in 2006 and 2007, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) and VKM in 2011 and the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (ASEAN) for use in food supplements in 2012 (AESAN, 2012; ANSES, 2011; EFSA, 2006; EFSA, 2007; IOM, 2005; VKM, 2011). N-acetyl-L-cysteine was evaluated for the use in foods for special medical purposes by EFSA in 2003 (EFSA, 2003).

The opinions from EFSA in 2006 and 2007 evaluate L-cysteine and L-cystine in amounts magnitude lower than the doses relevant for this opinion, and the conclusions from these EFSA-reports are therefore not relevant for our purpose. However, human and animal toxicological data from these opinions are listed in the tables below.

Opinion related to N-acetyl-L-cysteine for use in foods for particular nutritional uses and in foods for special medical purposes. EFSA, 2003

The opinion by EFSA in 2003 evaluated the extent to which NAC could be used as a source for L-cysteine in foods for particular nutritional uses and for special medical purposes. The opinion concluded that including NAC in the list of substances permitted for foods for special medical purposes was acceptable. However, due to some uncertainties regarding "a potential" prooxidative effect of NAC, they did not recommend NAC as general replacement for L-cysteine in foods (EFSA, 2003).

Dietary reference intakes, tolerable upper intake levels for individual amino acids, Institute of Medicine. USA, 2005

The IOM (2005) concluded that the data on adverse effects of L-cysteine and L-cystine intake from supplements were not sufficient for a dose-response assessment and derivation of a tolerable upper intake level (UL). L-cysteine has been classified as a neuro excitotoxin

because of its interaction with N-methyl D-aspartate receptors (Olney, 1994 cited in IOM, 2005). Single oral doses of 5-10 g of cysteine have produced nausea and light-headedness in healthy humans (Carlson et al., 1989 cited in IOM, 2005).

Opinion of the French Agency for Food, Environmental and Occupational Health & Safety on the assessment of the risks associated with substances with nutritional or physiological effects with a view to restricting or prohibiting their use in foodstuffs. France, 2011

ANSES (2011) elaborates on the topic of amino acid metabolism and states that "on a nutritional and metabolic level, consumption of an amino acid at levels much higher than other amino acids, and much higher than the corresponding quantitative requirements for protein synthesis, induces changes in circulating pools, changes in functions directly controlled by the amino acids, the substantial entry of these amino acids into catabolic pathways (sometimes in "secondary"metabolic pathways) and the activation of excretion pathways. " Specific for cysteine, and based on animal experiments, this ANSES report lists hypercholesterolemia, fatty liver and neurotoxicity as possible adverse effects of high intakes of cysteine.

VKM report on risk categorisation of amino acids. Norway, 2011

In 2011, VKM conducted a risk categorisation of about 30 amino acids and amino acid compounds based on potential health risks related to high intakes of the amino acids. Cysteine, cystine and NAC were suggested categorised as substances with moderate risk (VKM, 2011). This categorisation was based on no scientific documentation other than the general knowledge that amino acids in general are bioactive compounds. No studies with cysteine or cysteine were identified. It was emphasised that the VKM report from 2011 has several limitations and can only be regarded as an initial screening and not as risk assessment of the many amino acids.

Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition on the use conditions for certain substances other than vitamins, minerals and plants in food supplements. Spain, 2012

AESAN (2012) noted that a maximum daily amount of 300 mg of L-cysteine is lower than the requirements of L-methionine + L-cysteine established by WHO and is very far from the doses of L-cysteine that cause dizziness and nausea. ASEAN therefore considered it acceptable from the safety point of view for use as a food supplement. An explanation for the conclusion on 300 mg is not given in the report, but we assume that ASEAN was requested to evaluate if the dose of 300 mg was safe.

2.1.1.1 Overview of some toxicity studies in previous risk assessments

Table 2.1.1-1 - 2.1.1-3 gives an overview of various toxicity studies with cysteine or cystine cited in previous risk assessment.

Table 2.1.1-1: Overview of genotoxicity studies (in vitro) with cysteine or cystine cited in previous risk assessments.

Reference	Substance	Test cystem, test object	Concentration	Result
Sargentini & Smith, 1986	Cystine	Mutagenesis assay (plate method)	2 mM (481 µg/ml)	No effects observed
Stich et al., 1981	L-cysteine	Chromosomal aberration assay, Chinese hamster ovary cells	$5x10^{-4}$ M (61 µg/ml)	No effects observed
Speit et al., 1980	L-cysteine	Sister chromatid exchange, V79) Chinese hamster ovary cells	Up to 10^{-3} M (121 µg/ml) (2 doses tested)	No effects observed

Table 2.1.1-2: Overview of acute toxicity studies with cysteine or cystine cited in previous risk assessments.

Reference	Substance	Species; sex	Route	LD_{50} (mg/kg bw)
Kawai et al., 1978	Cystine	Rat; M, F	Oral	>25,000
Sprince et al., 1974	L-cysteine	Rat, M	Gavage	1890
Takasaki et al., 1973	L-cysteine	Rat; M, F	Oral	M:6350 F: 5580
Takasaki et al., 1973	L-cysteine	Mouse	Oral	M: 3550 F: 4200

M: male

F: female

Table 2.1.1-3: Overview of subacute/subchronic/chronic/carcinogenicity studies and developmental and reproductive toxicity studies with cysteine or cystine cited in previous risk assessments.

Reference	Substance	Species; sex No/group	Route	Dose level (mg/kg bw/day)	Duration	NOAEL (mg/kg bw/day)
Kawai et al., 1978	Cystine	Rat; M, F 4/14 or 20	Oral	0, 100, 300, 600 or 3000	93 days	600
Takasaki et al., 1973	L-cysteine	Mouse; 3/8-12	Gavage	0, 200, 1000 or 3000	30 days	No NOAEL derived, adverse effects observed at all doses tested
Takasaki et al., 1973	L-cysteine	Rat; 3/9-12	Gavage	0, 200, 1000 or 5000	30 days	No NOAEL derived, adverse effects observed at all doses tested
Takasaki et al., 1973	L-cysteine	Rat; M 3/10-12	Gavage	0, 100, 500 or 2000	6 months	No NOAEL derived, adverse effects observed at all doses tested

Reference	Substance	Species; sex No/group	Route	Dose level (mg/kg bw/day)	Duration	NOAEL (mg/kg bw/day)
Frape et al., 1971	L-cysteine	Rat; M, F 1-3/4-72	Diet	0 or 3500 ppm in diet (equivalent to 0 or 175 mg/kg bw/day L-cysteine)	6-generation reproduction	3500 ppm in diet (equivalent to 175 mg/kg bw/day)

M: male

F: female

We have not described these animal studies further than the information given in the tables in the present report. The original studies were not accessible and in Japanese and the information is taken from the EFSA report (EFSA2008). No further details on the adverse effects were given in the report. Animal studies are briefly discussed in chapter 2.4.

2.1.2 Literature search

Literature searches were performed in MEDLINE, EMBASE and Global Health in order to retrieve publications on adverse effects caused by cysteine and cystine. These databases were chosen to ensure comprehensive study retrieval. The literature searches were performed by a librarian 5 March 2015. The strategy for the search is included in Appendix 1.

2.1.2.1 Publication selection and data extraction

The study types for inclusion in this opinion have been human studies. Animal studies were not included in the literature search due to numerous human studies and because several animal studies were included in previous risk assessments. However, we critically reviewed animal studies included in the previous risk assessments. The criteria for inclusion were:

- Cysteine or cystine in relation to adverse effect must be addressed in the abstracts of the paper
- Outcome not affected by other substances than cysteine or cystine
- Oral route of exposure to cysteine or cystine in human studies
- Human studies were performed in apparently healthy individuals or patient groups who are assumed to have normal cysteine and cystine absorption and metabolism.

