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A critical review of the factors determining dermal absorption of nanomaterials and available tools for the assessment of dermal absorption

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Abbreviations

AD – adapalene
Ag - silver
$Al2O_3$ - aluminium oxide
ASO - anti-sense oligonucleotide
CAS number - Chemical Abstracts Service number
cm^2 – square centimetres
CO_3O_4 - cobalt oxide
(M)NM - (manufactured) nanomaterials
EC – European Commission
ECHA – European Chemicals Agency
EFSA - European Food Safety Authority
EPA – Environmental Protection Agency
EU – European union
EUON - European Union Observatory for Nanomaterials
h – hour
HA – hyaluronate
HPMC - hydroxypropyl methylcellulose
LPs - liposomes
L-DOPA – L-dopamine
µm – micrometre
µg – microgram
MEs - Microemulsions
mg – milligram
micro-XRF – micro X-ray Fluorescence Spectrometry
min – minute
mL – millilitre
MS Access – Microsoft Access
ng – nanogram
nm – nanometre
NEs - loaded nano-emulsions
NS - poly-ε -caprolactone nanospheres
OECD - Organisation for Economic Co-operation and Development
P - penetratin
PAMAM - poly(amidoamine)
PEG - polyethylene glycol
PTDs - nanocarriers containing protein transduction domains
QD - quantum dot
QSAR - quantitative structure-activity relationship
REACH - Registration, Evaluation, Authorisation and Restriction of Chemicals
RP - retinyl palmitate
SCCS - Scientific Committee on Consumer Safety
SiO - mesoporous silica
SiO_2 - Silicon dioxide
SLNs - solid lipid nanoparticles
T - transportan
TAT - HIV-1 transactivator of transcription
TG - test guideline
TiO_2 – titanium dioxide
UV – ultraviolet
VE - viable epidermis
WHO – World health organization
ZnO – zinc oxide

1. Abstract

Dermal absorption can lead to systemic availability of substances. The literature on dermal absorption of (manufactured) nanomaterials (MNM), was therefore studied starting with a previous report by Danish EPA in 2013. Studies were selected for analysis via criteria for inclusion, exclusion, quality and relevance.

Crucial information on particle size in the application medium or dissolution of the particles was missing in many studies. *Ex vivo* studies with human or porcine skin were found to be most relevant, while rodent skin studies were considered less relevant, due to differences in skin and lack of knowledge on penetration mechanisms.

Compromised skin integrity and formulations that increase permeability of the skin appear to increase penetration. Some indications were found for larger penetration of smaller particle sizes, lower penetration after agglomeration and an increase of penetration with positive surface charge of MNM. Lack of validated and standardized methods to measure the nanomaterials limits the drawing of conclusions.

It is recommended to use *ex vivo* studies with human or porcine skin to evaluate dermal absorption of nanomaterials and to perform proper studies with fully characterized MNM and comparable testing protocols to assess influential factors.

2. Executive summary

Dermal absorption is a possible route of entry for manufactured nanomaterials (MNM). In a previous study for Danish EPA, the literature on dermal absorption of MNM up to 2013 was analysed (Poland *et al.*, 2013). The current report provides an update of the information based on newly published studies.

Objectives

The objectives of the study were to perform a literature search for studies focussing on dermal absorption of manufactured nanomaterials (MNM), and to review the information available in the public domain.

The following questions were to be answered as far as possible from the available information:

- What studies are available on nanomaterials which are relevant to determine if dermal absorption is taking place?
    - *In vitro/ex vivo* and *in vivo* (animal studies including repeated dose toxicity studies, human case studies or epidemiological data, using intact or damaged skin)
- What are the guidelines followed for the studies (if any)? Are the results available in a structured way e.g. following OECD harmonised templates?
- What are the MNM properties that can affect dermal absorption and how does each property contribute to dermal absorption (e.g. physico-chemical properties, surface treatment, formulation etc.)?
- What are the factors associated with test methodology that can affect dermal absorption (e.g. sample preparation, species difference, vehicle, exposure conditions etc.)?
- What are the available tools to assess dermal absorption of MNM?
    - Are the existing methods (*in vitro*/*ex vivo*/in vivo) suitable for MNM?
    - What are the suitable detection methods to assess dermal absorption of MNM?
- What recommendations can be provided for future work on evaluating dermal absorption of MNM and are there still data gaps for assessing dermal absorption of MNM?

Methodology

The already mentioned report by the Danish EPA was used as the main starting point. The scope of the final review, was defined as follows:

To gather, evaluate and summarize the knowledge available regarding dermal penetration and absorption of nanomaterials used in consumer products and occupational settings.

Studies with experimental data including *in vivo* and *ex vivo* studies were within the scope. The influence of damaged versus intact skin and of different experimental set-up and methods was also investigated. Nanomaterial characteristics, including e.g. particle size and surface charge, were analysed for their influence on dermal penetration or absorption. The focus was on materials with low dissolution rates.

A literature search was done, mainly in Pubmed, using the following search strings: (Ultrafine OR nano-object OR nanoparticle OR nanoparticulate OR nanomaterial OR nanotube OR nanofiber OR nanofibre OR nanowire OR nanocomposite OR nanoplate OR nanorod OR fullerene OR "quantum dot") AND (dermal OR skin) AND (absorption OR penetration OR uptake) AND (***Keyword***). At the place of the word 'keyword' indicated in the search strings above, a large number of different keywords was used. A limited search of references

potentially missed in the search for the Danish report up to 2013, was also performed with specific keywords.

Inclusion and exclusion criteria were used to select studies for further analysis. The starting point for these criteria were those used in the GRACIOUS project (HORIZON2020). The criteria were evaluated per study, not per reference, because a reference may contain more than one study, e.g. a study with human and porcine *ex vivo* skin.

To conclude on penetration or absorption of nanomaterials through the skin, information on materials and the study design must be sufficiently described. A quality evaluation system was developed, using substance criteria and study design criteria. The criteria were derived from a previous project (Fernández-Cruz *et al.*, 2018), but adapted for dermal absorption of nanomaterials.

The S-score describes how well the studied material was characterized. Nanomaterial properties such as size and shape can greatly affect the behaviour of the materials and the better these properties are described, the higher the quality assigned to the study. The S-score, which is independent of the type of study, is based on a number of questions, of which some are considered critical. In the final S-score, information on primary particle shape and on dissolution rate in exposure medium was not considered absolutely necessary, because otherwise hardly any study would pass the criteria. A categorisation was made into accepted studies, supporting studies and rejected studies. The K-score describes the quality of the study design. Three different lists of questions were developed for calculating the K-score for studies in human subjects, *ex vivo* studies in human or porcine skin and studies in rodents.

Next to the quality of a study, the relevance was also assessed, *i.e.* how useful is the study for the purpose of investigating dermal absorption/penetration of nanomaterials. For studies with humans *in vivo*, a set of decision schemes was used to distinguish between non-relevant studies and studies of full or limited relevance. For studies with *ex vivo* skin (human or porcine) and for studies with rodents *in vivo* separate sets of questions were created to evaluate the relevance, some of which were considered critical.

Results

The searches performed resulted in close to 1380 references. Around 950 of these were excluded when checking title and abstract alone and there were 66 review articles. Further exclusions were made based on further in-depth reading of documents. Also, a (relatively small) number of references could not be accessed directly by the researchers via their various subscriptions. Because of the already large number of references available, it was decided to exclude those. A total of 123 references remained and were checked against quality criteria. Some of the references contain more than one study, therefore 150 separate studies (from 123 references) were checked for relevance. Based on the S-scores on information on the nanomaterials, 72 studies were accepted, 28 were considered supporting, while 50 were rejected. Applying the relevance criteria to the studies that passed the overall quality criteria (S- and K-scores), only 15 *ex vivo* studies with human or porcine skin were found to be of sufficient relevance for further analyses. Most of these were using skin preparations placed in Franz diffusion cells.

Some studies analysed both intact and damaged skin. It was generally found that dermal absorption through damaged skin is higher than through intact skin, e.g. in studies by Cardoso *et al.* (2019) and Mauro *et al.* (2015). Dermal absorption of actual MNM in particle form, *i.e.* particles reaching receptor fluid, was not detected or only in trace amounts in studies by Lewinski *et al.* (2017), Mauro *et al.* (2019) and Crosera *et al.* (2015). Some penetration to deeper skin layers and actual dermal absorption (all the way through to reception fluid) was observed for some substances, most clearly for silver when skin was exposed to silver nanoparticles (Bianco *et al.*, 2014; Bianco *et al.*, 2015). The fact that silver occurred in deeper

skin layers and was present in the form of silver-silver chloride aggregates indicates that part of the penetration will have occurred in ionic form.

The importance of skin condition on the penetration of nanomaterials was investigated by Lalloz *et al.* (2019), indicating that ions released from damaged skin reacted with polymeric particles, resulting in their aggregation, destabilization and deposition in the skin. This was not observed in intact skin, nor with particles that were stabilized with a polyethylene glycol coating. Kumar *et al.* (2016) showed increased skin permeation with decreasing size of emulsion droplets, indicating that size may play a role, although other components of the emulsion such as transcutol P also act as permeation enhancers.

Jatana *et al.* (2016) found large differences in skin penetration of quantum dots between different formulations, between mouse and human skin (both *ex vivo* using a petri dish exposure method) and between different types of experimental setups. A much higher presence of quantum dots in human skin, rested less than two hours after processing before exposure, could in the opinion of the original authors be in part due to anatomical differences between human and mouse skin, e.g. epidermal thickness, but also due to the processing of the skin, which is generally only done for human skin. There were large differences in maximum levels of quantum dot signals found in the stratum corneum, after application via different vehicles, between human skin that rested less than two hours after processing and human skin that rested 24 hours after processing before exposure. Also, the relative ordering of the vehicles in maximum levels of quantum dot signals found in the stratum corneum was different between human skin that rested less than two hours compared to at least 24 hours after processing before exposure occurred. This implies that the processing of the skin has a substantial effect on penetration.

In the study of Peira *et al.* (2014), drug skin permeation from titanium dioxide nanoparticles with a positive charge was increased substantially compared to the permeation from coated non-positively charged titanium dioxide nanoparticles. Various other studies also indicated that differences in either nanomaterial surface properties and/or vehicle's properties lead to differences in penetration of material into different skin layers. A positive surface charge and some types of formulation appear to increase the dermal penetration, as indicated by e.g. (Sallam and Marin Bosca (2017), Clares *et al.* (2014), Pepe *et al.* (2016). In many cases, the studied material was not the actual MNM, but a drug that was attached to the MNM, because the MNM was only used as a carrier. In those cases, it is not sure whether the MNM have the same penetration as the drug, because there is no information on potential removal of the drug from the MNM in a certain skin layer.

Several references studied both human or pig skin and rodent skin for dermal absorption studies, but the results did not lead to clear conclusions on the comparison of human or pig skin versus rodent skin. This is because the results between the models could often not be compared, e.g. because of differences in experimental setup such as mode of administration and exposure duration.

Discussion and conclusions

It was found that the use of stringent quality criteria leads to rejection of almost all studies. Therefore, some lenience had to be used. Even though several studies used protocols similar to OECD TG 428, much improvement on reporting of details on MNM used and methods of studying dermal absorption is needed to allow further analysis of various potential determinants of dermal penetration and absorption.

Skin integrity is an important factor affecting dermal absorption of NMN. Skin permeability can be influenced by the processing of the skin before the actual testing. Another major factor is the type of donor suspensions used for the exposure. Skin absorption and penetration is a complex interplay between the properties of the particles in the formulation and the skin condition. Nanoparticles often aggregated to a large extent in artificial sweat donor fluid,

probably preventing penetration of the stratum corneum. Penetration may be higher for particles that are embedded in a formulation which prevents their aggregation.

The lack of standardized, validated methods to measure nanoparticles and use of varying protocols hinders comparison of studies and evaluation of whether particles or ions have penetrated into the skin.

With a lack of *in silico* models, information on dermal absorption of MNM needs to be generated experimentally. *Ex vivo* studies using human skin are a promising tool and the use of porcine skin is considered to be an acceptable alternative, because it is known to be structurally similar to that of humans. Use of rodent skin or rodent *in vivo* studies is not recommended, as it is unknown how the differences between rodent and human skin affect the absorption of MNM. Development of a guideline for measuring dermal penetration and absorption of MNM is recommended, taking into account the test method factors that affect dermal absorption.

A few studies show results that suggest that particle size has some effect on dermal penetration of MNM, while there is also an indication of an influence of surface properties and surface charge (with positive charge leading to higher penetration) of the particles. These indications agree with similar indicative findings in earlier reviews of dermal absorption of nanomaterials, including Poland *et al.* (2013).

Quantitative numbers on percentage absorption of nanomaterials through the skin cannot be derived from the selected studies due to the lack of mass balance data. However, any findings of absorption of MNMs through intact skin can be considered very low, with the exception of silver, which probably penetrates the skin partly in ionic form. Absorption of MNMs does appear to occur to some extent in damaged skin, although no quantitative numbers on this can be derived.

More comparable studies are needed to shed more light on the relationship between MNM properties and their potential for dermal absorption. Due to the complex interplay between MNMs and the dispersion/formulation that is used for the exposure, different relationships may exist for a single MNM, depending on the formulation used.

Recommendations

New studies on dermal absorption of nanomaterials, with the aim to provide qualitative and quantitative proof of dermal penetration and dermal absorption, should be performed with *ex vivo* methods, comparable to OECD TG 428, with human or porcine skin. Rodent skin should not be used for assessing dermal penetration and absorption for MNM, because the differences in skin characteristics between rodents and humans that may affect dermal absorption are too large and the influence of these differences are not sufficiently known.

General aspects mentioned in dermal exposure guidance for conventional substances, related to e.g. using fresh skin with shown integrity, skin thickness to be used, number of samples and interpretation of the results to obtain quantitative dermal absorption values, should be taken into account.

Important aspects to account for, when testing nanomaterials for dermal absorption, include:

- General physicochemical characteristics as also determined for conventional substances
- Sufficient specification of the original primary nanomaterials studied should be reported; in accordance with (at least) the requirements for characterization of nanoforms at registration of substances under REACH (ECHA, 2019)

- Other characteristics that may be relevant for dermal absorption of nanomaterials should also be reported, e.g. Zeta potential (surface charge), dissolution rate and shape. Where relevant, these should be characterized in the exposure medium
- The level of agglomeration and the particle sizes in the formulation as applied in the test should be specified
- Testing should preferably be done with a number of different formulations, including artificial sweat, formulations expected to increase dermal penetration and at least the relevant formulation type for the expected uses of the nanomaterial
- Skin processing before testing should be done very carefully to prevent inadvertent flexing, or even damaging, of the skin that may influence the dermal absorption

As far as possible the tests should provide both qualitative and quantitative information on penetration and absorption of the actual nanomaterial.

It is recommended to perform a well-organised and structured research programme to obtain more and better information on the characteristics of nanomaterials and the factors in the test methodology that influence dermal absorption of nanomaterials.

For risk assessment of non-soluble nanoparticles it can be assumed that dermal absorption is very low if the formulation in which the nanoparticles are expected to reach the skin does not enhance the penetrability of the skin, the dissolution of the nanoparticles or the dispersion of particles in actual nanoform. For other situations, the dermal absorption of nanoparticles may be higher and may require specific testing.

3. Background

3.1 General background

Dermal exposure, penetration and absorption is one of the possible ways in which substances can become systemically available. This is also the case for nanomaterials. This report is the result of a study with the aim to review the factors that influence dermal absorption of nanomaterials and the tools that are available for the assessment of dermal absorption of nanomaterials.

In 2013, the Danish EPA published a report on "Dermal Absorption of Nanomaterials", resulting from the 'Better control of nano' initiative (Poland *et al.*, 2013). That report (which will be called 'the Danish report' in this report) includes literature up to 2013 addressing the use of MNM in consumer products in relation to dermal absorption and penetration.

Nanomaterials may occur naturally (e.g. in volcanic ash, sand and dust) but of particular interest for this study are the MNM. MNM are designed with very specific properties (including shape, size, surface properties, and chemistry etc.), that make them very attractive for commercial development. MNM are used in many industrial processes and they are also incorporated in many consumer products. It has been reported that there are more than 400 products containing metal nanomaterials that are intended for topical applications for consumers (Poland *et al.*, 2013); Robertson *et al.* (2010). Consequently, workers and consumers are increasingly exposed to nanomaterials and exposure via the skin is a relevant route of exposure to the MNM that may be contained in cosmetics and sunscreens, pharmaceuticals, stain resistant and antibacterial clothing, sports equipment, cleanings products, etc.

Systemic exposure via the dermal route is estimated to be quite low in comparison with inhalation or oral routes, due to the general low permeability of the skin for nanomaterials (Poland *et al.*, 2013). However, the skin is one of the largest organs of the human body and there is still debate in the scientific community regarding the ways in which MNM interact with the skin and their potential to cause adverse health effects. As the dermal absorption of MNM is a pre-requisite for any systemic availability following dermal exposure, it is very important to clarify the mechanisms by which MNM can be absorbed through the skin and what are the factors influencing dermal absorption of MNM.

The extensive review by The Danish Environmental Protection Agency investigating the dermal absorption of nanomaterials (Poland *et al.*, 2013) drew several important conclusions, which are briefly summarised below:

- **Particle size**: "*Overall, the conclusion that can be drawn is that penetration of particles in the nano-range into the skin is possible and that this may be greater than for larger particles although this still occurs only at low levels. However there is considerable variability outside of this broad view with little evidence of large deviations in absorption efficiency or distribution profile within the skin across different size fractions within the nano-range and this may depend on various factors such as surface chemistry and experimental conditions etc.*"

- **Surface chemistry:** "*In relation to surface charge, there is a slight tendency towards greater uptake of positively charged particles although there are conflicting studies in relation to this. Such conflicting information is even more apparent in the less well studied aspects of surface chemistry meaning that elucidating a clear relationship between surface chemistry components and dermal absorption/penetration is difficult.*"

- **Particle composition and shape**: "*Particle composition and shape tend to have little effect.*"

- **Solubility**: "*One prominent challenge relating to composition is the effect of solubility on detection of absorption whereby if a particle is soluble, such as silver, then it may be detected in the receiving fluid etc. (e.g. blood serum, Franz cell receptor chamber) either as a particle or as a soluble fraction such as the ionic form. Indeed, detection methods and especially those employed for large samples of complex composition such as blood or urine tend not to be able to differentiate between particulate and ionic forms thereby negating the ability to conclude if particle absorption does or does not occur.*"

- **Methodological approaches**: "*There are numerous methodical approaches that have been used to evaluate absorption ranging in terms of the nature of the model (e.g. in vitro or in vivo), species, sample type, sample preparation, vehicle, duration of experiment, doses used and methods of detection. …. an overriding theme evident from a holistic view of the literature is the need for harmonisation of experimental approaches (e.g. models), methods (e.g. sample preparation, vehicles, and doses) and reporting (e.g. in terms of penetration depth, particle frequency etc.).*"

The report by Danish EPA also described key gaps in relation to the physiochemical properties, test methods and detection methods related to dermal absorption leading to recommendations on methods and endpoints to assess dermal absorption for MNM. The identified gaps and the further recommendations have been further explored in the current study. Since the publication of the Danish study, new data has emerged. Particular focus in this study was on studies which are prescriptive in terms of harmonization of the methodology, including whether measured particles actually originate from the MNM tested, or possibly from other sources, vehicles used, concentrations applied between differing research projects with common aims. There is an identified need to conduct systematic evaluations of nanoparticle physicochemical properties to define quantitatively the role such properties play in dermal absorption.

