Scientific Committee on Consumer Safety

SCCS

OPINION ON

Titanium Dioxide (nano form)

COLIPA n° S75

The SCCS adopted this opinion by written procedure on 22 July 2013

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SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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1. BACKGROUND

The first scientific opinion on the safe use of titanium dioxide as a UV-filter at a maximum concentration of 25% in cosmetic products was adopted 24 October 2000 by the SCCNFP (SCCNFP/0005/98).
However, a review of the substance in its nanoform is deemed necessary according to the opinion on Safety of Nanomaterials in Cosmetic Products adopted on 18 December 2007 (SCCP/1147/07), where it is stated that:

"The SCCNFP opinion from 2000 (SCCNFP/0005/98) is on micro-crystalline preparations of TiO2 and preparations of coarse particles. However, since this opinion, new scientific data on nanosized particles including, TiO2 has become available. Therefore, the SCCP considers it necessary to review the safety of nanosized TiO2 in the light of recent information. Also, a safety assessment of nanosized TiO2, taking into account abnormal skin conditions and the possible impact of mechanical effects on skin penetration needs to be undertaken".

Supplementary information on nanosized Titanium dioxide was submitted following a meeting with stakeholders on 1 October 2008, where data requirements were agreed.

Titanium Dioxide is currently regulated - irrespectively of its form - as a UV-filter in a concentration up to 25% in cosmetic products in Annex VII, entry 27 of the Cosmetics Directive.

1.1 TERMS OF REFERENCE

1. *Does SCCS consider that use of titanium dioxide in its nanoform as a UV-filter in cosmetic products in a concentration up to maximum 25.0 % is safe for the consumers taken into account the scientific data provided?*

2. *In order for the COM to differentiate in the regulation between materials in its nanoform and its non-nano form, can the SCCS give quantitative and qualitative guidance on how this differentiation should be given based on the particle size distribution or other parameters?*

1.2 OPINION

1.3 Chemical and Physical Specifications

1.3.1 Chemical identity

Titanium Dioxide

1.3.1.1 Primary name and/or INCI name

Titanium Dioxide

1.3.1.2 Chemical names

Titanium Dioxide

1.3.1.3 Trade names and abbreviations

COLIPA No. S75

1.3.1.4 CAS / EC number

CAS number: 13463-67-7

EC: 236-675-5

Other registry numbers: 100292-32-8; 101239-53-6; 1025343-79-6; 116788-85-3; 12000-59-8; 1205638-49-8; 1236143-41-1; 12701-76-7; 12767-65-6; 12789-63-8; 1309-63-3; 1344-29-2; 1377807-26-5; 1393678-13-1; 1400974-17-5; 158518-86-6; 185323-71-1; 185828-91-5; 188357-76-8; 188357-79-1; 195740-11-5; 221548-98-7; 224963-00-2; 246178-32-5; 252962-41-7; 37230-92-5; 37230-94-7; 37230-95-8; 37230-96-9; 39320-58-6; 39360-64-0; 39379-02-7; 416845-43-7; 494848-07-6; 494848-23-6; 494851-77-3; 494851-98-8; 52624-13-2; 55068-84-3; 55068-85-4; 552316-51-5; 62338-64-1; 767341-00-4; 859528-12-4; 861455-28-9; 861455-30-3; 866531-40-0; 97929-50-5; 98084-96-9. [Source: ChemIdPlus]

1.3.1.5 Structural formula

TiO2

1.3.1.6 Empirical formula

Formula: TiO2

1.3.2 Physical form

Titanium Dioxide (TiO2¸ COLIPA No. S75, CAS No. 13463-67-7) is described as a solid, white, odourless powder. The TiO2 materials used in sunscreen products are reported to be composed of two crystalline types: rutile and anatase or a mixture of the two. The different materials included in the dossier have been reported to be needle, spherical, or lanceolate (longer than wide) in shape. The primary particle size of the TiO2 nanomaterials has been reported to range from around 20 to 100 nm.

Nanoparticles are generally known to have a tendency to stick together to form agglomerates and/or aggregates, and it is claimed by the Applicant that, in sunscreen products, TiO2 is not present in the form of primary nanoparticles but as aggregates of a

size between 30 nm to >150 nm. These aggregates are claimed to be formed during the manufacturing process.

SCCS Comment
For this opinion, the trade names of the nanomaterials under assessment have been coded by the SCCS in Table 1, and are subsequently referred to by the relevant codes.

Fifteen (15) TiO2 nanomaterials have been presented in the submission for evaluation. They include uncoated as well as surface-coated nanomaterials with various organic and inorganic coating materials. A range of coating materials has been used which include hydrophilic, hydrophobic and amphiphilic materials, such as alumina/silica, methicone/silica, aluminium hydroxide and dimethicone/methicone copolymer, trimethyloctylsilane, alumina/silicone and alumina/silica/silicone, dimethicone, simethicone, stearic acid, glycerol, dimethoxydiphenylsilane, triethoxycaprylylsilane (Table 1).

The coating materials have been stated by the Applicant to be those that are common cosmetic ingredients. The purpose of coatings has been stated to include improvement of the dispersion of TiO2 nanomaterials within the cosmetic formulation, inhibiting or controlling photoactivity, and improving compatibility with other ingredients in sunscreen formulations. The coatings applied to nanoparticle surface are also stated to be not UV absorbers themselves, and three studies have been provided (submission II – Ref 62 and 63, and Submission III – Ref 68) to indicate that the coatings (e.g. silica/alumina) are stable in formulation, as well as under different conditions of pH, temperature, shear force, etc.

SCCS Comment
It is stated by the Applicant that '[the stability of coating] is certainly less relevant from a human-safety aspect, especially since materials used as coating agents for TiO2 may be present as constitutive ingredients of the same cosmetic product'. This may be true for some materials, but it also needs to be considered that a range of materials has been used for coating the TiO2 nanomaterials under current assessment. Some of these materials have been used in a substantially high coating to nanomaterial ratios (e.g. 16% alumina). Although a few studies showing coating stability have been provided, it is important to know whether this, for example, could lead to the release of aluminium ions from alumina that may be present after the coating process and which may dissolve in the final formulation. Thus, where appropriate, safety of the coating materials should also be considered in their own right because a significant dissolution of a coating component, such as alumina, may require a separate safety assessment.
Other cosmetic ingredients applied as stable coatings on TiO2 nanomaterials can also be used if they are accepted by the regulatory authorities and are demonstrated to be safe for use as cosmetic ingredient.

Table-1: Form and composition TiO2 nanomaterials

Material code	TiO2 purity/form	Coating material	Doping material	Form	Bulk density (g/cm3)	VSSA (m2 cm-3)
S75-A	> 99.5% Rutile	6% silica, 16% alumina	None	Oil dispersion	0.35	460
S75-B	> 99.5% Rutile	6% silica, 16% alumina	None	Aqueous dispersion	0.35	460
S75-C	> 99.5% Rutile	7.5% alumina, 9,5% aluminium stearate	None	Oil dispersion	0.31	220
S75-D	> 99.5% Rutile	10% alumina, 13.5% stearate	None	Oil dispersion	0.58	300
S75-E	> 99.5% Rutile	10% alumina, 13.5% stearate	None	Aqueous dispersion	0.58	300
S75-F	Anatase 85%, Rutile 15%	7.5% trimethoxycaprylylsilane	None	Hydrophobic powder	0.2	192
S75-G	Anatase 85%, Rutile 15%	None	None	Hydrophilic powder	0.13	213
S75-H	> 99,5% Rutile	6% alumina, 1% glycerin	None	Hydrophilic powder	0.31	260
S75-I	> 99,5% Rutile	7% alumina 10% stearic acid	None	Hydrophobic powder	0.28	300
S75-J	> 99,5% Rutile	6% alumina 1% dimethicone	None	Hydrophobic powder	0.31	260
S75-K	> 94% Rutile	6-8% aluminium hydroxide, 3.5-4.5% dimethicone/methicone copolymer	None	Hydrophobic powder	0.12-0.28	426
S75-L	> 94% Rutile	6.5-8.5% hydrated silica, 2.5-4.5% aluminium hydroxide, 4.5-6.5% dimethicone/methicone copolymer	None	Hydrophobic powder	0.07-0.2	426
S75-M	> 98% Rutile, <2% anatase	17% silica	None	Hydrophilic powder	0.09	260
S75-N	> 95% Rutile, <5% anatase	Alumina 10% simethicone 2%	1000 ppm Fe	Amphiphilic powder	0.16	400
S75-O	100% Anatase	Simethicone 5%	None	Hydrophobic powder	0.75	400

1.3.3 Molecular weight

Molecular weight of TiO2: 79.9 g/mol.

1.3.4 Purity, composition and substance codes

According to the Applicant, the TiO2 nanomaterials have been produced according to USP 31 specifications, in high purity, with concentration of the active material ≥99.0 %. It is also stated that the materials do not contain heavy metals (e.g., Hg, Cd, Pb, As or Sb) beyond the generally accepted limits.

SCCS Comments

1. Two materials (S75-K and S75-L) are quoted to have TiO2 purity of >94%. No further information has been provided on the impurities in these two materials.
2. The nanomaterials included in the submission have been stated to be manufactured according to USP-31 specifications, with no heavy metals beyond the 'generally accepted limits'. The Applicant should provide the contents of heavy metals, such as Hg, Cd, Pb, As and Sb, which are considered 'acceptable' under USP-31, as they may or may not be considered acceptable under the EU regulations. In addition, impurities of well-known metallic contact allergens, such as Cr, Co, Ni, should also be reported.

1.3.5 Impurities / accompanying contaminants

Details not provided.

SCCS Comment

Information on the impurities has not been provided for any of the nanomaterials. This is especially important for the two materials (S75-K and S75-L) that have a stated TiO2 purity of >94%.

1.3.6 Solubility

TiO2 is insoluble in water and organic solvents. It also has a very low dissociation constant in water and aqueous systems, and thus can in practice be considered as insoluble also under the physiological conditions.

(Numerous references in open literature)

1.3.7 Partition coefficient (Log Pow)

Log P_{ow}: Not applicable for uncoated TiO2.

(Reference: 137)

SCCS Comment

A method to determine partition coefficient of nano particles coated with organic materials is not yet available. However, distribution of TiO2 nanomaterials coated with organic substances between polar and non polar phases should be described.

1.3.8 Additional physical and chemical specifications

Melting point:	Not provided
Boiling point:	Not applicable
Flash point:	Not applicable
Vapour pressure:	Not applicable
Density:	The Tap Density of the titanium dioxide powders was measured according to DIN ISO 787/11 (Table 1)
Viscosity:	Not provided
pKa:	Not applicable for uncoated TiO2
Refractive index:	Not provided

UV_Vis spectrum (200-800 nm): UV data only (see Table 3)

SCCS Comment
The dissociation kinetics of the materials in acidic media can be potentially modified by certain coatings. However, considering the physicochemical properties of TiO2, it is agreed that, for TiO2 nanomaterials, coatings are unlikely by definition to change the dissociation constant of TiO2 in water.

Table-2: Physicochemical properties of TiO2 nanomaterials

Material code	Crystal size	Aspect ratio	UV Absorption (Extinction coefficient)			Zeta potential	Photo-catalytic activity		Photo-stability	Coating stability
	(XRD)	(L /W)	E308	E360	E400	(IEP)	ΔE	% to Reference		
S75-A	15	3.8	44	20	11	7	3	9	Photo-stable	Stable
S75-B	15	3.8	51	22	12	N/A	3	9	Photo-stable	Stable
S75-C	15	3.7	54	16	7	N/A	7.8	23	Photo-stable	Stable
S75-D	9	4.5	48	7	3	N/A	7.2	21	Photo-stable	Stable
S75-E	9	4.5	50	10	4	N/A	7.2	21	Photo-stable	Stable
S75-F	21	1.2	45	15	8	N/A	11.8	35	Photo-stable	Stable
S75-G	21	1.2	38	16	9	7	25.1	74	Photo-stable	Stable
S75-H	21	1.7	30	17	9	7	0.3	1	Photo-stable	Stable
S75-I	15	3.2	38	14	6	N/A	0.8	2	Photo-stable	Stable
S75-J	21	1.5	36	16	9	N/A	0.6	2	Photo-stable	Stable
S75-K	15	3.9	60	12	1	N/A	2.3	7	Photo-stable	Stable
S75-L	15	4.3	55	14	2	N/A	0.8	2	Photo-stable	Stable
S75-M	20	2.6	26	12	5	2	0.6	2	Photo-stable	Stable
S75-N	13	4.1	45	13	5	9	0.7	2	Photo-stable	Stable
S75-O	18	1.2	20	8	5	N/A	15.7	46	Photo-stable	Stable

SCCS Comment
S75-G has been stated to have a stable coating although the material is in fact uncoated. The SCCS considers up to 10% photocatalytic activity of a coated or doped nanomaterial, compared to the corresponding non-coated or non-doped reference, as acceptable.

1.3.9 Droplet size in formulation

According to the information provided by the Applicant, sunscreen spray products containing nano-sized TiO2 are available on the EU market. These spray products are formulated with non-volatile ingredients in pump sprays (without propellant gas) to generate minimal aerosol cloud. It is stated that these products comply with current standards and requirements in terms of droplet size, Mass Median Aerodynamic Diameter (MMAD) of at least 30 µm, with no more than 1% of the droplets having an aerodynamic diameter of 10 µm or less. The Applicant has quoted the Technical Guidance Document on Risk Assessment of the European Chemical Bureau (2003), which considers aerosols with an MMAD >10-15 µm as not respirable for humans because of deposition mainly in the upper regions of the lungs (Reference 148). It is also quoted that the U.S. Silicones Environmental, Health and Safety Council (2001) suggests that a consumer aerosol application for any silicone-based

material, regardless of the method of aerosol generation, should have particle size MMAD at least 30 µm, with no more than 1% of the particles having an aerodynamic diameter of 10 µm or less (Reference 203). The Applicant has provided droplet size distribution measurements for a few sprayable products. The technique used for droplet size measurement was based on Laser Diffraction by Malvern method.

SCCS Comments

- The trade name of one sprayable product suggests that it may be for use by children.
- The droplet size of an aerosolised formulation would affect the entry and uptake of nanomaterial in the lung. It is therefore noteworthy that whilst droplet size would depend on nebulizer/ matrix, it may change due to evaporation/sublimation of the fluid used in the emulsion. Thus, the characteristic dimension of a nanomaterial contained in the formulation would have little relevance to the droplet size, which is typically much larger (tens of micron).
- Although the measurement results indicate that droplet sizes were largely above the respirable range (>10 µm), and only 0.24 to 0.37% of the droplets were in the size range below 20 µm, it should be noted that even a low fraction based on droplet weight is still relevant because it will contain a large number of nanoparticles. The possibility of droplets drying and becoming smaller in size following spraying, and the possible lung exposure to dried residual particles after inhalation also needs to be taken into account. The measurement of the droplet size distribution therefore needs to be complemented by measurements of the size distribution of the dried residual aerosol particles as well, if they can dry on the timescale in a practical use scenario.
- The size distribution of the droplets and dried droplets/ particles should be presented as number size distribution.

1.3.10 Particle size

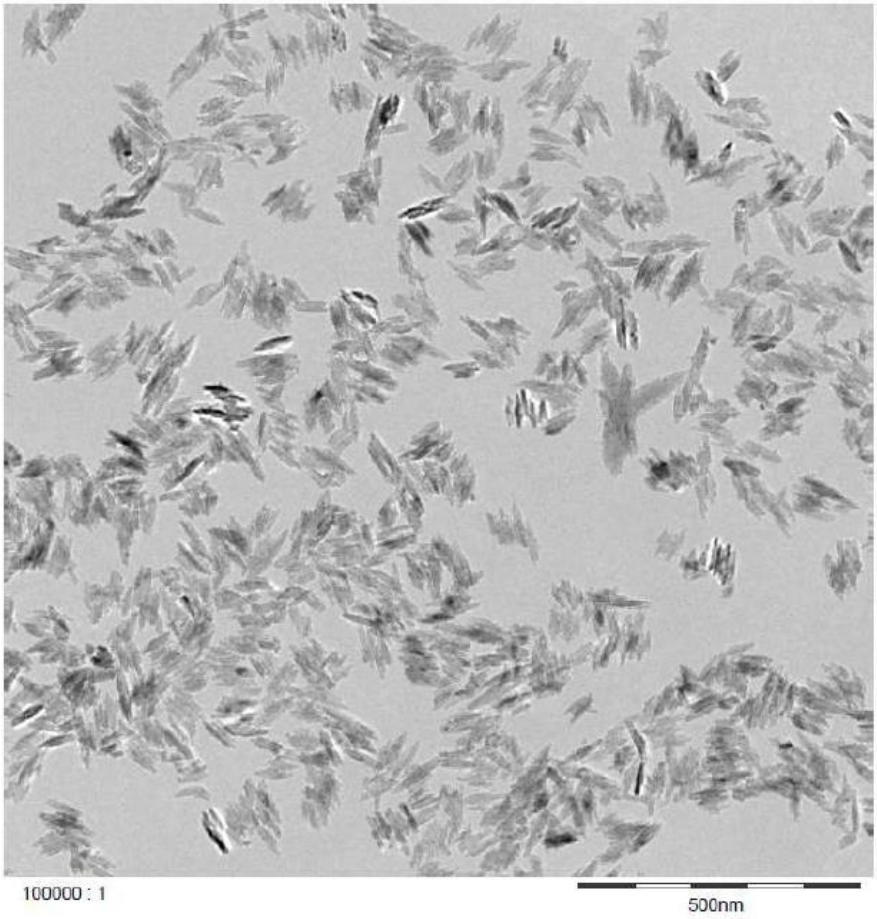
Table 3: Particle size of TiO2 nanomaterials

Material code	**Particle Size Distribution**											
	Lower Cut Off level (nm)				Volume weighted median, $X_{50,3}$ (nm)				Number weighted median, $X_{50,0}$ (nm)			
	CPS	LUMi-sizer	DLS	**Median**	CPS	LUMi-sizer	DLS	**Median**	CPS	LUMi-sizer	DLS	**Median**
S75-A	20	33	35	**33**	53	71	111	**71**	37	48	79	**48**
S75-B	28	34	47	**34**	68	76	145	**76**	47	56	105	**56**
S75-C	20	25	26	**25**	52	49	78	**52**	39	48	59	**48**
S75-D	17	23	15	**17**	35	44	56	**44**	28	34	34	**34**
S75-E	21	27	41	**27**	45	51	104	**51**	37	42	81	**42**
S75-F	35	49	63	**49**	75	92	139	**92**	55	70	115	**70**
S75-G	25	58	54	**54**	77	99	129	**99**	45	79	102	**79**
S75-H	29	63	41	**41**	71	120	112	**112**	50	79	82	**79**
S75-I	22	58	41	**41**	73	107	140	**107**	40	76	103	**76**
S75-J	33	52	35	**35**	71	103	125	**103**	48	69	85	**69**
S75-K	26	34	30	**30**	48	52	75	**52**	41	44	58	**44**
S75-L	33	37	41	**37**	56	64	103	**64**	46	53	80	**53**
S75-M	42	75	73	**73**	119	124	173	**124**	75	99	133	**99**
S75-N	21	37	26	**26**	51	61	91	**61**	41	51	65	**51**
S75-O	24	71	47	**47**	354	653	146	**354**	33	87	85	**85**

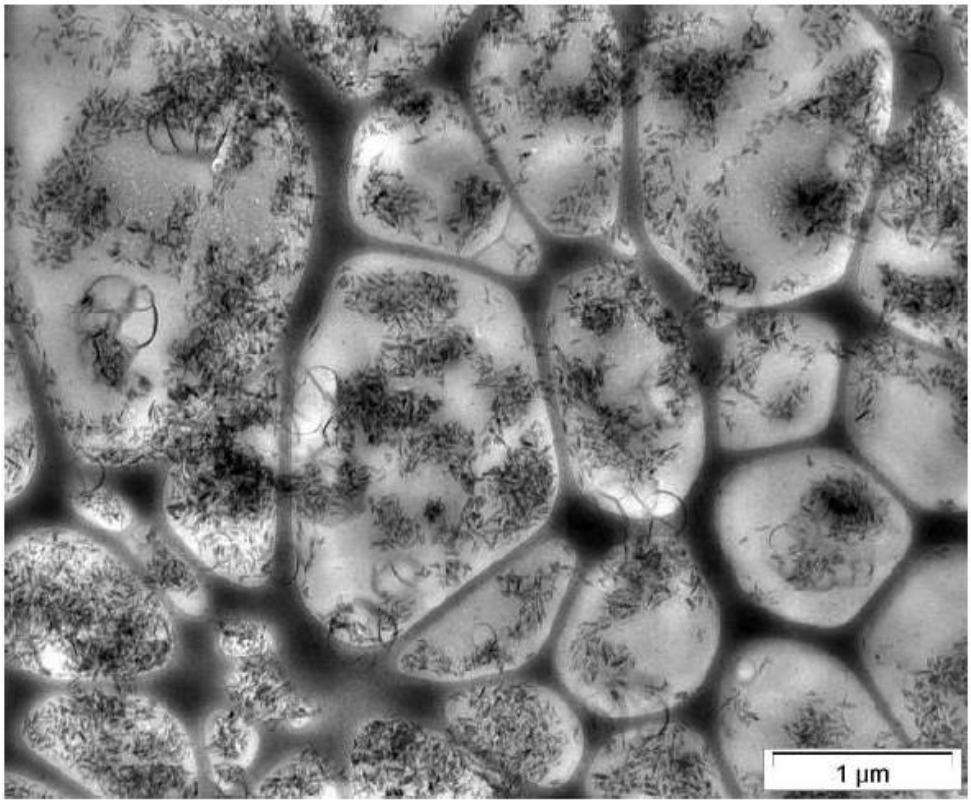
SCCS Comment
The different materials included in the dossier have different particle sizes. These range from ~44 nm to 354 nm on volume weighted median basis, and ~34 nm to ~99 nm on the basis of number weighted median. The lower size cut offs range between 17 nm and 73 nm.

1.3.11 Microscopy

An example transmission electron microscopy (TEM) image of TiO2 nanomaterial is shown below:



An example Cryo-TEM image of TiO2 nanomaterial in formulation is shown below:



SCCS Comment
The different nanomaterials included in the dossier have primary particles that have either spherical, needle, or lanceolate (longer than wide) shapes, and appear to be present in aggregated clusters.

1.3.12 Homogeneity and stability

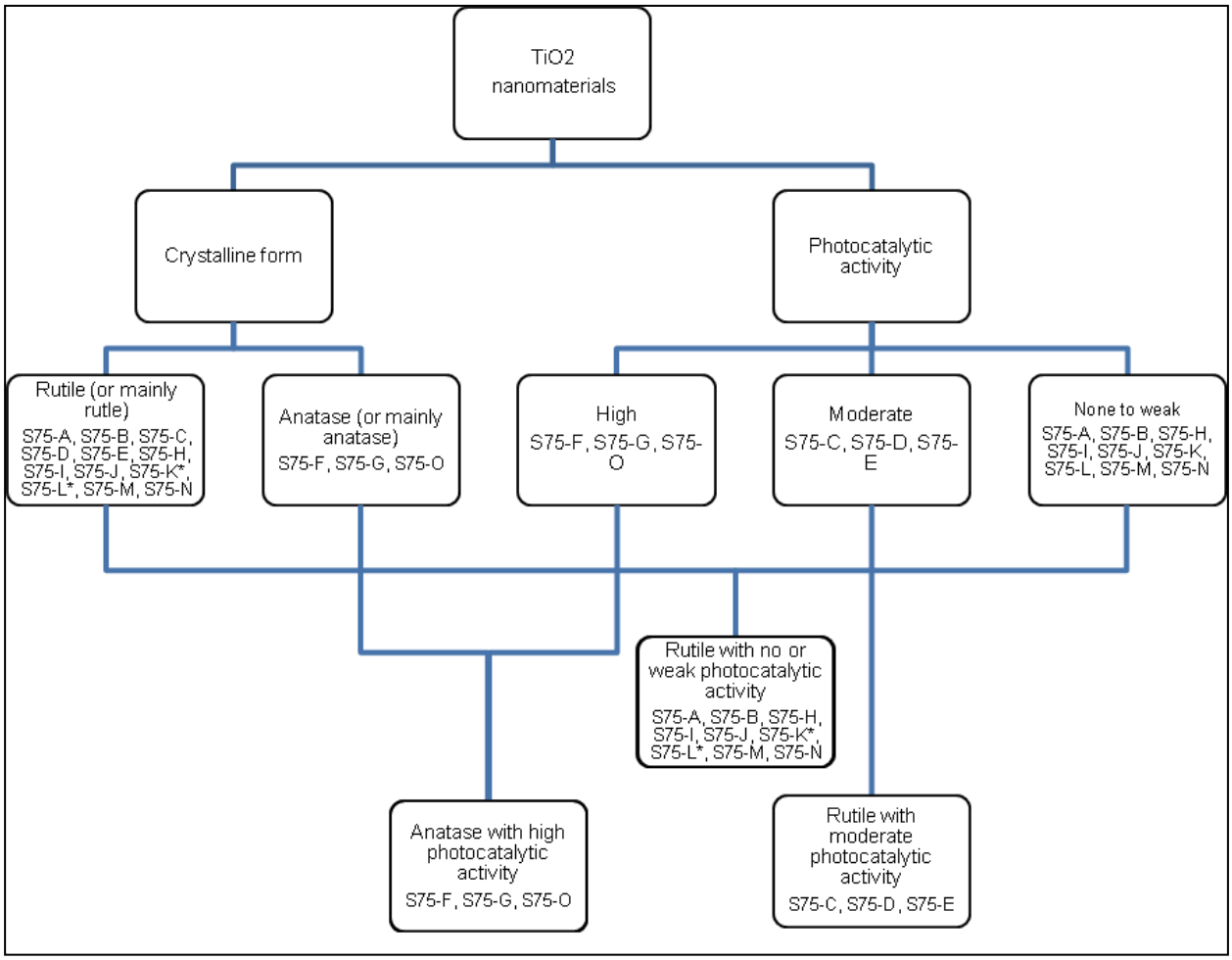
According to the Applicant, the term "dispersion" has been used in relation to the dispersion of TiO2 clusters/ aggregates in the cosmetic product, whereas aggregates bound by strong forces could not be dissociated. They also claim that coating materials on the TiO2 particle are stable under various conditions of pH, temperature and shear forces, and that the materials used as coating agents for TiO2 may also be present as constitutive ingredients of the same cosmetic product.

SCCS Comments on Physicochemical Characterisation
The physicochemical characterisation data provided in the dossier relates to fifteen (15) TiO2 nanomaterials. The data are reasonably extensive, which show that:

1. Two materials (S75-K and S75-L) have a purity of >94%. No further information has been provided on the impurities in these two materials.
2. Ten out of the 15 materials (S75-A, S75-B, S75-C, S75-D, S75-E, S75-H, S75-I, S75-J, S75-K, S75-L) are rutile. Two other materials (S75-M, S75-N) are mainly rutile with a small proportion (2-5%) of anatase.
3. One material (S75-O) is anatase. Two other materials (S75-F and S75-G) are mainly anatase (85%) with rutile (15%).
4. The median particle sizes of the different materials range from ~44 nm to 354 nm on volume weighted basis, and ~34 nm to ~99 nm on number weighted basis. The lower size cut offs range between 17 nm and 73 nm.

5. One material (S75-G) is uncoated, all other materials are surface coated with different coating materials (silica, alumina, organo-silanes).
6. All coatings are reported to be stable at least in the short-term *in vitro* test systems. It is worthy of note that coating on S75-G is also stated to be stable even though the material is in fact uncoated. In view of the diversity of the coating materials and some high coating to nanomaterial ratios, it is important to know the concentration of dissolved coating materials, e.g. alumina that could release aluminium ions, in the final formulation. A significant dissolution of a coating material (e.g. alumina) may require a separate safety assessment for the coating material.
7. One material (S75-N) is doped with 1000 ppm iron. All other materials are not doped.
8. The apparent bulk density of the materials ranges between 0.09 to 0.75 g/cm3. The SCCS notes that the lowest density reported for some materials does not fit in the normal range. As all materials have core particles of TiO2, with sizes in the nano-scale, it is not clear why there is such a large variation in their bulk densities. The Applicant needs to clarify whether the materials with low bulk densities have a porous structure, as in such a case they may have different physicochemical properties from the other TiO2 materials.
9. One material (S75-E) is in aqueous dispersion. All other materials are either hydrophilic or hydrophobic powders, or are in oil dispersions.
10. The VSSAs of the materials range between 192 to 460 m2/cm3 for the different materials, indicating that they are indeed nanomaterials (i.e. VSSA ≥60 m2/cm3).
11. Aspect ratios of the different materials range between 1.2 and 4.5, indicating that the high aspect ratio materials have needle or lanceolate shaped particle structures.
12. All materials are stated to be photostable.
13. UV absorption data for the materials have been provided.
14. Zeta potential measurements have been provided for some materials, and not for others due to difficulties in measuring zeta potential for hydrophobic nanomaterials.
15. Photocatalytic activity data have been provided for all materials. The data show that the materials have differing levels of photocatalytic activity, which ranges from insignificant to weak (S75-A, S75-B, S75-H, S75-I, S75-J, S75-K, S75-L, S75-M, S75-N), to moderate (S75-C, S75-D, S75-E), and strong (S75-F, S75-G; S75-O). All 3 nanomaterials with strong photocatalytic activity are also either anatase form of TiO2, or mainly anatase with some rutile.

From the physicochemical characterisation data provided, the materials could be broadly grouped as shown below for the purpose of this assessment. This grouping is based on the differences between physicochemical properties and the potential effects of anatase/rutile, coated/uncoated, and photocatalytic/non-photocatalytic forms of TiO2 nanomaterials. It is known that uncoated and non-doped TiO2 nanoparticles are photocatalytic when exposed to UV light. The anatase form has been shown to be more photoreactive than rutile or anatase-rutile mixtures (e.g. Sayes et al., 2006). Another indicator of catalytic activity of nanomaterials is the increased generation of reactive oxygen species (ROS) in biological systems and the resulting toxicological effects, such as cytotoxicity. Jiang et al. (2008) noted that the generation of ROS (per unit surface area) was the highest in amorphous nano-TiO2, followed by anatase, anatase/rutile mixture, and rutile. Anatase form of nano-TiO2 has also been reported to be 100 times more cytotoxic under UV than rutile of a similar size (e.g. Sayes et al., 2006).



* S75-K and S75L have stated purity of >94% with no impurity profile provided.

On the basis of above physicochemical considerations, the SCCS has considered the TiO2 nanomaterials in the following 3 groups of for the purpose of this assessment:

- 9 materials (S75-A, S75-B, S75-H, S75-I, S75-J, S75-K, S75-L, S75-M, S75-N) on the basis that they are (mainly) rutile with a relatively low photocatalytic activity. However, two of these materials (S75-K and S75-L) have a stated purity of >94%, with no impurity profile provided. These two materials (S75-K and S75-L) were considered by the SCCS to be not sufficiently pure to include in this opinion.
- 3 materials on the basis that they are rutile with a moderate photocatalytic activity (S75-C; S75-D; S75-E);
- 3 materials on the basis that they are (mainly) anatase, and also that they have a strong photocatalytic activity (S75-F, S75-G, S75-O).

In view of the foregoing, it is important to note that this opinion applies to thirteen (13) TiO2 nanomaterials (S75-A, S75-B, S75-C, S75-D, S75-E, S75-F, S75-G, S75-H, S75-I, S75-J, S75-M, S75-N, S75-O) out of the fifteen (15) nanomaterials presented in this submission. The opinion may, however, be also applicable to other TiO2 nanomaterials that have a close-similarity to the 13 nanomaterials in this submission in terms of the physicochemical parameters listed in Tables 1-3, and other specific provisions laid out in Section 2 below.

1.4 Function and uses

Titanium dioxide is used as an UV-filter in a concentration up to 25% in cosmetic products. It is regulated in Annex VII, entry 27 of the Cosmetics Directive

1.5 Toxicological Evaluation

1.5.1 Acute toxicity

1.5.1.1 Acute oral toxicity

Acute toxicity, single oral administration, rat

Guideline:	OECD Guidelines 401 and EEC Guidelines 92/32/EEC
Species/strain:	8 week old rats/Hsd-Win: WU
Group size:	5 male/ 5 female
Test substance:	TiO2 T805, hydrophobic fluffy white powder, CAS 100209-12-9
Batch:	27073
Purity:	TiO2 96.5%, SiO2 3%, carbon approx 4%.
Vehicle:	suspension in peanut oil
Dose levels:	2150 mg/kg
Dose volume:	21.5 ml/kg of 100 mg/ml
Route:	Oral
Administration:	single dose
GLP:	yes
Study period:	August 1993

References
Submission I - Evonik (Degussa) 1993 (5) and DHS Evonik (Degussa) 1993 (1)

Results
No signs of toxicity recorded during the observation period, no deaths recorded, necroscopy showed no alterations, LD50 for male and female rats >2150 mg/kg.

SCCS Comment
The study relates to S75-F material included in the dossier, which is anatase/rutile material, with organic coating of trimethoxy-caprylylsilane, in oily suspension. This study is relevant to the nanomaterial group (85% anatase, 15% rutile).

Acute toxicity, multiple oral administration, rat

Guideline:	OECD Guidelines 401 and EEC Guidelines 92/32/EEC
Species/strain:	7 week old male rats, 8 week old female rats /Hsd-Cpb: WU
Group size:	5 male/ 5 female
Test substance:	TiO2 T817, hydrophobic fluffy white powder, CAS 100209-12-9
Batch:	04095
Purity:	TiO2 >97%, Fe2O3 2±1%, carbon 3.5-4.5%.
Vehicle:	suspension in olive oil
Dose levels:	Total dose of 2150 mg/kg (dosed twice in equal amount)
Dose volume:	twice dose of 21.5 ml/kg of 50 mg/ml
Route:	Oral
Administration:	single dose
GLP: yes	
Study period:	

DHS Evonik (Degussa), 1993 (2)

Results
No signs of toxicity were recorded during the observation period, no deaths recorded, only signs of diarrhoea in 2 male and 1 female rats from day 1 until day 2 after administration. Necroscopy showed no alterations, LD50 for male and female rats were >2150 mg/kg.

SCCS Comment
The study relates to S75-F material included in the dossier, which is a coated, anatase/rutile material, with organic coating of trimethoxy-n-octyl-silane, in oily suspension. This study is relevant to the nanomaterial group (85% anatase, 15% rutile).