In vitro studies were not included. Also papers in languages other than English, Norwegian, Danish or Swedish were excluded.

The literature search 5 March 2015 identified 2525 articles.

Study titles and abstracts were first reviewed by the secretariat, followed by a further selection by the author of this report, resulting in selection of 55 full text articles. After

review of the available full text articles using the same inclusion criteria as above, 19 articles were included.

Because of the previously described effect of cysteine on cholesterol in animal studies, a separate, Pubmed search on studies on cholesterol and cysteine or cystine was performed 26 May 2015 (search terms: cholesterol OR Lipid OR lipoprotein AND cysteine or cysteine). One additional human study and two animal studies were identified.

A final total of 22 publications (20 human and 2 animal studies) were identified and included in the results in this report (see Figure 2.1.2.1-1).

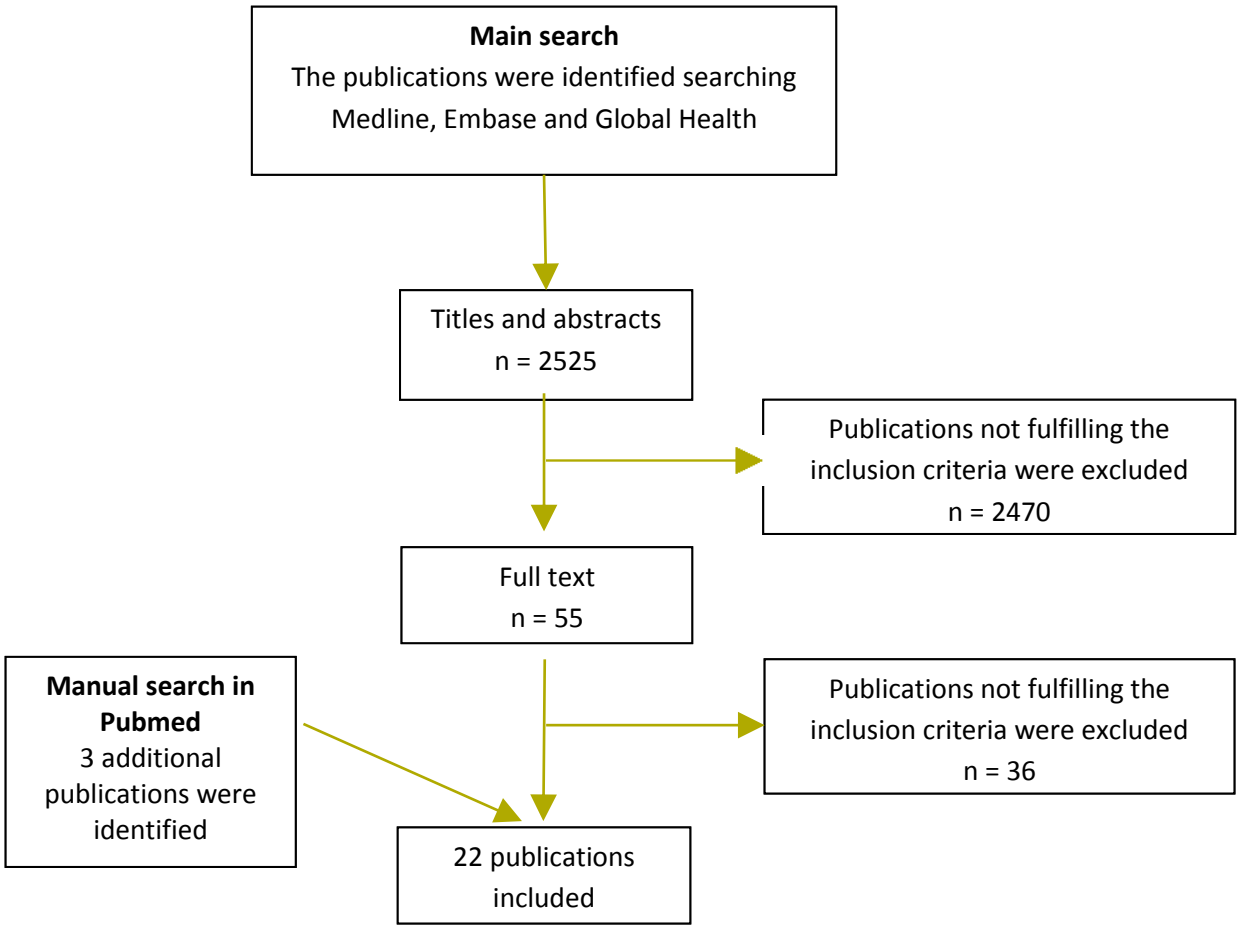

Figure 2.1.3.1-1: Flow chart for publication selection for cysteine and cystine literature search.

2.2 General information

2.2.1 Chemistry

L-cysteine $C_3H_7NO_2S$ (CH_2SH-$CHNH_2$-COOH) is a conditionally essential sulphur-containing amino acid. L-cystine is the dimer of L-cysteine. The CAS numbers are 52-90-4 (L-cysteine)

and 56-89-3 (L-cystine). Figure 2.2.1-1 shows the structural formulas for these amino acids and their interconvertion reaction.

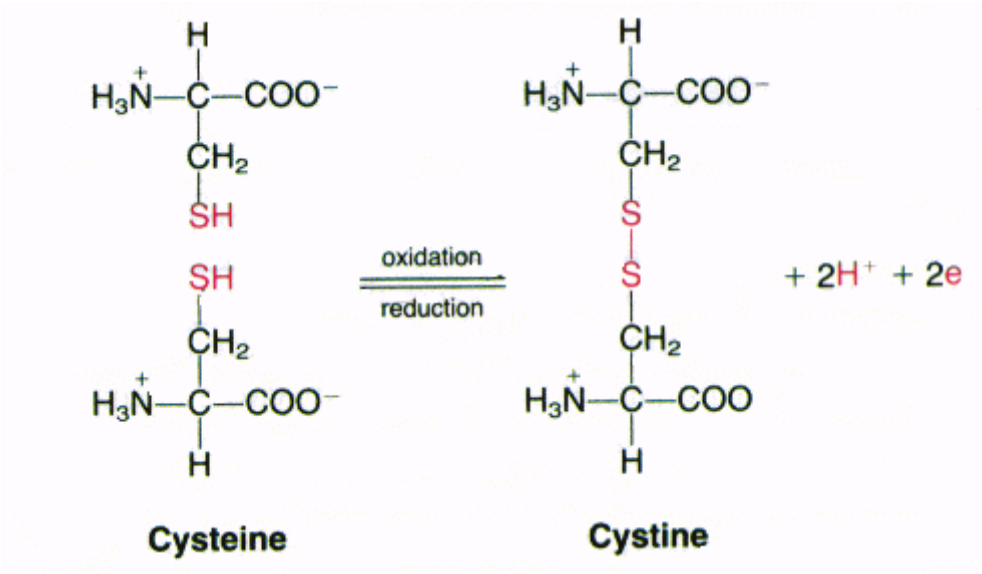

Figure 2.2.1-1: Structural formulas of cysteine and cystine and their interconversion reaction.

NAC $C_5H_9NO_3S$ is the N-acetyl derivate of cysteine. Figure 2.2.1-2 shows the structural formula for NAC.

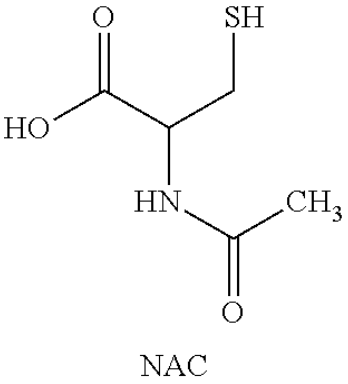

Figure 2.2.1-2: Structural formula of NAC.