There are also reports available on specific types of MNM such as TiO_2 (Scientific Committee on Consumer Safety, 2014) and ZnO (Scientific Committee on Consumer Safety, 2012) discussing their absorption in intact and compromised skin. Regarding TiO_2 absorption it was pointed out that currently there are certain knowledge gaps in relation to the possible dermal penetration of nano TiO_2 on repeated or long term use of cosmetic products, which may not only be used on flexed healthy skin but also on damaged skin (Scientific Committee on Consumer Safety, 2014).

3.2 Definitions

The EU adopted a definition for the term nanomaterial in 2011: Recommendation on the definition of a nanomaterial (2011/696/EU)). The basic definition is:

> "'*Nanomaterial'* means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm."

Fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm are also considered as nanomaterials.

For the purpose of the definition of nanomaterial, the following terms are also defined in 2011/696/EU:

- *particle* means a minute piece of matter with defined physical boundaries;

- *agglomerate* means a collection of weakly bound particles or aggregates where

the resulting external surface area is similar to the sum of the surface areas of the individual components;

- *aggregate* means a particle comprising strongly bound or fused particles.

While the European Union Observatory for Nanomaterials (EUON) applies this definition in its discussions on nanomaterials, it is recognized that the definition is not universally used. The existence of different definitions for the term nanomaterial complicates the understanding and interpretation of the studies on the topic of investigation in this study.

For the purposes of this study, the term "nanomaterial" is interpreted widely, in order to capture the largest possible sources of information and studies for further assessment. Studies that use a different definition compared to the EC Recommendation are still included within the scope of this study. Therefore, studies that used MNM at a size up to 150 nm will be taken into account. It is considered that beyond that size the MNM are not generally penetrating the skin. Also, some studies that used nano-emulsions as 'carrier' for medicinal substances to increase the absorption of those substances are taken into account, although emulsions could be considered outside the definition of 'particle' as provided above.

Nanomaterials may have different nanoforms of the same material with different physico-chemical properties (e.g. size, shape, surface treatment etc).

For the definition of terms related to dermal absorption, we use in this study the following definitions, which come from the Environmental Health Criteria 235, Dermal Absorption by WHO (2006):

- *Dermal (percutaneous, skin) absorption* is a global term that describes the transport of chemicals from the outer surface of the skin to the systemic circulation. This is often divided into:
    - *penetration*, which is the entry of a substance into a particular layer or structure, such as the entrance of a compound into the stratum corneum;
    - *permeation*, which is the penetration through one layer into a second layer that is both functionally and structurally different from the first layer; and
    - *resorption*, which is the uptake of a substance into the skin lymph and local vascular system and in most cases will lead to entry into the systemic circulation (systemic absorption).

Different documents, referenced in this study, may however use different definitions of these terms. Where the definitions are not clear in an original document, we will use the term as used in the original document.

Dermal penetration and absorption studies can be performed in various types of skin. In this report we use the following descriptions in relation to the source of the skin used:

- *in vivo* studies are studies in life humans or animals
- *ex vivo* studies are studies using actual skin surgically removed from humans or animals; (in many documents these types of studies are included in the term *in vitro*)
- *in vitro* studies are, in the scope of this report, studies with cell cultures or 3D reconstructed skin models, *i.e.* not skin surgically removed, but 'built' or 'grown' in a laboratory.

4. Objectives

The objectives of the study were to perform literature searches for studies focussing on dermal absorption of MNM, and to review the information available in the public domain.

The following questions were intended to be answered as far as possible from the available information:

- What studies are available on MNM which are relevant to determine if dermal absorption is taking place?
    - *In vitro/ex vivo* and *in vivo* (animal studies including repeated dose toxicity studies, human case studies or epidemiological data, using intact or damaged skin)
- What are the guidelines followed for the studies (if any)? Are the results available in a structured way e.g. following OECD harmonised templates?
- What are the manufactured nanomaterial (MNM) properties that can affect dermal absorption and how does each property contribute to dermal absorption (e.g. physico-chemical properties, surface treatment, formulation etc.)?
- What are the factors associated with test methodology that can affect dermal absorption (e.g. sample preparation, species difference, vehicle, exposure conditions etc.)?
- What are the available tools to assess dermal absorption of MNM?
    - Are the existing methods (*in vitro/ex vivo/in vivo*) suitable for MNM?
    - What are the suitable detection methods to assess dermal absorption of MNM?
- What recommendations can be provided for future work on evaluating dermal absorption of MNM and are there still data gaps for assessing dermal absorption of MNM?

5. Methodology

5.1 General set-up of the study

The study was performed in four Work Packages:

- Work Package 1: Definition of the scope of the review and methodology;
- Work Package 2: Literature search and review;
- Work Package 3: A transparent analysis of the data;
- Work Package 4: Final report.

An interim report was produced after Work Packages 1 to 3 and used as input to this final report.

5.2 Scope of the review

The current review used as a starting point the review documents published before the start of this study. The already mentioned report by the Danish EPA was used as the main starting point, because it was a very structured and most recent report that also used quality criteria to decide on studies to be taken into account.

The scope of the final review, was defined as follows:

To gather, evaluate and summarize the knowledge available regarding dermal penetration and absorption of MNM used in consumer products and occupational settings.

The intention was to analyse studies that provided experimental results on the use of different testing methods, such as the use of skin explants from animal or human sources (also called *ex vivo* studies), *in vivo* studies and/or *in vitro* studies (including cell cultures or 3D reconstructed skin models used as reconstructions of human skin). The influence of using damaged and intact skin in combination with different methods as an experimental set up was also investigated. In addition, attention was given to nanomaterial related aspects, and predominantly their physicochemical properties. Main focus was on low solubility and/or non-biodegradable MNM (e.g. TiO_2, fullerenes or quantum dots), MNM with high dissolution rates (e.g. Ag and ZnO) and soluble and/or biodegradable MNM (such as liposomes, micelles, nanovesicles or solid lipid nanoparticles). To come to a more targeted study, publications already included in the Danish EPA report were excluded. Publications with focus on nano-drug delivery systems and percutaneous administrations without information on factors influencing dermal penetration of MNMs, were also excluded.

This final report delivers the critical evaluation of the link between the properties of MNM and the dermal absorption values in humans as well as regulatory relevance of these findings. Furthermore, animal (mainly porcine) skin absorption of MNM *in vivo* and *in vitro* were analysed and compared with mechanisms found in humans. This was done in order to provide recommendations about extrapolating animal experimental data to humans for regulatory purposes.

5.3 Literature search

5.3.1 Search strategy

The recent report published by Danish EPA (Poland *et al.*, 2013) was used as a starting point for the screening, along with the following reviews:

- Interaction of inorganic nanoparticles with the skin barrier. Relevant studies for human dermal risk assessment of nanomaterials in consumer products (Wijnhoven, 2012);
- Grouping of nanomaterials. A strategy towards grouping and read-across (Sellers *et al.*, 2015);
- Description of a Nano Cosmetics Tool for Risk Assessment (de Jong *et al.*, 2015);
- A review of recent advances towards the Development of (Quantitative) Structure-Activity Relationships for Metallic Nanomaterials (Chen *et al.*, 2017);
- Quality evaluation of human and environmental toxicity studies performed with nanomaterials - the GUIDEnano approach (Fernández-Cruz *et al.*, 2018);
- Evaluation of *in vitro* methods for human hazard assessment applied in the OECD Testing Programme for the Safety of Manufactured Nanomaterials (OECD, 2018).

The basic approach for finding relevant studies published after the Danish report was to perform a literature search via relevant online literature databases. However, in order not to exclude studies that might not have been included in the report of the Danish EPA an additional screening in Pubmed and Toxnet for literature up to 2013 was performed using the search strings as chosen and keywords not used in that report.

The search and selection strategy was split in four stages:

1. Define the keywords, search strings, search databases, inclusion/exclusion criteria;
2. Use the keywords for a search; the search was split in a preliminary search and further search, based on preliminary and additional set of keywords and search strings;
3. Use inclusion and exclusion criteria to filter the literature chosen in the search;
4. Use quality and relevance criteria to filter the literature chosen in the previous step.

5.3.2 Databases, search strings and keywords

The main databases covered in the search were the scientific literature databases Pubmed and Toxnet and they were used to search for relevant original research data present in the public literature.

The following search strings were used:

> Pubmed and ToxNET: (Ultrafine OR nano-object OR nanoparticle OR nanoparticulate OR nanomaterial OR nanotube OR nanofiber OR nanofibre OR nanowire OR nanocomposite OR nanoplate OR nanorod OR fullerene OR "quantum dot") AND (dermal OR skin) AND (absorption OR penetration OR uptake) AND (***Keyword***).

At the place of the word 'keyword' indicated in the search strings above, the following keywords were used (

Table 1).

Table 1: The keywords used for the search strategy

Keywords			
assay	epidermis	occupation	sunscreen
bioavailability	epidemiology	occupational	surface chemistry
biodegradability	exposure	permeation	systemic
biodistribution	hair follicles	personal care	systemic absorption
characterisation	hazard assessment	physicochemical	test guidelines
characterization	hazard identification	physico-chemical	test methods
consumer products	human	QSAR	testing strategy
corrosion	*in silico*	sensitisation	toxicity assessment
cosmetics	*in vitro*	sensitization	toxicology assessment
dermal-abrasion	*in vivo*	size	translocation
dermis	irritation	solubility	worker
disease	modelling	sun block	workplace
dissolution rate	modelling	sun lotion	
distribution	models	susceptibility	

For the limited search of references potentially missed in the search for the Danish report up to 2013, the following keywords were added, that were not used in the Danish report: biodegradability, epidemiology, human, size, solubility and surface chemistry.

5.4 Inclusion and exclusion criteria

After the search based on keywords it was evaluated whether, based on the scope of the study, the references should be included or excluded from the analysis. In order to decide on inclusion of studies, carefully chosen specific criteria were set. The inclusion and exclusion criteria were determined using as starting point information generated through research performed by the HORIZON2020 project GRACIOUS (https://www.h2020gracious.eu/). The criteria used in that project were critically evaluated for their applicability to the current project. Further adaptation or modification was performed, where needed, and amendment of the criteria in order to determine the inclusion and exclusion criteria that were used in this project. The final inclusion and exclusion criteria are given in

Table 2.

Table 2: Inclusion and exclusion criteria used

Inclusion criteria		Explanation
1	*In vivo* studies and/or *ex vivo* studies with skin explants from animal or human sources, evaluating MNM skin penetration.	In addition to studies on human skin, useful information can also be obtained from some animal models, such as pig. Studies using rodent skin are much less relevant for evaluating skin absorption and penetration of MNM in humans. However, studies in which MNM were studied in multiple models, e.g. *in vivo* rats and *ex vivo* human skin, were included. This may enable understanding of the differences in penetration of MNM between rodents and humans.
2	Studies performed using low solubility and/or nonbiodegradable MNMs	MNM were considered that demonstrate very low values on dissolution or degradability, because the intention is to study penetration of particles and not diffusion of ions.
3	Studies based on MNM with high dissolution rates such as Ag and ZnO.	Dissolution rates of MNMs depend on time and experimental conditions. Some of these MNMs may still be intact throughout the test duration and could provide useful information.
4	Studies in which the substances were topically applied to intact skin or damaged skin via different methods such as tape stripping, mechanical abrasion, UV radiation or needle-punction.	The reason to include these studies is to look into possible differences in testing models that potential damage on the skin can have on the absorption of the MNM and the interpretation of the results
5	Review articles about MNM dermal penetration.	Review publications may be used to additionally find original publications not found in the preliminary search.
6	Studies investigating MNM *in vitro* penetration in 3D reconstituted skin models (e.g. Episkin® or Epiderm®).	These studies are particularly interesting if multiple materials or experimental conditions are included, as it will enable identification of the factors determining skin absorption. Factors established in a single study using these methods or the same methods used in different studies on substances with different characteristics may enable a comparison. These studies were also included to analyse the relevance of these tools for MNM dermal absorption testing.

7	Studies based on soluble and/or biodegradable MNM that are likely to change their conformation through the dermal barrier such as liposomes, micelles, nanovesicles or solid lipid nanoparticles.	It will be useful to know whether these types of materials are able to penetrate the skin or not, and if so, in what form.
Exclusion criteria		Explanation
1	Studies based on nano-drug delivery systems since these experiments are commonly based on monitoring translocation of the drug rather than the nanocarrier unless information on the nanocarrier itself is included in the study.	These studies were excluded, because they do not provide actual information on dermal penetration of MNM.
2	Studies with percutaneous administrations of MNM performed via iontophoresis, sonophoresis or assisted with microneedles.	This way of administration is a specified skin administration and not relevant for normal dermal exposure.
3	Studies already included in the Danish EPA report.	In order not to duplicate previously performed work.
4	Studies focused on the development of the chemical entity/MNM for application via the skin, e.g. by improving the linking of the coating to the primary MNM to stabilize the coating.	No specific information on penetration and absorption.
5	Studies focused on using different techniques for manufacturing of MNM and their benefits or drawbacks.	These studies do not contain data on penetration and absorption.
6	*Ex vivo* studies with rodent skin without analysis of factors determining dermal absorption of MNM (e.g. physic-chemical characterisation of MNM, testing conditions etc.) .	Actual information on dermal penetration or dermal absorption values from *ex vivo* rodent studies are not useful because the comparability of rodent skin and human skin is too low to take these values into account.
7	Studies without access to the full-text version.	In case of no access to the full publication the quality and the relevance of the study cannot be evaluated.
8	Studies investigating MNM *in vitro* penetration in simple cell line cultures.	Simple cell line cultures have very limited comparability with the complexity of the skin layers, making results not representative for skin.

5.5 Quality and relevance criteria

5.5.1 Introduction

Over the years, many studies have been published which investigate the skin penetration or

absorption of MNM. The value of these studies for risk assessment purposes is variable. In order to prioritize these studies, we distinguish two types of criteria: quality and relevance criteria.

- **Quality criteria** inform on the *completeness* of the experimental set-up and data reported in the study

- **Relevance criteria** inform on the *appropriateness* of the experimental model and set-up for the purpose of determining skin penetration and absorption of the MNM under investigation. For both types of criteria, scoring tables or decision trees have been developed, which are explained in more detail below.

5.5.2 Quality criteria

To conclude on the potential of MNM to absorb or penetrate the skin, it is essential that information on the materials as well as the study design is appropriately described. Previously, a quality assessment system was established specifically for studies with MNM (Fernández-Cruz *et al.*, 2018). This system consists of a list of substance criteria (the 'S-score'), describing information on the characteristics of the tested MNM as well as a list of study design criteria based on the Klimisch score and the ToxRtool (the 'K-score') (Fernández-Cruz *et al.*, 2018). For the current report, we have adapted this approach to assess the quality of absorption/penetration studies with nanomaterials.

The S-score depicts the completeness of the characterization of the MNM and is evaluated in a similar way for all types of studies. The K-score describes the quality of the study design, and the criteria depend on the study type, i.e. different criteria apply for *ex-vivo* skin studies compared to studies with human subjects.

5.5.2.1 The S-score

The **S-score** describes the quality of the study in terms of how well the studied material was characterized. Nanomaterial properties such as size and shape can greatly affect the behaviour of the materials and the better these properties are described, the higher the quality of the study. To determine the S-score, a number of questions need to be answered. Note that some questions are considered essential (depicted in red), i.e. without this information, the study should be considered of unacceptable quality. The other questions serve only to additionally improve the quality score of the study and can as such help to prioritize studies. Note that the S-scoring system is the same for different study types, i.e. studies with human subjects, *ex vivo* skin or rodents.

In the process of scoring the retrieved studies, it became apparent that with the original S-score as developed at the beginning of the project, only very few (if any) studies would pass the acceptability criteria (*i.e.* studies including sufficient information on all essential information elements). Information was found to be lacking mostly on **primary particle shape** and on **dissolution rate in exposure medium**. The lack of information on these properties may be due to current technical limitations to characterize these properties for many different materials. It was therefore decided that these two pieces of information would not be considered critical anymore. Rather than rejecting all studies that did not contain both pieces of information, three categories of S-scores have been created:

- Green studies contain all pieces of critical information listed above, including primary particle size and size distribution in exposure medium and are therefore 'Accepted studies'.

- Orange studies contain either information on primary particle size or information on the size distribution in the exposure medium, and all other critical information listed above.

These studies will be considered 'Supporting studies'.

- Red studies are missing one or more of the critical pieces of information listed above. These studies are rejected, and therefore not scored for their K-score and relevance score. They were not included (but 'Rejected') in the further analysis.

In

Table 3 the questions leading to the S-score are presented. The questions presented in orange are the ones that originally were considered critical, but for which it was decided that missing information would lead to studies being 'supporting' studies, instead of being rejected. The questions in red are the ones for which missing information leads to rejection of the studies.

Table 3: Questions leading to the S-score

No.	Question	Score (0 = no, 1 = yes)	Explanation
1	Was the chemical composition of the test material identified?	0 or 1	Chemical name, CAS number and/or chemical composition including coating
2	Is information on the source of the test material given?	0 or 1	
3	Is the purity of the test material given?	0 or 1	
4	Are protocols of dispersion and characterization in the exposure medium sufficiently stated?	0 or 1	
5	Is the primary particle size stated?	0 or 1	One single value or distribution
6	Is the surface area of the primary particles stated?	0 or 1	
7	Is the shape of the primary particles stated?	0 or 1	Sphere, rod, fibre etc.
8	Other primary particle characteristics given?	0 or 1	Many particle properties may affect skin absorption/ penetration, such as crystal structure, magnetic properties, redox potential, hydrophobicity, etc.
9	Is size (distribution) in exposure medium presented?	0 or 1	It is essential that the size distribution in the exposure medium is described because primary particles can have another size due to aggregation/ agglomeration in the exposure medium.

10	Is the dissolution rate in exposure medium given?	0 or 1	Studies that do not show presence of MNM in particulate form in the dermis or beyond need to provide information on the dissolution rate of the test material in the exposure medium. This is because analytical methods such as mass spectroscopy do not allow for the distinction between particles that are dissolved and particles that are intact. This is important, because MNM with a high dissolution rate such as certain metal oxides may have dissolved even before penetrating the skin. (For studies demonstrating MNM in particulate form in the dermis or beyond, e.g. by imaging, score 1)
11	Is the surface charge in exposure medium provided?	0 or 1	
	Maximum S-score	**11**	

5.5.2.2 The K-score

The **K-score** describes the quality of the study design. Three different lists of questions were developed for calculating the K score:

1. to assess the quality of absorption/penetration studies in human subjects
2. to assess the quality of absorption/penetration studies in human and porcine skin
3. to assess the quality of absorption/penetration studies in rodents

Studies in human subjects

The following questions and associated scoring system was developed for absorption/penetration studies in human subjects (Table 4). Note that some questions are considered essential (depicted in red), i.e. this information is essential to assess the quality (or later, the relevance) of the study. Other questions are not essential but improve the quality score of the study and can be used for prioritization of studies.

Table 4: Questions for the K-score for studies in human subjects

No.	Question	Score (0 = no, 1 = yes)	Explanation
1	Was written informed consent obtained from the test subjects?	0 or 1	A general indicator of the quality of the study
2	Is the number of test subjects given?	0 or 1	A general and essential indicator of the quality of the study.
3	Were inclusion and exclusion criteria for test subjects described?	0 or 1	A general indicator of the quality of the study.