Approximate Lethal Dose study, Intragastric intubation, Rats

Guideline:	OECD Guidelines 401 and EEC Guidelines 92/32/EEC
Species/strain:	7 week old Male rats/Crl-CD®BR
Group size:	not mentioned
Test substance:	TiO2 T805, white powder, CAS number 13463-67-7
Batch:	H-20762
Purity:	TiO2 100%.
Vehicle:	suspension in deionised water
Dose levels:	2,300 to 11,000 mg/kg
Dose volume:	not described
Route:	Oral
Administration:	single dose
GLP:	No (not mentioned)
Study period:	August-October 1994
Reference:	Submission I - DuPont, 1994 (1)

Results
No signs of toxicity were recorded during the observation period, no deaths recorded, pathological examination not performed, weight loss (up to 6%) in some animals after 1 day of dosing, ALD >11000 mg/kg, considered as very low toxicity.

SCCS Comment
The study relates to S75-F material included in the dossier, which is anatase/rutile material, with organic coating of trimethoxy-caprylylsilane, in oily suspension. This study is relevant to the nanomaterial group (85% anatase, 15% rutile).

Exploratory study, acute toxicity, oral, mice (Wang et al., 2007)

Guideline:	OECD Guidelines, No. 420
Species/strain:	mice/ CD-1 (ICR)
Group size:	80 (40 female, 40 male)
Test substance:	TiO2 nanoparticles (25, 80 and 155 nm) - not mentioned whether rutile or anatase
Batch:	not mentioned
Purity:	not mentioned
Vehicle:	0.5% hydroxypropylmethylcellulose K4M used as a suspending agent.
Dose levels:	5 gram/kg bw
Dose volume:	not mentioned
Route:	single oral gavage
Administration:	single high dose 5 g/kg bw gavage.
GLP:	
Study period:	

Reference 213: (Wang, J., Zhou, G., Chen, C., Yu, H., Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B., Sun, J., Li, Y., Jiao, F., Zhao, Y. and Chai, Z. 2007. Acute toxicity and biodistribution of

different sized titanium dioxide particles in mice after oral administration. Toxicol Lett 168 (2): 176-85).

Results
Retention of a small percentage of titanium (measured by ICP-MS) showed predominantly in the liver and spleen. Kidney, liver and heart pathology was observed with all sizes, with more pronounced effects for 80 and 155 nm particles. Changes in serum biochemical parameters (increased lactate dehydrogenase (LDH) and alphahydroxybutyrate dehydrogenase (alpha-HBDH) levels) were most pronounced for 80 nm particles.

SCCS Comment
The study has a number of flaws, and is therefore of little value to this assessment. Sufficient characterisation of the nanomaterials used was not carried out, the administered dose (5 g/kg/bw) was very high, frequent oesophageal ruptures were reported that led to animal deaths, translocation of TiO2 from GI tract was measured as titanium with no evidence that it was in nanoparticulate form. It is not clear whether any of the effects observed were due to TiO2 toxicity, or simply overloading the gut at high dose of the particulate material.

SCCS Comment on Acute Oral Toxicity
The TiO2 nanomaterials tested for this endpoint are mainly anatase/rutile mixtures, coated with trimethoxy-n-octyl-silane. The derived LD50 in rat is >2150 mg/kg. One study has determined the approximate lethal dose at >11000 mg/kg.

In addition, the following two articles have been provided on acute toxicity, but they are of no value to this assessment:

An article by Ferch, Habersang, 1982 (SI-3) is in fact an old review article (up to 1982) which focuses mainly on the possible health effects of amorphous and crystalline silica. It also includes literature review on possible effects of Degussa P25 TiO2 on the formation and induction of granulomatous changes in the lungs or the peritoneum. Since these were not found, the authors claim that P25 TiO2 is not toxic. As such the article does not provide experimental data, but is solely a review of the literature, with the main emphasis on SiO2 and only a few remarks on P25 TiO2.

An article by Warheit et al., 2007 (SI-II-215) is a review of different studies on ultrafine TiO2 particles to develop a base set of toxicity tests. As such it does not provide any details on the studies or any experimental data that could be used for this assessment.

From the limited data available, the acute oral toxicity of nano-TiO2 (anatase and rutile mixtures) appears to be very low.

1.5.1.2 Acute dermal toxicity

Exploratory study, Acute toxicity and Skin and Eye irritation tests, Mouse and Rabbit

Guideline:	OECD Guidelines 401 and EEC Guidelines 92/32/EEC
Species/strain:	Male albino mice (acute toxicity tests), and male albino rabbits (skin irritation tests), male albino rabbits (eye irritation tests)
Group size:	10 mice for toxicity tests, 4 rabbits for skin irritation tests, 3 rabbits for eye irritation tests
Test substance:	TiO2 (referred to as natural colour)
Batch:	
Purity:	not stated
Vehicle:	suspension in water
Dose levels:	up to 10 g/kg for toxicity study, 100mg/square inch for skin patch tests, 100 mg for eye irritation tests

Dose volume:
Route: Oral intubation for toxicity tests, skin patch for irritation test, instillation in lower conjunctival sac of eye,
Administration: 7 days for toxicity tests, 48 hours for skin irritation tests, eye washed after 5 minutes of instillation.
GLP: No
Study period:

Reference 2
(Roy, D. and Saha, J. (1981) Acute toxicity of dyes used in drugs and cosmetics, The Eastern Pharmacist, May 1981, pages 125-126)

Results
No mortality recorded in mice, even at 10 g/kg. No sign of skin irritation or eye irritation. LD50 >10,000 mg/kg, TiO2 regarded as non-toxic, non-irritant to both skin and eye.

SCCS Comment
The study is of little value in relation to the current assessment for nano-forms of TiO2 as characterisation data (particle size distribution) have not been provided to show that the tested materials were nanomaterials.

Acute dermal toxicity, limit test, rat

Guideline: OECD Guidelines 401 and EEC Guidelines 92/32/EEC
Species/strain: 8 week old rats/Sprague Dawley
Group size: 5 male/ 5 female
Test substance: TiO2 NP88/296 (ultrafine), fluffy white powder, CAS 100209-12-9
Batch: control No. 27073; July 27th, 93.
Purity: TiO2 96.5%, SiO2 3%, carbon approx 4%.
Vehicle: suspension in peanut oil
Dose levels: 2000 mg/kg
Dose volume:
Route: Dermal
Administration: single application under occlusion
GLP: yes
Study period: February 1989
Submission I
Croda (Tioxide UK), 1989 (Reference 6)

Results
No deaths recorded after 24 hour dermal administration, under occlusion, of NP 88/296 at 2000 mg/kg. Clinical signs noted only after day 1 of dosing, and included hypokinesia, ataxia, chromodacryorrhoea (eyes and nose), animals hot to the touch. All animals were normal 2 days after dosing. Median Dermal lethal dose (LD50) of NO 88/296 in rats is >2000 mg/kg. No significant abnormalities noted after post-mortem.

SCCS Comment
The study used ultrafine TiO2, and lacks data on characterisation (particle size distribution) of the tested material. According to the Applicant, the material used in this study relates to rutile material coated with alumina/silica (i.e. S75-A, S75-B, S75-C, S75-L). It is however not clear how the test material relates to those included in the dossier and what proportion of the micronized material was in the nano-scale.

SCCS Comment on Acute Dermal Toxicity
The TiO2 material tested in one study is described as 'natural colour'. The other study has used ultrafine TiO2, and it is not clear what proportion of the micronized material (coated

with alumina/silica) was in the nano-scale. Another reference provided in relation to acute toxicity (Submission I – ref 4, Trochimowicz et al., 1988) is in fact a secondary citation of the oral lethal dose cited in another article which relates to chronic inhalation toxicity.
From the provided test data, acute dermal LD50 of TiO2 has been derived at >2000 mg/kg (ultrafine material), and >10,000 mg/kg (natural colour material). However, the provided studies are of no value to the current assessment of nano forms of TiO2.

1.5.1.3 Acute inhalation toxicity

No study has been provided on acute inhalation toxicity. The SCCS has therefore considered relevant studies in the open literature:

Respiratory deposition of particles
Inhaled particulate materials may deposit in the lung depending on size (and shape) of the particles, structure of the lung, and breathing pattern (Sarangapani & Wexler, 2000). The mammalian respiratory tract is often divided into three regions - the extrathoracic (mouth or nose and throat), the trachea-bronchial and the alveolar regions with each having a typical structure and function. In general, particles >10 µm deposit in the extrathoracic region. Nanoparticles also mainly deposit in the extrathoracic region, but alveolar deposition has been noted for particles with a size of 300-200 nm down to 3-2 nm (ICRP 1994 – Oberdorster 2005, Cassee et al. 2002*).*

Particulate materials getting into the lung are generally cleared from the respiratory system. Large insoluble particles are cleared mechanically, whereas those that dissolve in the lung are removed via adsorption. Particles in the extrathoracic region are generally removed by coughing or swallowed into the gastrointestinal tract. Particles deposited into the trachea-bronchial region are in contact with the mucus layer covering the ciliated cells, and are generally cleared via the 'mucociliary escalator', which moves the mucus (and the particles) toward the epiglottis where they are subsequently swallowed and cleared via the GI-tract. Clearing of particles from the alveolar region is much slower and may take weeks to years. The most important pathway here involves alveolar macrophages. These phagocytic cells reside on the alveolar epithelium, and phagocytize the particles. The particle-laden macrophages can be removed via the mucociliary escalator, or can translocate to the interstitial tissue – together with free particles. These clearance mechanisms are similar in humans and most mammals, although clearance rates can significantly differ between species.

Some particles may be retained in the alveoli for long periods (months) before being cleared. A small fraction of the inhaled particles can reach the systemic circulation by passing the pulmonary epithelial barrier; another small fraction can probably reach the brain via olfactory nerve route. It has been shown that ultrafine (including nano) particles have a longer retention time in the alveoli compared to larger particles (Oberdorster, 1994). During chronic and/or cumulative exposure nanoparticles in the alveoli potentially accumulate in the tissue of the entire lungs.

Exposure to ultrafine particles has been linked to inflammatory and neurodegenerative changes in the olfactory mucosa, olfactory bulb, and cortical and subcortical brain structures (Oberdorster, 2005). So far there are no toxicological studies available which show extrapulmonary effects when the exposure was performed under relevant occupational or environmental conditions. Yet there exists a vast epidemiological literature which clearly indicates exposures to urban ambient aerosols containing nano-sized particles at high number concentrations are associated with cardiovascular morbidity and mortality (Pope et al., 2009).

4-Hour Acute Inhalation Toxicity Study in Rats
Authors: Dekker, U.
Reference: RCC-Report B25007, internal report

Guideline:	The following guidelines were considered: European Communities, Directive 92/69/EEC, Part B.2 "Acute Toxicity (Inhalation)", published December 29, 1992 and European Communities Directive 93/21/EEC, April 27, 1993 amending the aforementioned Directive. OECD Guidelines for Testing of Chemicals, Section 4, No. 403: "Acute Inhalation Toxicity", adopted May 12, 1981. U.S. Environmental Protection Agency, Health Effects Test Guidelines OPPTS 870.1300, Acute Inhalation Toxicity, August 1998.
Species/strain:	15 males and 15 females HanRcc:WIST(SPF) rats; 9-10 weeks old
Group size:	15 rats per group, one TiO2 exposed group, one placebo exposed group
Test substance:	TiO2;
Batch:	/
CAS No.	/
Purity:	unknown
Dose levels:	A mean TiO2 aerosol concentration of 4.877 mg/L was inhaled by the rats. TiO2 particles were resuspended in water and jet nebulized. Median aerodynamic diameters (MMADs) and geometric standard deviations (GSD) were 1.4 µm (GSD 2.10)
Route:	Acute 4-hour nose-only inhalation. After a 4-hour inhalation BAL was performed in satellite groups of 5 rats at 14 hours and 2 days after inhalation. The rats were studied at day 15 after exposure.
GLP:	No
Study period:	

Results
In BALF collected at 14 hours post end of exposure, total cell count (neutrophil numbers) and total protein were significantly elevated in both sexes of the exposed group compared to the control group. The changes in BALF were consistent with the histopathology findings of diffuse alveolar histiocytosis and alveolar lining cell activation seen in all animals of the exposed group. Significant increases of the absolute and relative lung weights and histopathology findings of diffuse alveolar histiocytosis and alveolar lining cell activation were found in the exposed group on day 2. These findings were consistent with TNFα and IL-6 levels in BALF higher in females of the exposed group than in control group on day 2.

SCCS Comments
It is not clear which of the three noted guidelines were followed. The distribution was not investigated. The deposited TiO2 particle dose was not determined. The exposed group showed signs of inflammation based on the methodology applied. The study was poorly performed and important control parameters are missing. This is by no means a comprehensive study and is of questionable value to this assessment.

Chronic inhalation Exposure of rats to titanium dioxide dust

Authors:	Trochimowicz, H.J. et al. (1988)
Reference:	Chronic inhalation study ref. No. 4
Guideline:	not specified
Species/strain:	3-6 months ChR-CD rats at the begin of the study
Group size:	11 males + 11 females
Test substance:	TiO2 not specified
Batch:	not specified.
Purity:	not specified
Dose levels:	250 mg/m³, 50 mg/m³, 10 mg/m³,0 mg/m³, 6h/day, 5 days/week, 104 weeks
Route:	chronic inhalation for 104 weeks;
Administration:	whole body exposure
GLP:	not specified
Study period:	/

Results
After 3 months: alveolar cell hyperplasia at doses of 250 mg/m³, 50 mg/m³,
After 6 months: alveolar cell hyperplasia at all dose levels
After 12 months: additionally minute areas of collagen fiber deposition at 250 mg/m³ dose
After 24 months: massive alveolar hyperplasia, focal patches of pneumonia, areas of collagenized fibrosis; only at 250 mg/m³ dose; occurrence of lung tumours
The authors conclude significant patho-physiological alterations at doses of 250 mg/m³, 50 mg/m³ but not at 10 mg/m³

SCCS Comment
This study is one of the early chronic inhalation studies on titanium dioxide which triggered later chronic inhalation studies in the 1980s and 1990s and later investigations into biokinetics and more toxicological endpoints.

Studies in open literature
Several sub-chronic (90 days) TiO2 inhalation exposure studies have been reported:

- Rats inhaled a TiO2 aerosol of 22 mg/m³ concentration consisting either of nanostructured or pigmentary TiO2 particles for 6h/d 5d/wk for 12 consecutive weeks and were followed up for 1 year (Ferin et al., 1992).
- Rats, mice and hamsters inhaled a nanostructured TiO2 aerosol at concentrations of 10, 50 or 250 mg/m³ for 6h/d 5d/wk for 13 consecutive weeks and were followed up for 1 year (Bermudez et al., 2002; Everitt et al., 2000).
- Rats, mice and hamsters inhaled a nanostructured TiO2 aerosol at concentrations of 0.5 or 2 or 10 mg/m³ for 6h/d 5d/wk for 13 consecutive weeks and were followed up for 1 year (Bermudez et al., 2004)

Common findings of these sub-chronic studies were: substantial responses of inflammation and overload associated with diminishing particle clearance in a dose dependent manner, and histologically clear indications of epithelial hypertrophy and hyperplasia. Most pathophysiological responses disappeared after 1 year of recovery and only the very high doses led to persistent adverse effects. Rats always responded more sensitively than mice; hamsters had the least responce. When nanostructured or pigmentary TiO2 particles were compared, stronger effects were observed for the nanostructured particles.

Two 5-day inhalation-exposure studies in rats with a follow-up of 28 days as a substitute of sub-chronic 90-days studies with a follow-up of 1 year have been conducted:

- TiO2 nanoparticles at a concentration of 100 mg/m³, and pigmentary TiO2 particles at a concentration of 250 mg/m³ - with a positive control exposure to quartz particles at 100 mg/m³ (van Ravenzwaay et al., 2009) were investigated. Mild inflammation was reported in lung histology and BAL with subsequent reversibility. All responses were transient but the quartz effects persisted. The authors suggested that the effects seen in these short term studies would be similar to those after 90-day exposure studies. It is however not clear to the SCCS how the major differences seen in these and the other studies can be equated.
- Nanostructured TiO2 particles at concentration of 2, 10 and 50 mg/m³ were investigated. Transient inflammatory responses were observed in lung histology and BAL. (Ma-Hock et al., 2009).

Another intratracheal instillation study used nanostructured anatase TiO2 particles of 5, 23 and 154 nm (actual hydrodynamic diameters of 19, 28 and 176 nm) at a concentration of 5 mg/kg bw administered to the rats and studied until three months after instillation. The results showed that the smaller the particles, the larger the inflammatory response and hypertrophy. However the effects were transient, (Kobayashi et al., 2009). Several other instillation studies have been published that used nano- and submicron-sized TiO2 particles

but they have not been considered here because the particles had already formed larger sized agglomerates.

SCCS Comment on acute inhalation toxicity
No study on acute inhalation toxicity was provided. Studies (including open literature) on acute and sub-chronic inhalation exposure to TiO2 nanomaterials have indicated substantial inflammatory responses, and histologically clear indications of epithelial hypertrophy and hyperplasia at high exposure dose. In view of this, the SCCS does not recommend the use of nano TiO2 in applications that would lead to any significant inhalation exposure (e.g. powder or sprayable products).

1.5.2 Irritation and corrosivity

1.5.2.1 Skin irritation

Skin irritation/corrosion, Patch test, Rabbit

Guideline:	OECD Guidelines 404 and EEC Guidelines 92/32/EEC
Species/strain:	11 month old Rabbit/white Russian
Group size:	3 male
Test substance:	TiO2 T805, hydrophobic fluffy white powder, CAS 100209-12-9
Batch:	27073.
Purity:	TiO2 96.5%, SiO2 3%, carbon approx 4%.
Vehicle:	Paraffin
Dose levels:	0.5 g in 0.64 ml paraffin to dorsal skin area patch 6.25 cm2.
Dose volume:	
Route:	skin patch
Administration:	single application, observation over 3 days
GLP:	yes
Study period:	August 1993

Submission I
Evonik (Degussa), 1993 (13)
DHS Evonik (Degussa), 1993 (5)

Results
Very slight erythema (grade 1 in 2 animals), very slight edema (one animal) after one day of exposure. Primary Irritation Index is 0.3, TiO2 was regarded non-irritant on rabbit skin.

SCCS Comment
The study relates to S75-F material included in the dossier, which is anatase/rutile material, with organic coating of trimethoxy-caprylylsilane, in oily suspension. This study is relevant to the nanomaterial group (85% anatase, 15% rutile).

Skin irritation/corrosion, Patch test, Rabbit

Guideline:	OECD Guidelines 404 and EEC Guidelines 92/69/EEC
Species/strain:	48 month old male, 43 month old female Rabbit/white Russian
Group size:	3 (1 male, 2 female)
Test substance:	TiO2 T817, hydrophobic fluffy white powder, CAS number 100209-12-9
Batch:	
Purity:	TiO2 > 97%, Fe2O3 2±1%, carbon approx 3.5-4.5%.
Vehicle:	
Dose levels:	0.5 g in peanut oil to dorsal skin area patch 6.25 cm2.
Dose volume:	
Route:	skin patch
Administration:	single application, observation over 3 days

GLP:	Yes
Study period:	February 1998
Reference:	DHS Evonik (Degussa), 1998 (6)

Results
No changes observed, neither erythema nor edema observed. Primary Irritation Index was 0.0, TiO2 regarded non-irritant on rabbit skin. No systemic effects observed.

SCCS Comment
The study relates to S75-F material included in the dossier, which is anatase/rutile material, with organic coating of trimethoxy-n-octyl-silane, in oily suspension. The study is relevant to the nanomaterial group (85% anatase, 15% rutile).

Skin irritation/corrosion, Patch test, Rabbit

Guideline:	OECD Guidelines 404 and EEC Guidelines 92/32/EEC
Species/strain:	11 month old Rabbit/ New Zealand white
Group size:	3 male, 3 female
Test substance:	TiO2 H20762, CAS number 13463-67-7
Batch:	
Purity:	TiO2 100%.
Vehicle:	
Dose levels:	0.5 g in pre-moistened patch (2 inch square gauze)
Dose volume:	
Route:	skin patch
Administration:	single application, observation over 3 days
GLP:	No (not mentioned)
Study period:	August-September 1994
Reference:	Submission I - DuPont, 1994 (10)

Results
Three rabbits showed no dermal irritation during the study, no to mild erythema by 1 hour after patch removal. By 24, 48 and 72 hours, no to slight erythema observed, no edema observed during the study. H-20762 is regarded a mild skin irritant.

SCCS Comment
The study is of little value in relation to assessment for nano-form of TiO2 as there is a lack of data on characterisation (particle size distribution) of the tested materials to show that they were nanomaterials.

Skin irritation/corrosion, Patch test, Rabbit

Guideline:	not mentioned
Species/strain:	Rabbit/ albino
Group size:	6 male
Test substance:	TiO2 - referred to as Haskell Nos. (H 12684, H 12685, H 12686)
Batch:	
Purity:	not mentioned
Vehicle:	
Dose levels:	0.5 g pre-moistened with physiological saline (1½ inch square gauze)
Dose volume:	
Route:	skin patch
Administration:	single application, observation over 2 days
GLP:	No (not mentioned)
Study period:	
Reference:	Submission I DuPont, 1978 (11)

Results
No skin irritation observed on intact rabbit skin.

SCCS Comment
The study is of little value in relation to assessment for nano-form of TiO2 as there is a lack of data on characterisation (particle size distribution) of the tested materials to show that they were nanomaterials.

Skin irritation/corrosion, Patch test, guinea pig

Guideline:	not mentioned
Species/strain:	guinea pig/ albino
Group size:	12 male
Test substance:	TiO2 - referred to as 99.5% active ingredient
Batch:	
Purity:	
Vehicle:	
Dose levels:	0.5 g powder and 0.1 g 28% paste were slightly rubbed into shaved back skin, covered with impervious film and wrapped.
Dose volume:	
Route:	skin patch
Administration:	single application, 24 hours, then rinsed in water, observation over 2 days
GLP:	No (not mentioned)
Study period:	
Reference:	Submission I - DuPont, 1969 (12)

Results
No skin irritation observed on intact guinea pig skin.

SCCS Comment
The study is of little value in relation to assessment for nano-form of TiO2 as there is a lack of data on characterisation (particle size distribution) of the tested materials to show that they were nanomaterials.

Skin irritation/corrosion, 5 day repeat application study, Rabbit

Guideline:	not mentioned
Species/strain:	Rabbit
Group size:	2 male, 1 female
Test substance:	TiO2 utrafine dispersion - referred to as NP 89/97, NP 89/98.
Batch:	
Purity:	not mentioned
Vehicle:	
Dose levels:	around 0.5 g (2.5 cm2 patch)
Dose volume:	around 0.5 ml
Route:	skin patch
Administration:	4x repeated (application, removal, skin observation)
GLP:	No
Study period:	
Reference:	Submission I - Croda (Tioxide UK), 1989 (14)

Results
One animal died on day 4 (unrelated to the test), 5 day repeat applications produced mean irritation scores of 1.58 and 1.92 for 89/97, NP 89/98 respectively. NP 89/98 considered slightly more irritant than NP 89/97.

SCCS Comment
The study used ultrafine TiO2, however, data on characterisation (particle size distribution) of the tested material has not been reported. It is therefore not clear whether the material had a nano-sized fraction, and if so, in what proportion.

Skin irritation/corrosion, 5 day repeat application study, Rabbit

Guideline:	not mentioned
Species/strain:	Rabbit/ New Zealand white
Group size:	3 (2 male, 1 female)
Test substance:	TiO2 utrafine dispersion - referred to as NP 88/296.
Batch:	
Purity:	not mentioned
Vehicle:	
Dose levels:	2 dispersions tested (40% A.I. and 10% A.I. which was diluted with carrier oil NP88/310)
Dose volume:	around 0.5 ml
Route:	skin patch
Administration:	4x repeated (application, removal, skin observation)
GLP:	No (not mentioned)
Study period:	
Reference:	Submission I - Croda (Tioxide UK), 1989 (15)

Results
5 day repeat applications produced mean irritation scores of 0.13 for both dispersions tested (i.e. no dose response). Neither the undiluted or diluted test material NP 88/296 produced significant reactions. One rabbit did not react, and the other 2 rabbits showed only slight to non persistent erythema.

SCCS Comment
The study used ultrafine TiO2. However, there is a lack of data on characterisation (particle size distribution) of the tested material. According to Applicant, the material used in this study relates to rutile material coated with alumina/silica (i.e. S75-A, S75-B, S75-C, S75-L). However it is not clear how the test material relates to the nanomaterials included in the dossier and what proportion of the micronized material was in the nano-scale.

SCCS Comment on Skin irritation
The study by Warheit et al., 2007 (SI-II-215) is of no use to this assessment because it is a detailed literature review on the possible effects of different TiO2 ultrafine particles. As such it does not provide details on the studies, or any experimental data, that could be used for this assessment.

Two studies provided in the submission are relevant to the TiO2 nanomaterials. They relate to anatase/rutile mixture, coated with trimethoxy-n-octyl-silane. In one of the studies, the test animals showed signs of very slight erythema and oedema. The primary irritation index was estimated to be zero and 0.3, and the materials regarded as non-irritant on rabbit skin.

Two other studies used ultrafine grade materials and showed the mean irritation scores of 0.3 and 1.58-1.92 during 5 day repeat applications on rabbit skin, but the proportion of nano-scale fraction in the materials used has not been reported.

The remaining 3 studies showing the tested materials as either mild irritant or non irritant to rabbit and guinea pig skin are of little value to this assessment because there is a lack of data on characterisation (particle size distribution) of the tested materials, and it is not clear whether they were in fact nanomaterials.

From the limited useful data presented in the dossier, it appears that the TiO2 nanomaterials are either mild or non-irritant to skin.

1.5.2.2 Mucous membrane irritation

Eye irritation, single application, rabbit

Guideline:	OECD Guidelines 405 (1) and EEC Guidelines 92/32/EEC
Species/strain:	10-11 month old Rabbits/ white Russian (albino)
Group size:	3 (males)
Test substance:	TiO2 T805, hydrophobic fluffy white powder, CAS 100209-12-9
Batch:	27073
Purity:	TiO2 96.5%, SiO2 3%, carbon approx 4%.
Vehicle:	
Dose levels:	22.8 to 24.3 mg
Dose volume:	0.1 ml
Route: eye instillation	
Administration:	single application, 3 days observation period
GLP:	Yes
Study period:	August 1993
Reference:	Submission I - Evonik (Degussa), 1993 (9); DHS Evonik (Degussa), 1993 (3)

Results
No alterations detected in cornea, iris and conjunctiva, primary irritation index is zero, TiO2 (805) regarded as non-irritant on rabbit eye. No systemic toxic effects detected.

SCCS Comment
The study relates to S75-F material included in the dossier, which is anatase/rutile material, with organic coating of trimethoxy-caprylylsilane, in oily suspension. This study is relevant to the nanomaterial group (85% anatase, 15% rutile).

Eye irritation, single application, rabbit

Guideline:	OECD Guidelines 405 (1) and EEC Guidelines 92/69/EEC
Species/strain:	35 month old Rabbits/ white Russian (albino)
Group size:	3 (females)
Test substance:	TiO2 T817, hydrophobic fluffy white powder, CAS number 100209-12-9
Batch:	04095
Purity:	TiO2 >97%, Fe2O3 2±1%, carbon 3.5-4.5%.
Vehicle:	
Dose levels:	11.5 to 16.8 mg
Dose volume:	0.1 ml
Route:	eye instillation
Administration:	single application, 3 days observation period
GLP:	Yes
Study period:	February 1998
Reference:	DHS Evonik (Degussa), 1993 (4)

Results
Some blood vessels definitely hyperaemic in two animals after one hours of application. Primary irritation index is 0.3, TiO2 regarded as non-irritant on rabbit eye. No systemic toxic effects detected.

SCCS Comment
The study relates to S75-F material included in the dossier, which is anatase/rutile material, coated with organic coating of trimethoxy-n-octyl-silane, in oily suspension. This study is relevant to the nanomaterial group (85% anatase, 15% rutile).

Eye irritation, single application, rabbit

Guideline:	OECD Guidelines 405 (1) and EEC Guidelines 92/69/EEC
Species/strain:	Rabbits/ New Zealand white
Group size:	2 (females)
Test substance:	TiO2 H-20762, CAS number 13463-67-7
Batch:	
Purity:	TiO2 100%.
Vehicle:	
Dose levels:	approx. 10 mg
Dose volume:	
Route:	eye instillation
Administration:	single application, eye washed after 20 seconds of application. 3 days observation period
GLP: yes	
Study period:	September 1994
Reference:	Submission I - DuPont, 1994 (7)

Results
Moderate redness and slight chemosis observed in both treated and untreated washed eyes (normal after 1 and 3 days respectively). No clinical signs of toxicity obeserved, TiO2 (H20762) regarded as moderate eye irritant but could be classified as non-irritant under the EEC Directive 93/21, Annex VI.

SCCS Comment
The study is of little value in relation to assessment for nano-form of TiO2 as there is a lack of data on characterisation (particle size distribution) of the tested materials to show that they were nanomaterials.

Eye irritation, single application, rabbit

Guideline:	OECD Guidelines 405 (1) and EEC Guidelines 92/69/EEC
Species/strain:	Rabbits/ New Zealand white
Group size:	3 (2 male, 1 female)
Test substance:	TiO2 NP 88/296 (ultrafine)
Batch:	not mentioned
Purity:	not mentioned
Vehicle:	
Dose levels:	not mentioned
Dose volume:	0.1 ml
Route:	eye instillation
Administration:	single application, eye washed after 20 seconds of application. 3 days observation period
GLP:	Yes
Study period:	
Reference:	Submission I - Croda (Tioxide UK), 1989 (8)

Results
No corneal or iridial reactions, slight conjunctival redness (score 1) which disappeared after 72 hours of treatment. TiO2 (NP88/296) is regarded slightly irritant to rabbbit eyes.

SCCS Comment
The study relates to ultrafine TiO2. However, information on the characterisation (particle size distribution) of the tested material has not been reported. According to the Applicant, the material used in this study relates to rutile material coated with alumina/silica (i.e. S75-A, S75-B, S75-C, S75-L). It is however not clear how the test material relates to the

nanomaterials included in the dossier and what proportion of the micronized material was in the nano-scale.

SCCS Comments on Eye Irritation
The following two articles provided with the submission on acute toxicity are of no value to this assessment:

1. An article by Frosch and Kligman (Reference 16 - S75 irritation skin) refers mainly to the development of a scarification chamber test for irritancy of materials. It does refer irritancy of titanium dioxide as low, but it is not clear whether the tested TiO2 was a nanomaterial.
2. An article by Warheit et al., 2007 (SI-II-215) is a review of different studies on ultrafine TiO2 particles to develop a base set of toxicity tests. As such it does not provide any details on the studies or any experimental data that could be used for this assessment.

Two other studies provided used TiO2 anatase/rutile mixtures, coated with trimethoxy-n-octyl-silane. From these studies, primary irritation index was between zero and 0.3. Another study has regarded the tested material (TiO2-NP88/296) as slightly irritant to rabbit eye. In this study, the material used has been described as ultrafine rutile material coated with alumina/silica (relating to S75-A, S75-B, S75-C, S75-L) but information on characterisation (particle size distribution) has not been reported to indicate what proportion was in the nano-scale. Similarly, another study has regarded the tested material (TiO2-H20762) moderately irritant to rabbbit eye, but it is not clear whether the tested material was a nanomaterial.

From the limited useful data provided, eye irritation potential of nano-TiO2 appears to be low.

1.5.3 Skin sensitisation

Skin sensitisation, Guinea Pig, maximisation test

Guideline:	OECD Guidelines 406 and EEC Guidelines 84/449/EEC
Species/strain:	8 week old 12 male, 10 female guinea pigs/Pirbright white
Group size:	3 (1 male, 2 female)
Test substance:	TiO2 T805, hydrophobic fluffy white powder, CAS 100209-12-9
Batch:	030492
Purity:	TiO2 96.5%, SiO2 3%, carbon approx 4%.
Vehicle:	paraffin oil, Freunds Complete Adjuvant for immunisation
Dose levels:	0.5 g in paraffin oil to dorsal skin area 5 cm2 patch.
Dose volume:	0.1 ml of 0.5% dispersion, 0.2 ml of 5% dispersion for challenge
Route:	Induction application intradermal and epidermal, challenge application epidermal
Administration:	single application, 48 hours, challenge on day 22 for 24 hours, observation over 48 hours
GLP:	yes
Study period:	June 1992
Reference:	Submission I Evonik (Degussa), 1992 (19); DHS Evonik (Degussa), 1992 (7)

Results
Following epidermal challenge neither treated nor control animals showed any changes at the skin. TiO2 regarded as non-sensitiser in maximisation test on guinea pig skin. No systemic effects observed.

SCCS Comment
The study relates to S75-F material included in the dossier, which is anatase/rutile material, with organic coating of trimethoxy-caprylylsilane, in oily suspension. This study is relevant to the nanomaterial group (85% anatase, 15% rutile).

Skin sensitisation, Guinea Pig, Buehler test

Guideline:	OECD Guidelines 406 and EC Guidelines 96/54/EC
Species/strain:	8 week old guinea pigs/PsdPCC: DH
Group size:	20 male, 20 female (2 vehicle control groups of 10, and 1 test group of 20)
Test substance:	TiO2 T817, hydrophobic fluffy white powder, CAS 100209-12-9
Batch:	04095
Purity:	TiO2 > 97%, Fe2O3 2±1%, carbon approx 3.5-4.5%.
Vehicle: paraffin oil	
Dose levels:	0.5 g applied, 3 applications on day 1,8,15.
Dose volume:	
Route:	Induction phase duration 15 days, epidermal, challenge application epidermal (occlusive patch)
Administration:	epidermal, 48 hours, challenge on day 29 for 6 hours, observation over 48 hours.
GLP:	yes
Study period:	November-December 1997
Reference:	DHS Evonik (Degussa), 1992 (8)

Results
Following first challenge, 3 out of 10 animals reacted with an erthyema and 1 in 10 animals showed edema. Following epidermal challenge neither treated nor control animals showed any changes at the skin. TiO2 regarded as non-sensitiser in Buehler test on guinea pig skin. No systemic effects observed.