2.2.2 Occurrence

In the normal diet, the amino acids are ingested as components of food proteins and not as free acids. Sulphur-containing amino acids are mainly found in cereal proteins and animal proteins, and less abundantly in pulses. Cysteine may occur in proteins either as cysteine itself or as cystine. In addition cysteine and cystine are available in food supplements.

According to Fukagawa et al. (1998), cysteine turnover in elderly is 35.6 µmol/kg/hour, corresponding to approximately 7 g/day in a 70 kg person.

Most of the commercially available L-cysteine is produced from hydrolysis of human hair, poultry feathers or pig hair (Berehoiu et al., 2013).

2.3 Absorption, distribution, metabolism and excretion

2.3.1 In humans

Cysteine may occur in proteins either as cysteine itself or as cystine. Cystine passes through the gastrointestinal (GI) tract and is immediately reduced to two cysteine molecules upon cell entry. L-cystine is converted to L-cysteine through cystine reductase, which requires NADH as cofactor.

L-cysteine is a central compound in sulfur metabolism in the human body. In humans, L-cysteine is synthesised from L-methionine and L-serine. L-cysteine is degraded to pyruvate in two steps, by desulphurisation and transamination. Cysteine can be metabolised to form taurine and carbon dioxide through the cysteinsulfinate pathway, where the initial step is oxidation of cysteine to cysteine sulfinate. This step is catalysed by cysteine dioxygenase. Cysteine sulfinate may then be decarboxylated to form taurine or it may be metabolised via the putative intermediate beta-sulfinylpyruvate to pyruvate and sulfite and then to carbondioxide and sulfate, illustrated in Figure 2.3.1-1 (Stipanuk et al., 2006).

L-cysteine is also involved in the synthesis of coenzyme A and glutathione.

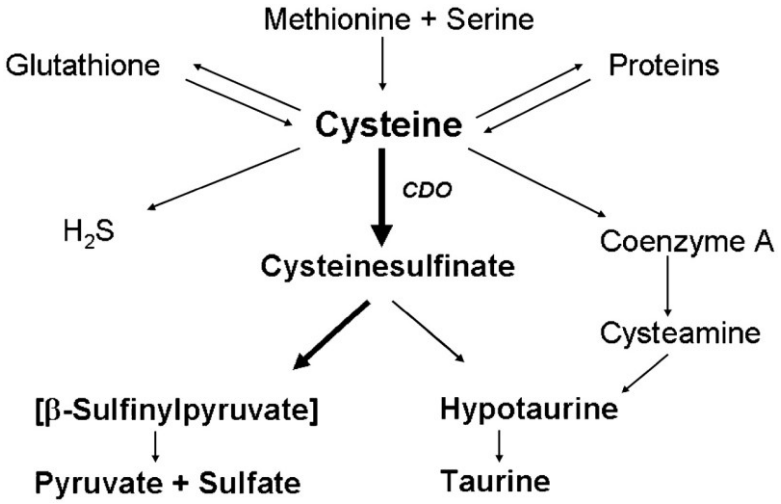

Figure 2.3.1-1: Metabolism of cysteine in the human body (Source:Stipanuk et al. (2006))

Absorption and metabolism of NAC

The EFSA opinion on N-Acetyl-L-cysteine for use in foods for particular nutritional uses and in foods for special medical purposes from 2003 elaborated on the absorption and metabolism of NAC (EFSA, 2003). The following is cited from this opinion: "In humans, single or multiple oral doses of NAC resulted in measurable plasma levels of NAC in most subjects and in

significant increases of plasma L-cysteine or L-cystine, total and protein SH groups, non-protein SH groups and disulphide-bound thiols, showing that NAC is metabolised to cysteine and further metabolites including incorporation into proteins. Only a small percentage of the intact molecule NAC arrives in plasma and tissues (Maddock, 1980). The oral bioavailability in humans, calculated as area under the curve (AUC): AUC (oral)/AUC (i.v.), varied between 6 and 10%, probably due to the fast metabolism in the gut wall and liver (Borgström et al., 1986)."

NAC is readily converted in cells to cysteine (Raftos et al., 2007). NAC is deacetylated in isolated hepatocytes and supports glutathione synthesis in these cells (Thor et al., 1979). Also, human endothelial cells, rat lung, intestinal, and liver homogenates, and human liver homogenates catalyse the deacetylation of NAC (Sheffner, 1966; Sjodin et al., 1989).

Bioequivalence NAC to L-cysteine

The following is cited from the EFSA opinion on NAC from 2003: "A direct comparison of plasma bioavailability of L-cysteine from oral NAC and L-cysteine has not been undertaken. However, NAC was found to be bioequivalent to an isomolar amount of L-cystine in stimulating growth of male weanling rats fed enteral formula (Baker and Han, 1993). In accordance with earlier studies, the partial conversion of orally administered NAC to plasma L-cysteine has been demonstrated in humans by showing that the oral administration of 600 mg NAC resulted not only in the appearance of NAC in plasma in a mean maximum concentration of 4.6 µM, but also in an increase of the mean cysteine plasma concentration from 10 µM to a maximum level of 18.6 µM after one hour (Tsikas et al., 1998)".

2.3.2 In animals

The following is also cited from the EFSA opinion on NAC from 2003: "NAC is almost completely absorbed, when orally administered to rats, only 3% of the radioactivity of 35 S-NAC being excreted in the faeces (Bonanomi and Gazzaniga, 1980). It is effectively deacetylated by intestinal epithelial cells of rats (Cotgreave et al., 1987) and rat liver, lung and intestine homogenates (Sjodin et al., 1989). A metabolic study with 35 S-NAC in rats showed that cysteine and cystine were the major metabolites in the liver and inorganic sulphate was the major urinary excretion product (Sheffner, 1966). Another study in rats demonstrated that the levels of cysteine and inorganic sulphite in the portal vein plasma were five and three times higher than that of NAC, respectively, following intraintestinal administration of NAC (Cotgreave et al., 1987)."

2.4 Toxicological data/Adverse effects

2.4.1 Human studies

In humans, oral doses of 5 and 10 g of L-cysteine provoked nausea and slight dizziness (Carlson et al., 1989). In addition, healthy people who were administered with increasing

doses of up to 20 g of L-cysteine (with tranylcypromine) exhibited fatigue, dizziness, nausea and insomnia depending on the dose (Davis et al., 1972).

Table 2.4.1-1: Overview of human studies investigating NAC and adverse health effects.

Reference	Participant characteristics, age groups	Country	Number in treatment group		Doses, mg/day	Main endpoints	Length of follow-up	Adverse effects
			NAC	Control				
RCTs								
Kopke et al. (2015)	Hearing loss in marine recruits, 18-35 years	USA	317	317	2700	Hearing	13 days	Several reported, but no differences between the groups. No severe adverse events
Mousavi et al. (2015)	Methamphetamine addicts, mean age 29 years	Iran	23	23	1200	Methamphetamine craving	4 weeks (crossover)	No differences between the groups. No severe adverse events
Berk et al. (2014)	Depressed, mean age 50 years	Australia	135	134	2000	Change in symptom scale	12 weeks	The NAC group had a greater rate of gastrointestinal and musculoskeletal adverse events
Zheng et al. (2014)	COPD patients, mean age 67 years	China	504	502	1200	Exacerbations	1 year	Several, reported. 9% had adverse events regarded by the investigators as possibly related to study products, as did 7% patients who received placebo
Tse et al. (2013)	COPD patients, 66 years	Hong Kong	58	62	1200	Lung function, symptom scale	1 year	Several reported, but no differences between the groups. No severe adverse events
Gray et al. (2012)	Cannabis dependent, mean age 19 years	USA	58	58	2400	Cannabis use	8 weeks	No differences between the groups. No severe adverse events
Hardan et al. (2012)	Children with autism 3-11 years	USA	14	15	900-2700	Symptom scale	12 weeks	Several reported, but no differences between the groups. No severe adverse events
Ferreira et al. (2011)	Healthy volunteers, mean age 30 years	USA	8	7	700-1500	Handgrip exercise	Short, days	No differences between the groups. No severe adverse events
Dauletbaev et al. (2009)	Cystic fibrosis patients, mean age 28 years		11	10	700 or 2800	Safety and clinical parameters	12 weeks	No differences between the groups. No severe adverse events
Grant et al. (2009)	Trichotillomania patients, mean age 34.3 years	USA	25	25	1200/ 2400	Symptom score	12 weeks	Some GI were reported. No differences between the groups. No severe adverse events
Berk et al. (2008a)	Schizophrenia patients, mean age 36.6 years	Australia	69	71	1000	Change in symptom scale	24 weeks	Several reported. None of these related to NAC