4	Is the age of the test subjects given?	0 or 1	A general indicator of the quality of the study.
5	Is the sex of the test subjects given?	0 or 1	A general indicator of the quality of the study.
6	Is the condition of the skin of the test subjects given?[a)]	0 or 1	Information on condition of the skin should contain whether the exposed skin was healthy, damaged or flexed.
7	Are frequency and duration of exposure as well as time-points of observations sufficiently explained?	0 or 1	Essential information includes how long the test material was in contact with the skin, and how long after exposure measurements were performed.
8	Are sufficient details of the exposure method given?	0 or 1	Necessary details consist of information on: • dilution of the test item in vehicle • the concentration and total volume applied • homogeneity of application media • location of the skin treatment • pre-treatment with irritant substance or not • type of occlusion and exposed skin area
9	Have the measurement / observation methods been described?	0 or 1	What technique was used to measure/observe MNM absorbed or penetrated through the skin, e.g. radiolabelling, spectrometry or microscopy methods
	Maximum K-score	**11**	

[a)] While this was considered a critical question during scoring, it was later agreed that, as long as the subjects are healthy, this can be considered a non-critical question for future scoring of studies.

Ex vivo skin studies

The following questions and associated scoring system were developed for absorption/ penetration studies in human or porcine skin (Table 5). The questions that are considered critical are indicated in red. Without this information, the relevance of the studies cannot be assessed.

Table 5: Questions for the K-score for studies with *ex vivo* human or porcine skin

No.	Question	Score (0 = no, 1 = yes)	Explanation
1	Is the species source of the skin given?	0 or 1	Human, porcine or other

2	Is information given on the source location of the skin?	0 or 1	For example, skin from back or abdomen.
3	Is information on skin properties given?	0 or 1	Necessary information includes: • skin integrity and viability • split thickness
4	Is information on conditions of cultivation and maintenance given?	0 or 1	Type and composition of culture media
5	Is the method of exposure described in sufficient detail?	0 or 1	Necessary details consist of information on: • dilution of the test item in vehicle • the concentration and total volume applied • homogeneity of application media • amount of skin area covered • temperature of skin • temperature, pH and composition of reception fluid
6	Is the duration of exposure provided?	0 or 1	Essential information includes how long the test material was in contact with the skin
7	Are the time-points of observations explained?	0 or 1	Essential information includes how long after exposure measurements were performed
8	Is the number of skin samples given?	0 or 1	A minimum number of skin samples is needed for a good quality result
9	Is the number of donors of skin samples given?	0 to 1	Skin of different donors may have different characteristics; therefore, using samples from multiple donors is preferred
10	Are the method(s) of detection of absorption/penetration of test materials clearly described?	0 or 1	It should be clear whether the methods used allow for the distinction of particles or not.
	Maximum K-score	**10**	

Studies in rodents

The following questions and associated scoring system were developed for absorption/ penetration studies in rodents *in vivo* (Table 6). The questions that are considered critical are indicated in red. Without this information, the relevance of the studies cannot be assessed.

Table 6: Questions for the K-score for *in vivo* studies in rodents

No.	Question	Score (0 = no, 1 = yes)	Explanation

1	Is the species and strain of animals given?	0 or 1	Species and strain determine whether the experimental model is similar enough to humans
2	Is the number of animals per treatment group given?	0 or 1	As variation is expected to be high, a minimum number of animals is needed
3	Is type of cage used specified?	0 or 1	Individual versus grouped caging, metabolism cage or not
4	Are details given on housing conditions?	0 or 1	Light/dark cycle, room temperature
5	Are details given on diet?	0 or 1	Type and availability of food and drinking water, measures to prevent spillage
6	Is information given on the location of the skin exposure?	0 or 1	For example, exposure took place on back
7	Is information on skin condition throughout the study given?	0 or 1	Necessary information includes: • skin integrity • hairless or shaven or neither • occurrence of irritation or dermal toxicity
8	Is the method of exposure described in sufficient detail?	0 or 1	Necessary details consist of information on: • dilution of the test item in vehicle • the concentration and total volume applied • homogeneity of application media • skin surface area covered • use of occlusion or not
10	Are timepoints of measurements defined	0 or 1	The time point of taking samples for measurement needs to be sufficiently long to allow for the penetration to occur
11	Is the number of samples for measurement given?	0 or 1	A minimum number of samples is required to make it a relevant study
12	Are the method(s) of detection of absorption/penetration of test materials clearly described?	0 or 1	It should be clear whether the methods used allow for the distinction of particles or not.
	Maximum K-score	**11**	

5.5.3 Relevance criteria

To determine the relevance of absorption/penetration studies of MNM for the purpose of this study, we have developed relevance criteria for experimental studies with human subjects, for studies using human or porcine skin and for rodent studies. By applying these criteria, the studies eventually can be assigned a score, which, along with its quality score (see above) determines the overall value of the study.

The relevance scores are divided into studies of high, limited or no relevance. Studies of no relevance get a score of 0 points. The value of the score further depends on the experimental model used, i.e. human subjects, human skin, porcine skin or rodents. The final numerical scores for studies of high and limited relevance are assigned according to Table 7 below.

Table 7: Assignment of relevance scores for different types of studies

	Type of study			
	Human subjects	Human skin *(ex vivo)*	Porcine skin *(ex vivo)*	Rodents
High relevance	5	4	3	2
Limited relevance	4	3	2	1

Note that these score values are arbitrary and only serve to facilitate prioritization of available studies according to their relevance.
The following paragraphs provide more explanation on the relevance criteria used.

5.5.3.1 Studies with human subjects

The relevance of absorption/penetration studies with human subjects depends on a number of factors related to the experimental set-up of the study. The following factors have been taken into account.

Exposure duration and timepoints of observation/measurement
In order for MNM to be able to penetrate the skin, the exposure time (i.e. the actual contact time of the test material with the skin) and the amount of time after exposure until observation/measurement of the particles in the dermis or beyond needs to be sufficiently long. As far as we know, studies investigating what should be the appropriate exposure and observation/measurement times are unavailable. Using expert judgment, an exposure time of at least 30 minutes as well as an observation/measurement time of 24h post exposure is considered to be the minimum, unless studies clearly show that the material has penetrated beyond the dermis after shorter periods of time.

Source of the observed/measured material
Materials such as TiO_2 and SiO_2 are omnipresent in our food and environment and background levels in human subjects may be high. Therefore, studies with such materials need to show that the observed or measured MNM in the dermis or beyond are not from a source other than the test material. This could be done by, for example, labelling the test materials or by demonstrating low background levels. This is most relevant for *in vivo* studies and measurements of actual absorbed amounts.

Aggregation or agglomeration in the exposure medium
For studies that have used sufficiently long exposure and observation/measurement times, and have not measured or observed MNM in the dermis or beyond, it needs to be ensured that the test material has not severely aggregated or agglomerated in the exposure/dispersion medium, both before and after application to the skin. Aggregation or agglomeration of the material in the exposure medium used in the study may have led to the formation of particles that were too large to penetrate the skin, while in real life or in different dispersion media, a lower aggregation or agglomeration level may have allowed these particles to penetrate the skin.

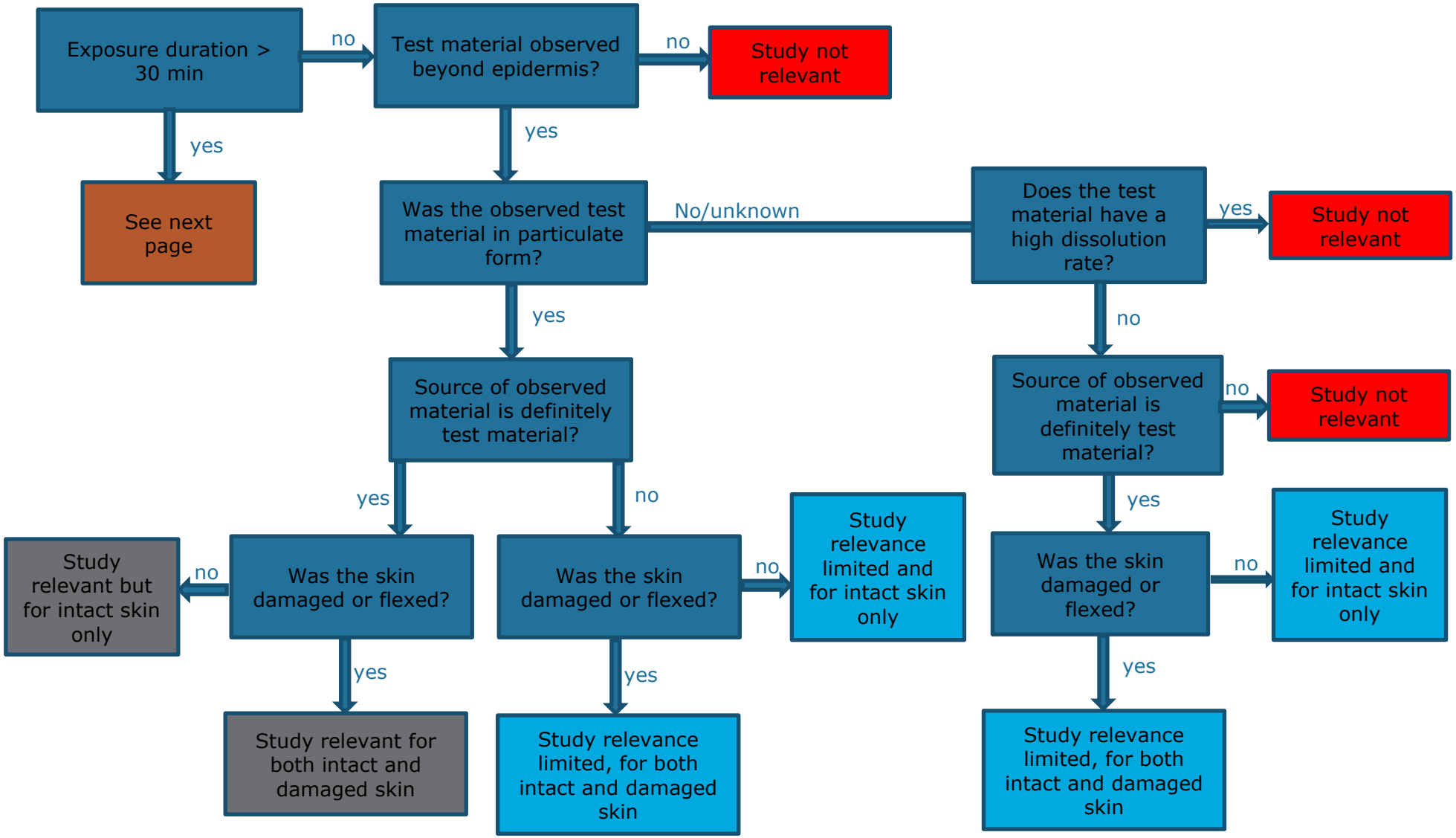
Application under occlusion
Another factor that needs to be taken into consideration especially for studies that have used sufficiently long exposure and observation/measurement times and have not measured or observed MNM in the dermis or beyond is that the material was applied under occlusion. This is important because materials that were not applied under occlusion may have come off before they were able to penetrate the skin.

Damaged or flexed skin
Studies that have been performed on intact skin may be just as relevant as studies that have been performed on flexed skin or skin that was damaged, e.g. by abrasion. However, the outcome is relevant for different situations. Outcomes of studies using intact skin are relevant only for skin exposure occurring on healthy skin, whereas outcomes of studies using damaged skin are relevant for sensitive groups (e.g. for humans with skin diseases) or exposure scenarios that may lead to skin damage such as UV light. Outcomes of studies using abraded skin present worst-case scenarios for humans with healthy, undamaged skin.

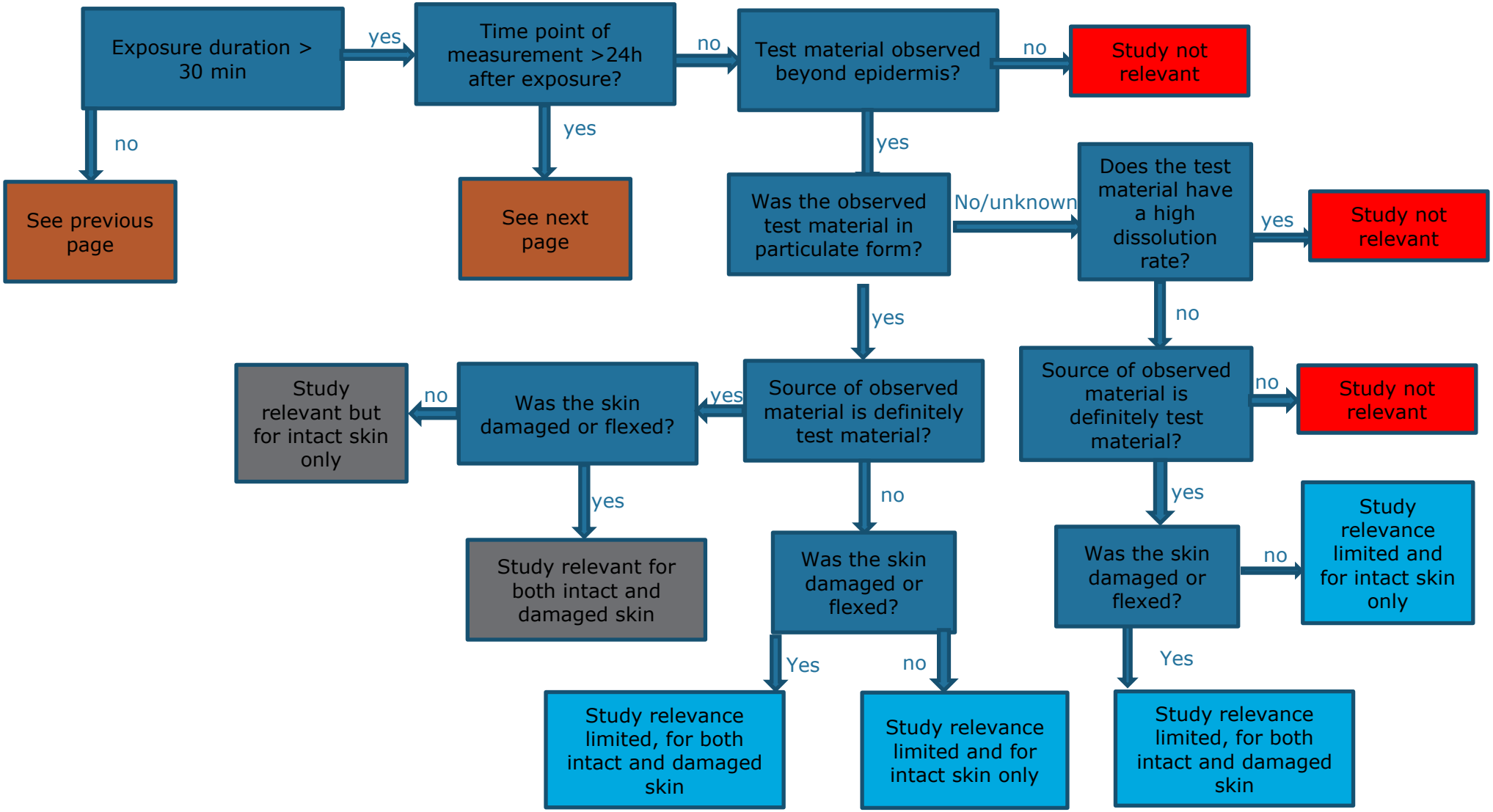
Decision tree to determine the relevance of nanomaterial absorption/penetration studies with human subjects

The factors listed above have been integrated in the decision tree depicted in Figure 1.

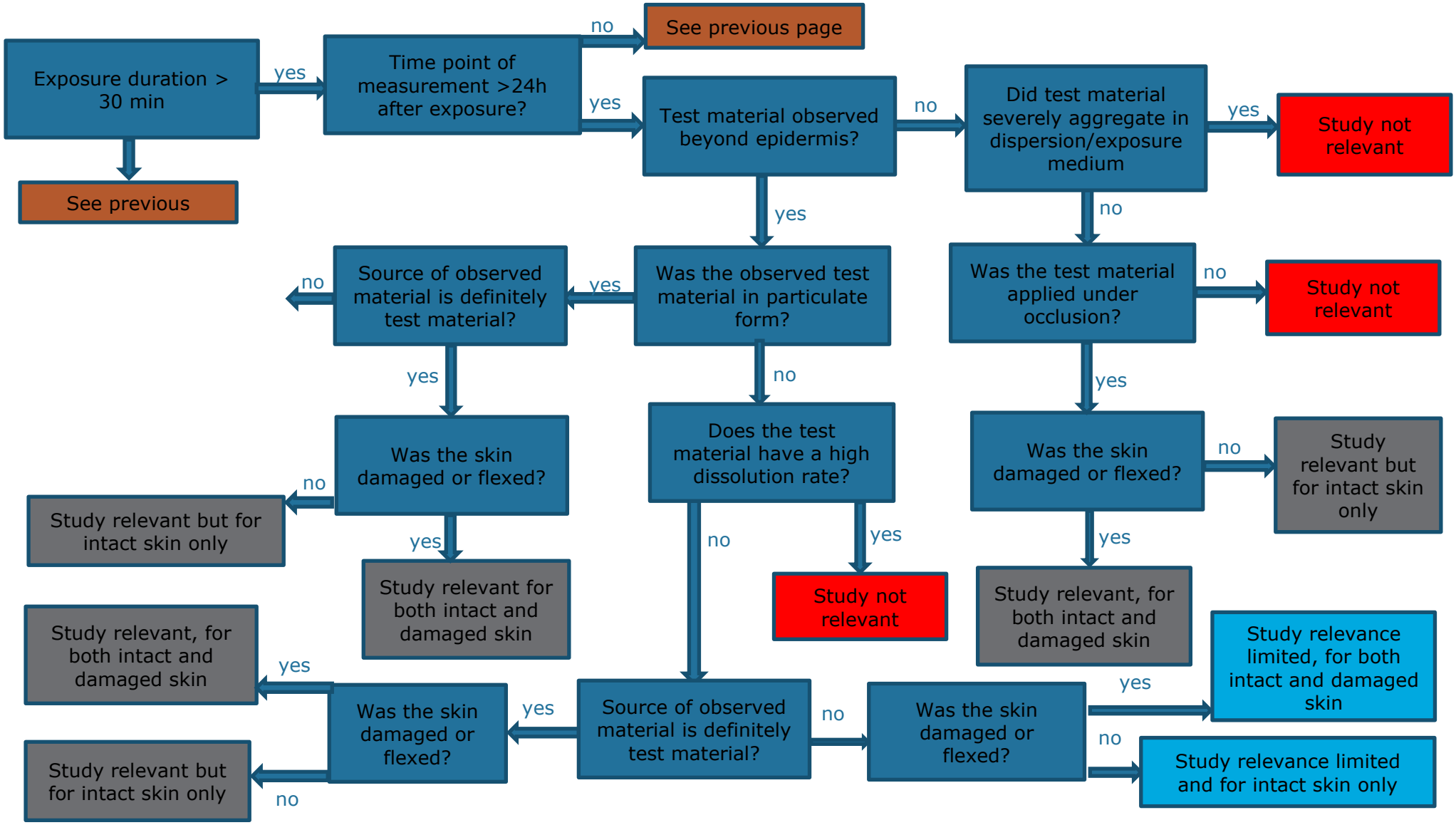
Figure 1: Relevance of nanomaterial absorption/penetration studies with human subjects



(**Figure 1** continued)



(**Figure 1** continued)



5.5.3.2 *Ex vivo* studies with human or porcine skin

Also, for absorption studies with skin from either human or pig, a number of factors related to the experimental set-up and available information is crucial for the relevance of the studies. The majority of factors is derived from OECD test guideline (TG) 428 for *in vitro* testing of skin absorption (OECD, 2004b) as well as from the SCCS basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients (Scientific Committee on Consumer Safety, 2010). (In the present report, such studies are called *ex vivo* studies.) Based on these criteria, the relevance scoring presented in Table 8 was developed.