SCCS Comment
The study relates to S75-F material included in the dossier, which is anatase/rutile material, coated with organic coating of trimethoxy-n-octyl-silane, in oily suspension. This study is relevant to the nanomaterial group (85% anatase, 15% rutile). Due to the absence of skin penetration of TiO2 as demonstrated by many studies included in this dossier, the usefulness of the Buehler test for assessing sensitisation potency of nanomaterials is doubtful as it is based on exposure to intact skin.

Skin sensitisation, Guinea Pig, Magnusson-Kligman maximisation test

Guideline:	
Species/strain:	guinea pigs/Dunkin Hatley strain
Group size:	20 test group, 16 control group
Test substance:	TiO2 NP89/145
Batch:	
Purity:	TiO2 96.5%, SiO2 3%, carbon approx 4%.
Vehicle:	Freunds Complete Adjuvant for immunisation
Dose levels:	2 cm x 4 cm patch, 2cm x 2 cm patch for challenge
Dose volume:	
Route:	Induction with NP 89/145 at 10% v/v in NP 88/310 (injection) and 100% (topical), challenge application at 100% and 50% v/v in NP88/310.
Administration:	Patch, 48 hours (induction patch), 24 hour (challenge patch), observation period 24 and 48 hours
GLP:	Yes
Study period:	April-May 1989
Reference:	Submission I - Croda (Tioxide, UK), 1989 (20)

Results
At challenge, none of the test or control group animals treated with NP 89/145 at 100% or 50% v/v (in NP 88/310) showed a positive response. No evidence that NP 89/145 is a sensitiser in guinea pigs. Classified as a weak sensitiser according to the Magnusson-Kligman classification. No clinical signs were noted, body weight gains were acceptable.

SCCS Comment
The study used ultrafine TiO2, however, there is a lack of information on the characterisation (particle size distribution) of the tested material. According to Applicant, the material used in this study relates to rutile material coated with alumina/silica (i.e. S75-A, S75-B, S75-C, S75-L). It is however not clear how the test material relates to the nanomaterials included in the dossier because the proportion of the nano fraction in the micronized material has not been provided.

SCCS Comment on Skin Sensitisation
The article by Warheit et al., 2007 (SI-II-215) is a review of different studies on ultrafine TiO2 particles to develop a base set of toxicity tests. As such it does not provide any details on the studies or any experimental data that could be used for this assessment.

From two of the other studies, TiO2 nanomaterials (anatase/ rutile mixture, coated with trimethoxy-caprylylsilane or trimethoxy-n-octyl-silane) have been regarded non-sensitiser. Another material (rutile, coated with alumina/silica) is classified as a weak sensitiser according to the Magnusson-Kligman classification (that considers 0 to 8% response a weak sensitizer category). The material used in this study is described as ultrafine rutile material coated with alumina/silica (relating to S75-A, S75-B, S75-C, S75-L) but information on characterisation (particle size distribution) of the tested materials has not been reported to indicate what proportion was in the nano-scale.

Due to the absence of skin penetration of TiO2 as demonstrated by many studies included in this dossier, the usefulness of the Buehler test for assessing sensitisation potency of nanomaterials is doubtful as it is based on exposure to intact skin.

From the limited useful data, TiO2 nanomaterials appear to be weak or non- skin sensitiser.

1.5.4 Dermal / percutaneous absorption

***In vitro* studies**:

Guideline/method:	
Species:	human abdominal epidermis
Test substances:	Titanium dioxide T805, comprising 5% micronized titanium dioxide; not radiolabelled.
Particle size:	not given
Group sizes:	2 female donors in experiment 1, 1 male and 1 female donor in experiment 2
Dose applied:	3.6g/cm2 of cream with a content of 5% micronized titanium dioxide (actual dose 3.55 mg/cm2)
Skin area:	0.32 cm2
Skin temperature:	30-32°C
Test chamber:	flow through diffusion cells
Receptor fluid:	0.9% saline
Exposure period:	6 hours
GLP:	yes
Published:	no
Study period:	1995
Reference:	Reference 24 submission 1

Method
The amount applied to each cell was 3.55 mg/cm2. Skin integrity was checked. The penetration through the skin membranes was determined over a period of 6 hours under non-occluded conditions. The receptor fluid was delivered at a flow rate of about 1.5 mL/h during the testing period. The perfusate from each cell was collected separately at ambient temperature for 0-8h post application. Eight hours post application the perfusate sampling was terminated. All skin membrane rinse fractions were combined according to the individual cells and added to the 0-8h perfusate.

Results
The perfusate samples were analysed by IPCMS, the TiO2 content ranged from 2.6 to 4.8 ng/ml. These concentrations were reported to be in the same range as the 'blind' solutions (2.-2.9 ng/ml). Transmission electronic microscopy of titanium dioxide in the skin samples showed presence only in the outer skin layers and not in the deeper layers of the epidermis. Thus TiO2 nanoparticles did not penetrate through human skin under the experimental conditions described above.

SCCS Comments
The study shows lack of detectable skin penetration of the test nanomaterial which relates to S75-F included in the dossier (anatase/rutile material, with organic coating of trimethoxy-caprylylsilane, in oily suspension). This study is relevant to the nanomaterial group (85% anatase, 15% rutile).
The particle size of the tested nano-material was not determined in this study. It is assumed that the particle size is similar to the data shown in Table 1.3. However most likely the particles were present as agglomerates as the test item was used in a cream formulation.

Study Design:

Guideline/method:	-
Species:	human abdominal epidermis
Test substances:	micronized TiO2: Eusolex TA (5% O/W lotion),
micronized TiO2:	Eusolex TC (5% W/O cream)
vehicle	(O/W lotion and W/O cream)
Particle size:	particle sizes not provided, Eusolex TA: BET= 84.2 m2/g Eusolex TC: BET= 58.8 m2/g
Group sizes:	4 cells per donor; 4 donors
Dose applied:	between 3.19 and 4.28 mg/cm2
Skin area:	0.32 cm2
Skin temperature:	30-32°C
Test chamber:	flow through diffusion cells
Receptor fluid:	0.9% saline
Exposure period:	6 hours
GLP:	yes
Published:	no
Study period:	1995
Reference:	Reference 25 submission 1

Method
The amount applied to each cell was 3.19-3.31 mg/cm2 (Eusolex TC and TA, respectively; applied amount of vehicle only was slightly higher). Skin integrity was checked. The penetration through the skin membranes was determined over a period of 6 hours under non-occluded conditions. The receptor fluid was delivered at a flow rate of about 1.5 mL/h during the testing period. The perfusate from each cell was collected separately at ambient temperature for 0-8h post application.
Eight hours post application the perfusate sampling was terminated. All skin membrane rinse fractions were combined according to the individual cells and added to the 0-8h perfusate.

Results
The perfusate samples were analysed by ICP-OES, the TiO2 content were below 0.05ug/sample. No, or only slight traces of TiO2 particles were detectable on the skin samples treated with Eusolex® TA under the light microscope. The refracting colourless TiO2 particles were localized on the outer surface of the stratum corneum. One skin sample revealed two particles sited intracellularly at one location at the stratum granulosum. Whether these were refracting particles of TiO2 could not be resolved unequivocally under the optical microscope. Multiple foci of TiO2 particles were observed on most of the skin samples that had been treated with Eusolex® TC. The refracting particles were localized on the outer surface of the stratum corneum. It was concluded that titanium dioxide nanoparticles did not penetrate through human skin under the experimental conditions described above.

SCCS Comments
The study shows lack of detectable dermal penetration of TiO2 nanoparticles. The test material possibly (as it is not clear from the different code) relates to S75-M, S75-N, and/or S75-O. The particle size of the tested nano-material was not determined in this study.

Test for penetration of micronized TiO2 through the egg membrane or the chorio-allantoic membrane (CAM).

Guideline:	
Species/strain:	White Leghorn chicken eggs, freshly fertilized
Group size:	3 eggs per group (control group: 2 eggs)
Test substance:	micronized Eusolex TC (TC);
Batch:	TO 118279
Purity:	not reported
Particle size:	not reported
GLP:	
Reference:	Reference 26 submission I

Method
The testing material was prepared on the day of exposure. The concentration was 5 g/100 ml carrier. The carrier used was water for injection to which 0.01 % of the cationic tenside UCARE 10 had been added. To enable the test material to be applied to the egg membrane, the eggshell was opened with the aid of a dentist's drill and the material was introduced with the aid of a needle. The volume introduced was 0.06 ml per egg. To enable the material to be applied to the CAM (chorio-allantoic membrane), the eggshell was taken off, the egg membrane removed and the material introduced onto the exposed CAM. The volume introduced was 0.3 ml. After the prescribed period of exposure, the treated surface was fixed for 24h with approximately 10% formaldehyde solution. The fixed CAM or egg membrane with CAM was removed, embedded in paraffin, sliced, and then stained with nuclear fast red and H. E. The sections were evaluated under an optical microscope.

Results
No signs of penetration by TiO2 through the egg membrane or the chorio-allantoic membrane were seen under an optical microscope. The introduction of TiO2 was fully tolerated in this sensitive model.

SCCS Comments
The test report is very concise. No positive control was used in this test. This test is therefore of very limited use for this assessment.

Study Design:
Guideline/method:

Species:	human abdominal skin
Test substance:	J&J Baby Sunblock SPF 30 (2723L) containing microfine titanium oxide (Hombifine 535) (conc unknown)
Particle size:	not reported.
Group sizes:	not reported (1 donor?)
Dose applied:	400 um formulation
Skin area:	not reported
Skin temperature:	not reported
Test chamber:	flow through diffusion cells
Receptor fluid:	0.9% saline
Exposure period:	24h hours
GLP:	no
Published:	no
Study period:	1990
Reference:	Reference 28 submission 1

Method
A layer of about 400 um of formulation was applied on each human cadaver skin sample and left to dry for 15 minutes. The treated skin samples with the epidermis side facing up were then mounted on each of the modified diffusion cells. The receptor compartment was filled with 0.9% NaCl adjusted to pH 7.4 and 5 respectively. The permeation was conducted for 24 hrs and the receptor solutions were collected at the end of the experiment. The amount of cream left on the skin surface was then recovered using wipes and rinsed with methanol (methanol washings).

Results
In these diffusion cell based tests, samples of stripped human cadaver skin and mouse skin were used. The stripped skin does not have a stratum corneum and can thus be regarded to simulate injured skin. The study showed that only a negligible amount of titanium permeated through either whole skin or the simulated "damaged skin". About 15% of titanium oxide was found in the skin tissue and most of the titanium (ca. 85%) was recovered from the skin surface for both whole skin and stripped skin when the receptor pH was adjusted at pH 7. 4. It appears that titanium has little tendency to permeate through the skin. The amount of titanium oxide recovered in the skin tissue may include the physical adsorption of titanium oxide to the skin surface, which was difficult to be rinsed off by methanol.
The effect of pH in the receptor fluid may play an important role towards the penetration of titanium oxide. The point of zero charge (pzc) of microfine titanium oxide (Hombifine S35) is 5.6. Therefore, the receptor fluid will provide better "sink" conditions if its pH is adjusted further away from 5. 6. Less titanium was found in the skin when the receptor pH was controlled at pH 5. This can be explained by the fact that pH 5 (0.6 pH unit away from pzc) is providing less "sink condition" compared to pH 7.4 (1.8 pH unit from zpc). It was concluded that the chance for titanium oxide to penetrate across human cadaver skin is slim.

SCCS Comments
This is a special and limited study to investigate the influence of different pH conditions. Reporting is very concise. Therefore this study provides some additional but limited information for the risk assessment.

Guideline/method:

Species:	human abdominal skin
Test substances:	Sunscreen cream with 5% UV-Titan M160 formulation containing 5% titanium dioxide Sunscreen cream without UV-Titan (ca 50 ml).
Particle size:	not given

Group sizes:	3 donors, 1 male and 2 females; 17 samples from 3 donors were treated with sunscreen cream with UV -titan M 160 formulation. A total of 4 samples of epidermis taken from the 3 different donors were treated with the control formulation
Dose applied:	2.06 mg/cm2 of cream with a content of 5% micronized titanium dioxide
Skin area:	0.32 cm2
Skin temperature:	30-32°C
Test chamber:	flow through diffusion cells
Receptor fluid:	0.9% saline
Exposure period:	8 hours
GLP:	yes
Published:	no
Study period:	1996
Reference:	Reference 30 submission 1

Method
The amount applied to each cell was 2.06 mg/cm2. Skin integrity was checked. The penetration through the skin membranes was determined over a period of 6 hours under non-occluded conditions. The receptor fluid was delivered at a flow rate of about 1.5 mL/h during the testing period. The perfusate from each cell was collected separately at an ambient temperature for 0-8h post application. Eight hours post application the perfusate sampling was terminated.

Results
The absorbed amount of Titanium Dioxide was below the detection limit of 5 ng (1ug/l in ICP-MS) in all samples. The analyses of the samples did not indicate significant penetration of Titanium Dioxide UV-TITAN within the detection limit of the method.

SCCS Comments
The study shows lack of detectable dermal penetration of TiO2 nanoparticles. The tested material is S75-I (>99.5% Rutile, coated with 7% alumina 10% stearic acid).

Guideline/method:	
Species:	Human (4 females, mean age 26), upper arm
Test substances:	A- Oil/Water lotion: 5% w/w Ti02 from 12.5% Tioveil AQG B- Water/Oil cream: 7.5% w/w Ti02 from 18.75% Tioveil TG
C- Oil/Water lotion:	7.5% w/w Ti02 from 18.75% Tioveil OP
Particle size:	not reported
Group sizes:	4 volunteers, 3 different locations of the upper arm (for application A, B and C)
Dose applied:	2.0 ul/cm2 : 8 ul spread over 4 cm2 area of skin.
Skin:	Intact human skin
Skin temperature:	37 °C
Exposure period:	8h under occlusion
GLP:	No
Study period:	1993
Reference:	Reference 29 submission 1

Method
The three test products were randomly allocated to three of the four test sites on the forearm. After the 8 hour occlusion, the dressings were removed. The sites were not wiped prior to removal of stratum corneum by the skin surface biopsy (SSB) procedure. Successive SSBs were taken from the same site such that a profile across the stratum corneum was obtained. Four SSBs were taken from each of the treated sites.

The migration of titanium dioxide from sunscreen formulas into the skin was investigated using a range of sunscreen formulas (A-C) and four subjects. Consecutive 4cm^2 skin samples (biopsies) were taken from test areas. A maximum of 4 biopsies were taken from any one skin area, providing 16-20 skin layers in total. Selected skin biopsies were then analysed using X-ray microanalysis to determine the concentration of titanium dioxide in the biopsy and to show the migration of the titanium dioxide through the skin.

Results
Emulsion A did not appear to have migrated past the first biopsies from subjects 1, 2 and 4 but had migrated to the second biopsy from subject 3.
Comparing the emulsions tested on subject 1, emulsions A and B showed little difference with titanium only present in the first biopsy, but the titanium from emulsion C had migrated to the second biopsy. These results have been confirmed by transmission electron microscopy examination of these samples where titanium dioxide crystals were shown to be present through the first biopsy and in the second skin biopsy of the area treated with emulsion C but not in the second biopsies of the areas treated with any of the other emulsions. Repeat analyses on selected samples showed that there was an error of ±0.2% in the measurement of titanium in these samples. This indicates that there is some variation across the samples possibly due to uneven migration of the sunscreen or uneven thickness of the biopsies. The detection limit of the analyser was -0.1% and though comparative results were obtained by this method it is not as accurate as observations made by transmission electron microscopy. Any measurements less than 0.3% were confirmed by repeat analyses. It was concluded that in all cases no Ti02 was detected beyond the top two (out of four) skin surface biopsies. No evidence of penetration to the viable epidermis was found.

SCCS Comments
The study shows some penetration of TiO2 nanoparticles to the outer layers of skin, but not to the viable epidermis. The tested material relates to S75-B (>99.5% Rutile, coated with 6% silica, 16% alumina).

Study Design:	
Guideline/method:	Comparative study according to an internal laboratory methodology considering real use conditions and recommendation of US FDA and COLIPA SPF requirements
Species:	Human (25- to 65-year-old adults)
Test substances:	Commercial products containing coated (Al2O3 and SiO2) nano-sized
titanium dioxide.	No information on size except for Eusolex T-2000 TiA: contained only TiO2 TiB: contained TiO2 plus ZnO TiHB: (Eusolex T-2000) contained coated rutile TiO2 (average size of 20 nm)
Particle size:	Nanoparticles of TiO2 needle-shaped; dimension not given
Group sizes:	TiA, TiHB: 8 volunteers (intact skin) TiB: 9 volunteers (intact skin) TiA, TiB, TiHB: 10 volunteers (stripped skin) TiA: 4 psoriatic patients
Controls:	6 volunteers for basal elemental concentration in the skin
Dose applied:	0.5 – 1.0 mg/cm² on an area of 25 cm²
Skin:	Intact and tape stripped human skin
Skin temperature:	37 °C
Exposure period:	2 h (intact) or 48 h (stripped skin and psoriatic patients)
GLP:	No
Published:	Yes
Study period:	Before 2009
Reference:	Filipe et al., 2009 (54, 155)

Method
The localization and possible skin penetration of TiO2 nanoparticles dispersed in three sunscreen formulations, in use under certain conditions were investigated in normal and altered skin. Commercial products containing nano-sized particles of coated TiO2 and ZnO dispersed in hydrophobic emulsions were used. One product contained only TiO2 (TiA), another TiO2 plus ZnO (TiB) and a third material (TiHB) contained nanoparticles of coated rutile form TiO2.
The nanoparticles were dispersed in hydrophobic basis gel composed by high pressure polyethylene and viscous paraffin with Al2O3 (8-11%) and SiO2 (1-3%). The coated preparations contained 76 – 82% TiO2. The size and shape of nanoparticles in the three formulations were inspected with transmission electron microscopy and X-ray microanalysis. Nanoparticles were needle-shaped and similar in both commercial and test formulation. The application protocol consisted of an open test. The formulation was applied on the sacral region and buttocks for 2 h, using a sunscreen application of approximately 0.5-1.0 mg/cm² within an area of 25 cm².
The 3 formulations used in the study were tested in normal skin: TiB was applied to 9 individuals and both TiA and TiHB to 8 individuals. Nanoparticle penetration (TiA, TiB, TiHB) was also evaluated in normal skin in an independent group of 10 individuals under non-physiological conditions induced by tape stripping and occlusive patches (48-hour application). Tape stripping consisted of series of strips until the tapes were free of corneocytes. A TiA-containing commercial sunscreen was further tested in involved skin areas of 4 psoriatic patients. A matched control group constituted by 6 individuals was used for the determination of basal elemental concentrations in skin including Ti.
Skin punch biopsies of 3 mm diameter were taken after application, quench-frozen and kept in containers until processing. One biopsy was taken from each volunteer. Sections of 14 µm thickness were cut from the frozen biopsy in a cryostat at -25 °C. Biopsies were mounted in mounting medium for microscopy. Sections were obtained from the non-immersed portion of the tissue, and sectioning performed from inside to outside to avoid tissue contamination. Tissue integrity and the efficacy of corneocyte removal after tape stripping were checked by preparing intercalary stained sections for optical microscopy purposes. Scanning Transmission Ion Microscopy technique and Particle Induced X-ray Emission technique were used for detection. The minimum detectable concentration of TiO2 in the skin was 0.31 µmol/g (24.8 µg/g tissue or 25 ppm).

Results
For imaging and localizing TiO2 and ZnO nanoparticles in intact skin, the coverage of the outer skin layer with the TiA and TiB sunscreen formulations was homogeneously distributed. The TiHB formulation showed a patchy distribution. Sunscreen formulations accumulated in skin wrinkles and depressions as well as infundibulum cavities. Exogenous Ti and Zn remained at the outer layers of the keratinized tissue that enfold the follicle i.e. outside the living skin.

The nanoparticles penetration profiles obtained with the treated skin groups (TiA, TiB and TiHB) were all similar. The high levels of TiO2 observed at the outer layers of stratum corneum sharply decreased within deeper layers to become undetectable (as Ti by x-ray emission technique). High Ti concentrations levels were only determined in the stratum corneum of skin treated with the three formulations. In the subcorneal regions Ti concentration was below the minimum detectable concentration estimated for the analytical technique. In non-treated skin Ti was below the minimum detection limit in all strata inspected. For the depth positions, where TiO2 nanoparticle penetration ended an estimated error of 10% was obtained, which approximately corresponds to 0.5 µm. In occluded skin, there was no significant difference in TiO2 nanoparticles distribution and penetration depth profiling.

Nanoparticle localization in damaged skin

Parts of the outer layers of the stratum corneum were removed by tape stripping (at least 15 strips) before sunscreen application. Removal of the stratum corneum was confirmed by histological examination and ultimately by nuclear microprobe examination. Under this condition there was negligible adhesion of the formulation tested (TiA). The TiO2 contents determined on the skin outer layers were unimportant suggesting that, in normal skin, the outer layers of stratum corneum trapped nanoparticles inside the desquamating corneocytes network.

Results
In psoriatic skin, where the horny layer is thicker and less compacted than in normal skin showed that the sunscreen formulation remained only, in the first layers of the stratum corneum. The Ti distribution was often non-uniform and in some "hot-spots" sunscreen was deposited at the outer layers of stratum corneum partly even in the hair follicle infundibulum region.

Conclusion
The authors concluded that following 2 h exposure period of normal human skin to nano-sized TiO2-containing sunscreens, detectable amounts of these physical UV filters were only present at the skin surface and in the upper most stratum corneum regions. Layers deeper than the stratum corneum were devoid of TiO2, even after 48 h exposure to the sunscreen under occlusion. Deposition of TiO2 and ZnO nanoparticles in the openings of the pilosebaceous follicles was also observed. Penetration of nanoparticles into viable skin tissue could not be detected.

SCCS Comments
The study is of good quality. Although for the TiO2 nanomaterial used in this study information on surface area, number of particles per mass was not provided, the results showed penetration of the nanoparticles only to the outer layers of Stratum corneum, but not to the viable epidermis. The tested material relates to S75-N (>95% Rutile, <5% anatase, coated with alumina 10% simethicone 2%, doped with 1000 ppm Fe).

Study Design:	
Guideline/method:	
Species:	Porcine and human skin
Test substances:	TiO2 uncoated nanoparticles, mixture of rutile and anatase, average primary particle size 21 nm, uncoated, approximately spherical platelets (Degussa-P25) TiO2 coated nanoparticles, rutile, composition 76-82% TiO2, 8-11% Al2O3 and 1-3% SiO2, primary particle size about 20-100 nm, needle shaped (Eusolex T-2000, Merck KGaA)
Formulations:	All formulations contained 5% TiO2 nanoparticles. 1. TiO2 uncoated: carbomergel, 20% propylenglycol, 0.5% carbomer 500,000, 0.3% trometamol, and 79.2% purified water. 2. TiO2 coated: hydrophobic basisgel, 5% high pressure polyethylene and 95% viscous paraffin 3. TiO2 coated: polyacrylategel, 20% propylenglycol, 0.5% carbopol 980, 0.3% trometamol, and 79.2% purified water.
Dose applied:	2 mg/cm²
Skin:	Porcine skin. The porcine skin specimens (n=12) were obtained from domestic pigs. Specimens were sampled from the inner parts of thighs in the form of punch biopsies. Human skin. The human skin was obtained from the dorsal region and buttocks of healthy adult volunteers (n=8). Human grafted skin samples were produced from normal human foreskins obtained from circumcision and grafted on a severe combined immunodeficient (SCID) mouse model (n=4).

Skin temperature:	Not stated
Exposure period:	Porcine and human skin 2 h under semi-occlusive conditions, human grafted skin 1 h, 24 h, and 48 h under occlusive conditions.
GLP:	No
Published:	Yes
Study period:	Before 2008
Reference	Gontier et al., 2008 (158)

Method
All three formulations were topically applied at 2 mg/cm2 and for 2 h to porcine and human skin under semi-occlusive conditions, i.e., a breathable plaster protected the area. In a previous pilot study with exposure times between 8 and 48 h no significant differences were found for different exposure times. The sunscreen was applied to human skin grafted on SCID mice for 1 h, 24 h, and 48 h under occlusive conditions. Untreated control samples were also prepared for each analysis.
The skin biopsies (3 mm in diameter) were studied by Transmission Electron Microscopy (HRTEM) and Scanning Transmission Ion Microscopy (STIM) combined with Rutherford Backscattering Spectrometry (RBS) and Particle Induced X-Ray Emission (PIXE) on ultra-thin and thin cross-sections, respectively.

Results
Porcine skin
TiO2 uncoated. By superimposing the titanium distribution obtained by the PIXE map on to the STIM map, it was possible to unambiguously determine the distribution of TiO2 particles via their chemical fingerprint with a close correlation to the epidermal layers. TiO2 particles were exclusively localized on the surface of the outermost SC layer. No titanium could be found in the layers containing vital cells. The porcine skin after application of hydrophobic basisgel exhibited a similar titanium distribution. To quantify the penetration depth of TiO2 particles, a region of interest was chosen to extract the titanium depth profile displayed. The extent of the profile was about 30 µm. A clear titanium peak is visible at the skin surface, the titanium being strictly limited to the SC. The nuclear microprobe observations were cross-checked by the results obtained on the same type of samples studied by HRTEM. Apart from corneocyte layers, nanoparticles and agglomerates on and in between the corneocytes are clearly visible. Electron X-ray microanalysis on individual nanoparticles proved that they contain Ti. In addition, morphological features of the TiO2 particles were examined. The TiO2 particles sometimes appear as individual particles, but more frequently agglomerated to clusters of different sizes.

TiO2 coated. An average size of 12 nm in width and of 60 nm for the length was estimated for the primary needle-shaped particles. The large amount of the titanium particles for both test emulsions, carbomergel and hydrophobic basisgel, was strictly located at the surface of the last corneocyte layer with the possible exception of agglomerates below the first and third corneocyte layer.

Human skin
The STIM map exhibits a thick SC and a well delineated SS containing keratinocyte cell bodies. The Ti PIXE-maps are superimposed onto the STIM image, demonstrating that the particles were exclusively located on the outermost layers of the SC. This observation is corroborated by the superimposition of the same titanium PIXE-map onto the RBS carbon map. The depth profile of titanium extracted from the region of interest demonstrates that the presence of this element is limited to a layer with a thickness of about 20 µm.

On the STIM map obtained for the commercial formulation, the SC is easily observable due to its high density despite its unusually low thickness. In the titanium PIXE-map is superimposed onto the STIM image. Ti is exclusively localized on the surface of the horny

layer. From the titanium depth profile, extracted from the region of interest, titanium was found to penetrate into a 10 µm thickness layer of the SC only, but no titanium was detected in the SS.

In the HRTEM micrograph, TiO2 particles were identified by the presence of large homogeneous electron dense objects on the surface of the horny layer. At low magnification the particles appear to be spread in a very homogeneous thin layer. With a high magnification, the particles occasionally appeared as needle-shaped individual particles, but most frequently aggregated in clusters of different sizes. The primary particles have a width of 12 nm and an average length of 60 nm. Some particles were seen four to five layers deeper, apparently only when a passage exists due to the looseness of corneocytes.

Human skin grafted to SCID-mice
The murine SCID model allows human skin to be grafted without any rejection. The commercial product was applied for 2 h under occlusive conditions. Here, the STIM image enables to delineate the SC from the large SS by its high density. In addition it shows the papillary dermal-epidermal junction and the dermis. When the PIXE-titanium maps were superimposed onto the STIM images obtained from the two different areas of interest a microlesion, i.e., a partly detached horny layer, with Ti in the cleft was seen. The result seemed to indicate that in some areas of the SC titanium penetrated more deeply compared to other skin samples. The HRTEM micrographs revealed a thinner SC constituted by two or three layers of corneocytes only. In fact, this sample was taken from the border between mouse and human skin. The corneocytes are separated by larger spaces which have allowed the product to penetrate down to the innermost corneocyte layer. The TiO2 particles seem to be attached to the corneocyte layers. Nevertheless no TiO2 particles were observed in the very close SG.

Conclusion
The authors concluded that whereas the HRTEM and STIM/PIXE images reveal clear differences – mainly related to the different thickness of the cross-sections – they unambiguously show that penetration of TiO2 nanoparticles is restricted to the topmost 3–5 corneocyte layers of the stratum corneum.

SCCS Comments
The study is of good quality. Although for the TiO2 nanomaterial used in this study information on surface area, number of particles per mass was not provided, the results showed penetration of the nanoparticles only to the outer layers of Stratum corneum, but not to the viable epidermis. The tested material relates to S75-G (uncoated, anatase 85%, rutile 15%), and S75-N (>95% Rutile, <5% anatase, coated with alumina 10% simethicone 2%, doped with 1000 ppm Fe).

Compromised skin

Study Design:	
Guideline/method:	Exploratory comparative percutaneous skin penetration study *in vitro* after UVB radiation *in vivo* (sunburn simulation)
Test system:	Skin of weanling Yorkshire pigs (approximately 20–30 kg)
Test substances:	O/W and W/O sunscreen formulations A: T-Lite SF (coated, 10% O/W formulation, CM 630) B: T-Lite SF (coated, 10% W/O formulation, CM 634) CM 630 and CM 634 consist of TiO2 (rutile, crystallite of 14–16 nm) coated with hydrated silica, dimethicone/methicone copolymer, and aluminium hydroxide for a primary particle size of 10 x 50 nm and specific surface area of 100 m2/g. The mean size of the agglomerates was 200 nm with a range of ca. 90–460 nm
Batch:	Not stated (source: BASF SE, Germany)

UVB exposure: A Fiber optic UVB lamp (Lightning cure 200 UV-Spot light) was used.
Reference: Monteiro-Riviere et al., (2011) (181, 182).

Method
On day 1 a pig was sedated and the hair clipped. The minimal erythemic dose (MED) was determined by sequential exposure to UVB light (30 – 110 mJ/cm², - 22 sec.). On day 2 the exposed sites were analyzed to determine the UVB dose required to produce 1 MED. The pig was subsequently sedated and multiple sites (52 sites) on the back were exposed to the UVB dose that caused a consistent +2 erythema, a pale red in a defined area of the skin. Twenty-four hours after UVB exposure (Day 3), the pig was sedated, sites visually analyzed for consistency, and the pig euthanatized. The UVB-exposed sites were dermatomed to a thickness of approximately 400-500 µm and placed dermis side down on paper towels saturated with physiological saline.
The skin prepared for the *in vitro* or *in vivo* studies.
UVB dose: 100, 110 and 120 mJ/cm² (pig 1, 2, 3 for MED of about 2.5)

In vitro part:

Dose level:	50 µl of each formulation on 0.64 cm² dermatomed pig skin
Skin preparation:	Exposed and unexposed skin sites were dermatomed to 400 µm. Dermatomed skin, placed dermis side down on towels saturated with physiological saline, was cut into with a 19 mm circular punch.
Cells:	Formulation A and B: 4 with UVB exposed skin, 2 with unexposed skin
Control:	2 with UVB exposed skin, 2 with unexposed skin
Skin temperature:	37 °C
Test chamber:	Flow-through diffusion cells
Route:	Topical application
Exposure time:	24 hours
Sampling time points:	Every 2 h for the first 12 h, every 4 h thereafter up to 24 h
Examinations:	Light microscopy (LM). Transmission electron microscopy (TEM) plus X-ray microanalysis (EDS) Scanning electron microscopy (SEM). Time-of-flight secondary ion mass spectrometry (TOF-SIMS)

In vivo part:

Dose level:	250 µl of each formulation on exposed sites (n = 3 per formulation) on 2 pigs on 1.0 cm² pad Hill Top chamber
Controls:	Normal pig skin (no UVB, no sunscreen, no Hill Top chamber (n = 2 per pig)
UV-B exposed:	No sunscreen, dry chamber (n = 2 per pig)
Sunscreen in a Hill Top chamber:	No UVB (n = 2 per formulation)
Route:	Topical application
Exposure time:	2x 24 h and termination after 48 h
Sampling:	Skin was removed by 8-mm biopsy punch
Examinations:	As *in vitro* part
GLP:	No
Published:	Yes

Method
The purpose of the study was to determine whether skin damaged by UVB radiation inducing moderate sunburn with a +2 erythema reaction, enhanced the penetration of TiO2 or ZnO nanoparticles (see Opinion on ZnO (nanoform)) present in sunscreen formulations.
Weanling Yorkshire pigs (approximately 20–30 kg) were sedated and multiple sites (about 52) on the back were exposed to the UVB dose that caused a consistent +2 erythema (a pale red in a defined area of the skin).

Twenty-four hours after UVB exposure, the pig was sedated, sites visually analyzed for consistency, and the skin prepared for *in vivo* or *in vitro* studies.
For the *in vitro* studies, the UVB exposed and non exposed sites were dermatomed to a thickness of approximately 400–500 µm. The dermatomed skin was mounted in the flow-through diffusion cells with a dosing area of 0.64 cm2 and maintained at 37°C. The skin was equilibrated in perfusate and a flow rate of 2 ml/h for 30 min prior to dosing. The skin was subsequently dosed with 50 µL of each formulation (CM 630: (n=4 UVB exposed skin, n=2 unexposed skin; CM 634: n=4 UVB exposed skin, n=2 unexposed skin; and control: n=2 UVB exposed skin, n=2 unexposed skin). After completion of dosing, the perfusion was resumed and the perfusate collected every 2 hours for the first 12 hours and every 4 hours thereafter up to 24 hours. After 24 hours, the perfusion was terminated and the skin was removed from the diffusion cells.

The dose site was removed with an 8 mm biopsy punch and cut into thirds. One third was placed in Trump's fixative and stored at 4°C for later processing by light microscopy (LM; flow-through 1 and 2 only) and transmission electron microscopy (TEM). The remaining third of the skin was cut in half and immediately frozen and stored at -20°C for later elemental analysis. The vials containing perfusate from each timed collection were capped and the samples immediately stored at 4°C.

For *in vivo* treatment exposed sites (n = 3 per formulation) on two pigs were treated with 250 µl of each formulation; 200 µl was loaded onto the pad of the Hill Top chamber (1.0 cm² area) and 50 µl was placed directly on the skin within a template. Controls included normal pig skin (no UVB, no sunscreen, no Hill Top chamber; n = 2 per pig), UVB-exposed (no sunscreen, dry chamber; n = 2 per pig), and sunscreen in a Hill Top chamber (no UVB; n = 2 per pig per formulation). Sites were redosed with new Hill Top chambers after 24 h, and the treatment was terminated after 48 h. Erythema was scored for each site, and the pigs were euthanatized as above. Skin from all of these sites was removed with an 8-mm biopsy punch for microscopy studies as stated above.