Reference	Participant characteristics, age groups	Country	Number in treatment group		Doses, mg/day	Main endpoints	Length of follow-up	Adverse effects
			NAC	Control				
Berk et al. (2008b)	Bipolar disorder subjects, 45 years	Australia	38	37	2000	Change in symptom score	24 weeks	Several reported. No severe related to NAC
Bachh et al. (2007)	COPD patients, mean age 62 years	India	50	50	600	Lung function and exacerbations	1 year	There were no differences between the groups, but the type of adverse effects were not mentioned
LaRowe et al. (2006)	Cocaine dependent, 37 years	USA	11	11	1200	Safety and biochemical parameters	2 days (crossover)	Several reported, but no differences between groups. No severe adverse events
Zuin et al. (2005)	COPD patients, mean age 66 years	Italia	42	39/41	600/1200 mg	Lung function, symptoms, CRP- and interleukin-levels	10 days	Several reported, but no differences between the groups. No severe adverse events
Pela et al. (1999)	COPD, patients, mean age 66 years	Italia	85	84	600	Exacerbations	6 months	No differences between the groups. No severe adverse events
De Flora et al. (1997)	Elderly with chronic illness	Italia	133	129	1200	Episodes of influenza	6 months	No differences between the groups. No severe adverse events
Meta-analysis								
Chalumeau and Duijvestijn (2013)	Children with respiratory infections	World-wide	831	249	Approx 500 mg (max)	Efficacy of mucolytic drugs given during acute respiratory infections		Several biochemical and clinical parameters were recorded. Only mild GI symptoms were associated with NAC
Other human studies								
Mallet et al. (2011)	Children with respiratory infections, 0-2 years	France	30	No	Approx 500 mg (max)	Respiratory Paradoxical Adverse Drug Reactions between 1989 and 2008	Short, therapeutic use of NAC	Only paradoxical drug reactions were examined
Franceschini et al. (1993)	Adults with hypercholesterolemia, 31-61 years	Italy	10	No	Max 3600	Plasma lipids, plasma lipoproteins	4 weeks per dose	No adverse effects were recorded. No increase in lipoprotein concentrations

2.4.1.1 Randomised controlled trials (RCT)

Efficacy and safety of N-acetylcysteine in prevention of noise induced hearing loss: A randomized clinical trial. Kopke et al., 2015

In this randomised, double-blind, placebo controlled trial NAC was given to prevent noise induced hearing loss in US military personnel (Kopke et al., 2015). Safety was a pre-specified outcome of this study that enrolled 566 subjects and randomised them into daily NAC (2700 mg) or placebo for 16 days. There were small positive and borderline significant effects on the hearing measurements. NAC was not associated with (mild or severe) adverse effects. The study participants completed questionnaires regarding adverse events prior to weapons training on each of the 16 days of dosing, and at study completion (approximately 10 days after their last dose). The principal investigator and medical monitor each received daily reports of subject adverse events and medical clinic visits for immediate review and determination of the subject's medical needs and ability to continue in the study.

The efficacy of N-acetylcysteine in the treatment of methamphetamine Dependence: A Double-blind Controlled, Crossover Study. Mousavi et al., 2015

This was a double blind crossover trial in 32 methamphetamine dependent volunteers who were randomised to receive 1200 mg NAC or placebo (Mousavi et al., 2015). Following a wash out period of three days, all participants changed to the alternative treatment. Twenty-three participants completed the study. Adverse events were self-reported and only mild adverse events were observed. The frequency of these events was not different according to placebo or NAC administration.

The efficacy of adjunctive N-acetylcysteine in major depressive disorder: A double-blind, randomized, placebo-controlled trial. Berk et al., 2014

In this study 269 patients with severe depressive symptoms were randomised to receive 2 g NAC or placebo daily (Berk et al., 2014). The mean age of the patients was approximately 50 years. The participants randomised to NAC experienced more often mild gastrointestinal symptoms and musculoskeletal pain than the placebo group. Adverse events were self-reported, three participants (two placebo and one in the NAC group) withdrew because of non-severe adverse events. NAC was not associated with increased risk of severe adverse events.

Twice daily N-acetylcysteine 600 mg for exacerbations of chronic obstructive pulmonary disease (PANTHEON): a randomised, double-blind placebo-controlled trial. Zheng et al., 2014

This is the largest included randomised, placebo-controlled trial on the effect of NAC. From 34 hospitals in China, 1006 patients with chronic obstructive pulmonary disease (COPD) were randomised to receive placebo or 1200 mg NAC daily for a year (Zheng et al., 2014). The mean age of the study participants was 67 years. According to the authors, adverse events

were monitored carefully and 26% of the participants in the placebo group and 29% in the NAC group experienced adverse events, however serious adverse events was more common in the placebo group than in the NAC group. It should be noted that these differences did not reach statistical significance. The most frequently reported serious adverse event was severe COPD, which was an outcome of the trial. Other severe adverse events were coronary artery disease, cerebral infarction, lower respiratory infections, upper respiratory tract infection, and osteoarthopathy. No notable laboratory abnormalities were reported, however, they did not mention which laboratory parameters that were recorded.

High-dose N-acetylcysteine in stable COPD. The 1-year, double-blind, randomized, placebo-controlled HIACE study. Tse et al., 2013

In Hong Kong, 120 patients with COPD were randomised to receive placebo or 600 mg daily for one year (Tse et al., 2013). The mean age of the participants was 71 years. Self-reported adverse events were recorded and there was no increase in incidence of adverse events with NAC, no severe adverse event occurred in either group. The most common adverse events reported were GI discomfort including diarrhea and gastroesophageal reflux disease symptoms. Three patients died during the year NAC was given: one receiving placebo and two receiving NAC and all three were unrelated to this treatment.

A double-blind randomized controlled trial of N-acetylcysteine in cannabis-dependent adolescents. Gray et al., 2012

Cannabis dependent adolescents aged 15-21 years of age were randomised to receive 2400 mg NAC or placebo daily for eight weeks (Gray et al., 2012). The main outcome of this double-blind, randomised placebo-controlled trial was to measure the efficacy of NAC on abstaining from cannabis use. Adverse events were recorded through an open-ended interview. There were no FDA-defined serious adverse events, and there were no significant differences between the two treatment groups in the occurrence of any adverse events (38 events in the NAC group and 46 events in the placebo group). The most common adverse event was upper respiratory infection, which occurred in 19 participants (11 in the NAC group and eight in the placebo group).

A randomized controlled pilot trial of oral N-acetylcysteine in children with autism. Hardan et al., 2012

Thirty-four children (3-11 years) with autism were randomised to receive placebo or NAC in increasing doses from 900 mg at study start till 2700 mg over a 12-week period (Hardan et al., 2012). Main outcome was autism symptoms measured by standard scales. Adverse events were recorded by a standardised, structured questionnaire. Oral NAC was well tolerated with limited side effects. Most adverse effects were gastrointestinal and with a slightly higher non-significant incidence among the NAC participants.

N-Acetylcysteine in handgrip exercise: plasma thiols and adverse reactions. Ferreira et al., 2011.