Table 8: Relevance scoring criteria for studies with human or porcine skin *ex vivo*

No.	Criteria	Score (0 = no, 1 = yes)	Explanation
1	Study follows OECD TG 428	0 or 1	OECD TG 428 addresses the *in vitro* testing of skin absorption. When the performed study is compliant to this test guideline, which is part of a series of most relevant internationally agreed testing methods, a proper test design is guaranteed. However, not all criteria in this guideline are relevant and crucial for MNM.
2	Standardized skin preparation of known source and location is used	0 or 1	The skin preparations should be chosen and treated with care. Human skin from an appropriate site (abdomen or back) remains the gold standard. If not available, porcine skin is an alternative. The selection of species, anatomical site and preparative technique must be justified
3	8 skin samples of 4 donors are used, 2 replicates per donor	0 or 1	According to the SCCS Notes of Guidance (SCCS/1602/18), a dermal absorption study should be performed with 8 skin samples from at least 4 different donors. Because of the variability between individuals and between explanted skin samples, replications in both donor and samples per donor are needed
4	Human skin studies use epidermal membranes (enzymically, heat or chemically separated) or split thickness skin (200-500 µm) For porcine skin studies, full thickness skin can be used	0 or 1	According to the SCCS Notes of Guidance (SCCS/1602/18) and OECD TG 428, human skin should be split to a maximum thickness of 200-500 µm. Pig skin is thinner and can be used completely

5	Skin integrity is warranted	0 or 1	Poor barrier quality may lead to unrealistic high dermal absorption values. Therefore, skin integrity is of key importance and should be verified. Skin integrity can be measured using a variety of methods, e.g. transepithelial electrical resistance (TEER).
6	The temperature of the skin and the receptor fluid is controlled	0 or 1	The temperature should be physiologically relevant.
7	Appropriate exposure medium and receptor fluid are used	0 or 1	The stability of the test material in the exposure medium should be demonstrated (i.e. no severe aggregation). Furthermore, the exposure medium and receptor fluid should not interfere with the skin integrity and with the analytical method.
8	Exposure takes place using a diffusion cell	0 or 1	A diffusion cell is a donor chamber and receptor chamber between which the skin is positioned
9	The minimum skin area covered is 0.64cm^2	0 or 1	It is important to use a sufficiently large surface area of skin.
10	Test material does not dissolve in exposure medium in <24h	0 or 1	MNM with a high dissolution rate such as certain metal oxides may have dissolved even before penetrating the skin. (For studies demonstrating MNM in particulate form in the receptor fluid, e.g. by imaging, score 1)
11	Minimum exposure concentration is 0.1 mg/cm^2, maximum does not exceed stable dispersion concentration	0 or 1	The exposure concentration of NM should not be too low, but also not higher than the maximum concentration at which a stable dispersion is reached in the exposure medium.
12	Exposure duration is at least 30 minutes	0 or 1	An exposure time of at least 30 minutes exposure is considered to be the minimum, unless studies clearly show that the material has penetrated beyond the dermis after shorter periods of time.

13	Sampling takes place up to at most 24h	0 or 1	Skin integrity may start to deteriorate beyond 24 hours, and so sampling times should not normally exceed 24 hours. However, much shorter sampling times may result in material still being in skin layers that could still be absorbed.
14	Sampling of receptor fluid takes place at multiple timepoints	0 or 1	Regular sampling (i.e. at multiple time points) within these 24 hours is preferred, taking into account delayed penetration into skin layers.
15	Mass balance is provided, test material is accounted for 85-115%	0 or 1	The absorption needs to be calculated for each single diffusion cell and these values should be used to derive the mean absorption. The overall recovery of test material (including metabolites, although these may not be relevant for most MNM) should be within the range of 85-115%.
	Maximum relevance score	**15**	

Based on the scoring of the table above, the distinction between studies of no, limited or high relevance are determined as follows:

- Studies that do not score 1 on all red questions are considered not relevant.
- Studies that score 1 on all red questions and score 1 on 12 questions in total are considered of limited relevance.
- Studies that score 1 on all red questions and score 1 on more than 12 questions in total are considered of high relevance.

Note that again, the values of the scores are arbitrary and only serve to enable prioritization studies according to their relevance.

5.5.3.3 Studies with rodents

There are significant anatomical differences between human and rodent dermis (Sundberg *et al.*, 2012). First, the human dermis is a highly vascularized system with both a rich papillary network and a deep dermal network, whereas the mouse dermis is poorly vascularized, and the predominant capillary networks are associated with hair bulbs. Second, there is a clear difference in hair density. Although not explicitly mentioned by the authors, it is likely that what holds for the mouse dermis in this respect, also holds for the rat dermis. A comparison of human, rat, and mouse skin showed a thickness of stratum corneum (SC), viable epidermis (VE) and dermis (D) of 17 µm, 47 µm, and 2.9 mm, respectively, for human abdomen; 18 µm, 32 µm, and 2.0 mm, respectively for rat dorsum; and 9 µm, 29 µm, and 0.7 mm, respectively for mouse dorsum (Wei *et al.*, 2017). In conclusion, the skin thickness for rat is lower than for humans and for mouse clearly lower still. For conventional substances, species differences may not pose much of a problem as they can be dissolved in various different exposure vehicles that allow for maximum penetration of the skin, which generally takes place by passive diffusion. A maximized, diffusion-led penetration is not readily possible for NMN, because of their tendency to aggregate. On the other hand, where MNM are in particulate form and not very aggregated, they may use penetration routes not available to larger particles,

such as hair follicles, making the difference in hair density an important factor. Therefore, anatomical differences between species are likely to play a much bigger role in species differences in penetration. Therefore, dermal absorption/penetration studies with MNM in rodents are considered to be of limited relevance for humans. Nevertheless, since many of the nanomaterial dermal absorption/penetration studies available are executed in rodents, we have developed relevance criteria to use for studies in rodents comparing various MNM. These criteria are presented in **Error! Reference source not found.**.

Table 9. Relevance scoring criteria for studies with rodents

No.	Criteria	Score (0 = no, 1 = yes)	Explanation
1	Study follows OECD TG 427 (2004a)	0 or 1	OECD TG 427 addresses the in vivo testing of skin absorption. When the performed study is compliant to this test guideline, which is part of a series of most relevant internationally agreed testing methods, a proper test design is guaranteed. However, not all criteria in this guideline are relevant and crucial for MNM.
2	Study is performed in young adult rodents of a single sex of commonly used laboratory strains, for which it has earlier been shown experimentally, that their skin absorption rates are similar to those in humans	0 or 1	Though there is too limited information to know whether their skin absorption rates are actually similar to humans, it is expected that the similarity is higher for hairless rodents than for normal rodents.
3	Number of animals per group (one sex) is at least four	0 or 1	The expected variation is large. Therefore, a group of at least four animals of one sex should be used for each test preparation and each scheduled termination time.
4	Animals are individually housed in metabolism cages	0 or 1	To prevent animals from licking each other's skin, and to allow for a correct mass balance calculation, the animals should be individually housed in metabolism cages throughout the study.
5	Housing conditions are properly controlled.	0 or 1	The temperature in the experimental animal room is 22°C (± 3°C). The relative humidity is 50-60% (at least 30% and preferably not exceed 70%). Lighting is artificial, the sequence being 12 hours light, 12 hours dark.

6	The feeding conditions are properly controlled.	0 or 1	Conventional laboratory diets may be used and should be freely available together with an unlimited supply of drinking water. Since food and water spillage would compromise the results, the probability of such events should be minimised.
7	Test material in exposure vehicle is in particulate form throughout exposure duration	0 or 1	Test materials that dissolve within the duration of the exposure do not give any information on the skin penetration/absorption potential of MNM
8	Test material application site is protected from grooming	0 or 1	If application site is unprotected, materials may be ingested and give false positive results on skin penetration/absorption potential
9	Skin area application site is at least 10 cm^2	0 or 1	The 10 cm2 skin area of the application site does not need to be fully covered but does need to be sufficiently large for materials to absorb/penetrate.
10	Exposure duration is at least six hours	0 or 1	The duration of exposure is the time interval between application and removal of test preparation by skin washing. Six hours is probably sufficiently long for MNM to cross the skin barrier, although data to confirm this is lacking. Note: this question may be scored with 1 if shorter exposure durations are used, but skin penetration in the dermis or beyond is demonstrated.
11	Minimum exposure concentration is 1 mg/cm^2, maximum does not exceed stable dispersion concentration in exposure vehicle	0 or 1	Concentrations need to be sufficiently high to be above detection limits, but not so high that the material aggregates in the exposure vehicle, preventing skin absorption/penetration
12	At least three time points of measurement from directly after exposure up to 72h after exposure are included	0 or 1	Multiple timepoints help with the mass balance calculation.

13	Method of detection allows for completion of mass balance and distinction between particulate and dissolved material	0 or 1	Commonly used methods for conventional substances are radiolabelled substances. For MNM, it needs to be demonstrated that for absorbed/penetrated materials, the label is attached to the test material in particulate form, not in dissolved form.
	Maximum relevance score	**13**	

Based on the scoring of the table above, the distinction between studies of no, limited or high relevance are determined as follows:

- Studies that do not score 1 on all red questions are considered not relevant.
- Studies that score 1 on all red questions and score 1 on 11 questions in total are considered of limited relevance.
- Studies that score 1 on all red questions and score 1 on more than 11 questions in total are considered of high relevance.

Note that again, the values of the scores are arbitrary and only serve to enable prioritization of the studies according to their relevance.

6. Results

6.1 Results of searches and references scored for quality

A summary of the results of the searches performed with the search strings and keywords as indicated earlier is given in Table 10.

Table 10: Results of literature searches

	Number of references obtained by using the search strategy		
	(Ultrafine OR nano-object OR nanoparticle OR nanoparticulate OR nanomaterial OR nanotube OR nanofiber OR nanofibre OR nanowire OR nanocomposite OR nanoplate OR nanorod OR fullerene OR "quantum dot") AND (dermal OR skin) AND (absorption OR penetration OR uptake) AND (Keyword)		
	Keyword	**Pubmed**	**Toxline (excluding PUBMED records)**
1	assay	318	14
2	bioavailability	110	0
3	biodegradability	7	0
4	biodistribution	31	0
5	characterisation	12	0
6	characterization	379	0
7	consumer products	21	0
8	corrosion	3	0
9	cosmetics	138	2
10	dermal abrasion[a)]	0	0
11	dermis	97	2
12	disease	117	55
13	dissolution rate	8	0
14	distribution	214	0
15	epidemiology	6	0
16	epidermis	197	7
17	exposure	177	0
18	hair follicles	78	0
19	hazard assessment	4	0

20	hazard identification	4	0
21	human	716	0
22	*in silico*	9	0
23	*in vitro*	594	0
24	*in vivo*	395	0
25	irritation	78	6
26	modeling	17	0
27	modelling	5	51
28	models	397	5
29	occupation	2	0
30	occupational	31	0
31	permeation	420	0
32	personal care	12	0
33	physicochemical	147	26
34	physico-chemical	17	1
35	QSAR	0	0
36	sensitization	6	0
37	sensitization	0	0
38	size	580	0
39	solubility	146	0
40	sun block	1	4
41	sun lotion	1	0
42	sunscreen	83	0
43	surface chemistry	306	0
44	susceptibility	2	0
45	systemic	128	17
46	systemic absorption	633	5
47	test guidelines	1	0
48	test methods	74	10

49	testing strategy	8	12
50	toxicity assessment	34	14
51	toxicology assessment	13	0
52	translocation	25	0
53	worker	1	0
54	workplace	5	0
55	published before 2013	15	0
	Total references	**6823**	**173**
	Total references excluding duplicates from all databases	**1377**	
	Manual exclusion of titles and abstracts	**951**	
	Review articles included	**66**	
	Final total number of literature citations for further analysis	**360**	
	Further exclusion after more in-depth check of content [b)]	**218**	
	Exclusion of non-accessible papers[c)]	**19**	
	Studies checked for quality	**123**	
	Relevant studies	**37**	

[a)] searching for 'dermal abrasion' in the keywords also resulted in zero included references. However, a combined search of 'abra' AND 'damag' resulted in 7 included references. ECHA provided results of a search using Google. Four of these were reviews. One experimental study is included in this report, one experimental study was considered not to fit the inclusion criteria and the final experimental study was in a journal not accessible for the authors.
[b)] After checking the full content of papers, quite a substantial number were found to not fit the inclusion criteria after all.
[c)] The researchers have access to a large number of relevant journals, either via open access or via paid subscriptions to collections of journals. However, the researching institutes do not have paid subscriptions to all journals. Given the large number of papers that could be accessed directly, it was decided not to pursue access to the relatively small number of papers without direct access.

Of course, there was substantial overlap in references if single keywords were counted. After removing duplicates, 1377 references were left. These were screened on title and, if necessary, abstract to deselect references did not fit the inclusion criteria for this study (see 5.4). A large number of references was excluded: 951 on the basis of title and abstract, 218 because an in-depth look at the paper showed that they did not fit the inclusion criteria and 19 because they were published in journals not in the large selections for which the researcher

institutes have direct access (either open access or paid subscriptions). Furthermore, 66 review articles were found, which were only checked and used for comparing the findings with the findings from analysis of original studies in this report. A total of 123 references with original studies were included in the analysis of relevance criteria.

Some references contain more than one type of dermal absorption test, specifically in the case of *ex vivo* tests, where e.g. both human skin and porcine skin have been studied. These different tests in one reference are considered to be different 'studies' in this report. There are therefore more studies (150) than references (123) included in the check against quality criteria. Information on the number of 'studies' included in the database for further evaluation that used *ex vivo* 'studies' or used combination of models can be found in Table 11 and Table 12.

Table 11: Number of 'studies' that used different types of *ex vivo* 'studies'

Skin origin for *ex vivo* studies		Number of 'studies'
Human skin		52
Porcine skin		67
Rodent skin	Rat	2
	Mouse	3

Table 12: Number of 'studies' that used combination of 'studies'

Combination of 'studies'	Number of 'studies' included in the analysis
Rodent *in vivo* and *ex vivo* model	2
Human *ex vivo* and rodent models	7
Porcine *ex vivo* and rodent models	3
Human subjects and rodent *in vivo* model	1
Human and porcine *ex vivo* models	6
Human subjects and *ex vivo* human model	2
Human subjects and *ex vivo* porcine model	4
Human and pig *ex vivo* and rodent *in vivo*	1
Total	**26**

6.2 Overview of available studies of acceptable quality and relevance

6.2.1 S-scores

The S-scores are independent of the type of study, because they are only dependent on the information on the MNM tested. Therefore, the results of the application of the S-score criteria

is here presented for all the references that were included. In total, 132 references with 150 'studies' or separate datasets were included. The S-scores were checked for all the separate 'studies'. The results are indicated in Table 13.

Table 13: Results of applying the criteria for the S-score for all studies

	Human subjects	Human and porcine Ex vivo	Rodents In vivo	Total
Accepted	3	61	8	**72**
Supporting	4	22	1	**27**
Rejected	4	36	10	**50**
Total	**11**	**119**	**19**	**149**

6.2.2 Studies with human subjects: K-scores and relevance

Seven studies with human subjects were found that were of acceptable or supporting quality in relation to the S-score. Four of these were rejected based on the K-score (Table 14). They were missing essential information on the study details: frequency and duration of exposure, and timepoints of observation. Since this information really is needed to determine if, for example, exposure was long enough for absorption or penetration of MNM to occur, we decided not to adjust these criteria. As a consequence, only three human studies were not rejected. Of these, two studies were of acceptable quality (Esposito *et al.*, 2017; Vieira *et al.*, 2019). The third study is considered a supporting study, due to the lack of information on primary size (Erdal *et al.*, 2016).

Table 14: Results of applying the criteria for the S- and K-score for all human studies

	Accepted K-score	Rejected K-score	Total
Acceptable S-score	2	1	**3**
Supporting S-score	1	3	**4**
Total	**3**	**4**	**7**

The relevance criteria for studies with human subjects, as defined earlier (see 5.5.3) consist of a decision tree with questions on the experimental set up of the study which eventually leads to the conclusion on whether a study is relevant or not, or of limited relevance. In addition, for (limited) relevant studies, further distinction is made on whether the study is relevant for intact skin only or also for damaged skin.

The three studies of sufficient quality investigating MNM applied to the skin of human subjects were checked for their relevance to study skin absorption or penetration. Because of the small number of sufficient quality studies with human subjects, and because studies with human subjects are closest to real exposure situation, a brief description of these few studies is given below.

In the study by **Vieira *et al.*** (2019), human subjects were exposed to mesoporous silica (SiO), zinc oxide (ZnO) or a composite of SiO and ZnO MNM (~12-140 nm) for one to four hours, followed by immediate detection of the particles in various layers of the viable epidermis using Fluorescence Lifetime Imaging Microscopy. The MNM were not observed beyond the intact or barrier impaired stratum corneum. However, the timepoints of observation (up to 4h post topical application) are too short to conclude on potential

absorption or penetration in the long run.

In the study by **Esposito *et al.*** (2017) human subjects were exposed to progesterone lipid nanoparticles for six hours, after which the stratum corneum was investigated for the presence of progesterone at various timepoints up to six hours after exposure. Based on the progesterone depletion from the stratum corneum, the authors conclude that the progesterone lipid nanoparticles penetrate into the stratum corneum and deeper skin layers. From this, it can be concluded that the stratum corneum was fully penetrated. The study did not include an investigation of the presence of the MNM or of progesterone in the viable epidermis or beyond.

In the supporting study by **Erdal *et al.*** (2016), human subjects were exposed to microemulsions containing the drug naftifine for one hour, followed directly by tape stripping to determine the content of naftifine in various layers of the stratum corneum. The authors measured skin barrier function and found that after 20 times tape stripping the barrier function was almost lost, suggesting the authors collected most or all of the stratum corneum. They show naftifine concentrations per 4 subsequent tape strips (so, 5 groups). A gradual decrease is seen, with naftifine still present in the last 4 tape strips. So, at least part of the naftifine fully penetrated the stratum corneum. Similar to the study by Esposito *et al.* (2017), the study did not include an investigation of the presence of the microemulsion or of naftifine in the viable epidermis or beyond.

Using the defined relevance criteria, the three studies with human subjects give very little information on the ability of MNM to absorb or penetrate human skin, either because the timepoints of observation were too short or because the skin layers beyond the upper stratum corneum were not investigated in the study.

6.2.3 Studies with *ex vivo* human or porcine skin: K-score and relevance

The K-score for *ex vivo* skin studies, as defined in section 0, is created from a list of ten questions, eight of which are considered critical, after changing the consideration on two questions for which hardly any reference provides information.

Application of the final criteria to the studies resulted in the following K-score quality scoring as indicated in Table 15.

Table 15: Results of applying the criteria for the K-score for *ex vivo* studies

	Accepted K-score	Rejected K-score	Total
Acceptable S-score	27	34	**61**
Supporting S-score	9	13	**22**
Total	**36**	**47**	**83**

The relevance criteria, as defined in 5.5.3.2, for *ex vivo* studies using human or porcine skin consist of fifteen questions, seven of which were considered critical, after reconsidering to avoid too many rejections. All questions are to be answered with yes or no. For a study to be considered relevant, at least all critical questions need to be answered with yes. The more questions that are answered with yes, the more relevant.

Applying these final relevance criteria gives the outcome of studies with regard to their acceptable or supporting quality and their relevance as provided in **Error! Reference source not found.**.

Table 16. Results of applying the relevance criteria for ex vivo studies

	Relevant	Not relevant	Total
Studies of acceptable quality	12	16	**28**
Supporting studies	3	6	**9**
Total	**15**	**22**	**37**

Because of the relatively large number of studies of sufficient quality that were not sufficiently relevant, these will not be discussed at all. The ones with sufficient relevance are discussed in 6.3.2.

6.2.4 Rodent studies

Based on the S-score, eight rodent studies were of acceptable quality and three of supporting quality.