Results
For the *in vitro* studies, light microscopy showed that UVB exposed skin showed focal intracellular epidermal oedema, sunburn cells, dermal inflammation and focal microblister and residual sunscreen containing TiO2 limited to the stratum corneum. The morphology of the normal and the UVB-exposed skin was not affected by topical treatment with the sunscreen formulations. The TiO2 in each formulation was confirmed by TEM and elemental analysis. EDS found the presence of Ti and Cu (copper grid)) in CM 630 and CM 634. Si for the coating, Pb for lead citrate and U for uranyl acetate staining.
In the *in vitro* flow-through studies, TEM/EDS found penetration of Ti to a depth of 9 layers in the stratum corneum of normal skin and 17 layers in the stratum corneum of UVB-exposed skin. TEM/energy dispersive x-ray spectroscopy or inductively coupled plasma mass spectrometry detected no Ti or Zn, indicating minimal transdermal absorption.
For *in vivo* tests, skin was dosed at 24 h occluded with formulations and at 48 h. TiO2 NP in o/w formulation penetrated 13 layers into UVB-damaged SC, whereas only 7 layers in normal skin; TiO2 in w/o penetrated deeper in UVB-damaged SC. Coated and uncoated ZnO NP in o/w were localized to the upper one to two SC layers in all skin. TOF-SIMS showed Ti within epidermis and superficial dermis, whereas Zn was limited to SC and upper epidermis in both treatments. In summary, UVB-damaged skin slightly enhanced TiO2 NP or ZnO NP penetration in sunscreen formulations but no transdermal absorption was detected.

Conclusion
In summary, UVB-sunburned skin slightly enhanced the *in vitro* or *in vivo* penetration of the TiO2 or ZnO NPs present in the sunscreen formulations into the stratum corneam (SC). Although penetration of the two NPs into the SC was shown by TEM, and into the epidermis and dermis by TOF-SIMS, there was no definitive evidence that they penetrated the skin *in vitro* into the perfusate. In most cases, TiO2 penetration into the SC was greater than ZnO. These results viewed together suggest minimal penetration of TiO2 and ZnO NPs into the

upper epidermal layers when applied topically in sunscreen formulations to normal and UVB-sunburned skin, with no evidence of systemic absorption.

SCCS Comments

The study is of a good quality. The test material relates to S75-K (>94% rutile, coated with 6-8% aluminium hydroxide, 3.5-4.5% dimethicone/ methicone copolymer). The results of transmission electron microscopy indicated penetration of TiO2 nanoparticles into stratum corneum, whereas TOF-SIMS analysis indicated penetration into the epidermis and dermis. However, analysis of perfusate by TEM/Energy Dispersive Analysis or ICP-MS did not detect Ti or Zn indicating nanoparticles did not penetrate the skin *in vitro*.

***In Vitro* study (Senzui et al., 2010 - Ref 204)**

Study Design:	
Guideline/method:	
Species:	Yucatan micropig skin
Test substances:	All TiO2 are rutile-type T-35. size 35 nm, uncoated TC-35, size 35 nm, coated alumina + silica + silicone T-disp, size 10 x 100 nm, mixture of alumina coated and silicone coated T-250, size 250 nm, uncoated
Formulations:	All formulations contained 10% TiO2 nanoparticles. Cyclopentasiloxane (silicone, KF-995) used as dispersing medium)
Dose applied:	2 µl/cm²
Skin:	Yucatan micropig skin removed the subdermal tissue and fat was used as full-thickness skin (intact skin). The SC was removed from intact skin with adhesive tape (Scotch 313, 3M) (stripped skin). Hair was removed from intact skin using tweezers (hair removed skin)
Skin temperature:	Not stated
Exposure period:	24 h
GLP:	No
Published:	Yes
Study period:	Before 2010
Reference:	Senzui et al., 2010 (204)

Method

The TiO2 was suspended in a volatile silicone fluid used for cosmetics, cyclopentasiloxane, at a concentration of 10%. The suspension was applied at a dose of 2 mg/cm2 for 24 h.

The skin penetration was investigated *in vitro* with intact skin and with stripped skin (the SC removed from intact skin with adhesive tape) as a model of injured skin. In addition hair-removed skin (hair was removed from intact skin using tweezers) was used to represent skin damaged by hair-removal treatment.

Two µl of suspension were applied to an area of skin of approximately 1 cm2. Then the skin was placed on a modified Franz-type diffusion cell. After 24 h, the receptor phase (pH 7.1 isotonic phosphate buffer solution) was collected, the skin was removed from the diffusion cell and cut off at the rim for mounting the cell. Residues on the skin surface were removed by two cyanoacrylate stripping and Ti in the skin was determined. For some samples, the epidermis and dermis were separated by heating after cyanoacrylate stripping.

Skin conditions after application of TiO2 was observed using two methods. After application and drying, the skin surface was observed by digital fine scope microscopy. The epidermis of the skin prepared by a heat separation method was mounted on a scanning electron microscope (SEM) stage with adhesive tape.

Results
The particle size distribution of TiO2 in silicone was determined. The mean particle size of T-35 was 1700 nm, which was larger than that of T-250, 1200 nm. In contrast, suspensions of the coated TC-35 and T-disp contained nanoparticles with mean diameter of 80 and 130 nm, respectively.
Ti concentration in the receptor phase was similar in all skin conditions and formulation applied. For intact and stripped skin, no significant difference in Ti concentration was found between the control and suspension applied, which indicates TiO2 did not penetrate into the skin regardless of particles size and even when the SC was removed. For hair-removed skin, Ti concentration in skin after application of TC-35 suspension was significantly higher than that of the control, and after application of T-disp suspension, tended to be high. The Ti concentration in the dermis was not different from the control.
Ti concentration in the epidermis after application of TiO2 nanoparticles tended to be greater than that of the control, but the difference was not significant. The epidermis consists of SC, viable epidermis and hair follicles. Ti was detected in the hair follicle pockets of hair-removed skin, but not in the surrounding viable skin. The radius of a hair follicle is 0.05 – 0.2 mm which allow solvent to enter the hair shaft and sebum did not fill the follicle space. When fluid enters a small space by capillary action, small particles of Ti in fluid may be able to enter the follicle. Large particles cannot be moved by such small force, but TC-35 well dispersed in solvent might enter a follicle more easily than other types of TiO2. For T-disp, the dispersing agent had some effect, resulting in particles left in the skin after drying of the suspension

Conclusion of the Applicant
The authors concluded that TiO2 does not penetrate into viable skin, even if the particle size is less than 100 nm and the SC is damaged. However, immediately after hair removal the concentration of Ti in skin was higher when TC-35 was applied, which was most probably caused by dispersion. SEM-EDS observation showed that Ti penetrated into vacant hair follicles but in any case did not penetrate into dermis or viable epidermis. It was noted that since this was an *in vitro* study, inflammation could affect the results and further *in vivo* studies on viable skin with hair removal are needed.

SCCS Comments
The quality of the study is difficult to evaluate. Moreover, the study was performed with skin from Yucatan micropigs and experience with this skin type in skin absorbance studies is limited.

In vitro exploratory study - percutaneous skin penetration - pig skin (Ref 70)

Study design.

Guideline/method:	exploratory study
Species:	pigs
Test substances:	T805 (Degussa), hydrophobically coated with trimethyloctylsilane
Particle size:	about 20 nm
Group sizes:	n=2 skin samples
Dose applied:	0.8 mg total (20 mg with 4% TiO_2), 0.16 mg TiO_2 per cm^2
Skin:	fresh skin obtained from pigs used within 3 h after collection
Skin area	4.9 cm^2
Skin temperature:	32°C
Test chamber:	custom-made Franz-type diffusion cells
Receptor fluid:	0.9% w/v NaCl, 0.1% w/v gentamycin sulfate, 1% w/v bovine serum albumin in bi-distilled water
Exposure period:	24 h
GLP:	no
Published:	yes
Study period:	1999

Reference: Reference 70 submission III+IV Pflücker et al., 1999

Method
Fresh pig skin was obtained from the butcher, and used within 3 hours after collection. Skin samples were punched (5 cm in diameter). The dermal absorption study was performed with custom-made Franz-type diffusion cells. The lower cell was placed on a magnetic stirrer (Variomag, Germany) and connected by tygon tubes to a thermostat (Type CS-C6, Lauda, Germany) set at a temperature of 32°C (*in vivo* skin temperature). Magnetic stirring bars were placed in the lower cells, which were filled with the receptor fluid (0.9% w/v NaCl, 0.1% w/v gentamycin sulfate, 1% w/v bovine serum albumin in bidistilled water). 20 mg of the test emulsion, which contained 4% titanium dioxide, were topically applied with a gloved finger to two excised pig skin discs (area 4.9 cm2, 2.5 cm in diameter, giving a concentration of 4 mg cm2). After 24 h incubation 2 mm punch biopsies were obtained for histological evaluation (TEM and SEM). SEM micrographs were recorded to evaluate the morphology of the freeze-dried skin samples and the stripped stratum corneum sheets. Freeze dried skin samples were investigated before and after 10–fold tape stripping.

Results
TiO_2 was found exclusively on the outermost SC layer. No titanium dioxide could be found in the living cell layers of the stratum granulosum. The surface deposit, as displayed by TEM, featured clearly distinguishable agglomerates as well as single particles with a characteristic cubic shape and a primary particle size of about 20–50 nm. Concurrently, SEM/EDXA micrographs first showed an even distribution of TiO2 on the skin surface. After 10-fold stripping, however, TiO2 was found to be localized only in the furrows and not on the partially removed ridges of the skin surface. In the upper part of the hair follicle TiO_2 was demonstrated.

SCCS Comments
The actual TiO_2 dose was 0.16 mg, and not 20 mg as mentioned in the paper. The study does not show quantitative results but demonstrates by electron microscopy that the TiO_2 nanoparticles are present on the skin mainly as aggregates. The study is of limited value with number of samples investigated was only 2, but can be considered as supporting evidence that TiO_2 nanoparticles do not penetrate to the viable cell layers of the dermis.

***In vitro* exploratory study - percutaneous skin penetration and *in vivo* - human skin (Ref 78)**

Study design.

Guideline/method:	exploratory study
Species:	human healthy volunteers (female)
Test substances:	Mixture of broad spectrum UV water-in –oil emulsions containing water, glycerin, dimethicone, ethylhexyl methoxycinnamate, isododecane, cyclomethicone, C12-15 alkyl benzoate, PEG-30 dipolyhydroxystearate, decyl glucoside, dodecyl glycol copolymer, magnesium aluminium silicate, preservatives, zinc oxide, tocopheryl acetate, *o* -cymen-5-ol, fragrance, xanthan gum and 3% ultrafine TiO2 (**T805**, Degussa, Germany) and 8% methylene bis-benzotriazoyl tetramethylbutylphenol (MBBT) in a dispersion of decyl glucoside. TiO_2 was coated with trimethyloctylsilane.
Particle size:	TiO_2 20 nm
Group sizes:	n=3
Dose applied:	2 mg/cm^2 of formulation, 60 µg TiO_2 / cm^2
Skin:	*in vitro* abdominal and face skin frozen until use, *in vivo* skin of upper arm
Skin area	*in vivo* 10 cm^2 (2x5 cm) Teflon static diffusion cell 10 cm^2 (2x5 cm)

	Franz diffusion cell 1.13 cm^2
Test chamber:	Teflon® homemade static diffusion cell with a 10 cm^2 (5x2 cm) surface and a receptor volume of 8 ml. Franz diffusion cell with a 1.13-cm^2 surface and 5 ml of receptor fluid.
Receptor fluid:	0.9% NaCl water solution with 3% bovine serum albumin
Skin temperature:	32°C
Exposure period:	5 h
GLP:	no
Published:	yes
Study period:	2007
Reference:	Reference 78 Submission VII Mavon et al., 2007.

Method

Samples of the mixture of broad spectrum UV water-in –oil emulsions were applied on skin of volunteers (10 cm^2, 2x5 cm) and on two types of diffusion chambers, one Teflon® homemade static diffusion cell with a 10 cm^2 surface allowing tape stripping of the test system, and a Franz diffusion cell with a 1.13-cm^2 surface. The applied dose for the *in vitro* study was 60.6 ± 3.1 µg/cm^2, and for the *in vivo* study 58.4 ± 1.9 µg/cm^2.

The distribution of the sunscreens in the skin was directly assessed by the tape stripping method, using adhesive tape (Scotch TM No. 6204, 3M Corp.). A total of 15 tape strippings were applied onto the surface of the skin, and each was pressed on the skin 10 times with a roller. Each strip was removed with 1 quick movement. No washing procedure was used.

The titanium analysis in the tape strippings and skin samples (epidermis, dermis and receptor fluid) was based on a microwave assisted treatment, which digested the organic components in the presence of sulphuric and nitric acid. The samples were then analyzed by colorimetric assay, using diantipyrylmethane (0.5 g in 20 ml HCl 1 N). One ml of the colored solution and 1 ml of the solution to be tested were mixed. The absorbance was read at 390 nm with a spectrophotometer (Anthelie Advanced, France) 30 min later. Using this technique, a LOD of 0.2 µg/ml was obtained.

Transmission electron microscopy and particle-induced X-ray emission (PIXE) techniques were used to localize the TiO 2 in skin sections. Punch biopsies of 6 mm in diameter were made on skin samples, consecutively after 1, 8 and 15 tape strippings and were fixed with 2% glutaraldehyde in a Sorensen buffer for TEM analysis.

Results

For the *in vitro* experiments with n=3 >94.2% of the recovered TiO_2 was found in the 15 tape strippings and in the stratum corneum. In the epidermis 5.6% was found, and <0.1% was found in the dermal compartment. No TiO_2 was found in the receptor fluid (below LOD). The amount recovered accounted for 88.8% of the applied dose of TiO_2. In the *in vivo* study (n=3) the recovery was 93% of the TiO_2 dose. Most of the recovered dose was in the first three tape strippings. After 15 tape strippings a few grains could be distinguished in the TEM samples (amplification x 15,000), attributed to TiO 2 nanoparticles, but they were very few and isolated in the stratum corneum (SC) layer. Deeper in the SC, no particles could be observed, which suggested an absence of penetration into the viable skin tissue.

The 2-dimensional mapping of titanium using Micro-PIXE analysis of the skin showed that most of the Ti applied at the skin surface remained there or penetrated only into the opened infundibulum. Quantitative analysis revealed a concentration of Ti at the LOD, in the underlying layer of the epidermis, the dermis, the follicle and the sebaceous glands.

It was concluded by the authors that the study confirms that TiO_2 accumulates in the uppermost layers of the SC and in the opened infundibulum only. No TiO_2 was detected in the viable skin layers through either transcorneal or transfollicular pathways. From these data the authors concluded that the amount of TiO 2 found in the *in vitro* 'epidermal' compartment is located mainly in the furrows or the opened infundibulum and does not represent actual transcorneal penetration.

SCCS Comments
Both TiO2 and MBBT were present in the broad spectrum UV water-in–oil emulsions. Lack of penetration of TiO_2 was supported by both *in vitro* and *in vivo* studies. Whether the detected particles were attributed to TiO2 or not has not been identified by the study.

***In vitro* study - Percutaneous skin penetration pig skin (Ref 56)**

Study design.

Guideline/method:	yes (OECD 428, SCCNFP/0750/03) skin absorption *in vitro* method
Species:	pig
Test substances:	T-Lite SF-S coated with silica (2%-5% wt%) and methicone (4.5%-6.5%) T-Lite SF coated with methicone (3.5%-5.5%)
Particle size:	T-Lite SF-S, needle like with a size of 30-60x10 nm T-Lite SF, needle like with a size of 30-60x10 nm Both TiO_2 materials were present including aggregates up to 200 nm and higher (1 µm)
Group sizes:	skin from 3 pigs, and per sample 3 skin preparations (n=9)
Dose applied:	4mg/cm^2 corresponding to nominal doses of about 400 µg/cm^2 of titanium dioxide or to nominal doses of 240 µg/cm^2 of titanium,
Skin:	full thickness skin samples from lateral abdominal region
Skin area	about 1 cm^2
Skin temperature:	32 ± 1°C
Test chamber:	modified Franz static dermal penetration cells
Receptor fluid:	physiological saline containing 5% bovine serum albumin
Exposure period:	24 h, sampling at various time intervals (3, 6, 12, and 24 h)
GLP:	yes
Published:	yes
Study period:	2007
Reference:	Reference 56 Submission VII Gamer et al., 2007

Method
After removal of the receptor fluid the skin was removed from the diffusion cell and put onto parafilm. Titanium was removed from the skin preparations by washings with sponge pieces dipped into soap solution, and subsequent tape stripping was used to remove titanium together with the superficial layers of the stratum corneum. Ti was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) or ICP-mass spectrometry (ICP-MS).

Results
For the titanium dioxide formulations T-Lite SF-S and Tlite SF, mean total recoveries of Ti ranged from 98% to 100% and 86% to 93% of the total Ti applied, respectively. Virtually the total amount of applied Ti could be removed from the skin surface by washing. The amounts of titanium found in the tape strips and skin preparations were in the order of the analytical determination limit. No Ti was found in the receptor fluid at any sampling time.

SCCS Comments
This is a GLP study with three independent measurements indicating lack of TiO_2 penetration in an *in vitro* assay using pig skin. Although the number of measurements (n=3) per skin is limited, it was repeated in skin samples of three different pigs.

In vitro exploratory study percutaneous skin penetration, and _in vivo_ study on human skin (Ref 130)

Study design.	
Guideline/method:	exploratory study
Species:	human (male and female)
Test substances:	commercial microfine TiO2 dispersion either in octyl palmitate or in water (Tioxide Specialities Ltd)
Particle size:	not reported
Group sizes:	n=3
Dose applied:	_in vitro_ 150 µl/cm^2 of commercial preparation (5% TiO_2, 7.5mg/cm^2) in aqueous or oily dispersion _In vivo_ 2 µl/cm^2 (5% TiO_2, 0.1 mg/cm^2) in aqueous or oily dispersion
Skin:	_in vitro_ human skin from abdominal area (samples stirred at -20°C), and skin equivalents with cultivated human keratinocytes and fibroblasts _In vivo_ ventral side of forearm of male and female volunteers
Skin area	not reported
Skin temperature:	32°C
Test chamber:	penetration cells identified with figure.
Receptor fluid:	phosphate buffer pH 7.4
Exposure period:	24 h for _in vitro_ studies 45 minutes for the _in vivo_ studies
GLP:	no
Published:	yes
Study period:	2000
Reference:	Reference 130 Submission VII Bennat and Müller-Goymann 2000

Method
A penetration cell was used for both skin samples and the human skin equivalent studies. For _in vitro_ test the amount added was 150 µl per skin sample, for the _in vivo_ tests 2 µl per skin area. This results in TiO_2 administrations of 7.5 mg/cm^2 and 0.1 mg/cm^2, respectively. All dispersions were removed after the exposure period (_in vitro_ 24 h, _in vivo_ 45 minutes) with a paper towel. Both _in vivo_ and _in vitro_ Tesa® were used for collection of cell layers of the skin treated with the TiO_2 formulations. Atomic absorption spectrometry (AAS) was used for determination of the Ti content. Tests were performed in triplicate. The formulations investigated were: an oil/water emulsion with carboxymethylcellulose (CMC), and dimethicon and silicon oil; a liposomal formulation with phospholipid and water.

Results
The amounts of Ti observed after the _in vitro_ and _in vivo_ exposure of skin was in the µg range. In the sequential tape strips starting at about 25-35 µg/cm^2 in the first tape strip and declining just above the limit of detection (0.1 µg/cm^2) level at tape strip #6-#12. For the oily dispersion having the highest Ti levels were measured in the first tape strips. For the _in vivo_ exposure the Ti recovery started at about 7.5 µg/cm^2 and declined in the following tape strips. Microfine TiO_2 was found to penetrate deeper in the human skin from an oily dispersion than from an aqueous one.

SCCS Comments
No information was provided on the actual size of the used microfine TiO_2, so the study can only be considered as supporting evidence.

***In vitro* exploratory study percutaneous skin penetration - human skin (Ref 142)**

Study design.

Guideline/method:	exploratory study
Species:	human (female Caucasian)
Test substances:	Solaveil CT10W 3% W/Si emulsion, 3% W/O emulsion, both used as sprayable product (Uniqema, UK)
Particle size:	not reported in M&M section. Mentioned in Discussion to be between 20-70 nm
Group sizes:	n=6 (experiments)
Dose applied:	2 mg/cm^2
Skin:	abdominal skin from plastic surgery stored at -25°C for maximally 6 months
Skin area	5.31 cm^2
Skin temperature:	32 ± 1 °C
Test chamber:	static Franz-type diffusion cell,
Receptor fluid:	PBS with 4% bovine serum albumin
Exposure period:	1, 2, 4, 6, 8, 12 and 24 h
GLP:	no
Published:	yes
Study period:	2009
Reference:	Reference 142 submission VII Durand et al., 2009

Method

The incubation in the diffusion cells was performed in wells covered with Parafilm paper to avoid drying, and the whole system was protected from sunlight by opaque paper. Receptor fluid was removed at several time points and replaced immediately with fresh solution. Ti level was quantified and determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). Samples were digested and dissolved before Ti determination.

At the end of the experiment, the skin samples were removed from the cell and rinsed with PBS solution and tetrahydrofuran/ acetonitrile (THF/CAN, 80 : 20, v : v) until no product was left on the skin. The skin was then ground and mixed with a THF/ACN (80 : 20, v : v) solution and placed in an ultrasonic bath for 30 min. Each solution was then divided in two parts: one part was kept at -25°C for the further analysis of the TiO_2 by spectrometric methods. Three types of sample were taken and analysed:

1 The receptor fluid (5 mL).

2 A solution of the recovered product remaining on the skin (after evaporation of all liquid).

3 The mixture of ground skin (after evaporation of all the liquid).

The samples were heated at 450°C in a muffle furnace for 10–12 h. They were then fused with 5 g of $K_2S_2O_7$ in a flame. The resulting substance was dissolved in 10 mL hot H_2SO_4 solution (1 : 1 v/v) and diluted with ultra-pure water to 100 mL.

The solutions obtained were then injected into the ICP-OES apparatus.

Results

The recovery of the TiO_2 from the emulsions and spiked PBS solution with 4% BSA was 92.5% (W/O emulsion), 92.4% (W/Si emulsion, and 96.8% for the BSA-PBS solution , respectively, demonstrating the validity of the method for determination of Ti. In each part of the skin and in the receptor fluid for W/O and W/Si, respectively. the levels of recovery were between 76% and 86% for Ti present in the skin and/or on the skin. The limits of detection and of quantification are respectively 0.01 ppm (0.01 µg/g) and 0.1 ppm (0.1 µg/g) for the W/O and W/Si emulsion. Presence on skin after washing was about 40% and 50% for the W/O and W/Si emulsion, respectively. Approximately 20% (W/O) to 40% (W/Si) of the TiO_2 was observed in the skin. After 24 h of experiment titanium levels were below the limit of detection. So it was considered that no TiO_2 passed into the receptor fluid. There was a loss in the recovery up to 25% of the administered dose.

SCCS Comment
No characterization data for TiO_2 is presented - only size has been indicated in discussion section of the paper. Presence of coating indicated in Table 4.2 of supplicant but not mentioned in the paper. Data on receptor fluid indicated in text but not shown in paper. Level of Ti at 24 h mentioned to be below limit of detection but data on recovery/determinations at various time points are not presented in the paper. Results are of limited value for the evaluation of skin penetration of TiO_2 as no data on the receptor fluid were presented. It was demonstrated that approximately 20% to 40% of the TiO_2 was observed in the skin. No further evaluation of localization was done.

***In vitro* exploratory study percutaneous skin penetration - human skin (Ref 143)**

Study design.

Guideline/method:	exploratory study
Species:	human
Test substances:	Titanium dioxide T805, and Spectra veil MOTG, a 60% dispersion of zinc oxide in mineral oil/triglyceride.
Particle size:	not reported
Group sizes:	not reported
Dose applied:	1 mg/cm^2 *in vitro*
Skin:	abdominal skin recovered from plastic surgery
Skin area	not reported
Skin temperature:	room temperature
Test chamber:	not reported
Receptor fluid:	not reported
Exposure period:	not reported
GLP:	no
Published:	yes
Study period:	1997
Reference:	Reference 143 submission VII Dussert et al., 1997.

Method
The presence of TiO_2 in the skin was evaluated by TEM. At TEM characterization the TiO_2 was identified as a mixture of rutile and anatase crystal forms. The sunscreen formulation investigated was a mixture of both TiO_2 and ZnO. The test formulation was a w/o emulsion formulated with ultrafine titanium dioxide (11% wt), and zinc oxide (2.5% wt). The formulation was used as topical administration *in vitro* with a dose of 1 mg/cm^2 Skin penetration was evaluated by TEM.

Results
Cross-sections of the horny layer of human epidermis, after topical application of the sunscreen emulsion, show an almost regular mineral-coating of the stratum corneum. The crystals appear to surround the desquamating corneocytes. However, neither intercellular nor intracellular penetration of crystallites is evident in transmission electron microscopy. The TEM evaluation shows the presence of particles above the stratum corneum and between desquamating stratum corneum cells.

SCCS Comments
Although this study provides some evidence that there is no penetration of the nanoparticles from the formulation into the skin, the information on the study itself is rather limited, e.g. time of incubation and surface area of treated skin were not indicated. A mixture of TiO_2 and ZnO nanoparticles was used in the formulation. In the TEM evaluations the TiO_2 and ZnO could not be identified separately. This study is of no value for the evaluation of skin penetration of TiO_2 nanoparticles. Presence of coating is indicated in Table 4.2 of supplicant but not mentioned in the paper.

Kertesz et al. 2004, Ion-microscopic evaluation of porcine or human skin after treatment with TiO2 samples (Ref 66)

Samples investigated by ion microscopy are 14-16 μm thick porcine and human skin.

Quantitative elemental concentrations and distributions a new measurement setup and data evaluation system has been developed.

The penetration studies using different formulations were started on domestic pig skin, which resembles human skin closest. In a next step, human skin xenografts transplanted into SCID mice were used.

22 pig skin, 11 transplanted human skin and 13 human skin samples were investigated.

Results
The results obtained by ion microscopy or electron microscopy show that in the case of healthy skin the nanoparticles penetrate into the deepest corneocyte layer of the skin, but never reach the vital layers.

Conclusion
No penetration of the test material into viable porcine or human skin

Nanoderm - Quality of skin as a barrier to ultra-fine particles (ref 67)

Penetration of TiO2-nanoparticles through the epidermis of human foreskin grafts transplanted into SCID (Severe Combined Immune Deficiency) mice.

The skin grafts were treated with a hydrophobic emulsion (Antheil's XL F60) containing micronized TiO2-nanoparticles in occlusion, for 1, 24 and 48 h.

Quantitative elemental concentrations and distributions have been determined in 14-16 µm thick freeze-dried sections obtained from quick frozen punch biopsies using PIXE (Particle Induced X-ray Emission), STIM (Scanning Transmission Ion Microscopy) and RBS (Rutherford Backscattering) analytical methods.

Result
In most cases it was found that the remnant of the liposome crème together with the outermost stratum corneum was removed during the sample preparation. When the crème remained on the skin the Ti was quasi homogeneously distributed in the outermost layers, and the penetration seemed to be limited to the outermost part of the stratum corneum. However, in two cases, both after 48 h exposure, penetration through the stratum corneum to the limit of the vital stratum granulosum was observed. The sample originates from the entry of a sweat gland.

Conclusions
No penetration to the viable skin was reported except for some limited observations of material entering sweat glands.

Adachi et al., 2010, *In vivo* effect of industrial titanium dioxide nanoparticles experimentally exposed to hairless rat skin (Ref 126)

Guideline/method:	No specific guidelines followed
Test system:	Hairless Rat (Male Westar Yogi Rats) 8 weeks old, weighing 202–267 g, (Japan SLC, Hamamatsu)
Test items:	Uncoated anatase TiO2 nanoparticles (ST-01) from Ishihara Sangyo, Ltd, Japan.
Formulation	White water/oil (W/O) emulsion containing 10 wt% TiO2, 4 wt% Nikko Nikkomulese WO (cyclopentasiloxane, PEG-10 dimethicone, dosteardionium hectrite), 50.0 wt% decamethylcyslopentasiloxane

	KF-995 and 0.55 wt% acetic acid, and purified water was added to a final volume of 100 wt%
Concentrations:	Four mg/cm2 emulsion (0.4 mg/cm2 TiO2) was applied to a 15 cm2 area on the rat dorsal skin in the absence of ultraviolet (UV) radiation.
Exposure:	Skin samples at 4 h after exposure were observed using light, electron, and confocal laser scanning microscopy over 48 hrs. Time course study for light microscopic evaluation in the other groups of rats (10 TiO2-treated and five control rats) was carried out at 24, 72 and 168 h after exposure.

Results
After 24 h, no particles were observed in keratinized layers of the follicular infundibulum, but a small amount of particles remained in the superficial part of the stratum disjunctum. After 72 h, the particles were still observed in upper keratinized layers of the infundibulum but were not found in the interfollicular horny cell layer (Figure 3d). After 168 h, small crops of particles were found in the uppermost keratinized layer of only a few follicular openings.

Conclusion
The study shows no penetration of TiO2 in water / oil emulsion into viable skin through either the transcorneal or transfollicular pathway.

Gopee et al., 2009, Lack of dermal penetration following topical application of coated and uncoated nano- and micron-sized titanium dioxide to intact and dermabraded skin in mice (Ref 162 - poster presentation)

Guideline/method:	No
Test system:	Mice (hairless)
Test item:	TiO2 (Unreported batch) roughly spherical uncoated particles, with 25.1 ± 8.2 nm diameter (minimum particle size was 13 nm and maximum particle size was 71 nm). Formulation consisted of titanium dioxide suspended in polyglyceryl-3 distearate, cetearyl alcohol, light mineral oil, propylene glycol, k-phosphate buffer, methyl paraben, propyl paraben, and propylene glycol:water (1:4, v:v).
Treatment:	Mice (hairless) were treated with 5 uL of 5% uncoated anatase TiO2 (intact or dermabraded skin). At 6 and 24 hr post-application, mice were sacrificed and skin, right regional lymph nodes, blood, liver, kidney and spleen were collected and analyzed for titanium (Ti) by ICP-MS. Tissues of one mouse was analyzed microscopically.

Result
No significant elevations in Ti levels were observed in any of the organs analyzed for Ti.

Conclusion
The results suggest that both intact and compromised skin of hairless mice may be an effective barrier for nano-sized TiO2.

Kiss et al. 2008, Investigation of micronized titanium dioxide penetration in human skin xenografts and its effect on cellular functions of human skin-derived cells (Ref 167)

Guideline/method:	No

Test system:	*In vivo* SCID mice, grafts area, 6-mm diameter human foreskin punch biopsies were taken.
In vitro:	human immortalized HaCaT keratinocyte cells, human dermal fibroblasts (HDFs) & human immortalized sebaceous gland cell line SZ95.
Test items:	TiO2, 9 nm Anatase (gift from Prof. Z. Stachura, Krakow, Poland)
Vehicle:	hydrophobic emulsion ('TiO2-emulsion') was used (Anthelios XL SPF 60, La Roche Posay, La Roche Posay, France)
Concentrations:	2 mg / cm^3
Exposure:	24 h

Result
TiO2 particles did not penetrate through the stratum corneum of human skin transplants. TiO2 nanoparticles are internalized by *in vitro* cultured fibroblasts and melanocytes but not by keratinocytes and sebocytes.

Conclusions
This type of TiO2 (custom made, anatase) does not penetrate human foreskin grafts. *In vitro* uptake is cell type dependent.

Pinheiro et al. 2007, The influence of corneocyte structure on the interpretation of permeation profiles of nanoparticles across skin (Ref 191)

Guideline/method:	No
Test system:	Healthy and psoriatic human skin was collected by .punch biopsy (3 mm diameter) at lumbar-sacral region,
Test material:	Commercial sunscreen formulation (unknown source), containing nano TiO2.
Concentrations:	Unknown
Exposure:	2h

Results
The TiO2 permeation in psoriatic skin reached deeper regions of the stratum corneum than in healthy skin. However, for both cases TiO2 nanoparticles did not reach the living layers of the granulosa or spinosum strata.

Conclusion
Psoriasis seems to have only a limited effect on the permeation profile of TiO2 nanoparticles. It has to be mentioned that the source and concentration of the particles is not specified in this study.

Popov et al. 2005, 2005, 2010 (Ref 192, 193, 194)

Test system:	human skin (volunteers). Sunscreen including rutile TiO2 particles (100 nm) was administered five times over a period of 4 days onto the surface area of flexor forearm skin. The tape-stripping procedure started on the fourth day, 1 h after last application. The surface density of TiO2 particles on the tape strips was analyzed by x-ray fluorescent measurements.
Test material:	Sunscreen including rutile TiO2 particles (100 nm), this was not further specified.

Concentration: 2 mg/cm² sunscreen. skin area of 10 X 8 cm (160 mg sunscreen).

Results
Approximately 14 µg/cm2 of TiO2 was found on the first tape strip and almost zero on those taken at the depth of 15 µm. The particles were mainly located at a depth range of 0 to 3 µm.

Conclusions
No penetration into living layer of skin. The source and nature of TiO2 is not well reported. Three different papers all presenting the same experiment as an original study.

Sadrieh et al. 2010, Lack of significant dermal penetration of titanium dioxide (TiO2) from sunscreen formulations containing nano- and sub-micron-size TiO2 particles (Ref 199)

Test system: Female Yucatan minipigs (~4 months of age; n ¼ 12) from Sinclair Research Center (Auxvasse, MO, USA).

Test items: Uncoated nano titanium dioxide (Degussa Aeroxide P25, a mixture of anatase and rutile and known to be photocatalytic;

1. coated (aluminum hydroxide/dimethicone copolymer) nano titanium dioxide (BASF T-Lite SF obtained from BASF, Shreveport, LA; rutile; ''coated nano'')
2. uncoated submicron titanium dioxide (treated with aluminum hydroxide, Ishihara Tipaque CR-50 obtained from Ishihara Corporation, San Francisco, CA; rutile; ''submicron'')

Vehicle All used particles were added to the same sunscreen preparation, preparation without particles was used as control.

Concentrations: Approximately 5% preparations were achieved.