In this experiment 17 participants were divided into two groups who were given NAC capsules or NAC solutions at different doses (Ferreira et al., 2011). The highest dose was 140 mg/kg (i.e. 9800 mg in an individual of 70 kg). The main outcome of the study was to measure the response of the different doses of NAC on plasma thiol availability and on the performance of a "handgrip exercise". The report did not explicitly define the primary and secondary outcomes of the experiment. Each dose was only given once and adverse reactions were observed for maximum three days. Subjects completed a questionnaire about adverse reactions experienced during the trial. The intensity of adverse reactions was mostly mild, but some individuals experienced GI reactions of moderate intensity after the highest dose of NAC.

A phase II study on safety and efficacy of high-dose N-acetylcysteine in patients with cystic fibrosis. Dauletbaev et al., 2009

Twenty one patients with cystic fibrosis with a mean age of 28 years were randomised to receive 700 or 2800 mg NAC daily for 12 weeks (Dauletbaev et al., 2009). No study participants were given placebo only. The clinical efficacy was similar for these two doses and the high dose did not result in higher rates of adverse events. Several adverse events as well as markers of inflammation and liver function were recorded in this study. However, the power to detect differences between the groups was low and the effects on the biochemical parameters listed in the methods were not reported in the results.

N-acetylcysteine, a glutamate modulator, in the treatment of trichotillomania: A double-blind, placebo-controlled study. Grant et al., 2009

In this double blind, placebo controlled trial that lasted for 12 weeks, 50 individuals (mean age 34.3 years) with trichotillomania were randomised to receive 1200 to 2400 mg of NAC per day throughout the study period (Grant et al., 2009). The main outcome of the study was the effect of NAC on trichotillomania. Safety assessments at each visit included evaluations of blood pressure, heart rate, and weight. Adverse effects were documented and included time of onset and resolution, severity, action taken, and outcome. No adverse events occurred in the NAC group, and NAC was well tolerated.

N-Acetyl Cysteine as a Glutathione Precursor for Schizophrenia-A Double-Blind, Randomized, Placebo-Controlled Trial. Berk et al., 2008

In this study 140 schizophrenia patients with an average age of 36.5 years were randomised to placebo or 1000 mg NAC daily for 24 weeks (Berk et al., 2008a). Adverse effects were self-reported; there were no increased risk of adverse events in the group receiving NAC.

N-Acetyl Cysteine for Depressive Symptoms in Bipolar Disorder-A Double-Blind Randomized Placebo-Controlled Trial. Berk et al., 2008

In this RCT, 75 patients with an average age of approximately 45 years were randomised to receive 2000 mg NAC or placebo daily for 12 weeks (Berk et al., 2008b). Adverse events were self-reported and included fatigue (21% NAC, 27% placebo), headaches (18% NAC, 8% placebo), heartburn (16% NAC, 8% placebo), and increased pain in joints (16% NAC, 8% placebo). No reported event was significantly more common in the NAC group compared with the placebo group. There were seven serious adverse events reported during the study, three were in the NAC group and four were in the placebo group. All were hospitalisations, and all, except a victim of a motorcycle accident, were due to deteriorations in mental state.

Effect of oral N-acetylcysteine in COPD - A randomised controlled trial. Bachh et al., 2007

One hundred patients with COPD were given 600 mg NAC or placebo every day for one year (Bachh et al., 2007). The main outcome of this trial was lung function measured by spirometry and number of exacerbations. The authors reported that there were no adverse effects related to NAC. However, it was not stated which adverse effects were measured and how this was done.

Safety and tolerability of N-acetylcysteine in cocaine-dependent individuals. LaRowe et al., 2006

This crossover study included 11 cocaine dependent individuals who were given 1200 mg NAC or placebo daily during a three-day hospital stay (LaRowe et al., 2006). Each participant was hospitalised twice and randomised to receive either of the treatments during each stay. NAC was well tolerated, no severe adverse effects were reported and there were no differences in adverse effects between the study groups. The manuscript does not give a good description of the design and does not include a figure that indicates the flow of participants through the study. It was not easy to identify the duration of the intervention, the daily dose, as well as the total number of participants of the study.

High-dose N-acetylcysteine in patients with exacerbations of chronic obstructive pulmonary disease. Zuin et al., 2005

In this randomised, double blind placebo controlled trial 123 patients with COPD (mean age 67 years) were randomised in a 1:1:1 ratio to receive placebo, 600, or 1200 mg NAC daily for 10 days (Zuin et al., 2005). Very few, and no severe, adverse events were observed and they were not associated with NAC. Furthermore, no significant changes were observed in vital signs, blood chemistry, whole blood cell count or urinalysis. It is however, unclear how the adverse effects were recorded and which parameters were included in the laboratory assessments.

N-acetylcysteine reduces the exacerbation rate in patients with moderate to severe COPD. Pela et al., 1999

In this study 169 patients with COPD were randomised to receive placebo or NAC (600mg) daily for six months (Pela et al., 1999). NAC treatment resulted in a substantial and significant improvement in lung function and a reduction of exacerbation rate. The study apparently monitored adverse effects carefully; however, how these were registered is not clearly described. The events were self-reported and none were severe or related to NAC.

Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term N-acetylcysteine treatment. De Flora et al., 1997

Two hundred and sixty two Italian elders were recruited from 20 medical centres and randomised in a 1:1 ratio to receive 1200 mg NAC or placebo for six months, the main outcome of the trial was the incidence of influenza (De Flora et al., 1997). NAC was well tolerated and there were no serious adverse events and not a significantly increased risk of self-reported adverse events in the NAC group. None of the laboratory end-points monitored and main vital parameters, such as cardiac frequency and arterial pressure, were different between the groups following the intervention. However, the authors only refer to "routine haematological and biochemical parameters" and do not mention the actual biomarkers assessed.

2.4.1.2 Other human studies

Respiratory paradoxical adverse drug reactions associated with acetylcysteine and carbocysteine systemic use in paediatric patients: A national survey. Mallet et al., 2011

In this French survey, the safety of acetylcysteine in childhood respiratory infection was assessed using voluntary case reports from French physicians between 1989 and 2008 (Mallet et al., 2011). In 2010, the French drug agency, and then the Italian drug agency, withdrew the licenses for children younger than two years old for NAC because their use was associated with paradoxically increased bronchorrhea (increased secretions from the peripheral airways) and acute respiratory distress during respiratory tract infections. In this survey they analysed the data for 30 cases of paradoxical respiratory adverse drug reactions in children younger than six years after an exposure of >200 mg NAC. They concluded "Parents, physicians, pharmacists, and drug regulatory agencies should know that the benefit risk ratio of mucolytic drugs is at least null and most probably negative in infants according to available evidence".

Dose-related increase of HDL-cholesterol levels after N-acetylcysteine in man. Franceschini et al., 1993

In this study 10 hypercholesterolic patients (age 31-61 years) were given graded doses (1200, 2400 and 3600 mg) of NAC daily for four weeks and plasma lipids and lipoproteins

were measured after the three four week regimes (Franceschini et al., 1993). Plasma total, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol and triglyceride concentrations were not affected by either of the NAC doses. However, high density lipoprotein (HDL) cholesterol increased progressively by increasing NAC doses. The authors concluded that the study "failed to note any significant change in plasma lipoprotein (a) levels, even after very high daily doses of NAC".

2.4.1.3 Meta analyses

Acetylcysteine and carbocysteine for acute upper and lower respiratory tract infections in paediatric patients without chronic broncho-pulmonary disease (Cochrane Review). Chalumeau and Duijvestijn, 2013

Efficacy and safety of NAC have been evaluated in a Cochrane meta-analysis and concluded: "This report included the results from nine controlled trials where acetylcysteine was given orally at doses up to approximately 500 mg per day for up to 14 days. The trials that reported safety studied clinical or biological tolerance, or both. The biological tests consisted usually of full blood tests and the monitoring of hepatic and renal function. In two studies, safety was also evaluated using radiographic or pulmonary function tests. The included studies all showed good clinical safety, except mild gastrointestinal tract adverse events and vomiting (Chalumeau and Duijvestijn, 2013).