These 11 studies in total were scored for the K-score, leading to the results in

Table 17.

Table 17: Results of applying the criteria for the K-score to the rodent studies

	Accepted K-score	Rejected K-score	Total
Acceptable S-score	1	7	**8**
Supporting S-score	0	1	**1**
Total	**1**	**8**	**9**

One rodent study passed the quality criteria and was checked against the relevance criteria for rodent studies. The number of studies of sufficient quality is quite low for different reasons including the information on housing. Only 1 study gave information on individual housing of the animals and 8 out of 9 did not give any information on housing whatsoever. None of the studies was reported to be performed according to OECD TG 427, which requires individual housing in metabolism cages. The only sufficient quality rodent study did not pass the relevance criteria.

6.3 Overview of findings in acceptable and relevant studies

6.3.1 Human studies

None of the (three) human studies with sufficient quality was considered to be sufficiently relevant. A brief discussion of the three studies is presented in section 6.2.2.

6.3.2 *Ex vivo* studies with human or porcine skin

Cardoso *et al.* (2019) investigated the percutaneous skin penetration of phenytoin-loaded polymeric nano-capsules and nano-emulsions to determine if the drug would become systemically available in this formulation when used as a cutaneous healing agent. Intact and

damaged (by tape stripping) skin preparations from porcine ear were histologically analysed for their integrity. The skin preparations were placed in a Franz diffusion cell and exposed to 300 µL aqueous dispersion of nanocarriers (125-161 nm) or 300 µg of an emulsion containing nanocarriers, both containing 75 µg of phenytoin, for 2, 4, 6, 8 and 12 h. The actual concentration of nanocarriers themselves in the dispersion and in the emulsion was not determined. No phenytoin was detected in the receptor fluid of experiments with intact skin up to 12 h after topical application. Phenytoin was retained in the stratum corneum of the skin. On the other hand, approximately 7.5 and 6 µg·cm^2 of phenytoin in nano-emulsions and nano capsules respectively penetrated to the dermis and similar amounts to the receptor medium.

Lewinski et al. (2017) investigated the effectiveness of hand washing on the removal of two types of iron oxide MNM (~30 nm). Human abdominal skin samples were obtained from surgical waste (both fresh and frozen) and their integrity was verified by trans-epidermal water loss measurements. The experiment followed OECD TG 428. Skins were placed in a diffusion cell and exposed to 1 mg of dry nanoparticle dust powder for 1 or 20 h. Subsequently, skin samples were cleaned using soap and rinsed with MilliQ water. Presence of iron oxide in skin samples, receptor fluids and wash fluids were determined by UV/Vis spectrophotometry, inductively coupled plasma-optical emission spectroscopy and/or magnetic susceptibility. The concentration of iron oxide MNM in the receptor fluid was below the detection limit. After washing, approximately 80-95% of the applied particles was found to be removed from the skin by washing. It is reported that less than 15% of the dose was "measured on the skin after two wash steps with water and soap". It was also reported that iron oxide nanoparticles were observed in the tape strips. However, this was studied only qualitatively. The authors focus on the washing efficacy and do not provide further conclusions on dermal penetration.

Mauro *et al.* (2015) studied the skin penetration of cobalt oxide (CO_3O_4) MNM (~17 nm) in both intact and damaged (needle abraded) abdominal human skin obtained as surgical waste. Skin integrity was tested using electrical conductibility and experiments were performed using Franz diffusion cells. The intact or abraded skin was exposed to 1 mg/mL MNM in synthetic sweat up to 24 h. The amount of cobalt oxide in the receptor fluid and in skin sections was determined using inductively coupled plasma mass spectrometry. Transmission Electron Microscope images revealed significant aggregation of the cobalt particles of >800 nm in the synthetic sweat. Ion dissolution of the cobalt particles was very low (0.1%). Permeation of cobalt through the skin (through epidermis and dermis) was only observed in the damaged skin samples. The amount of cobalt permeated through skin (*i.e.* found in the receptor fluid) in 24 h was 57 ± 38 ng·cm^2 with a flux of 2.1 ± 2.0 ng·cm^2·per hour and a lag time of 4.3 ± 2.1 h. In the intact skin samples, most of the cobalt was found in the epidermis (approximately 15 ng·cm^2), although approximately 1 ng·cm^2 was also found in the dermis. In damaged skin, approximately 12 ng·cm^2 was retained in the epidermis and dermis.

A second, similar study from this research group investigated the skin penetration of aluminium oxide (Al_2O_3) MNM (~ 53 nm) in both intact and damaged (needle abraded) abdominal human skin obtained as surgical waste (**Mauro *et al.***, 2019). Skin integrity was tested using electrical conductibility and experiments were performed using Franz diffusion cells. The intact or abraded skin was exposed to 20 mg/mL MNM in synthetic sweat up to 24 h. Transmission Electron Microscope images revealed significant aggregation to aggregates in the micrometre range of the aluminium oxide particles in synthetic sweat. Ion dissolution of the particles was very low (0.1%). Only traces of aluminium were found in receptor fluids of intact and damaged skin and were not significantly different from those in non-exposed cells. The percentage of the applied dose that permeated through all skin layers and was measured in the receptor fluid was 0.0011% for intact skin and 0.0028% for damaged skin. For both intact and damaged skin, approximately 2 µg·cm^2 remained in the dermis and 2 µg·cm^2 in the epidermis. This amounts to 0.12-0.13% of the applied dose retained in the skin (dermis plus epidermis).

A third study from this research group investigated the skin penetration of (predominantly

anatase) titanium dioxide (TiO_2) MNM (~38 nm) in both intact and damaged (needle abraded) abdominal human skin obtained as surgical waste (**Crosera *et al.***, 2015). Skin integrity was tested using electrical conductibility and experiments were performed using Franz diffusion cells. The intact or abraded skin was exposed to 1 mg/mL MNM in synthetic sweat up to 24 h. Transmission Electron Microscope images revealed significant aggregation of the titanium dioxide particles in the synthetic sweat, to aggregates of ~1254 nm after 24 h. There was no detectable ion dissolution. The amount of Ti in the receptor fluids was below the detection limit for both intact and damaged skin. Traces of Ti could only be detected in the epidermal layer (0.47 µg/cm^2), not in the dermal layer.

A study by **Bianco *et al.*** (2015) followed a similar experimental setup as the previous studies, investigating the skin penetration of silver MNM (Crosera *et al.*, 2015; Mauro *et al.*, 2019; Mauro *et al.*, 2015), but using intact skin only. The silver MNM were derived from soaking three different textiles in a synthetic sweat solution in the donor fluid of the Franz diffusion cell for 24h. The resulting aggregates consisted of silver-silver chloride, indicating that the silver was released from the textiles mostly in ionic form. The aggregates in the donor solution had sizes ranging from 16 to 700 nm and reached concentrations ranging from 0.7 to 4.7 µg/mL. The silver concentration in the receptor fluid sampled up to 12 h was below the detection limit. However, after 24 h, silver levels of 0.13-0.19 µg/L were detected in the receptor fluid and higher levels of silver were found in the epidermal and the dermal layers of the skin, indicating the potential of silver to penetrate the skin. Soaking the three textiles resulted in silver concentrations in the donor fluids of 4.0, 0.6, and 1.8 µg/cm^2, respectively. In the epidermis the concentrations were 1.05 (26%), 0.26 (43%), and 0.33 (18%) µg/cm^2, respectively, and in the dermis 0.30 (8%), 0.03 (5%), and 0.07 (4%) µg/ml, respectively. In the epidermal and dermal layers, silver was present in large silver chloride aggregates which probably formed in these deeper layers in the skin since they were too large to pass the stratum corneum. There were large differences in the presence of silver between the skin samples of the two donors, which was speculated by the authors to be due to the higher abundance of hair in one of the donor skins.

Silver percutaneous absorption after exposure to polyvinylpyrrolidone coated silver MNM (~19 nm) was compared for three human skin graft samples: fresh, glycosylated and cryopreserved skin (**Bianco *et al.***, 2014). The study used the same experimental setup as the previous studies, i.e. skin integrity was checked with electrical conductivity, exposure (113 µg/cm2 in synthetic sweat) took place up to 24 h in a Franz diffusion cell with sampling at selected intervals. Subsequently, silver content was determined in the receptor fluid, and in epidermal and dermal layers of the skin. The silver particles aggregated significantly in the artificial sweat, but silver content was detected in the receptor fluid. After 24 h, the silver penetration was 0.2 ng/cm^2,h for fresh skin, 0.3 ng/cm^2,h for cryopreserved skin, and 3.8 ng/cm^2,h for glycerolized skin. This suggested that the use of cryopreserved, but not glycerolized, human skin for *in vitro* experiments could be a good model for skin permeation evaluation, because the results of cryopreserved skin were not significantly different from that of fresh skin, but the glycerolized skin has morphological and structural analogies with necrotic skin. There were no differences in silver content between fresh and cryopreserved skin, indicating that silver permeation through the skin could be achieved through passive diffusion rather than active uptake which is much lower in cryopreserved skin. Silver content in the dermis layer of glycerolized skin samples was significantly higher.

Another study demonstrating the importance of skin condition on the penetration of MNM was presented by **Lalloz *et al.*** (2019). For this study, porcine flank skin was used, and skin integrity was confirmed using the trans-epidermal water loss method. Intact skin and damaged skin (tape stripped to remove the stratum corneum) were placed in Franz diffusion cells and exposed to ~100 nm cholecalciferol loaded polymeric MNM (approximately 35 µg/cm^2) with increasing polyethylene glycol (PEG) coating for 24 h. The skin penetration of cholecalciferol to the receptor fluid in intact skin was below the detection limit. The drug was deposited mostly in the stratum corneum (132 ng/cm^2), to a lesser extent in the viable epidermis (80 ng/cm^2), and very low amounts were absorbed by the dermis (25 ng/nm^2). In case of damaged skin,

these concentrations were 567 and 258 ng/cm^2 for viable epidermis and dermis, respectively. Skin absorption was slightly higher for the more hydrophilic nanocarriers with a high PEG content, but differences were small. In damaged skin, penetration to the receptor fluid was still very low, but detectable. In addition, skin absorption was higher than for intact skin. A strong dependence on PEG content was observed in damaged skin. In contrast to intact skin, absorption of the drug was highest for hydrophobic, non-PEGylated nanocarriers. This was due to increased aggregation and destabilization of the non-PEGylated particles upon exposure to ions released from the damaged skin, resulting in adhesion of the destabilized particles to the skin and increased deposition of the drug compared to the more stable PEGylated particles.

Kumar *et al.* (2016) studied the skin permeation of four different formulations of nano-emulsions (size range 118-170 nm) containing the anti-inflammatory drug thiocolchicoside for the purpose of increasing its bioavailability through transdermal delivery. Epidermis retrieved from porcine abdominal skin was exposed to the 1 mL nano-emulsions for 24 h using the Franz diffusion cell method, and the receptor fluid was sampled at various time intervals. Epidermal permeation of thiocolchicoside was observed for all formulations and was higher than for an aqueous solution of thiocolchicoside. For the optimized nano-emulsion, the steady-state flux and permeability coefficient were 31 µg/cm^2/h and 15 x 10-3 cm^2/h, respectively, while for the unformulated control they were 6 µg/cm^2/h and 3 x 10-3 cm^2/h, respectively. The permeation increased with decreasing size of emulsion droplets, indicating that size may play a role, although other components of the emulsion such as transcutol P also act as permeation enhancers.

Jatana *et al.* (2016) investigated the effect of five different commercial cosmetic formulations, glycerol and water on nanoparticle (quantum dot (QD)) permeation in mouse and human skin (derived from mammoplasty and processed) using two different methods. In the first method, the skin is placed in a petri dish on a gauze sponge, exposed for 24 h to the formulation and photographed with a UV lamp immediately after and 24 h after the application. The second method used a Franz diffusion cell as described previously with a 24 h exposure duration and was performed with two of the commercial formulations and glycerol only. There were large differences in skin penetration between the different formulations; Eucerin smoothing lotion and glycerol enhanced penetration of QD into the skin, compared to the other formulations. Various formulations showed significant presence of QDs in the viable epidermis. When testing human skin within 2 hrs from processing, the curves relating intensity of QD signal to depth of stratum corneum showed a similar form in both human and mouse skin, *i.e.* a peak around 5 to 20 µm and a strong decrease up to 40 µm. In both models a formulation with Eucerin smoothing lotion clearly had the highest peak, but the order of other formulations was different. Unexpectedly, the absolute intensity of the signal after 24 hours exposure was around 4 times higher in human skin, than in mouse skin. A reduced barrier function of the human skin, due to processing, may be a partial explanation of this difference. This was supported via an additional experiment by the fact that for most (but not all) formulations there was a much higher QD signal intensity in human skin tested immediately after processing, compared to human skin tested after a 24-hour resting period.

In the study of **Peira *et al.*** (2014), the impact of nano-TiO_2 surface properties on drug permeation was investigated in an ex vivo pig skin model under indoor light. To this end, amphotericin (a model drug that normally does not penetrate through the skin) was applied on the skin in two different media in the presence of three differently coated TiO_2 samples, after which the drug flux was measured. The three nano-TiO_2 samples were made of mixed phases of anatase and rutile, at a 9:1 ratio, either with no coating, a silane coating or a silane and silica coating. Suspensions containing nano-TiO_2 were put in contact with full-thickness pig ear skin using vertical Franz-type diffusion cells. After 24 h, the skin surface was washed 5 times with ethanol and normal saline solution. After that, the skins were collected to perform Differential scanning calorimetry
or micro-XRF and Raman studies. The naked, but not the coated nano-TiO_2 showed enhanced

properties, with a fourfold increase of the drug flux. Only the positively charged, naked TiO_2 strongly adhered to the stratum corneum and altered its structure. To enhance the drug permeation, both a surface charge-driven adhesion and an oxidative disorganisation of the stratum corneum lipids are required. The data were obtained under controlled light illumination with a very low UV content such that the strong photochemical activity of TiO_2 is not activated. Full sunlight could further enhance the skin permeability.

Sallam and Marin Bosca (2017) studied the incorporation of the anti-acne drug adapalene (AD) into poly-ε -caprolactone nanospheres (NS) in order to develop a biocompatible, non-oily nanomedicine for follicular delivery of AD. This is important for a convenient localized topical treatment of acne by ameliorating the irritation potential of the drug. The AD-NS were embedded in either hydroxypropyl methylcellulose (HPMC) or hyaluronate (HA) gel and subsequently the *ex vivo* human skin dermato-kinetics of AD from each system was studied. The amount applied was not specifically reported. Full-thickness human skin was cut into pieces suitable to be placed on the Franz-type diffusion cell. Experiment duration was 24 h. The MNM dispersion showed, according to the authors, significantly higher AD retention in the epidermis and dermis than the AD suspension alone. However, the values were not specifically reported. Furthermore, the NS-HPMC decreased the retainment of AD in all cell layers, whereas NS-HA increased this retainment. The HPMC gel restricted the presence of NS to the stratum corneum and epidermis, whereas the HA gel enhanced the penetration of NS to all the skin layers. The authors do not provide an explanation for these differences.

The aim of a study of **Clares *et al.*** (2014) was to develop biocompatible lipid-based nanocarriers for retinyl palmitate (RP) to improve its skin delivery, photostability and biocompatibility, and to avoid undesirable topical side effects (that were not specified by the authors). RP loaded nano-emulsions (NEs), liposomes (LPs) and solid lipid nanoparticles (SLNs) were characterized. The permeation study was performed on vertical amber glass Franz-type diffusion cells. At the end of the permeation study, the amounts of RP remaining on the skin were quantified. The cumulative amount of drug permeated through human skin after 38 h was highest for NE, then LP and SLNs, the values being respectively 6.67 - 1.58 mg, 4.36 - 0.21 mg and 3.64- 0.28 mg for NEs, LPs and SLNs, respectively. NEs flux (mass per time through the skin sample) was significantly higher than SLNs and LPs: NEs (0.37 - 0.12 mg/h) > LPs (0.15 - 0.09 mg/h) > SLNs (0.10 - 0.05 mg/h). LPs offered significant higher skin retention than NEs and SLNs. Finally, even though all developed nanocarriers were found to be biocompatible, NE was the system that, according to the authors, most disrupted the skin and therefore effected the dermal penetration. The study shows that penetration and retention characteristics of drugs (such as retinyl palmitate), as well as stratum corneum properties can be modified by the selection of appropriate nanocarriers. In this line, the cosmetic or dermatologic action of the drug on the skin is also influenced since the ingredient or drug expulsion from SLNs becomes more likely in superficial skin layers, while LPs or NEs improve the permeation to deeper skin layers.

The study of **Pepe *et al.*** (2016) focused on the ability of nanocarriers containing protein transduction domains (PTDs) of various classes to improve cutaneous paclitaxel delivery and efficacy in skin tumor models. Microemulsions (MEs) (concentrations not specified) were prepared and added to the different PTDs transportan (T), penetratin (P), or HIV-1 transactivator of transcription (TAT), resulting in ME-T, ME-P, or ME-TAT. Skin penetration of paclitaxel from the MEs was assessed for 1–12 hours using porcine skin and Franz diffusion cells. Among the PTD-containing formulations, paclitaxel skin (stratum corneum, epidermis and dermis) penetration into stratum corneum at 12 hours was highest with ME-T, and lowest with ME-TAT (1.6-fold less). According to a graphical presentation of the results, the values were in the order of 15 to 40 $\mu g/cm^2$. This is consistent with the stronger ability of ME-T to increase trans-epidermal water loss (2.4-fold compared to water) and tissue permeability. Paclitaxel-containing ME-T reduced cell and tissue viability by two-fold compared to drug solutions, suggesting the potential clinical usefulness of the formulation for the treatment of cutaneous tumors.

Venuganti *et al.* (2015) investigated the feasibility of using the dendrimer poly(amidoamine) (PAMAM) as a carrier for topical iontophoretic delivery of an anti-sense oligonucleotide (ASO). Iontophoretic delivery employs a small electrical current to transport charged molecules through the skin. The donor chamber of a vertical Franz diffusion cell was loaded with 0.2 ml of 15 µM free ASO or ASO/PAMAM complex. Confocal laser scanning microscopy showed that in case of iontophoretical delivery of ASO/PAMAM complex, 50% could reach the viable epidermis (VE) in porcine skin, whereas only 6% of passively delivered free ASO or 10% of passively delivered ASO/PAMAM complex reached the VE and were therefore mainly localized in the stratum corneum. Only 10% of iontophoretically delivered of free ASO reached the VE and so, the cell uptake of ASO was significantly enhanced by the PAMAM complex.

6.3.3 Rodent studies

One rodent study by Guo *et al.* (2015) was found to be of sufficient quality. However, in this study only one rat was used, and the amount of skin exposed was very small (1.77cm^2 rather than the standard 10 cm^2). Therefore, this study did not pass the relevance criteria and was rejected for further analysis.

Studies comparing absorption or penetration in rodent skin and human or porcine skin

Some studies had information that compared results in rodent studies with result in human skin (or porcine skin) studies. These are discussed here.

Five publications were found that studied skin absorption/penetration in both human or pig skin and in rodent skin. None of these studies passed both quality and relevance criteria as set out previously in this report. Nevertheless, we analysed these studies to determine whether a trend could be observed with regard to the magnitude of skin absorption/penetration of MNM, i.e. whether rodent skin is always more permeable than human or porcine skin, or vice versa. The studies are summarized below.