Exposure: Topical application four times daily, 5 days a week, for a total of 22 days. Dose of 2 mg/cm², each animal received a total of 176 mg/cm² cream resulting in a average of ~1.32 l of cream per animal

Negative control: cream without TiO2

Result
The epidermis from minipigs treated with sunscreens containing TiO2 showed elevated titanium levels. Increased titanium was detected in abdominal and neck dermis of minipigs treated with uncoated and coated nano TiO2. EM-energy dispersive x-ray analysis showed that TiO2 particles were found in the stratum corneum and upper follicular lumens in all treated skin samples. Isolated titanium particles were present at various locations in the dermis of animals treated with any of the three types of TiO2 sunscreens; however, there was no pattern of distribution or pathology.

Conclusion
These findings indicate that there is some, though probably not significant, penetration of TiO2 nanoparticles through the intact normal epidermis in minipigs. The quantification of the concentration in the dermis is difficult since the removal of the epidermis is almost never perfect (resulting in possible false positive results).

Exploratory study, dermal penetration and toxicity, hairless mice and porcine skin, subchronic dermal exposure (Wu et al., 2009)

The paper has its focus on the penetration of TiO2 nanoparticles through the skin after dermal exposure.

- No penetration in *in vitro* porcine skin model of TiO2 (4, 10, 25, 60 and 90nm). The amount of TiO2 was below detection limit, but materials and methods stated that TiO2 was not removed. Not clear whether the TiO2 was removed before tape stripping. Results indicate that tape stripping most probably was done after removal of TiO2, hence there was a low levels in the tape strip pools.
- Pig skin in vivo: TiO2 present in stratum corneum, stratum granulosum, prickle cell layer and stratum basale of the epidermis but not in dermis. Only 4nm TiO2 in basal cell layer. Figure 2 does NOT clearly show presence of TiO2 nanoparticles in epidermis.
- Hairless mice: Effect of TiO2 on body weight observed. Decreased growth compared to control mice and mice treated with normal sized TiO2. 10-25- and 21 (P25) nm TO2 induced growth retardation.
- Biochemical parameters for skin and liver malondialdehyde (MDA) increase (10-25-21nm), superoxide dismutase (SOD) skin and liver decrease (10-21nm), skin hydroxyproline (HYP) decrease (10-25-21-60nm)
- Organ distribution after 60 days skin exposure showed 10, 25, 21, 60nm TiO2 in skin, sub muscles, heart, liver, spleen, 21, 60nm TiO2 in lung, 21nm TiO2 in brain, whereas TiO2 in kidney was similar to control. However the differences were not significant.

Conclusion
Local effects on skin are demonstrated by biochemical parameters SOD, MDA, and HYP, and histopathology (keratinization). Systemic effects are not clearly identified because of possible alternative route of exposure by oral uptake. Also the lesions shown in various organs may be due to background lesions present in animal strain. This is not excluded by scoring of lesions in control versus treated animals. However, the treatment resulted in growth retardation of the animals.

Studies with limited information

In vivo study (Gottbrath et al., 2003; FitzGeral, 2005)
Penetration of nano-sized titanium dioxide (Tioveil AQ N; (rutile, coated with alumina/silica) into human stratum corneum after *in vivo* application of two formulations was studied. Penetration was measured by tape stripping of skin (10 strips). Tape strips from the titanium dioxide-treated skin sites were assayed for titanium by atomic absorption spectrometry. Tape strips from the vehicle control treated sites were viewed with an inverted microscope to estimate the amount of corneocyte aggregates. Titanium dioxide nanoparticles in the formulations and tape strips were visualized by transmission electron microscopy (TEM). The authors concluded that, after application of the liposomal formulation, a fraction of the TiO2 nanoparticles penetrated into the stratum corneum and did not remain in shallow valleys formed by the corneocytes, explaining the water resistance of the liposomal formulation, i.e. the deposition of TiO2 nanoparticles depends on the formulation used.

In vivo study (Tan et al., 1996; FitzGeral, 2005)
Review of recent literature on safety of nanomaterials in cosmetics with special references to skin absorption and resorption of ultrafine titanium dioxide and zinc oxide, prepared for Physical Sunscreens Manufacturers Association (PSMA), European Cosmetic, Toiletry and Perfumery Association and BASF AG, 28 September 2005.

A study with 10-50 nm TiO2 particles was performed in order to evaluate if the particles could penetrate the stratum corneum to the dermis following repeated application in volunteers (13 patients with compromised skin scheduled to have surgery for skin lesions).

The patients received repeated application (twice a day for 2-6 weeks) of a sunscreen lotion containing 8% microfine TiO2. Chemical analysis (ICPMS) were performed on skin biopsies. The authors concluded that non-statistically significantly higher Ti levels in the dermis of treated subject vs. controls (cadaver skin) were found.

In vivo study (Lademann et al., 1999)
The dermal penetration of 20 nm TiO2 nanoparticles (Titan M 160, coated, rutile) (assumed particle size, based on description of product used) in a sunscreen formulation (o/w emulsion) was studied. The sunscreen was applied repeatedly (11 times) over 4 days to the forearm skin (2 mg/cm2) of human volunteers. UV/Vis spectroscopic evaluation, X-ray fluorescence measurements LIFM, SRLSM and Raman spectroscopy of skin tape strips and histological evaluation of skin biopsies were performed. The only significant finding concerning a potential penetration of TiO2 beyond the upper skin layers was their deposition in single hair follicle openings, although there was no evidence that these residues were located within the living skin. The concentration of Ti in the hair follicle openings was two orders of magnitude lower than that in the upper skin layers. The authors concluded that that there was no penetration of TiO2 particles in living skin and that the TiO2 particles were mainly located in the outer layers of the SC.

In vivo study (Schulz et al., 2002)
The influence of particle size on the dermal absorption of three TiO2 preparations was investigated (T805 [20 nm, cubic, Ti/Si coating, rutile/anatase], Eusolex T-2000 [rutile, 10-15 nm NPs in 100 nm aggregates, needles, Ti/ Al2O3/SiO2 coated] Tioveil AQ-1 0P [100 nm, needles, Ti/Al/Si coated]). Each had a different primary particle size (10-15 nm, 20 nm and 100 nm), shape (cubic or needles) and hydrophobic/hydrophilic characteristics. The preparations were topically applied (4 mg/cm2) in an oil-in water emulsion containing 4% TiO2 to the forearm skin of human volunteers for 6 hours. Skin biopsies were examined by scanning electron microscopy to visualize the distribution of particles within the skin layers. TiO2 particles were only deposited on the outermost surface of the SC, and were not detected in deeper SC layers, the human epidermis and dermis. The authors concluded that none of the particles penetrated beyond the outer layer of the stratum corneum.

Another study provided under dermal penetration (Reference 10, submission 1) seems to be an irritation study and has therefore not been reviewed.

SCCS Comments on Dermal/ Percutaneous Absorption
The studies presented in the submission cover a range of nanomaterials of which some relate to the materials under assessment. The studies range from *in vitro* to ex vivo and *in vivo* experimental conditions, and intact and UV damaged skin. The results from these studies suggest that TiO2 nanoparticles, when applied to skin in a sunscreen formulation, are likely to stay largely on the skin, whilst a small proportion of the particles may penetrate to the outer layers of stratum corneum. A few reports have suggested the possibility that TiO2 nanoparticles may penetrate deeper to reach stratum granulosum – e.g. in human foreskin grafts transplanted onto SCID mice (Kertész et al., 2005) - or to dermis of minipigs treated with nano TiO2 (Sadrieh et al., 2010 (Ref 199)). There is, however, a consistent and large body of evidence from the submitted studies, and other studies published in open literature (e.g. NANODERM, 2007; Nohynek et al., 2007), which shows that nanoparticles do not penetrate deep enough to reach the viable epidermis or dermis cells of healthy skin. In psoriatic skin, Pinheiro et al. (2007) showed that nano-TiO2 in a sunscreen formulation penetrated into deeper areas of the stratum corneum than in healthy skin, but did not reach living cells in either psoriatic or healthy skin. Some in vitro test systems, however, lack a stratum corneum layer, which can block penetration of TiO2 nanoparticles. Toxicological effects from such tests therefore need a careful consideration since they may be difficult to extrapolate to the effects *in vivo* (Nohynek et al., 2007).

Contrary to the strong evidence suggesting a lack of penetration of TiO2 nanoparticles to viable epidermis or dermis cells, there are a number of studies (in this submission and

published elsewhere), which indicate that nanoparticles can enter hair follicles. According to SCCP opinion (2007) and NANODERM report (2007), adverse effects are not expected from dermal exposure of healthy unflexed skin to photostable nano-TiO2 in sunscreens. However, if photocatalytic nano-TiO2 is present in a sunscreen, it can potentially lead to generation of reactive oxygen species (ROS) on exposure to UV light.

Most, if not all, studies provided in the submission were performed with nano TiO2 as present in sunscreen formulations depicting consumer use. The studies were not directed towards hazard identification using either a dose response approach or a worst case scenario (overdosing situation). It is also of note that currently there are certain knowledge gaps in relation to the possible dermal penetration of nano TiO2 on repeated or long term use of cosmetic products, which may not only be used on flexed healthy skin but also on skin that may have lesions or cuts. Studies provided in support of this submission have shown that TiO2 nanoparticles do not penetrate the (simulated) sunburnt skin, whereas such information on flexed or damaged skin is currently not available.

1.5.5 Repeated dose toxicity

1.5.5.1 Repeated Dose (30 days) oral toxicity

Exploratory subchronic oral study – Mice 30 day oral (gavage)

Guideline:	No guideline
Species/strain:	Mice/CD-1
Group size:	20 females per group
Test substance:	TiO2 (Anatase, prepared from hydrolysis of Ti-tetrabutoxide, Primary particle size 5 nm)
Batch:	
Purity:	
Vehicle:	
Dose levels:	0, 62.5, 125 and 250 mg/kg bw/day
Dose volume:	
Route:	Oral
Administration:	Intragastric administration every other day for 30 days
GLP:	No
Study period:	2009
Reference:	SI-II-Duan et al., 2010, (140)

Results
Mice treated with doses ≥125 mg/kg bw/d showed body weight reduction, an increase in coefficients of the liver and increased coefficients of the liver, kidney, spleen and thymus and serious damage to liver function as shown by:

- A decrease in interleukin-2 activity, white blood cells, red blood cells, haemoglobin, mean corpuscular haemoglobin concentration, thrombocytes, reticulocytes, T lymphocytes (CD3+, CD4+, CD8+), NK lymphocytes, B lymphocytes, and the ratio of CD4 to CD8 of mice.
- An increase in NO level, mean corpuscular volume, mean corpuscular haemoglobin, red (cell) distribution width, platelets, hematocrit, mean platelet volume of mice.
- Disruption of the liver function in terms of enhanced activities of alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase and cholinesterase, increase of total protein, and reduction of albumin to globulin ratio, total bilirubin, triglycerides, and the total cholesterol levels.

No such effects were seen at low dose, and the NOAEL appears to be 62.5 mg/kg bw/d.

SCCS Comment
NOAEL derived from this study is 62.5 mg/kg bw/d.

1.5.5.2 Sub-chronic (90 days) toxicity (oral, dermal)

Subchronic oral toxicity – Rat 90 day oral (diet)

Guideline:	No guideline
Species/strain:	Rat/F344
Group size:	10 m, 10 f per group
Test substance:	TiO2 (uncoated, Unitane®, Anatase), CAS No. 13463-67-7
Batch:	402110C46
Purity:	98%
Vehicle:	
Dose levels:	6250, 12500, 25000, 50000, 100000 ppm
Dose volume:	
Route:	Oral
Administration:	Diet
GLP:	No
Study period:	1978
Reference:	I-NCI, 1979 (22); DHS-NCI, 1979 (9)

Results
No deaths, no differences in body weight gains, no substance-related gross or microscopic pathological finding, NOAEL: 100000 ppm.

SCCS Comment
No information has been provided on the particle size profile of the material tested in this study. The study is therefore of little value in relation to the current assessment for nano-forms of TiO2.

Note
The two references provided (I-NCI, 1979 (22) and DHS-NCI, 1979 (9)) are in fact the same.

Subchronic oral toxicity – Mouse 90 day oral (diet)

Guideline:	No guideline
Species/strain:	Mouse/B6C3Fi
Group size:	10 m, 10 f per group
Test substance:	TiO2 (uncoated, Unitane®, Anatase), CAS No. 13463-67-7
Batch:	402110C46
Purity:	98%
Vehicle:	
Dose levels:	6250, 12500, 25000, 50000, 100000 ppm
Dose volume:	
Route:	Oral
Administration:	Diet
GLP:	No
Study period:	1978
Reference:	I-NCI, 1979 (22); DHS-NCI, 1979 (9)

Results
No deaths, no differences in body weight gains, no substance-related gross or microscopic pathological finding, NOAEL: 100000 ppm.

SCCS Comment
No information has been provided on the particle size profile of the material tested in this study. The study is therefore of little value in relation to the current assessment for nano-forms of TiO2.
Note: Two references provided (I-NCI, 1979 (22); DHS-NCI, 1979 (9)) are in fact the same.

Exploratory subchronic oral study – Mice 60 day oral (gavage)

Guideline:	No guideline
Species/strain:	Mice/CD-1
Group size:	20 females per group
Test substance:	TiO2 (Anatase, prepared from hydrolysis of Ti-tetrabutoxide, Primary particle size 5 nm)
Batch:	
Purity:	
Vehicle:	
Dose levels:	0, 5, 10, 50 mg/kg bw/d
Dose volume:	
Route:	Oral
Administration:	Intragastric administration every day for 60 days
GLP:	No
Study period:	2010
Reference:	SI-II- Hu et al., 2010 (163)

Results
Potential effects on nervous system function, significant impairment of the behaviours of spatial recognition memory. Indications for impaired neurofunction and behaviour at all dose levels, indicated by:

- Significantly altered levels of Ca, Mg, Na, K, Fe and Zn in brain
- Inhibition of the activities of Na+/K+-ATPase, Ca2+-ATPase, Ca2+/Mg2+-ATPase, acetylcholine esterase, and nitric oxide synthase;
- Disturbed function of the central cholinergic system – significantly decreased levels of monoamines neurotransmitters such as norepinephrine, dopamine and its metabolite 3, 4- dihydroxyphenylacetic acid, 5-hydroxytryptamine and its metabolite 5-hydroxyindoleacetic acid,
- Increased levels of acetylcholine, glutamate, and nitric oxide.

SCCS Comment
From the 60 day oral (gavage) study in mice, a LOAEL of 5 mg/kg bw/d may be derived.

1.5.5.3 Chronic (> 12 months) toxicity

No study provided

SCCS Comment on Repeated Dose Toxicity:
Two out of the 4 subchronic studies provided are of little value to the assessment of nano-forms of TiO2 because particle size distribution of the tested materials is not provided. The other two studies used anatase nanomaterials. From the 60 day oral (gavage) study in mice, a LOAEL of 5 mg/kg bw/d may be derived.

1.5.6 Mutagenicity / Genotoxicity

There are a number of issues in regard to *in vitro* testing of nanomaterials for mutagenicity. Bacterial mutagenicity assays are considered to be less appropriate for the testing of nanoparticles compared to mammalian cell systems due to the lack of endocytosis by bacterial cells (EFSA, 2011). Therefore, for a negative outcome of such tests to be acceptable, it is essential that contact of the test materials with bacterial DNA (*i.e.* nanoparticle uptake by bacteria) is demonstrated. Furthermore, for testing of (conventional) chemical substances, generally accepted positive controls are used for the various *Salmonella* strains. The use of such chemical positive controls in testing nanomaterials would not provide a proof for a negative response of the nanomaterial. Currently, there is no accepted nanoparticle positive control that can demonstrate whether the assay is suitable for the mutagenicity testing of insoluble/poorly soluble nanoparticles.

It is of note that the following studies have not been reviewed as part of this assessment because they relate to test materials that are either not nanomaterials, or lack data on material characterisation to establish whether they were relevant nanomaterials for this assessment.

SI-Dunkel et al., 1985 (32); SI-Tennant et al. 1987 (33 (i, ii)); SI-Ivett et al., 1989 (35); SIII-Lu et al., 1998 (56c), Nohynek, 1999 (56), PSMA statement, 1999 (66); SI-II Warheit et al., 2007 (215); SIII-Lu et al., 1998 (56c), SI-Myhr, Caspary, 1991 (34); SI-Poole et al. 1986, (36); SI-Lemaire et al., 1982 (37); SI-II Msiska et al., 2010 (183); SI-Casto et al., 1979 (38); SI-Mikalsen et al., 1988 (39); SI-DiPole, Casto, 1979 (40); SI-Tripathy et al., 1990 (44); SI-Kitchin, Brown, 1989 (43); SI-II Pan et al., 2010 (189), SI-II DiVirglilio et al., 2010 (139), Osman et at 2012 (188).

1.5.6.1 Mutagenicity / Genotoxicity in vitro

Bacterial gene mutation test

Guideline/method:	OECD 471 (1997)
Test system:	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA. Tests were performed in absence or presence of S9-mix
Replicates:	Triplicate cultures in 2 independent experiments
Test items:	T805 (coated, A/R, PSMA 1 type)
Batch:	/
Solvent:	Ethanol
Concentrations:	8, 40, 200, 1000 and 5000 µg/plate in 1st experiment (range findings experiment); 312.5, 625, 1250, 2500 and 5000 µg/plate in 2nd experiment
Exposure:	48 h using the direct plate incorporation method
Negative control:	yes (vehicle)
Positive control:	ENNG for WP2uvrA, TA100 and TA1535; 9AA for TA1537 and 4NQO for TA98; 2AA in all strains in experiments with S9-mix.
GLP:	in compliance
Date of report:	19 June 1994 – 25 August 1994
Reference:	Submission DHS (11), II(67)

The test substance was tested for mutagenicity in bacterial gene mutation assays with and without metabolic activation (S9-mix prepared from Arochlor 1254 induced male Sprague Dawley rat livers) using the direct plate incorporation method. Test concentrations were based on the results of a preliminary toxicity study. The *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and the *E. coli* strain WP2uvrA$^-$ were exposed for 48 h to the

test substance (suspended in ethanol) in concentrations ranging from 8 - 5000 µg/plate (1st experiment) and 312.5 - 5000 µg/plate (2nd experiment).

Results
The test substance caused no visible growth reductions. Precipitation was observed at concentrations of 625 µg/plate and above. All positive controls showed marked effects on revertant colony numbers and the ethanol vehicle tested negative. Exposure to the test substance did not result in biologically relevant increases in revertant colony numbers.

Conclusion
Under the experimental conditions used T805 was not mutagenic in this gene mutation tests in bacteria.

SCCS Comment
See comments under 3.3.6 on the issues relating to the suitability of bacterial mutagenicity assays for nanomaterials.

Bacterial gene mutation test

Guideline/method:	OECD 471 (1983)
Test system:	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537. Tests were performed in absence or presence of S9-mix
Replicates:	Triplicate cultures in 2 independent experiments
Test items:	T817 (coated, A/R, PSMA 1 type)
Batch:	04095
Solvent:	Ethanol
Concentrations:	33.3, 100, 333.3, 1000, 2500 and 5000 µg/plate
Exposure:	48 h using the direct plate incorporation method
Negative control:	vehicle
Positive control:	NaN_3 for TA100 and TA1535; 4-NOPD for TA1537 and TA98; 2AA in all strains in experiments with S9-mix.
GLP:	in compliance
Date of report:	1997
Reference:	Submission DHS (12), II(67)

The test substance was tested for mutagenicity in a bacterial gene mutation test with and without metabolic activation (S9-mix was prepared from phenobarbital/β-naphtoflavone induced male Wistar Rat livers). The *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 were exposed for 48 h to the test substance (suspended in ethanol) at concentrations ranging from 33.3 to 5000 µg/plate.

Results
Normal background growth was observed up to 5000 µg/plate. All positive controls showed distinct increases in revertant colony numbers. Exposure to the test substance did not result in biologically relevant increases in revertant colony numbers.

Conclusion
Under the experimental conditions used T817 was not mutagenic in this gene mutation tests in bacteria.

SCCS Comment
The study is on T817 (coated, A/R, 95%, PSMA 1 type) which relates to Eusolex T in the dossier. This study is relevant to the nanomaterial group (anatase).
See comments under 3.3.6 on the issues relating to the suitability of bacterial mutagenicity assays for nanomaterials.

Bacterial Gene Mutation Test

Guideline/method:	OECD 471 (1997)
Test system:	*Salmonella typhimurium* strains T 98, T 100, T 102, T 1535 and TA1537, in presence or absence of S9-mix
Replicates:	Triplicate plates
Test items:	T-Lite™ SF, pure rutile, primary particle size 10 x 50 nm, mean agglomerates approximately 200 nm (d10: 90 nm, d90: 460 nm); coating consisting of aluminium hydroxide and dimethicone/methicone copolymer T-Lite™ MAX, pure rutile, primary particle size 10 x 50 nm, mean agglomerates approximately 200 nm (d10: 90 nm, d90: 460 nm); coating consisting of dimethoxydiphenylsilane, triethoxycaprylylsilane crosspolymer, hydrated silica and aluminium hydroxide
Batch:	/
Solvent:	DMSO (SPT), FCS (PIT)
Concentrations:	0, 20, 100, 500, 2500, 5000 µg/plate
Exposure:	Standard plate test and or preincubation test
GLP:	in compliance
Reference:	Landsiedel *et al.,* 2010

The test substances were tested for mutagenicity in the reverse mutation assay in bacteria with and without metabolic activation. The S9 fraction was prepared from phenobarbital/β-naphthoflavone induced male Wistar rat liver. Both the standard plate test (SPT) and the plate incorporation test (PIT) were used. The *S/ typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were exposed to the test substance (dissolved in DMSO (SPT) or fetal calf serum (PIT)) at concentrations ranging from 20–5000 µg/plate. For control purposes, DMSO) as negative control and the positive controls (NOPD, MNNG, AAC, MIT.C, 2-AA) were also investigated.

Results
With the T-Lite™ SF a weak bacteriotoxicity was occasionally observed from 2500 µg/plate onward in the presence of S9-mix only. With T-Lite™ MAX no bacteriotoxicity was noted. Precipitation of the test substance was recorded from 100 µg/plate onward for T-Lite™ SF and from 2500 µg/plate with T-Lite™ MAX.
The test substances did not induce a biologically relevant increase in revertant colony numbers in the bacterial strains at any concentration tested in the presence or absence of metabolic activation.

Conclusion
Under the experimental conditions used T-Lite™ SFand T-Lite™ MAX were not mutagenic in this gene mutation tests in bacteria.

SCCS Comment
The tested materials relate to S75-K (94% rutile, coated with aluminium hydroxide, dimethicone/methicone copolymer). See comments under 3.3.6 on the issues relating to the suitability of bacterial mutagenicity assays for nanomaterials.

Chromosome aberration test in mammalian cells

Guideline/method:	OECD 473 (1997)	
Test system:	CHO cells. Tests were performed in absence or presence of S9-mix	
Replicates:	Duplicate cultures in 2 independent experiments	
Test items:	T805 (coated A/R, PSMA 1 type)	
Batch:	0510067	
Solvent:	Ethanol	
Concentrations:	Experiment 1:	86.72, 209.7 and 800 µg/ml without S9 mix 167.8, 640 and 800 µg/ml with S9-mix
	Experiment 2:	167.8, 512 and 800 µg/ml

Exposure:	Experiment 1:	20 h treatment without S9 mix
		3 h treatment and 17 h recovery with S9-mix
	Experiment 2:	3 h treatment and 17 h recovery with S9-mix
Negative control:	Vehicle	
Positive control:	NQO (without S9), CPA (with S9)	
GLP:	yes	
Date of report:	17 November 1998 – 11 January 1999	
Reference:	Submission DHS (13), II(67)	

The test substance was evaluated for potential cytogenetic effects in Chinese hamster ovary (CHO) cells in the absence or presence of S9-mix. The S9 fraction was prepared from livers of rats treated with Arochlor 1254 (experiment 1) or phenobarbital/β-naphtoflavone (experimen2t). Cytotoxicity was measured as a reduction in cell number compared to the solvent control. In the absence of S9-mix only one experiment was performed. 4-nitroquilonine 1-oxide and cyclophosphamide were used as positive controls in the experiments without and with S9-mix respectively. For each culture cells with structural aberrations excluding gaps, and polyploidy, endoreduplication or hyperdiploidy were categorized.

Results
The number of cells with structural aberrations in the negative control cultures were within normal range. A biologically relevant increase in the number of cells with chromosome aberrations was not observed due to exposure to T805 both without and with S9-mix. The positive controls NQO and CPA induced statistically significant increases in the number of cells with structural aberrations in the absence or presence of S9 mix respectively.

Conclusion
Under the experimental conditions used T805 was not genotoxic (clastogenic) in this chromosome aberration test in mammalian cells.

SCCS Comment
The experiment in the absence of S9-mix was performed only once.

Chromosome aberration test in mammalian cells

Guideline/method:	OECD 473 (1997)	
Test system:	CHO cells. Tests were performed in absence or presence of S9-mix	
Replicates:	Duplicate cultures in 2 independent experiments	
Test items:	T817 (coated A/R, PSMA 1 type)	
Batch:	04095	
Solvent:	Ethanol	
Concentrations:	Experiment 1:	85.9, 640 and 800 µg/ml without S9-mix
		167.8, 512 and 800 µg/ml with S9-mix
	Experiment 2:	209.7, 512 and 800 µg/ml with S9-mix
Exposure:	Experiment 1:	20 h treatment without S9 mix
		3 h treatment and 17 h recovery with S9-mix
	Experiment 2:	3 h treatment and 17 h recovery with S9-mix
Negative control:	Vehicle	
Positive control:	NQO (without S9), CPA (with S9)	
GLP:	yes	
Date of report:	June 1999	
Reference:	Submission DHS (14), II(67)	

The test substance was evaluated for potential cytogenetic effects in Chinese hamster ovary (CHO) cells in the absence or presence of S9-mix. The S9-mix was prepared from livers of rats treated with Arochlor 1254 (experiment 1) or phenobarbital/β-naphtoflavone (experiment 2). In the absence of S9-mix only one experiment was performed. Cytotoxicity

was measured as a reduction in cell number compared to the solvent control. 4-nitroquilonine 1-oxide and cyclophosphamide were used as positive controls in the experiments without and with S9-mix respectively. For each culture cells with structural aberrations excluding gaps, and polyploidy, endoreduplication or hyperdiploidy were categorized.

Results
The number of cells with structural aberrations in the negative control cultures was within normal range. In the experiment without S9-mix, a slight but not statistically significant increase in the number of cells with chromosomal aberrations was observed. In the experiments with S9-mix no biologically relevant increase in the number of cells with chromosomal aberrations was observed. The positive controls NQO and CPA induced statistically significant increases in the number of cells with structural aberrations in the absence or presence of S9-mix, respectively.

Conclusion
Under the experimental conditions used T805 was not genotoxic (clastogenic) in this chromosome aberration test in mammalian cells.

SCCS Comment
The experiment in the absence of S9 mix was performed only once. A tendency of an increasing number of cells with structural aberrations was noted in the experiment without S9-mix.

In vitro micronucleus test in human epidermal cells

Guideline/method:	According to an generally accepted published protocol
Test system:	Human epidermal cell line, A431
Replicates:	3 independent experiments
Test item:	TiO_2 NP (Anatase, 99.7%), commercial
Batch:	/
Solvent:	DMEM with 10% FBS
Concentrations:	0.008, 0.08, 0.8, 8, 80 µg/ml
Exposure:	6 h treatment without S9-mix, harvest time 24 h after the start of treatment
Negative control:	Vehicle
Positive control:	Ethyl methanesulfonate (6 mM)
GLP:	Not in compliance
Published:	Shukla *et al.,* 2011
Reference:	Submission SI-II, (205)

The cytokinesis-block micronucleus (CBMN) assay was carried out to determine the potential genotoxicity of TiO_2 NP in the human epidermal cell line A431. The cells were treated for 6 h with different concentrations of TiO_2 NP (0, 0.008, 0.08, 0.8, 8, and 80 µg/ml). Ethyl methanesulfonate was used as positive control. After the 6 h exposure, the NPs were removed by washing with medium and cells were grown for additional 18 h in fresh DMEM medium containing Cytochalasin-B (3 µg/ml medium). Cytospin preparations were examined for the presence of micronuclei in binucleate cells. From each concentration 2000 binucleate cells were scored; the cytokinesis block proliferation index (CBPI) was calculated from 500 cells/concentration as recommended in OECD Guideline 487. Transmission electron microscopy (TEM) was used to evaluate uptake of the TiO_2 NP into the cells.

Results
CBPI was not significantly different from the control treatments. TEM analysis showed that NPs were taken up by the cells. The NPs were found to be distributed mostly in cytoplasm,

some NP were also localised in the nucleus. A statistically significant induction in the number of cells with micronuclei was observed after 6 h exposure to TiO_2 NP.
The particles were also found to induce oxidative stress in the cells indicated by a significant depletion of glutathione, induction of lipid peroxidation and reactive oxygen species generation.

Conclusion
Under the experimental conditions used TiO_2 NPs induced an increase in the number of cells with micronuclei and, consequently, TiO_2 NPs is genotoxic (clastogenic and/or aneugenic) in the human epidermal cell line A431.

Fpg modified Comet assay in human epidermal cells

Guideline/method:	According to an generally accepted published protocol
Test system:	Human epidermal cell line A431
Replicates:	2 cultures
Test item:	TiO_2 NP (Anatase, 99.7%), commercial
Batch:	/
Solvent:	DMEM with 10% FBS
Concentrations:	0.008, 0.08, 0.8, 8, 80 µg/ml
Exposure:	6 h treatment
Negative control:	Vehicle
Positive control:	25 µM hydrogen peroxide
GLP:	Not in compliance
Published:	Shukla et al., 2011
Reference:	Submission SI-II, (205)

TiO_2 NP was assayed for DNA damage in the human epidermal cell line A431 with the Comet assay. The cells were treated for 6 hours with TiO_2 NP in a concentration range up to 80 µg/ml. DNA damage was evaluated by formamidopyrimidine DNA glycosylase (fpg) modified Comet assay. The fpg allows for detection of oxidative DNA base damage lesions, in particular, 8-OH guanine. Hydrogen peroxide was included as a positive control and cytotoxicity was evaluated by MTT and NRU assay.

Results
The TiO_2 NP caused a significant concentration-dependent induction of DNA damage. Effects were statistically significant at the two highest testing concentrations. These concentrations were not cytotoxic after 6 or 24 h treatment in the MTT or NRU assay. Significant cytotoxicity for both concentrations was found in these assays after 48 h treatment. Uptake of NP into the A431 cells was shown by TEM analysis. Particles were observed mostly in the cytoplasm, but occasionally also in the nucleus. Oxidative stress in the cells was indicated from the significant depletion of glutathione, induction of lipid peroxidation and reactive oxygen species generation.

Conclusion
Under the experimental conditions used the results of the study indicate that TiO_2 NPs possess DNA damaging potential in human epidermal cells.

Comet assay in human lymphocytes

Guideline/method:	According to an generally accepted published protocol for the alkaline Comet assay
Test system:	Human lymphocytes
Replicates:	triplicate culture in 2 independent experiments
Test items:	TiO_2 NP commercial, declared size of 100 nm and surface area of 14.0 m^2/g

Batch:	/
Solvent:	RPMI-1640
Concentrations:	0, 0.25, 0.50, 0.75, 1, 1.25, 1.50, 1.75, 2 mM
Exposure:	3 hour treatment
GLP:	Not in compliance
Published:	Ghosh et al., 2010
Reference:	Submission SI-II, (157)

The DNA damaging potential of TiO_2 NP was evaluated using the Comet assay in human lymphocytes obtained by venipuncture from peripheral blood of healthy volunteers. Cells were isolated by gradient centrifugation using Histopaque and resuspended in RPMI-1640 culture medium. Cells were treated for 3 hours with the TiO_2 NP at a concentration range of 0 to 2mM. The Comet assay was performed according to published methods. DNA damage was reported as % tail DNA in treated lymphocytes. Slides were prepared in triplicates per concentration and each experiment was repeated twice. Viability was determined by trypan blue dye exclusion, MTT assay and WST-1 assay in the same concentration range as used for the Comet assay.

Results
Trypan blue indicated viability above 80% at the highest treatment concentrations. MTT and WST-1 assay showed increased toxicity, with an LC50 in the range of 1.0 to 1.25 mM. A statistically significant increase in DNA damage was observed in lymphocytes treated with the TiO_2 NP at a concentration of 0.25 mM. No concentration dependent effect and no statistically significant effects were found at any of the other testing concentrations.

Conclusion:
Under the experimental conditions used, the results of the study indicate that TiO_2 NPs possess DNA damaging potential in human epidermal cells.

SCCS Comment
The authors of the paper conclude that TiO_2 NP were genotoxic to human lymphocytes. They propose that the absence of a dose-dependent effect on DNA damage may be due to the agglomeration behaviour of the nanoparticles.
SCCS concludes that in view of the absence of a dose-dependent effect, this study does not provide evidence for the genotoxicity of TiO_2 in human lymphocytes.

In vitro mammalian cell gene mutation test

Guideline/method:	/
Test system:	*gpt* delta transgenic mouse primary embryo fibroblasts
Replicates:	/
Test items:	TiO_2 NP anatase (5 nm, 114m^2/g), Sigma Aldrich, TiO_2 NP anatase (40 nm, 38 m^2/g), Inframat Advanced Materials LLC Fine TiO_2 (325 mesh, 8.9m^2/g), Sigma-Aldrich
Batch:	/
Solvent:	/
Concentrations:	0, 0.1, 1, 10 and 30 µg/ml
Exposure:	24 h treatment
Solvent:	Distilled water (sonicated and then further diluted in culture medium)
GLP:	not in compliance
Reference:	Xu *et al.,* 2009

Mutant frequencies in *red/gam* loci by *Spi-* detection by two nano-sized and one fine TiO_2 materials were evaluated in *gpt* delta transgenic mouse primary embryo fibroblasts. The samples were suspended in distilled water, subsequently sonicated for 30 min, sonicated on

ice, and diluted in medium before addition to the cells. S9-mix was not included in the assay.

Results
Concurrent cytotoxicity of the samples was evaluated by the MTT assay and uptake of the NP in the cells was assessed by flow-cytometry. Increased mutants frequencies were observed with both the nanosize TiO_2 samples, but not with the fine TiO_2, demonstrating that these nanomaterials can cause kilo-base pair deletion mutations. These effects could be abrogated by co-treatment of the endocytosis inhibitor (lipid raft/caveolae disrupting agent) Nystatin, the nitric oxide synthase inhibitor, NG-methyl-L-arginine (L-NMMA) and the cyclooxygenase-2 activity inhibitor NS-398.