2.4.1.4 Interactions, allergic sensitisation and adjuvant effects

Treatment with NAC may strengthen the effects of immune suppressants, such as azathioprine, cyclophosphamide, or prednisone. L-cysteine should be used with caution with these medications and such combinations should be discussed with the doctors.

Nitroglycerin and isosorbide: NAC may strengthen the effect of nitroglycerin and isosorbide; two medications commonly used to angina pectoris. But this combination may also raise the risk of side effects, such as severe headache, and may lead to abnormally low blood pressure.

Anaphylactoid reactions have been observed when oral NAC has been given in high doses for treatment of paracetamol toxicity. These reactions have been considered nonimmunological and accordingly not classified as allergic. The gastrointestinal side effects may also, in part, be mediated though the same mechanisms and not by an allergic reaction to NAC or L-cysteine.

2.4.2 Animal studies

According to ANSES, animal studies have shown that high doses cysteine can result in fatty liver and hypercholesterolemia and that it can be neurotoxic in young rodents (ANSES, 2011). However, subsequent animal studies have not been able to reproduce this adverse

effect of cysteine containing compounds on lipid metabolism or hypercholesterolemia (Korou et al., 2014; Lin and Yin, 2008).

2.4.3 Mode of action for adverse effects

Except for the anaphylactoid reactions related to high doses of NAC, no specific or definite mechanism for adverse effects have been described.

2.4.4 Vulnerable and high intake groups

No tolerance level is set for cysteine or cystine specifically for children or adolescents, but an assumption is made that these age groups have similar tolerance as adults.

NAC may enhance the effect of nitroglycerin and isosorbide; two medications commonly used to angina pectoris. But this combination may also raise the risk of side effects, such as severe headaches, and may lead to abnormally low blood pressure.

In the hereditary disease cystinuria, kidney stones are formed from circulating cystine. People with this disease should consult their physician before they take supplemental cysteine or cystine.

In the included literature, no information was available about other vulnerable groups from human studies, including pregnant or nursing women.

2.5 Summary of hazard identification and characterisation

There are several RCTs that have measured the efficacy of NAC at relatively high doses for up to one year. The study groups have been various patient groups ranging from children, adolescents, adults and elderly, but also some healthy subjects. In the RCTs there were no differences in severe adverse events between the placebo and NAC-groups. The following adverse effects were investigated: dizziness, fatigue, energy level, gastrointestinal discomfort, allergic reactions and muscle pain among others. In most of the studies, the results for adverse effects were based on self-reporting systems or clinical examination. A few studies also included analyses of biomarkers from blood or urine samples.

The majority of the studies have been conducted in adults. The included studies demonstrate that it is well documented that the dose 1200 mg, and in some studies even up to 2400 mg NAC per day, do not cause adverse effects. These doses of NAC correspond to 900 and 1800 mg of cysteine and cystine. This is equivalent to 13 and 26 mg cysteine or cystine per kg bw in an adult per day (70 kg as default weight). In the large, recent study by Zheng et al. (2014), where 1200 mg NAC (i.e. 900 mg cysteine) or placebo was given daily for a year to 1000 people, NAC was not associated with increased risk of severe adverse events. These results correspond with those of the other RCTs using NAC.

Studies with doses of 500 mg NAC have been conducted in children (corresponding to 375 mg cysteine or cystine). The few studies that included children and adolescents were of relatively short duration.

Animal studies have shown that high doses can result in fatty liver and hypercholesterolemia and that it can be neurotoxic in young rodents. There are, to our knowledge, no reports from studies in humans that confirm these findings. On the contrary, we were able to identify one study that demonstrated that NAC in increasing doses increased the levels of HDL while not affecting the concentration of other lipoproteins and lipids.

Animal studies included in previous reports with high doses of cysteine over six generations in rats found a NOAEL of 175 mg/kg bw/day at the highest dose.

As value for comparison used in the risk characterisation of cysteine and cystine, VKM will use 900 mg/day corresponding to 13 mg/kg bw/day. This is based on doses used in many studies in various population groups.

3 Exposure / Intake

Exposure of cysteine and cystine was estimated from the intake of food supplements. For food supplements, the intake of cysteine and cystine was estimated for the age groups 10-14 years, 14-18 years and adults (≥18 years).

3.1 Food supplements

The Norwegian Food Safety Authority has requested a risk assessment of 10 mg/day cysteine and 250, 500, 750 and 1000 mg/day cystine in food supplements for children 10 years and above, adolescents and adults. The default body weights (bw) for age groups determined by the EFSA were used: 10 to <14 years=43.4 kg, 14 to <18 years=61.3 kg, and adults=70 kg. The intakes per kg bw is given in (Table 3.1-1).

Table 3.1-1: Estimated exposure of cysteine or cystine from specified doses in food supplements in children, adolescents and adults.

Groups	Daily doses, mg	Body weight	Exposures (mg/kg bw per day)
Children (10 to <14years)	10, 250, 500, 750 and 1000	43.4	0.2, 6, 12, 17 and 23
Adolescent (14 to <18 years)	10, 250, 500, 750 and 1000	61.3	0.2, 4, 8, 12 and 16
Adults (≥18 years)	10, 250, 500, 750 and 1000	70.0	0.1, 4, 7, 11 and 14

3.2 Other sources

Based on distribution data from the 1988–1994 National Health and Nutrition Examination Survey (NHANES III), the common mean daily intake for all life stage and gender groups of cysteine is 1.0 g/day in USA. Men 51 through 70 years of age had the highest intakes at the 99th percentile of 2.2 g/day (IOM, 2005).

4 Risk characterisation

The doses received from NFSA are 10 mg/day L-cysteine and 250, 500, 750 and 1000 mg/day L-cystine in food supplements, and the exposures for adults, adolescents and children above 10 years are given in chapter 3.

The value for comparison used in this risk characterisation is 900 mg/day (corresponding to 13 mg/kg bw/day in a 70 kg adult).

The few studies that included children and adolescents were of relatively short duration. However, there are no data indicating that children and adolescent are more vulnerable than adults for cysteine or cystine. No tolerance level is set for cysteine or cystine specifically for children or adolescents. Assuming similar tolerance for these age groups as for adults, doses below 13 mg/ kg bw in children and adolescents are considered to be unlikely to cause adverse health effects.

VKM considers that:

In adults (≥18 years), the specified doses 10 mg/day L-cysteine and 250, 500 and 750 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects, whereas the dose 1000 mg L-cystine per day may represent a risk of adverse health effects.

In adolescents (14 to <18 years), the specified doses 10 mg/day L-cysteine and 250, 500 and 750 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects in adolescents, whereas the dose 1000 mg L-cystine per day may represent a risk of adverse health effects.

In children (10 to <14 years), the specified doses 10 mg/day L-cysteine and 250 and 500 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects, whereas the doses 750 and 1000 mg L-cystine per day may represent a risk of adverse health effects

5 Uncertainties

All of the human studies included in this report are from randomised placebo controlled trials with NAC. NAC is an acetylated and stable form of cysteine and we have assumed that it has identical biological properties as cysteine and cystine.

Similarly, cystine consists of two cysteine molecules covalently bound to each other via a disulfide bond. Cystine is stable and is metabolised to two cysteine molecules intracellularly. We have therefore also assumed cystine and cysteine to have identical biological properties, which may not be appropriate in all circumstances.

Several of the studies referred to are RCTs, specifically designed to investigate the positive effects of NAC, and not negative effects. Adverse effects are reported, but often based on self-reporting questionnaires or physical examination, and to a lesser extent biomarkers for negative health effects e.g. blood lipids.