A study by **Fernandes *et al.*** (2015) systematically compared the skin penetration of gold MNM with different shape and surface properties in mouse and human skin samples. It should be noted that the exposure durations for the different skins were different (6h and 24h for mouse and human skin, respectively) because the mouse skin's integrity was found to be compromised faster than that of human skin. Exact exposure concentrations were not given in the paper, apart from them being 'high'. For nanorods, and for nanospheres functionalized with skin or cell penetrating peptides, the skin penetration was higher in mouse skin compared to human skin. The highest skin penetration was observed in mouse skin for positively charged gold nanorods, amounting up to 10% of the initial concentration. The highest skin penetration in human skin was found for the same material and amounted to approximately 3% of the initial concentration. For positively charged nanospheres, the skin penetration was lower in mice compared to human skin, with levels of skin penetration for human skin of approximately 1% of the initial concentration applied.

Jatana *et al.* (2016) studied the penetration of quantum dots (QD, 0.01 mg QDs per cm^2 skin in 0.05g vehicle) in human and mouse skin using a number of methods. With QD exposure after less than 2 hrs resting of the processed human skin, the vehicle-dependent penetration trends in skin, *i.e.* the curves relating QD signal to depth from the stratum corneum, were similar between mice and human skin. The QD signals in human skin were however, as seen in graphs, approximately two to six times higher than in mouse skin. According to the researchers, this difference was in part due to anatomical differences between human and mouse skin, e.g. epidermal thickness. However, they were also in part due to the processing of the skin, which is generally only done for human skin. Significantly higher QD penetration was observed in human skin that was used within 2 hours after processing to skin that was allowed to rest for 24h after processing for most of the vehicles, but not for glycerol. This also resulted

in a different order of vehicle-dependent skin penetration of human skins rested for 24 hours compared to human skins rested for less than two hours and mouse skins.

In a study by **Sintov *et al.*** (2017) permeation of 0.5 g of L-DOPA in silica hydrogel through rat (200-250 µg/cm^2)and mouse (50-100 µg/cm^2) skin was found to be lower than the permeation through the frozen-thawed porcine skin (approx. 400 µg/cm^2) , and higher compared to fresh porcine skin (approximately 50 µg/cm^2), after 24h of exposure. The authors note that frozen-thawed porcine skin has been shown by various groups to be more permeable than fresh excised porcine skin. In rat and mouse skin, the permeation of the drug was lower when administered in a hydrogel formulation of silica MNM compared to a liquid formulation. In porcine skin (fresh and frozen-thawed), permeation of the drug was similar between the liquid and hydrogel formulations.

In the study by **Shiva *et al.*** (2012) the differences between human and mouse skin permeation could not be compared. Permeation of solid lipid nanoparticles of isotretinoin in the mouse skin was studied in a Franz diffusion cell, while permeation in the human skin was studied by tape stripping. Similarly, in the study by **Wiraja *et al.*** (2019), skin permeation of framework nucleic acids was studied in mice *in vivo* and in humans *ex vivo*, and does not allow for comparison of the magnitudes of penetration between the two models.

Conclusion on studies comparing rodent skin and human or porcine skin
After further analysis, only three of the five studies allowed for a more or less direct comparison of rodent models with human or skin models, but even in these studies, experimental set ups were not fully similar. Based on this limited data, no conclusions can be drawn about whether either one or the other model always presents a worst-case situation. Apart from the skin model used, the magnitude of skin permeation appears to depend on particle properties (positively vs negatively charged), exposure time and skin processing and integrity.

6.4 Database with information from acceptable studies

An MS Access database was set up to capture the relevant information on the literature selected for further evaluation. Every reference assessed for quality or quality and relevance is included in the Access database. The set-up of the final version of the database is described below. The initial literature screening started from the Danish report, that also contained a database (Poland *et al.*, 2013). This database was used as an example to build our own database. The database itself is based on an Excel file into which all the relevant information has been entered previously. The Excel file was imported into MS Access, modified in order to make it more transparent and saved as an Access database.

The final version contains experimental studies only; the review articles are not included. The main database body consists of the following fields:

- General information on the test material:
  - Chemical composition of the test material
  - Source of the test material
  - Purity of the test material
  - Protocols of dispersion and characterization in the exposure medium
  - Primary particle size
  - Surface area of the primary particles
  - Shape of the primary particles
  - Other primary particle characteristics
  - Size (distribution) in exposure medium
  - Dissolution rate in exposure medium
  - Surface charge in exposure medium

Furthermore, the fields corresponding to the quality criteria were added as a separate form, linked to the main database body. They were divided into the following forms:

- Information on human subjects:
  - Was written informed consent obtained from the test subjects?
  - Number of the test subjects
  - Description of inclusion and exclusion criteria for test subjects
  - Age of the test subjects
  - Sex of the test subjects
  - The condition of the skin of the test subjects
  - Frequency and duration of exposure as well as time-points of observations
  - Details of the exposure method
  - Have the measurement/observation methods been described
- Information on human or porcine skin *ex vivo*:
  - Species source of the skin
  - The source location of the skin
  - Skin properties, conditions of cultivation and maintenance
  - Details of the method of exposure
  - Duration of exposure and time-points of observations
  - Number of skin samples and donors
  - The method of detection of absorption/penetration of test materials
- Information on animal experiments:
  - Species and strain of animals
  - The number of animals per treatment group given
  - The type of cage used
  - The details on housing conditions
  - Details on diet
  - Location of the skin exposure
  - Skin condition throughout the study
  - Details on the method of exposure
  - Duration of exposure
  - Timepoints
  - Number of samples for measurement
  - Method(s) of detection of absorption/penetration of test materials

In case of studies that fulfilled the quality criteria, the following fields corresponding to studies' relevance were included. These are contained in a separate form linked to the main database body:

- Human or porcine skin *ex vivo*:
  - Study follows OECD TG 428
  - Standardized skin preparation of known source and location is used
  - 8 skin samples of 4 donors are used, 2 replicates per donor
  - Human skin studies use epidermal membranes (enzymically, heat or chemically separated) or split thickness skin (200-500 µm)
  - Skin integrity is warranted
  - The temperature of the skin and the receptor fluid is controlled
  - Appropriate exposure medium and receptor fluid are used
  - Exposure is done using a diffusion cell
  - The minimum skin area covered is 0.64 cm^2
  - Test material does not dissolve in exposure medium in <24h
  - Minimum exposure concentration is 0.1 mg/cm^2, maximum does not exceed stable dispersion concentration
  - Exposure duration is at least 30 minutes
  - Sampling of receptor fluid takes place at multiple timepoints and up to 24 h
  - Mass balance is provided, test material is accounted for 85-115%
- Animal studies:

  - Study follows OECD TG 427
  - Study is performed in young adult rodents of a single sex of commonly used laboratory strains
  - Number of animals per group (one sex) is at least four
  - Animals are individually housed in metabolism cages
  - Housing conditions are properly controlled.
  - The feeding conditions are properly controlled.
  - Test material in exposure vehicle is in particulate form throughout exposure duration
  - Test material application site is protected from grooming
  - Skin area application site is at least 10 cm^2
  - Exposure duration is at least six hours
  - Minimum exposure concentration is 1 mg/cm^2, maximum does not exceed stable dispersion concentration in exposure vehicle
  - At least three time points of measurement from directly after exposure up to 72h after exposure are included
  - Method of detection allows for completion of mass balance and distinction between particulate and dissolved material
- Human studies *in vivo*:
  - Exposure duration
  - Test material observed beyond epidermis
  - Test material observed in particulate form
  - Dissolution rate
  - Source of the observed material is definitely test material
  - Skin damaged or flexed
  - Time point measurement > 24 h after exposure
  - Aggregation in exposure or dispersion medium
  - Applied under conclusion
  - Exposure scenario (i.e. use of UV or other techniques used before exposure)

Some of the MNM characteristics appear, next to in the fields of characterisation of the tested material, also in the fields for human studies *in vivo*, because the results influence the flow in the assessment of relevance of human studies *in vivo*.

Figures showing the structure of the database along with the description of use can be found in Appendix 1.

7. Discussion and conclusions

7.1 Evaluation of the information gathering and results

7.1.1 Search strategy

The information gathering was performed via searches in Pubmed and Toxline (from Toxnet). Original publications and reviews were searched. The searches with the search strings and the large number of keywords resulted in a very large number of potentially interesting publications. By far the largest number of these publications was (also) found in Pubmed. In the meantime, Toxline and other Toxnet information is no longer separately hosted. Toxline has become part of Pubmed. Therefore, in future searches, only Pubmed needs to be searched. Of course, it is possible that some publications exist in journals not covered by Pubmed. However, given the very large number of potentially interesting publications from 2013 onward in Pubmed alone (almost 1380), it was decided not to perform any additional searches outside Pubmed, because it was considered that these would not result in substantial new information.

A large number of keywords was used in the searches. Of course, there was very substantial overlap between the results of the different searches, because publications often were tagged with two or more of the keywords. It is remarkable, that there were no publications found with the search string using the term 'dermal abrasion' as keyword. Because this was unexpected, some additional searches for similar terms (e.g. 'dermal abr*') were done, but these again did not result in hits. However, this does not imply that no studies with damaged skin were found. Several of these studies were found, but apparently the keyword 'dermal abrasion' was not tagged to these publications. The publications were already found via other keywords and via the partial words 'abra*' and 'damag*'. It is also not to be expected that any relevant article on dermal penetration or absorption will have only keywords 'abra*' or 'damag*' and not one of the other keywords in our search strategy.

Some other keywords also resulted in no or hardly any hits. For example, 'sun lotion' resulted in no hits. However, 'sunscreen' resulted in 83 hits, so apparently that is the more usual keyword for this product in these kinds of publications.

Not surprisingly, the keywords '*in silico*' and 'QSAR' resulted in very limited number of hits. The knowledge on factors influencing dermal absorption of MNM is so limited, that it cannot be expected that *in silico* tools or QSARs have already been developed.

Thanks to the fact that we used a large number of, often rather comparable, keywords, the number of total hits was extensive. A much smaller number of keywords might have missed several interesting publications.

7.1.2 Search results

In the searches with various keywords a large number of potentially interesting references was found. The limited search for publications before 2013 that were not yet included in the Danish report revealed only 15 potentially interesting publications. This indicates that the Danish report was very complete in its searches.

A substantial number of review articles was found (66). The large majority of these describes the developments of methods to enhance drug penetration using (amongst others) nano-emulsions or nanoparticles, generally without apparent attention to the dermal absorption of the MNM themselves. Quite a few also discuss potential mechanisms or pathways of nanoparticle dermal absorption with generally limited definitive conclusions. There are a few reviews that provide at least some insight in factors involved in nanoparticle dermal

absorption, together with some indicative support, or some indication of whether penetration occurs or not for specific nanoparticle types. Reviews with clear quantitative conclusions on dermal absorption of MNM were not found. This is not surprising, since the original papers described in the Danish report and in this study also did not find substantial numbers of clear quantitative conclusions on dermal absorption.

The majority of the individual original research papers found in our search, just like the majority of reviews, focus on transdermal delivery of medicines. In general, they attempt to measure the penetration of the medicines into skin layers or the absorption through the skin. The goal of these studies is not to derive conclusions on nanoparticle skin penetration or dermal absorption. Therefore, while these studies provide indications of the relevance of MNM in relation to dermal absorption, they do not provide extensive information on how and how much the actual MNM penetrate beyond the stratum corneum or are dermally absorbed.

The number of acceptable or supporting quality *in vivo* studies with humans found was very low. None of these was found to be relevant according to our criteria. This is caused largely by too short periods of observation of lack of study of layers beyond the stratum corneum. Of course, studies with human volunteers with longer duration and more intensive study of full skin are complex and costly and therefore cannot be expected to be performed very often.

The majority of acceptable or supporting quality studies found were *ex vivo* studies with human or porcine skin. These studies are relatively well standardised, partly because such studies are not uncommon to be performed for conventional substances using OECD guidelines. The OECD guidelines are however reported not to be directly applicable to dermal absorption studies of MNM. The fact that various laboratories are experienced in the OECD studies on dermal absorption and have methods and protocols available, may be one of the reasons why such studies are relatively common also for MNM. Of course, there is also the issue of ethical approval, which is needed for studies with human volunteers, but not with *ex vivo* studies. And the possibilities to determine penetration into various skin layers are more extensive in *ex vivo* studies too.

Several studies were also found on dermal absorption in rodents (*in vivo*). There are OECD guidelines for *in vivo* dermal absorption studies with rodents for conventional substances and several labs have experience and equipment to perform such studies. However, the OECD clearly indicates that the present guidelines are not readily applicable for MNM. Unfortunately, only one the rodent study that was found had an acceptable or supporting quality. Single housing is prescribed in OECD TG 427. However, the publications did not mention that this guideline was followed, so that did not provide the necessary information. The most important information missing was the lack of description of housing (single housing is critical to avoid oral absorption via licking). Without information on the type of cage used, the relevance of oral absorption cannot be assessed. However, not only information on housing was generally missing in the publications, but also some other critical quality criteria were not fulfilled.

The single rodent study that passed the quality criteria did go through to evaluation of relevance. However, this study did not pass the relevance criteria because it was performed with a single animal and on a very small area of skin.

A few studies were found that contained information on both human or porcine skin and rodent skin. Even though the rodent skin studies (and sometimes also the human or porcine skin studies) in these publications did not fulfil the quality criteria, these studies were used to see whether there were any qualitative indications on the differences between nanomaterial dermal penetration or absorption between human and porcine skin on the one hand and rodent skin on the other.

Three studies were found that used 3D reconstituted skin models (*in vitro*). However, when scoring quality criteria, it was found that one of them did not attempt to measure dermal absorption or penetration, but irritation. This study was not further scored. The other two

studies failed to pass the criteria for the S-score of the quality criteria, because either no information on chemical composition and/or no information on dispersion or dissolution in exposure medium was provided.

7.2 Evaluation of the quality and relevance criteria

Relevant conclusions from studies can only be drawn if the studies have a good quality and provide relevant results. Therefore, it was decided to use a set of quality criteria and relevance criteria to select studies with sufficient quality and relevance.

In general, it was found that the original criteria developed were too stringent to allow a reasonable number of publications to go forward to analysis. It was therefore assessed what specific aspects were most often insufficiently reported and whether considering these aspects as 'not critical' would completely disable drawing relevant conclusions. In some cases, it was decided that some criteria could be considered 'not critical', although this leads to limitations in the aspects that can be studied and the conclusions that can be drawn. This is further described in the following paragraphs.

7.2.1 Evaluation of the quality criteria

The quality criteria were based on a system originally developed by Fernández-Cruz *et al.* (2018). In this system, the quality is assessed based on two aspects: the quality of information on the MNM studied (S-score) and the quality of information on the actual study performance (K-score). The S-score is independent of the type of study, because it is only about the MNM studied. In fact, this or a similar score can also be used for other toxicological studies on MNM, because the factors influencing the dermal absorption are probably also relevant for e.g. inhalation absorption, toxicokinetics and actual effects. The K-score is dependent on the type of study. However, some general aspects are relevant in the K-score of each type of study. Very important or all studies are aspects related to duration of the exposure and of the observations after exposure. Dermal absorption can be slow and therefore there must be sufficient duration of exposure and observations. Of course, the skin condition is relevant in all cases as well. It is expected that damaged skin will have a different penetrability to MNM than intact skin and therefore the skin condition is critical. Because it was the aim of this study to study dermal absorption and penetration of MNM (and not of dissolved ions), it was relevant for all types of studies to have information on the actual MNM penetrated or absorbed. This was an issue that often led to a low-quality score, because of a lack of information.

The quality criteria were developed at the beginning of the project. For both S-score and for K-score, for all types of studies, a number of critical aspects were defined as well as a number of less critical aspects. After the screening of the available literature, it was found that a stringent adherence to the original quality criteria and to require information for all aspects considered critical, would lead to very low numbers of studies to be analysed. It was therefore decided to be less stringent for a number of aspects.

A lack of information on primary particle shape and on dissolution rate in exposure medium was no longer considered critical for the S-score. The effect thereof, of course, is that it is not possible to study differences in dermal penetration or dermal absorption in relation to shape. Also, because dissolution rates are unknown, it was also not possible to assess how much of any measured amount was in the form of particles or in the form of ions. The fact that only very few publications clearly reported both the primary particle size and the size distribution in the exposure medium leads to the issue that it is not clear from results whether they are relevant for primary particles or for aggregates/agglomerates. According to Bruinink *et al.* (2015), agglomeration greatly reduces exposure and also reduces translocation across barriers, including skin. Therefore, it would be very relevant to know the particle size as in the

exposure medium. Without such knowledge, it is possible that conclusions are drawn about lack of penetration and absorption, which is partly caused by agglomeration. Such conclusions may then not be fully valid for primary MNM.

Nevertheless, without some substantial lenience on a number of criteria, it would be almost impossible to provide any result and therefore, the above aspects were for pragmatic reasons not considered critical for further analysis of the information in this study.

For the K-score for human studies, information on frequency and duration of exposure and on timepoints of observation was missing in about half of the studies. It was decided that this information is too critical to be disregarded and therefore no modifications for the K-score for human studies were made. With the small number of human studies found in the first place, the result was a very small number of acceptable or supportive quality studies left for assessment of relevance.

With regard to *ex vivo* studies, it was decided to not reject studies if there was no information on the number of donors and skin samples used. Originally, this information was considered critical. However, by being strict on these criteria, the number of remaining studies would be too limited. The result of the decision to no longer require that information is mainly a substantial level of uncertainty on the results. Different skin samples from the same or different donors are (for conventional substances) known to sometimes have rather different dermal absorption. Therefore, the OECD TG 428 requires a minimum number of samples and the guideline on dermal absorption of the European Food Safety Authority (EFSA) (Buist *et al.*, 2017) has rules for accounting for the number of samples and the variability in the results between samples. Still, without information on number of samples, it is still possible to obtain at least indicative results on dermal penetration and dermal absorption and therefore these criteria were no longer considered critical.

When analysing the rodent studies against the original criteria for K-score, it was found that only two rodent study passed the quality criteria. It was therefore studied whether disregarding the question on skin condition and the question on type of cage would change this. It was found that all the rodent studies that did not pass the K-score, next to generally having no information on the housing, also had no information on at least one other aspect considered critical. Therefore, no changes in the criteria were made and it was decided that most of the rodent studies did not pass the quality criteria and were not eligible for further analysis.

Of course, the quality of a study can only be assessed based on the available information in the reports or publications of the study. Therefore, the assessment of the quality is partly an assessment of the quality and completeness of reporting. It is possible that some of the studies that were found to be of insufficient quality for our study in fact did note all the information for the quality criteria but did not report them in the publications. Also, the quality criteria set for this study are not necessarily very relevant for the original purpose of the studies found in literature. Therefore, the fact that many studies did not pass the quality criteria in this study does not imply that the studies themselves were of low quality.

7.2.2 Evaluation of the relevance criteria

For the human studies, it was found that the timepoints of observation were too short or the skin layers beyond the upper part of the stratum corneum were not investigated. These aspects were considered too critical to be lenient about them and therefore, the relevance criteria for human studies were not modified. None of the human studies was therefore considered sufficiently relevant.

The relevance criteria originally defined for *ex vivo* studies using human or porcine skin consisted of fifteen questions, nine of which were considered critical. All questions are to be answered with yes or no. For a study to be considered relevant, at least all critical questions

need to be answered with yes. The more questions that are answered with yes, the more relevant the study is.

Applying these relevance criteria to the studies that passed the quality check using the S-score and K-score, the far majority of the studies were rejected. Upon closer examination, the criteria that led to most rejections were the critical questions on dissolution in the exposure medium and on the mass balance. With regard to the dissolution in the exposure medium, the quality criteria already revealed that information on this is largely lacking in the studies. At least half of the studies that passed the quality criteria with an acceptable S-score and K-score did not contain information on whether particles dissolved in the exposure medium. Even fewer studies contained information on the mass balance of the materials recovered.