Conclusions
TiO_2 NP were taken up by the cells and induced kilo-base pair deletion mutations in a transgenic mouse mutation system.
It was suggested that induction of [ONOO]-, triggered by the signalling events associated with the transporting of nanoparticles into the cells, rather than the chemical composition/surface area combination of the nanoparticles may be a critical event for the observed genotoxicity.

SCCS Comment
Translocation and contact of the test items with the fibroblast DNA has not been demonstrated in the tests. The effects of the applied inhibitors suggest an indirect effect mediated by TiO_2 triggered formation of reactive oxidants. Uptake cannot be verified by flow-cytometry on the basis of side-scattering, as particles may merely be adhered to the cell membranes.

In vitro micronucleus test in mammalian cells

Guideline/method:	Draft OECD 487
Test system:	V79 cells
Replicates:	Quadruplicate cultures
Test item:	T-Lite™ SF, pure rutile, primary particle size 10 x 50 nm, mean agglomerates approximately 200 nm (d10: 90nm, d90: 460 nm); coating consisting of aluminium hydroxide and dimethicone/methicone copolymere
Batch:	/
Solvent:	FCS
Concentrations:	75, 150 and 300 µg/ml with 4 h exposure 18.8, 37.5 and 75 µg/ml with 24 h exposure
Exposure:	4 h treatment and harvest 24 h after start of the treatment 24 h treatment and harvest immediately after the end of treatment
Positive control:	Ethyl methanesulfonate 500 µg/ml
GLP:	not in compliance
Reference:	Landsiedel *et al.,* 2010

Micronucleus formation was evaluated in V79 cells after treatment with TiO_2 rutile NP coated with aluminium hydroxide and dimethicone/methicone copolymere (T-LiteTM SF rutile). The study was performed without S9 mix which was considered scientifically justified because of the nanoparticulate nature of the material. Cells were either treated for 4 hours with 75, 150 and 300 µg/ml, followed by 24 h recovery, or for 24 h at concentrations of 18.8, 37.5 and 75 µg/ml without recovery. The concentrations were selected on the basis of pilot experiment on cytotoxicity. Ethyl methanesulfonate was used as positive control.

Results
The occurrence of precipitation at higher concentrations influenced the toxicity assessment. Concurrent evaluation of cytotoxicity by analysis of proliferation index (PI) demonstrated

the absence of cytotoxicity up to highest scorable concentrations. A biologically relevant increase in the number of cells with micronuclei was not observed after exposure to T-Lite™ SF.

Conclusions
Under the experimental conditions used T-Lite™ SF did not induce an increase in the number of cells with micronuclei and, consequently, T-Lite™ SF is not genotoxic (clastogenic and/or aneugenic) in V79 cells.

SCCS Comment
The test material relates to S75-K (94% rutile, coated with aluminium hydroxide and dimethicone/methicone copolymer). Translocation and contact of the test material with the V79 cells and its possible translocation into the nucleus and interaction with DNA has not been demonstrated.

Alkaline Comet assay in mammalian lung cells

Test system:	A549 human lung carcinoma cells
Replicates:	Triplicate cultures
Test items:	TiO_2 synthesized by laser pyrolysis (spherical, 12 nm, 92 m^2/g, 95% anatase, PZC (point of zero charge) = 6.4) TiO_2 synthesized by laser pyrolysis (spherical, 21 nm, 73 m^2/g, 90% rutile) TiO_2-A25 AEROXIDE-P25 (spherical, 24 nm, 46 m^2/g, 86% anatase, PZC = 7.0) uncoated TiO_2 ref. 637262 from Sigma-Aldrich (Elongated, L: 68 nm, d: 9nm, 118 m^2/g, 100% rutile) TiO_2 ref. T8141 from Sigma-Aldrich (spherical, 142 nm, 10 m^2/g, 100% anatase, PZC = 5.2)
Batch:	/
Solvent:	Ultrapure sterile water (pH5.5), suspended at 10 mg/ml, further diluted in cell culture medium
Concentrations:	0 and 100 µg/ml
Exposure:	4 h, 24h and 48 h after start of the exposure
GLP:	not in compliance
Reference:	Jugan et al., 2011

DNA damage of five different types of TiO_2 particles (which included AEROXIDE-P25) was evaluated by alkaline Comet assay in A549 cells. No S9-mix was added to the test system. Cells were treated with one concentration (100 µg/ml) for 4 h, 24 h and 48 h. Cytotoxicity was evaluated by the MTT-assay. Electron microscopy was performed to evaluate uptake of the test samples into the A549 cells after 4 h.

Results
Electron microscopic evaluation demonstrated a rapid uptake of the various test materials into the cytoplasm of the A549 cells. Samples were tested only at one concentration.
Cytotoxicity, evaluated by MTT-test, revealed that cell death was less than 25% for all samples after 48 h of exposure.
DNA damage was significantly increased with all samples at 4 h, with three out of the five samples at 24 h. After 48 h no significant increase was detected with the exception of one sample (i.e. laser pyrolysis synthesized rutile, 21 nm). The uncoated sample (AEROXIDE-P25) caused a significant increase in DNA single strand breakage at treatment times of 4 and 24 h. For all smallest, including the uncoated sample cellular internalization and accumulation into cytoplasm was reported. For one sample (i.e. 12 nm laser pyrolysis synthesized), nanoparticles were found located in the nucleus.
It was concluded that several types of TiO_2 can cause DNA single strand breaks. In parallel investigations, they also showed capacity of TiO_2 to cause formation of the oxidative DNA

damage lesion 8-OHdG as well as an inhibition of DNA (base excision) repair activity. In contrast, they did not detect double strand breaks evaluated by γH2AX immunohistochemistry or clastogenic/aneugenic effects evaluated by micronucleus assay in the same cells.

Conclusions
Under the experimental conditions used it was concluded that TiO_2 nanoparticles have a genotoxic potential in this alkaline Comet assay in mammalian lung cells.

Alkaline Comet assay in mammalian liver cells

Guideline/method:	According to generally accepted and published protocols
Test system:	Human hepatoblastoma cell line C3A
Replicates:	Triplicate cultures
Test items:	NM101 Anatase 9 nm (XRD), 4-8/50-100 nm; two different particle types (TEM), 322 m^2/g NRCWE001 Rutile 10 nm (XRD), 80-400 (TEM), 99 m^2/g NRCWE002 Rutile 10 nm (XRD), 80-400 (TEM), 84 m^2/g, negative charged NRCWE003 Rutile 10 nm (XRD), 80-400 (TEM), 84 m^2/g, positive charged NRCWE004 Rutile approx. 100 nm (XRD), 1-4/10-100/100-200/1000-2000; five different types of particles (TEM)
Batch:	/
Solvent:	Distilled water with FCS
Concentrations:	Three concentrations, i.e. LC_{20}, ½ of LC_{20} and 2x LC_{20}
Exposure:	4 h treatment
Positive controls:	H_2O_2
GLP:	not in compliance
Reference:	Kermanizadeh *et al.,* 2012

DNA damage in human hepatoblastoma C3A cell line was evaluated by the alkaline Comet assay (evaluated as % tail DNA), with inclusion of fpg enzyme to detect oxidative DNA damage. A total of five different types of TiO_2 were tested at a concentration that caused 20% viability loss (LC_{20}), as well as twice or half of this concentration. The toxicity was evaluated by WST-1 assay (24 h treatment), the treatment time for the Comet assay was 4 hours. S9 mix was not included in the assays.

Results
Biologically relevant and small but statistically significant increases in DNA damage were found with several of the samples. The most pronounced effects were seen with NM101 and RWCE001. No biologically relevant increase in DNA damage was observed with the negatively charged RCWE003. In view of the observed effects in the presence of fpg (as well as based on further analysis of oxidative stress markers in the study), the authors suggest that the DNA damage effects are mediated by reactive oxygen species (ROS).

Conclusions
Under the experimental conditions used, it was concluded that short term exposure of liver cells to some TiO_2 particles caused small but significant increases in DNA damage.

SCCS Comments
Translocation and contact of the test material with the hepatoblastoma cells and its' possible translocation into the nucleus and interaction with DNA have not been demonstrated. Some of the effects are minor but are concentration dependent, this might become significant at a certain exposure level.

Further mutagenicity/genotoxicity in vitro studies (open literature):

The *in vitro* mutagenicity genotoxicity studies on TiO2 nanomaterials have been recently reviewed by Magdolenova et al. (2013). In many of these studies, particle size (and chemistry) is not, or poorly specified in the publications. As such, these studies do not allow for evaluation of the potential effects of the nanosize aspect of the potential genotoxicity of TiO2 (Le Boeuf et al., 1996; Endo-Capron et al., 1993; Pelin et al., 1995; Miller et al., 1995; Lu et al., 1998; Kamp et al., 1995; Dunford et al., 1997; Wamer et al., 1997). In several studies only fine TiO2 was used (e.g. Driscoll et al., 1997; Van Maanen et al., 1999, in both these studies TiO2 anatase 180nm with a BET value of 8.8 m2/g was used; Notably however, one may argue that this sample contains a particle distribution "tail" in the nanosize range).

Nagakawa *et al.* (1997) tested four TiO_2 samples, *i.e.* 21 nm and 255 nm anatase and 255 nm and 420 nm rutile for DNA strand breaks by alkaline Comet assay in the mouse lymphoma cell clone L5178Y/$tk^{+/-}$. In the presence of UV/light all samples showed enhanced DNA strand breaks at concentrations which also elicited cell death. Without irradiation only the 255 nm anatase showed enhanced strand breakage. The 21 nm anatase sample was also evaluated for the induction of chromosomal aberrations in the Chinese Hamster cell line CHL/IU, for mutagenicity in the *Salmonella typhimurium* strains TA100, TA98 and TA102, and colony formation in the L5178Y/$tk^{+/-}$ cells. Chromosomal aberrations (mainly polyploidy, chromatid breaks and chromatid exchanges) were found only in the presence of UV/visible light, and occurred at cyotoxic concentrations. In the absence of light the 21 nm anatase did not elicit chromosomal aberrations in contrast to the positive control (ofloxacin). Irrespective of UV/light irradiation, the 21 nm anatase failed to enhance the frequencies of revertant *Salmonella* colonies or mutant L5178Y colonies, in contrast to the positive control methyl methanesulfonate (MMS).

Linnainmaa *et al*. (1997) investigated micronucleus formation in rat liver epithelial cells after treatment with various TiO_2 samples in the presence or absence of UV light. Mitomycin C was used as positive control. TiO_2 samples were a 170 nm and a 20 nm anatase sample, and a 20 nm coated rutile sample. The coated sample was prepared with aluminium hydroxide and stearic acid. The sample was ethanol washed to remove the stearic acid before treatment of the cells. In contrast to the positive control, none of the samples induced an increase in cells with micronuclei.

Rahman *et al.* (2002) studied micronucleus formation in SHE fibroblasts after treatment with fine TiO_2 (>200nm) and nanosize TiO_2 (20nm). Apart from size, no further details of the samples were provided. Increased micronuclei were found only with the ultrafine TiO_2. The authors reported (but did not show in the manuscript) that further kinetochore-staining experiments revealed indications for chromosomal non-disjunction during mitosis. The nanosize TiO_2 also elicited apoptosis shown by DNA fragmentation analysis and the appearance of apoptotic bodies (transmission electron microscopy evaluation).

Gurr *et al.* (2005) tested a variety of TiO_2 samples for micronucleus formation as well as the induction of oxidative DNA damage using the Fpg-modified Comet assay in BEAS-2B human bronchial epithelial cells. The samples used were four different anatase samples, with respective sizes of 10, 20, 200 and >200 nm, and one rutile sample with the size of 200 nm. Micronucleus induction was found with the 10 and 200 nm anatase sample, but not with the >200 nm anatase and the 200 nm rutile samples. For the 20 nm anatase sample no data were provided. Enhanced oxidative DNA damage (fpg-Comet assay) was observed with the 10 and 20 nm anatase samples and with the 200 nm rutile. All other samples were negative. Finally, the authors showed that a 1:1 mixture of 200 nm anatase and 200 nm rutile caused stronger oxidative DNA damage than the 200 nm anatase or 200 nm rutile alone.

Bhattacharya *et al.* (2009) investigated the genotoxicity of anatase TiO_2 in BEAS-2B human bronchial epithelial cells and IMR-90 human lung fibroblasts. The TiO_2 nanoparticles caused induction of the oxidative DNA adduct 8-OHdG in IMR-90 cells (measured by an ELISA method), but did not cause increased strand breaks (measured by Comet assay) in the IMR-

90 and BEAS-2B cells. Electron microscopy demonstrated that both particles translocated near to nucleus, but were not found inside the nucleus, mitochondria or ribosomes.

Falck *et al.* (2009) investigated the genotoxicity of three TiO_2 samples in BEAS-2B human bronchial epithelial cells by the alkaline Comet assay and the micronucleus test. The samples were a nanosize rutile sample coated with <5 SiO_2 (10x40nm needle shaped, BET 132 m^2/g), a fine rutile sample (<5 µm, 2 m^2/g), and a nanosize anatase sample (<25 nm, 222 m^2/g). Hydrogen peroxide and mitomycin-C were used as respective positive controls. All samples showed mild but significant DNA damaging effects. The effects of the nanosize rutile were much weaker than those of the nanosize anatase and fine rutile sample. The nanosize anatase, in contrast to both other samples, also caused increased micronuclei. For the observed DNA damaging and micronucleus effects mostly no clear dose-dependency could be observed. It was also reported that the micronucleus scoring was difficult due to the presence of the particles during microscopy.

Magdolenova *et al.* (2012a) showed in human TK6, EUE and Cos-1 cells that genotoxicity of TiO_2 (DNA damage and oxidised DNA lesions) measured by the Comet assay (with and without fpg) depends on the stock dispersion protocol. The same TiO_2 (Aeroxide P25, primary particle size 21 nm, mixture of anatase /rutile), but prepared with different stock dispersion protocol, following further with the same media and exposure conditions resulted in differed state of agglomeration and gave different results. Larger agglomerates gave positive results. Thus differences in stock dispersion preparation could explain contradictory results published on the same nanoparticles. Magdolenova *et al.* (2012b) studied the possible interference of TiO_2 and other nanoparticles with the fpg enzyme in the Comet assay but did not find this to cause any artefacts.

1.5.6.2 Mutagenicity/Genotoxicity in vivo

Open literature studies

Micronuclei in peripheral blood erythrocytes after oral uptake

Guideline/method:	/
Species/strain:	C57Bl/6Jp_{un}/p_{un} mice.
Group size:	5 mice/treatment group
Test substance:	Aeroxide P25, Degussa/Evonik, primary particle size 21 nm, BET surface area 50 m^2/g, DLS in water: 21-1446 nm)
Batch:	/
Vehicle:	water
Dose levels:	0, 50, 100, 250, and 500 mg/kg bw (estimated dose)
Treatment:	/
GLP:	not in compliance
Reference:	Trouiller et al., 2009

Methods

C57Bl/6Jp_{un}/p_{un} mice, containing naturally occurring 70-kb internal duplication in the *pink-eyed dilution* (p) gene, were exposed via drinking water to the TiO_2 NP. The suspensions were ultrasonicated for 15 min before providing to animals. Water (with/without the NP) was provided *ad libitum* during 5 days. Peripheral blood was collected and erythrocytes were evaluated for the presence of micronuclei. The estimated exposures were 0, 50, 100, 250 and 500 mg/kg bw. The doses were estimated on the basis of estimated drinking water consumption (set at 5 ml) and the average weight of the animals. The authors also evaluated DNA damage, measured as 8-hydroxy-2'-deoxyguanosine in liver tissue by HPLC/ECD analysis, and alkaline Comet assay in blood cells, but these were tested only at one concentration (500 mg/kg bw). Moreover, DNA deletions were evaluated in the offspring of pregnant C57Bl/6Jp_{un}/p_{un} mice treated for 10 days at 500 mg/kg bw/day, to evaluate in utero effects.

Results
A biologically relevant increase in the number of peripheral blood erythrocytes after oral administration of TiO_2 NP was found in mice at the highest treatment dose only (500 mg/kg). This concentration also caused increased DNA strand breakage in white blood cells (Comet assay), γ-H2AX foci in bone marrow cells, and 8-hydroxy-2'-deoxyguanosine formation in liver cells. A 10-day exposure in pregnant mice also led to DNA deletions in offspring. The TiO_2 NP exposure also caused a mild but statistically significant increase in systemic inflammation, as shown by qRT-PCR analysis of the mRNA expression of proinflammatory genes (*TNFalpha, IFNgamma, KC/IL-8*) in peripheral blood. It was concluded that oral TiO_2 NP exposure causes genotoxicity in mice, possibly caused by a secondary genotoxic mechanism associated with inflammation and/or oxidative stress.

Conclusions:
Under the experimental conditions used, Aeroxide P25 was genotoxic (clastogenic and/or aneugenic) in human lymphocytes *in vitro*.

SCCS Comments
The test material relates to S75-G (anatase/rutile, not coated). However, the description of the test material given in the paper suggests a different proportion of anatase and rutile (75%:25%) than the proportion specified for S75-G. Data indicate genotoxic effects of TiO_2 NP after oral exposure in mice in organs/tissues other than those that are in direct contact via the exposure route (*i.e.* effects in blood, bone marrow, liver and foetuses). Insufficient details have been provided in the article regarding methodology. This makes the findings of the study of limited value to this risk assessment.
Further limitations of the study are:

- The work does not contain biokinetics, *i.e.* dosimetry cannot be accurately determined. Actual intake of the NP is not measured, only indirect by calculation of the amount of drinking water. Translocation of particles and accumulation in different organs was also not determined.
- Potential local effects (histopathology, genotoxicity assays) in gastrointestinal tract target cells are not provided, and thus do not allow for assessment of potential effects on epithelial barrier integrity, inflammation and local mutagenicity.
- The effects were observed at a rather high dose (calculated cumulative oral dose of 500 mg/kg). The authors do not report whether these concentrations affect intestinal physiology. The high surface burden of TiO_2 NP in the G.I. tract may have significant impact on the adsorption and transport of nutrients.

DNA double strand breakage in bone marrow cells after oral uptake

Guideline/method:	According to published protocols
Species/strain:	C57Bl/6Jp_{un}/p_{un} mice.
Group size:	5 / treatment group
Test substance:	Aeroxide P25, Degussa/Evonik, primary particle size 21nm, BET surface area 50m2/g, DLS in water: 21-1446nm)
Batch:	/
Vehicle:	water
Dose levels:	0, 50, 100, 250, and 500 mg/kg bw (estimated dose)
Treatment:	/
GLP:	not in compliance
Reference:	Trouiller et al., 2009

Methods
DNA double strand breaks were analysed by immunohistochemical detection of γ-H2AX foci in C57Bl/6Jpun/pun mice exposed to TiO_2 NP via drinking water. Bone marrow smears were analysed after 5 exposure days for γ-H2AX foci, at estimated exposure of the mice to 0, 50, 100, 250, and 500 mg/kg bw TiO_2 NP.

Results
Oral TiO_2 NP caused increased γ-H2AX foci in a clear dose dependent manner, being significant from the lowest dose (50 mg/kg bw) onwards. DNA double-strand break was considered the most sensitive parameter among a variety of genotoxicity endpoints. It was therefore concluded that oral TiO_2 NP exposure causes DNA double strand breaks in bone marrow of the mice and suggest that this may be caused by a secondary genotoxic mechanism associated with inflammation and/or oxidative stress.
The TiO_2 NP exposure also caused mild but significantly increased systemic inflammation, as shown by qRT-PCR analysis of the mRNA expression of proinflammatory genes (TNFalpha , IFNgamma, KC/IL-8) in peripheral blood.

Conclusions:
Under the experimental conditions used, Aeroxide P25 was genotoxic rats causing DNA double strand breaks in bone marrow cells.

SCCS Comments

The test material relates to S75-G (anatase/rutile, not coated). Marked dose dependent effects are observed, suggesting that the bone marrow may be a sensitive target for TiO_2 nanoparticle (after oral uptake). Whether the nanoparticles actually reached this target is not shown in the study. The effects were observed at high concentrations. Other limitations of the study are:

- The work does not contain biokinetics, *i.e.* dosimetry cannot be accurately determined. Actual intake of the NP is not measured, only indirect by calculation of the amount of drinking water. Translocation of particles and accumulation in different organs was also not determined.
- Potential local effects (histopathology, genotoxicity assays) in gastrointestinal tract target cells are not provided, and thus do not allow for assessment potential effects on epithelial barrier integrity, inflammation and local mutagenicity.
- The effects were observed at a rather high dose (calculated cumulative oral dose of 500 mg/kg bw). The authors have not reported whether these concentrations affect intestinal physiology. The high surface burden of TiO_2 NP in the G.I. tract may have significant impact on the adsorption and transport of nutrients.

Comet assay *in vivo* in rat lungs (five day inhalation study)

Guideline/method:	According to generally accepted and published protocols
Species/strain:	Male Wistar Crl:W1 Han rats
Group size:	3 animals per group
Test substance:	T-Lite™ SF, pure rutile, primary particle size 10 x 50 nm, mean agglomerates approximately 200 nm (d10: 90 nm, d90: 460 nm); coating consisting of aluminium hydroxide and dimethicone/methicone copolymere
Batch:	/
Vehicle:	/
Dose levels:	0 and 10 mg/m^3/treatment/day
Treatment:	6 h/day for 5 consecutive days
GLP:	not in compliance
Reference:	Landsiedel et al., 2010

Rats were exposed by inhalation (head-nose exposure) for 6 hours on five consecutive days to 0 or 10 mg/m^3/treatment/day. DNA damage was evaluated by alkaline Comet assay in the rat lung cells (isolated by *in situ* perfusion) from three animals per group. Viability of the isolated cells was determined by trypan blue dye exclusion. Further parameters evaluated included body weight, and bronchoalveolar lavage levels of LDH and ALP.

Results
The treated animals showed significantly increased LDH and ALP concentrations in BAL. Average viability of the cells isolated for the Comet assay were 95% and 88.7% respectively for air and TiO_2 exposed animals. A biologically relevant increase in DNA damage was not detected by the Comet assay.

Conclusion
Under the experimental conditions used it was concluded that T-Lite^{TM} SF has a genotoxic potential in this alkaline Comet assay in lung cells.

SCCS Comment
The test material relates to S75-K (94% rutile, coated with aluminium hydroxide and dimethicone/methicone copolymere). The applied method is not yet validated, but represents tissue that at least in part is directly exposed to the testing material. The isolation procedure may have affected the background damage in the cells from the animals.

Further mutagenicity/genotoxicity studies *in vivo* (open literature)
In specific animal studies no information is provided on the size of the particles used, or only non-ultrafine samples were used for effects of nano-sized TiO_2 (Shelby, 1993; Driscoll *et al.,* 1997).

Rehn *et al.* (2003) investigated oxidative DNA damage induction by two samples of TiO_2 in rat lungs after intratracheal instillation at the dosages of 0, 0.15, 0.3, 0.6 and 1.2 mg/kg bw/day. The samples used were an untreated TiO_2 and a trimethoxyoctylsilane-treated TiO_2 sample, both approximately 20 nm. DQ12 crystalline silica was used as a positive control at 0.6 mg/kg. Oxidative damage induction was determined after 90 days by immunohistochemical analysis of lung sections using an 8-oxoguanine antibody. Enhanced oxidative DNA damage was not observed with the untreated or silanised TiO_2 nanoparticles, in contrast to the DQ12 crystalline silica. Analysis of markers of pulmonary inflammation and toxicity at 3, 21, and 90 days indicated a strong progressing inflammation with DQ12 crystalline silica, whereas for both TiO_2 samples only mild inflammatory effects were noticed. Proliferation in lung tissue, as determined using Ki-67 staining, showed only minor differences between control and TiO_2 treated rats in contrast to DQ12 treated rats which showed strong increase in % Ki-67 positive cells after 90 days. The contrasting observations with regard to oxidative DNA damage induction and proliferation were considered to be due to the marked contrasts in severity and persistence of pulmonary inflammation.

Similar to these observations, Driscoll *et al*. (1997) have demonstrated the likely role of pulmonary inflammation in driving mutagenesis in rat lungs after *in vivo* instillation of different particles. These included a fine crystalline silica sample, a nano-sized carbon black sample and a fine anatase TiO_2 sample (180 nm median diameter, 8.8 m2/g). Mutagenicity was studied by *hprt*-analysis of lung epithelial cells isolated from the lungs of female SPF F334 Fischer rats, 15 months after intratracheal instillation of each of the particles at 10 mg/kg or 100 mg/kg. For the fine TiO_2 sample, enhanced *hprt*-mutagenesis was observed with 100 mg/kg, the dose which also elicited persistent lung inflammation, but not with the 10 mg/kg dose. Similar for the other particles used (carbon black, silica) *in vivo* mutagenicity was only observed at doses that also caused persistent inflammation. The inflammatory cells obtained by bronchoalveolar lavage from the particle-treated animals were found to induce *hprt*-mutagenesis in a rat lung epithelia cell line *in vitro*.

SCCS Comments on Mutagenicity/Genotoxicity

From the studies discussed above, the potential to cause DNA damage has been clearly demonstrated for some TiO_2 nanomaterials. However, it is not clear how this relates to the other nanomaterials presented in the submission.

1.5.7 Carcinogenicity

Two stage skin painting carcinogenicity studies

Study Design:	Two stage mouse skin carcinogenicity (Initiator: DMBA)
Date of publication:	Available online 30 November 2010.
Guideline/method:	Two stage muse skin carcinogenicity test. Coated and uncoated titanium dioxide nanoparticles were used as promoter with 7,12-dimethylbenz[a]anthracene (DMBA) as initiator.
Test system:	CD1 (ICR) female mice.
Test substance:	Industrial material-grades of coated (alumina and stearic acid) titanium dioxide nanoparticles (CTDN, titanium dioxide content: 79.2%, spindle shape, long axis of 50–100 nm, short axis of 10–20 nm) and uncoated titanium dioxide nanoparticles (UCTDN, titanium dioxide content: 96.0%, spindle shape, long axis of 50–100 nm, short axis of 10–20 nm) from Ishihara Sangyo Kaisha, Ltd., Osaka, Japan.
Batch:	No data
Concentrations:	CTDN and UCTDN dispersed in Pentalan 408 (pentaerythrityl tetraethylhexanoate) at concentrations of 5 mg/0.1 g, 10 mg/0.1 g and 20 mg/0.1 g on ultra sonic cleaner.
Exposure:	Twice weekly for 19 weeks
Solvent:	Pentalan 408 (pentaerythrityl tetraethylhexanoate)
Negative control:	Solvent
Positive Controls:	12-o-tetradecanoylphorbol 13-acetate (TPA)
GLP:	No
Reference:	Furukawa *et al.,* 2011

This study was conducted to examine the promoter potential of coated and uncoated titanium dioxide nanoparticles (CTDN and UCTDN) in a two-stage mouse skin carcinogenesis model using 7 week old CD1 (ICR) female mice. Initiation treatment: 0.1 ml (0.1 mg) DMBA or vehicle alone was applied to furclipped back skin one time, using a micropipetter with disposable tips. Starting 1 week after the initiation treatment, aliquots of 5, 10 and 20 mg of CTDN or UCTDN in 0.1–0.09 ml of Pentalan were applied using a disposable syringe and glass spreader daily, or 0.2 ml (4 µg) of TPA were applied using a micropipetter twice weekly for 19 weeks to the animals as post-initiation treatments. TPA was used as a positive control promoter. Pentalan 408 served as a vehicle control as well as negative control.

No changes in survival rate, general condition and body weight related to the test materials were observed. On macroscopic observation, 1–2 nodules/group on the skin were observed in each group applied CTDN and UCTDN as well as the control group after DMBA initiation. The nodules were histopathologically diagnosed as squamous cell hyperplasia, sebaceous gland hyperplasia, squamous cell papilloma and keratoacanthoma. While in CTDN and UCTDN experiments enlargement of the mandibular, pancreatic, lumbar region and inguinofemoral lymph nodes, spleen and thymus was observed in mice given 5 and 10 mg but not 20 mg, the lack of dose-dependence suggests no biological significance.

The study authors concluded that CTDN and UCTDN applied as promoter at doses of up to 20 mg/mouse did not increase the development of nodules. There were no significant differences between the number of nodules in the negative control (no initiator) and the experiments with TiO_2 as promoter. In the positive control, DMBA as initiator and TPA as

promoter, 100% of the animals developed nodules. The authors concluded that titanium dioxide nanoparticles do not possess promoter activity for mouse skin carcinogenesis.

SCCS Comment
The test material used in this study might be comparable to one type of materials included in this dossier. It was a good experiment with a procedure that is generally accepted for studying initiation and promoter activity. SCCS agree that under the experimental conditions uncoated and alumina- and stearic acid- coated nano TiO_2 do not show any carcinogenic promoter activity.

Study Design:	Two stage mouse skin carcinogenicity
Date of publication:	Published 2012
Guideline/method:	Two stage mouse skin carcinogenicity test. Coated and uncoated titanium dioxide nanoparticles were used as promoter with 7,12-dimethylbenz[a]anthracene (DMBA) as initiator.
Test system:	Female rasH2 mice and their wild-type counterparts CB6F1 mice and CD1 mice.
Test substance:	$sTiO_2$ particles (rutile type, silicone coated, mean particulate diameter 35 nm) and $ncTiO_2$ rutile type mean particulate diameter 20 nm) were provided by Japan Cosmetics Association, Tokyo.
Batch:	No data
Concentrations:	0, 50 and 100 mg/ml
Exposure:	$sTiO_2$ rasH2 mice 5 times a week for 8 weeks, CB6F1 mice 5 times a week for 40 weeks. $ncTiO_2$ CD1 mice 2 times a week for 52 weeks
Solvent:	$sTiO_2$ silicon oil, $ncTiO_2$ Pentalan 408 (pentaerythrityl tetraethylhexanoate)
Negative control:	Solvent
Positive Controls:	$sTiO_2$ no positive control, $ncTiO_2$, 12-o-tetradecanoylphorbol 13-acetate (TPA)
GLP:	No
Reference:	Sagawa et al., 2012

TEM analysis showed that the shape of $sTiO_2$ particles was generally round to oval while $ncTiO_2$ particles were more club shaped. The mean length of $sTiO_2$ particles suspended in silicone was 0.28±0.22 µm. The mean length of $ncTiO_2$ particles suspended in Pentalan 408 was 4.97±0.50 µm.

$sTiO_2$ nano particles
The skin on the backs of 7-week old female rasH2 mice and wild type CB6F1 mice was shaved and the animals received a single topical application of 0.1 ml DMBA (0.2 mg). Two weeks later the animals were divided into 3 groups. Group 1 (control, only initiation than vehicle) (15 mice of each strain) were painted with 0.2 ml silicone oil. Group 2 (15 mice of each strain) were painted with 0.2 ml of 50 mg/ml $sTiO_2$ suspended in silicone oil. Group 3 (15 mice of each strain) were painted with 0.2 ml of 100 mg/ml $sTiO_2$ suspended in silicone oil. Group 4 (control, no initiation)(15 mice of each strain) were painted with 0.2 ml of 100 mg/ml $sTiO_2$ suspended in silicone oil without prior DMBA treatment. The mice were painted 5 times a week. The rasH2 mice were killed after 8 weeks and the wild-type CB6F1 mice after 40 weeks.

rasH2 mice
The incidence of squamous cell papillomas was 100% in all groups (Group 1 – 3) of rasH2 mice treated with DMBA. No skin tumours were found in the group (Group 4) which was only treated with $sTiO_2$. The incidence of squamous cell carcinomas was 33% in Group 1 (only DMBA and silicone oil), 60% in Group 2 (DMBA + 10 mg TiO_2), and 53% in Group 3 (DMBA + 20 mg TiO_2). The difference in carcinomas was not significant. No difference was found in the multiplicity of tumours.

CB6F1 mice
The incidence of squamous cell papillomas was 7% (1 mouse) in Group 1 (only DMBA and silicone oil) and 13% (2 mice) in Group 2 and 3 (DMBA + 10 and 20 mg TiO_2). No skin tumours were found in the group (Group 4) which was only treated with $sTiO_2$. The incidence of squamous cell carcinomas was 7% (1 mouse in Group 1 (only DMBA and silicone oil). No squamous cell carcinomas were found in any of the other groups.

$ncTiO_2$ nano particles
The skin on the backs of 10-week old female CD1 mice was shaved and the animals received a single topical application of 0.1 ml DMBA (0.2 mg). Two weeks later the animals were divided into 4 groups. Group 1 (control, only initiation than vehicle) (16 mice) were painted with 0.2 ml Pentalan 408. Group 2 (16 mice) were painted with 0.2 ml of 50 mg/ml $ncTiO_2$ suspended in Pentalan 408. Group 3 (15 mice) were painted with 0.2 ml of 100 mg/ml $ncTiO_2$ suspended in Pentalan 408. Group 4 (positive control)(15 mice) were painted with 0.2 ml of TPA 200 nmol/ml in acetone. Groups 1 – 3 were painted 2 times a week and killed after 52 weeks. Group 4 was painted 4 times a week and killed after 40 weeks.

CD1 mice
The incidence of squamous cell papillomas was 19% (3 mice) in Group 1 (only DMBA and silicone oil), 6% (1 mice) in Group 2 (DMBA + 10 mg TiO_2) and 13% (2 mice) in Group 3 (DMBA + 20 mg TiO_2). None of the mice in Groups 1 – 3 had developed squamous cell carcinomas. In the positive control (DMBA + TPA), 87% (13 mice) had developed squamous cell papillomas and 13% (2 mice) had squamous cell carcinomas.

SCCS Comment
The results indicate that $ncTiO_2$ does not promote skin tumours in mice. With $sTiO_2$ an increase in the number of tumours was found among mice initiated with DMBA. The increase was not significant and no conclusion can be drawn.