There is a lack of studies that have measured the effect of high doses for longer periods. The longest, randomised placebo controlled trials that have measured the effect of daily administration of NAC at doses of 1200 mg per day have been carried out for six to 12 months and there have, to our knowledge, been no attempts to measure adverse or beneficial effects beyond the time of supplementation. The data for children and adolescents are more scarce than for adults.

Animal studies have shown that high doses can result in fatty liver and hypercholesterolemia and that it can be neurotoxic in young rodents. There are, to our knowledge, no reports from studies in humans that confirm these findings.

There is also a risk that our search strategy has failed to identify studies that have reported adverse events as not all RCTs report adverse events in a similar manner and according to existing guidelines such as those suggested by the "Consort group" (http://www.consort-statement.org).

6 Data gaps

There is a lack of studies of adverse effects as primary outcomes of cysteine and cystine in humans. The studies which have reported negative health effects related to NAC in adults have high heterogeneity both in design, target population, and results.

There are few studies on negative health effects related to NAC or L-cysteine / L-cystine in children and adolescents.

7 Conclusions with answers to the terms of reference

The Norwegian Food Safety Authority (NFSA) requested the Norwegian Scientific Committee for Food Safety (VKM) to assess the safety of L-cysteine and L-cystine in food supplements at the following doses: L-cysteine 10 mg and L-cystine 250, 500, 750 and 1000 mg/day for the general population, ages 10 years and above.

In adults, it is well documented that doses up to 900 mg per day L-cysteine or L-cystine for one year is unlikely to cause adverse health effects. The adverse effects reported in a small number of subjects in human studies are generally limited to mild gastrointestinal discomfort. Data for doses above 900 mg/day are more scarce.

Studies on children (10-14 years) and adolescents (14-18 years) were identified. Based on these there was no evidence indicating that children or adolescents are more vulnerable than adults for cysteine or cystine. Therefore, in this risk characterisation a tolerance as for adults, based on body weight, were assumed for these age groups.

We were not able to identify any particular vulnerable groups. However it is possible that cysteine or cystine can modify the effect of certain drugs such as nitroglycerin. The extent to which this represents a risk for individuals on these drugs is uncertain.

VKM concludes that:

- In adults (≥18 years), the specified doses 10 mg/day L-cysteine and 250, 500 and 750 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects, whereas the dose 1000 mg L-cystine per day may represent a risk of adverse health effects.
- In adolescents (14 to <18 years), the specified doses 10 mg/day L-cysteine and 250, 500 and 750 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects in adolescents, whereas the dose 1000 mg L-cystine per day may represent a risk of adverse health effects.
- In children (10 to <14 years), the specified doses 10 mg/day L-cysteine and 250 and 500 mg/day L-cystine in food supplements are considered to be unlikely to cause adverse health effects, whereas the doses 750 and 1000 mg L-cystine per day may represent a risk of adverse health effects.

An overview of the conclusions is presented in Table 6.1.

Table 6.1: An overview of the conclusions for L-cysteine and L-cystine in food supplements.
Green: Estimated exposures to L-cysteine and L-cystine are unlikely to cause adverse health effects.
Red: Estimated exposures to L-cystine may represent a risk of adverse health effects.

	L-Cysteine	**L-Cystine**			
Doses / Age groups	10 mg/day	250 mg/day	500 mg/day	750 mg/day	1000 mg/day
Children (10 to <14 years)					
Adolescents (14 to <18 years)					
Adults (≥18 years)					

8 Referanser

AESAN. (2012) Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on the use conditions for certain substances other than vitamins, minerals and plants in food supplements - 1 Scientific Committee of the Spanish Agency for Food Safety and Nutrition, Spain.

ANSES. (2011) Opinion of the French Agency for Food, Environmental and Occupational Health & Safety on the assessment of the risks associated with substances with nutritional or physiological effects with a view to restricting or prohibiting their use in foodstuffs, French Agency for Food, Environmental and Occupational Health & Safety, Maisons-Alfort, France.

Bachh A.A., Shah N.N., Bhargava R., Ahmed Z., Pandey D.K., Dar K.A., Haq I. (2007) Effect of oral N-acetylcysteine in COPD - A randomised controlled trial. JK Pract 14:12-16.

Berehoiu R.M.T., Popa C.N., Popescu S. (2013) Assessment of the E 920 additive (L-cysteine) in relation to some problems of modern food industry. Scientific Papers Series Management, Economic Engineering in Agriculture and Rural Development 13:413-418.

Berk M., Copolov D., Dean O., Lu K., Jeavons S., Schapkaitz I., Anderson-Hunt M., Judd F., Katz F., Katz P., Ording-Jespersen S., Little J., Conus P., Cuenod M., Do K.Q., Bush A.I. (2008a) N-Acetyl Cysteine as a Glutathione Precursor for Schizophrenia-A Double-Blind, Randomized, Placebo-Controlled Trial. Biol Psychiatry 64:361-368. DOI: http://dx.doi.org/10.1016/j.biopsych.2008.03.004.

Berk M., Copolov D.L., Dean O., Lu K., Jeavons S., Schapkaitz I., Anderson-Hunt M., Bush A.I. (2008b) N-Acetyl Cysteine for Depressive Symptoms in Bipolar Disorder-A Double-Blind Randomized Placebo-Controlled Trial. Biol Psychiatry 64:468-475. DOI: http://dx.doi.org/10.1016/j.biopsych.2008.04.022.

Berk M., Dean O.M., Cotton S.M., Jeavons S., Tanious M., Kohlmann K., Hewitt K., Moss K., Allwang C., Schapkaitz I., Robbins J., Cobb H., Ng F., Dodd S., Bush A.I., Malhi G.S. (2014) The efficacy of adjunctive N-acetylcysteine in major depressive disorder: A double-blind, randomized, placebo-controlled trial. J Clin Psychiatry 75:628-636. DOI: http://dx.doi.org/10.4088/JCP.13m08454.

Carlson H.E., Miglietta J.T., Roginsky M.S., Stegink L.D. (1989) Stimulation of pituitary hormone secretion by neurotransmitter amino acids in humans. Metabolism 38:1179-82.

Chalumeau M., Duijvestijn Y.C. (2013) Acetylcysteine and carbocysteine for acute upper and lower respiratory tract infections in paediatric patients without chronic broncho-pulmonary disease. Cochrane Database Syst Rev 5:CD003124. DOI: 10.1002/14651858.CD003124.pub4.

Dauletbaev N., Fischer P., Aulbach B., Gross J., Kusche W., Thyroff-Friesinger U., Wagner T.O.F., Bargon J. (2009) A phase II study on safety and efficacy of high-dose N-acetylcysteine in patients with cystic fibrosis. Eur J Med Res 14:352-358.

Davis J.M., Spaide J.K., Himwich H.E. (1972) Effects of tranylcypromine and L-cysteine on plasma amino acids in controls and schizophrenic patients. Am J Clin Nutr 25:302-10.

De Flora S., Grassi C., Carati L. (1997) Attenuation of influenza-like symptomatology and improvement of cell- mediated immunity with long-term N-acetylcysteine treatment. Eur Respir J 10:1535-1541. DOI: http://dx.doi.org/10.1183/09031936.97.10071535.

EFSA. (2003) Opinion related to N-Acetyl-L-cysteine for use in foods for particular nutritional uses and in foods for special medical purposes. EFSA Journal 21:1-8.

EFSA. (2006) Opinion related to the use of L-cysteine in foods intended for infants and young children. EFSA Journal 390:1-7.

EFSA. (2007) Amino acids from chemical group 34. Flavouring Group Evaluation 26, Revision 1. Scientific opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food. EFSA Journal 790:1-51.