To obtain a complete, quantitative picture on absorption and penetration, studies ideally should include information on the fate of the full amount of MNM applied to the skin, i.e. the proportion present in the different layers of the skin, the proportion that was washed off and the proportion in the receptor fluid. In OECD TG 428 studies this is typically reflected in a mass balance, in which 85-115% of the test material needs to be accounted for. The consequence of the lack of mass balance data is that the information on absorption and penetration of MNM is mostly of a qualitative rather than a quantitative nature. The lack of dissolution data in exposure medium has consequences mostly for studies that find absorption or penetration of materials beyond the stratum corneum. Without supporting evidence from imaging techniques, it is not possible to conclude whether the materials were in particulate form.

Moving forward, the dissolution question and the mass balance question were no longer considered critical questions, keeping in mind the limitations as outlined above.

7.3 Discussion and conclusions on the findings of the analysed studies

7.3.1 Studies available

The studies available that passed our quality and relevance criteria were all *ex vivo* studies using human or porcine derived skin samples. The studies with human subjects and rodent studies were of insufficient quality and relevance to investigate skin penetration/absorption of MNM. Many of these studies were missing critical information on the characterization of the materials, or on the experimental design. A large number of *ex vivo* studies also did not pass the quality and relevance criteria. This does not mean that the studies are all of poor general quality. Many studies simply were designed for a different purpose than investigating the skin penetrating potential of MNM. For example, the purpose of a lot of studies was to investigate the ability of nanocarriers to improve the transdermal delivery of pharmaceutical ingredients. As a result, little information on the nanocarrier itself was described in the study, resulting in a low S-score of the quality check.

The types of MNM investigated in the accepted studies included lipidic or polymeric nanocarriers, metallic or metal oxide MNM, quantum dots and nano-emulsions.
The skin was obtained from different sources. Human skin was often obtained from surgical waste of hospitals and was often taken from the abdomen. Porcine skin generally was taken from the ear. The skins were placed in a Franz diffusion cell and exposed to the materials for different periods of time, but largely with a maximum duration of 24h. For the exposure medium, the materials were dispersed in artificial sweat, physiological fluid or simply in the dispersion that was used to produce the materials. The nanomaterial permeation was often determined at multiple timepoints after the exposure, and at the very least at the end of the experiment.
The analytical methods used to quantify the content of MNM in skin and in the matrices used in the experiment (i.e. exposure formulation and receptor fluid) varied widely between studies. Some studies did not measure the actual content of MNM, but determined this by proxy, for

example, by quantifying the amount of pharmaceutical ingredient that was included in a nanocarrier. In studies where the nanomaterial itself was quantified, electron microscopy imaging techniques were used by some research groups, while others used mass spectrometry, or labelling with fluorescent probes.
The large variety of models, materials and analytical methods used complicates comparison of quantitative results between the studies.

7.3.2 Guidelines followed

Various guidelines exist on studying absorption or penetration of chemicals through the skin. OECD published technical guidelines for *in vivo* and *ex vivo* dermal penetration studies of chemicals (TG 427 and 428, respectively, OECD 2004 (OECD, 2004a, 2004b)). The EFSA (Buist *et al.*, 2017) also published a guideline on dermal absorption, and the EU Scientific Commission on Consumer Safety (Scientific Committee on Consumer Safety, 2010) published an opinion describing basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients. The guidelines on *ex vivo/in vitro* studies are largely overlapping, but none of them specifically provide guidance on studying MNM.

The experimental set ups of the final selection of *ex vivo* studies varied, but there was some overlap as well. A number of studies were conducted within the framework of the EU project EDETOX (2004) and these studies followed a protocol that was similar to the one described in OECD TG 428. In this protocol, exposure takes place in a Franz diffusion cell for up to 24 h, where particles are dispersed in artificial sweat and subsequently, the particle content in receptor fluid, epidermal and dermal skin samples are analyzed at multiple timepoints.

7.3.3 Test methodology factors affecting dermal absorption

One of the most important factors affecting dermal absorption of MNM is the skin integrity. This was evident in studies demonstrating penetration of MNM through skin that was intentionally damaged compared to intact skin, where penetration of MNM was very low or not detected at all. This finding underlines the importance of demonstrating skin integrity before the start of the experiments. In addition, it is important to know how long the skin integrity can be maintained, as the condition of the skin may start to deteriorate throughout the duration of the study. Knowledge on the skin integrity is also of relevance for the type of skin processing taking place. OECD, EFSA and SCCS guidelines all recommend using 200-400 µm dermatomed skin for human *in vitro/ex vivo* studies. A study by Jatana *et al.* (2016) demonstrated that the skin was much more permeable to MNM shortly after the processing compared to 24h later.

Most studies use the Franz diffusion cell for their experiment. One study used an additional method for studying skin penetration of quantum dots, i.e. applying cosmetic formulations containing quantum dots to skin mounted on a gauze in a petri dish and photographing with a UV lamp (Jatana *et al.*, 2016). Large differences in levels of skin absorption were found between the two protocols, indicating that quantitative results may vary depending on the experimental protocols used.

Another major factor determining the results of absorption studies with MNM is the type of donor suspensions used for the exposure. Skin absorption and penetration is a complex interplay between the properties of the particles in the formulation and the skin condition. Ions released from damaged skin may result in aggregation and skin deposition of some particles, depending on their surface coating. PEGylated particles appear to be less prone for this destabilization (Lalloz *et al.*, 2019). MNM often aggregated to a large extent in the artificial sweat donor fluid used in the EDETOX studies. Such aggregation likely prevents their penetration of the stratum corneum and explains the general observation of the lack of skin penetration of MNM. While this exposure may reflect the real-life situation for particles which are in direct contact with the skin, this may not be the case for particles that are embedded in a formulation which prevents their aggregation. One study investigating the skin penetration of

silver MNM in fresh, cryopreserved and glycerolized skin demonstrated that skin absorption in the dermal layers was much higher in glycerolized skin compared to the other skin grafts (Bianco *et al.*, 2014). This may indicate that particles are able to absorb or penetrate the skin in case they are dispersed in a formulation with glycerol-like properties, such as in certain cosmetics. Indeed, a study by Jatana *et al.* (2016) demonstrated the enhancement of the permeation of quantum dots in the viable epidermis of intact skin when applied in glycerol and in some commercially used cosmetic formulations. Also, various nano-emulsions were able to penetrate intact epidermis in the presence of permeation enhancers in the formulation, such as transcutol P (Kumar *et al.*, 2016).

As discussed, the method used for analysing the nanomaterial content in the skin or receptor fluid varied between studies. Standardized, validated methods are often not available for every material, so even when the same general method is used, protocols may be different. The method of choice likely depended on the type of material and on the equipment available to the research groups. Different methods yield a different type of result. When only the pharmaceutical ingredient in a nanocarrier is quantified, the amount of nanocarrier can only be roughly estimated and involves the assumption that the pharmaceutical ingredient is still associated with the nanocarrier. Methods based on mass spectrometry often only give information on the mass of an element (not particles) present per mass of skin or volume of receptor fluid. Electron microscopy does detect particles, and yields results in the form of number of particles per surface area of skin, or per volume of receptor fluid.
Not all methods are compatible with all types of materials, or available to all research groups, as each method has its own limitations. For example, labelling of materials with a fluorescent probe involves the risk of the probe leaching out of the nanomaterial. Mass spectrometry does not allow to distinguish between particles and ions unless it is coupled to additional specialized equipment such as a field flow fractionator. Electron microscopy set-ups will have limitations to the size of particles they are able to detect, depending in part on the chemical composition and density of the particles. In addition, electron imaging is expensive and quantifying the amount of particles in, for example, a section of skin would require a large number of images.

7.3.4 Other available tools

Given the limited amount of high quality and relevant studies on absorption of MNM, it is not surprising that currently, no *in silico* tools exist that can be used to estimate the potential of MNM to pass the skin barrier. Such tools can only be developed once more data is generated in comparable experimental studies. This would facilitate more insight into the type of nanomaterial properties that affect dermal absorption.
For now, information on dermal absorption therefore needs to be generated experimentally. When sufficient information will become available and reliable data on MNM with similar properties will be produced, read-across could be applied. ECHA has published a best practices document that provides guidance on how to use toxicity data from similar materials (ECHA, 2016) and further considerations on determining the similarity between MNM can be found in a study by Park *et al.* (2018). The ongoing EU project GRACIOUS is also developing read-across guidance for MNM to be used for regulatory purposes. These initiatives may also be helpful in performing read across on dermal absorption data with MNM.
Various tools are available for generating experimental data on dermal absorption of MNM. Of course, generating data from studies with human subjects would be ideal, but this is not feasible in most cases. Alternatively, *ex vivo* studies using human skin are a promising tool to study dermal absorption of MNM. Many hospitals may be able to provide human abdominal skin from surgical waste. In case this is not feasible, the use of porcine skin should be considered over that of other species, as porcine skin is known to be structurally similar to that of humans. Slaughterhouses may be able to provide porcine skin for this purpose. Use of rodent skin or rodent *in vivo* studies is not recommended, as it is unknown how the differences between rodent and human skin affect the absorption of MNM. This also holds for hairless rodents, which are actually born with hair, but lose their hair shortly after birth, because they still have many more (disintegrated) hair follicles than human skin.

As discussed, no guideline exists that specifically describes an experimental protocol for studying the dermal absorption of MNM. Development of such a guideline is recommended, taking into account the test method factors that affect dermal absorption of MNM as discussed above.

7.3.5 MNM properties affecting dermal absorption

Despite the fact that the final selection of studies included a large variety of materials, no further insight into the MNM properties affecting dermal absorption was obtained from this review. Comparison of the results across studies was complicated by the use of different skin models, formulations and dispersions used for exposure, test protocols and detection methods. As a consequence, the absorption potential of different MNM could only be compared if they were included in the same study. However, most studies only included one type of material.

One study on nano-emulsions did confirm previous findings that size affects dermal absorption (Kumar *et al.*, 2016). However, the data does not allow for a cut-off size above which absorption no longer occurs.

Another study confirmed previous findings that the surface properties of MNM affect dermal absorption (Lalloz *et al.*, 2019). In this study, the dermal absorption of particles depended on the amount of PEG on the surface with MNM with a high PEG-content showing a higher penetration into the skin than MNM with a low PEG-content. Whether the presence of PEG on the surface increased or decreased dermal absorption depended on the skin integrity, again demonstrating the complex interplay between the nanomaterial and its direct environment.

Quantitative numbers on what percentage of the applied dose of MNMs absorbed through the skin cannot be derived from the selected studies due to the lack of mass balance data. However, any findings of absorption of MNMs through intact skin can be considered very low, since the measurements in these studies were around or below the detection limit. Particles that are not washed off generally remain in the upper layer of the epidermis, although some particles of some substances also reach the dermis. This may in part be due to the fact that particles tend to aggregate and become too large to penetrate the stratum corneum. Low levels of the element silver were detected in the receptor fluid of skin exposed to silver MNM (Bianco *et al.*, 2015), implying that it passed both epidermis and dermis. However, since this silver was in the form of silver chloride, it is likely that the silver detected had penetrated the skin in the form of ions.

Absorption of MNMs does appear to occur to some extent in damaged skin, although again, no quantitative numbers on this can be derived.

More comparable studies are needed to shed more light on the relationship between MNM properties and their potential for dermal absorption. Due to the complex interplay between MNMs and the dispersion/formulation that is used for the exposure, different relationships may exist for a single MNM, depending on the formulation used.

7.3.6 Relevance of rodent studies

Prior available information
In the past, several studies have addressed skin penetration/absorption of MNM in rodents (Adachi *et al.*, 2013; Furukawa *et al.*, 2011; Labouta & Schneider, 2013; Wu *et al.*, 2009; Xu *et al.*, 2011). In this paragraph we will briefly discuss arguments in favor or against testing nanomaterial absorption in rodents, compared to *in vivo* human studies, *in vivo* pig studies, *ex vivo* human studies and *ex vivo* pig studies. Regarding *in vivo* testing of skin absorption, the OECD has published OECD TG 427 (OECD, 2004a). It should be noted, however, that this test guideline is not readily applicable to MNM and no current initiatives exist to adapt this guideline in this respect, neither with regard to the OECD Guidance Note 156 (OECD, 2011), which states that the effects of nanotechnology have not been addressed or to the OECD Test

Guidelines Program (OECD, 2019).

Regarding *in vitro* testing of skin absorption, the OECD has published OECD TG 428 (OECD, 2004b). Similar to TG 427, this test guideline is not readily applicable to MNM and no current initiatives exist to adapt this guideline in this respect. Moreover, the OECD Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured MNM states for this guideline "*The use of the in-vitro method has been questioned for MNM since it has been claimed that mechanical aspects such as flexing may be important*".

Dermal penetration/absorption of compounds is dependent on their physicochemical characteristics and also on various skin characteristics. Due to species differences in terms of skin structure, especially dermal layer thickness, lipid composition, and pelage density, differences in terms of dermal penetration/absorption can be marked (Labouta & Schneider, 2013; *Principles and Practice of Skin Toxicology*, 2008). For conventional chemicals, it is generally considered that dermal permeability between species can be ranked as follows from more permeable to less permeable: Rabbit > Rat > Pig > Monkey > Human (Magnusson *et al.*, 2001). A comparison of human, rat, and mouse skin showed a thickness of stratum corneum (SC), viable epidermis (VE) and dermis (D) of 17 µm, 47 µm, and 2.9 mm, respectively, for human abdomen; 18 µm, 32 µm, and 2.0 mm, respectively for rat dorsum; and 9 µm, 29 µm, and 0.7 mm, respectively for mouse dorsum (Wei *et al.*, 2017). Given that mouse skin is less thick than rat skin, it likely has a more permeable skin for conventional chemicals. Rat skin, at least viable epidermis and dermis are also about two-thirds of the thickness of human skin, but clearly thicker than mouse skin. Therefore, it is expected that the dermal absorption of conventional chemicals, for which absorption can be considered a diffusion-like process, is lower for rat skin than for mouse skin. It should be kept in mind that this is considered for general chemicals, which does not necessarily mean it is true also for MNM. Depending on the compound studied, these differences in terms of skin permeability could range from more than 4 times for the pig and up to 9 times more permeable for the rat compared to human skin. Therefore, in terms of animal models, rodents, which have a dense pelage, are not necessarily a relevant model to predict dermal penetration/ absorption in humans, since these species generally present a higher permeability. Overall, human skin remains the "gold standard" to evaluate dermal penetration/ absorption of compounds for humans.

The rat is the most commonly used animal for these studies, and hairless strains are also available. The best animal alternative seems to be pig skin which displays the most common characteristics with humans and as such, pigs and minipigs are commonly accepted as models for dermal absorption studies to extrapolate results to humans, although as mentioned previously their permeability can be up to 4 times higher than human skin. In terms of experiments with MNM, where deposition (if not penetration/ absorption) occurs along the hair follicles, the density of hair on the skin may also be expected to be important. So, keeping the differences between rodent and human skin in mind, if rodents are used, they should at least be hairless (and not shaven).

There are anatomical differences other than thickness between the dermal composition of humans and that of mice. Other notable features that differ between human dermis and mouse skin are vascularization patterns and hair density. The human dermis is a highly vascularized system with both a rich papillary network and a deep dermal network. In contrast, mouse dermis is poorly vascularized compared to human dermis, and the predominant capillary networks are associated with hair bulbs (Sundberg *et al.*, 2012). Though the author does not mention rats, it is known that rats also have a much higher hair density.

The skin of hairless rodents may be less different from human skin in one relevant aspect, although they originally still have more hair follicles per area than human skin, but they shed their hairs after birth and their hair follicles disintegrate (Panteleyev *et al.*, 1999). It may be expected that this implies that hairless rodents have a more similar dermal penetration and absorption of MNM than normal rodents. However, whether this is actually the case, has not

yet been studied and therefore also information from studies with hairless rodents cannot be properly interpreted.

Discussion
The choice whether or not to include rodent studies in this report is not based on a single (or a few) decisive factor, but rather on a reasoning. The differences in skin thickness, vascularity and hair density between humans and rodents may affect permeability, and this cannot be simply corrected for due to the unknown underlying mechanisms that affect permeability and that may differ between different MNM. Although it could be argued that this is intrinsic to animal testing and in fact an OECD TG exists, pig and human skin data are available that suffer less or not at all from these interspecies differences.

Langerhans cells take up MNM and migrate to the lymph nodes (see e.g. Lee *et al.* (2010)), after which MNM become systemically available. Since Langerhans cells reside in the epidermis (most prominently in the stratum spinosum) differences in epidermal thickness may be less important. Indeed, skin thickness per se is not an argument to not include rodent studies. However, differences in vasculature (by means of substance removal) and hair density (by means of short-cutting the epidermis) may affect dermal absorption (Jatana & DeLouise, 2014)

Therefore, while for conventional, soluble chemicals, a comparison between human and rodent skin may be feasible, the uptake mechanisms of particulate materials through the skin are expected to be very different from soluble chemicals. These potential differences in the uptake mechanisms of particulate materials between human and rodent skin have not been studied and therefore it is unknown whether results from rodent skin can be translated to human skin. Actual comparisons between uptake of the same nanomaterial under the same conditions from the same medium via human and rodent skin might shed light on the relative difference for some MNM, but such comparisons of good quality are very limited so far.

The limited number of references found in this project with both a rodent skin and human or porcine skin model (see 6.3.3) did not allow drawing any conclusion on the comparability or on whether rodent skin systematically allows higher or lower penetration or absorption of MNM.

7.4 Comparison of the findings of this study with previous reviews

The Danish report was used as the basis of this study. It is therefore relevant to evaluate how the findings of this study relate to those in the Danish report (Poland *et al.*, 2013).

A number of conclusions or aspects reported in the conclusions of the Danish report will be specifically discussed below.

7.4.1 Limitation of reporting

The Danish report indicates: "*However despite the relative abundance of publications, there is a limitation on the reporting of physicochemical data and/or the alteration of multiple experimental parameters in a non-systematic way that hampers true comparisons of MNM or their physicochemical properties and the drawing of robust conclusions*."

This general conclusion is still true. In our study also a relative abundance of publications was found, though many of them were on dermal penetration or absorption of drugs when linked to MNM and not on dermal penetration or absorption of MNM themselves. And also, in the present study, it was found that various physicochemical data on the MNM and formulations used were often not reported. For example, the dissolution in exposure medium and the primary particle size as well as the particle size in exposure medium were often not reported. It was also found that there were quite some differences in parameters in different experiments, such as duration of exposure, timepoints of observation and whether MNM were investigated in the stratum corneum, in some layers of the stratum corneum only or also in other skin layers and

even in reception fluid. Many studies on dermal penetration of drugs compared two or more forms of application, e.g. nano emulsions of two different compositions, but did not specifically analyse the parameters that could explain potential differences in results.

Due to these factors, it is still very difficult to draw robust conclusions from the large number of studies.

7.4.2 Influence of solubility

The Danish report indicates: "*Of those properties considered within this report, size and surface chemistry appear to play the most significant roles in dermal penetration whilst particle composition and shape tend to have little effect. However one prominent challenge relating to composition is the effect of solubility on detection of absorption whereby if a particle is soluble, such as silver, then it may be detected in the receiving fluid etc. (e.g. blood serum, Franz cell receptor chamber) either as a particle or as a soluble fraction such as the ionic form*."

In the present study, it was also found that dissolution in exposure medium was often not reported. Therefore, just like concluded in the Danish report, the lack of knowledge on dissolution, together with the detection methods that do not distinguish between particles and ions, hinders the possibility to conclude on dermal penetration or absorption of MNM in particle form. However, when no penetration or absorption is found at all, it must be concluded that at least also no penetration or absorption in particle form was seen.