Two stage rat skin carcinogenicity

Study Design

Date of publication:	Published 2012
Guideline/method:	Two stage rat skin carcinogenicity test. Uncoated titanium dioxide nanoparticles ($ncTiO_2$) was used as promoter with 7,12-dimethylbenz[a]anthracene (DMBA) as initiator.
Test system:	Male Hras128 rats and their wild-type counterparts SD rats.
Test substance:	$ncTiO_2$ rutile type mean particulate diameter 20 nm) were provided by Japan Cosmetics Association, Tokyo.
Batch:	No data
Concentrations:	0, 100 and 200 mg/ml
Exposure:	$ncTiO_2$ Hras128 rats 2 times a week for 28 weeks and SD rats 2 times a week for 40 weeks.
Solvent:	Pentalan 408 (pentaerythrityl tetraethylhexanoate)
Negative control:	Solvent
Positive Controls:	None
GLP:	No
Reference:	Sagawa et al., 2012

TEM analysis showed that the shape $ncTiO_2$ particles were clubbed shaped. The mean length of the $ncTiO_2$ particles suspended in Pentalan 408 was 4.97±0.50 µm.

$ncTiO_2$ nano particles
The skin on the backs of 10-week old male Hras128 rats and wild type SD rats was shaved and the animals received a single topical application of 0.5 ml DMBA (2.5 mg). Two weeks

later the animals were divided into 3 groups. Group 1 (control, only initiation than vehicle) (17 Hras128 rats and 12 SD rats) was painted with 0.5 ml Pentalan 408. Group 2 (16 Hras128 rats and 12 SD rats) was painted with 0.5 ml (50 mg) $ncTiO_2$ suspended in Pentalan 408. Group 3 (17 Hras128 rats and 12 SD rats) was painted with 0.5 ml (100 mg) $ncTiO_2$ suspended in Pentalan 408. The rats were painted twice a week. The Hras128 rats were killed after 28 weeks and the SD rats after painting for 40 weeks.

Hras128 rats
The incidence of squamous cell papillomas was 94% (16 rats) in Group 1 (only DMBA and Pentalan 408), 88% (14 rats) in Group 2 (DMBA + 50 mg TiO_2) and 94% (16 rats) in Group 3 (DMBA + 100 mg TiO_2). None of the rats Groups 1 had developed squamous cell carcinomas, while 13% (2 rats) in both Group 2 and Group 3 had developed squamous cell carcinomas.

SD rats
The incidence of squamous cell papillomas was 25% (3 rats) in Group 1 (only DMBA and Pentalan 408), 17% (2 rats) in Group 2 (DMBA + 50 mg TiO_2) and 8% (1 rat) in Group 3 (DMBA + 100 mg TiO_2). None of the rats in Groups 1 and 3 had developed squamous cell carcinomas, while 17% (2 rats) in both Group 2 had developed squamous cell carcinomas.

SCCS Comment
This rat model is less developed than the mouse two-stage carcinogenicity model. Since 94% of the Hras rats treated with DMBA only developed tumours, the model is not adequate and no conclusion can be drawn from the study.

Study Design:	Two stage rat skin carcinogenicity (Initiator: UV-B irradiation)
Date of publication:	2011
Guideline/method:	Exploratory Dermal UV-B initiated skin carcinogenesis promotion study.
Test system:	Rat/Sprague-Dawley (wild-type and transgenic Hras128). 10 weeks old
Group size:	5 – 8 male and 5 – 8 female per group.
Test substance:	TiO_2 NP (uncoated, rutile type, R, PPS: 20 nm, Ishihara Sangyo Kaisha, Japan)
Batch:	No data
Concentrations:	0, 100 mg/ml per rat (0.5 ml on 9 cm^2)
Route:	Topical application
Exposure:	42 weeks with/without pre-irradiation with UV-B for 10 weeks
Source of UV-light:	UV-B radiation unit, Dermaray 100, Eisai-Toshiba, Tokyo, Japan
Irradiation: UV-B:	800 mJ/cm^2P, 2x/week for 10 weeks
Solvent:	Pentalan 408 (pentaerythrityl tetraethylhexanoate)
Negative control:	Solvent
Positive Controls:	None
GLP:	No
Reference:	Xu *et al.*, 2011

The potential of TiO_2 NPs (uncoated, R, PPS: 20 nm) to promote skin tumours after dermal application after UV-B irradiation was studied in transgenic rats carrying the human c-Ha-ras proto-oncogene (Hras128 rats), known to be sensitive to chemically induced skin carcinogenesis in males and mammary carcinogenesis in females, and their wild-type counterparts. A total of 80 Hras128 rats and their wild-type siblings were investigated.

The size of TiO_2 particles suspended in Pentalan 408 ranged from 10 nm to 300 µm (mean size of 5.0 µm, median size of 4.6 µm) indicating that a large majority of the particles formed aggregates in the Pentalan 408 suspension.

Group 1 (initiation and promotion) received ultraviolet B (UV-B) radiation (UV-B radiation unit, Dermaray 100, Eisai-Toshiba, Tokyo, Japan) 2 times per week for 10 weeks at 800 mJ/cm^2, on the shaved target skin, followed by painting with 0.5 ml of TiO_2 suspended in Pentalan 408 at 100 mg/ml on the shaved (9 cm²) area twice a week until sacrifice. Group 2 (negative control, initiation + vehicle) received UV-B radiation and painting with the vehicle Pentalan 408 on the shaved area twice a week until sacrifice, and Group 3 (no initiation, only TiO_2 as promoter) received painting with 0.5 ml of TiO_2 suspension as in Group1 but without prior UV-B radiation.

Any grossly visible papilloma lesions were carefully examined every day. All the animals were sacrificed at week 52 (after 42 weeks painting) except for the female Hras128 rats, which were terminated at week 16 (after 6 weeks painting) due to early mammary tumour development. The skin, brain, lung, liver, mammary gland, mesenterial lymph nodes, spleen and kidney, were excised, fixed and processed for light microscopic examination.

In male Hras128 rats, papillomas on the back skin developed from week 32 and the incidence of skin papillomas was 12.5% (1/8) in Groups 1 and Group 3. No skin tumours were observed on the targeted back skin in female Hras128 rats or wild-type rats of either sex. Eye lid squamous cell papillomas were found in wild type female rats exposed to UVB (Groups 1 and 2) with incidences of 12.5% (1/8) and 14.3% (1/7). No statistically significant inter-group differences in incidence, multiplicity or weight were found. Mammary tumours (adenocarcinomas) were induced with high incidence in Hras128 rats of both sexes. Wild-type female rats also had an increased incidence of mammary tumours but no statistically significant inter-group differences in incidence, multiplicity or weight were observed.

Conclusions by the authors
TiO_2 particles were detected in the upper *stratum corneum* but not in the underlying skin tissue layers. TiO2 did not induce or promote skin carcinogenesis in transgenic (Hras128) and wild-type Sprague-Dawley rats under the conditions of this study. The data suggest that TiO_2 does not cause skin carcinogenesis, probably due to its inability to penetrate through the epidermis and reach underlying skin structures.

SCCS Comment
This is not a generally accepted model for studying initiation and promotion of skin tumours. Since no positive control was included it is not possible to make any conclusion with regard to potential carcinogenic properties of TiO_2 from the study.

Study Design:	Intra-pulmonary spraying
Date of publication:	Advance Access publication February 25, 2010.
Guideline/method:	Two stage rat skin carcinogenicity test. Uncoated titanium dioxide nanoparticles (ncTiO_2) were used as promoter with DHPN as initiator.
Test system:	Female transgenic rats carrying the human c-Ha-ras gen (Hras128 rats) and female wild-type SD rats were obtained from CLEA Japan Co., Ltd (Tokyo, Japan)
Test substance:	ncTiO_2 rutile type mean particulate diameter 20 nm) were provided by Japan Cosmetics Association, Tokyo.
Batch:	No data
Concentrations:	TiO_2 particles were suspended in saline at 250 µg/ml or 500 µg/ml.
Exposure:	Initiation: 0.2% DHPN (N-nitrosobis(2-hydroxypropyl)amine), (Wako Chemicals Co., Ltd Osaka, Japan) in the drinking water for 2 weeks. Promotion: Two weeks after DHPN treatment, the rats were exposed intratracheally every second week to TiO_2 suspensions under isoflurane anesthesia for a total of 7 times. The rats were killed 3 days after the last exposure.
Solvent:	Saline
Negative control:	Only DHPN in drinking water

Positive Controls: None
GLP: No
Reference: Xu *et al*., 2011

Female transgenic Hras128 rats and female wild-type SD rats were used in the study. TiO_2 particles were suspended in saline at 250 µg/ml or 500 µg/ml. The TiO2 suspension was intratracheally administered to animals under isoflurane anesthesia using a Microsprayer (Series IA-1B Intratracheal Aerosolizer, Penn-Century, Philadelphia, PA) connected to a 1 ml syringe; the nozzle of the sprayer was inserted into the trachea through the larynx and a total of 0.5 ml suspension was sprayed into the lungs synchronizing with spontaneous respiratory inhalation (IPS).

IPS-initiation–promotion protocol
Female Hras128 rats aged 6 weeks were given 0.2% DHPN, in the drinking water for 2 weeks. Two weeks later, the rats were divided into four groups. Group 1 (9 rats). DHPN alone. Group 2 (10 rats). DHPN followed by 250 µg/ml TiO_2. Group 3 (11 rats). DHPN followed by 500 µg/ml TiO_2. Group 4 (9 rats). 500 µg/ml TiO_2 without DHPN initiation.

The TiO_2 particle preparations were administered by IPS once every 2 weeks from the end of week 4 to week 16 (a total of seven exposures). The total amount of TiO_2 administered to Groups 1, 2, 3 and 4 were 0, 0.875, 1.75 and 1.75 mg per rat, respectively. Three days after the last treatment, animals were killed and the organs (brain, lung, liver, spleen, kidney, mammary gland, ovaries, uterus and neck lymph nodes) were excised

TiO_2 was distributed primarily to the lung, but minor amounts of TiO_2 were also found in other organs. Various sizes of TiO2 aggregates were observed in alveolar macrophages. The TiO_2-laden macrophages were evenly scattered throughout the lung alveoli. Of 452 particle aggregates examined, 362 (80.1%) were nanosized, i.e.100 nm. Overall, the average size was 84.9 nm and the median size was 44.4 nm.

The author concluded that TiO_2 treatment significantly increased the multiplicity of DHPN-induced alveolar cell hyperplasias and adenomas in the lung. In the rats, which received TiO_2 treatment without prior DHPN treatment, alveolar proliferative lesions were not observed although slight inflammatory lesions were observed. TiO_2 aggregates were localized exclusively in alveolar macrophages and had a mean diameter of 107.4 nm.

In the mammary gland, TiO_2 treatment significantly increased the multiplicity of adenocarcinomas from about 3 tumours per rat in Group 1 to about 6 tumours per rat in Group 2 and 3. The treatment did also tend to increase the weight of the mammary tumors from about 6 g per tumour in Group 1 to about 12 – 15 g per tumour in Group 2 and 3 (only shown in Figure with no Table).

IPS 9 day protocol
Twenty female SD rats (wild-type counterpart of Hras128) aged 10 weeks were treated by IPS with 0.5 ml suspension of 500 µg/ml TiO_2 particles in saline five times over a 9 day period. The total amount of TiO_2 administered was 1.25 mg per rat. Six hours after the last dose, animals were killed and the lungs and inguinal mammary glands were excised. Fatty tissue surrounding the mammary gland was removed as much as possible. The left lungs and inguinal mammary glands were used for biochemical analysis, and the right lungs were fixed in 4% paraformaldehyde solution in PBS adjusted at pH 7.3 and processed for histopathological examination and immunohistochemistry.

Morphologically, TiO_2 particles were observed as yellowish, polygonal bodies in the cytoplasm of cells. These cells are morphologically distinct from neutrophils and strongly positive for CD68, indicating that the TiO_2 engulfing cells were macrophages. TiO_2 aggregates of various sizes were found in macrophages, and aggregates larger than a single macrophage were surrounded by multiple macrophages. Of 2571 particle aggregates

examined, 1970 (76.6%) were <100 nm and five particles were >4000 nm in size. Overall, the average size was 107.4 nm and the median size was 48.1 nm.

TiO_2 treatment significantly increased 8-hydroxydeoxy guanosine level, superoxide dismutase activity and macrophage inflammatory protein 1a (MIP1a) expression in the lung

Comment by the authors
TiO_2 treatment significantly increased 8-hydroxydeoxy guanosine level, superoxide dismutase activity, and macrophage inflammatory protein 1a (MIP1a) expression in the lung. MIP1a, detected in the cytoplasm of TiO_2-laden alveolar macrophages *in vivo* and in the media of rat primary alveolar macrophages treated with TiO_2 *in vitro*, enhanced proliferation of human lung cancer cells. Furthermore, MIP1a, also detected in the sera and mammary adenocarcinomas of TiO_2-treated Hras128 rats, enhanced proliferation of rat mammary carcinoma cells. These data indicate that secreted MIP1a from TiO_2-laden alveolar macrophages can cause cell proliferation in the alveoli and mammary gland and suggest that TiO_2 tumor promotion is mediated by MIP1a acting locally in the alveoli and distantly in the mammary gland after transport via the circulation.

SCCS Comment
TiO_2 treatment significantly increased the multiplicity of DHPN-induced alveolar cell hyperplasias and adenomas in the lung, and the multiplicity of mammary adenocarcinomas. Thus, non-coated TiO_2 administered intratracheally had tumour promoter activity.

Oral carcinogenicity studies in non-nano TiO_2

Oral study with F344 rats. Each groups consisted of 60 male and 60 female rats. The control diet contained 1% corn oil, while experimental diets contained 1.0, 2.0, or 5.0% titanium dioxide-coated mica and 1% corn oil.

The article states: "TiO_2-coated mica is a nonfibrous, naturally occurring silicate, which, when coated with TiO_2 is used as a pearlescent pigment in plastics, industrial coatings, simulated leather, and cosmetic preparations. Annual worldwide production of TiO_2-coated mica exceeds 1 million pounds and the potential for human exposure is great."

The test material consisted of a 1:1 blend of two samples of titanium dioxide-coated mica. The material was in the form of flat platelet with the longest dimension ranging from 10 to 35 µm. The final blend of test material contained 28% TiO_2 and 72% mica. A purity of 100% was assumed for purposes of diet formulations.

The rats (6 week old) received the TiO_2 containing for up to 130 weeks. The study authors stated that "there was no evidence that TiO_2-coated mica produced either toxicologic or carcinogenic effects at dietary concentrations as high as 5.0%.

Ref.: Bernard *et al.,* 1990

Groups of 50 male and 50 female B6C3F1 mice, 5 weeks of age, were fed diets containing 0, 2.5 or 5% titanium dioxide (size unspecified; anatase; purity, ≥98%) daily for 103 weeks. Mice were killed at 109 weeks of age, at which time no significant difference in survival was observed between treated and control males. In females, a dose-related trend in decreased survival was noted. No significant differences in body weights or incidence of tumours were observed between treated and control groups.

Groups of 50 male and 50 female Fischer rats, 9 weeks of age, were fed diets containing 0, 2.5 or 5% titanium dioxide (size unspecified; anatase; purity, ≥98%) daily for 103 weeks. The rats were killed at 113 weeks of age, at which time no significant difference in survival was observed between treated and control groups of either sex. No significant differences in body weights or incidence of tumours were observed between treated and control groups.

Ref.: National Cancer Institute, 1979

SCCS Comment
From the studies, exposure to non-nano titanium dioxide via the oral route does not appear to lead to carcinogenic effects.

1.5.8 Photo-carcinogenicity

Photo-carcinogenicity studies in non-nano TiO_2

The ability of MTD (titanium dioxide, not further specified) and 2-EHMC (2-ethylhexyl-p-methoxycinnamate) to protect mice from the "promotion phase" of tumorigenesis was studied.

The dorsal trunks of inbred female C3H/HeJ mice (10 – 12 weeks old) were shaved and the relevant groups (15 mice) initiated with 10 nmol DMBA. Five days later UV-irradiation and/or sunscreen treatment was commenced and this was continued for 32 weeks. The mice were monitored for a further 14 weeks after cessation of irradiation.

The sunscreens were in oil-in-water emulsion and contained MTD (7.2%) or 2-EHMC (8%). The MTD was a broad-spectrum-reflecting physical sunscreen with an SPF of 7, while the 2-EHMC was shown to be a UVB-absorbing sunscreen with an SPF of 4. The sunscreens or base lotion (BL) were applied at least 10 min prior to UV exposure at approximately 2 mg/cm^2. The integrated irradiance was 1.7 W/m^2 for UVB and 34 W/m^2 for UVA.

The mice were irradiated 5 days per week for 32 weeks, i.e. until 50% of the DMBA plus UV irradiated groups had tumors. The average cumulative dose was 571 kJ/m^2 for UVB and 11.4 mJ/m^2.

The DMBA-initiation alone and DMBA-initiated sunscreen-treated groups did not develop tumours. UV alone induced tumours in 46% of the mice at week 48. Initiation with DMBA prior to UV irradiation enhanced tumour formation such that 87% had tumours at week 48. Both MTD and 2-EHMC completely protected the mice from UV-induced tumour formation.

Ref.: Bestak and Haliday, 1996.

Groups of female inbred mice (hr/hr, strain Skh:HR-1) treated with an SPF 15 sunscreen formulated with MT100T microfine titanium dioxide coated with aluminium stearate (not further specified) were exposed daily to minimally skin reddening UV radiation over 12 weeks. Throughout a 200 day observation period substantial protection was afforded from the induction of skin cancer compared to unprotected controls.

Two groups of sunscreen protected mice were treated immediately following the radiation regime with the tumour promoter croton oil. UV + croton oil induced tumours in 100% of the mice. The mice protected by a sunscreen showed only 3.7% with tumours, which was less than with treatment with croton oil alone. However, where sunscreen protected mice were exposed to croton oil about 25% proved to have been initiated.

The authors concluded that the superfine titanium dioxide sunscreen provided a high level of protection similar to that by conventional sunscreen formulations.

Ref.: Greenoak *et al.*, 1993.

SCCS Comment
The studies above are of little value because size and specifications of the titanium dioxide particles are unknown.

SCCS Comments on Carcinogenicity
Pigmentary and ultrafine titanium dioxide has been tested for carcinogenicity by oral administration in mice and rats, by inhalation exposure in rats and female mice, by intratracheal administration in hamsters and female rats and mice, by subcutaneous injection in rats, and by intraperitoneal administration in male mice and female rats.

- According to the evaluation of titanium dioxide by IARC (2010), induction of lung tumours was observed in two inhalation studies with rats while two inhalation studies in rats and one in female mice gave negative results.
- Intratracheally instilled female rats showed an increased incidence of lung tumours following treatment with two types of titanium dioxide. Tumour incidence was not increased in intratracheally instilled hamsters and female mice.
- Oral, subcutaneous and intraperitoneal administration did not produce a significant increase in the frequency of any type of tumour in mice or rats.
- IARC concluded that there is *inadequate evidence* in humans for the carcinogenicity of titanium dioxide but *sufficient evidence* in experimental animals for the carcinogenicity of titanium dioxide. Titanium dioxide was classified as a Group 2B carcinogen (P*ossibly carcinogenic to humans).*
- In their recent evaluation of TiO2 NIOSH has determined that ultrafine TiO2 with equal nano-sized TiO2 is a potential occupational carcinogen and, that there is insufficient data to classify fine TiO2 as potential occupational carcinogen after inhalation (NIOSH 2011).
- Nano titanium dioxide has been studied in 2 two-stage skin carcinogenicity studies with mice, 2 two-stage skin carcinogenicity studies with rats, and one two-stage lung study with rats.
- Both non-coated ($ncTiO_2$) and coated titanium dioxide have been studied in the two-stage mouse skin carcinogenicity studies with CD1 mice and a transgenic mouse strain (rasH2). In one well performed study with non-coated and alumina and stearic acid coated titanium dioxide, no promoter activity was found (Furukawa et al., 2011). Promoter activity was also not found for $ncTiO_2$ in the other study (Sagawa *et al.*, 2012). However, it is difficult to draw a firm conclusion from this study with silica coated titanium dioxide due to lack of positive controls and very high tumour activity in the "initiated" mice.
- Non-coated titanium dioxide was studied in 2 two-stage rat skin carcinogenicity studies. Although, no tumour promoter activity was observed, it is difficult to draw any conclusion since little experience with the model used is available and no positive controls have been used in the studies.
- One two-stage rat lung carcinogenicity study has been carried out with non-coated titanium dioxide. The rats were "initiated" by DHPN in the drinking water prior to intra-pulmonary spraying with $ncTiO_2$. The experiment demonstrated promoter activity of $ncTiO_2$ (Xu *et al*., 2011).

Since TiO_2 particles have shown carcinogenic activity and since nano $ncTiO_2$ also showed promoter activity after intra-pulmonary spraying, the use of nano TiO_2 in sprayable applications needs specific considerations.

1.5.9 Reproductive toxicity

In the submission, no studies have been provided with reproductive toxicity data relevant to the nanomaterials under assessment. A review of reproductive and developmental toxicity studies of manufactured nanomaterials (including TiO2) has been provided (Ema et al., 2010 - Reference 146). The two TiO2 materials referred to include a TiO2 material with particle size <10µm (no further information), and a TiO2 nanomaterial with primary particle

size 25-70 nm (20–25m2/g surface area, anatase). Relevant studies in the review by Ema et al. (2010) showed that:

- Pregnant BALB/c mice administered on gestational day 14 with <10 μm TiO2 suspended in phosphate-buffered saline at 50 μg/mouse by a single intranasal insufflation had higher serum levels of cytokines, including interleukin-1β, tumor necrosis factor-α, interleukin-6 and chemokine, 48 h after exposure compared with nonpregnant mice. The offspring of the dams exposed to TiO2 showed increased airway hyperresponsiveness, increased percentage of eosinophils, and pulmonary inflammation. These findings showed that TiO2 caused acute cellular inflammation in pregnant mice and increased allergic susceptibility in their pups.
- Pregnant Slc:ICR mice administered on gestational days 6, 9, 12 and 15 with TiO2 nanomaterial suspended in saline with 0.05% Tween 80 via subcutaneous injection at 100μg/mouse/day caused changes in the expression of genes associated with brain development, cell death, response to oxidative stress, and mitochondria in the brain during the prenatal period, and genes associated with inflammation and neurotransmitters in the later stages of the offsprings.
- *In vitro* exposure of testis-constituent cells (mouse Leydig cell line TM3) to nano-TiO2 showed uptake of the nanoparticles after incubation of cells at 30μg/mL for 48h, and a remarkable inhibition of viability and transient reduction in proliferation of the cells at 100μg/mL after 24 h.

The article is, however, a review of exploratory studies, and as such is of a limited usefulness to this assessment.

Other studies in open literature, including some of those reviewed by Ema et al. (2010) have demonstrated the possibility of placental transport of different manufactured nanomaterials in pregnant animals into the fetus, or found effects in the offspring. Yamashita et al. (2011) reported on the presence of nano-TiO2 in fetuses after the intravenous administration of nano-TiO2 in pregnant mice. Nano-TiO2 was detected by TEM in the placenta, fetal liver and fetal brain, and induced a decrease in uterine weight and higher fetal absorption. A limitation of the study was that relatively high doses (about 32 mg/kg body weight on gestation days 16 and 17) were used. In addition, the chemical nature of the nanomaterials observed in the organs was not confirmed. For the silica nanoparticles investigated in the same paper a size dependency of transplacental migration was demonstrated as 70 nm nanoparticles did show placental transport while 300 nm and 1000 nm silica nanoparticles did not (Yamashita et al., 2011).

After subcutaneous administration to dams (Slc:ICR mice) on gestation days 3, 7, 10 and 14 at 100μg/mouse/day, Takeda et al. (2009) observed TiO2 particle aggregates (identified by Energy Dispersive X-ray Spectroscopy, EDS) in the testis of male offsprings at day 4 and week 6 after birth. Also histopathological alterations were observed in the testis. In addition, nano-TiO2 particles were demonstrated in the brain of offspring mice (Takeda et al., 2009), suggesting that nano-TiO2 might have passed through undeveloped or developing Blood Brain Barrier (BBB) in embryos of the young mice. However, since mice were tested at 4 days or 6 weeks of age, it is not clear whether exposure to nano-TiO2 occurs in utero via the placenta or through milk. A previous study of the same research group observed alterations in gene expression in the brain (Shimizu et al., 2009). The gene expression alterations were already observed in 16 days old embryos. As only the mother animals were exposed to nano-TiO2 it seems likely that the offspring received the Ti via the mother either during pregnancy or in the weaning period via the milk (Takeda et al., 2009). For some effects, like reduced pup weight and gene alterations, indirect mechanisms due to effects on the pregnant animals themselves could not be excluded.

After inhalation exposure to nano-TiO2 during gestation days 8-18 moderate behavioural effects were observed in the offspring (Hougaard et al., 2010). Time to first litter was prolonged after mating the exposed male offspring to unexposed mice but did not reach statistical significance. For females there was no difference. After inhalation of a surface coated nano-TiO2 by pregnant mice, no effects were seen on DNA damage in

bronchoalveolar lavage fluid (BALF) cells and liver cells (Jackson et al., 2011), nor in offspring that had been prenatally exposed. Some changes were noted in liver gene expression profiles of female offspring. However, as in general the exposure of the fetuses would be rather low, the observed alterations might have been caused as a secondary response to the maternal inflammation in the lungs.

Shimuzu et al. (2009), from the same research group as Takeda et al. (2009) performed a similar study in which pregnant mice were injected subcutaneously (100 µl of 1 mg/ml TiO2 solution) with nano-TiO2 (25-70 nm, anatase) on gestational days 6, 9, 12, and 15. This study also investigated the effects of maternal exposure to nano-TiO2 on gene expression in brain during the developmental period using cDNA analysis. Expression levels of the genes associated with apoptosis were altered in the brain of newborn pups, whereas genes associated with brain development were altered in early age. The genes associated with response to oxidative stress were changed in the brains of 2 and 3 weeks old mice. Using Medical Subject Headings (MeSH) terms information, the changes of the expression of genes was found to be associated with neurotransmitters and psychiatric diseases.

In conclusion, although after inhalation or subcutaneous exposure of pregnant mice the exposure of offspring in the uterus has been reported, exposure through this route is likely to be low and some of the effects might be secondary to maternal toxicity induced by the nanomaterials. The reported fetal effects were observed after high doses of intravenously administered nano-TiO2, which are unlikely to occur in real life with the use of sunscreen products.

SCCS Comment
No relevant study on reproductive toxicity is provided. One review article covering exploratory studies has been provided (SI-II, Ema et al., 2010 (146)). Overall information on this endpoint is as yet patchy and inconclusive.

1.5.9.1 Two generation reproduction toxicity

SCCS Comment
No data on two-generation reproductive toxicity is provided

1.5.9.2 Teratogenicity

SCCS Comment
No data on teratogenicity is provided

1.5.10 Toxicokinetics

The following studies on toxicokinetics and metabolism have been provided:

Exploratory distribution, excretion study in rat

Reference: Fabian et al., Arch Toxicol. 2007 ref. No. 28 + 53; and Fabian E. + Landsiedel R. ref. No. 28)

Guideline: Study considered a number of guidelines: EC Commission Directive 87/302/EEC (EC Commission Directive 1988), OECD Guidelines for Testing of Chemicals (Method No. 417) (OECD Guidelines 1984), U.S. EPA, Health EVects Guidelines, OPPTS 870.7485 (U.S. EPA 1998), and the Japan/MAFF: Guidelines on the Compiling of Test Results on Toxicity (Japan/MAFF2001).

Species/strain:	male Wistar rat, 7–12 weeks old and weighed 200– 300g
Group size:	12 rats; 3 rats per group
Test substance:	TiO2; 06/0489; P25 consisted of both anatase and rutile forms (70/30), had no surface coating, the TiO2 primary particles were in the size range 20–30 nm; approximately 10 wt.% of the particle agglomerates/aggregates are found in the nano-size range. BET specific surface area of 48.6 m2/g.
Batch:	4165012298 (FI);
CAS No.	13463-67-7
Purity:	unknown
Dose levels:	5 mg/kg body weight, TiO2 particles suspended in serum
Route:	A single intravenous injection followed by biokinetics study
GLP:	not applied
Study period:	

Results
Analysis was performed on ICP-AES. According to the analytical method there were no detectable levels of TiO2 in blood cells, plasma, brain, or lymph nodes. There were no changes in the cytokines and enzymes measured in blood samples. Highest Ti retention was observed in the liver at about 100-150 µg/g of organ with a limited clearance during the next four weeks. Ti concentrations in spleen were only slightly lower than in the liver, but Ti concentrations in kidneys and in lungs were about one order of magnitude lower with rather remarkable clearance of about 66% during the next 14 days.

SCCS Comments
It is not clear which of the numerous noted guidelines were followed. Ti contents of the organs were not corrected for background levels but untreated rats were analysed as well. This means only 3 rats per group were analysed. Questions arise where the rest of the administered TiO2 particles went, since an estimated dose of about 1.25 mg per rat were injected and liver, spleen, lungs and kidneys amounted only to 600-700 µg per rat providing no information on the remainder 500 µg.

Exploratory distribution, excretion study in rat

Reference:	Sugibayashi K., Todo H., Kimura E Safety evaluation of titanium dioxide nanoparticles by their absorption and elimination profiles. *Journal of Toxicological Sciences* **33**(3), 293-8 (2008).
Guideline:	not specified
Species/strain:	mouse of unspecified strain
Group size:	not clearly identified, probably 3-5 mice at each time point
Test substance:	rod-shaped TiO2 rutile surface-coated with silica; (primary particle diameter: 15 nm; agglomerated particle size: 220 nm);
Batch:	HD-AW-150 from a Japanese company
Purity:	rutile analysis by XRD, 27.5% silica content from surface modification, no further analysis on impurities
Dose levels:	no dose levels specified
Route:	intravenous injection followed by biokinetics study in mice;
Administration:	intravenous injection of titanium dioxide nanoparticles, single intravenous injection; biokinetics after 5 min, 72 h and 30 d
GLP:	not specified
Study period:	

Results
Distribution of TiO2 (measured as Ti) was in blood and several tissues (primarily liver) but not in brain. A slow decrease of TiO2 in liver was observed over time (~30% decrease in one month). Observation of substantial amounts of Ti found in untreated mice prior to any treatment due to significant natural food contamination; this led to an estimated dose of 90 µg/ Ti per day. After i.v. injection the Ti level was significantly increased in blood and tissues. Ti concentrations per organs are provided but it is not clear whether or not these were corrected for background Ti in all organs nor is the administered dose given.

SCCS Comment
Neither the strain nor the number of mice is clearly identified. The i.v. injected dose of TiO2 NP is also not specified. This study is therefore of no use to the current assessment.

Open literature

There are other toxicokinetic data of inhaled agglomerated TiO2 nanoparticles (Ma Hock et al. 2008, 2009) showing oxidative stress and inflammatory reactions similar to previously described 90-days exposure investigations. As far as toxicokinetic parameters were evaluated, due to the detection limits, extrapulmonary TiO2 particles were not detected.

There are also toxicokinetic studies in which TiO2 NP were intravenously injected into the vein of rodents (Fabian et al., 2007, and other papers). Retention was highest in the liver followed by spleen, lungs, kidneys and it was highest at the first day compared to days 14 and 28. Cytokine levels remained unchanged indicating no detectable toxicity.

There are no new toxicokinetic data on the absorption of TiO2 NP after administration to the gastrointestinal-tract (GIT). The most recent study from Wang et al. 2008 used unrealistically high doses of 5 g/kg BW in rats such that their findings are not useful and may even be modulated by uncontrolled other forms of intake like inhalation of aspiration. Their biodistribution data showed the highest retention in the liver followed by spleen, kidneys and lungs. Thus toxicokinetics data after GIT administration still rely on the studies of the group of Alexander Florence, in the 1990ies. These suggest that about 5-7% of the administered 500nm TiO2 particles were absorbed and retained in the body, mainly in the liver.

Applicant's conclusions
Intravenous administration of large doses of nano TiO2 did not result in adverse effects or signs of toxicity in rodents. A non-specific and expected tissue distribution of TiO2 was observed. No TiO2 was detected in brain, and the levels in other organs decreased over time.

SCCS Comment
The limited available evidence suggests that if TiO2 nanoparticles become systemically-available, they may accumulate mainly in liver with a very slow clearance.

1.5.11 Photo-induced toxicity

1.5.11.1 Phototoxicity / photoirritation and photosensitisation

Photo- irritation

Guidelines: OECD good laboratory principles

Product tested:	TiO2 T805 (1992 batch 030492)
Species:	SPF NZ white rabbits (Ch. River), Female
Groups:	3 animals/group
Dosing:	3, 10, 30% in ethanol 96% during 100 min
Exposure area:	15 – 7.5 cm total, each exposure side spot approximately 2 cm diameter
UVA-light:	310-420 nm peak 365nm total dose 10J/cm² (approx. 50 min dosing)
Readings:	30 min, 24h, 48h, 72 h after UV-exposure
Observations:	No irritation found, neither non-irradiated as irradiated TiO2 treated animals.
Reference:	15
Conclusion:	TiO2 (T805) is not photo-irritating for rabbit skin under the assay conditions after UVA irradiation up to 10 J/cm².

Guidelines:	OECD good laboratory principles
Product tested:	TiO2 T805 (1992 batch 030492)
Species:	SPF albino guinea pigs (Ch. River)
Sex:	Males & Female
Experimental protocol:	Following Ichikawa, Armstrong & Harber 1981, Induction treatment followed by challenge 12 days later
Groups:	Test groups 5 animals of each sex
Dosing:	30% TiO2 in ethanol (96%) at induction treatment & challenge treatment (day 12)

Induction protocol:

- 6-8 cm area cleared from fur
- Area is subcutaneously pre-treated with Freund adjuvant and exposed to 0.2 ml of suspension followed by UVA-light: 310-420 nm (peak 365 nm) total dose 10 J/cm²
- In total 5 treatment over 2 weeks (only first time Freund adjuvant was used).
- Skin was not cleared after treatment
- Reading after each treatment

Challenge protocol:

- 12 days after last induction
- 5-10 cm area cleared from fur
- Exposed to 0.5 ml of 30% TiO2 (T805) suspension direct followed by light: 310-420 nm (peak 365 nm) total dose 10 J/cm² - 37 min

Observations:	No irritation found, neither during induction phase or challenge phase, in both non-irradiated as irradiated TiO2 treated animals.
Conclusions:	TiO2 (T805) is not photo-sensitizer for guinea pigs under the assay conditions after UVA irradiation up to 10 J/cm².
Reference:	17

Human data:

Product tested: 0685115 (No other info in the document)

Species: 60 volunteers (19-77 y) of which 50 completed the study

Sex: Males & Female

Protocol:

- Induction: 3 patches per week (Mon, Wed, Fri) during 3 weeks (0.2 ml TiO2 suspension per patch – no concentration reported). Patches remain at place 24 h (removal by volunteers). If reaction, next patch was moved to adjacent area (testing was discontinued if severe reaction was noted)
- Challenge: 2 weeks after last induction at different spot

Result: No effects observed, in any of the volunteers

Conclusion: Product 0685115 is not a sensitizer for humans under the assay conditions

Reference: 27

SCCS Comment
The study is not a photosensitisation study but is only sensitization study.