Ferreira L.F., Campbell K.S., Reid M.B. (2011) N-acetylcysteine in handgrip exercise: plasma thiols and adverse reactions. Int J Sport Nutr Exerc Metab 21:146-154.

Franceschini G., Werba J.P., Safa O., Gikalov I., Sirtori C.R. (1993) Dose-related increase of HDL-cholesterol levels after N-acetylcysteine in man. Pharmacol Res 28:213-8. DOI: 10.1006/phrs.1993.1124.

Fukagawa N.K., Yu Y.M., Young V.R. (1998) Methionine and cysteine kinetics at different intakes of methionine and cystine in elderly men and women. Am J Clin Nutr 68:380-388.

Grant J.E., Odlaug B.L., Suck W.K. (2009) N-acetylcysteine, a glutamate modulator, in the treatment of trichotillomania: A double-blind, placebo-controlled study. Arch Gen Psychiatry 66:756-763. DOI: http://dx.doi.org/10.1001/archgenpsychiatry.2009.60.

Gray K.M., Carpenter M.J., Baker N.L., DeSantis S.M., Kryway E., Hartwell K.J., McRae-Clark A.L., Brady K.T. (2012) A double-blind randomized controlled trial of N-acetylcysteine in cannabis-dependent adolescents. Am J Psychiatry 169:805-812. DOI: http://dx.doi.org/10.1176/appi.ajp.2012.12010055.

Hardan A.Y., Fung L.K., Libove R.A., Obukhanych T.V., Nair S., Herzenberg L.A., Frazier T.W., Tirouvanziam R. (2012) A randomized controlled pilot trial of oral N-acetylcysteine in children with autism. Biol Psychiatry 71:956-961. DOI: http://dx.doi.org/10.1016/j.biopsych.2012.01.014.

IOM. (2005) Protein and Amino Acids, in: I. O. M. (IOM) (Ed.), Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids, The National Academies Press, Washington DC. pp. 589-768.

Kopke R., Slade M.D., Jackson R., Hammill T., Fausti S., Lonsbury-Martin B., Sanderson A., Dreisbach L., Rabinowitz P., Torre P., Balough B. (2015) Efficacy and safety of N-acetylcysteine in prevention of noise induced hearing loss: A randomized clinical trial. Hear Res 323:40-50. DOI: http://dx.doi.org/10.1016/j.heares.2015.01.002.

Korou L.M., Agrogiannis G., Koros C., Kitraki E., Vlachos I.S., Tzanetakou I., Karatzas T., Pergialiotis V., Dimitroulis D., Perrea D.N. (2014) Impact of N-acetylcysteine and sesame oil on lipid metabolism and hypothalamic-pituitary-adrenal axis homeostasis in middle-aged hypercholesterolemic mice. Sci Rep 4. DOI: Artn 6806

Doi 10.1038/Srep06806.

LaRowe S.D., Mardikian P., Malcolm R., Myrick H., Kalivas P., McFarland K., Saladin M., McRae A., Brady K. (2006) Safety and tolerability of N-acetylcysteine in cocaine-dependent individuals. Am J Addict 15:105-110. DOI: http://dx.doi.org/10.1080/10550490500419169.

Lin C.C., Yin M.C. (2008) Effects of cysteine-containing compounds on biosynthesis of triacylglycerol and cholesterol and anti-oxidative protection in liver from mice consuming a high-fat diet. Br J Nutr 99:37-43. DOI: Doi 10.1017/S0007114507793881.

Mallet P., Mourdi N., Dubus J.C., Bavoux F., Boyer-Gervoise M.J., Jean-Pastor M.J., Chalumeau M. (2011) Respiratory paradoxical adverse drug reactions associated with acetylcysteine and carbocysteine systemic use in paediatric patients: A national survey. PLoS One 6. DOI: http://dx.doi.org/10.1371/journal.pone.0022792.

Mousavi S.G., Sharbafchi M.R., Salehi M., Peykanpour M., Sichani N.K., Maracy M. (2015) The efficacy of N-acetylcysteine in the treatment of methamphetamine dependence: A double-blind controlled, crossover study. Arch Iran Med 18:28-33.

Pela R., Calcagni A.M., Subiaco S., Isidori P., Tubaldi A., Sanguinetti C.M. (1999) N-acetylcysteine reduces the exacerbation rate in patients with moderate to severe COPD. Respiration 66:495-500.

Raftos J.E., Whillier S., Chapman B.E., Kuchel P.W. (2007) Kinetics of uptake and deacetylation of N-acetylcysteine by human erythrocytes. Int J Biochem Cell Biol 39:1698-1706. DOI: DOI 10.1016/j.biocel.2007.04.014.

Sheffner A.L. (1966) Metabolic studies with acetylcysteine. Biochem Pharmacol 15:1523-1535.

Sjodin K., Nilsson E., Hallberg A., Tunek A. (1989) Metabolism of N-acetyl-L-cysteine. Some structural requirements for the deacetylation and consequences for the oral bioavailability. Biochem Pharmacol 38:3981-5.

Stipanuk M.H., Dominy J.E., Lee J.I., Coloso R.M. (2006) Mammalian cysteine metabolism: New insights into regulation of cysteine metabolism. J Nutr 136:1652s-1659s.

Thor H., Moldeus P., Orrenius S. (1979) Metabolic activation and hepatotoxicity. Effect of cysteine, N-acetylcysteine, and methionine on glutathione biosynthesis and

bromobenzene toxicity in isolated rat hepatocytes. Arch Biochem Biophys 192:405-13.

Tse H.N., Raiteri L., Wong K.Y., Yee K.S., Ng L.Y., Wai K.Y., Loo C.K., Chan M.H. (2013) High-dose N-acetylcysteine in stable COPD: The 1-year, double-blind, randomized, placebo-controlled HIACE study. Chest 144:106-118. DOI: http://dx.doi.org/10.1378/chest.12-2357.

VKM. (2011) Risk categorisation of amino acids, Vitenskapskomiteen for mattrygghet, Oslo, Norway.

WHO. (1994) Assessing human health risks of chemicals: derivation of guidance values for health-based exposure limits, Environmental Health Criteria 170, World Health Organization.

Zheng J.P., Wen F.Q., Bai C.X., Wan H.Y., Kang J., Chen P., Yao W.Z., Ma L.J., Li X., Raiteri L., Sardina M., Gao Y., Wang B.S., Zhong N.S. (2014) Twice daily N-acetylcysteine 600 mg for exacerbations of chronic obstructive pulmonary disease (PANTHEON): A randomised, double-blind placebo-controlled trial. The Lancet Respiratory Medicine 2:187-194. DOI: http://dx.doi.org/10.1016/S2213-2600%2813%2970286-8.

Zuin R., Palamidese A., Negrin R., Catozzo L., Scarda A., Balbinot M. (2005) High-dose N-acetylcysteine in patients with exacerbations of chronic obstructive pulmonary disease. Clin Drug Investig 25:401-408. DOI: http://dx.doi.org/10.2165/00044011-200525060-00005.

9 Appendix

Search strategy for this risk assessment:

---

1. (cystein* or l cysteine* or cystin* or acetylcystein* or n acetylcystein* or n acetyl cysteine*).ti. (51581)
2. (risk* or safety or adverse or side-effect*1 or hazard* or harm* or negative or contraindicat* or contra-indicat* or interact* or toxicity or toxic).tw. (9677036)
3. 1 and 2 (10038)
4. (homocysteine* not (homocysteine* and (cystein* or cystin* or acetylcystein* or n acetylcystein* or n acetyl cystein*))).mp. (48526)
5. 3 not 4 (10038)
6. (conference abstract* or letter* or editorial*).pt. (4356439)
7. 5 not 6 (9497)
8. limit 7 to (danish or english or norwegian or swedish) (9180)
9. limit 8 to human [Limit not valid in Global Health; records were retained] (4050)
10. remove duplicates from 9 (2525)