7.4.3 Influence of particle size

The broad view on the influence of particle size in the Danish report is the following: "*As indicated, our result showed that the role of size is considered to be a critical component of dermal penetration but this is still subject to the influences of other properties as well as changes in particle size in a dynamic fashion (i.e. agglomeration/ aggregation state). Overall, the conclusion that can be drawn is that penetration of particles in the nano-range into the skin is possible and that this may be greater than for larger particles although this still occurs only at low levels.* It also states that *"However there is considerable variability outside of this broad view with little evidence of large deviations in absorption efficiency or distribution profile within the skin across different size fractions within the nano-range and this may depend on various factors such as surface chemistry and experimental conditions etc."* While the word 'critical' in the referenced text appears to suggest a clear conclusion on the relevance of particle size in the Danish report, the other statements in the reference indicate that the actual finding is not very strong. The present study did not change that conclusion. Actually, there was generally a lack of clear presentation of both primary and (potentially) agglomerated particle size in exposure medium in the analysed studies. Therefore, whether particle size is a very relevant parameter in dermal penetration and absorption of MNM is difficult to evaluate. Some studies, e.g. Kumar *et al.* (2016) provide indications that particle size may play a role, but this is too limited information to draw robust conclusions. The review by Liang *et al*. (2013) also suggests that smaller sizes are more likely to penetrate, but this review is probably based on the same publications as the Danish report and therefore the similarity in conclusions is to be expected.

One aspect that hinders conclusions on the effect of particle size is the fact that MNM tend to agglomerate or aggregate. In some studies, e.g. Mauro *et al.* (2015) and (2015) substantial aggregation was observed in the exposure medium. According to general expectations, and also concluded in a review on agglomeration (and aggregation) by Bruinink *et al.* (2015), agglomeration may reduce translocation of MNM across primary barriers, such as the skin. Therefore, the observation that agglomeration or aggregation has occurred substantially questions any finding on the influence of particle size in studies where agglomeration and aggregation is not specifically investigated.

One possible mechanism of dermal penetration (and possibly subsequent absorption) of MNM is follicular penetration. According to the review by Fang *et al.* (2014), the influence of nanoparticulate size on follicular uptake is controversial. There are studies that suggest that a decrease in size leads to an increase in follicular uptake, but there are also studies suggesting a feasible size range for absorption via the follicles in the range of 300-600 nm (*i.e.* clearly higher than the size range in the proposed EU definition). Liang *et al.* (2013) suggest an optimum size for hair follicle penetration in human skin of around 530 nm.

All-in-all, the new information does not provide much additional support for robust conclusions on the influence of particle size on dermal penetration or absorption.

7.4.4 Influence of surface chemistry and formulation

The Danish report mentions that there is a slight tendency towards greater uptake of positively charged particles. However, it also reports that the studies on this lead to conflicting results. Liang *et al.* (2013) similarly suggest that positively charged particles could be preferred for penetration, but again the literature base for this review will be largely the same as for the Danish report. A study by Peira *et al.* (2014) supports the notion that a positive charge of the particles increases skin penetration. In this study positively charged TiO_2 particles adhered strongly to the skin and altered its structure. Coated non-charged particles did not. The flux of drugs was increased fourfold from the positively charged particles compared to the non-charged particles.

Skin absorption and penetration is a complex interplay between the surface of the particles in the formulation and the skin condition. Ions released from damaged skin may result in aggregation and skin deposition of some particles, depending on their surface coating. PEGylated particles appear to be less prone for this destabilization (Lalloz *et al.*, 2019). In our study, there were a number of references studying the possible influence of the formulation in which the nanomaterial is applied to the skin. These included Kumar *et al.* (2016), who observed higher epidermal permeation of a drug from nano-emulsions than from an aqueous solution and Jatana *et al*. (2016) who observed large differences in skin penetration between different formulation. Pepe *et al.* (2016) also found differences between different microemulsions in penetration of a drug.

The chemistry of the MNM, the skin and the formulation in which the MNM are present of course interacts, as indicated above. Liang *et al.* (2013) also report that formulations and their interaction with the skin are important. Chemical surface modifications of MNM may lead to changes in the skin, but they will also influence the interaction with the formulation that in itself may influence the skin. Damaged skin may further lead to chemical modifications at the surface or in the formulation on the skin. Predictions of qualitative directions of this influence are not really possible yet and calculating quantitative influence of surface or formulation chemistry on dermal penetration or absorption is as yet absolutely impossible.

7.4.5 The need for guidelines

The Danish report concludes that there is considerable variability in approaches for studying dermal absorption and that harmonisation of experimental approaches, methods and reporting is needed. The development of 'test guidelines' for elements such as sample preparation is recommended. Jatana and Delouise (2014) in their review conclude that the ability to reach consensus on the ability of MNM to penetrate beyond the stratum corneum is limited by instrumentation detection sensitivity. Liang *et al.* (2013) conclude that many factors in relation to penetration of MNM in and through the skin are unknown and that future studies should be well designed to allow for the elucidation of the effects of various factors on skin penetration of MNM.

Our results clearly support these findings and the need for more harmonisation and guidelines in the field of dermal penetration and absorption of MNM. It is not surprising that there is no

substantial harmonisation between the various studies found, because they all have their own goals, which were often quite exploratory. However, a number of studies followed the (not precisely defined) guidelines from the EDETOX project and were therefore more or less comparable. These internal project guidelines, together with guidelines from other sources, can be used as a starting point to develop more specific guidelines.

An important aspect in the need for guidelines is the need for clear reporting of the nanomaterial characteristics. Many studies analysed for this report did not fulfil the critical criteria for information on MNM tested, specifically in regard to dissolution in exposure medium and to particle size, both of the original primary particles and the particles (agglomerates) in the exposure medium.

More technical aspects that require additional attention are the duration of exposure and the time points of observation after exposure as well as the skin samples and media tested both quantitatively (for the relevant substance) and qualitatively (to show actual MNM in particle form). As indicated by Jatana and DeLouise (2014) the methods of detection may need to be further improved to allow both quantification and qualification as particles.

A relevant aspect for the studies with *ex vivo* skin is the fact that preparation of the skin may alter the characteristics. Specifically the flexing of the skin may influence dermal penetration, e.g. by increasing the importance of hair follicles as collection sites, as reported by Liang *et al.* (2013). The OECD Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured MNM states for TG 428 states: "*The use of the in-vitro method has been questioned for MNM since it has been claimed that mechanical aspects such as flexing may be important*". In general, the OECD documents, where they do refer to MNM and dermal absorption, indicate that the present guidelines do not specifically address MNM.

The number of samples and the number of donors was often not reported in the analysed studies. In the assessment of dermal absorption of conventional substances, this is considered quite an important aspect, at least when reasonably accurate quantitative conclusions are to be drawn. The EFSA guideline on dermal absorption (Buist *et al.*, 2017) gives specific attention and rules for e.g. discarding samples that have deviating values from the other samples and for accounting for variability, which is dependent on the number of valid samples.

8. Recommendations

The following recommendations are derived from this study.

8.1 Testing of dermal absorption of MNM

New studies on dermal absorption of MNM, with the aim to provide qualitative and quantitative proof of dermal penetration and dermal absorption, should be performed with *ex vivo* methods with human or porcine skin. Rodent skin should not be used, because the differences in skin characteristics between rodents and humans that may affect dermal absorption are too large and the influence of these differences are not sufficiently known to allow any kind of extrapolation from rodent skin to human skin. Any conclusions on these differences from conventional (non-nanoparticle) substances cannot be extrapolated to MNM, because the relevant penetration and absorption processes for MNM are expected to be substantially different from those for conventional substances. *Ex vivo* studies present the best option to study both the quantitative absorption and the actual penetration of MNM through skin layers.

The *ex vivo* tests should be done with a vertical diffusion cell, e.g. the Franz diffusion cell (Franz, 1975; Franz, 1978). However, other vertical diffusion cells (both static and flow-through) can be used as well, as indicated in OECD TG 428 (2004b).

Tests should generally be performed as described in OECD TG 428 and as also performed in the EDETOX project (EDETOX, 2004). Modifications to account for the fact that these guidelines were not designed for MNM may be necessary.

General aspects related to e.g. using fresh skin, the appropriate skin thickness, number of samples and interpretation of the results to obtain quantitative dermal absorption values should be taken into account as described in the guidance document OECD TG 428, in the EDETOX project report and in the EFSA Guidance on Dermal Absorption (Buist *et al.*, 2017).

Important aspects to be taken into account, when testing MNM for dermal absorption, and reporting the data include:

- General physicochemical characteristics as also determined for conventional substances, including:
  - Dissolution rate in water and artificial sweat.
- Sufficient specification of the original primary MNM studied should be reported; in accordance with (at least) the requirements for characterization of nanoforms at registration of substances under REACH (ECHA, 2019), including:
  - Particle size distribution and number fraction of constituent particles in the size range 1 nm to 100 nm;
  - Shape, aspect ratio and other morphological characterization;
  - Crystallinity;
  - Surface functionalization or treatment and identification of each agent;
  - Surface area (by volume, by mass or both)
- Other characteristics that may be relevant for dermal absorption of MNM should also be reported, e.g.:
  - Zeta potential (surface charge)
- The level of agglomeration and the particle sizes in the formulation as applied in the test should be specified
- Testing should preferably be done with a number of different formulations, including:
  - Artificial sweat
  - Formulations expected to increase dermal penetration, e.g. containing glycerol or PEG

    - At least the relevant formulation type for the expected uses of the nanomaterial should be included
- The dissolution in the exposure medium should be studied and reported
- Skin processing before testing should be done very carefully to prevent inadvertent flexing, or even damaging, of the skin that may influence the dermal absorption
    - A processing method should be used that has been shown not to significantly influence the penetrability of the skin
    - Any effect of skin processing should preferably be studied specifically, before drawing final conclusions from the study
- Intentionally damaged skin should also be tested, because this may be relevant for some nanomaterial applications

As far as possible the tests should provide both qualitative and quantitative information on penetration and absorption of the actual nanomaterial. Qualitative information should show, using appropriate techniques, whether the nanomaterial itself penetrates relevantly into and beyond specific skin layers in the form of MNM. Quantitative information, using appropriate techniques, should provide a quantification of the amounts of the chemical in the various fractions in the study, see OECD TG 428 (OECD, 2004b) and EFSA Guidance (Buist *et al.*, 2017). If quantification will not be able to distinguish between particle forms and ionic or dissolved forms, combining the qualitative and quantitative information can lead to conclusions on relevance of actual nanoparticle penetration and absorption.

8.2 Future research

It is recommended to perform a well-organised and structured research programme to obtain more and better information on the characteristics of MNM and the factors in the test methodology that influence dermal absorption of MNM. Such a study should be performed with *ex vivo* human skin of good skin condition. Possibly also the effect of damaged skin (abraded) should be taken into account. It should use the recommended methods as mentioned above and have sufficient samples per situation to draw statistically valid conclusions. It should study differences in characteristics that are most likely relevant for dermal absorption, including:

- Particle size (of the original MNM and of agglomerates in the applied formulation)
- Particle shape
- Surface charge
- Surface functionalisation (e.g. functions limiting aggregation and agglomeration of MNM)
- Effect of formulations via effect on aggregation/agglomeration of MNM
- Effect of formulations via effect on the skin

Appropriate qualitative methods should be used to determine the penetration of MNM in different layers of the tested skin (and the presence in receptor fluid).
Appropriate quantitative methods should be used to allow conclusions on amounts of substance (*i.e.* the chemical that forms the core of the MNM, not an attached chemical) in different layers of the tested skin and other relevant samples.

8.3 Use of existing information on dermal absorption of MNM

It is recommended in general not to base any conclusions on either qualitative or quantitative absorption of MNM through human skin only on rodent skin studies (either *in vivo* or *ex vivo*). The differences between human and rodent skin in relation to factors related to possibly relevant mechanisms of dermal penetration and absorption of MNM are too large to draw any conclusions from data gathered with only rodent skin. Because penetration and absorption of MNM through any type of skin is generally found be (very) low, the uncertainty caused by differences between human and rodent skin will have a very large impact.

In some exceptional cases, it may be possible to draw indicative conclusions based only on studies with rodent skin:

- If a proper *ex vivo* study with rodent skin, with formulations that are expected to enhance dermal absorption, does find no MNM in the skin beyond the stratum corneum and the quantitative analyses do not find any values above very low limits of detection in samples to be counted for dermal absorption, except the first set of tape strips of the stratum corneum, it can be concluded that the dermal absorption via human skin will be very, very low. In that case, it might be concluded that dermal absorption test with human skin is not necessary and dermal absorption might either be neglected in risk assessment or a very low value for percentage dermal absorption could be used.
- If quantitative analyses of a properly conducted *ex vivo* study in rodent skin shows substantial amounts of the substance in the reception fluid and qualitative analyses show clear presence of MNM in particle form in layers of skin beyond the epidermis, it can be concluded that actual dermal absorption via human skin is very likely. Follow-up with an appropriate dermal exposure study in human skin is then warranted.
- If in a properly conducted *ex vivo* dermal absorption study in rodent skin relevant amounts of substance are measured in fractions that count for dermal absorption (except the upper tape strips of the stratum corneum), but no MNM in particle form are discovered at all in layers beyond the stratum corneum and if it can be excluded that this is caused by e.g. errors in the tests or inappropriate materials used, it can be concluded that the MNM apparently dissolve in the application formulation and/or in the skin and that absorption is at least largely in the form of dissolved material. Again, performing an *ex vivo* human skin dermal absorption study is warranted.

Hairless rodents may have their advantages, specifically with regard to dosing, since no shaving is needed and therefore the risk of inadvertent damage of the skin is smaller. Whether the fact that they are hairless also implies that the dermal penetration of MNM is closer to that of humans is uncertain, because hairless rodents still have many (degraded) hair follicles. It is not clear how that influences dermal penetration.

Existing information on dermal absorption of MNM from well-performed *ex vivo* human or porcine skin studies, that have been performed according to the guidelines mentioned and referred to above, and that contain sufficient information on the characteristics of the MNM and on the methods used, can be used.

8.4 Use of MNM dermal absorption data in regulatory risk assessment

For risk assessment of non-soluble MNM the following assumptions can be used:

- Dermal absorption of MNM is very low if:
    - The MNM are not soluble in water or in artificial sweat;
    - The formulation in which the nanoparticles will reach the skin is expected to
        - a) not significantly enhance the penetrability of the skin
        - b) not ensure almost complete dispersion of MNM in actual nano-form
        - c) not lead to significant dissolution of the MNM;
    - The skin is expected not to be seriously damaged in the situation to be assessed.

In these cases, though no percentage of dermal absorption can be indicated, due to lack of knowledge, it can be assumed that the percentage is much lower than that of many conventional chemicals.

- Dermal absorption of MNM is probably still very low if the above conditions are fulfilled, but there is some damage to the skin to be expected

It is recommended that the above conditions are justified in regulatory risk assessments of the relevant MNM.

The justification of the solubility in various media should be based on testing, while the justification of the type of formulation can be based on general argumentations related to the types of formulations expected.

For other situations, e.g. if the MNM are in a formulation, such as a cosmetic, or a plant protection product, that has characteristics that suggest enhanced skin penetrability or effective dispersion of the MNM in the formulation, the dermal absorption of MNM may be higher and may require specific testing.

For MNM that are substantially dissolved in the formulation used or in water or artificial sweat, it should be considered that dermal absorption may occur in dissolved (ionic) form and appropriate testing may be needed.

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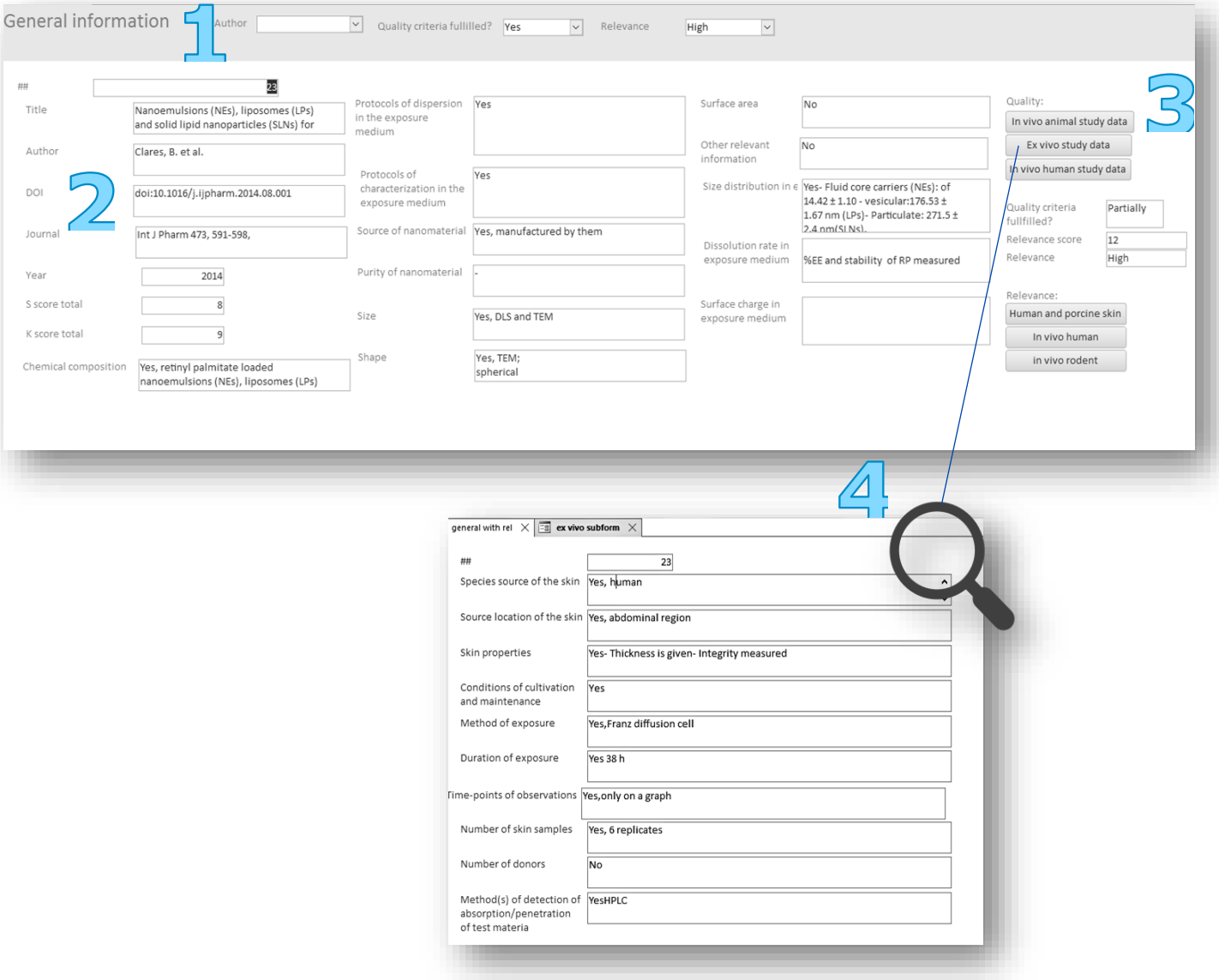
Appendix 1. Illustration of the database with information

This Appendix presents the Access database structure. The main body of the database consists of the quick search bar (1), main form with information on the properties of nanoparticles and the publication's administrative data (2) as well as links to the details of the study (3) corresponding to quality and relevance criteria discussed in the report. These links open a separate form where the specifics of each model used in the study (*i.e.* human *ex vivo*, human *in vivo*, animal *in vivo*) are outlined (4).
The search bar (1) facilitates quick choice of studies that fulfil the quality criteria and show high relevance. It is also possible to search for the first author of the publication.



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