Product tested: 0685115 (No other information in the document)

Species: 29 human volunteers (18-60 y) of which 25 finished the whole study (drop-out were not related to the test)

Sex: Males & Female

Pre-testing: MED (Minimal Erythemal Dose) of unprotected skin of each volunteer was assessed. [MED = time interval or dose of UV sufficient to produce minimal perceptive erythema]

Light source: UV A (320-400 nm), 3 min(approximately 10.08 Joules)

Protocol:

- Induction: 2 spot prepared for exposure to compound 0685115, of which one is irradiated while the other is not be irradiated.
  The areas cleared from hair of 1 inch/ 1 inch, and 0.2 ml (no concentration of TiO2 suspension reported) of test material is placed on the spots. Exposed side is kept under patch during 24 h.
  2 applications applied per week for 3 weeks (total 6 applications).
  After removal of patch spots irradiated with a dose of 2x MED of the volunteer
- Challenge: 2 weeks after last induction at different spots on the back. Spots are under patch for 24 h, then irradiated for 3 min (non erythemogenic dose). Reading after 24, 48 & 72 h

Result: No effects observed, in any of the volunteers

Conclusion: Product 0685115 is not a photo-sensitizer for humans under the assay conditions

Reference: 28

SCCS Comments
Ref 16 and 18 could not be found. The given references are not correct, as they do not report photo-irritation (Sonnenschutzformulierungen: Lotions und Cremes)

1.5.11.2 Phototoxicity / photomutagenicity / photoclastogenicity

A number of studies has not been reviewed as part of this assessment, because the experiments were performed with bacterial cells. As discussed in section 3.3.6, bacterial mutagenicity assays are not considered to be appropriate for the testing of nanoparticles compared to mammalian cell systems. Other studies were not reviewed because they are related to test materials that are either not nanomaterials, or they lack data on material characterisation to establish whether they were relevant nanomaterials to this assessment.

Phototoxicity test *in vitro*

Guideline/method:	OECD TG432
Test system:	Balb/c 3T3 fibroblasts, neutral red uptake (NRU)
Replicates:	no replicates
Test item:	T805 (coated, A/R, PSMA 1 type), T817 (coated, A/R, PSMA 1 type), TiO2 P25 (non coated)
Batch:	05 10067 (T805), 04095 (T817), P1S-3087 (p25)
Vehicle:	EBSS wit 1% ethanol
Concentrations:	0.78 t0 1—mg/L UV-A: 5.0 J/cm²
Exposure:	0, 0.79, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 mg/L
Negative control:	vehicle
Positive control:	not included
GLP:	no
Date of report:	1999
Reference:	Submission DHS, 24 and 25

Balb/c 3T3 cells were pre-incubated with eight different concentrations (0.79, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100) of the nanoparticles in two 96-well plates, one plate was subsequently exposed to 5 J/cm^2 UVA while the other plate was kept in the dark. Medium was then replaced and after 24 h cell viability was determined by spectrophotometrical evaluation of neutral red dye uptake (3 h incubation of neutral red). The phototoxic potential was determined by calculation of the ratio of the nanoparticle concentration that reduced viability by 50% (NR50) in presence versus absence of UV irradiation.

Results
T805 and T817 showed neither cytotoxicity nor phototoxicity up to a concentration of 100mg/L. The p25 (non coated NP) sample also was not cytotoxic up to the highest concentrations, but in the presence of irradiation a viability reduction of 82 % (at 50 mg/L) and 44% (at 100 mg/ml) was observed.

Conclusion
p25 sample is phototoxic towards Balb/c 3T3 cells, while T805 is not phototoxic.

SCCS Comment
This study is indicative of the importance of coating on the phototoxic properties of TiO2 nanoparticles.

Photoclastogenicity test *in vitro*

Guideline/method:	Chromosomal aberration test in presence or absence of UV treatment
Test system:	CHO-WBL cells
Replicates:	Duplicate
Test item:	See table
Batch:	-
Vehicle:	Ethanol (sample A), PBS (samples B and C), DMSO (D, E,F,G and H)
Concentrations:	Three concentrations for each sample with as higest concentration either 5000 µg/ml or a dose that resulted in less than 50% cytotoxicity

Exposure: 3 h followed by 17 h recovery
UV dose: 750 mJ/cm^2 (provided 15 min after NP treatment initiation)
Negative control: Vehicle
Positive control: 8-methoxypsoralen (8-MOP), 4-nitroquinoline-1-oxide (NQO)
GLP: -
Published: yes
Reference: Theogaraj et al., 2007

Test items used:

Table 1
Description of ultrafine titanium dioxide particles tested

Sample code	Crystal type	Inorganic coating	Organic coating	Particle size
A	Anatase (80%), rutile (20%)	None	Trimethoxy caprylylsilane	Approximately 21 nm[a]
B	Anatase (80%), rutile (20%)	None, doped di-iron trioxide (2 ± 1%)	None	Approximately 21 nm[a]
C	Anatase (80%), rutile (20%)	None	None	Approximately 21 nm[a]
D	Rutile (100%)	Alumina (8–11%)	Simethicone (1–3%)	14 nm[b]
E	Anatase (100%)	Alumina (37%), silica (12–18%)	None	60 nm[c]
F	Rutile (100%)	Alumina (5–6.5%)	Dimethicone (1–4%)	20 nm[b]
G	Rutile (100%)	Alumina (3–8%)	Stearic acid (5–11%	15 nm[a]
H	Rutile (100%)	Alumina (10.5–12.5%), silica (3.5–5%)	None	20–22 nm[b]

[a] Primary particle size determined by transmission electron microscopy (TEM).
[b] Primary particle size determined by X-ray diffraction.
[c] Characterisation by X-ray disc centrifugation (XDC) giving an aggregate rather than particle size.

The photoclastogenicity of TiO2 was determined in CHO cells. S9 mix was not included in the protocol. Cells were treated in the dark for 15 min and then UV radiated. After irradiation the cultures were incubated in the dark, after which the medium was removed. Cultures were washed and fresh medium was added for a further 17 h. Cells were then harvested and stained slides were then evaluated for the presence of chromosomal aberrations.

Results
No increases in chromosomal aberration frequencies were found either in the presence or absence of UV up to the highest treatment concentrations.

Conclusion
No photogenotoxicity was observed under the applied testing conditions.

SCCS Comment
Uptake of the NP into the cells was not evaluated. The UV treatment was performed shortly after initial exposure to the particles (15min). At this time uptake may have been limited.

1.5.12 Human data

A number of human studies have been quoted on different versions of skin patch test. Some of the studies have used TiO2 materials for which no information on material characterisation has been provided, whist others have been reviewed in relevant sections.

1.5.13 Special investigations

A number of studies have been provided, relating to cytotoxicity, coating stability and photostability of TiO2 materials. Many of these studies have used TiO2 materials for which information on material characterisation has not been provided.

1.5.14 Human safety evaluation (including calculation of MoS)

Given the very low, if any, dermal penetration of nano-TiO2 when applied on skin, and in consideration of the low toxicity observed, the calculation of a margin of safety (MoS) is not relevant for this assessment.

Any exposure to nano-TiO2 via oral route from a dermally applied product is also likely to be insignificantly low. Again in consideration of the low toxicity observed, the calculation of a margin of safety (MoS) for the oral route is not relevant.

In view of the concerns over safety of nano-TiO2 via inhalation route, its use in applications that might lead to inhalation exposure (such as powders or sprayable products) is not recommended and therefore has not been considered in the calculation of MoS.

1.5.15 Discussion

General considerations:
The submission consists of fifteen (15) TiO2 nanomaterials that vary in terms of various physicochemical parameters. The studies provided in support of the submission range from old to recent ones. A major proportion of the (old) studies are on materials for which little or no information on characterisation has been provided, which makes it difficult to relate many of them to the nanomaterials under current assessment.

The evaluation by the SCCS of these and other studies provided in this submission has shown that many of them are not relevant to the nanomaterials in the submission. Therefore the relevance and usefulness of the data provided for this evaluation is poor and patchy. It is difficult (in some cases impossible) to relate the studies to the types of nanomaterials under evaluation. It would have been more productive if a complete set of supporting data was provided on one (or a few) rather than several different TiO2 nanomaterials in a single submission.

Despite the fact that the materials used as coatings to TiO2 nanomaterials have a wide diversity, and some of them have been used in substantially high proportions (e.g. 16% alumina), putative exposure to the coating materials has not been considered in the assessment. Although a few studies showing coating stability have been provided, it is important to know the concentration of any dissolved coating materials, e.g. aluminium ions, in the final formulation. A significant dissolution of a coating material (e.g. alumina) may require a separate safety assessment for the coating material.

Physicochemical properties:

- The studies provided in the submission relate to a range of TiO2 materials that comprise micronized, ultrafine, or nano-sized particles. The physicochemical characterisation data include coated and non-coated materials, composed of rutile and/or anatase forms of TiO2. On the basis of the physicochemical data provided, the SCCS has considered the materials in three broad groups on the basis of crystalline form and photocatalytic activity.
- The SCCS agrees that TiO2 nanoparticles, due to agglomerative behaviour, are likely to be present in the final sunscreen products mainly in the form of agglomerates, which can also be in the nanoscale. It can therefore be assumed that the consumer is likely to

be exposed mainly to TiO2 agglomerates. However, it is also possible for the agglomerates to de-agglomerate under certain conditions of formulation/use. Therefore, the SCCS has considered the size of the primary particles more important than the size of agglomerates for the purposes of risk assessment.

- As nanoparticles may have different properties and biokinetic behaviour than their soluble equivalents, it is important to know the exact purity/impurity profile of a nanomaterial intended for use in a cosmetic product (SCCS Guidance, SCCS/1484/12). Due to the lack of such information, this opinion does not cover TiO2 nanomaterials that have TiO2 purity less than 99.5%, and for which an acceptable impurity profile has not been provided. In the current submission, this applies to two of the nanomaterials (S75-K and S75-L). It is, therefore, important to note that this opinion applies to thirteen (13) (S75-A, S75-B, S75-C, S75-D, S75-E, S75-F, S75-G, S75-H, S75-I, S75-J, S75-M, S75-N, S75-O) out of the fifteen (15) TiO2 nanomaterials presented in this submission. The opinion may, however, be also applicable to other TiO2 nanomaterials that have a close-similarity to the 13 nanomaterials considered by the SCCS in this submission, in terms of the physicochemical parameters listed in Tables 1-3, and other specific provisions laid out in Section 2 below.
- Zeta potential measurements have been provided for some materials, and not for others due to difficulties in measuring zeta potential for hydrophobic nanomaterials.
- Among the 13 nanomaterials assessed, the SCCS has noted a potential concern in relation to photocatalytic activity, and stability of the coating, of some of the materials. It is stated by the Applicant that all coatings on the materials included in the submission are stable. Three (3) studies have been provided, which show that coatings are stable. However, from the other physicochemical data provided, it is less clear how stable the coatings are in final formulations. The photocatalytic activity data, which is measured in formulations, clearly indicate that either some of the materials were not completely coated, or some of the coatings (e.g. organic, organosilanes) were not so stable in the formulations. This is an important aspect to ascertain because application of a formulation containing a nanomaterial that has a significant photocatalytic activity may lead to local effects on sun-exposed skin. Such effects may or may not manifest during the immediate use, and it is important to investigate the possibility of latent effects following the use of a skin product that contained photocatalytic nanoparticles. This is because, whilst most studies on dermal absorption indicate that TiO2 nanoparticles are not able to penetrate the skin deep enough to reach live cells of the epidermis/dermis, they do show that nanoparticles can penetrate into stratum corneum, and can also enter hair follicles and sweat glands. It is therefore possible that a trace amount of nanoparticles may remain embedded in stratum corneum, in hair follicles, and/or sweat glands, potentially over several days after skin application of a product and washing off. If the nanoparticles have a significant photocatalytic activity, there is a possibility that they may cause generation of reactive radical species on exposure to sunlight, long after the skin formulation had been applied and washed off. This, in a close proximity of living cells, raises a concern over the possibility of harmful effects. Generally metal(oxide) nanomaterials which exhibit a high photocatalytic activity are those that are either uncoated, partially coated, or have not been quenched by other means (e.g. doping) to adequately reduce photoreactivity. The TiO2 nanomaterials in the current submission that have a high photocatalytic activity include anatase materials in uncoated (S75-G) and coated forms (S75-F, S75-O). Three (3) other rutile coated nanomaterials also have comparatively lower but still significant levels of photocatalytic activity (S75-C, S75-D, S75-E).
- The SCCS considers up to 10% photocatalytic activity compared to corresponding non-coated or non-doped reference as acceptable.
- In view of this, the SCCS does not recommend the use of nanomaterials that have a high photocatalytic activity (S75-F, S75-G, S75-O) in dermal formulations. These materials can only be recommended after appropriate coating/doping has been applied to quench their photocatalytic activity down to acceptable levels.

- Three rutile materials (S75-C, S75-D, S75-E) with relatively lower but still significant levels of photocatalytic activity may be used in dermal formulations, but further investigations over longer post-application periods may be necessary to ascertain that they do not pose a risk due to photocatalytic activity.

Acute toxicity:

- The studies provided on acute oral toxicity in the submission mainly relate to TiO2 nanomaterials that are anatase/rutile mixtures, coated with trimethoxy-n-octyl-silane. From the limited relevant information provided, and considering that oral intake is not likely to be the major route of exposure to TiO2 nanomaterials from dermal application of formulations, the acute oral toxicity of TiO2 is unlikely to be of a concern.
- The studies provided on acute dermal toxicity relate to an ultrafine TiO2 material and a material described as 'natural colour', and are therefore of no relevance to the assessment of nanomaterials.
- No study has been provided on acute inhalation toxicity. Sub-chronic (inhalation) and chronic (instillation) studies have indicated substantial inflammatory responses and overload associated with diminishing particle clearance in a dose dependent manner, and histological indications of epithelial hypertrophy and hyperplasia.
- The limited relevant information provided in the submission, and other information in the open literature, indicates that TiO2 nanomaterials are likely to be non-toxic via oral or dermal application routes. However, inhalation exposure to TiO2 nanoparticles is likely to cause substantial inflammatory effects in the lung.

Skin irritation:

- Only two of the studies provided are relevant to the TiO2 nanomaterials. They relate to anatase/rutile mixtures, coated with trimethoxy-n-octyl-silane. The results showed primary irritation index between zero and 0.3. Two studies using ultrafine grade materials showed the mean irritation scores of 0.3 and 1.58-1.92 during 5 day repeat applications on rabbit skin. Other studies also showed the tested materials to be either mild- or non- irritant to rabbit and guinea pig skin, but it is not clear whether the tested materials were nanomaterials.
- From the limited relevant information, it can be considered that TiO2 nanomaterials are likely to mild- or non- irritant to skin.

Eye irritation:

- Two studies tested TiO2 anatase/rutile mixtures, coated with trimethoxy-n-octyl-silane. From the studies, the derived primary irritation index was between zero and 0.3. A different study used ultrafine rutile material coated with alumina/silica and regarded the tested material as slightly irritant to rabbit eye. Another study found the tested TiO2 materials to be moderately irritant to rabbbit eye, but it is not clear whether the material was a nanomaterial.
- From the limited relevant data provided, eye irritation potential of nano-TiO2 appears to be low.

Skin sensitisation:

- Two of the provided studies have regarded TiO2 nanomaterials (anatase/ rutile mixture, coated with trimethoxy-caprylylsilane or trimethoxy-n-octyl-silane) as non-sensitiser. Another ultrafine material (rutile, coated with alumina/silica) is classified as a weak sensitiser, but characterisation data (particle size distribution) has not been reported to indicate what proportion of the particles was in the nano-scale.
- Due to the absence of skin penetration of TiO2 as demonstrated by many studies included in this dossier, the usefulness of the Buehler test for assessing sensitisation potency of nanomaterials is doubtful as it is based on exposure to intact skin.

- From the limited relevant data provided, TiO2 nanomaterials appear to be non- or weak skin sensitisers.

Dermal absorption:

- A number of *in vitro* and *in vivo* dermal penetration studies have been provided with the submission. In addition, there is a body of open literature on this subject. The evidence from these studies supports the conclusion that TiO2 nanoparticles are unlikely to penetrate across the skin to reach viable cells of the epidermis. In these studies, TiO2 nanoparticles have been shown to penetrate only to the outer layers of the stratum corneum, and there is as yet no conclusive evidence to show that they do reach living cells of the epidermis/dermis. Studies have also shown that TiO2 nanoparticles do not penetrate the (simulated) sunburnt skin.
- Despite the extensive database showing a general lack of TiO2 nanoparticle absorption via the dermal route, there are a few gaps in the knowledge. For example, it is not clear whether TiO2 nanoparticles will be able to penetrate through cuts and bruises, or over repeated or long term applications of a sunscreen formulation.
- A number of studies have indicated that TiO2 nanoparticle can enter the hair follicles and sweat glands, and that they may remain there for a number of days. This is a scenario in which TiO2 nanoparticles are likely to get and remain in a close proximity to the living cells for a length of time. A photocatalytic nanoparticle in such a situation may cause generation of reactive oxyradical species (ROS) and potential harmful effects when exposed to sunlight. As mentioned before, more data would be needed to justify the use of those TiO2 nanoparticles in skin applications that have a considerable level of photocatalytic activity.

Repeated dose toxicity:

- Only two of the four provided subchronic studies on repeated dose toxicity are relevant to the TiO2 nanomaterials under evaluation. However, these studies relate to oral exposure only, from which a LOAEL of 5 mg/kg bw/d has been derived.
- No chronic toxicity study (>12 months) is provided, although a chronic inhalation study has been provided (Section 3.3.1.3).

Inhalation toxicity:

- Studies in open literature indicate that subacute repeated dose respiratory toxicity studies with nano size TiO2 induce an acute inflammation in the lungs that may be reversible depending on the dose and the time evaluated after exposure. In view of this, acute inflammation (spray) applications, which may result in inhalation exposure is not recommended by the SCCS.

Mutagenicity/ Genotoxicity:

- Although an extensive range of studies on mutagenicity has been provided in the submission, most of them have not been conducted in any special consideration of the nano-related properties of the test materials.
- Several studies have been performed mainly to investigate mechanistic effects relating to DNA damage and genotoxic properties. These studies are usually not performed according to specific genotoxicity guidelines (e.g. OECD). Many of the studies have not evaluated the effects in a dose- and/or time- dependent manner. Those that have addressed this, often reveal no clear dose- or time- dependent effects.
- From the provided studies, and open literature, TiO2 particles have also been reported, or suggested, to interfere with the assays, because:

- Micronucleus scoring is difficult in the presence of TiO2 particles. This effect was suggested to explain for the occasionally observed decreases in MN counts after TiO2 treatment (Falck et al., 2009).
- It has been suggested (although not shown) that artefacts may be caused in relation to the use of cytochalasin B for micronucleus testing. On one hand, it is suggested that nanoparticles may interfere with cytochalasin B (binding), and on the other, that the cytochalasin B may act as an inhibitor of the uptake of nanoparticles in cells potentially leading to false negatives (Landsiedel et al., 2010).
- Due to the current lack of information on the possible cellular uptake and subsequent translocation of TiO2 nanoparticles to nucleus, it is not possible to draw a conclusion on whether or not exposure to TiO2 nanomaterials can lead to mutagenic effects.

- Overall in a number of assays, TiO2 nano particles were observed to induce DNA damage, so TiO2 nano particles have to be considered genotoxic.
- It is also of note that appropriate coating of nanomaterial to quench surface photocatalytic activity will also reduce the likelihood of generation of reactive oxygen species (ROS), which may in turn reduce the chances of genotoxicity.

Carcinogenicity:

- Pigmentary and ultrafine TIO2 materials have been tested for carcinogenicity by oral administration in mice and rats, by inhalation exposure in rats and female mice, by intratracheal administration in hamsters and female rats and mice, and by subcutaneous injection in rats and by intraperitoneal administration in male mice and female rats.
- According to the evaluation of TiO2 by IARC (2010), induction of lung tumours was observed in two inhalation studies with rats. Two other inhalation studies in rats, and one in female mice gave negative results. Intratracheally instilled female rats showed an increased incidence of lung tumours following treatment with two types of titanium dioxide. Tumour incidence was not increased in intratracheally instilled hamsters and female mice. Oral, subcutaneous and intraperitoneal administration did not produce a significant increase in the frequency of any type of tumour in mice or rats. IARC concluded that there is inadequate evidence in humans for the carcinogenicity of titanium dioxide but sufficient evidence in experimental animals for the carcinogenicity of titanium dioxide. Both nano and non nano size Titanium dioxide was classified as a Group 2B carcinogen (Possibly carcinogenic to humans).
- In their recent evaluation of TiO2 NIOSH has determined that ultrafine TiO2 which contains nano-sized TiO2 is a potential occupational carcinogen and, that there is insufficient data to classify fine TiO2 as potential occupational carcinogen after inhalation (NIOSH 2011).
- Nano titanium dioxide has been studied in 2 two-stage skin carcinogenicity studies with mice, 2 two-stage skin carcinogenicity studies with rats, and one two-stage lung study with rats. Both noncoated (ncTiO2) and coated titanium dioxide have been studied in the two-stage mouse skin carcinogenicity studies with CD1 mice and a transgenic mouse strain (rasH2). In one well performed study with non-coated and alumina and stearic acid coated TiO2, no promoter activity was found (Furukawa et al., 2011). Promoter activity was also not found for ncTiO2 in the other study (Sagawa et al., 2012). However, it is difficult to draw a firm conclusion from this study with silica coated titanium dioxide due to lack of positive controls and very high tumour incidence in the 'initiated' mice.
- Non-coated titanium dioxide was studied in 2 two-stage rat skin carcinogenicity studies. Although, no tumour promoter activity was observed, it is difficult to draw any

conclusion since little experience with the model used is available and no positive controls have been used in the studies.

- One, two-stage rat lung carcinogenicity study has been carried out with non coated titanium dioxide. The rats were 'initiated' by DHPN in the drinking water prior to intra-pulmonary spraying with ncTiO2. The experiment demonstrated promoter activity of ncTiO2 (Xu et al., 2011).
- Since TiO2 particles have shown carcinogenic activity (after inhalation) and since nano ncTiO2 showed promoter activity after intra-pulmonary spraying, the use of nano TiO2 in sprayable applications is not recommended by the SCCS.

Reproductive toxicity

- No study has been provided on reproductive toxicity that is relevant to the nanomaterials under assessment. A review article covering exploratory studies in mice has been provided, which relates to the use of a TiO2 material which is <10µm (with no further information), and a TiO2 nanomaterial with primary particle size 25-70 nm (no further information).
- Other studies in open literature have indicated the possibility of placental transport in pregnant animals into the foetus, or found effects in the offspring for various manufactured nanomaterials including nano-TiO2. However, the information relating to this endpoint is patchy and therefore inconclusive.

Photo-induced toxicity

- Only a few studies have been provided that are relevant to the nanomaterials under assessment.
- These indicate that TiO2 materials may not be photo-sensitisers. However, concerns regarding the use of photocatalytic nanomaterials in dermal formulations discussed above need to be taken into consideration.
- Several studies have specifically addressed photo-sensitization effects TiO2. However, the outcomes of these studies need to differentiate between photo-sensitization and other local effects on skin (taking into account the aspect of penetration), versus potential effects at other target sites.

Toxicokinetics:

- Two studies have been provided in the submission on toxicokinetics of TiO2 following intravenous injection in rats and mice. In addition, there are few other relevant studies in the open literature relating to inhalation and intravenous, as well as limited (questionable) information on oral administration routes.
- The available evidence suggests that, if TiO2 particles become systemically available by the oral and inhalation uptake pathway, they are likely to accumulate mainly in the liver, followed by a very slow rate of clearance.

Special investigations:

No relevant specific studies have been provided apart from those already discussed above under relevant endpoints.

2. CONCLUSIONS

This opinion is based on the risk assessment of nano-sized titanium dioxide (TiO2) for use as a UV filter in sunscreen formulations. It is important to note that risk assessment of nanomaterials in general still has certain gaps in the knowledge - for instance in relation to the behaviour of nanoparticles in a test medium, or in the animals. This has led to uncertainties over whether the nanoparticles are able to reach and interact with various moieties and biological target sites, and whether, on dermal application, they may penetrate through damaged skin, or during repeated or long term applications. There are also uncertainties over the validity of the currently available tests used for nanomaterials. However, a positive toxic response in these tests is still considered valid for risk assessment as it would indicate a hazard potential.

As discussed above, the safety data provided in support of the fifteen (15) nanomaterials is quite patchy, and is only partially useful for any of the given nanomaterials. However, the SCCS took the view that this submission could be considered for evaluation as an exception. This is because some additional information on TiO2 nanomaterials is available in open literature which is relevant for this evaluation. Also, for example, although the safety data provided in the submission on rutile nanomaterials is insufficient, the studies on anatase form (or rutile/anatase mixtures) could be considered as a surrogate because published studies in open literature have regarded anatase a greater safety concern than the rutile form. However, as the evaluation is still based on limited information which could be related to specific nanomaterial types in the submission, this opinion is limited to the nanomaterials indicated below:

- On the basis of physicochemical considerations discussed above, this opinion applies to thirteen (13) of the TiO2 nanomaterials (S75-A, S75-B, S75-C, S75-D, S75-E, S75-F, S75-G, S75-H, S75-I, S75-J, S75-M, S75-N, S75-O) presented in this submission. In addition, the opinion may also be applicable to other TiO2 nanomaterials that have a close-similarity to the 13 nanomaterials in terms of the physicochemical parameters listed in Tables 1-3, and the specific provisions laid out in the sub-section below.
- It needs to be stressed that the main consideration in the current assessment is the apparent lack of penetration of TiO2 nanoparticles through skin, which is supported by a body of evidence both in the form of studies provided by the Applicant and other studies reported in open literature. In the absence of a systemic exposure, a margin of safety (MoS) could not be calculated for TiO2 nanomaterials in this assessment. From the limited relevant information provided in the submission, and the information from open literature, the SCCS considers that TiO2 nanomaterials in a sunscreen formulation are unlikely to lead to:
    - systemic exposure to nanoparticles through human skin to reach viable cells of the epidermis, dermis, or other organs;
    - acute toxicity via dermal application or incidental oral ingestion. This, however, does not apply to sprayable applications that may lead to inhalation exposure of TiO2 nanomaterials, which may result in lung inflammation;
    - skin irritation, eye irritation, or skin sensitisation when (repeatedly) applied on healthy skin (except possible photoxicity of insufficiently coated nanomaterials);
    - reproductive effects when applied on healthy skin.
- Some TiO2 nanoparticles have been shown to be able to damage DNA and should be considered genotoxic. However as negative results have also been reported, the current evidence in relation to potential genotoxicity of TiO2 nanomaterials is not conclusive. TiO2 particles have also shown to lead to carcinogenic effects after inhalation. These manifestations are a major hazard concern. However, no penetration was found through the stratum corneum of reconstructed human full thickness skin models and no DNA damage was detected by the Comet assay in these cells in contrast to epidermal cell line. Considering the absence of a systemic exposure, the SCCS considers that the use

of nano TiO2 in dermally applied cosmetic products should not pose any significant risk to the consumer.

- Evidence on acute and sub-chronic inhalation toxicity does not support the overall safety of use of TiO2 nanomaterial formulations for spray applications. In addition, tumour promoter activity of nano (non-coated) TiO2 has been shown after intra-pulmonary spraying. Therefore the SCCS does not recommend the use of nano TiO2 in sprayable applications. This may be reconsidered if further evidence is provided to rule out the possibility that the nanoparticles can reach the lower respiratory tract during spray applications.
- Although there is no conclusive evidence at present to indicate penetration of TiO2 nanoparticles through the skin to viable cells of the epidermis, a number of studies have shown that they can penetrate into the outer layers of the stratum corneum, and can also enter hair follicles and sweat glands. It is therefore recommended not to use TiO2 with substantially high photocatalytic activity (e.g. S75-F, S75-G, S75-O) in sunscreen formulations. Other TiO2 nanomaterials that have a relatively lower but still significant level of photocatalytic activity (e.g. S75-C, S75-D, S75-E) may be used, but further investigations over longer post-application periods may be necessary to ascertain that they do not pose a risk due to photocatalytic activity.

Overall conclusion

1. Does SCCS consider that use of titanium dioxide in its nanoform as an UV-filter in cosmetic products in a concentration up to maximum 25.0 % is safe for the consumers taken into account the scientific data provided?

On the basis of the available evidence, the SCCS has concluded that the use of TiO2 nanomaterials with the characteristics as indicated below, at a concentration up to 25% as a UV-filter in sunscreens, can be considered to not pose any risk of adverse effects in humans after application on healthy, intact or sunburnt skin. This, however, does not apply to applications that might lead to inhalation exposure to TiO2 nanoparticles (such as powders or sprayable products). Furthermore, this assessment applies to 13 out of the 15 TiO2 nanoparticles presented in the submission, but may also be applicable to other TiO2 nanomaterials that have a close-similarity to the parameters in Tables 1-3, i.e. TiO2 nanomaterials that:

- have TiO2 purity of ≥99.5%, or in case of a lesser purity, have an impurity profile which is acceptable to the regulatory authorities;
- are composed of mainly the rutile form, or rutile with up to 15% anatase, with crystalline structure and physical appearance as described in the current submission, i.e. clusters of spherical, needle, or lanceolate shapes;
- have a median primary particle size based on number size distribution of 30 to 100 nm as submitted in the dossier, or larger;
- have an aspect ratio from 1.5 and up to 4.5, and volume specific surface area up to 460 m2/cm3;
- are coated with one of the coating materials described in Table 1, and the coatings are stable in the final formulation and during use. Other cosmetic ingredients applied as stable coatings on TiO2 nanomaterials can also be used if they are accepted by the regulatory authorities and are demonstrated to be safe for use as cosmetic ingredient.
- are photostable in the final formulation;
- do not have a substantially high photocatalytic activity. The SCCS considers up to 10% photocatalytic activity compared to corresponding non-coated or non-doped reference as acceptable.

It is also worth highlighting again that this opinion is based on the currently available scientific evidence which shows an overall lack of dermal absorption of TiO2 nanoparticles. If any new evidence emerges in the future to show that the TiO2 nanoparticles used in a sunscreen formulation can penetrate skin (healthy, compromised, or damaged skin) to reach viable cells, then the SCCS may consider revising this assessment.

It should also be noted that the risk assessment of nanomaterials is currently evolving. In particular, the toxicokinetics aspects have not yet been fully explored in the context of nanoparticles (e.g. the size dependency). Also, long term stability of the coatings remains unclear. At the moment, testing of nanomaterials and the present assessment, are both based on the methodologies developed for substances in non-nano form, and the currently available knowledge on properties, behaviour and effects of nanomaterials. This assessment is, therefore, not intended to provide a blue-print for future assessments of other nanomaterials, where depending on the developments in methodological risk assessment approaches and nano-specific testing requirements, additional/different data may be required and/or requested on a case-by-case basis.

It is also important to note that the potential ecotoxicological impacts of nano TiO2 when released into the environment have not been considered in this opinion.

2. In order for the COM to differentiate in the regulation between materials in its nanoform and its non-nano form, can the SCCS give quantitative and qualitative guidance on how this differentiation should be given based on the particle size distribution or other parameters?

A detailed SCCS guidance on risk assessment of nanomaterials in cosmetics has recently been published (SCCS/1484/12). The guidance provides a detailed account of the important nano-related parameters that should be considered in relation to physicochemical characterisation, hazard identification, exposure assessment and risk assessment of nanomaterials.

3. MINORITY OPINION

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ABBREVIATIONS AND GLOSSARY OF TERMS

BET	Brunauer-Emmett-Teller method based on nitrogen gas absorption
CAS	A chemical registry system established by the Chemical Abstracts Service (CAS)
ECVAM	European Centre for the Validation of Alternative Methods
EDX	Energy Dispersive X-ray
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
In vitro test method	Biological method that uses organs, tissue sections and tissue cultures, isolated cells and their cultures, cell lines and subcellular fractions, or non-biological method that uses chemical interaction studies, receptor binding studies, etc [Rogiers and Beken 2000]
ISO	International Organization for Standardization
IARC	International Agency for Research against Cancer
IUPAC	A system of chemical nomenclature established by the International Union of Pure and Applied Chemistry (IUPAC)
Local effects	A Local effect refers to an adverse health effect that takes place at the point or area of contact. The site may be skin, mucous membranes, the respiratory tract, gastrointestinal system, eyes, etc. Absorption does not necessarily occur.
Nanomaterial	An insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm [Regulation (EC) No 1223/2009]
Nanoparticle	A nano-object with all three external dimensions in the nanoscale [ISO/TS 27687:2008, Nanotechnologies -- Terminology and definitions for nano]. For the purpose of this assessment the term 'nanoparticle' is used to also include other forms of nano-object, such as nano-rods, nano-tubes, etc.
NPs	Nanoparticles
Nanoscale	Size range from approximately 1 nm to 100 nm [ISO/TS 80004-1:2010, Nanotechnologies -- Vocabulary]
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
ROS	Reactive Oxygen Species
SCCNFP	Scientific Committee on Cosmetic products and Non-Food Products intended for consumers
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SED	Systemic Exposure Dosage
SEM	Scanning electron microscopy
Solubility	The terms 'solubility' and 'persistence' are often used to describe the rate of "degradation". As such there are a number of definitions of solubility (see SCENIHR Opinion 'Scientific Basis for the Definition of the Term "Nanomaterial", 8 December 2010). In the context of this assessment, solubility means disintegration of a nanomaterial in an

	aqueous medium or biological environment into molecular components with the loss of nano features.
Systemic effects	Systemic effect refers to an adverse health effect that takes place at a location distant from the body's initial point of contact and presupposes absorption has taken place.
TEM	Transmission electron microscopy
TiO2:	Titanium Dioxide
UV-Vis	Ultraviolet-visible spectrophotometry
Validated method	A standard method for which the relevance and reliability have been established for a particular purpose, usually through an inter-lab comparison, which found uncertainties in the measurements acceptable..
VSSA	Volume specific surface area (see Kreyling et al., 2010)
XRD:	X-ray diffraction